UNIVERSIDAD DE GRANADA

Facultad de Medicina
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NOVEL STRATEGIES FOR THE TREATMENT OF VISCERAL PAIN.

Role of voltage-gated sodium channels and enhancement of the opioid-induced analgesia by selective blockade of sigma-1 receptor.

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Trabajo en opción al grado de DOCTOR por la Universidad de Granada. Programa de Doctorado en Biomedicina

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Donde la vida comienza y
el amor nunca termina

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Ya sé que me juras que hay un futuro,
y el tiempo futuro yo creo que es este.
Era solo un último intento más,
el tiempo del futuro está aquí.

EYLSQ, "El tiempo futuro".

Contents

1 Introduction

1.1 Pain

1.1.1 Neuroanatomy and neurophysiology

According to the International Association for the Study of Pain (IASP, 2020), pain is "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage".

The ability to detect and interpret a wide range of noxious stimuli (thermal, mechanical and chemical) is crucial for an organisms survival and comfort. These stimuli can generate acute pain and transmit an electric stimuli via the nociceptive system, a peripheral and central nervous system (PNS and CNS, respectively) pain pathway. Acute pain has evolved as a key physiological alert system for avoiding noxious stimuli and protecting damaged regions of the body by discouraging physical contact and movement [1]. The pain pathway shows plasticity, enhances pain signals and produces hypersensitivity; or undergoes chronic pain condition if the changes persist [2]. Chronic pain has been recognized as pain that persists beyond normal healing time and hence lacks the acute warning function of physiological nociception. Chronic pain, defined as lasting pain if it reoccurs for more than 3-6 months, may be associated with many common diseases or considered a disease in itself.

1.1.1.1 Pain ascending pathways

Peripherally, stimulus intensities that reach the noxious range activate specific primary nociceptive neurons that act as receptors (nociceptors) and whose axons project into the dorsal horn (DH) of the spinal cord. In the DH, the axons of specific neurons cross the midline within one or two segments and ascend via spinal pathways. The most important via is the spinothalamic tract (STT). The

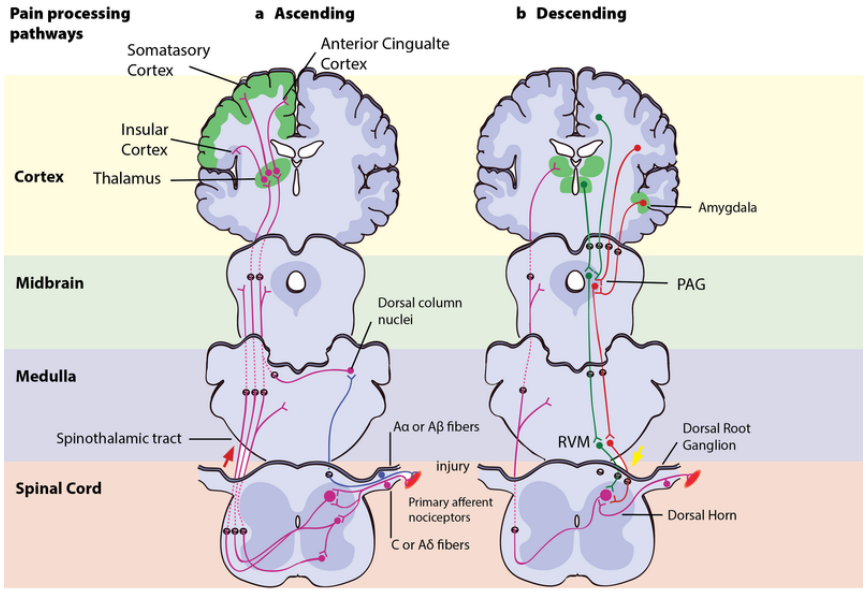

Figure 1.1: Schematic view of pain processing pathways. (a) ascending pain pathways; and (b) descending pain modulation pathways. Periaqueductal gray (PAG); rostral ventral medulla (RVM). Reproduced from [3].

STT cells project to the thalamus (lateral and medial nuclei) and neurons in these thalamic nuclei project to areas of the cortex (Figure 1.1, left panel a).

Nociceptors have free nerve endings and their cell bodies are located in the dorsal root ganglia (DRG), nodose ganglia (NG), trigeminal ganglia, jugular and petrosal ganglia and relay sensory signals from skin, muscle, joints, and viscera to the CNS [4, 5]. These neurons are highly diverse in soma sizes, axon diameters, expression of different ion channels and receptors, electrophysiological properties, and innervation territories [5]. All this sensory specificity is possible due to the differential expression of specific transducers, which can be activated by mechanical, thermal (heat or cold) or chemical stimuli [6].

The nociceptors can be divided in three major groups:

i) The first includes small diameter unmyelinated "C" fibers. They relay second or slow pain and are in turn subdivided into polymodal and silent nociceptors. Polymodal fibers respond to noxious mechanical, thermal, and

chemical stimuli [7] and silent fibers are heat responsive, more responsive to chemical stimuli compared to the polymodal neurons but mechanically insensitive (develop mechanical sensitivity only in the setting of injury) [8].

ii) The second are medium diameter myelinated Aδ afferents that mediate acute, well-localized and fast pain [2].

iii) The third are the large diameter myelinated Aβ fibers that stimulate low-threshold mechanoreceptors [6].

Most of the molecules synthesized by the nociceptor cell body (such as pH, lipids, and neurotransmitters) are distributed to both central and peripheral terminals. Whereas only the nociceptor peripheral terminal will respond to primary stimuli, both the peripheral and central terminals can be targeted by those endogenous molecules that regulate its sensitivity [2].

In the DH, primary afferent nerve fibers are projected. Anatomically, the DH is organized into distinct laminae (I to X) where, with a remarkable stratification, terminate the different population of primary afferent axons [9]. The anatomic distribution of primary afferent endings is defined as a function of location (skin, viscera, muscle or joints) and the type of nociceptor (Aδ or C).

There are three main classes of neurons in the spinal cord:

i) Projection neurons. These are in turn divided into nociceptive specific and wide dynamic range (WDR) neurons. The first ones respond specifically to noxious stimuli and are mainly located in the superficial aspects of the DH (laminae I-II). WDR neurons respond to both noxious and non-noxious stimuli and are predominantly located in the deep DH laminae IV-V [2, 9].

ii) Propriospinal neurons.

iii) Interneurons.

Spinal cord neurons do not appear isolated from each other yet in a group, so that sensitive information is coordinated by some of them and projected to higher levels. This input of stimuli from somatic and visceral nociceptors, as well as their coordination, contributes to the phenomenon of referred pain.

The axons of neurons cross the midline in one or two segments and ascend by the STT or trigeminothalamic tract.

Axons in the anterior STT project to the medial thalamus and limbic structures and are believed to mediate the emotional and aversive components of pain [9, 10].

Cells in thalamus in turn project to various distributed control cortical areas (somatosensory cortex (SSC), anterior cingulate gyrus, insular cortex, prefrontal cortical areas, basal ganglia and cerebellum). Here, the pain perceptive input is integrated with information about the general state of the body to provide cognitive information on different aspects of the sensory system, such as the sensory discrimination properties and the emotional aspects of pain [2, 11, 12].

1.1.1.2 Pain descending pathways

All the steps in the ascending pain pathway of the nociceptive system can be modulated by descending projections. Unlike the nociceptive transmission system with centripetal and ascending characteristics, this endogenous inhibitor system is descending and centrifugal. For example, analgesia is not only the interruption of nociceptive transmission. This is a coordinated and highly complex function that regulates, controls and limits nociceptive transmission preventing chaos and instability that can occur if only excitatory mechanisms exist. There is a common neural mechanism for antinociception in these regions.

Two of the most important control areas are rostral ventral medulla (RVM), and midbrain periaqueductal gray (PAG) [10].

Three types of neurons have been found in those areas [13]:

i) ON neurons are excited by the noxious stimulus and have a net facilitating influence on nociceptive transmission (exciting the spinal neuron or inhibiting an interneuron of an inhibitory nature).

ii) OFF neurons are inhibited by harmful stimuli and have a net inhibitory action on the pain transmission.

iii) NEUTRAL neurons show a variable response and do not respond to injurious stimuli.

Descending impulses are generated from the PAG to the RVM where endorphins and enkephalins are released. From the RVM, excitatory impulses are generated descending the dorsolateral spinal cord and ending in the second lamina

of the DH, where serotonin is released. Serotonin from the OFF neurons of the RVM contacts an interneuronal inhibitor that releases enkephalins, which, contacts the projection neuron, thus inhibiting nociceptive transmission by a primarily presynaptic mechanism [14], (Figure 1.1, right panel b).

1.2 Visceral pain

1.2.1 Visceral *vs* somatic pain.

Visceral pain is more common than somatic pain and originates in the internal organs of the chest, abdomen or pelvis. This disorder may be the result of direct inflammation of a visceral organ (e.g., inflammatory bowel disease (IBD), pancreatitis, appendicitis), occlusion of bile or urine flow (e.g., kidney stones), or from functional visceral disorders (e.g., irritable bowel syndrome (IBS)). Angina, bladder pain syndrome (BPS) (interstitial cystitis), gastroesophageal reflux disease, endometriosis, and dyspepsia [15] can also be included in this list; although functional gastrointestinal (GI) disorders underlie the most prevalent forms of visceral pain [16].

Most studies in the field of pain and nociceptors have focused only on the somatic sensory system, but the processing of pain originating from visceral organs is very different from somatic nociception (see [17] for a more in-depth analysis).

The visceral system includes multiple ion channels, neurotransmitters and receptors that are qualitatively and/or quantitatively different from those involved in somatic or neuropathic pain [15, 17] and there are a large number of organs and systems with two extrinsic innervations (vagal and spinal), as well as of numerous intrinsic neurons (the enteric nervous system (ENS)). Noxious and non-noxious inputs are propagated by Aδ and C fibers, and it is believed that the visceral painful perception is dependent on the intensity of the stimulus due to the low intensity of the electric stimuli that raise sensations of fullness and nausea, while stimuli of high intensity cause pain [18].

Since a *noxious stimulus* is semantically distinct from a *painful stimulus*, a new definition for the visceral nociceptor has been proposed: "a sensory receptor that, when activated, can produce a reflex or response that is protective or adaptive (e.g., withdrawal, guarding, vocalization); can encode stimulus intensity in the noxious range; and can sensitize (i.e., give increased responses to noxious in-

tensities of stimulation after insult or exposure to chemical mediators such as those produced during inflammation)" [15].

Cutaneous nociceptors have many different sensory endings (e.g., Merkel cells, Ruffini endings, Pacinian corpuscles), whereas internal organs are innervated by low threshold, high threshold, silent and mucosal varicose nerve endings [19].

Even though visceral pain may be the response to noxious stimuli as distension or inflammation, the severity of pain does not always reflect the severity of the condition causing the pain [20] probably because:

1. The viscera are poorly innervated[1].
2. There are convergent inputs from spinal neurons in the skin (eliciting the referred pain) [21, 22, 23].
3. Visceral pain is commonly associated with emotions [15].

Sub-regional differences in visceral and cutaneous pain processing have been demonstrated. Although the primary and secondary SSC, anterior cingulate cortex (ACC), and insular cortex are activated in both, this occurs at different loci in one or the other pain process [11, 24].

1.2.2 Neuroanatomy of visceral pain

The internal organs are innervated by three main groups of receptors:

i) High threshold or phasic receptors that respond mostly to mechanical stimuli within the noxious range.

ii) Low threshold, tonic or WDR mechanonociceptors. Mainly intraganglionic laminar endings (IGLEs), located in the myenteric or submucosal plexus and intramuscular arrays (IMAs), on the circular and longitudinal muscle layers. IGLEs are sensitive to distension and muscle contraction, and IMAs respond to muscle stretch. Generally, WDR mechanonociceptors respond again to mechanical stimuli but with an encoding function that covers the range of stimulation intensity from innocuous to noxious.

[1] It is estimated that < 7 % of the spinal afferents in the DRG project to the viscera and only a fraction of these inputs are recognized by the CNS.

iii) Silent nociceptors, activated by inflammatory mediators and perhaps with a similar role to somatic pain.

In addition to these three main ones, there are also mucosal endings, which are involved in chemoreception and are located:

1. in the stomach mucosa (afferent endings of the gastric mucosa),
2. in the villi (afferent villi, which detect substances released by the epithelium), and
3. in the crypts (afferent crypts) of the small intestine mucosa [15, 25, 26, 27, 28].

1.2.2.1 Extrinsic innervation

An useful division of extrinsic innervation is into (i) cervical (vagus) and (ii) spinal (thoracolumbar and pelvic) visceral afferent fibers [15, 25, 26, 29] (Figure 1.2). Most vagal and spinal nerves axons are unmyelinated (C fibers) and a minority have fine myelinization (Aδ fibers) [25]. Broadly, parasympathetic afferents (vagal and pelvic nerves) subserve homeostatic functions (via chemonociception pathways) whereas sympathetic splanchnic afferents control pain evoked by distension of the upper GI tract [30]. Although visceral pain originates in the internal organs of the thorax, abdomen or pelvis, functional and inflammatory GI disorders underlie the most prevalent forms of visceral pain, especially IBS and IBD [16].

- Cervical innervation. The vagus nerve provides sensory innervation and efferent control pathways from thoracic and upper abdominal viscera including the entire gut except the urinary bladder and transverse and distal colon. Their axons project directly into the brainstem and their cell bodies are located in the NG and jugular ganglia [21]. Those axons terminate in the brainstem nucleus tractus solitarius in the dorsal medulla [15] which, in turn, projects to the thalamus and, directly, the hypothalamus, locus coeruleus, amygdala, and PAG [19, 26].

  Vagal afferents have been shown to facilitate nociceptive transmission whereas the vagal nerve participates in an antinociceptive descending pathway mediated by nanomolecules such as, but not limited to, opioids [31, 32], whatever modulate visceral pain [33]. This may be due to differences in stimu-

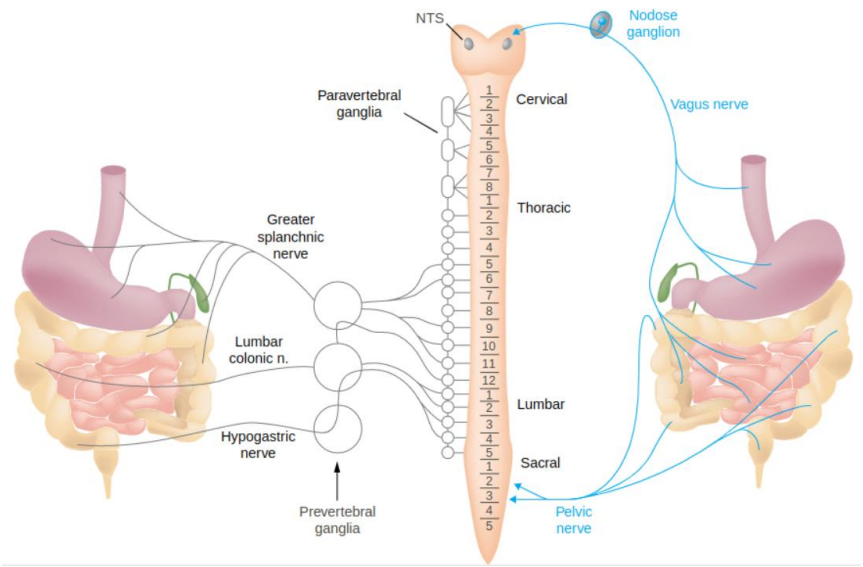

Figure 1.2: Extrinsic innervation of the gastrointestinal tract. Nucleus tractus solitarius (NTS). Reproduced from [21].

lation parameters: low intensity stimulation of vagal afferents facilitates, while high intensity stimulation inhibits nociception [32, 34]. Efferent vagal pathways originate in the dorsal motor nucleus of the vagus and the nucleus ambiguous of the brain stem, and underlie control of motor and secretory gut functions [35].

- Spinal innervation. Thoracolumbar and sacral inputs. The afferent endings of the spine are spread across the entire intestine and travel via the splanchnic, lumbar colonic and hypogastric nerves to the thoracic and lumbar spinal cord and via the pelvic nerves and sacral plexus (innervating the distal colon and rectum, bladder and reproductive organs) to the sacral cord [21, 36].

  With the exception of the input to the sacral cord, the visceral afferents are located across, but do not synapse in the pre- and para-vertebral ganglia. Both, thoracolumbar and sacral fibers enter the spinal cord through the DRG and project mainly into the superficial part of laminae I-II, and to the deeper laminae V-VII and X of the DH of spinal cord [15, 37, 38, 39] where they converge with fibers from other organs and somatic inputs [29,

39, 40]. Second-order neurons project to the brain through the spinoreticular, spinomesencephalic, spinohypothalamic, spinoparabrachial and STT [18]. From these tracts, information reaches the emotional and behavioral control areas (including the hypothalamus, locus coeruleus, amygdala and PAG), and the SSC (somatosensory I/II lateral pain system, ACC and the insula) via the sensory nuclei of the thalamus [18, 25, 41]. See Figure 1.3.

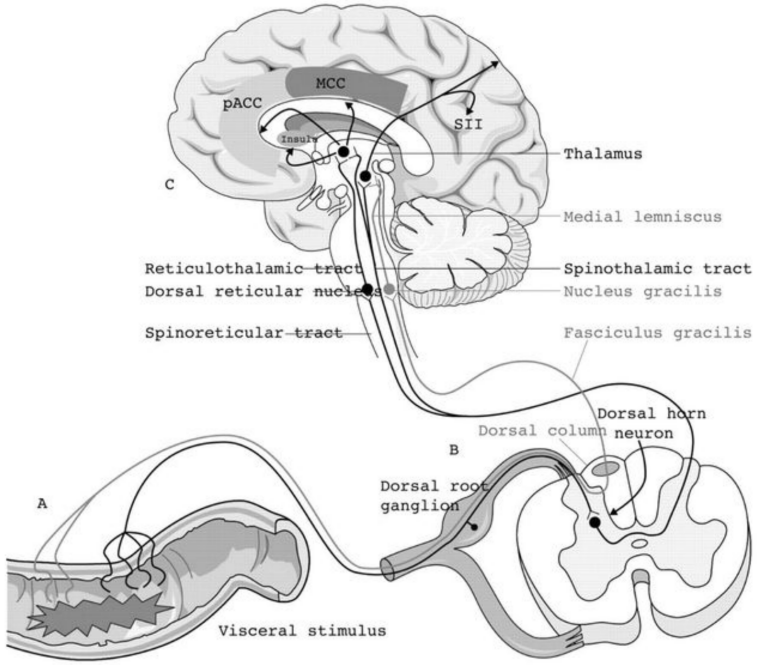

Figure 1.3: Main connections of the gastrointestinal pain pathways (except the spinomesencephalic and spinohypothalamic vias) to the central nervous system. Perigenual anterior cingulated cortex (pACC); mid-cingulate cortex (MCC). Reproduced from [42].

1.2.2.2 Enteric nervous system

The ENS is the extensive system of neurons located in the wall of the GI tract, gallbladder, and pancreas. The autonomously active ENS controls motility, gastric and mucosal secretion and absorption, mucosal growth, local blood flow and immune function in the gut [19, 43]. Modulation of these functions occurs through integration with extrinsic innervation reflexes [19, 34].

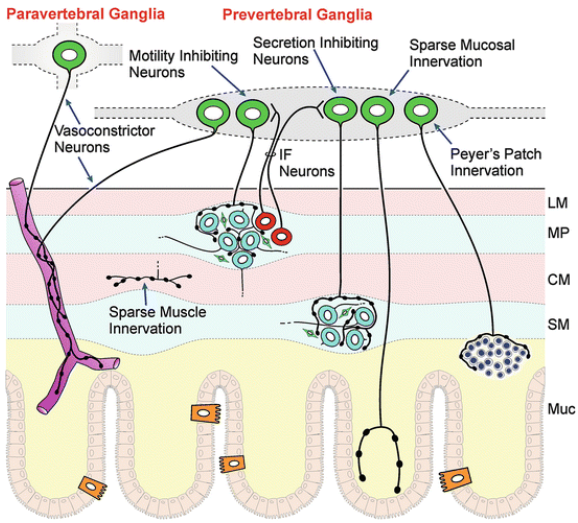

Figure 1.4: Sympathetic innervation of the gastrointestinal tract. Reproduced from [44].

The ENS contains many of the neurotransmitters and neuromodulators found in the CNS. It is structured in specific circuits of sensory neurons, inter and motor neurons grouped in ganglia (intrinsic primary afferent neurons (IPANs)) forming plexuses.

Branched in various layers of the lower GI tract, 82% of spinal afferent nerve endings respond to mechanical stimuli. Attending to a structural division, gut innervation can be classified as (schematic view in Figure 1.4) :

i) Mucosa. Type II neurons. In addition to responding to mechanical stimuli, they are sensitive to enteroendocrine cell mediators.

ii) Submucosa. Type III neurons form Meissner's plexus. They are mainly mechanically responsive.

iii) Circular and longitudinal muscle. The muscle layer contains intramuscular or type IV afferents. They also respond mainly to mechanical stimulation. The myenteric ganglia (Auerbach's plexus) are located between the circular and smooth muscle. They are mostly intra-ganglionic laminar or varicose (IGLEs or IGVEs) or type I endings. Their position may provide a structural basis for potential bidirectional communication between enteric nerves and extrinsic spinal afferents.

iv) Serosa. Blood vessels have vascular or V-type afferents, which are also sensitive to mechanical stimuli, and are modulated by chemical mediators such as capsaicin and those released during ischaemia and hypoxia [15, 20, 39, 45, 46, 47].

1.2.3 Neurophysiology of visceral pain

The precise neuropathophysiology of visceral pain is still far from being clarified, in contrast to somatic pain. This disorder may be associated with a multi-level dysregulation of the gut-brain axis.

This involves neuronal circuits from the CNS/PNS and ENS. Nociceptors are known to undergo regulation and/or modification of their functions, resulting in aberrant signalling of visceral nociception. Pain sensations from the gut are thought to be mediated by afferent impulses transmitted to the thoracolumbar spinal cord. Pain mediators released by nociceptors reduce the transduction threshold of a series of cation channels expressed in the peripheral terminals of Aδ and C-fibers. As a result, the pharmaceutical industry is increasingly focused on exploring possible specific targets for the treatment of this form of pain. [16, 48, 49].

In addition to neural circuit activity, immune signals and emotional descending spinal pathways including the hypothalamic-pituitary-adrenal neuroendocrine axis appear to play important roles in the neurophysiology of visceral pain [16, 26, 29, 35, 39].

1.2.3.1 Neural circuits

The main source of GI nociception is known to be visceral afferents arising from the mesentery. These are bare nerve endings with thinly or unmyelinated axon, pseudo-unipolar with cell bodies at the DRG and synapses in the DH [25].

Peripheral receptor activation:

Direct activation of peripheral nociceptor terminals by opening of the voltage-gated Na^{2+} channel (VGSC) (a.k.a., Na_v) triggers potential generators by depolarizing stimuli and ends up with voltage inactivation of these channels and

opening of the voltage-gated K^+ channel (VGKC) (a.k.a., K_v) [50], see Figure 1.5, step 1. Both of them determine the excitability of sensory neurons [51]. Of particular importance is $K_v1.4$ channel, the only subtype of K_v1 that is expressed in small-diameter DRG neurons and is therefore responsible for K^+ conduction in A-δ and C fibers [52].

The control of pain occurs at the neuronal level via voltage-gated Ca^{2+} channel (VGCC) (a.k.a., Ca_v channels) signalling, but is a complex and heterogeneous process. On the one hand, in the DH, Ca_v channels control neurotransmitter release, and their blockade results in reduced neurotransmission and thus pain relief. But elsewhere, especially in the periphery, inhibition of Ca_v channels results in inhibition of Ca^{2+}-activated K^{2+} channels. These channels control subsequent hyperpolarisation, so their inhibition increases membrane excitability and firing frequency, leading to the opposite result. Ca^{2+} also activates a number of second messengers [53, 54].

Secondary activation of transduction channels in response to noxious stimuli requires the expression of ion channels that are capable of responding to a high threshold of particular changes in the mechanical [2], chemical [3], and thermal [4] environment [51, 52, 55, 56, 57, 58], Figure 1.5 step 2.

The key ion channels involved in visceral pain are (see Figure 1.6 for a schematic distribution):

i) TRPs are thermo- and mechano- receptors found on afferents from the DRG, NG, and the CNS [59]. Among them, TRPV1 is a non-selective cation channel with high permeability for Ca^{2+}, is activated by capsaicin and its analogues, lipids, resiniferatoxin, endocannabinoids and acidosis; and plays an important role in visceral hypersensitivity and inflammation [17, 51, 60]. TRPV1 allows the inflow of cations in a non-selective way, and are activated by various stimuli, these range from high temperatures to irritant components to changes in both intra- and extracellular pH [61]. TRPA1 is an excitatory Ca^{2+} channel directly activated by formalin [62] or by low doses of mustard oil [63].

[2] transient receptor potential ankyrin subtype 1 (TRPA1), Piezo1/2, P2X3, and acid sensing ion channels (ASICs).

[3] transient receptor potential ion channel for vanilloid 1 (TRPV1), P2X3 and ASICs.

[4] cold: transient receptor potential melastatin 8 (TRPM8); and heat: TRPV1-2.

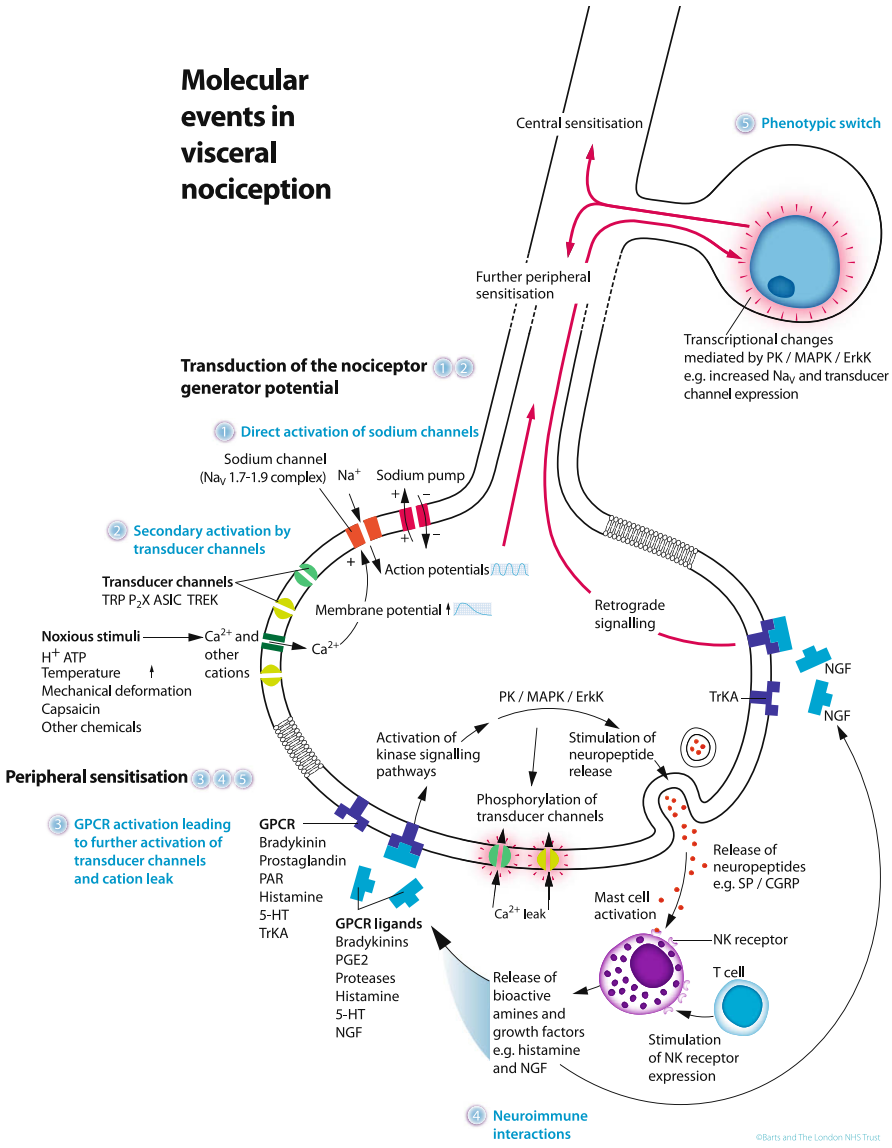

Figure 1.5: Molecular basis of peripheral visceral nociceptive bias before (steps 1 and 2) and after (steps 3, 4 and 5) sensitization. Reproduced from [25].

ii) N-methyl- D aspartate (NMDA) are essential for determining the incoming signal received during synapses. Sustained glutamate release triggers

the activation of NMDA receptors which, in addition to transporting Na^+, also transport Ca^{2+}. This increase in intracellular Ca^{2+} levels leads to cell death and failure of Ca^{2+} homeostasis, which contribute to neurodegeneration and are also implicated in chronic diseases (reviewed by [56]).

iii) ASICs are Na^+ selective and are expressed in human enteric neurons in the intestine [64]. They are involved in mechanosensation [57] and chemonociception [60] and may play a role in the perception of acidosis-induced pain in humans [64].

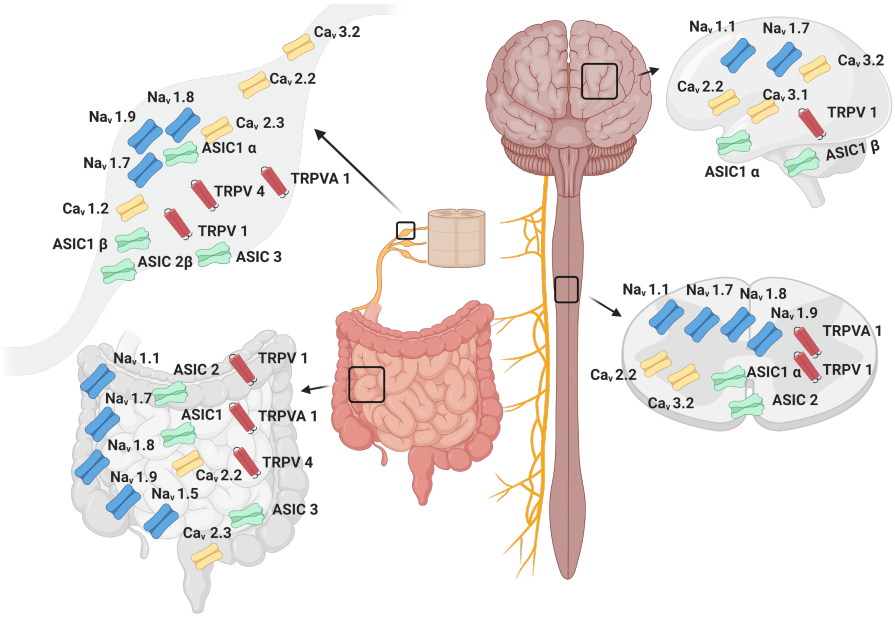

Figure 1.6: Distribution of the main ionic channels involved in visceral pain along the gut-brain axis. They are located in the ENS, in the primary afferent spinal and vagal fibers, in the intermediate spinal cord, as well as in the brain stem and higher brain areas where they participate in the modulation of visceral pain perception.

SENSITIZATION is the result of increased excitability and synaptic facilitation (temporal, spatial, and threshold changes in sensitivity), leading to the development of hypersensitivity [54], Figure 1.5 step 3.

Peripheral sensitization is a form of nociceptor plasticity caused by longer stimulation and leads to a change in the chemical environment of the nociceptor. This allows activation of nociceptors at lower thresholds than those required for an acute noxious stimulus and leads to a lowering of pain thresholds [25].

Central sensitization is ultimately caused by plasticity in DH neurons, the first point of integration of somatosensory information. DH neurons respond to activity, inflammation and neural injury, and are a key region where plasticity has been demonstrated. Several events occur in the DH that account for central sensitization. These include primary afferent inputs, interneurons, projection neurons and downward modulation from the brain [65]. Sensory neurons respond to a wide variety of mediators. The three major neuronal components regulating DH neurons are: i) glutamate, the main excitatory neurotransmitter of primary activating afferent inputs that allows the activation of NMDA receptors[5]; ii) gamma amino butyric acid (GABA), for local inhibitory DH interneurons; and iii) noradrenaline for supraspinal descending inhibitory modulation of the DH.

1.2.3.2 Immune signals

Sensitization can be further enhanced by a series of interactions with surrounding cells (keratinocytes and immune cells), Figure 1.5 step 3. Mediators responsible for the immune response include kinins (bradykinin) [67], nitric oxide [68]; prostanoids (e.g., prostaglandin E_2), biogenic amines (histamine and 5- hydroxytryptamine (5-HT)), chemokines, growth factors (e.g., nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF)), neuropeptides such as substance P (SP) and calcitonin gene related peptide (CGRP), proteases, lipids, endothelins, cannabinoids and opioids, among others. In addition, sensory neurons can respond to shifts in pH and adenosine triphosphate (ATP) [40, 41], which act directly to increase sensitivity to mechanical and chemical stimuli, or indirectly by binding a number of specific G-protein coupled receptors (GPCRs) in the nociceptor membrane. Through transcriptional alterations in nocicep-

[5] And to a limited extent of AMPA and kainite as ionotropic and mGlu1 as metabotropic glutamate receptors) [66].

tors, mediators promote phenotypic changes conducted by different molecular effectors including protein kinase A/C (PKA/C), Ca^{2+}/ CalModulin (Ca^{2+}/CaM)-dependent protein kinase II (CaMKII), and ERK1/2 kinases. These enzymes control the threshold, activation kinetics and membrane trafficking of the receptor by enhancing the response to presynaptic neurotransmitter release. Reviewed by [40, 69], among others.

1.2.3.3 Emotional descending pathways

As in somatic pain, it is known that human visceral pain can be modulated by other types of non-harmful neurons, as well as by non-neuronal factors. So much so that negative emotions have been linked to unpleasant visceral sensations for many years [70, 71]. The response to a distressing factor is driven by a network of integrative structures that can inhibit or facilitate depending on the nature of visceral stimulus [25, 72].

First, there are descending spinal pathways comprising cortical structures (ACC), subcortical regions (PAG and amygdala), ventromedial medulla and dorsolateral pontine tegmentum, and spinal cord. The latter selectively modulates nociceptive transmission due to its anatomical proximity to primary afferent nociceptor terminals and DH neurons that respond to noxious stimulation [25].

The last component of the emotional motor system is the hypothalamic-pituitary-adrenal neuroendocrine axis. Following activation of the cortical (where the ACC is prominent) and subcortical (hypothalamus, amygdala, and PAG) regions, corticotropin-releasing hormone (CRH) is discharged, inducing the release of adrenocorticotropin hormone (ACTH) from the pituitary gland, which in turn triggers the release of glucocorticoids from the adrenal cortex and catecholamines (adrenaline and noradrenaline) from adrenal medulla cells. This modulates enteric neuronal and intestinal immunocyte activity [25].

1.2.4 Animal models of visceral pain

For preclinical and translational models of visceral pain, animal studies are the standard of choice because only whole animal physiology can approximate clinical pathology and the effects of therapeutics. Care must be taken when interpreting the effects of a therapy when evaluated in a single animal model, as it should not be considered representative of an entire phenotype and each ani-

mal species has its own strengths and weaknesses that will affect the relevance of translation. Rodents (mice, rats, guinea pigs) have a GI tract and ENS very similar to humans. Large numbers of individuals as well as genetically modified strains are available to assess specific disease mechanisms.

- IBS is a complex and heterogeneous disorder characterized by different peripheral and central pathophysiological mechanisms behind the symptoms in various subsets of patients [73]. In clinical practice, the disorder is considered to have no underlying structural or biochemical explanation. In the case of rodent studies, most experiments can accelerate colonic transit to induce diarrhoea, but there are few models for induced constipation as well as for the mixed gut response seen in some IBS patients. In addition, disorders that are often associated with IBS, such as anxiety and depression, are difficult to assess in animal models, and often require more animals in each experiment, as well as multiple tests to study behavior. Finally, most of the studies conducted have used male animals, although the disease occurs predominantly in females. The issue of sex differences within models has not been given much consideration, although this seems to be changing [74]. Nevertheless, many animal models have been developed that alter visceral sensitivity and may be useful in understanding different aspects of IBS aetiology. These include limited nesting, maternal separation, odor attachment learning, neonatal colonic irritation, water restriction and avoidance stress, brain or spinal cord manipulation, enemas (acetic acid, butyrate, capsaicin, mustard oil or zymosan) and post-inflammatory hypersensitivity (reviewed by [35]).

- IBD, also known as Crohn's disease and ulcerative colitis, is a complex, multifactorial, immune-mediated disorder of the GI tract characterized by chronic inflammation. Although the exact aetiopathogenesis remains unknown, recent studies have linked genetic (more than 150 genes have been identified as involved), environmental (such as smoking, diet, pollution, stress and sleep) and immunological factors, as well as the involvement of the gut microbiome. A prerequisite in animal models of IBD is transmural inflammation, although this is usually limited to the colon and may not be homogeneous. The colitis-inducing agents in the study of this disease are dextran sulfate sodium (DSS), 2,4,6-trinitrobenzene-sulfonic acid (TNBS) and some human pathogens [75].

- BPS includes the heterogeneous spectrum of painful interstitial cystitis. BPS presents with severe supra-pubic/pelvic pain together with increased urination frequency and urgency. It manifests with cystoscopic abnormalities such as petechial hemorrhages or ulcers and loss of urothelium [76]. No effective treatment has yet been found and multiple therapeutic options are often necessary to achieve symptom control [77]. Animal models have been useful in investigating and evaluating the mechanisms underlying the symptoms associated with lower urinary tract inflammation [78]. These include the cyclophosphamide-induced cystitis model in mice, which produces several histopathological alterations in the urinary bladder (including oedema and hemorrhage [79]).

1.2.4.1 Pain-related behavioral markers

The most important phenomenon related to visceral pain are the following:

- Hyperalgesia. Primary hyperalgesia is described as a peripheral sensitization of primary sensory afferents innervating the viscera, as described in the previous section 1.2.3.1. It is due to the lowering of the threshold of high-threshold receptors and the stimulation of silent receptors. In behavioral experiments, it occurs when stimulated by overdistention and/or after application of irritants (mustard oil, turpentine), algogens (capsaicin, ATP, NGF) or inflammatory substances (5-HT, histamine, prostaglandin E_2) [29, 39].

- Referred somatic pain occurs when visceral pain develops as pain at somatic sites and is due to the involvement of convergent viscero-somatic inputs to the same spinal sensory neurons. Related to referred hyperalgesia, these may persist after the primary stimuli have ceased [29, 40, 80].

- Viscero-visceral hyperalgesia (cross-organ sensitization). Similar to the referred hyperalgesia, it consists of an increase in pain due to second order neurons receiving convergent inputs from two different internal organs that share at least part of their afferent circuitry [29, 40, 80].

- Hyperexcitability of ascending spinal neurons receiving inputs from nociceptors. This leads to neuroplastic changes in the CNS. While the development of visceral pain is considered an important defensive mechanism, the development of hypersensitivity represents a major clinical prob-

lem [29, 51]. These neuroplastic changes in the CNS in turn lead to aberrant central processing of descending pathways that modulate spinal nociceptive transmission [16, 29].

1.2.5 Visceral pain management

1.2.5.1 General overview

Acute pain is a protective mechanism against potential environmental hazard, however chronic pain does not have that protective function and this is why chronic pain should be considered a real disease state by itself. Central sensitization plays a role in the maintenance of visceral pain [81]. As result, uncontrolled acute visceral pain is likely to lead to central neuroplasticity and chronic pain despite resolution of the underlying cause of the pain.

There are several options for the treatment of acute visceral pain, but these therapies are generally not effective in relieving chronic pain because the mechanisms associated with both of them are different. It is unlikely that a single analgesic or targeted agent will significantly reduce most ailments, and this is why combinations of analgesics are commonly used. In recent years, however, considerable efforts have been made at preclinical and clinical level to find an effective treatment.

Visceral pain includes quite different disorders in terms of its physio-pathology. According to Rome Foundation [82], the main disorders are classified as:

1. Structural GI disorders (e.g., IBD). They are classified in terms of the morphology of the organs and the approach to a disease is the pathology of the organs.
2. Disorders of motility (e.g., intestinal pseudo-obstruction), classified in terms of organ function.
3. Functional GI disorders (FGIDs), (e.g., IBS). Related to the interpretation and feedback of a patient's experience of a disease, they are classified primarily in terms of symptoms.

Between the bowel disorders, two of the most common are:

- IBD. A typical structural disorder. It is difficult to discover a curative therapy for this sickness. The therapeutic objectives are to attain the clinical

remission together with the healing of the mucosa, to avoid complications such as surgeries and to improve the quality of life, using different classes of drugs such as (see Appendix A, Table 1 and Table 2): anti-inflammatory agents[6], opioids, antidepressants [7], anticonvulsants [8], antispasmodics, immunosuppressants, and antibiotics [9].

- IBS. Contrary to other organic GI diseases, where treatments are often developed on the basis of pathophysiology, IBS treatment is often selected individually and focuses on the predominant or most problematic symptom experienced by the patient (mainly related to secretion and motility). Therefore, the development of the effective treatments remain dissatisfied, especially for the pain component. Among the most recommended pharmacological treatments are (see Appendix A, Table 1 and Table 2): antispasmodics, secretagogues, anti-inflammatory agents, drugs acting on opioid or serotonin receptors, and TCAs [10].

Anyway, none of them have been shown to change the long-term nature of the disease and have been associated with unsatisfactory results in terms of both pain control and side effects, like addiction, analgesic tolerance and constipation [16, 84]. Other recommended treatment include lifestyle changes: e.g., dietary elimination or modifications and exercise and alternative therapies (pre- and probiotics) [85].

In the most common case where a single treatment is ineffective and/or its side effects make it unfeasible, augmentation treatment can be considered. Augmentation treatment takes place by adding a central with a peripheral or two central agents as positive synergistic effects have been seen with drugs that have complementary effects of action. Between the augmentation treatment highlights the addition to TCAs of atypical antipsychotics, azapirones, δ ligand agents or atypical antidepressants.

[6] Paracetamol, corticosteroids and the non-steroidal anti-inflammatory drugs (NSAID).
[7] Tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs) and atypical antidepressants.
[8] δ-ligand agents.
[9] It is crucial to control this aspect as it can lead to an increase in the antibiotic resistance of the intestinal flora [83].
[10] In the first line treatment, SSRIs, SNRIs and tetracyclic antidepressants.

1.2.5.2 Alternatives and novel therapeutic agents

Whatever the type of intestinal disorder, patients with increasing acceptance by medical professionals, tend to seek complementary and alternative medicine based on modulating intestinal microbiota through:

1. Nutraceuticals, among which are prebiotics, polysaccharides, phytochemicals and also remains in the exploratory phase medical cannabis use [86].
2. Mind-microbe balance, interrupted by psychological changes such as stress).
3. Dietetic management, where B and D vitamins are included, as well as the introduction of restrictive diets in certain foods.
4. Microbiome-therapy, which involves the removal or addition of a specific group of bacterial species to restore the host's microbial homeostasis, the use of probiotics and, more recently, the transplantation of faecal microbes.
5. And others, e.g., acupuncture, meditation and hypnotherapy. For a more in-depth analysis, see the reviews from [87, 88, 89].

Novel therapeutic agents include compounds which alter gut-brain pathways and local neuroimmune pathways, among others (see Appendix A, Table 3). Rome IV [90] introduced pharmacogenomics (for the selection of an optimal neuromodulator based on the variability of the expression of individual genes) as a potential new tool for diagnosis and management of patients with disorders of gut-brain interaction.

Experimental animal models for visceral pain, however, provide a simpler scenario that allows researchers to test experimental therapies without interference, even though it is not certain that this treatment is effective when the scenario becomes more complex.

1.3 The Opioid system

Opioid receptors are widely distributed throughout the body [91, 92]. Those related to pain modulation are expressed by:

i) Central and peripheral neurons (in pain-modulating descending pathways, i.e. in medulla, locus coeruleus, PAG and limbic, midbrain, and cortical structures).

ii) Neuroendocrine cells (pituitary, adrenal).

iii) Immune and ectodermal cells.

iv) Gut of rodents and humans: pacemaker and smooth muscle cells [93], where they directly inhibit neurons, which in turn inhibit spinal cord pain transmission [94, 95, 96, 97].

Exogenous and endogenous opioids produce their physiological effects mainly through the activation of the μ-opioid receptor (MOR), δ-opioid receptor (DOR) and κ-opioid receptor (KOR), belonging to the GPCR family [96]. The effects of opioids on the gut have been known for centuries to treat diarrhoea and pain. Their distribution in the intestine varies between regions, layers and species [84, 98, 99]. Enteric neurons also release many of the same transmitters and neuropeptides (endogenous opioids e.g., endorphins, enkephalins and dynorphins) as the brain. This explains why exogenous opioid analgesics inhibit GI function [96].

Several systems are involved in the mode of action of opioids:

1. After the binding of a ligand, a conformational change occurs that promotes the coupling of the trimeric G protein complex to the C-terminal end of the receptor. At the Gα subunit, guanosine triphosphate (GTP) replaces guanosine diphosphate (GDP) and dissociation of the G protein complex into Gα and G$\beta\gamma$ subunits, inhibiting the production of adenylcyclases (ACs) and cyclic adenosine monophosphate (cAMP), interacting directly with different ion channels in the membrane, and/or selective activation of one or another kinase dependent pathway (PKA/C) [100].

2. Reduced excitability of neurons is due to opioid modulation of ionic conductance:

    a) Opioid receptors can modulate pre- and post-synaptic Ca_v channels, suppress Ca^{2+} influx which attenuates the neuronal excitability and/or reduce the release of pro-nociceptive neuropeptides [101].

    b) Furthermore, the triggering of the receptors results in the opening of G-protein coupled inwardly rectifying K^+ (GIRK) channels, hence preventing neuronal excitation and/or propagation of action potentials [100, 102]. Several μ-opioid agonists increase K^+ conductance [103] that results in membrane hyper-polarization thus preventing action potential generation.

c) Opioids also inhibit Na_v channels. Studies in isolated myenteric neurons have shown that morphine inhibits tetrodotoxin resistant (TTX-R) Na_v channels on neuronal cell bodies [104, 105].

d) They also can modulate TRPV1 and ASICs channels in DRG neurons, as well as glutamate receptors in the spinal cord [106, 107].

All these events contribute to the decrease of the activity of the neurons and a deep reduction in pain perception when are stimulated by opioid agonists [94, 108].

3. It has also been shown that opioid receptors can interact with other second messenger systems. For example, opioid receptor agonists may recruit β arrestin which results in decoupling of the G-protein from the receptor. This mechanism is called biased agonism and could explain some of the differences in the effects on behavior of agonists acting on μ-opioid receptors, including those related to abuse, pain relief, production of dependence and respiratory depressants effects [109].

As a pharmacological treatment, the agonists used (mainly morphine, fentanyl, oxycodone and methadone), preferably bind to MORs (reviewed in [108]).

In the case of morphine, the most widely μ-opioid used, it is known that in the afferent system, morphine interacts at the spinal level with opioid receptors found at the endings of the primary sensitive fibers. From there, morphine penetrates into the posterior horn of the spinal cord, as well as into dendrites and somas of the spinothalamic neurons of laminae I and V, decreasing the activity of the spinothalamic pathway. In the midbrain and diencephalon, it depresses afferent activity in the PAG, periventricular gray and the intralaminar nuclei of the thalamus (structures that are part of the spinorreticular and spinomesencephalic pathways).

In the efferent system, the activation of opioid receptors mainly located in the midbrain and bulb causes the activation of a neuronal system inhibiting nociceptive transmission.

Morphine also acts at the limbic and cortical levels, where there are abundant opioid receptors. In this way, the opioid not only suppresses or reduces painful sensitivity but also attenuates the perception of the unpleasant or distressing

tone of pain, sometimes even replacing it with a feeling of well-being or pleasure.

Other effects of morphine are: i) respiratory depression (bulbar nuclei of the respiratory centre); ii) neuroendocrine actions (stimulates the secretion of ACTH, somatotropin, prolactin, β-melanocyte stimulating hormone (β-MSH) and antidiuretic hormone (ADH) and inhibits the secretion of thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and folicle-stimulating hormone (FSH) by acting on the hypothalamus and hypophysis); iii) hypothermia (hypothalamic origin); iv) myosis (due to the disinhibiting action of the Edinger-Westphal nucleus in the oculomotor system); v) muscular hypertonia; vi) bradycardia (vagal origin); vii) hypotension (due to the release of histamine); viii) vasodilation; and ix) and increased intracranial tension (due to increased CO_2 pressure).

At a strictly GI level, it causes: i) nausea; ii) vomiting; iii) increased myogenic tone; and iv) inhibition of neurogenic activity. They are due to a delay of gastric emptying, constipation and increased pressure in the bile ducts by both central and peripheral actions [110]. Central actions are carried out by receptors at the spinal and supra-spinal level, while peripheral ones occur at the level of the myenteric plexus, where morphine inhibits the release of neurotransmitters involved in the local reflections of the GI wall.

In a same class of opioid receptors, differences in affinity and function can be distinguished. This is the case of the opioid receptor μ1, which has a greater affinity for morphine, acts as a mediator of supra-spinal analgesia and is selectively blocked by the opioid μ antagonist naloxone; and the receptor μ2 has a lower affinity for morphine but plays a more important role in mediating spinal analgesia and respiratory depression.

This variety of receptor characteristics is linked to the capacity of opioid receptors to homo- (may lead to signal amplification) or hetero- (modulates the ligand binding profile of both receptors) -oligomerize in various combinations, which results in the generation of new "opioid receptors" with unique affinities of agonists and/or antagonists [111, 112]. This latter hetero-oligomeric composition makes it possible to separate the desired effects of opioids (analgesia) from some of their undesirable effects, such as tolerance, constipation and addiction [97]. Evidence documenting GPRCs form hetero-dimeric (DOR/MOR) complexes in the gut is not available. Nevertheless, both receptors are expressed in the ENS. MOR or DOR ligands can bind to the heteromeric receptor complex to activate

it individually, but the binding of a DOR antagonist will increase the activity of the agonists at the MOR binding site [113].

1.3.1 Opioid drugs

According to the opioid drug intrinsic activity, they can be classified as:

1.3.1.1 Pure agonists

The commonly available drugs in this group includes morphine, oxycodone, fentanyl, and their derivatives with systemic site of action; and loperamide as the prototype peripheral neuromodulator.

A. *Morphine*. As explained in the previous section 1.3, morphine is the prototype drug most commonly used for therapeutic purposes, and is characterized by activating with great affinity and potency the MORs along the neuroaxis. Analgesia is the most important therapeutic property and keeps strict relationship with dosage. Serves to alleviate or suppress severe pain, both acute and chronic, whatever its location [110].

B. *Oxycodone*. There is some controversy regarding the analgesic effect of oxycodone. It is believed that the central antinociceptive effect of oxycodone (and its active metabolites oxymorphone and noroxymorphone) is mainly through its interaction with the MOR, while the peripheral effects occurs through interaction mainly with KORs, but also with MORs, and possibly DORs in peripheral tissue [114]. Interestingly, a study of human volunteers found that oxycodone significantly blocked visceral pain better than morphine which has little KOR activity [115].

C. *Fentanyl* is 50 to 150 times more potent than morphine and has low cardiotoxicity, which makes it the drug of choice for modern opioid anaesthesia techniques in cardiovascular surgery and intensive care units and more often to control acute and chronic pain. Through the spinal cord, its high liposolubility facilitates rapid penetration into the medulla, where it reaches high concentrations, but also the exit is faster, as well as the escape of the opioid into the medullary, perimedullary and peridural blood vessels. For all these reasons, analgesia is fast and deep, but less long-lasting (maximum: 1-4 hours) than morphine [110].

D. *Loperamide* is a MOR agonist (no activity of DORs or KORs) [116] used to treat occasional episodes of diarrhoea and some IBS patients with diarrhoea as their predominant symptom due to its capacity to inhibit GI peristalsis and secretion [117, 118]. Loperamide has oral bioavailability and blood-brain barrier permeability limited [17].

1.3.1.2 Partial agonists

A. *Buprenorphine* is a partial agonist with effects such as euphoria or respiratory depression weaker than those of complete opioid agonists such as heroin and methadone.

B. *ORP-101* is a buprenorphine dimer that is an agonist in MORs and an antagonist in KORs, and an example of the new experimental therapeutic approach for visceral pain [119].

1.3.1.3 Agonists/Antagonists

A. *Eluxadoline*, known as μ/δ in the earlier literature, is a MOR and KOR agonist and DOR antagonist [120] located in the ENS and recently approved for the treatment of diarrhoea-predominant IBS [121, 122]. Preclinical studies showed that the actions of eluxadoline were restricted to the GI tract (limited systemic bioavailability after oral administration) and the beneficial effects may be related to biased signalling due to the mixed MOR agonist/DOR antagonist properties of the drug [118, 123].

1.3.1.4 Agonists with additional mechanisms

A. *Tramadol* has a weak-moderate affinity for opioid receptors (its analgesic action is moderate and partially antagonized by naloxone). This drug inhibits the reuptake of both serotonin and noradrenalin mediated by inhibition of 5-HT and noradrenaline [124]. Tramadol has a very low potential for abuse and respiratory depression [125] but it does have GI side effects.

1.3.1.5 Antagonists

A. *Naloxone* is a potent and very selective antagonist of opioid receptors (mostly μ). Naloxone blocks central (crosses the blood-brain barrier) and peripheral (including the enteric nervous system) sites of action of opioid drugs [123].

B. A naloxone derivate, *naloxegol* (a substrate for the blood-brain barrier p-glycoprotein transporter and together with its large molecular weight limits its naloxegol penetration across the blood-brain barrier), demonstrate in clinical trials the ability to control morphine-induced constipation while maintaining analgesic effects [126], so naloxegol is approved for treatment of opioid induced constipation especially in non-cancer pain patients [118, 127].

C. *Naloxone methiodide* is another naloxone analogue that impedes their access to the CNS. Widely used in preclinical studies [128, 129].

D. *Methylnaltrexone* is a naltrexone analogue with a quaternary amine group that is positively charged, limiting its blood-brain barrier permeability (can block peripheral MOR without affecting centrally mediated analgesia) (reviewed in [17, 118, 130]).

E. *Naldemedine* also has a structure similar to that of naltrexone (lateral chain added to increase its molecular weight and polar surface) [131]. It also has limited passage of the blood-brain barrier.

1.3.2 Complications of opioid treatment. Side effects.

The clinical utility of opioid receptor agonists for the treatment of pain continues to be limited by a compromise between efficacy and side effects. According to the site of action, they can be divided into peripheral and central side effects. The most common peripheral side effects includes those related with the GI system (i.e., increase of sphincter contraction and gastroesophageal reflux, and decrease in gastric motility, intestinal secretion and peristaltic waves in the colon) and cardiovascular system (hypotension).

Central side effects include nausea and vomiting, psychotomimetic disturbances, confusion, sedation, increased urinary retention, pruritus and rigidity of the trunk, and decreased respiratory rate.

GI peripheral side effects of opioids include constipation. In addition to a central component, constipation is mainly induced at opioid receptors located in the ENS whose activation causes inhibition of GI motility, intestinal secretion and sphincter contraction.

Stimulation of the vomiting chemoreceptor centre in the postrema area induces nausea and vomiting, central side effects that, together with constipation, can impair patients' quality of life.

They may be severe enough to contribute to under-dosing and inadequate analgesia, even leading to the discontinuation of treatment [94, 132].

At the clinical level, there are two strategies that have been suggested to minimize the adverse effects of opioids. On the one hand, an adjuvant drug (e.g., a NSAID or an anticonvulsant drug) with synergistic analgesic effects is administered in order to reduce the opiate dose and thus its potential side effects, whilst maintaining analgesic equivalency. On the other hand, it has also been shown that the peripheral antinociceptive capacity of opiates reduce undesirable effects mediated by the central system. This is because stimulation of peripheral opioid receptors means that analgesic activity occurs without activation of central opioid receptors and therefore CNS-mediated side effects (i.e., respiratory depression, mental cloudiness, altered consciousness or addiction) would not develop [96, 129, 133].

1.3.2.1 Tolerance

Tolerance to opioids, a loss of analgesic potency that leads to an increase in the required dose of opioids, occurs in two ways:

1. Innate, genetically determined and usually present in the early stages of treatment.

2. Acquired, dependent on learning (due to compensatory mechanisms) and/or pharmacokinetic (due to changes in the metabolism of a drug after repeated administration) and pharmacodynamic (related to up-regulation of receptors) parameters.

They can exacerbate the perpetual problem of the side effects mentioned above [94, 132].

Agonist binding to μ-opioid receptors can result in the activation of multiple downstream pathways. Generally, they can be divided into two ways:

a) G protein-dependent processes. Include the regulation of ion channels (Ca^{2+}, K^{+} and Na^{+}), inhibition of AC, and/or selective activation of PKA/C. In this case, the proposed mechanism of antinociceptive tolerance is through desensitization and down-regulation of the functional receptors present in the target neurons.

b) G protein-independent processes. Includes the steps leading to endocytosis and interactions with scaffolding molecules and kinases. Phosphorylation of MOR by G-protein receptor kinases results in binding of β arrestin2. The recruitment of β arrestin2 is known to follow activation of additional receptor signalling pathways that promote internalization of receptors by endocytosis. The receptors can then be de-phosphorylated and recycled on the cell surface to become useful again, but if opioid exposure is continuous, endocytosis can lead to receptor degradation and is one of the basic pathways for the development of tolerance (reviewed in [134, 135]).

However, it is known that different agonists behave differently with respect to these receptor desensitization mechanisms. An example of this is morphine, which induces a minimal internalization of the receptor, as opposed to [D-Ala^2, N-MePhe^4, Gly-Ol] - enkephalin (DAMGO), in which it is very pronounced [136].

In the mechanism of tolerance, in addition to changes in the opioid receptor, changes in second messengers have also been shown to occur. Such is the case of the enhanced expression of CGRP and SP via protein kinase activation throughout extracellular signal-regulated kinase (ERK)/ mitogen activated protein kinases (MAPKs), or the enhanced released of excitatory neurotrasmitters (including CGRP, SP, and glutamate) via the nitric oxide (NO) - NMDA pathway [94, 132, 134, 135, 136, 137].

1.3.2.2 Dependence

Physical dependence refers to the need to use opioids to maintain normal function. It is responsible for the opioid withdrawal symptoms seen when the dose is quickly reduced [138]. Physical dependence is due to a situation of hyperactivity or hyper-excitability of various brain nuclei caused by the permanent action of

the opioid. Following the abrupt cessation or rapid dose reduction of an opioid, molecular phenomena can be observed in these hyper-activated neurons, to counteract the acute action of the opioid and thus also increase tolerance: increased activity of G-proteins and AC (with the consequent increase in cAMP), phosphorylation of proteins by kinases and formation of genes of immediate action (c-fos, c-jun, etc.), facilitation of Na^+ outflows and Ca^{2+} inflows with increased bioelectrical activity [110].

Withdrawal symptoms include somatic (e.g., bone pain, muscular spasms, changes in body temperature, hyperalgesia, insomnia, hypertension and tachycardia) and affective (anxiety, irritability and emotional pain, among others) symptoms, with type and severity experienced varying widely [139].

1.4 Voltage-gated sodium channels

1.4.1 General overview

The Na_v channel family initiate action potentials and regulate the excitability of the neuron [140].

These channels are widely distributed in the nervous system (CNS and PNS), immune cells and colonic (enterochromaffin and smooth muscle) cells in humans and other species (review by [141]).

The Na_v channels are categorized according to their sensitivity to TTX, a potent neurotoxin. Most of these Na^+ channels ($Na_v1.1$ to $Na_v1.4$ and $Na_v1.6$ to $Na_v1.7$ isoforms) are blocked by nanomolar concentrations of TTX and are defined as TTX-sensitive (TTX-S) Na_v channels, while others ($Na_v1.5$ and $Na_v1.8$ - $Na_v1.9$) require micromolar concentrations and are defined as TTX-R Na_v channels [142]. Schematic view in Figure 1.7.

In humans, $Na_v1.1$ to $Na_v1.3$ and $Na_v1.6$ isoforms are predominant in the CNS; and $Na_v1.7$ to $Na_v1.9$ are preferentially expressed in the PNS [143]. The remaining two are located in muscle, with $Na_v1.4$ predominating in skeletal muscle and $Na_v1.5$ in the heart [144].

Numerous studies have shown the effects of TTX as antinociceptive and pain reliever, both in visceral and somatic pain through the inhibition of Na^+ ion flux (reviewed in [145]). They have linked Na_v channelopathies as the primary

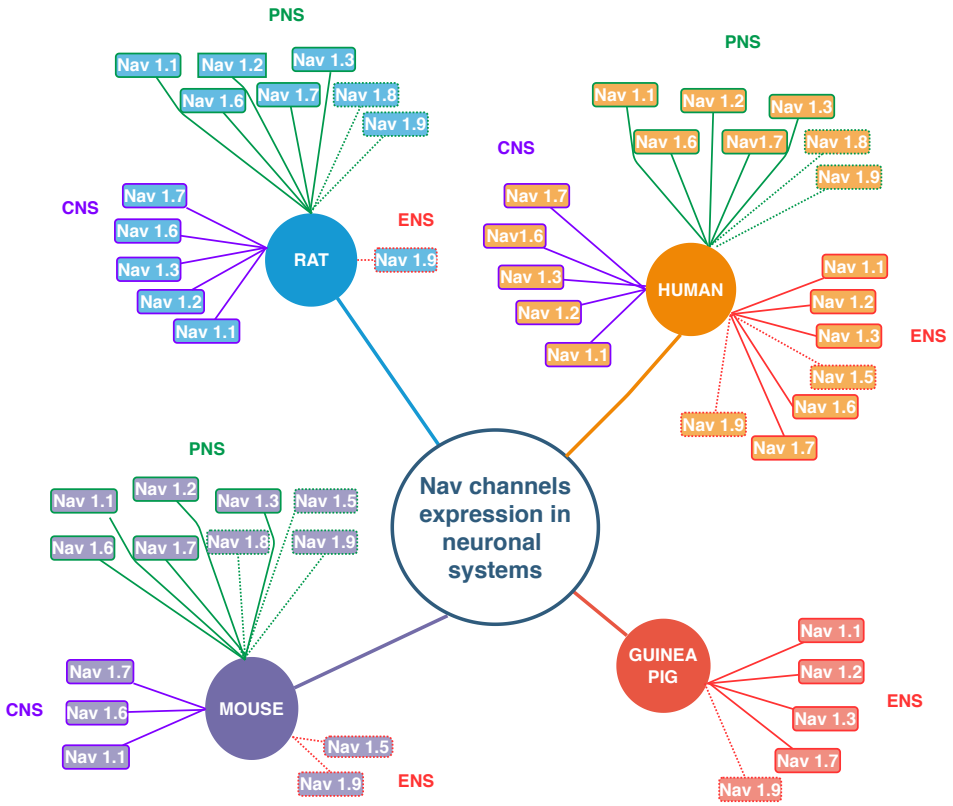

Figure 1.7: Na_v channels expression in neuronal systems (central nervous system (CNS), peripheral nervous system (PNS) and enteric nervous system (ENS)) in different species (human, rat, mouse and guinea pig) differentiating between tetrodotoxin sensitive (TTX-S) and tetrodotoxin resistant (TTX-R) (dotted line).

cause of pain control problems in humans, especially for the peripheral $Na_v1.7$ to $Na_v1.9$. (review by [141]). One example is the administration of Na_v selective agents in humans, where the response to various types of pain, including visceral pain, is decreased (e.g., TTX [146] and neosaxitoxin, a blocker of TTX-S Na_v channels- [147].

Similar results have been found in rodent animal models of inflammatory and neuropathic pain using TTX [148].

1.4.2 Na_v expression in visceral organs

Recent studies reveal the diversity of Na_v isoform expression in visceral organs, ranging from organ-specific to neuronal cells (see Figure 1.8). Among them, $Na_v1.1$, $Na_v1.6$, $Na_v1.8$ and $Na_v1.9$ highlights to contribute to visceral hypersensitivity. Despite the high expression of $Na_v1.2$ and $Na_v1.5$ mRNA in the viscera, they have been related to visceral functions and not to afferent pain pathways. $Na_v1.3$ and $Na_v1.4$ have not yet been shown to be related to visceral pain. For $Na_v1.7$, it has not yet been associated with visceral pain as has been done with somatic pain. ([141, 149]).

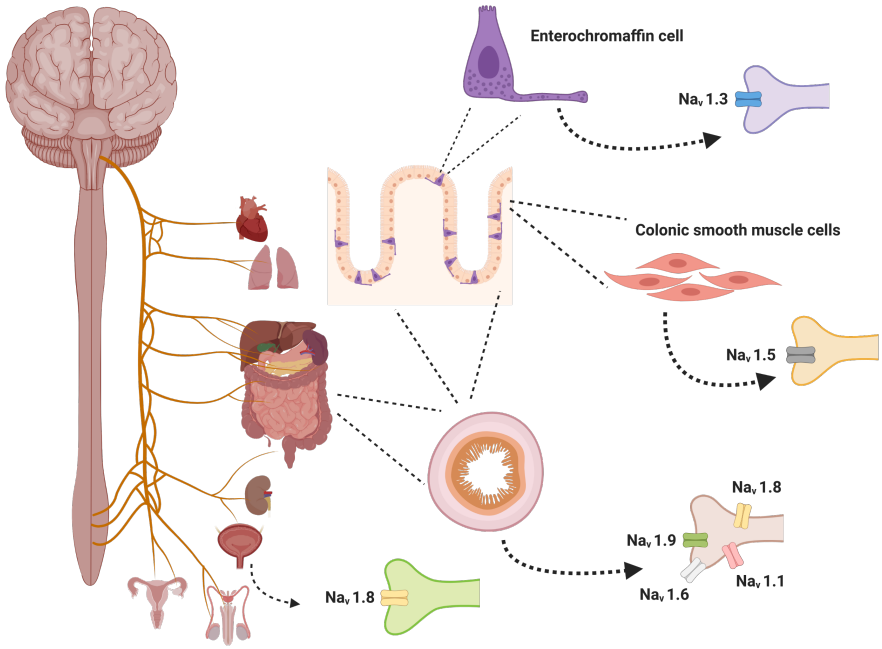

Figure 1.8: Diagram of the specific Na_v channels in both neurons and non-neuronal cells within the visceral organs.

- *$Na_v1.1$.* Predominantly expressed in several areas of the CNS (cell bodies, axon initial segments and the nodes of Ranvier), is also expressed in PNS (in humans L3-L5, in mice T10-L1 and L5, and in rats L4-L5 DRG neurons which innervates the colon, rectum, bladder and skin; and myenteric plexus [150]). When compared with skin innervation studies it can be seen that there are differences in the expression of this channel. In skin, they

are located primarily in large A-fibers and are almost absent in C-fibers. In contrast, in the colon they appear mainly in C-fibers. There are still no studies linking the $Na_v1.1$ to sensory bladder neurons.

- *$Na_v1.6$.* Is located in roughly the same areas of the CNS and PNS as the $Na_v1.1$ (but mainly located at the nodes of Ranvier). The predominance in the nodes of Ranvier suggests that they play a role in the transmission of the nerve impulse [149]. There are still no studies linking the $Na_v1.1$ to sensory bladder neurons.

- *$Na_v1.8$.* Is the most abundant isoform in the mouse lumbar DRGs neurons [143] where co-expresses with $Na_v1.7$ [149]. It is believed to mediate pain sensations under both physiological [11] and pathological conditions [12].

  In the case of cyclophosphamide induced cystitis, $Na_v1.8$-null mice develop normal pain and inflammatory responses [151]; and pain behaviors are maintained in rats after administration of a $Na_v1.8$ antagonists. It is also interesting to know the mechanisms of visceral connection (cross-organ sensitization), where a relationship of the TTX-R channels have been found in bladder pain after inflammation of the GI tract.

- *$Na_v1.9$.* This peripheral expressed channel controls neuronal excitability by bringing the resting membrane potential closer to the threshold. Unlike the previous, null mice $Na_v1.9$ shows normal sensitivity to acute noxious colonic distension but reduced visceral hypersensitivity under pathophysiological conditions. The role of this channel in the bladder is still not clearly understood [141].

Among the isoforms of the Na_v channel related to pain, $Na_v1.7$ has been extensively studied in recent years and stands out due to the relationship in humans of several mutations in the gene SCN9A. This gene encodes the α subunit and the appearance of phenotypes due to:

1. Gain of function. Erythromelalgia, small-fiber neuropathy and paroxysmal extreme pain disorder (originally called "familial rectal pain syndrome").

[11] Null mice $Na_v1.8$ shows normal sensitivity to acute noxious colonic distension [151].

[12] Visceral hypersensitivity models show increased expression of $Na_v1.8$ in sensory DRG neurons that innervate the colon. Visceromotor responses are reversed with $Na_v1.8$ antagonists, and null mice do not develop hypersensitivity after intracolonic (i.cl.) administration of chemical stimulants (reviewed by [141]).

2. Loss of function. Congenital insensitivity to pain, defined as the inability to feel pain in the absence of other sensory impairments).

3. Polymorphism. Found in a subset of patients with interstitial cystitis/bladder pain syndrome. Reviewed by [141].

It is believed that pharmacological inhibition could prevent or treat a wide variety of types of pain. Coupled this with its extensive expression in the PNS, the selective inhibition of $Na_v1.7$ represents a potentially new analgesic strategy that is expected to be devoid of the skills associated with current treatments. In addition, it is possible to maintain high levels of efficacy and security [152].

1.4.3 TTX as a therapeutic strategy for visceral pain

TTX is a powerful neurotoxin found in puffer fish and other marine animals which use it as a defence against predators. [153]. As described above, section 1.4.1, TTX selectively blocks some Na_v channels and has therefore been widely used as a pharmacological approach in a wide range of physiological and pathophysiological processes in the nervous system [154, 155]. TTX appears to act by stabilizing neuronal membranes by inhibiting the influx of Na^+ ions required for the initiation and propagation of nociceptive impulses, especially in those pain conditions where up-regulation of TTX-S Na_v channels occurs in the periphery of the nervous system [156].

It has been reported that TTX has analgesic and antihyperalgesic effects in several somatic pain conditions, including nociceptive [148], inflammatory [148, 157, 158], muscle [159], and neuropathic [148, 160, 161, 162] pain models. In addition, TTX has been tested in humans in several clinical trials for counteracting cancer-related pain and patients with chemotherapy-induced neuropathic pain [146, 163, 164], but the contribution of TTX-S Na_v channels to visceral pain has never been investigated in a pure visceral pain model.

Since the $Na_v1.7$ is the only one of the TTX-S channel and at the same time located mainly in the PNS [143] and ENS [150] in humans [143], one of the goals of this Thesis was to study the antinociceptive effects of TTX and the possible involvement ot the $Na_v1.7$ in different visceral pain models in mice as a potential strategy for the treatment of visceral pain.

1.5 Sigma-1 receptor

1.5.1 General overview

Nowadays, the sigma-1 receptor (σ1R) is considered a non-opioid intracellular chaperone with 223 amino acids that comprise one transmembrane domain [165] and high homology between species [166] [13].

With the absence of a GPCR structure [169], σ1Rs have no precedent, homology and are functionally different from other known mammalian proteins [170]. σ1R s are considered a new molecular entity distinct to any other known protein, and recently have been proposed as a pluripotent modulator in the living system [171]. At the cellular level, σ1R has been located as highly clustered globular structures enriched in cholesterol and neutral lipids at the endoplasmic reticulum (ER)-mitochondrion interface called mitochondrion associated ER membrane (MAM) [172]. The σ1R is widely distributed in various tissues including heart, liver, testis, spleen, GI tract, retina, immune cells, as well as the nervous system (see [53, 173] for reviews) and cancer cells [174].

The story of σ1R knowledge begins in 1976 when σRs were first classified as a subclass of opioid receptors based on the N- allylnormetazocine (SKF-10047) and other benzomorphans [175] actions.

In 1989, two subtypes of σRs were identified, σ1R and σ2R [176]. The molecular entity and structure were totally unclear until 1996 when σ1R was first cloned [167]. The σ2R has taken 2 more decades to be cloned. Initially classified as the progesterone receptor membrane component 1 (PGRMC1) [177], is TMEM97, an ER-resident transmembrane protein implicated in cancer and a binding partner of Niemann-Pick disease protein NPC1 [178]. From there, very useful tools have been developed to deepen the knowledge of σ1R, highlighting the design of antisense oligodeoxynucleotides [179, 180, 181] and the development of σ1 receptor Knockout (σ1R-KO) mice [182].

The σ1R is considered a potential therapeutic target for behaviors related to neurological (neuropsychiatric and neurodegenerative) disorders, stroke, sub-

[13] Although it has always been considered as a two-membrane domain protein by in vitro approach [167, 168], Schmidt and cols. [165] recently published the first crystal structure of the full-length human σ1R where they reported σ1R as a one transmembrane domain protein. This suggests that the *in vitro* crystal structure does not accurately represent the *in vivo* structure of σ1R, which may indicate that the σ1R has an amorphous structure that is inherently disordered *in vivo*.

stance addiction, retinal disease, amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease, cancer and nociception (reviewed in [179, 183, 184, 185, 186, 187, 188, 189, 190]). Currently, σ1R ligands are in clinical trials for the treatment of chemotherapy-induced neuropathic pain [191], Alzheimer's disease (an extension study of ANAVEX2-73 in patients with mild to moderate Alzheimer's Disease), and ischaemic stroke [192].

1.5.2 Functions

The ER is a multifunctional organelle with an important role in protein synthesis, folding and translation as well as cellular homeostasis (Ca^{2+} homeostasis and oxidative/ nitrosactive stress response). The MAMs are important for multiple aspects of normal mitochondrial and cellular functions. MAM maintains lipid synthesis and trafficking, Ca^{2+} homeostasis, and regulation of mitochondrial-dependent apoptosis [186].

Under physiological conditions, newly synthesized proteins are translocated into the ER lumen, where they are folded into proper conformations by aid of several molecular chaperones [193], and a majority of those ER chaperones also serve to store ER Ca^{2+} [194]. However, under different perturbations such as Ca^{2+} deregulation or stress, the ER accumulates unfolded proteins and transmits signals. This initiates a stress response known as unfolded protein response through an up-regulation of chaperones to facilitate protein refolding and control the ER response [194, 195]. Schematic view in Figure 1.9.

From a functional point of view, σ1Rs are considered as ligand-regulated molecular chaperones that regulate the stability and function (protein folding/ degradation) of specific signalling molecules. One of the associated problems with the σ1R is precisely the number of intracellular partners it has, and therefore the cellular pathways it can affect after being activated.

σ1R physically interacts with a variety of proteins including receptors, ion channels, other chaperones or elements involved in gene expression [172, 202, 203, 204, 205, 206] (see next section 1.5.3), for ultimately engage cellular functions such as:

i) Cell survival (growth and cell death/apoptosis).

ii) Neuronal firing and differentiation.

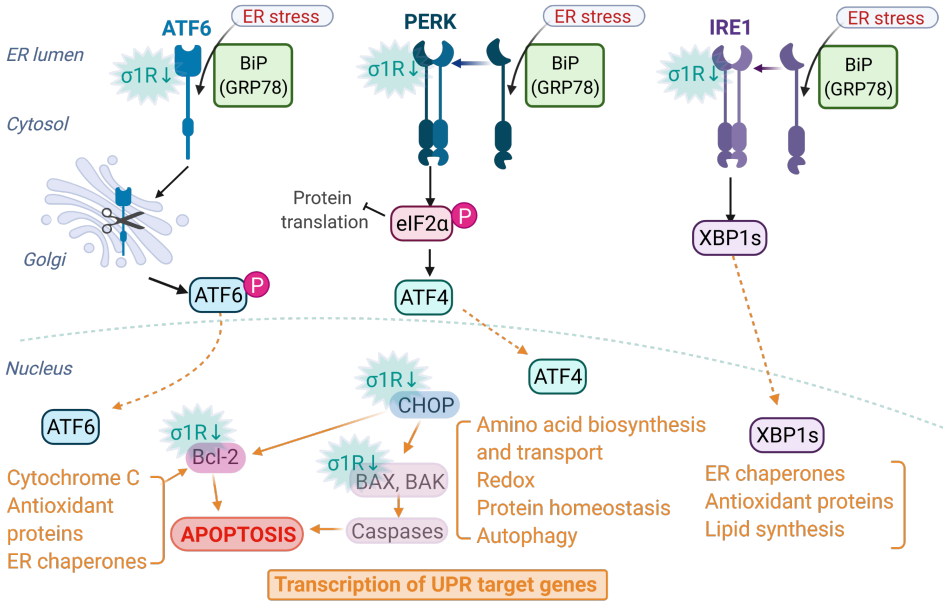

Figure 1.9: The stress response. Under normal conditions, BiP/GRP78 binds to the three ER-stress sensors (PERK [196], IRE1 [197] and ATF6 [198] (reviewed by [195]), but under ER-stress BiP dissociates from sensors. PERK and IRE1α are phosphorylated and oligomerized, ATF6 translocates to the Golgi to be activated. In the central area of the drawing is represented how throughout the phosphorylation of eIF2α by the stress sensor PERK, the protein translation is reduced, thus leading to a decrease of protein synthesis [196, 199]. To the contrary, the activated eIF2α can increase the translation of ATF4 which translocates to the nucleus to induce the expression of several genes involved in pro-apoptotic response (CHOP, BAX, BAK, and Bcl-2 among others, related to amino acid metabolism and transport, protection against oxidative stress, protein homeostasis, and autophagy) [200, 201]. At both ends of the picture, the activation of IRE1 (right) leads to the splicing of XBP1 and jointly to the phosphorylated ATF6 (left), translocate into the nucleus to promote the transcription of target proteins involved in the adaptative process to restore ER homeostasis (ER chaperones, antioxidant proteins or enzymes, and Bcl-2) [197, 198]. σ1Rs activation modulates the activity and/or levels of PERK, ATF6 and IRE1α; decreases pro-apoptotic response throughout CHOP, BAX, and caspases; and increases anti-apoptotic response by dint of Bcl-2. (PERK, protein kinase RNA like ER-kinase; IRE1, inositol requiring enzyme 1α; ATF6, activating transcription factor; eIF2α, eukaryotic translation initiation factor 2α; ATF4, transcription factor 4).

iii) Gene expression (and cancer), (see [207] for a review).

iv) ER stress (free radical damage).

v) Bioenergetics.

These cellular functions are carried out through two main intracellular functions:

i) σ1R modulates the intracellular Ca^{2+} signal [172]. σ1R stabilizes inositol 1,4,5- trisphosphate receptors (IP_3Rs) at the MAM [207] and associates with STIM1 in the ER; reducing the binding of STIM1 to Orai1, and inhibiting the store-operated Ca^{2+} entry [208]).

ii) σ1R regulates the stress response. On one side, σ1R activates the antioxidant response elements via NAD(P)H quinone oxidoreductase 1 and superoxide dismutase [209]. On the other side, σ1R suppresses the apoptosis caused by ER stress [210]. Moreover, σ1R stabilizes the ER stress sensor IRE1 [207] and suppresses reactive oxygen species (ROS) generation [204]). Figure 1.9.

   The σ1R is also involved, among others, in the regulation of K_v [186, 211], and the release of various neurotransmitters such as acetylcholine and glutamate [212].

σ1R is regulated by ligands in an agonist/antagonist manner (reviewed by [171]). It is the C-terminal of the σ1R which are supposed to be linking sites for ligands [165, 213]. σ1Rs endogenous ligands include steroids [214], sphingolipids [215], dimethyltryptamine [216], and myristic acid [217], but although these substances show affinity for σ1Rs, they are not selective for it.

As exogenous ligands, σ1Rs binds with a wide spectrum of drugs with different pharmacological applications, including, among others, the treatment of neurodegenerative disorders (e.g., donepezil), antidepressants (e.g., fluvoxamine), antipsychotics (e.g., haloperidol), antitussives (e.g., carbetapentane), Ca^{2+} channel blockers (e.g., verapamil), antihistamines (e.g., chlorpheniramine), drugs of abuse (e.g., cocaine) and some opioids (e.g., pentazocine) (reviewed by [189, 218]).

In addition to those, other compounds have been designed specifically to bind σ1Rs and are extensively employed to study the σ1R function, with the PRE-084 standing out as an agonist and BD-1047, BD-1063, NE-100 and S1RA (a.k.a.,

E-52862 and MR309) as antagonists [218, 219]. The latest is in Phase II of a clinical trial showing efficacy in reducing chemotherapy-induced neuropathic pain in patients treated for colorectal cancer [191].

As a pluripotent modulator in the living systems, the unique nature of the σ1R ligands lies in their mode of action: ligands of σ1R have been proposed to have no effect by themselves and/or in healthy conditions, but normalize physiological or behavioral functions of other systems since under pathophysiological conditions σ1R target proteins become conformationally unstable and need the chaperone aid of σ1R [189, 211, 220, 221].

1.5.3 σ1 receptor interacting proteins

MAMs/ER σ1Rs can, upon agonist stimulation, translocate from the MAM to the plasma membrane and nuclear envelope [222]. At the plasma membrane, σ1Rs interact with and/or affect the function of many other proteins [223], and at the nuclear envelope σ1Rs recruit chromatin-remodeling factors to regulate the gene transcription. Furthermore, σ1Rs can interact with other partners in other parts of the cell such as cytosol and other organelle membranes [171]. Schematic view in Figure 1.10.

1. *σ1R at the ER-MAM-mitochondria.* It has been demonstrated that σ1R forms oligomers (dimers and/or monomers at the ER membrane) that creates what has been called the σ1Rs ligand-binding pocket [165] and σ1Rs ligands tend to cause oligomer dissociation and stabilization (agonists and antagonists, respectively) [224, 225]. In its dormant state, σ1R forms a homo-oligomer with BiP (an ER chaperone) [172]. Upon binding σ1R agonists or the lowering of the local Ca^{2+} concentration such as the efflux of Ca^{2+} from IP_3R, it happens the homo-oligomers destabilization and σ1R translocate to other parts of the cell to interact with and regulate the function of other proteins thus exercising its functions [172, 226, 227]. See Appendix A, Table 4.

2. *σ1R at the nucleus.* The nuclear membrane is in lipidic continuation with the ER membrane, so it is considered to form a continuous network [171]. There are old and more recent studies that confirm the presence of σ1R en the nuclear envelope. One of them, based on protein co-localization, sites σ1R and sterol isomerase co-located in ER and nuclear envelopes [228]. The other, based on electron microscopy, identified the precise subcellu-

lar localization of the σ1R, detected not only in the ER, but also in the nuclear envelope [229].This corroborates the σ1R as a transcriptional regulator again. See Appendix A, Table 5.

3. *σ1R at the plasma membrane.* Although most studies are based on the assumption that σ1R forms physical interactions with these proteins and regulates their activities in the plasma membrane itself, they have not yet been determined by immunoprecipitation or Western Blotting due to the lack of a high affinity σ1R antibody and control IgG interference (because IgG has the same molecular weight as σ1R). This is the reason why most of these studies have used over-expression techniques of both σ1R and target proteins, so there may be an over-saturation and aberrant localization of proteins. Electron microscopy studies have shown that σ1R could be located in the proximity of the plasma membrane and that σ1R can interact with these proteins due to this proximity [185, 229]. See Appendix A, Table 6.

Cellular biology studies continue to add to the list of the σ1R chaperone associating partners and precise its intracellular functions.

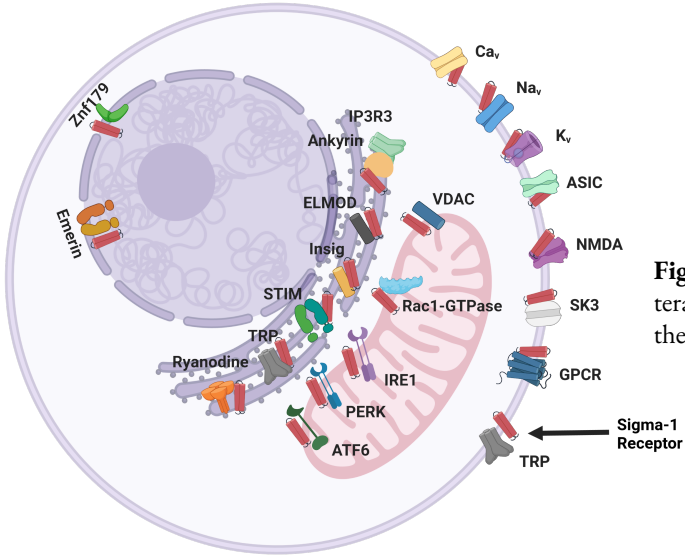

Figure 1.10: σ1R interacting proteins at the cell.

1.6 Sigma-1 receptor and pain

Although its structure as an opioid receptor has been ruled out, σ1R is related to the opioid system as described by Chien and Pasternak [230] at the end of the last century. It exerts a tonic anti-opioid effect [179] and modulates the sensitization induced by nociceptive stimuli in a wide range of pain-sensitive conditions [53, 218, 227, 231]. Some σ1R ligands have been shown to enhance the anti-nociceptive effects of certain commercial μ-opioids (such as morphine, fentanyl, oxycodone, codeine, buprenorphine and tramadol) [179, 230, 231, 232], without increasing the side effects associated with the use of them.

At the therapeutic level, new analgesics that act through different mechanisms of action than the current ones are needed, and thus would help to increase the efficacy of these therapies or reduce their side effects. Preclinical evidence for a modulatory role of the σ1R on pain of different aetiology makes it one of the most promising pharmacological target for this role. σ1R antagonists, in the absence of sensitizing stimuli, do not exert antinociceptive effects, nor do they modify normal mechanical and thermal sensory perception as do opioids. For a further study see [53, 233].

In relation to pain transmission, σ1R expression is widely reported in peripheral organs and in several areas of the CNS. In the PNS, σ1R expression is roughly one order of magnitude higher than in several areas of the CNS involved in pain signalling and is located in the soma of all peripheral sensory neurons in the DRG [185, 232, 234], but with a non-homogeneous distribution. Recent work published by our group [235] shows that expression levels of the σ1Rs were much higher in IB4$^+$ neurons (which mainly encode mechanical nociception) than in the rest of the small nociceptive neurons of the DRG. In the CNS, the σ1Rs are located in areas specialized in processing nociceptive signalling, memory and emotion, such as the spinal cord DH, thalamus, PAG, basolateral amygdala and RVM, among others [223, 236] (see [53, 173] for reviews).

1.6.1 σ1 receptor regulates neuronal activity.

As described briefly in a previous section 1.2.3.1, the course of information transmission through the neuronal network is managed by constant interactions between synapses and cellular excitability factors to control action potential generation, conduction along the axon, neurotransmitter release and post-synaptic

receptor sensitivity, and depends on both pre-synaptic neurotransmitter release and the functions of postsynaptic ionotropic receptors. It has been shown that the σ1R directly associates with and regulates primary sentinel transducers as well as secondary channels in the neuronal response to a stimulus, thus making the σ1R a pluripotent regulator of neuronal activity and sensitization [141, 237].

A) *Presynaptic* neurotransmitters release. σ1Rs, through heteromeric complexes, modulate several pre -synaptic metabotropic receptors that play a role in pre-synaptic glutamate release. On the one hand, σ1Rs associated with dopamine 1 [238] or serotonin [239] receptors promote presynaptic glutamate release in the rat prelimbic cortex, and on the other hand, the histamine 3 receptor and dopamine 1 receptor can heterodimerize through direct binding. σ1R activation also regulates dopamine 1-histamine 3 receptors signalling [240].

B) Most studies have shown that the σ1R regulates excitatory *post-synaptic* transmission, which depends mainly on the activation of ionotropic glutamate receptors and the modulation of some post-synaptic channels.

- **NMDARs**. The σ1R ligands regulates phosphorylation of NMDAR. In this regard, σ1R agonists increase and σ1R antagonists/genetic blockade [241] decrease NMDAR currents and Ca^{2+} flow through the channel. Differential alteration of the tridimensional structure upon binding of ligands has been suggested to modulate the affinity of σ1Rs for GluN1 and GluN2A/B subunits binding [242, 243, 244, 245] [14], HINT1 and Ca^{2+}/CaM complex [15].

- **SKs**. The mechanism by which the σ1R enhances NMDAR currents is not yet fully known, but another approach may be through preventing small conductance SK channels to open [246].

- **GPCRs**. Several GPCRs (involved in pain such as the cannabinoid CB1 and MOR) are associated with NMDARs by a dynamic process under the control of the histidine triad nucleotide-binding protein 1 (HINT1)-σ1R [245, 247].

[14] NMDAR-GluN1-σ1R interaction is a Ca^{2+} dependent binding that also competes with other regulators of NMDAR function.

[15] Both HINT1 and Ca^{2+}/CaM are negative regulators of NMDAR function and establish a weaker link than the NMDAR-GluN1-σ1R interaction.

- **TRPs**. It has been demonstrated [248] recently that σ1R associates to TRPV1 in a direct protein-protein interaction and this interaction regulates the membrane concentration of TRPV1. Therefore, σ1R antagonists can regulate downwards the number of TRPV1 channels without affecting the transcription of them. In this study, they explain the pathway by which σ1R reduces pain produced by capsaicin activated TRPV1 channels. In the absence of σ1R antagonists, the σ1R can positively regulate the expression of the TRPV1 protein in the plasma membrane. This occurs via the Golgi apparatus where TRPV1, after interacting with σ1R, is hyperglycated and then transported to the plasma membrane. σ1R antagonists promote the association of σ1R with BiP, leading to the inactivation of σ1R, and eventually to the degradation of TRPV1 via the proteasome, which in turn leads to decreased levels of expression of TRPV1 that resulted in diminished capsaicin-evoked TRPV1 currents and capsaicin-induced pain [249]. This protein-protein association is most prominent at the ER compartment and occurs less at the level of the plasma membrane.

- **ASICs**. σ1R agonists decrease ASIC1a induced Ca^{2+} flows [250] while antagonists (BD-1047) co-administered with ASIC blockers (amiloride) reduced ischaemic pain induced- mechanical allodynia, suggesting that σ1R activation facilitates pain via ASIC [251].

- **K_v channels**. σ1R has been proposed to be considered a ligand-regulated auxiliary K^+ channel subunit [202]. Its association with client proteins is dynamic, so unlike typical auxiliary subunits, σ1R is not believed to be stably associated with pore-forming subunits present in purified channel complexes [252], nor is it a protein found only at the plasma membrane level [253].

- **Ca_v channels**. As mentioned above, N-type $Ca_v2.2$ is the predominant synaptic Ca_v channel. It has been shown that σ1R agonists (SKF-10047 and pentazocine) are capable of inhibiting the influx of Ca^{2+} while the antagonists (BD-1047) enhanced the influx and blocked the effect of the agonists [254, 255]. In addition to ion fluxes, it has also been shown that σ1Rs are able to modify certain biophysical properties of these channels such as the acceleration of the inac-

tivation rate and the need for more negative potentials to activate-deactivate the channels [256].

- **Na_v channels**. σ1R interacts with Na_v channels [242] and σ1R agonists probably exert inhibitory effect on the action potential initiation and propagation [205]. See section 1.4 for more detail.

C) *Immune mediators* (inflammatory pain):

- σ1R activation enhances both bradykinin-induced Ca^{2+} signalling in neuronal-like cell cultures and nitric oxide signalling [220, 257, 258].
- Kinases (including ERK1/2) are known to be modulated by σ1R [259, 260, 261].

In vitro sensitization studies

In addition to studies that have provided insight into the relationship of σ1Rs to the modulation of synaptic transmission, the effect of σ1R antagonism on central sensitization is supported by other research (review of [53]). The subsequent increase in pain sensitivity after the initial intense C-fiber activity is thought to be due to increased excitability of DH neurons as a consequence of central sensitization. Therefore, the modulation of σ1R in the amplification of the spinal response has been studied in *isolated spinal cords* (wind-up response). The selective σ1R antagonist S1RA dose-dependently inhibited the spinal wind-up phenomenon when nociceptive afferent C-fibers were repeatedly stimulated [219, 262]. Genetic blockade of the receptor led to the same results [259].

1.6.1.1 Behavioral studies

Behavioral studies have also demonstrated an initial and intense activation of peripheral C-fibers of nociceptors after intradermal exposure of the plantar skin of the mouse hind paw to certain chemical irritants such as capsaicin and formalin. Moreover, the use of different animal models has also contributed to the study of the role of peripheral σ1R.

- *Capsaicin*, a component of hot chilli peppers, acts mainly on the polymodal receptor TRPV1. Capsaicin receptor is present in C-fibers and some Aδ-fibers [263]) and induces mechanical hypersensitivity. Capsaicin was unable to induce mechanical hypersensitivity in σ1R-KO mice, and the

effect in σ1R-KO mice was mimicked in WT animals treated with several *σ1R antagonists* (BD-1063, BD-1047 and NE-100 [264], haloperidol and its metabolites I and II [265], S1RA [219] and some spirocyclic thiophene bioisosteres [266], 10-benzyl- 3-methoxy- 3H-spiro [[2]benzofuran -1,40-piperidine] [267], and a 1,3-dioxane ligand 2 [268]), whilst the *σ1R agonist* PRE-084 reversed the effects of antagonists [264, 265]. This again supports the role played by σ1R in central sensitization phenomena.

- *Formalin* is a formaldehyde solution that activates TRPA1 that is highly expressed by a subset of C-fiber nociceptors [62] and induces acute nociceptive behaviors in the two phases formalin-induced pain mice model. The initial phase is due to the Ca^{2+} inflow through the TRPA1 channels at the nociceptor ends following direct activation of primary afferent sensory neurons in the periphery, and the second is due to central sensitization.

  – There are studies in which both phases of pain were reduced in genetic [269] and pharmacological (haloperidol and its metabolites I and II [270], S1RA [219, 271], and others [272, 273]) σ1R blockade.

  – It has also been shown that intrathecal (BD-1047) [274] or intracerebroventricular (S1RA) [271] σ1R antagonists administration reduced formalin-induced pain behaviors only in the second phase. BD-1047 dose-dependently reduced the second phase concomitant with a reduction of phosphorylation of GluN1 at PKA/C-dependent sites [274]. To the contrary, activation of spinal σ1R by several σ1R agonists (PRE-084, carbetapentane or dehydroepiandrosterone sulfate) facilitated nociception, enhanced NMDA-induced pain behavior, and promoted the phosphorylation of GluN1 (via PKA/C) in the DH [275, 276]. Following paw formalin injection, the activation of afferent glutamatergic nociceptive fibers lead to an enhancement of glutamate levels in the DH spinal cord. Systemic administration of S1RA reduces peripheral activating glutamatergic nociceptive inputs and enhances noradrenergic descending inhibitory inputs to the DH, but it does not modify the activity of GABAergic inhibitory DH interneurons [271].

  These studies suggest that the spinal cord and supraspinal regions of the CNS are sites of modulation of σ1R- mediated formalin sensitization.

- The role of peripheral σ1R with regards to *inflammatory pain* has also been evaluated (reviewed in [277, 278]). Systemic administration of several σ1R antagonists (BD-1063 and S1RA) was effective in the carrageenan pain model and the local administration of a σ1R agonist (PRE-084) abolished the systemic effect of the σ1R antagonists. Furthermore, local administration of S1RA to the inflamed paw was sufficient to reverse the inflammatory hyperalgesia being reversed again with PRE-084, and absent in σ1R-KO mice [279]. With respect to inflammatory pain, it is possible that the mechanism of peripheral modulation by the σ1R is more important than in other pain models, precisely because inflammatory pain has a high peripheral sensitization due to inflammatory mediators released at the site of inflammation [177].

- As mentioned in the previous section 1.6.1, in the rat model of thrombus-induced *ischaemic pain*, a relationship of the σ1R to the modulation of ASICs and P2X receptors has been found. In this study, i.pl. injection of the σ1R antagonist BD-1047 reduced mechanical allodynia synergistically with the ASIC blocker amiloride and the P2X antagonist TNP-ATP [251].

- In *neuropathic pain*, it has been reported that σ1R antagonists have a neuroprotective role against peripheral neuropathy. Research from our group has demonstrated the relationship of σ1R antagonism (pharmacological and genetic blockade) to prevent paclitaxel-induced sensory nerve mitochondrial abnormalities, and prevention of cold/ mechanical allodynia in the paclitaxel-induced model in mice [280]; and neuropathic cold, heat and tactile hypersensitivity in the spared nerve injury model in mice [281]. Conversely, Thomohisa and cols., [282] found antinociceptive effects against chemotherapeutic induced paclitaxel and oxaliplatin neuropathic pain by the σ1R agonist SA4503, but not by the σ1R antagonist NE-100 in the rat's spinal cord[16].

  In rats with chronic constriction injury of the sciatic nerve, the σ1R antagonist BD-1047 blocked the chronic constriction injury-induced increase in NMDAR GluN1 subunit expression and phosphorylation, so significantly attenuated mechanical allodynia; and PRE-084 reverses the effects [275].

[16] These differences in agonist/antagonist effects may be due to different σ1R location or expression levels. In the rat spinal cord, it has been shown that, under neuropathic conditions induced by sciatic constriction, endogenous ligands [283] and σ1R levels [275] increase, but σ1R levels decrease with oxaliplatin, paclitaxel [282] or peripheral nerve injury [234].

1.6.2 σ1 receptor and visceral pain

The role of σ1R in somatic pain control is well documented, but as explained above (see section 1.2.1), the associated symptoms, pathophysiological mechanisms and response to pharmacological treatment of visceral and somatic pain are different. Knowledge about somatic pain cannot be directly extrapolated to the field of visceral pain. Unfortunately, very few studies have linked σ1R to visceral pain.

In this sense, the most important investigations have been carried out by our group, and involve a pure model of visceral pain induced by i.cl. capsaicin [284], and a model of cystitis induced by an intraperitoneal chemotherapeutic agent, cyclophosphamide [285], both conducted in mice. These models are comparable in that they both assess the role of σ1R by measuring two types of responses to abdominal pain: pain-related behaviors (capsaicin)/ spontaneous pain (cyclophosphamide) and mechanical hyperalgesia referred to the painful stimulus in the abdominal wall. Both studies evaluated the behavior of wild-type (WT) mice treated with σ1R antagonists (BD-1063, NE-100 and S1RA) and σ1R-KO mice.

As a particular feature of the models, both i.cl. capsaicin and intraperitoneal (i.p.) cyclophosphamide induce both types of responses in WT and σ1R-KO mice but in different ways.

On the one hand, the number of pain-related behaviors/spontaneous pain, is significantly lower in σ1R-KO mice than in WT mice (pain attenuation in σ1R-KO), in agreement with previous studies by:

- *Chemical sensitization:* phase I and II of i.pl. formalin [219, 269, 271] and mechanical allodynia in i.pl. capsaicin [219, 264] pain induction.
- *Inflammatory pain:* mechanical hyperalgesia in i.pl. carrageenan pain induction [279].
- *Neuropathic pain:* mechanical and cold allodynia both in paclitaxel [280, 286] and partial sciatic nerve ligation [219, 259].

Conversely, referred mechanical hyperalgesia was similar between WT and σ1R-KO animals (pain development similar in WT and σ1R-KO), that may be attributable to the development of compensatory mechanisms in σ1R-KOs mice [287] and in line with other studies:

- *Nociceptive pain:* the tail-flick [259], *von Frey* [264], hot plate [259], paw pressure [129].
- *Inflammatory pain:* mechanical allodynia and thermal hyperalgesia induced by i.pl. carrageenan [279] and complete Freud's adjuvant [288].
- *Neuropathic pain:* thermal hyperalgesia in partial sciatic nerve ligation [219, 259].

Otherwise, σ1R antagonists reduced painful responses of both types in WT mice and were inactive in σ1R-KOs, confirming a σ1R-mediated effect. This is in line with most studies, but differs with nociceptive pain, suggesting that these analgesic actions may depend on the type of pain and the nociceptive stimulus applied. For a more detailed analysis, see [53, 289].

1.6.3 Enhancement of opioid-induced analgesia

At a molecular level, with regard to *μ-opioid analgesia*, the best known mechanism is:

a) Starting from the NMDAR-σ1R-MOR-HINT complex (see Figure 1.11):

- Upon binding of a MOR agonist, it transports HINT1, leading to activation of PKCγ, which phosphorylates NMDARs in the NR1 subunit. Once phosphorylated, NMDARs are released from the MOR-HINT1 complex, and their activity increases (Ca^{2+} levels rise) and thus NMDAR-mediated nociception increases *(MOR activation induces a positive modulation of NMDARs)* (reviewed in [290, 291]).
- This increase in Ca^{2+} levels: (i) activates the Ca^{2+}-CaM complex, which in turn increases CaMKII activity. This acts on the MOR by decreasing its activity *(NMDAR activation induces a negative modulation of the MOR*, opioid analgesia decreases and promotes tolerance) and (ii) promotes the transfer of the σ1R from the MOR to the GluN1 subunit of the activated NMDAR, thus keeping it active as this binding prevents NMDAR to CaM, (reviewed in [290, 291]). (iii) Although to a lesser extent due to the weaker binding, elevated Ca^{2+} levels also promote the binding of the Ca^{2+}- CaM complex to the NMDAR to decrease its activity, constituting a negative feedback mechanism that prevents excessive Ca^{2+} entry into the cytosol [292].

b) In the absence of σ1Rs (in σ1R-KO animals or treatment with a σ1R antagonist)

- Induction of NMDAR by MOR occurs as above, increasing Ca^{2+} levels.
- This increase in Ca^{2+} levels promotes the transfer of σ1R from the NMDAR to the MOR and facilitates the transfer of HINT1 to NMDARs. (iii) In this situation, NMDARs bind negative regulators to NMDARs (Ca^{2+}- CaM) as the main pathway, thereby reducing the function of NMDARs and *inducing positive modulation of MOR* [244, 293].

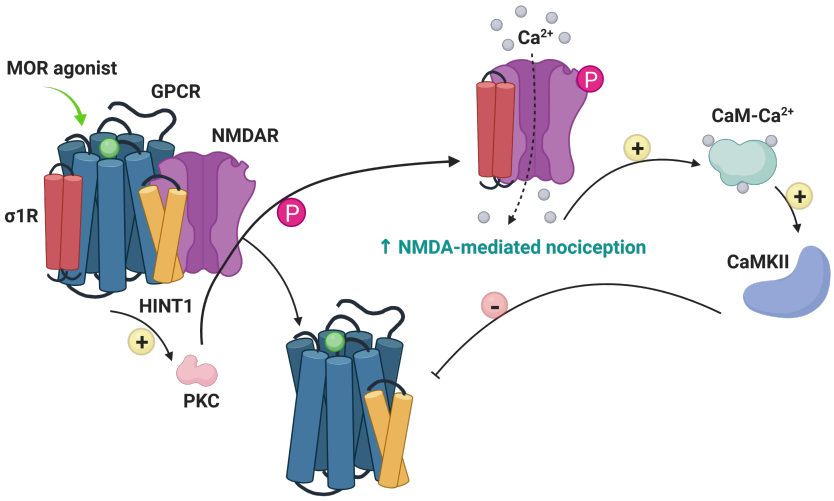

Figure 1.11: NMDAR- σ1R -MOR-HINT complex. The diagram shows the interaction between the components of the complex. After activation of the GPCR component by an agonist, NMDAR is activated through phosphorylation by PKC. The activation of NMDAR promotes Ca^{2+} influx, thus activating the CaM-Ca^{2+} complex, which, through CaMKII, exerts an inhibitory effect on MOR. Modified from [53, 294].

For this reason, *σ1R antagonists* have been proposed to enhance opioid analgesia by releasing the MOR from the negative influence of the NMDAR [206]. This MOR-NMDAR-HINT1-σ1R complex probably occurs in specific subsets of

neurons involved in pain pathways, as differences in the modulation of analgesia and opioid side effects have been described (reviewed by [53, 233, 294]).

In relation to animal behavioral model studies, in terms of modulation of opioid analgesia, there are many studies that support supraspinal sites to exert the modulatory effects on opioid analgesia (reviewed by [221]). The group of Pasternak have identified the PAG, RVM and locus coeruleus as supraspinal sites for σ1R ligands to exert their modulatory effects on opioid analgesia in rats [180].

- The *radiant heat tail-flick* test is the best studied. An enhanced antinociceptive effect of systemic morphine has been proved by S1RA administered intracerebroventricularly, but not intrathecally in rats [271]. The σ1R agonist (+)pentazocine decreased antinociception not only in μ, but also in δ, κ1, and κ3 opioid analgesia in mice [179].
- In the *warm water tail-flick* test, analgesic activity increase when morphine and several σ1R antagonist (S1RA, BD-1047, NE-100 and progesterone) were intracerebroventricular administered in mice. The σ1R agonist PRE-084 did not affect morphine-induced analgesia but did prevent S1RA from enhancing opioid antinociception when also administered intravenously [244].

It is also known that inhibition of peripheral σ1R enhances opioid antinociception.

- In the *paw pressure* test in mice, and in the absence of sensitizing conditions, σ1R antagonists (BD-1063, BD-1047, NE-100 and S1RA) administered systemically or locally showed no effect. Under sensitized conditions, local administration of morphine had no antinociceptive effect either against mechanical stimulation of WT mice, but when morphine and σ1R antagonists were administered jointly and peripherally, the enhancement of opioid analgesia was observed [129]. An extension [232] of the previous study with other opioids (fentanyl, oxycodone, buprenorphine) demonstrates the peripheral nature of the opioid agonist-σ1R antagonist interaction. The study concluded that, on the one hand, the greatest antinociceptive effect of the combination occurs when both are administered locally and the most potent effect is reversed by a peripheral opioid antagonist (naloxone methiodide); and, on the other hand, when a peripheral opioid (loperamide), which is totally ineffective when administered alone, is administered, an antinociceptive effect appears when combined with

S1RA. Obviously, the effect is reversed with the σ1R agonist PRE-084, and the lack of effect of the σ1R antagonists tested in σ1R-KO mice. As a complement to the above, opioid-induced side effects (such as hyperlocomotion and fentanyl- or loperamide- induced inhibition of GI transit) were also studied. The result was that these side effects were not potentiated in σ1R-KO mice or on co-administration of the σ1R antagonist.

- In a study using the *radiant heat tail-flick* test in rats [271], the peripheral opioid agonist loperamide was also found to have no effect on its own. When loperamide was combined with the σ1R antagonist S1RA, an *induction of analgesia* was observed and, again, its effect was reversed by the administration of the peripheral opioid antagonist (naloxone methiodide).

Therefore, since σ1R is known to localize mainly in the PNS and its modulation and enhancement of peripheral opioid analgesia in somatic pain models has been extensively studied, another aim of this Thesis is to study the peripheral modulation of σ1R as a potential therapeutic strategy to reduce opioid dosage and thus avoid its undesirable side effects for the treatment of visceral pain.

2 Rationale and Goals

Visceral pain is a major clinical disorder in humans. Most studies in the field of pain and nociceptors have only focussed on somatic/neuropathic sensory system but visceral pain processing is different from other forms of nociception [17]. The visceral system include multiple ion channels, neurotransmitters and receptors that are qualitatively and/or quantitatively different from those involved in somatic or neuropathic pain and nevertheless have a large number of organs and systems with unique intrinsic and extrinsic innervations. That is why the mechanisms of visceral pain are expected to differ from those of somatic pain [15, 17] and the results obtained in models of cutaneous/somatic pain cannot be extrapolated to visceral pain.

The treatment of visceral pain is complex and the current available pharmacological treatments have limited efficacy, therefore making it necessary to develop effective drugs against this painful condition [17]. The development of animal models of visceral pain is making it possible to investigate the mechanisms involved [295].

Antinociceptive properties of TTX are thought to be due to the stabilization of neuronal membranes through the inhibition of Na^+ ion flux required for initiation and propagation of nociceptive impulses, especially in those pain conditions in which an up-regulation of TTX-sensitive Na_v channels in the PNS takes place [156].

TTX has been shown to have analgesic and antihyperalgesic effects in several somatic pain conditions, including nociceptive [148], inflammatory [148, 157, 158], muscle [159], and neuropathic [148, 160, 162] pain models. However, the contribution of TTX-S Na_v channels to visceral pain has never been investigated in a pure visceral pain model.

The *first goal* of this Thesis was to **evaluate the antinociceptive effects of TTX in three different visceral pain models** in mice: the intracolonic administration of both capsaicin and mustard oil; and a model of cyclophosphamide-induced cystitis.

On the other hand, the σ1R is a small protein that is structurally unrelated to any other known protein in mammals. σ1R has a chaperone domain within its structure [172], which may explain part of its pharmacological properties. It has been reported that the σ1R is also present in the PNS where σ1R are found at a much higher density than in pain-related areas of the CNS.

This receptor has been studied by both genetic (σ1R-KO mice) and pharmacological (σ1R antagonists) blockade in several models of pain [182, 218, 219, 227, 259, 264, 269]. σ1R has also been associated with opioids to synergistically enhance their peripheral antinociceptive effects and avoid undesired side effects [129, 232].

In the field of visceral pain, σ1R were shown to play an important role in the intracolonic administration of capsaicin model in mice [284].

Nonetheless, the involvement of σ1R blockade in the potentiation of opioid-induced analgesia on visceral pain and the modulation of peripheral opioid analgesia in this type of pain remains unclear.

The *second goal* of this Thesis was to **evaluate the potentiation of morphine-induced analgesia by genetic and pharmacological blockade of σ1R and to to assess the modulation of the peripheral μ-opioid analgesia (*per se* or associated to σ1R antagonists) in a pure model of visceral pain**, the intracolonic administration of capsaicin.

Related to the previous goal, the *third goal* of this Thesis was to **corroborate that the enhancement of the morphine-induced analgesia is not specific for this drug and is extensible to other opioids commonly used in clinical practice (oxycodone and fentanyl) and also to study the contribution of peripheral receptors to such analgesia.**

3 Material and Methods

3.1 Animals and drugs

3.1.1 Experimental animals

The experiments were carried out, on the one hand, for studies of Na_v channel blockage in WT C57Bl/6 adult mice of both sexes; conditional $Na_v1.7$ Knockout ($Na_v1.7$-KO) mice and their littermate controls stored on a C57Bl/6 background and generated as described above [296]. On the other hand, in the case of studies with the σ1R, we used female WT CD1 mice (Esteve Pharmaceuticals S.A., Barcelona, Spain) and homozygous σ1R-KO ($\sigma1R^{-/-}$) mice as described previously [264].

In both cases the weights were between 20 and 30 g. All mice were acclimated in our animal facilities for at least 1 week before testing and were housed in a room under controlled environmental conditions: 12/12h day/night cycle, constant temperature (22 ± 2 °C), air replacement every 20 minutes, and they were fed a standard laboratory diet (Harlan Teklad Research Diet, Madison, WI, USA) and tap water *ad libitum* until the beginning of the experiments. Behavioral test was conducted during the light phase (from 9.00h to 15.00h), and randomly throughout the oestrous cycle. They were maintained at the Biomedical Research Centre (University of Granada, Spain).

All experiments were carried out following institutional (Research Ethics Committee of the University of Granada, Spain) and international standards (European Communities Council directive 86/609). Each animal was used only once and received a single concentration of algogen (or its vehicle) and an unique pharmacological treatment (a single dose of one drug or two doses for the association experiments). All experimental groups were run in parallel and the experimenters were blind to the pharmacological treatment and the genotypes of the animals.

3.1.2 Drugs and drug administration

- The **Na$_v$ channel blocker** TTX was supplied by Tocris (Bristol, UK).

We used the **selective σ1R ligands**:

- 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine (S1RA), (σ1R antagonist).
- N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl] ethylamine (NE-100), (σ1R antagonist).
- 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride (BD-1063), (σ1R antagonist).
- N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino) ethylamine dihydrobromide (BD-1047), (σ1R antagonist).
- [2-(4-morpholinethyl)1]-phenyl cyclohexane carboxylate hydrochloride (PRE-084), (σ1R agonist).

BD-1063, BD-1047 and PRE-084 were supplied by Tocris Cookson Ltd. (Bristol, UK). NE-100 and S1RA was synthesized and supplied by Esteve Pharmaceuticals S.A. (Barcelona, Spain) as reported previously [219, 297].

As **μ-opioid ligands**, we used:

- Morphine hydrochloride, (μ-opioid agonist).
- Fentanyl citrate, (μ-opioid agonist).
- Oxycodone hydrochloride, (μ-opioid agonist).
- Naloxone hydrochloride, (μ-opioid antagonist).
- Naloxone methiodide, (μ-opioid antagonist).

Morphine hydrochloride was obtained from the General Directorate of Pharmacy and Drugs, Spanish Ministry of Health (Madrid, Spain); fentanyl citrate, oxycodone hydrochloride, naloxone hydrochloride and naloxone methiodide were supplied by Sigma- Aldrich (Madrid, Spain).

All the drugs were dissolved in sterile physiologic saline (0.9% NaCl). The σ1R ligands, S1RA, NE-100, BD-1063, BD-1047 and PRE-084 were properly alka-

linized with sodium hydroxide (NaOH). Drug solutions were prepared immediately before the start of the experiments, and 5 ml/kg of the drug or its solvent were injected subcutaneous (s.c.) into the inter-scapular area. When the systemic effect of the association of two drugs was assessed, each injection was performed in different areas of the inter-scapular zone to avoid mixture of the drug solutions and any physico-chemical interaction between them, and with a 5 minute time interval between the drug under study and the agonist or antagonist. When we study the drug together with an agonist and an antagonist, the latter two are injected at the same time and always 5 minutes after the study drug.

To induce i.cl. pain, we used:

- 8-methyl-N-vanillyl 6-nonamide (Capsaicin) (Sigma-Aldrich).

- Mustard oil (Sigma-Aldrich).

Capsaicin was dissolved in a 1% (for TTX studies) or 0.1% (for σ1R ligands) weight/volume stock solution in a solvent comprising 10% absolute ethanol (Panreac Química SA, Barcelona, Spain), 10% Tween 80 (Sigma-Aldrich), and 80% sterile saline. This capsaicin solution was prepared once a week and stored at -20 °C in aliquots which were thawed and diluted at the appropriate concentrations on the day of the experiment. Mustard oil was dissolved in 70% absolute ethanol and 30% sterile saline and prepared at 0.1%. The capsaicin or mustard oil solutions (50 μL) were instilled into the colon by introducing through the anus a fine round-tip cannula (external diameter 0.61 mm; 4 cm long) connected to a 1710 TLL Hamilton micro-syringe (Teknokroma, Barcelona, Spain). Control animals were i.cl. instilled with the same volume of vehicle.

- Cyclophosphamide 100 mg/kg (Sigma-Aldrich), which was used to induce cystitis, was dissolved in saline and injected i.p. at the volume of 10 ml/kg.

Control animals were injected i.p. with the same volume of saline.

3.2 Experimental approach

3.2.1 Mouse models to test the effects of TTX

3.2.1.1 Mouse models of visceral pain

3.2.1.1.1 Chemical stimulation of the colon: intracolonic administration of capsaicin and mustard oil Visceral pain can be measured as the number of spontaneous visceral pain-related behaviors expressed by the animals and it is known that the referred hyperalgesia is proportional to the intensity of these spontaneous visceral pain-related behaviors [298]. Therefore we evaluated, in two experimental approaches in the same mice, spontaneous pain-related behaviors and referred mechanical hyperalgesia induced by i.cl. capsaicin or mustard oil following the methodology described previously [284, 298].

Animals were placed in the experimental room for a 40 min acclimation period before starting the experiments. They were housed in individual transparent plastic boxes (7×7×13 cm) on an elevated platform. They were removed from the compartments after the habituation period to inject s.c. the drugs (or its solvent) into the interscapular area. Likewise, mice were removed again 30 min later from the compartments and, after application of petroleum jelly on the perianal area to avoid stimulation of somatic areas through contact with the algogen, the capsaicin/mustard oil solution (or its solvent) was instilled i.cl., and once again they were returned to the compartment. Visceral pain-related behaviors (licking of the abdomen, stretching and abdominal retractions) were counted for 20 min in 5 min intervals.

Twenty minutes after the algogen administration (or its solvent), forces ranging from 0.02 to 2 g (0.19-19.6 mN) were applied to the abdomen to measure the frequency of the withdrawal responses to a punctate mechanical stimuli with a series of calibrated von Frey filaments (Touch-Test Sensory Evaluators; North Coast Medical Inc., Gilroy, CA), trying to avoid perianal and external genitalia areas and reproducing the up-down paradigm [299]. All the filaments were applied three times for 2-3 s, with inter-application intervals of 3 s. We considered a positive response if we observe immediate jumping, licking/scratching of the application site or retraction of the abdomen as reported previously [284, 298].

3.2.1.1.2 Cyclophosphamide-induced cystitis Cyclophosphamide-evoked visceral pain and referred hyperalgesia were examined following a previously de-

scribed protocol [149, 300]. Animals were placed in the experimental room for a 40 min acclimation period before starting the experiments. They were housed in individual transparent plastic boxes (7×7×13 cm) on an elevated platform. They were removed from the compartments after the 40 min habituation period to inject with intraperitoneal cyclophosphamide (100 mg/kg) or saline.

Drugs or saline were s.c. injected 2h after the cyclophosphamide injection into the interscapular area and the pain behaviors manifested by the animals were recorded for 2 min every 30 min over a 2h observation period.

The recorded pain-related behaviors were coded according to the following scale: 0=normal, 1=piloerection, 2=strong piloerection, 3=labored breathing, 4=licking of the abdomen, and 5=stretching and contractions of the abdomen [149]. If more than one of these pain behaviors was observed in one period, the sum of the corresponding points to the different types of behaviors was assigned and an overall score was obtained by summing the scores assigned at each time point.

At the end of the 2h observation period (i.e., 4h after the cyclophosphamide injection), the referred mechanical hyperalgesia was determined using the von Frey filaments as described in the previous section 3.2.1.1.1.

3.2.1.1.3 Comparison of drug effects

3.2.1.1.3.1 Na_v channel blockade The objective was to determine the effect of TTX concentration *versus* the number of behaviors or mechanical threshold induced in the three experimental models (capsaicin, mustard oil and cyclophosphamide). To this end, treatment with TTX (1-6 μg/kg, s.c. in chemical stimulation of the colon, and 3-6 μg/kg, s.c. on cyclophosphamide-induced visceral pain) and morphine (8 mg/kg, s.c. as a positive control) was carried out.

3.2.1.1.3.2 Na_v channel lock down To study the possible involvement of the Na^+ channel $Na_v1.7$ in the visceral pain models tested, we used WT C57Bl/6 and KO-$Na_v1.7$ (which possess a specific ablation of channels in $Na_v1.8$-positive neurons) mice. The aim was to test the maximum dose of TTX used (6 μg/kg, s.c.) in the number of behaviors an mechanical threshold induced by the three experimental models.

3.2.1.2 Locomotor coordination evaluated on the Rotarod test

3.2.1.2.1 General procedures Alterations in motor coordination were assayed as previously described [301] with a Rotarod device for mice (Ugo Basile, Comerio, Italy). Animals were placed in the experimental room for a 40 min acclimation period before starting the experiments. They were removed from the mouse cage after the 40 min habituation period to inject s.c. TTX or morphine and then placed to the Rotarod. The Rotarod apparatus was set to accelerate from 4-40 rpm over 5 min. Three training sessions separated by 30-min intervals were performed 1 day before drug testing. Rotarod latencies were measured before the administration of the drugs or saline (time 0) and 30, 60, and 120 min after treatment. A 300-s cut-off time was established in all experiments.

3.2.1.2.2 Comparison of drug effects The latency period to fall from the Rotarod apparatus before (time 0) and after the treatment with TTX (6 μg/kg, s.c., the highest dose used in the pain experiments), morphine (8 mg/kg, s.c., as a positive control), or saline was assessed to test the possible induction of locomotor disturbing effect by the TTX.

3.2.2 Capsaicin-induced visceral pain to study the role of σ1R on opioid-induced analgesia

Visceral pain and referred hyperalgesia were evoked by the i.cl. instillation of 0.1% capsaicin as described in the previous section 3.2.1.1.1.

3.2.2.1 Comparison of drug effects

Visceral antinociception (pain related behaviors and referred hyperalgesia to abdominal mechanical stimulation) induced by the administration of the μ-opioid agonists morphine (doses 0.5-16 mg/kg, s.c.), oxycodone (1-6 mg/kg, s.c.) and fentanyl (0.04-0.2 mg/kg, s.c.); and the selective σ1R antagonists S1RA (8-32 mg/kg, s.c.), NE-100 (2-8 mg/kg, s.c.), BD-1063 (4-16 mg/kg, s.c.) and BD-1047(4-16 mg/kg, s.c.) was assessed to construct concentration-response curves (concentration *vs* number of behaviors or mechanical threshold) in WT mice and both in WT and σ1R-KO mice in the case of morphine plus σ1R antagonists. In these experiments all drugs were administered 30 min before the capsaicin.

3.2.2.2 Enhancement of the opioid antinociceptive effects with selective σ1R antagonists

WT and σ1R-KO mice were used to test the potentiation effects of the administration of different selective σ1R antagonists together with the μ-opioid agonists. We administered σ1R antagonists S1RA (8-32 mg/kg, s.c.), NE-100 (2-8 mg/kg, s.c.), BD-1063 (4-16 mg/kg, s.c.) and BD-1047 (4-16 mg/kg, s.c.) associated to morphine (0.5-2 mg/kg, s.c.), oxycodone (1-2 mg/kg, s.c.) and fentanyl (0.04-0.08 mg/kg, s.c.). In these experiments σ1R antagonists or it solvent were administered 5 min before the μ-opioid agonists and therefore 35 min before the capsaicin.

3.2.2.3 Role of the central and peripheral opioid receptors on the antinociception induced by opioid agonists used in clinical practice

We have carried out the study of the role of the central and peripheral opioid receptors in the antinociception induced by μ-opioids in WT mice. For this, we administered several doses of the opioid antagonists naloxone hydrochloride (0.031-1 mg/kg, s.c.) and naloxone methiodide (2-8 mg/kg, s.c.) associated to morphine (3-4 mg/kg, s.c.), oxycodone (3-5 mg/kg, s.c.) and fentanyl (0.12-0.16 mg/kg, s.c.). In addition, we studied the effect of the σ1R agonist PRE-084 (32 mg/kg, s.c.) associated with the same doses of oxycodone and fentanyl to test the selectivity of the activity. In these experiments, opioid antagonists and the σ1R agonist were administered 5 min before the μ-opioid agonists, and therefore 35 min before the capsaicin.

3.2.2.4 Reversion of the enhancement of morphine-induced antinociceptive effects

Next, we study the selectivity in the enhancement of the antinociceptive opioid effects described in previous sections. For this purpose, we add to the morphine-σ1R antagonists associations the opioid antagonists naloxone hydrochloride and naloxone methiodide in different doses (1 mg/kg and 2-4mg/kg, s.c. respectively). In this experimental approach, opioid antagonists were jointly administered with the σ1R antagonists, 5 min before the morphine, and therefore 35 min before the capsaicin.

3.3 Data analysis

The mechanical threshold that produces 50% of responses is a measure of the degree of referred pain [302].

The values of the effective dose of the drug that produces the desired effect in 50% of the population (ED_{50}) and their standard errors of the mean (SEM) were calculated using non-linear regression analysis of the equation for a sigmoid plot and were compared by means of Snedecor's F test. A value of * $p < 0.05$ was considered statistically significant.

To compare the number of pain behaviors and mechanical thresholds across experimental groups we carried out one/two-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test; and when two means were compared we used a Student's t test for non-paired values. Each bar/point and vertical line represents the mean $\pm$ SEM of values obtained in at least eight animals per group. The dashed lines indicate the mean value of behaviors in saline-injected mice treated with capsaicin or the 50% threshold force in naïve mice. We consider statistically significant differences between values when * $p < 0.05$ and ** $p < 0.01$ after the test.

We used the Sigma Plot 12.0 program (Systat Software Inc., San Jose, CA, USA) and the GraphPad Prism 5.00 program (GraphPad Software Inc., San Diego, CA, USA).

4 Results

4.1 Effects of TTX in mouse models of visceral pain and locomotor coordination

4.1.1 Effects of TTX on visceral pain induced by chemical stimulation of the colon: intracolonic administration of capsaicin 1% and mustard oil 0.1%

To evaluate the effects of TTX on pure visceral pain, we used two common models: the intracolonic instillation of capsaicin and mustard oil. The i.cl. administration of capsaicin and mustard oil vehicle elicited a small and a moderate number of abdominal licking behaviors (saline bar in Figure 4.1 A and Figure 4.2 A, respectively). By contrast, the i.cl. instillation of capsaicin (1%) and mustard oil (0.1%) evoked a greater number of multiple types of pain-related behaviors (i.e., licking, stretching, and contraction of the abdomen) in control animals (saline plus capsaicin 1% and saline plus mustard oil 0.1% bars in Figure 4.1 A and Figure 4.2 A).

The s.c. administration of TTX (1-6 μg/kg) 30 min before the i.cl. instillation of capsaicin (1%) significantly reduced the number of pain-related responses in a dose-dependent manner (Figure 4.1 A). Similarly, the s.c. treatment with TTX (3 and 6 μg/kg) dose-dependently ameliorated the number of pain-related responses induced by i.cl. mustard oil (Figure 4.2 A). As a control analgesic drug, we used morphine (8 mg/kg), which fully abolished the pain-related behaviors produced by capsaicin and mustard oil, even below those observed in the vehicle plus saline group (Figure 4.1 A and Figure 4.2 A).

The i.cl. administration of capsaicin (1%) and mustard oil (0.1%) also produced a strong referred mechanical hyperalgesia in the saline-treated group, as it decreased the mechanical threshold in those mice, with respect to naïve mice (represented with a dashed line, Figure 4.1 B and Figure 4.2 B). In contrast, the vehicles of both algogens produced a slight reduction of the mechanical threshold in

the saline-treated animals (Figure 4.1 B and Figure 4.2 B). The s.c. injection of TTX (1-6 μg/kg) also reversed the mechanical hypersensitivity induced by capsaicin in a dose-dependent manner, abolishing it completely with the dose of 6 μg/kg (Figure 4.1 B). However, TTX (3 and 6 μg/kg) was completely ineffective on the mechanical referred hyperalgesia induced by i.cl. mustard oil at any of the doses tested (Figure 4.2 B). As expected, morphine (8 mg/kg) reversed the referred hyperalgesia in both models, even above the values of naïve mice (Figure 4.1 B and Figure 4.2 B).

In summary, TTX was able to decrease the number of pain-related behaviors produced by the i.cl. administration of both capsaicin and mustard oil. However, whereas TTX reversed the mechanical referred hyperalgesia produced by capsaicin, it had no effect on the mechanical referred hyperalgesia in the mustard oil model.

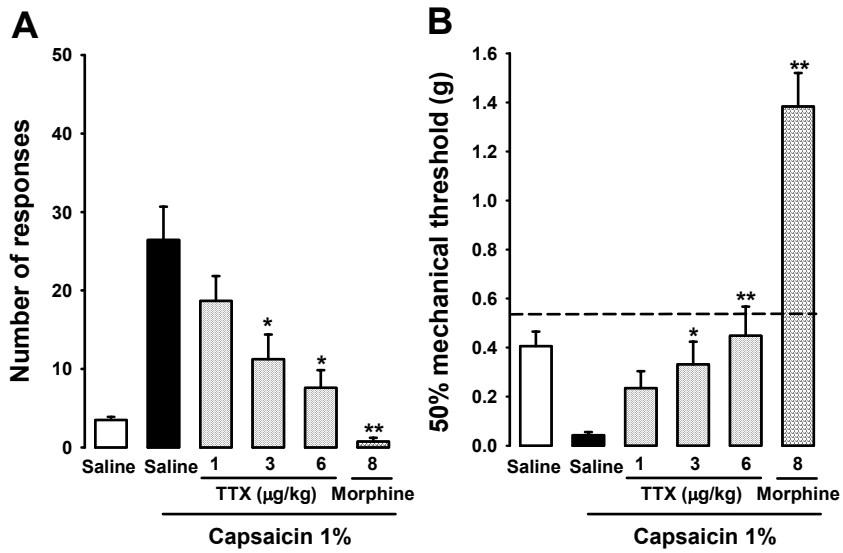

Figure 4.1: Effects of TTX and morphine on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of capsaicin 1% in wild-type (WT) mice. The subcutaneous (s.c.) administration of the drugs or their solvent was performed 30 min before the i.cl. administration of the algogen or its vehicle. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in drug- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

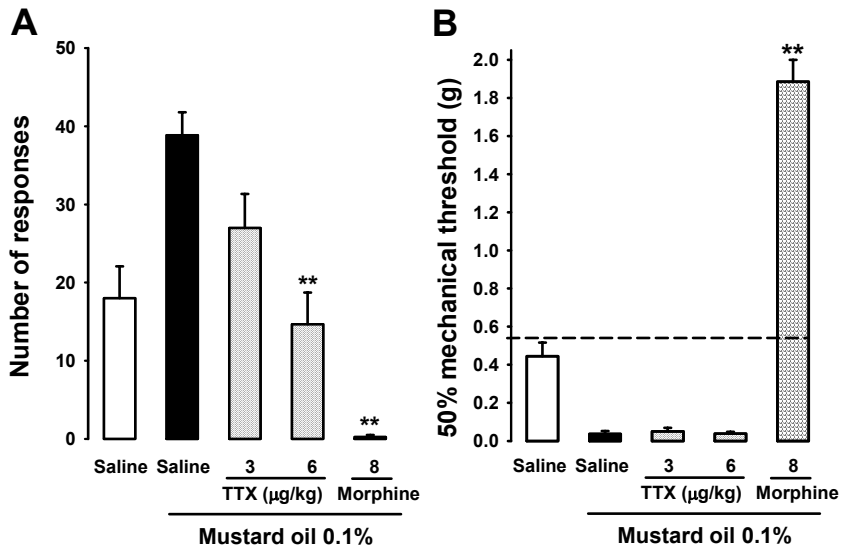

Figure 4.2: Effects of TTX and morphine on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of mustard oil 0.1% in wild-type (WT) mice. The subcutaneous (s.c.) administration of the drugs or their solvent was performed 30 min before the i.cl. administration of the algogen or its vehicle. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in drug- and saline-injected mice treated with mustard oil: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

4.1.2 Effects of TTX on visceral pain evoked by cyclophosphamide-induced cystitis

To test the effect of TTX on the pain originating in a different visceral organ, we used the model of bladder pain/cystitis induced by cyclophosphamide. The solution of cyclophosphamide (100 mg/kg) was administered i.p. and produced a progressive development of visceral pain behaviors. Mice treated with cyclophosphamide showed a significantly higher painful score than mice treated with the vehicle (Figure 4.3 A). The s.c. administration of TTX (3 and 6 μg/kg) significantly reduced this pain-related score in a dose-dependent manner, but none of them were enough to completely abolish the pain responses (Figure 4.3 A). The control drug, morphine (8 mg/kg), highly reduced the pain score, but it was also unable to eliminate the pain responses (Figure 4.3 A). The cyclophosphamide vehicle (saline) barely provoked pain-related responses in the evaluated animals (saline bar in Figure 4.3 A).

On the mechanical threshold, animals administered with the cyclophosphamide vehicle showed similar values as naïve mice (Figure 4.3 B). However, mice showed a marked reduction on their mechanical thresholds with respect to naïve (dashed line) and cyclophosphamide vehicle-injected animals when they were tested 4 h after cyclophosphamide treatment (Figure 4.3 B). The treatment with TTX (3 and 6 μg/kg) reversed in a dose-dependent manner the mechanical referred hyperalgesia evoked by cyclophosphamide with respect to saline-injected mice but did not produce a complete recovery (Figure 4.3 B). Morphine administration fully reversed the referred mechanical hyperalgesia and produced a pronounced analgesic effect (Figure 4.3 B).

4.1.3 Effects of TTX in mouse models of visceral pain studied on the $Na_v1.7$-KO mice

To study the possible involvement of the Na^+ channel $Na_v1.7$ in the visceral pain models tested, we used KO-$Na_v1.7$ mice, which possess a specific ablation of these channels in $Na_v1.8$-positive neurons. These animals, treated with saline s.c., did not show any differences in pain-related responses and referred hyperalgesia with respect to their control mice littermates when they were instilled i.cl. with capsaicin and mustard oil (Figure 4.4 A, left panel, zero bars) or treated i.p. with cyclophosphamide (Figure 4.4 A, right panel, zero bars). When the maximum dose of TTX used (6 μg/kg) was administered in KO-$Na_v1.7$ mice in the

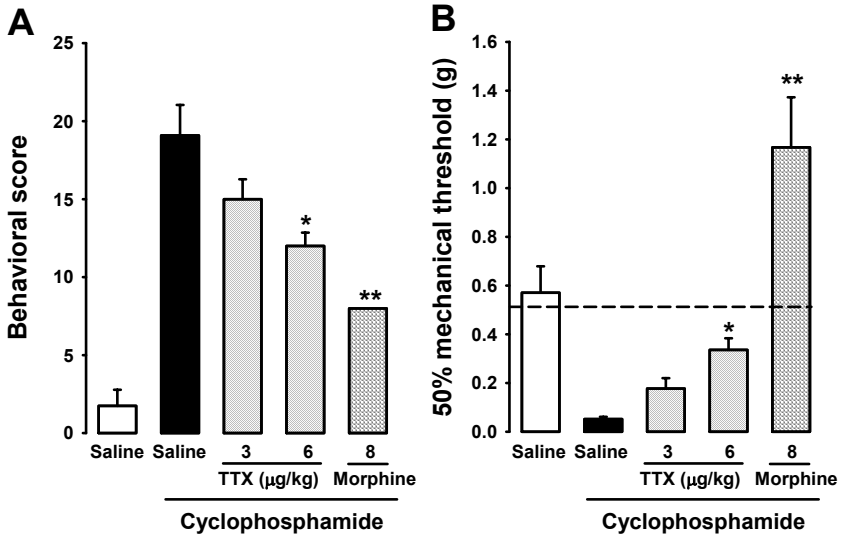

Figure 4.3: Effects of TTX and morphine on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by the intraperitoneal (i.p.) administration of cyclophosphamide (100 mg/kg) in wild-type (WT) mice. The subcutaneous (s.c.) administration of the drugs or their solvent was performed 2h after the administration of cyclophosphamide or its vehicle and 30 min before the beginning of the behavioral score record. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in drug- and saline-injected mice treated with cyclophosphamide: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

different pain models, we also did not find a difference between both types of animals, and TTX reversed both the pain responses and the referred hyperalgesia in the same way that it did in the control animal littermates (Figure 4.4 A and B). These results indicate that $Na_v1.7$ in sensory neurons expressing nociceptive markers is not necessary for the effect of TTX.

4.1.4 TTX does not alter locomotor coordination

Animals treated with TTX and morphine were tested with a Rotarod device to detect effects on the motor coordination of the mice. We tested the highest dose of TTX used in the pain experiments (6 μg/kg). The latency period to fall from the Rotarod apparatus before the treatment with TTX, morphine, or saline (time 0) was very similar in all groups. The Rotarod latency time values during all the evaluation periods following the administration of saline or TTX (6 μg/kg) were not significantly different from the baseline values (time 0) and there were no differences between the Rotarod values of mice treated with saline and TTX at any time after administration (Figure 4.5). Similarly, animals treated with morphine (8 mg/kg) showed no motor incoordination and even induced higher values in the Rotarod test after 120 min in comparison to their own values at time 0 (Figure 4.5). Therefore, TTX did not induce any locomotor disturbing effect.

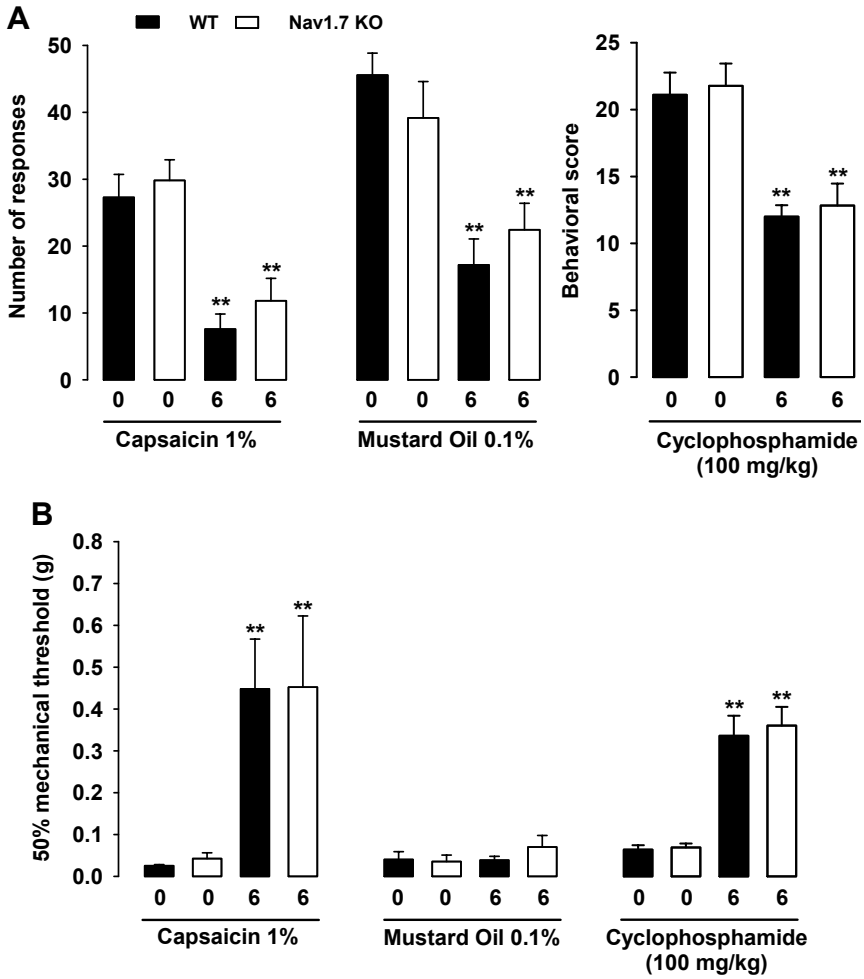

Figure 4.4: Comparison of the effects of TTX (6 μg/kg) and saline (0) on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of capsaicin 1%, mustard oil 0.1% or the intraperitoneal (i.p.) administration of cyclophosphamide (100 mg/kg) in wild-type (WT) and KO $Na_v1.7$ mice. TTX or saline was injected subcutaneously (s.c.) 30 min before the instillation of capsaicin and mustard oil and 2h after the administration of cyclophosphamide. Each bar and vertical line represents the mean $\pm$ SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in TTX- and saline-injected mice treated with the algogens: $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

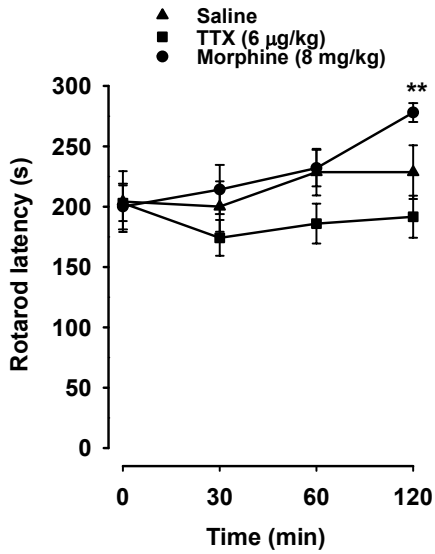

Figure 4.5: Effects of TTX, morphine, and saline on the rotarod test in wild-type (WT) mice. The latency time to fall down from the rotarod apparatus was recorded in each mouse before (time 0) and 30, 60 and 120 min after the subcutaneous (s.c.) injection of the drugs or saline. Each point and vertical line represent the mean ± S.E.M. of the values obtained in at least eight animals per group. Statistically significant differences between the values at time 0 and time 120 min after the s.c. injection of morphine: $^{**}p < 0.01$ (two-way repeated measures ANOVA followed by Bonferroni test).

4.2 Potentiation of morphine-induced analgesia by σ1R blockade on visceral pain induced by intracolonic administration of capsaicin 0.1% in mice

4.2.1 Effects of morphine-induced analgesia on capsaicin-evoked visceral pain in WT and σ1R-KO mice

We evaluated the antinociceptive effects induced by the s.c. administration of the selective μ-opioid agonist morphine or its solvent on the visceral pain induced by i.cl instillation of capsaicin 0.1% in WT and σ1R-KO mice.

The number of pain-related behaviors (e.g., licking, stretching, and contraction of the abdomen) in solvent-treated mice was statistically significant higher in WT (20.92$\pm$0.45) than in σ1R-KO (14.17$\pm$0.39) mice (Figure 4.6 A, dose 0), whereas the capsaicin-induced referred mechanical hyperalgesia did not show significant differences between WT and σ1R-KO in morphine solvent-treated mice (Figure 4.6 B, dose 0).

The s.c. administration of morphine (0.5-16 mg/kg) induced a dose-dependent reversion of pain-related behavioral responses (Figure 4.6 A) and capsaicin-induced referred hyperalgesia (Figure 4.6 B) in WT and σ1R-KO mice. Morphine induced a significant reduction of the spontaneous pain behaviors from doses of 0.5 mg/kg and 1 mg/kg, in WT and σ1R-KO mice, respectively (Figure 4.6 A). When we calculated the ED_{50}, we found that morphine was significantly more potent ($p < 0.01$, Snedecor's F test) in σ1R-KO mice (0.61$\pm$0.1 mg/kg) than in WT mice (1.99$\pm$0.09 mg/kg). On the referred mechanical hyperalgesia, the antihyperalgesic effects were statistically significant at doses of 3 mg/kg or higher in WT mice. By contrast, σ1R-KO mice showed significant antihyperalgesic effects from a dose of 2 mg/kg (Figure 4.6 B). Likewise, in terms of ED_{50}, we found that morphine was significantly more potent ($p < 0.01$, Snedecor's F test) in σ1R-KO mice (3.21$\pm$0.11 mg/kg) than in WT mice (6.09$\pm$0.11 mg/kg).

Therefore, the absence of σ1R evoked a clear and marked enhancement of systemic morphine-induced analgesia.

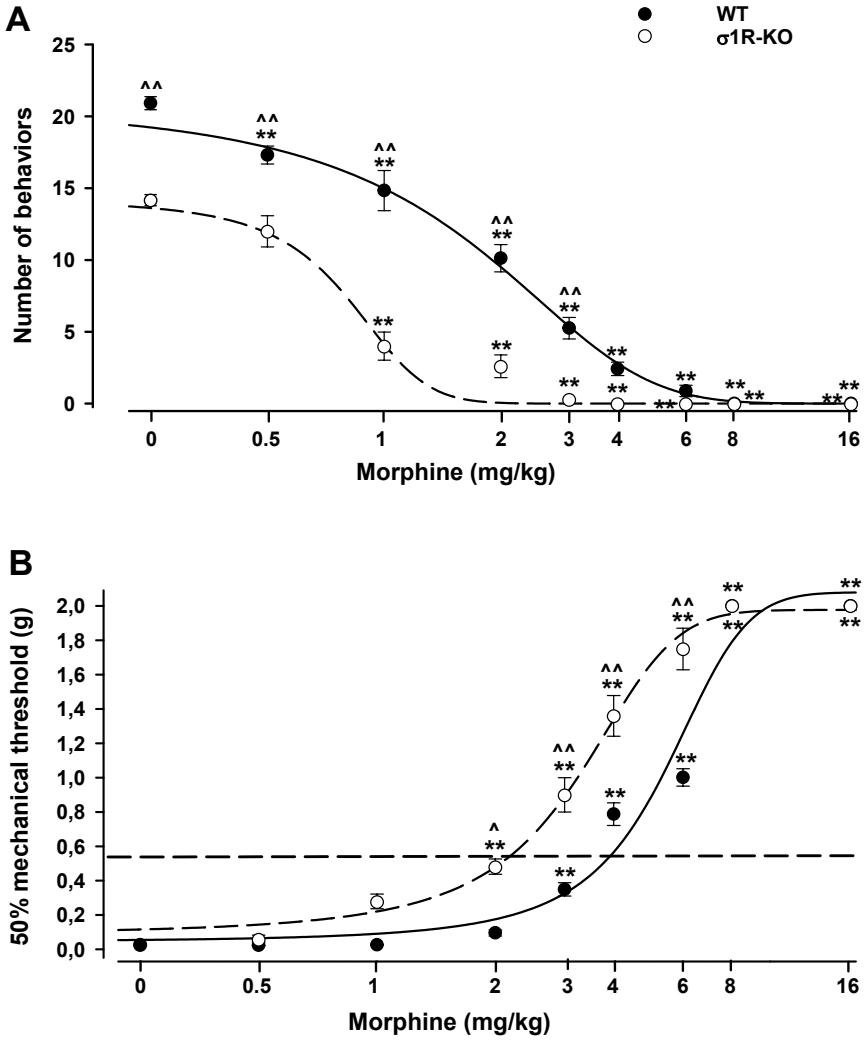

Figure 4.6: Effects of the morphine (0.5-16 mg/kg) on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) and sigma-1 receptor (σ1R)-KO mice. Subcutaneous administration of morphine or its solvent was performed 30 min before i.cl. administration of capsaicin. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each point and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in morphine- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$, and between the values obtained in WT and σ1R-KO mice at the same dose of morphine: $\Lambda p < 0.05$; $\Lambda\Lambda p < 0.01$ (two-way ANOVA followed by Bonferroni test).

4.2.2 Potentiation of morphine effect by pharmacological blockade of σ1R in WT mice

Since we were evaluating two different types of visceral pain induced by i.cl. capsaicin (spontaneous pain responses and referred mechanical hyperalgesia) in the same animal, we had to choose a dose of morphine that evoked an antinociceptive effect that could be sensitive to the modulation by the σ1R antagonists. As reported above, 2 mg/kg of morphine produced a moderate but statistically significant reduction of the spontaneous pain responses but had no analgesic effects on the referred mechanical hyperalgesia in WT mice (see Figure 4.6). In spite of the analgesic effect induced by morphine 2 mg/kg on the pain-related behaviors, still there was sufficient range of response to observe a reduction or increment of the pain responses in WT mice. Therefore, we chose this dose to perform the association studies with σ1R antagonists. To determine the optimal doses of σ1R antagonists to potentiate the analgesic effects of morphine, we evaluated the effects *per se* of several selective σ1R antagonists in WT mice. The s.c. administration of S1RA (8-32 mg/kg), NE-100 (2-8 mg/kg), BD-1063 (4-16 mg/kg), and BD-1047 (4-16 mg/kg) reduced significantly the number of pain-related behaviors at the highest doses tested (Figure 4.7 A), but none of them was able to modify at all the referred mechanical hyperalgesia induced by i.cl. instillation of capsaicin 0.1% at the administered doses (Figure 4.7 B).

When we evaluated the systemic administration of 2 mg/kg of morphine associated with the selective σ1R antagonists, we found a significant dose-dependent enhancement of morphine-induced analgesia in WT mice in the two experimental approaches used (acute visceral pain and referred mechanical hyperalgesia) (Figure 4.8). Thus, the association with all these σ1R antagonists decreased markedly the number of pain-related behaviors obtained in comparison with morphine 2 mg/kg alone (Figure 4.8 A). Similarly, the referred mechanical hyperalgesia induced by 0.1 % capsaicin was completely reversed by pretreatment of 2 mg/kg morphine with all these σ1R antagonists (Figure 4.8 B). It is noteworthy that BD-1063 was less effective but nevertheless has a significant effect at all doses tested; and mice co-administered with the highest doses of S1RA (32 mg/kg) and NE-100 (4-8 mg/kg) exerted a clear analgesic action, yielding a higher mechanical threshold than that observed in naïve mice (Figure 4.8 B).

Thus, the pharmacological blockade of σ1R potentiated considerably the effects induced by morphine 2 mg/kg on the spontaneous pain responses and referred mechanical hyperalgesia.

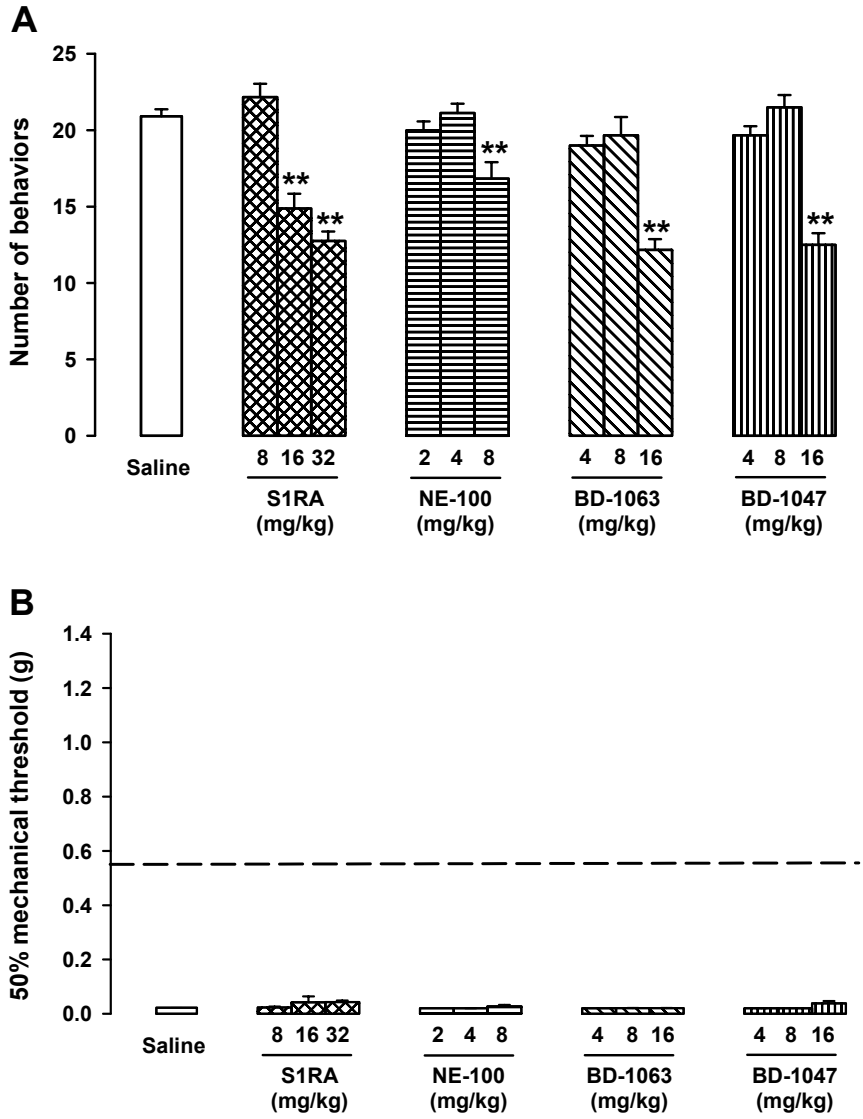

Figure 4.7: Effects of S1RA (8-32 mg/kg), NE-100 (2-8 mg/kg), BD-1063 (4-16 mg/kg) and BD-1047 (4-16 mg/kg) on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) mice. Subcutaneous administration of each sigma-1 receptor (σ1R) antagonist or their solvent was performed 30 min before i.cl. administration of capsaicin. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in drug- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

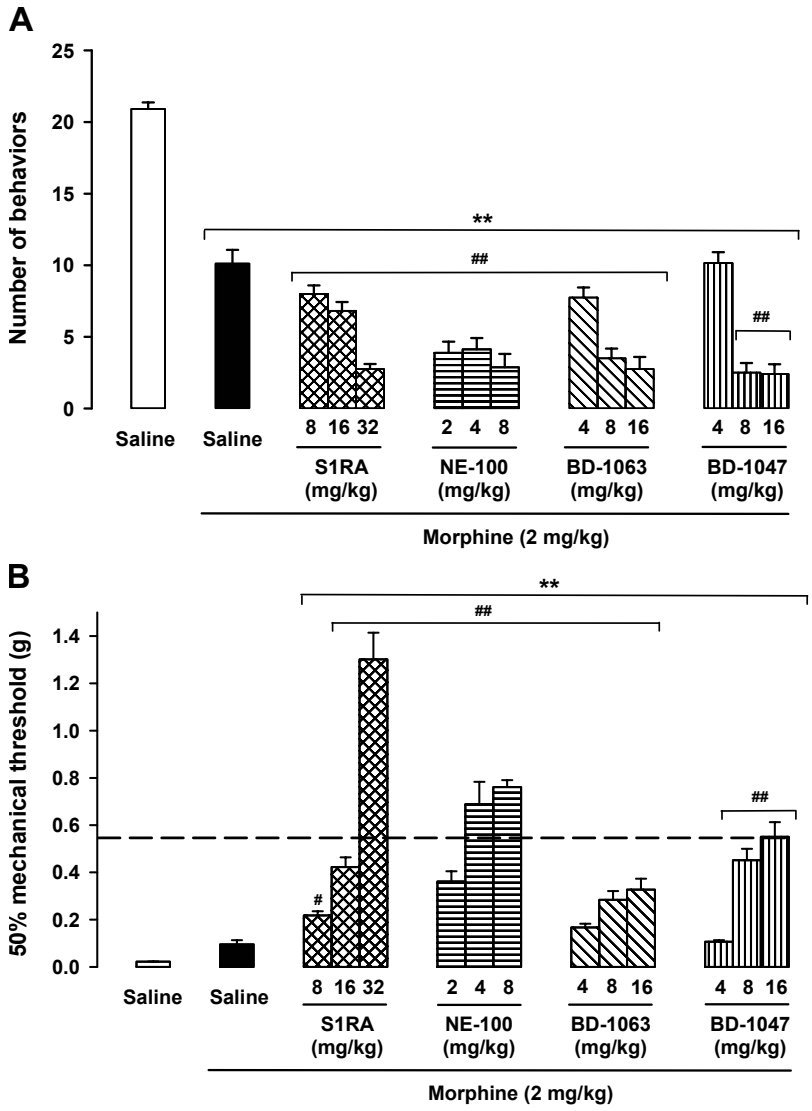

Figure 4.8: Effects of S1RA (8-32 mg/kg), NE-100 (2-8 mg/kg), BD-1063 (4-16 mg/kg) and BD-1047 (4-16 mg/kg) associated with morphine (2 mg/kg) on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) mice. Subcutaneous (s.c.) administration of morphine or its solvent was performed 30 min before the i.cl. administration of capsaicin; whereas the s.c. administration of each sigma-1 receptor (σ1R) antagonist was performed 5 min before the morphine administration. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in drug- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in co-administrated drugs- and morphine-injected mice treated with capsaicin: $\#p < 0.05$; $\#\#p < 0.01$ (one-way ANOVA followed by Bonferroni test).

4.2.3 Effects of the association of morphine with σ1R antagonists in σ1R-KO mice.

Once we identified the dose of each selective σ1R antagonist that produced the maximun potentiation of 2 mg/kg morphine (S1RA 32 mg/kg, NE-100 8 mg/kg, BD-1063 16 mg/kg, and BD-1047 16 mg/kg), we assessed the selectivity of the effects induced by the σ1R antagonists by evaluating their action on morphine-induced visceral analgesia in σ1R-KO mice (Figure 4.9). The co-administration of morphine 2 mg/kg and σ1R antagonists did not exert any change on the visceral pain-related behaviors (Figure 4.9 A, central panel) neither on the referred mechanical hyperalgesia in σ1R-KO mice (Figure 4.9 B, central panel).

We further confirmed no change in morphine-induced analgesia by selective σ1R antagonists by testing an equivalent morphine dose (0.5 mg/kg) in terms of efficacy in σ1R-KO mice (Figure 4.9, right panel). The antinociceptive effects induced by 0.5 mg/kg of morphine in σ1R-KO mice on the visceral pain (spontaneous pain-related behaviors and referred mechanical hyperalgesia) evoked by the instillation of 0.1% capsaicin were very similar to those observed in WT mice treated with 2 mg/kg morphine (see Figure 4.6).

Therefore, when we co-administered morphine 0.5 mg/kg plus the σ1R antagonists at the same doses tested, we obtained no potentiation of morphine-induced analgesia (Figure 4.9, right panel). These results confirm the selectivity of the effects induced by the σ1R antagonists given their lack of effect in σ1R-KO mice.

4.2.4 Effects of the opioid antagonists on the morphine analgesia in WT mice

We tested the non-selective opioid receptor antagonist naloxone injected s.c. 5 min before the systemic morphine, to determine the central mechanism of action of the morphine-induced analgesia in visceral pain induced by i.cl. capsaicin in WT mice.

As expected, naloxone (0.031-1 mg/kg) induced a dose-dependent reversion of the analgesia induced by morphine 4 mg/kg on both the pain-related behaviors and the referred mechanical hyperalgesia (Figure 4.10, central panel). Likewise, to test the role of peripheral opioid receptors on the capsaicin-induced visceral pain we tested the peripherally restricted opioid antagonist naloxone methiodide injected 5 min before the opioid. When considering the activity of morphine (4

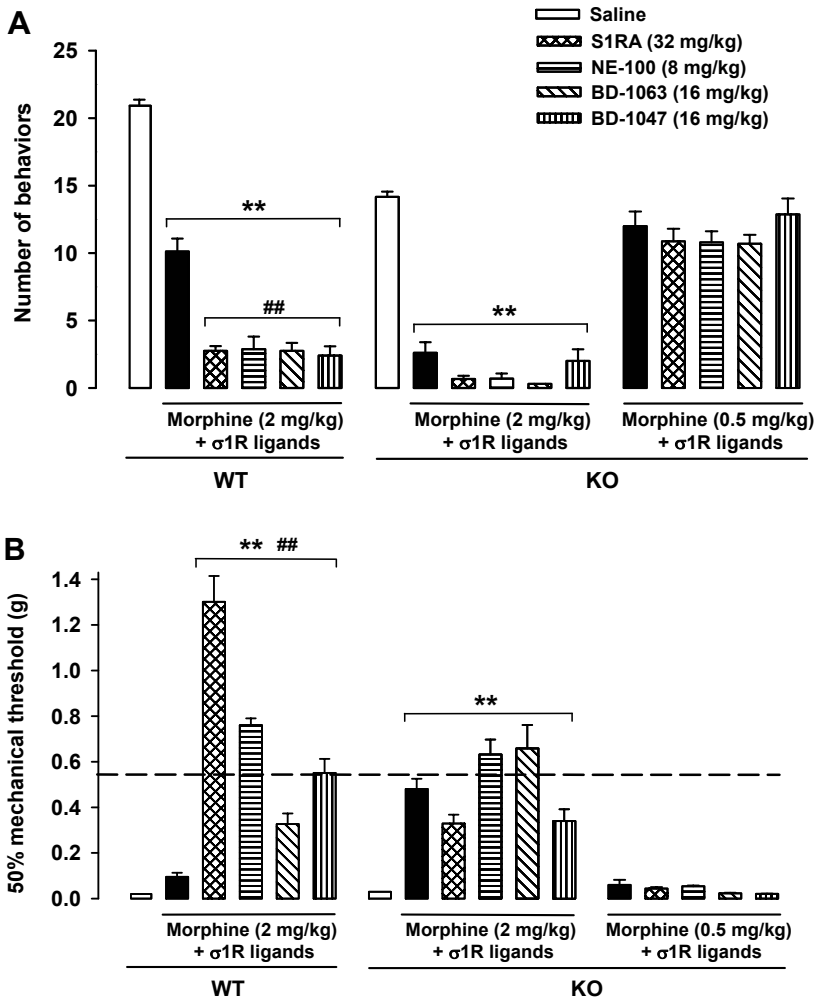

Figure 4.9: Effects of S1RA (32 mg/kg), NE-100 (8 mg/kg), BD-1063 (16 mg/kg) and BD-1047 (16 mg/kg) associated with morphine (0.5-2 mg/kg) on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) and sigma-1 receptor (σ1R)-KO mice. Subcutaneous (s.c.) administration of the morphine or its solvent was performed 30 min before i.cl. administration of capsaicin; whereas the s.c. administration of each selective σ1R antagonist was performed 5 min before morphine administration. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in drug- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in co-administrated drugs- and morphine-injected mice treated with capsaicin: $\#p < 0.05$; $\#\#p < 0.01$ (one-way ANOVA followed by Bonferroni test).

mg/kg) on pain-related behaviors, only the highest tested dose of naloxone methiodide (8 mg/kg) has a significant effect. Regarding the referred mechanical hyperalgesia, no tested dose of naloxone methiodide had any effect (Figure 4.10, central panel). However, when we evaluated a dose of morphine (3 mg/kg) that on the referred hyperalgesia induced an antihyperalgesic effect instead of an analgesic effect, the opioid antagonist actions were different (Figure 4.10, right panel). The antinociceptive effect of this dose of morphine was totally reversed by naloxone 1 mg/kg in both types of pain. On the other hand, only a dose of 2 mg/kg of naloxone methiodide was needed to achieve statistical significance in the referred mechanical hyperalgesia whereas on pain-related behaviors naloxone methiodide did not modify the effect of morphine 3 mg/kg (Figure 4.10, right panel). In addition, we also evaluated each opioid antagonist alone and the result was that none of them had effect *per se* at the highest dose tested (Figure 4.10).

4.2.5 Effects of the opioid antagonism on the morphine analgesia induced by the association with σ1R antagonists in WT mice

Similarly, to study the central mechanism of action of the interaction between the drugs we administered naloxone plus the σ1R antagonists associated to morphine (Figure 4.11 A and B). On the other hand, to test the sensitivity of these effects to the peripheral activity, we used the same experimental approach with naloxone methiodide (Figure 4.11 C and D). Naloxone (1 mg/kg) or naloxone methiodide (2-4 mg/kg) were s.c. co-administered with the highest doses of σ1R antagonists tested before (i.e., S1RA 32 mg/kg, NE-100 8 mg/kg, BD-1063 16 mg/kg, and BD-1047 16 mg/kg) 5 min before the morphine (2 mg/kg). Naloxone partially antagonized the analgesic effects on the pain-related behaviors induced by the co-administered treatment almost to the level of σ1R antagonists *per se* (Figure 4.11 A). On the referred mechanical hyperalgesia, naloxone completely reversed the analgesic effect induced by the combination of morphine and σ1R antagonists (Figure 4.11 B). Likewise to the study with naloxone, pretreatment with naloxone methiodide, was able to partially reverse the morphine-enhanced analgesic activity on the pain-related behaviors (Figure 4.11 C) but fully blocked the analgesic effects of morphine on referred mechanical hyperalgesia (Figure 4.11 D).

Hence, these results suggest that the enhancement of morphine analgesia by σ1R inhibition is mainly due to peripheral opioid receptors.

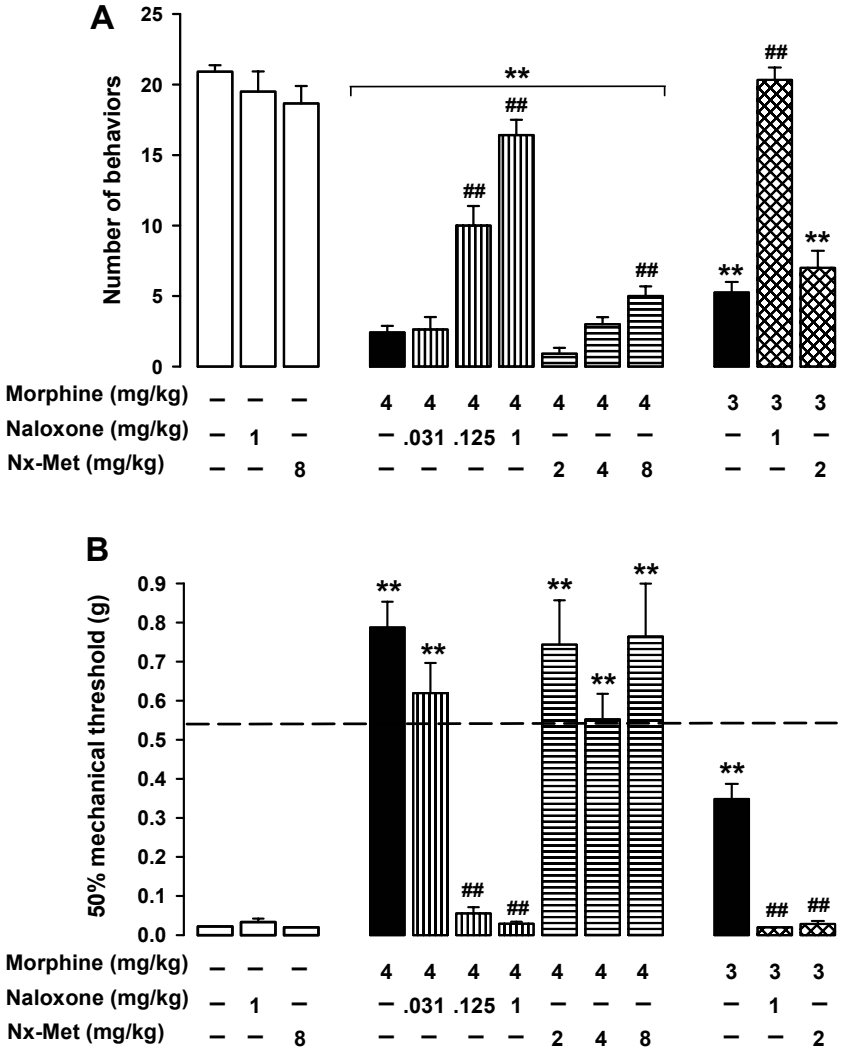

Figure 4.10: Effects of naloxone (0.031-1 mg/kg) and naloxone methiodide (2-8 mg/kg) on the antinociception induced by morphine (3-4 mg/kg) on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) mice. Subcutaneous (s.c.) administration of morphine or its solvent was performed 30 min before i.cl. administration of capsaicin. Opioid antagonists were s.c. administered 5 min before morphine. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in drug- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in co-administrated drugs- and morphine-injected mice treated with capsaicin: $\#p < 0.05$; $\#\#p < 0.01$ (one-way ANOVA followed by Bonferroni test).

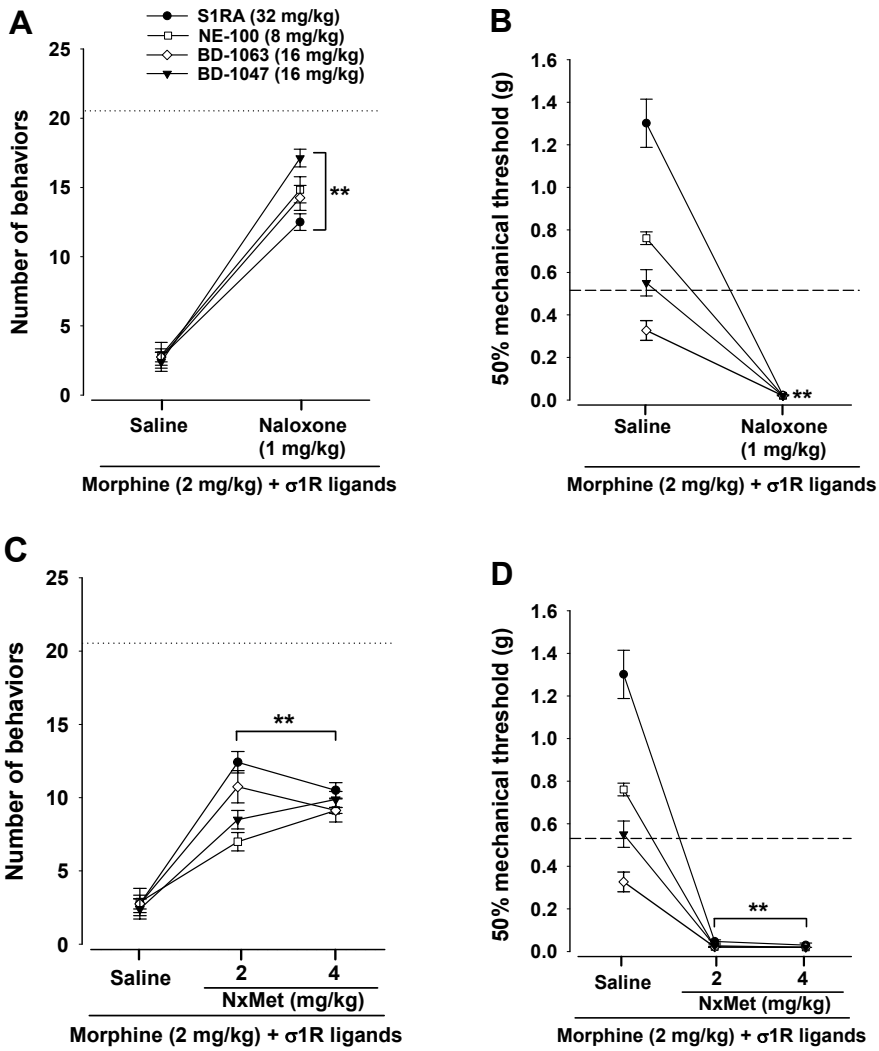

Figure 4.11: Effects of naloxone (1 mg/kg) (A and B) and naloxone methiodide (2-4 mg/kg) (C and D) on the antinociception induced by the enhancement of morphine analgesia (2 mg/kg) with S1RA (32 mg/kg), NE-100 (8 mg/kg), BD-1063 (16 mg/kg) and BD-1047 (16 mg/kg) on the pain-related behaviors (A and C) and the referred mechanical hyperalgesia (B and D) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) mice. Subcutaneous (s.c.) administration of morphine was performed 30 min before i.cl. administration of capsaicin. Naloxone/naloxone methiodide were s.c. co-administered with sigma-1 receptor (σ1R) antagonists 5 min before the morphine. The dashed line in the graphs indicates: (A and C) mean value in saline-injected mice treated with capsaicin; (B and D) 50% threshold force in naïve WT mice. Each point and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in opioid antagonists plus morphine-σ1R antagonists- and morphine-σ1R antagonists injected groups treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$ (t-Student test between groups (A and B); and one-way ANOVA (C and D) followed by Bonferroni test).

4.3 Comparison of the effects of the clinically relevant μ-opioid agonists oxycodone and fentanyl on 0.1% capsaicin-evoked visceral pain in mice.

4.3.1 Effects of s.c. administration of oxycodone and fentanyl

To study the antinociceptive effects of additional μ-opioid agonists used in clinical practice as analgesics on the i.cl. capsaicin-evoked visceral pain model, we tested oxycodone and fentanyl in WT mice. The s.c. administration of the μ-opioids oxycodone (1-6 mg/kg) and fentanyl (0.04-0.2 mg/kg), totally abolished, in a dose-dependent manner, the pain-related behaviors (Figure 4.12 A and B) and the mechanical referred hyperalgesia induced by i.cl. capsaicin 0.1% (Figure 4.12 C and D).

The number of pain responses was significantly reduced at the lowest doses tested, i.e., 1 mg/kg of oxycodone and 0.04 mg/kg of fentanyl (Figure 4.12 A and B). However, the referred mechanical hyperalgesia required to reach statistical significance 0.1 mg/kg of fentanyl whereas a dose of 3 mg/kg of oxycodone was needed (Figure 4.12 C and D). Interestingly, we found that the opioids under study were significantly more potent against the number of behaviors than in the mechanical threshold. Thus, oxycodone showed an ED_{50} = 1.52±0.25 mg/kg *vs* 3.26±0.15 mg/kg, and fentanyl 0.08±0.09 mg/kg *vs* 0.12±0.14 mg/kg, for the pain-related behaviors and referred mechanical hyperalgesia, respectively (Figure 4.12).

Therefore, the μ-opioid agonists evaluated totally inhibited in a dose-dependent way the visceral pain evoked by 0.1% capsaicin, indicating that this model is sensitive and suitable to study drugs with potential for the treatment of visceral pain.

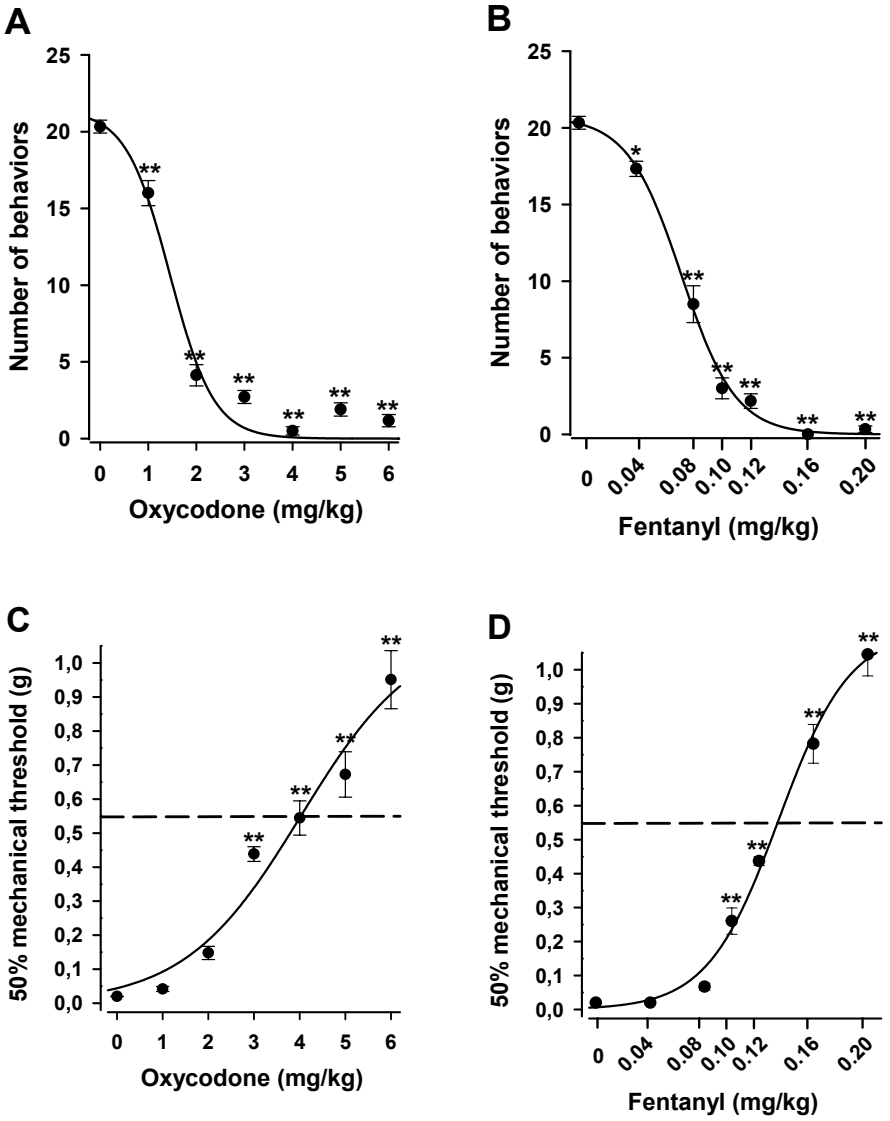

Figure 4.12: Dose-response effects of oxycodone (1-6 mg/kg) and fentanyl (0.04-0.2 mg/kg) on the pain-related behaviors (A and B) and the referred mechanical hyperalgesia (B and C) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) mice. Subcutaneous administration of the opioids or its solvent was performed 30 min before i.cl. administration of capsaicin. The dashed line in the graphs indicates the 50% threshold force in naïve WT mice. Each point and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in opioid- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test)

4.3.2 Potentiation of the effect of oxycodone and fentanyl by pharmacological inhibition of σ1R

To explore whether the observed enhancement of systemic morphine antinociception induced by the σ1R blockade was shared by other μ agonists on the visceral pain induced by 0.1 % capsaicin, we co-administered s.c. the highest doses tested of σ1R antagonists (S1RA 32 mg/kg, NE-100 8 mg/kg, BD-1063 16 mg/kg, and BD-1047 16 mg/kg) plus oxycodone and fentanyl (Figure 4.13). We used doses of μ agonists that *per se* induced little (oxycodone 1 mg/kg and fentanyl 0.04 mg/kg) or marked (oxycodone 2 mg/kg and fentanyl 0.08 mg/kg) antinociception on the spontaneous pain responses (Figure 4.12 A and B) but had no effects on the referred mechanical hyperalgesia (Figure 4.12 C and D). This experimental approach was used in order to test possible differences in the potentiation depending on the effect induced by the opioids before the coadministration.

All the σ1R antagonists co-administered with oxycodone and fentanyl (at low or high doses) enhanced their antinociceptive effects in both types of pain (Figure 4.13). On the pain responses, the potentiation was more evident after the association of the σ1R antagonists with the low dose of the opioids (oxycodone 1 mg/kg and fentanyl 0.04 mg/kg), since the high doses of μ agonists (oxycodone 2 mg/kg and fentanyl 0.08 mg/kg) induced a patent reduction of the pain responses *per se* (Figure 4.13 A). On the referred mechanical hyperalgesia, the association of the low doses of opioids with the σ1R antagonists exerted a clear potentiation of fentanyl (0.04 mg/kg) and oxycodone (1 mg/kg) (Figure 4.13 B). The co-administration of σ1R antagonists with the high dose of oxycodone and fentanyl produced a strong analgesic action (Figure 4.13 B).

Therefore, we confirmed that the enhancement of the μ-opioid analgesia by the selective σ1R blockade is a general pattern and could be a clinical strategy for the treatment of visceral pain.

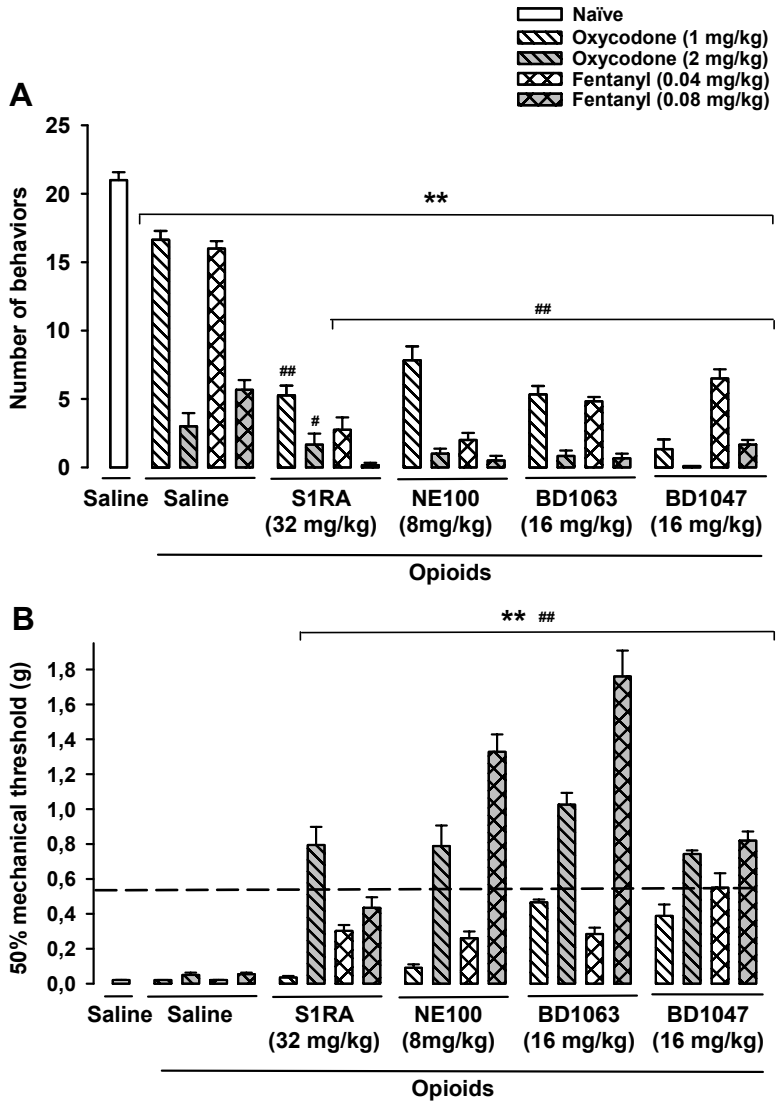

Figure 4.13: Effects of oxycodone (1-2 mg/kg) and fentanyl (0.04-0.08 mg/kg) associated with S1RA (32 mg/kg), NE-100 (8 mg/kg), BD-1063 (16 mg/kg) and BD-1047 (16 mg/kg) on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) mice. Subcutaneous (s.c.) administration of opioids or its solvent was performed 30 min before i.cl. administration of capsaicin, whereas the s.c. administration of the σ1R antagonists was performed 5 min before the opioid administration. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in co-administrated drugs- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in co-administrated drugs- and opioid-injected mice treated with capsaicin: $\#p < 0.05$; $\#\#p < 0.01$ (one-way ANOVA followed by Bonferroni test).

4.3.3 Effects of the μ-opioid antagonists naloxone and naloxone methiodide, and the selective σ1R agonist PRE-084 on the antinociception induced by oxycodone and fentanyl

In a complementary study, we investigated the role of the central and peripheral opioid receptors in the antinociception induced by oxycodone and fentanyl. In addition, we studied the effect of the σ1R agonist PRE-084 associated with these μ-opioid agonists. Similarly to the previous experiment with morphine, we used naloxone and naloxone methiodide to study the central or peripheral opioid receptors in the antinociception induced by the μ-opioid analgesics tested. Also, we tested doses of the μ-agonists that *per se* induced an antihyperalgesic effect (oxycodone 3 mg/kg and fentanyl 0.12 mg/kg) or an analgesic effect (oxycodone 5 mg/kg and fentanyl 0.16 mg/kg) on the referred mechanical hyperalgesia (see Figure 4.12 C and D).

The pretreatment with naloxone (1 mg/kg) reversed the antinociceptive effects induced by the μ-opioid agonists at all doses tested in both the spontaneous pain responses and mechanical hyperalgesia induced by i.cl. capsaicin (Figure 4.14). However, the association with naloxone methiodide (2mg/kg) partially reverses the effects of both doses of the two μ-opioid agonists on the spontaneous pain responses (Figure 4.14 A). On mechanical hyperalgesia, naloxone methiodide (2mg/kg) also partially blocked oxycodone analgesic effects (5 mg/kg) whereas oxycodone- and fentanyl-induced antihyperalgesia (3 mg/kg and 0.12 mg/kg, respectively), and fentanyl-induced analgesia (0.16 mg/kg) were blocked by pretreatment with naloxone methiodide (Figure 4.14 B). The administration of the selective σ1R agonist PRE-084 (32 mg/kg) did not modify the effects of oxycodone and fentanyl at any dose tested in either pain-related responses or referred mechanical hyperalgesia (Figure 4.14 A and B, left panel).

Hence, in agreement with morphine, these results indicate that peripheral opioid receptors contribute, at least in part, to antinociception induced by oxycodone and fentanyl, and the activation of σ1R do not play any role in their pharmacological actions (differentially to the σ1R blocked).

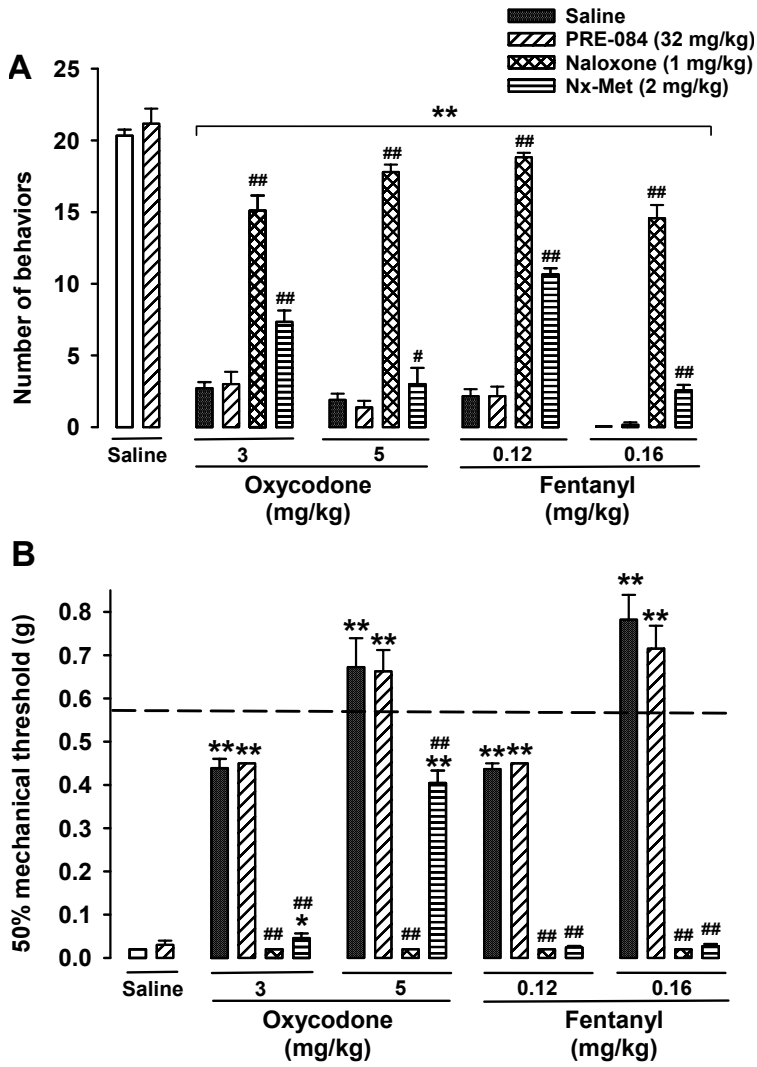

Figure 4.14: Effects of PRE-084 (32 mg/kg), naloxone (1 mg/kg) and naloxone methiodide (2 mg/kg) on the antinociception induced by oxycodone (3-5 mg/kg) and fentanyl (0.12-0.16 mg/kg) on the pain-related behaviors (A) and the referred mechanical hyperalgesia (B) induced by intracolonic (i.cl.) administration of 0.1% capsaicin in wild-type (WT) mice. Subcutaneous (s.c.) administration of opioids or its solvent was performed 30 min before i.cl. administration of capsaicin; whereas the s.c. administration of each agonist/antagonist was performed 5 min before the opioid administration. The dashed line in the B graph indicates the 50% threshold force in naïve WT mice. Each bar and vertical line represents the mean ± SEM of values obtained in at least eight animals per group. Statistically significant differences between the values obtained in co-administrated drugs- and saline-injected mice treated with capsaicin: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in co-administrated drugs- and opioid-injected mice treated with capsaicin: $\#p < 0.05$; $\#\#p < 0.01$ (one-way ANOVA followed by Bonferroni test).

5 Discussion

5.1 Effects of TTX in mouse models of visceral pain

This section of the Thesis is the first study detailing the actions of systemic TTX in pure visceral pain models. The main findings were that TTX administration reduced both the pain responses and the referred mechanical hyperalgesia in colonic and cystitis pain models, and that the TTX-S channel $Na_v1.7$ was not involved in those effects.

First, our results show that the subcutaneous administration of TTX at the doses tested (1-6 μg/kg) herein ameliorated the visceral pain. These doses of TTX were chosen based on the literature and previous studies showing safety and lack of toxicity [148, 162]. In mice, the toxicity of TTX depends on the route of of administration, and the reported lethal values after s.c. administration of TTX were 12.5-16 μg/kg and 8-10 μg/kg for the lethal dose LD_{50} and the minimal lethal dose, respectively [303, 304]. We found no signs of toxicity or motor incoordination in the Rotarod test after the administration of the highest dose tested. These results are in agreement with previous data on the Rotarod test using mice [162] and rats [160].

TTX has been tested before in the acetic acid-writhing test where it significantly reduced the number of abdominal contractions [148]. This test is a widely considered model of inflammatory and visceral pain, although this irritant combines visceral and somatic mechanisms of peritoneal pain [298] and exhibits a lack of pharmacological specificity (i.e., non-analgesic drugs can inhibit the writhings) [305]. Also, this model generates only brief acute reactions and does not reproduce any clinically relevant condition of visceral pain observed in humans such as the referred pain to the abdominal wall. By contrast, the animal models used in the present study permits exploration of both visceral pain-related responses and referred hyperalgesia, so they are considered appropriate translational models of visceral pain. In particular, the intracolonic capsaicin model in mice resembles

the responses observed in a human experimental model after the application of capsaicin to the human gut. The cyclophosphamide cystitis in rodents derives from the observation of the human patients treated with this anti-tumoral agent, thus it similarly mimics a human visceral pain condition.

We found in our study that TTX dose-dependently inhibited the number of pain-related behaviors in both colonic models (capsaicin 1% and mustard oil 0.1%) but only reversed the referred mechanical hyperalgesia induced by i.cl. capsaicin. The pain responses induced by i.cl. capsaicin and mustard oil are attributable to the direct stimulation of colonic nociceptors [298, 306]. Thus, the spontaneous pain behaviors induced by capsaicin and mustard oil are sustained by ongoing activity in nociceptors sensitized by the initial application of the irritants and, as such, can be partially considered as acute pain responses. It has been shown that systemic administration of TTX had no effect on somatic acute pain induced by thermal, mechanical or chemical stimuli [156]. Apart from the results in the acetic acid test, to our knowledge, the effect of TTX has been only tested in one model of chemical pain, the formalin test [148]. The formalin test is a commonly used animal model and comprises a first phase (acute pain) driven by nociceptor activation followed by a second phase associated with inflammation and spinal cord hypersensitivity [270]. In this test, Marcil and co-workers [148] found that TTX had no effect in the early or acute phase but decreased the pain scores in the second phase in rats. The early phase of the formalin test occurs typically in the first 5 min and the second phase starts from 10-15 min and lasts about 40-60 min after the injection [305]. In our study, the i.cl. capsaicin- and mustard oil-induced responses were evaluated for a time period much longer (20 min) than that of acute pain induced by i.pl. formalin (5 min) and, therefore, TTX might be acting in an inflammatory or sensitized pain state. In fact, TTX has been shown previously to reduce the pain behaviors in the second phase of the formalin test and the mechanical hyperalgesia induced by carrageenan in rats [148, 157]. In any case, there are no reported results of TTX in spontaneous/acute pain models using chemical stimulus in mice to compare with our results and further studies in somatic pain could be help to clarify this issue.

In contrast to the pain-related behaviors data, s.c. administration of TTX only reversed the referred mechanical hyperalgesia induced by i.cl. capsaicin but had no effect on the mustard oil-induced referred pain. Besides the inflammation pain models (i.e., formalin and carrageenan tests), the actions of TTX in pain hypersensitivity have been previously documented in somatic pain models [156].

In particular, the reduction of mechanical hypersensitivity induced by i.pl. capsaicin [264] and the neuropathic pain responses induced by mechanical stimulation [161, 162] have been well established. Thus, the inhibition of the mechanical referred pain induced by i.cl. capsaicin is in agreement with previous reports showing that TTX exerts antihyperalgesic effects in rodents [156].

Regarding the inability of TTX to inhibit the referred hyperalgesia in response to i.cl. mustard oil, there are differences between the two irritants that could explain this differential effect. Both compounds activate different subtypes of the TRP channel family, notably capsaicin is a TRPV1 agonist whereas mustard oil is a TRPA1 [307]. Thus, the differential effects of TTX against the referred mechanical hyperalgesia induced by these compounds may be related with the differential noxious activation via TRPV1 or TRPA1. However, it has been shown that mustard oil activates TRPV1 in nociceptive neurons, supporting the role of TRPV1 as a direct mediator of mustard oil-induced irritation [308]. Since TTX reverses the referred hyperalgesia induced by capsaicin through TRPV1 stimulation, and mustard oil also activates this channel, TTX might inhibit the mustard oil-induced referred hyperalgesia, but this has not been observed in our study. Another possible explanation of this lack of effect of TTX in reversing the hyperalgesia evoked by mustard oil may be related with the differential severity or type of the injury caused by both algogens. Although the i.cl. administration of either capsaicin or mustard oil evoked similar referred hyperalgesia in control animals, mustard oil produces direct tissue damage and a very pronounced inflammatory response, whereas capsaicin evokes a pure neurogenic inflammation [151, 298]. Thus, it could be possible that the type of lesion generated by each irritant can influence the antihyperalgesic efficacy of TTX. Previous studies have reported that the administration in mice of the non-steroidal anti-inflammatory drug ketoprofen suppresses the pain-related behaviors but not the referred pain after i.cl. mustard oil [309], whereas ketoprofen was reported to have no effect in either type of pain after the instillation of capsaicin [298]. By contrast, morphine was able to inhibit pain behaviors and referred hyperalgesia after the i.cl. administration of both mustard oil and capsaicin in these studies [298, 309]. These results indicate that the same drug can exert distinct efficacy for alleviating visceral pain depending on the algogen used and the pain responses recorded. Taken together, along with our data, it seems likely that the difference in the antihyperalgesic efficacy of TTX might be due to the damage produced by mustard oil compared to capsaicin.

TTX reduced the behavioral pain score and the referred mechanical hyperalgesia induced by the systemic administration of cyclophosphamide. cyclophosphamide produces cystitis by gradual accumulation of a toxic metabolite (acrolein) in the bladder, and thus is a model of tonic noxious chemical stimulation and inflammatory visceral pain [300]. After the administration of cyclophosphamide, the acrolein accumulates during the 4h of observation, and this slow accumulation is accompanied by a progressive increase in pain behaviors and a considerable bladder inflammation. As mentioned above, it was previously reported that TTX plays a role in reducing inflammatory pain in somatic pain models. Here, TTX was injected 2h after the cyclophosphamide and the behavioral pain score was recorded during the next 2h, hence, the effect of TTX in this model could be associated with its proved capacity to attenuate somatic inflammatory pain. Furthermore, cyclophosphamide also induces a neurogenic inflammation and sensitization [151]. Therefore, the antihyperalgesic effect of TTX in this model is consistent with the reduction of the referred hyperalgesia observed after the i.cl. capsaicin since it also induces a neurogenic inflammation.

According to previous studies, morphine totally abolished the spontaneous pain and induced a clear analgesic effect on the referred pain (i.e., evoked a much higher threshold than that observed in naïve animals) in both capsaicin [284] and mustard oil models [298]. Also, the responses and mechanical hyperalgesia were greatly attenuated in the cyclophosphamide model after the administration of morphine, as was previously reported [310]. Consequently, these results suggest that all types of behaviors evaluated were pain-related.

Since we administered TTX systemically, the present effects can be peripherally or centrally mediated. Our data in the Rotarod test indicate that TTX did not affect the CNS, thus suggesting a peripheral action. Also, a higher concentration (8 μg/kg) than that used here did not alter the contralateral paw withdrawal responses in a burn wound pain model in rat [311], supporting a lack of central effects. In addition, TTX is a hydrophilic compound which barely crosses the blood-brain barrier, thus entry to the CNS is limited [303], and we have previously reported peripheral effects using the same doses of TTX (1-6 μg/kg) in a model of neuropathic pain induced by paclitaxel [162]. Accordingly, the inhibition of pain responses and antihyperalgesic effects of TTX observed in the present study might be interpreted through peripheral actions.

The effects of TTX in the present study could be attributable to one or several TTX-sensitive VGSCs such as $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.4$, $Na_v1.6$, and

$Na_v1.7$. However, our data using a conditional nociceptor-specific $Na_v1.7$ knockout mouse (KO-$Na_v1.7$) suggest that subtype $Na_v1.7$ expressed in $Na_v1.8$ positive neurons is not fully required for visceral pain. In agreement with this finding, it has been recently reported that $Na_v1.7$ does not play a role in visceral pain and that these KO-$Na_v1.7$ mice have lost almost all visceral sensory neurons [149]. Then, the actions of TTX herein must be theoretically mediated by one or more different VGSCs subtypes, since the highest dose administered (6 μg/kg) evoked the same responses in both KO-$Na_v1.7$ and littermate controls. Moreover, the application of TTX did fully block afferent firing to noxious phasic distension in KO-$Na_v1.7$ mice [149]. Nevertheless, we cannot discard the possibility that some $Na_v1.7$-positive neurons lacking $Na_v1.8$ expression remain active, which may be enough to sustain pain responses. Among the remaining TTX-sensitive subtypes, $Na_v1.3$ has been proposed to play a role in pain, although contradictory data between several animal studies have been published [156, 312, 313]. $Na_v1.6$ [314, 315, 316] and $Na_v1.1$ [317] have also been reported to play a role in several pain conditions including visceral pain. $Na_v1.2$ is abundantly expressed in the adult CNS but does not seem to be involved in pain, whereas $Na_v1.4$ is almost restricted to the skeletal myocyte [156, 312]. All these TTX-sensitive VGSCs have been found to be present in significant proportions (except for $Na_v1.4$, which showed very low expression) in lumbosacral and thoracolumbar colonic sensory neurons in mice [149]. However, we cannot determine whether the effect of TTX was produced by the blockade of one or various of these TTX-sensitive subtypes, and further research is needed to elucidate this issue.

In summary, our data indicate that systemic administration of TTX could have a potential therapeutic use for treating clinical visceral pain, since the animal pain models used herein have translational value and they have been validated in humans.

5.2 Improving opioid analgesia by blocking the σ1R in capsaicin-induced visceral pain

The second section of the Thesis includes two complementary studies employing the visceral pain model evoked by i.cl. administration of capsaicin 0.1%: 1) the assessment of the modulation of morphine-induced visceral analgesia by σ1R blockade using genetic (σ1R-KO mice) and pharmacological (σ1R antagonists) approaches, and 2) the modulatory role of pharmacological σ1R inhibi-

tion on the visceral antinociception induced by the μ-opioid agonists fentanyl and oxycodone.

To our knowledge, this is the first study demonstrating the proposal of a new therapeutic strategy based on the synergy between opioids and σ1R antagonists in the control of visceral pain. This study extends the results found in previous studies reporting the σ1R blockade as a possible therapeutic strategy in the treatment of visceral pain [284, 285]. The main findings of this study were that the association of selective σ1R antagonists with μ-opioid agonists, commonly used as therapies in the treatment of visceral pain, potentiates the antinociceptive effect of those and that this potentiation is mediated, at least in part, by peripheral opioid receptors. First, we found that the drug combination induced an improvement of opioid analgesia through a dose-dependent inhibition of spontaneous pain-related behaviors and referred mechanical hyperalgesia evoked by i.cl. capsaicin. In addition, we found that there were differences on the central or peripheral regulation of morphine-induced analgesia when the morphine was administered alone or it was associated to the σ1R antagonists.

The lack of σ1R in KO mice has been shown to not interfere with the pain development process in nociceptive (tail-flick [259], von Frey [264], hot plate [259] and paw pressure [129] tests) and inflammatory pain (thermal hyperalgesia and mechanical allodynia in carrageenan [279] and complete Freund's Adjuvant [288] tests); thermal hyperalgesia in the model of neuropathic pain based in partial sciatic nerve ligation [219, 259]; and referred mechanical hyperalgesia in capsaicin and cyclophosphamide induced visceral pain [284]. On the contrary, σ1R-KO mice showed attenuated pain responses following chemical sensitization in both phases of the formalin test [219, 269, 271] and did not develop mechanical allodynia following intraplantar capsaicin sensitization [219, 264] or mechanical hyperalgesia following paw pressure test in the inflammatory carrageenan model [279]. The cold and mechanical hypersensitivity were strongly attenuated in σ1R-KO mice treated with paclitaxel [280, 286] or exposed to partial sciatic nerve ligation [219, 259]. Regarding visceral pain, the number of pain-related behaviors and spontaneous pain in capsaicin and cyclophosphamide induced visceral pain [284, 285] respectively, were also significantly lower in σ1R-KO mice than in WT mice.

We found that morphine produced a dose-dependent reduction of capsaicin-induced behavioral responses and mechanical hyperalgesia and, the analgesic effects of the opioid were greater in σ1R-KO than in WT mice. This is in agreement with previous studies in models of somatic and visceral pain [129, 232, 284,

285, 318]. In the case of the other μ-opioid agonists tested in WT mice (oxycodone and fentanyl), they also induced marked antinociceptive effects in a dose-dependent manner. Oxycodone and fentanyl are also common in clinical practice for the treatment of pain [114, 319], but they had not been tested before in a pure visceral pain model. These results are consistent with other studies on somatic and visceral pain [232, 318, 320, 321]. Although all opioids achieved 100% efficacy, the potency of each drug was different and varied also between each of the experimental approaches tested (i.e., acute pain and referred pain). In all cases the pharmacological potency was higher in the number of responses than in the referred mechanical hyperalgesia. Then, we tested the reduction of behavioral manifestations induced by capsaicin due to σ1R inhibition administering σ1R antagonists alone in WT mice. We found a dose-dependently inhibition of the number of behaviors evoked by capsaicin, but none of the σ1R antagonists at the administered doses was able to modify the referred mechanical hyperalgesia. These effects of σ1R antagonists are consistent with previous findings in several pain models [129, 219, 279], including visceral pain [284, 285].

The modulation of opioid antinociception by σ1R was described decades ago by Chien and Pasternak [322]. The physical interaction between σ1R and μ-opioids has also been studied, identifying the modulation of opioid G-protein coupled signal transduction as a mechanism underlying the σ1R modulation of the effects of opioids [206]. A previous study by our group in somatic pain supported a functional link between peripheral σ1R and the μ-opioid system, rather than interactions of σ1R ligands with μ-opioid receptors or opioid drugs with σ1R [232]. When we examined the association of the tested opioids with the σ1R antagonists in the present model of visceral pain, the result was in line with the previous data observed in somatic pain. The subcutaneous association of σ1R antagonists (S1RA, NE-100, BD-1063 and BD-1047) to a sub-analgesic dose of morphine (2 mg/kg) exerted a further enhancement of the opioid-induced analgesia in WT mice. All the σ1R antagonists tested showed almost the same inhibition of pain-related behaviors. Noteworthy, in referred mechanical hyperalgesia, the selective σ1R antagonist S1RA (32 mg/kg) exerted the highest potentiation on morphine-induced analgesia. This may be due to the major selective affinity by S1RA for σ1R than the other σ1R ligands assessed [219]. This result is in concordance with other report involving the same σ1R antagonists associated to cannabinoids, where the maximum potentiation was also observed with S1RA [245]. Morphine is the opioid of choice for the most studies evaluating its synergy with σ1R antagonists, but only it has been previously studied in so-

matic (thermal [318] and mechanical [232]) pain models. It has been reported that several central penetrance opioids (morphine, oxycodone, fentanyl, buprenorphine and tramadol) used at doses that induce little or no antinociception have a marked antinociceptive effect when coupled with σ1R blockade (using the σ1R antagonists S1RA and BD-1063). This is the reason to assess additionally the association of oxycodone and fentanyl with σ1R antagonists in a pure visceral pain model. The s.c. co-administration of the highest doses tested of σ1R antagonists with doses of oxycodone and fentanyl (non-analgesic on the referred mechanical hyperalgesia or that induced little or marked analgesia on the pain-related behaviors) reduced both the acute and the referred pain with all the selective σ1R antagonists. These results clearly show that the modulation of μ-opioid antinociception by the selective σ1R blockade is not restricted only to an specific opioid but is a general pattern for this type of pain. We found that none of the σ1R antagonists enhanced morphine-induced analgesia in σ1R-KO mice. This lack of potentiation of morphine analgesia in σ1R-KO mice supports that the effects of the σ1R antagonists are specifically mediated by their interaction with σ1R. In addition, since the dose of morphine used (2 mg/kg) showed a different analgesic pattern in both types of mice (higher effectiveness in σ1R-KO than in WT mice) we administered an equivalent morphine dose (0.5 mg/kg) in terms of efficacy in σ1R-KO mice (compared to the effect of WT mice treated with 2 mg/kg) plus the same dose of σ1R antagonists and we found no change in morphine analgesia. Thus, these results confirm the selectivity of the effects induced by the σ1R antagonists given their lack of effect in σ1R-KO mice. On the other hand, and since, to the best of our knowledge, it has not been proven before in any other visceral pain model, we conducted a complementary study to demonstrate the specificity of μ-opioid antinociception induced by oxycodone and fentanyl. We demonstrated that the analgesic/antihyperalgesic effect of opioids is not due to σ1R activation. To this end, we administered a dose of 32 mg/kg of the selective σ1R agonist PRE084, that has been shown to block the effect of σ1R antagonists in other models of somatic pain [294]. We found no differences regarding analgesia per se for both oxycodone and fentanyl. This demonstrates that σ1R agonism does not affect the antinociception induced by these opioids, unlike in other somatic pain models where the 32 mg/kg dose of PRE-084 has been shown to possess pronociceptive effects and thus potentiate mechanical hyperalgesia [323].

In an attempt to elucidate the central and/or peripheral localization of the mechanism of action of the tested opioids in the presence or absence of pharmaco-

logical blockade of the σ1R, we conducted studies with the opioid antagonists naloxone and naloxone methiodide.

As expected, naloxone completely reverses the effect of opioids on both referred mechanical hyperalgesia and pain-related behaviors. Then, we studied the role of peripheral opioid receptors in capsaicin-induced visceral pain by administering the peripheral opioid antagonist naloxone methiodide associated to a dose of morphine (4 mg/kg) that produced analgesia on the referred pain. We found, in agreement with previous studies in somatic [232, 235, 324] and visceral [325] pain, that the analgesic effects of high doses of morphine were not reversed by naloxone methiodide. This is probably due to a preferential localization at central levels of the analgesic effects of μ-opioids [232, 324, 325]. For acute pain responses, when we increased the dose of naloxone methiodide (8 mg/kg), we observed a significant reversion, although the referred mechanical hyperalgesia was not affected. This may be in line with the results obtained by Montilla-García [235]. They demonstrated, when comparing mechanical and thermal stimuli, that high doses of morphine with any dose of naloxone methiodide tested was not blocked in mechanical tests and, only a low dose of naloxone methiodide was sufficient to reverse thermal stimuli. Sánchez-Fernández [232] also demonstrated in a mechanical somatic pain test the blockade of a low dose of morphine with naloxone methiodide. Taking all this into account and to try to shed light on this different behavior, we administered a lower dose of morphine, which induced an antihyperalgesic effect instead of an analgesic effect (3 mg/kg). The result was that we still found that naloxone methiodide did not affect morphine in acute responses, but on the referred pain there was a full reversion. The differential responses observed in behavior administering the same dose of the opioid in each experimental approach could be explained by the different characteristics of the experiment. In our case, the record procedure for the pain-related behaviors is only experimental, whereas the determination of the referred mechanical hyperalgesia requires the experimenter intervention. Furthermore, it should be emphasized that the first comprises the mean of the responses that occur during a period of time that ranges from 30 to 50 minutes after the administration of the opioid whereas the second is just performed at the end of the first, i.e. 50 minutes after the drug has been administered. Similarly, we also extended the study to different doses of the other two opioids. We selected two different doses for each opioid: one low, with antihyperalgesic effects; and the other, a higher dose, that produced analgesia on the referred mechanical pain. The result was that naloxone methiodide reversed significantly the effect of the opioids

in both pain conditions. On the one hand, both doses of oxycodone showed partial sensitivity to naloxone methiodide. This has also been described previously in somatic pain models [232, 235] and is consistent with clinical data [326]. For fentanyl, a similar situation occurred for the number of pain-related behaviors while on the referred mechanical hyperalgesia there was a total reversal of opioid analgesia. The last may be due to the fact that fentanyl penetrates very quickly into the CNS, reaching a maximum of central action in 4-5 minutes, then it is redistributed to plasma, muscle and fat tissues, where it accumulates, constituting deposit organs from which it will spread again according to the gradients. All this means that the analgesia and other central actions disappear in 30 minutes but when high or successive doses of fentanyl are administered the elimination half-life increases. Therefore, the effects are longer lasting and there may even be accumulation, so some of the analgesic effects may be mediated peripherally [110].

To shed more light on the mechanism involved in the μ-opioid agonist - σ1R antagonist interaction, we tested morphine combinations with naloxone and naloxone methiodide. The opioid antagonist naloxone fully reversed the potentiation of morphine-induced analgesia by all σ1R antagonists tested on the referred mechanical hyperalgesia. However, naloxone was only able to partially antagonize the analgesic effects of morphine potentiation, at approximately the level of σ1R antagonists *per se* on pain-related behaviors. The results of naloxone methiodide on morphine analgesia potentiated by σ1R inhibition were very similar to the obtained with naloxone, i.e., full reversion of the referred mechanical hyperalgesia, and partial reversion of the pain-related behaviors. Thus, this fact may indicate that the antagonistic effects on the μ receptor, when it is potentiated by σ1R blockade, occur at the peripheral level. This is again consistent with the studies performed by our group in somatic pain [232, 235].

In recent years, considerable preclinical and clinical efforts have been made to find an effective treatment for visceral pain. The clinical utility of opioid receptor agonists for the treatment of pain continues to be limited by a compromise between efficacy and side effects (including constipation, sedation, respiratory depression, hyperlocomotion and nausea) that cause patients to discontinue treatment (reviewed by [94]). Nowadays, at the clinical level, several strategies have been suggested to minimize the adverse effects of opioids and improve the patient's quality of life. On the one hand, an adjuvant drug is usually administered with synergistic analgesic effects for the opioid dose, i.e., trying to reduce its pos-

sible side effects while maintaining analgesic equivalence. As discussed above, this study in a pure visceral pain model corroborates the results obtained in somatic pain in which opioids used in clinical practice are also potentiated by σ1R blockade [129], without increasing the most limiting morphine-induced side effects in mice (i.e., hyperlocomotion and inhibition of gastrointestinal transit). We used a dose of morphine (2 mg/kg) which, although it has some constipation effect, does not affect the locomotor activity (published by [129]). A different approach to reducing opioid side effects is to target peripheral opioid antinociception to minimize centrally mediated undesirable effects (reviewed by [133]). In somatic pain models, it has been demonstrated that σ1R inhibition can enhance the local analgesia of morphine, thus increasing its antinociceptive effect compared to that induced by systemic morphine [232]. Similarly, our results seem to indicate that the antagonistic effects on the μ receptor, when it is potentiated by σ1R blockade, occur at the peripheral level highlighting that the modulation of peripherally mediated opioid analgesia may provide safer and improved therapeutic responses for the treatment of visceral pain.

Finally, on the one hand, i.cl. capsaicin in mice evokes activation of capsaicin receptor TRPV1 and this receptor is known to be expressed throughout the upper and lower gastrointestinal tract in myenteric ganglia, muscle layers, and mucosa in patients with IBS [64]. On the other hand, as it has been above-mentioned the opioid agonists are used in the treatment of gastroenterological pain disorders, but they have undesirable side effects [327] Therefore, the study in this visceral pain model of the systemic combination of opioid activation with σ1R blockade may represent a potential strategy to improve the analgesic profile of opioids in gastrointestinal disorders.

6 Conclusions

6.1 Specific conclusions

1. The subcutaneous administration of the voltage-gated Na^{2+} channel (VGSC) blocker tetrodotoxin (TTX) tested in models of chemical stimulation of the colon (intracolonic instillation of capsaicin and mustard oil) and intraperitoneal cyclophosphamide-induced cystitis, dose-dependently inhibited the number of pain-related behaviors in all evaluated pain models and reversed the referred mechanical hyperalgesia induced by capsaicin and cyclophosphamide, but not that induced by mustard oil.

2. The subcutaneous administration of a high dose of morphine (8 mg/kg) inhibited both spontaneous pain responses and the referred mechanical hyperalgesia in all pain models tested. Consequently, these results suggest that all types of behaviors evaluated were pain-related.

3. No motor incoordination (tested with a Rotarod device) after the administration of TTX was observed. Therefore, the inhibition of pain responses and antihyperalgesic effects of TTX observed in the present study might be interpreted through peripheral actions.

4. The Na^{2+} channel subtype $Na_v1.7$ (expressed in $Na_v1.8$ positive neurons) is not entirely necessary for visceral pain, since the conditional nociceptor-specific $Na_v1.7$ Knockout mice treated with TTX showed the same responses as littermate controls after the administration of the algogens.

5. The subcutaneous administration to wild-type (WT) mice of several μ-opioid analgesics (morphine, oxycodone and fentanyl) reduces dose-dependently both the number of spontaneous pain responses and the referred mechanical hyperalgesia induced by intracolonic administration of capsaicin 0.1%.

6. The subcutaneous co-administration of the selective sigma-1 receptor (σ1R) antagonists (S1RA, NE-100, BD-1047 and BD-1063) dose-dependently potentiates the morphine-induced analgesia in WT mice. Similarly, the association of the σ1R antagonists (at the highest doses tested) with the μ-opioid agonists oxycodone and fentanyl enhances their antinociceptive effects in both pain-related behaviors and referred mechanical hyperalgesia in WT mice.

7. The antinociceptive effects of morphine alone are increased in σ1 receptor Knockout (σ1R-KO) mice indicating that the genetic σ1R blockade also enhances the morphine-induced analgesia. The association of the highest doses of the σ1R antagonists with morphine in σ1R-KO mice do not potentiate its analgesia, confirming the selectivity of the effects induced by the σ1R antagonists.

8. The analgesic effects induced by systemic administration of the μ-opioid agonists *per se* (i.e., in the absence of σ1R inhibition) in WT mice are produced mainly at peripheral levels. This is supported by the administration of the peripherally restricted opioid antagonist naloxone methiodide that antagonizes all the analgesia induced by the tested opioids (except for morphine 3 and 4 mg/kg in the acute pain and referred pain, respectively).

9. The potentiation of morphine analgesia in treated KO-σ1R mice systemically with σ1R antagonists depends on the activation of peripheral opioid receptors as it is abolished by naloxone methiodide.

6.2 General conclusions

1. The systemic administration of TTX could have a potential therapeutic use for treating clinical visceral pain, since the animal pain models used herein have translational value and they have been validated in humans.

2. The study of the systemic combination of opioid activation with σ1R blockade in the capsaicin induced visceral pain model may represent a potential strategy to improve the analgesic profile of opioids in gastrointestinal disorders.

Resumen

1 Antecedentes

El dolor visceral es un trastorno clínico importante en los humanos. La mayoría de los estudios en el campo del dolor y los nociceptores se han centrado únicamente en el sistema sensorial somático/neuropático, pero el procesamiento del dolor visceral es diferente de otras formas de nocicepción [17].
El sistema visceral incluye múltiples canales de iones, neurotransmisores y receptores que son cualitativa y/o cuantitativamente diferentes de los implicados en el dolor somático o neuropático y, por otra parte, tienen un gran número de órganos y sistemas con inervaciones intrínsecas y extrínsecas únicas. Por ello, se espera que los mecanismos del dolor visceral difieran de los del dolor somático [15, 17] y por tanto, los resultados obtenidos en los modelos de dolor somático y/o neuropático no pueden extrapolarse al dolor visceral.

El tratamiento del dolor visceral es complejo y los tratamientos farmacológicos actualmente disponibles tienen una eficacia limitada, por lo que es necesario desarrollar medicamentos eficaces frente a ello [17]. El desarrollo de modelos animales de dolor visceral está permitiendo investigar los mecanismos implicados [295].

La tetrodotoxina (TTX) es una potente neurotoxina. Se cree que las propiedades antinociceptivas de la TTX se deben a la estabilización de las membranas neuronales mediante la inhibición del flujo de iones Na^+ necesario para la iniciación y propagación de los impulsos nociceptivos, especialmente en las condiciones de dolor en las que se produce un aumento de los canales de Na^+ dependientes de voltaje sensibles a la TTX en el sistema nervioso periférico [156]. Se ha demostrado que esta neurotoxina tiene efectos analgésicos y antihiperalgésicos en varias condiciones de dolor somático, incluidos los modelos de dolor nociceptivo [148], inflamatorio [148, 157, 158], muscular [159] y neuropático [148, 160, 162]. Sin embargo, la contribución al dolor visceral de estos canales sensibles a la TTX nunca ha sido investigada en un modelo de dolor visceral puro.

Por otro lado, el receptor $\sigma 1$ es una pequeña proteína cuya estructura no está relacionada con ninguna otra proteína conocida en los mamíferos. El receptor $\sigma 1$ tiene un dominio chaperona dentro de su estructura [172], lo que puede explicar parte de sus propiedades farmacológicas. Se ha demostrado que el receptor $\sigma 1$ está presente en el sistema nervioso periférico, donde se encuentra en una densidad mucho mayor que en las zonas del sistema nervioso central relacionadas con el dolor. Este receptor ha sido estudiado tanto por bloqueo genético como farmacológico en varios modelos de dolor [182, 218, 219, 227, 259, 264, 269]. El receptor $\sigma 1$ también se ha asociado con opioides para mejorar sinérgicamente sus efectos antinociceptivos periféricos y evitar efectos secundarios [129, 232]. En el campo del dolor visceral, se demostró que el receptor $\sigma 1$ juega un papel importante en el modelo de administración intracolónica de capsaicina en ratón [284]. Sin embargo, la implicación del receptor $\sigma 1$ en la analgesia opioide, así como su modulación periférica sigue sin estar clara.

2 Objetivos

- El primer objetivo de esta Tesis fue evaluar los efectos antinociceptivos de la TTX en tres modelos diferentes de dolor visceral en ratón: estimulación química del colon mediante los algógenos capsaicina y aceite de mostaza administrados intracolónicamente; y el antineoplásico ciclofosfamida intraperitoneal como inductor de cistitis.
- El segundo, evaluar la potenciación de la analgesia inducida por morfina mediante el bloqueo genético y farmacológico del receptor $\sigma 1$ y estudiar la modulación periférica de la analgesia μ-opioide *per se* o asociada a los antagonistas del receptor $\sigma 1$ en un modelo puro de dolor visceral, la administración intracolónica de capsaicina.
- Relacionado con el anterior, el tercer objetivo fue corroborar que la potenciación de la analgesia inducida por morfina no es específica de este fármaco y es extensible a otros opioides comúnmente usados en la práctica clínica (oxicodona y fentanilo), además de estudiar la contribución de los receptores periféricos a esta analgesia.

3 Material y métodos

3.1 Material

Los experimentos se llevaron a cabo, por un lado, para los estudios de bloqueo de los canales de Na^+, en ratones adultos de ambos sexos con genotipos salvajes C57Bl/6 y deficientes del receptor $Na_v1.7$ ($Na_v1.7$-KO) así como sus controles compañeros de camada mantenidos en un bagaje C57Bl/6 tal y como se describió previamente [296]. En cuanto a los estudios con el receptor $\sigma 1$, los animales usados fueron ratones hembra adultas de la cepa CD1 tipo salvaje y homozigotos para dicho receptor ($\sigma 1^{-/-}$, σ1-KO) [264]. En ambos casos, los ratones fueron mantenidos en el Centro de Investigación Biomédica de la Universidad de Granada, usados con un peso comprendido entre los 20 y 30 g y aclimatados al menos una semana antes en nuestro laboratorio. Las condiciones ambientales fueron controladas, con ciclos de 12/12h día/noche, temperatura constante (22 $\pm$ 2 °C), reemplazo de aire cada 20 min, y alimentados *ad libitum* con agua y una dieta estándar de laboratorio (*Harlan Teklad Research Diet, Madison, WI, USA*) hasta el comienzo de los experimentos. Los experimentos se llevaron a cabo durante la fase de luz (de 9.00h a 15.00h), y al azar durante el ciclo estral.

En cuanto a los fármacos, se usó el bloqueante de los canales de Na^+ dependientes de voltaje TTX, antagonistas selectivos del receptor $\sigma 1$ (S1RA, NE-100, BD-1063 y BD-1047), el agonista selectivo también del receptor $\sigma 1$ PRE-084, agonistas (morfina, oxicodona, fentanilo) y antagonistas (naloxona y naloxona metiodida) opioides μ. Todos ellos fueron disueltos en salino fisiológico estéril (0.9% NaCl), y los ligandos del receptor $\sigma 1$, además, alcanilizados a un pH neutro mediante NaOH. Todos fueron preparados inmediatamente antes de comenzar los experimentos, y se inyectaron subcutáneamente en el área interescapular. En el caso de los algégenos usamos: capsaicina al 1% o 0,1% según el caso, disuelta en una solución *stock* compuesta por un 10% de etanol absoluto más otro 10% de *Tween* 80 y un 80% de solución salina; aceite de mostaza disuelto en una solución al 70% de etanol absoluto y un 30% de solución salina; y ciclofosfamida disuelta en solución salina. La capsaicina y el aceite de mostaza se instilaron a través del colon (50 μL) y la ciclofosfamida fue inyectada intraperitonealmente en un volumen de 10 ml/kg.

3.2 Métodos

3.2.1 Estimulación química del colon

El dolor visceral se puede medir como número de respuestas espontáneas al dolor que expresan los animales; y la hiperalgesia mecánica referida es proporcional a la intensidad de dichas respuestas [298]. Es por ello que evaluamos simultáneamente en el mismo ratón tanto uno como otro tras haber administrado a través del colon capsaicina (1 ó 0,1% para los estudios del efecto de la TTX y los de la potenciación de la analgesia opioide, respectivamente) o aceite de mostaza (0,1%) como ya se describió previamente [284, 298].

Los animales se situaron en la habitación experimental durante un periodo de habituación de 40 min colocados en unos compartimentos de plástico individuales (7×7×13 cm) situados sobre una plataforma elevada. Después fueron inyectados con los fármacos o solventes de estudio y devueltos al compartimento. Tras 30 min, y después de aplicar baselina en la zona perianal para evitar la estimulación de áreas somáticas tras el contacto con el algógeno, se le inyectó intracolónicamente capsaicina, aceite de mostaza o sus solventes y se devolvieron a sus compartimentos. Las respuestas espontáneas (lamido del abdomen, estiramientos y contracciones abdominales) se contabilizaron durante 20 min en cuatro intervalos de 5 min cada uno. Inmediatamente después de midió la hiperalgesia mecánica referida mediante la aplicación en el abdomen de los filamentos calibrados de *von Frey (Touch-Test Sensory Evaluators; North Coast Medical Inc., Gilroy, CA)*, usando una fuerza de 0,02 a 2 g [299]. Se aplicaron tres veces durante 2-3 s cada una, y dejando un descanso entre ellas de otros 3 s. Se consideraron respuestas positivas el salto, el lamido/estiramiento de la zona de aplicación o la retracción del abdomen [284, 298].

3.2.2 Cistitis inducida por ciclofosfamida

El dolor visceral y la hiperalgesia mecánica referida inducidas por ciclofosfamida se evaluaron según el protocolo descrito [149, 300]. El periodo de habituación de 40 min se llevó a cabo del mismo modo que en el caso de la estimulación del colon. En este caso, tras dicho periodo se inyectaron intraperitonealmente con una solución de 100 mg/kg de ciclofosfamida o salino. Dos horas después se inyectaron subcutáneamente los fármacos o sus solventes y se devolvieron a sus compartimentos. A partir de aquí se registraron las respuestas manifestadas por los animales durante 2 min cada intervalo de 30 min durante un período de 2h.

Estas respuestas se codificaron mediante la escala definida anteriormente [149]. Tras este periodo de observación (4h tras la inyección de ciclofosfamida) se midió la hiperalgesia mecánica referida con los filamentos de *von Frey* tal y como se describió en la sección anterior 3.2.1.

3.2.3 Evaluación de la coordinación locomotora

Las alteraciones en la coordinación motora se evaluaron mediante el test del Rotarod (Ugo Basile, Comerio, Italia) tal y como se describió previamente [301]. Tras el mismo protocolo de habituación que en el resto de experimentos pero situados en las mismas cajas en las que comúnmente se encuentran, los animales fueron inyectados con los fármacos o sus solventes y colocados en el Rotarod. El Rotarod se programó para que acelerase desde 4 a 40 rpm durante 5 min, con un tiempo de corte de 300 s. Se evaluaron a los tiempos 0, 30, 60 y 120 min tras el tratamiento. Previo a esto se llevaron a cabo tres sesiones de entrenamiento separadas en intervalos de 30 min el día anterior al ensayo.

3.3 Análisis de datos

El grado de dolor referido se midió como el umbral mecánico que produce el 50% de las respuestas [302]. Los valores de la dosis efectiva del medicamento que produce el efecto deseado en el 50% de la población y sus errores estándar de la media (ED_{50} y SEM por sus siglas en inglés, respectivamente) se calcularon usando análisis de regresión no-lineal sobre la sigmoidal y se compararon las medias mediante el test F de *Snedecor*. Se consideró significancia estadística a valores de * $p < 0{,}05$. Cuando se comparó el número de respuestas dolorosas y los umbrales mecánicos entre grupos experimentales se hicieron ANOVAs de una o dos vías seguidas de test de *Bonferroni*; y cuando se compararon dos medias se usó el test de la t de *Student* para valores no pareados. Cada barra/punto y sus barras verticales representan la media $\pm$ SEM de los valores obtenidos en al menos ocho animales por grupo. Las líneas punteadas indican el número de respuestas en ratones tratados con salino e inyectados con capsaicina o el 50% del umbral mecánico en ratones tratados con salino. Se consideraron estadísticamente significativas las diferencias entre los valores cuando * $p < 0{,}05$ y ** $p < 0{,}01$ tras el test. Para ello, usamos los programas *Sigma Plot 12.0 (Systat Software Inc., San José, CA, USA)* y *GraphPad Prism 5.00 program (GraphPad Software Inc., San Diego, CA, USA)*.

4 Resultados

4.1 Efectos de la TTX en distintos modelos animales de dolor visceral y en la coordinación locomotora

4.1.1 Efectos de la TTX tras la estimulación química del colon: administración intracolónica de capsaicina 1% y aceite de mostaza 0,1%

Para evaluar el efecto de la TTX en el dolor visceral puro usamos dos modelos animales distintos: la administración intracolónica de capsaicina (1%) (Figure 4.1) y de aceite de mostaza (0,1%) (Figure 4.2) .

En ambos casos la neurotoxina (1-6 μg/kg) produjo una reducción en el número de comportamientos dolorosos (lamidos, estiramientos y contracciones del abdomen) siguiendo una curva dosis-respuesta en animales controles de genotipo salvaje. Como fármaco control analgésico usamos la morfina en una dosis de 8 mg/kg, capaz de abolir completamente las respuestas dolorosas tanto en un modelo como en el otro. En el caso de la hiperalgesia mecánica referida, la neurotoxina revirtió la hipersensibilidad mecánica inducida por capsaicina también de forma dosis-dependiente mientras que en el caso del aceite de mostaza fue incapaz de hacerlo a las dosis estudiadas. Como era de esperar, la morfina también fue capaz de abolir el efecto del algógeno en ambos casos.

4.1.2 Efectos de la TTX sobre la cistitis inducida por ciclofosfamida

Para evaluar el efecto de la TTX en el dolor originado en un órgano visceral distinto, usamos el modelo de dolor de la vejiga/cistitis inducidos por ciclofosfamida. La ciclofosfamida administrada vía intraperitoneal produce un desarrollo progresivo de comportamientos dolorosos viscerales.

La administración de TTX redujo de forma dosis-dependiente la puntuación de respuestas dolorosas, pero ninguna de las dosis estudiadas fue capaz de abolirlas completamente (Figure 4.3). Esto ocurrió también con el fármaco control, la morfina, a dosis de 8 mg/kg, que aunque las reduce significativamente no es capaz de abolirlas como ocurría en los modelos de estimulación química del colon. En el caso del umbral mecánico, la neurotoxina tampoco es capaz de reducirlo completamente, pero sí se puede comprobar con el fármaco control que en este caso, además de revertir el umbral, muestra un pronunciado efecto analgésico.

4.1.3 Efectos de la TTX en los modelos de dolor visceral estudiados en ratones $Na_v1.7$-KO

Con la finalidad de estudiar el posible papel de los canales de Na^+ $Na_v1.7$ en estos modelos de dolor visceral usamos los ratones $Na_v1.7$-KO que poseen una rotura específica de estos canales en las neuronas positivas para el canal $Na_v1.8$.

Estos animales, así como sus compañeros de camada usados como controles, se comportaron de igual forma que los de genotipo salvaje cuando fueron sometidos a una inducción de dolor visceral con cada uno de los algógenos estudiados tanto en presencia como en ausencia de TTX (Figure 4.4). Estos resultados inducen a pensar que los canales $Na_v1.7$ expresados en las neuronas sensitivas no son necesarios para el efecto de la TTX.

4.1.4 Coordinación locomotora tras la administración de TTX

Los animales tratados tanto con neurotoxina como con morfina se evaluaron en el test de coordinación locomotora Rotarod. El resultado fue que los animales tratados con las dosis más altas evaluadas en los otros modelos de TTX, así como con morfina, nuevamente en una dosis de 8 mg/kg, no mostraron valores de latencia diferentes a los basales o a los controles salinos (Figure 4.5). Esto significa que la TTX no induce ningún efecto disruptor de la locomoción.

4.2 Potenciación de la analgesia inducida por morfina mediante el bloqueo de los receptores $\sigma 1$ en el dolor visceral inducido por capsaicina 0,1% intracolónica

4.2.1 Efectos de la analgesia morfínica en ratones de genotipo salvaje y KO para el receptor $\sigma 1$

Evaluamos los efectos antinociceptivos inducidos por la administración subcutánea de morfina en el modelo de dolor visceral inducido por capsaicina 0,1% tanto en animales de genotipo salvaje como de genotipo KO para el receptor $\sigma 1$.

La morfina (0,5-16 mg/kg) revirtió de forma dosis-dependiente las respuestas dolorosas y la hiperalgesia referida tanto en un genotipo de ratones como en el otro, aunque con un patrón diferente (Figure 4.6). En el caso de las repuestas dolorosas, la morfina indujo efectos antihiperalgésicos significativos a partir de dosis de 0,5 mg/kg en ratones salvajes, mientras que fueron necesarias dosis de

1 mg/kg en ratones de genotipo KO. En la hiperalgesia mecánica referida, los efectos antihiperalgésicos comenzaron a ser significativos a dosis de 3 mg/kg en ratones salvajes y de 2 mg/kg en KOs. Por lo tanto, se demuestra que el bloqueo genético del receptor σ1 provoca una marcada potenciación de la analgesia inducida por morfina.

4.2.2 Potenciación del efecto de la morfina mediante el bloqueo farmacológico del receptor σ1 en ratones salvajes

Ya que estamos evaluando dos tipos diferentes de dolor visceral en el mismo animal, tuvimos que escoger una dosis de morfina con efectos antinociceptivos y a la vez sensible a la modulación por los antagonistas del receptor σ1. Usamos la dosis de 2 mg/kg de morfina para asociar con los antagonistas del receptor σ1. De igual forma, determinamos la dosis idónea de varios antagonistas del receptor σ1 (S1RA (8-32 mg/kg), NE-100 (2-8 mg/kg), BD-1063 (4-16 mg/kg), y BD-1047 (4-16 mg/kg) con la que potenciar los efectos analgésicos de la morfina (Figure 4.7).

Al asociar la morfina junto con los antagonistas del receptor σ1 conseguimos una potenciación del efecto de la morfina de forma dosis dependiente tanto en un modelo como en el otro con todos y cada uno de los antagonistas testados (Figure 4.8).

4.2.3 Efectos de la asociación de morfina con antagonistas del receptor σ1 en ratones KO para el receptor σ1

A continuación, evaluamos la selectividad del efecto de dichos antagonistas testándolos en el mismo modelo pero con ratones con el receptor σ1 bloqueado genéticamente (Figure 4.9). El resultado fue que ningún antagonista del receptor σ1 fue capaz de potenciar la analgesia opioide en nunguna de las dos aproximaciones experimentales. Para evaluar la posibilidad de que no haya modificación en la analgesia morfínica debido a la mayor potencia frente al dolor visceral en ratones con bloqueo genético que en ratones salvajes probamos los efectos de los antagonistas selectivos con una dosis equivalente de morfina en términos de eficacia en los ratones KO. Para ello sumamos la dosis de 0,5 mg/kg de morfina a cada uno de los antagonistas. No encontramos diferencias en la analgesia. Estos resultados confirman, por tanto, la selectividad de los efectos inducidos por los antagonistas del receptor σ1 dada su falta de efecto en los ratones KOs.

4.2.4 Efectos de los antagonistas opioides en la analgesia morfínica en ratones salvajes

Con la finalidad de estudiar los mecanismos centrales de actividad analgésica de la morfina inyectamos naloxona (0,031-1 mg/kg) junto con el opioide, obteniendo una antagonización completa del efecto analgésico de la morfina 4 mg/kg tanto en un modelo como en el otro (Figure 4.10). De la misma forma, para estudiar los mecanismos de acción periférica, usamos el antagonista no-selectivo del receptor opioide limitado periféricamente naloxona metiodida (2-8 mg/kg). El resultado fue que cuando consideramos la actividad de la morfina sobre las respuestas agudas, solo la dosis más alta de naloxona metiodide tuvo algún efecto, no teniendo efecto alguno sobre la hiperalgesia mecánica (Figure 4.10).

Sin embargo, cuando evaluamos una dosis de morfina (3 mg/kg), que sobre la hiperalgesia mecánica indujo un efecto antihiperalgésico en lugar de analgésico, la actividad de los antagonistas fue diferente . El efecto antinociceptivo de esta dosis de morfina fue totalmente revertido por la naloxona 1 mg/kg en ambos tipos de dolor. Por otra parte, sólo se necesitó una dosis de 2 mg/kg de naloxona metiodida para lograr alcanzar significación estadística en la hiperalgesia mecánica referida, mientras que en los comportamientos relacionados con el dolor la naloxona metiodide no modificó el efecto de la morfina.

4.2.5 Efectos de los antagonistas opioides en la potenciación de la analgesia morfínica inducida por la asociación con antagonistas del receptor $\sigma 1$ en ratones salvajes

De forma similar, para estudiar el mecanismo de acción central de la interacción farmacológica los testamos junto al antagonista naloxona (1 mg/kg). Y para testar la sensibilidad de estos efectos a la actividad periférica hacemos lo equivalente con la naloxona metiodida (2-4 mg/kg). En el caso de las respuestas espontáneas, la naloxona revirtió el efecto analgésico de la asociación solo parcialmente, hasta el nivel de los antagonistas *per se*. En el caso de la hiperalgesia mecánica referida, se consiguió una reversión completa de la analgesia. Por su parte, la naloxona metiodida fue capaz de revertir parcialmente la actividad analgésica potenciada sobre las respuestas espontáneas pero bloqueó completamente los efectos analgésicos de la morfina sobre la hiperalgesia mecánica referida (Figure 4.11).

Por lo tanto, estos resultados sugieren que la mejora de la analgesia de la morfina mediante la inhibición de los receptores σ1 se debe fundamentalmente a los receptores opioides periféricos.

4.3 Comparación de los efectos de agonistas opioides μ clínicamente relevantes oxicodona y fentanilo en el dolor visceral inducido por capsaicina 0,1% en ratones de genotipo salvaje

4.3.1 Efectos de la administración subcutánea de oxicodona y fentanilo

Para estudiar los efectos antinociceptivos de otros agonistas opioides μ usados en la práctica clínica como analgésicos en este modelo de dolor visceral, testamos la oxicodona (1-6 mg/kg) y el fentanilo (0,04-0,2 mg/kg) en animales salvajes. Ambos agonistas opioides revirtieron de forma dosis dependiente tanto las respuestas espontáneas de dolor como la hiperalgesia mecánica referida (Figure 4.12). Las dosis más bajas de ambos opioides fueron suficientes para reducir significativamente las respuestas espontáneas de dolor (1 mg/kg para la oxicodona y 0,04 mg/kg para fentanilo), mientras que la hiperalgesia mecánica requirió dosis más elevadas para alcanzar la misma significancia estadística (3 mg/kg para la oxicodona y 0,1 mg/kg para fentanilo).

4.3.2 Potenciación del efecto de la oxicodona y el fentanilo mediante la inhibición farmacológica del receptor σ1

Para explorar si esta potenciación de la anlgesia era compartida por otros agonistas opioides μ, co-administramos la dosis más alta testada de los antagonistas del receptor σ1 con varias dosis de oxicodona y fentanilo (Figure 4.13). En este caso usamos dosis de los agonistas μ que ya inducían una antinocicepción ligera (oxicodona 1 mg/kg y fentanilo 0,04 mg/kg) o marcada (oxicodona 2 mg/kg y fentanilo 0,08 mg/kg) sobre el número de respuestas agudas pero sin efecto en la hiperalgesia mecánica referida. Todos los antagonistas del receptor σ1 potenciaron los efectos de los dos agonistas opioides en los dos tipos de dolor.

Por tanto, confirmamos que el incremento en la analgesia opioide mediante el bloqueo selectivo del receptor σ1 es un patrón general que podría considerarse una estrategia clínica para el tratamiento del dolor visceral.

4.3.3 Efectos de los antagonistas opioides μ (naloxona y naloxona metiodida) y del agonista selectivo del receptor σ1 PRE-084 en la antinocicepción inducida por oxicodona y fentanilo.

En un estudio complementario, estudiamos el papel de los receptores opioides centrales y periféricos, así como el efecto del agonista del receptor σ1 PRE-084 en la antinocicepción inducida por oxicodona y fentanilo. Usamos dosis de los opioides que inducían *per se* efectos antihiperalgésicos (oxicodona 3 mg/kg y fentanilo 0,12 mg/kg) o analgésicos (oxicodona 5 mg/kg y fentanilo 0,16 mg/kg) en la hiperalgesia mecánica referida.

El resultado fue que la naloxona revirtió los efectos antinociceptivos de todas las dosis de opioides testadas y en ambas aproximaciones experimentales. Sin embargo, la naloxona metiodida solo revirtió parcialmente dichos efectos en las respuestas agudas de dolor. En la hiperalgesia mecánica revirtió parcialmente los efectos analgésicos de la oxicodona (5 mg/kg) mientras que revirtió por completo el resto de los casos (Figure 4.14). La administración de PRE-084 no modificó la actividad de la oxicodona ni del fentanilo en ningún caso.

Por tanto, estos resutados junto a los obtenidos tras el estudio de la morfina indican que los receptores opioides periféricos contribuyen, al menos en parte, a la antinocicepción inducida por varios de los opioides más comúnmente utilizados en la clínica y que la activación del receptor σ1 no tiene tingún papel en estas acciones farmacológicas.

5 Conclusiones

5.1 Conclusiones específicas

1. La administración subcutánea del bloqueante del canal de Na^{2+} dependiente de voltaje tetrodotoxina (TTX) probada en modelos de estimulación química del colon (instilación intracolónica de capsaicina y aceite de mostaza) y cistitis inducida por ciclofosfamida intraperitoneal, inhibió de forma dosis-dependiente el número de conductas relacionadas con el dolor en todos los modelos de dolor evaluados y revirtió la hiperalgesia mecánica referida inducida por capsaicina y ciclofosfamida, pero no la inducida por aceite de mostaza.

2. La administración subcutánea de una dosis elevada de morfina (8 mg/kg) inhibió tanto las respuestas espontáneas al dolor como la hiperalgesia mecánica referida en todos los modelos de dolor probados. En consecuencia, estos resultados sugieren que todos los tipos de comportamientos evaluados estaban relacionados con el dolor.

3. No se observó ninguna incoordinación motora (probada con un dispositivo Rotarod) tras la administración de TTX. Por lo tanto, la inhibición de las respuestas al dolor y los efectos antihiperalgésicos de la TTX observados en el presente estudio podrían interpretarse a través de acciones periféricas.

4. El subtipo de canal de Na^{2+} $Na_v1.7$ (expresado en las neuronas $Na_v1.8$ positivas) no es del todo necesario para el dolor visceral, ya que los ratones de genotipo *Knockout* condicionales específicos de este nociceptor ($Na_v1.7$-KO) tratados con TTX mostraron las mismas respuestas que los controles de camada tras la administración de los algógenos.

5. La administración subcutánea de varios opioides analgésicos (morfina, oxicodona y fentanilo) a ratones de genotipo salvaje reduce, de forma dosis-dependiente, tanto el número de respuestas espontáneas al dolor como la hiperalgesia mecánica referida inducida por la administración intracolónica de capsaicina al 0,1%.

6. La coadministración subcutánea de los antagonistas selectivos del receptor $\sigma1$ (S1RA, NE-100, BD-1047 y BD-1063) potencia de forma dosis-dependiente la analgesia inducida por la morfina en ratones de genotipo salvaje. Del mismo modo, la asociación de los antagonistas del receptor $\sigma1$ (a las dosis más altas probadas) con los agonistas opioides oxicodona y fentanilo potencia sus efectos antinociceptivos tanto en las conductas relacionadas con el dolor como en la hiperalgesia mecánica referida en ratones de genotipo salvaje.

7. Los efectos antinociceptivos de la morfina sola aumentan en los ratones de genotipo *Knockout* para el receptor $\sigma1$, lo que indica que el bloqueo genético del receptor $\sigma1$ también potencia la analgesia inducida por la morfina. La asociación de las dosis más altas de los antagonistas del receptor $\sigma1$ con la morfina en los ratones de genotipo *Knockout* para el receptor $\sigma1$ no potencia su analgesia, confirmando la selectividad de los efectos inducidos por los antagonistas del receptor $\sigma1$.

8. Los efectos analgésicos inducidos por la administración sistémica de los agonistas opiáceos *per se* (es decir, en ausencia de inhibición del receptor σ1) en ratones de genotipo salvaje se producen principalmente a nivel periférico. Esto se ve respaldado por la administración del antagonista opioide restringido periféricamente naloxona metiodide que antagoniza toda la analgesia inducida por los opioides probados (excepto la morfina 3 y 4 mg/kg en el dolor agudo y el dolor referido, respectivamente).

9. La potenciación de la analgesia de la morfina en ratones de genotipo *Knock-out* para el receptor σ1 tratados sistémicamente con antagonistas del receptor σ1 depende de la activación de los receptores opioides periféricos, ya que es abolida por la naloxona metiodide.

5.2 Conclusiones generales

1. La administración sistémica de TTX podría tener un potencial uso terapéutico para tratar el dolor visceral clínico, ya que los modelos de dolor animal aquí utilizados tienen valor traslacional y han sido validados en humanos.

2. El estudio de la combinación sistémica de la activación de los opioides con el bloqueo del receptor σ1 en el modelo de dolor visceral inducido por capsaicina puede representar una estrategia potencial para mejorar el perfil analgésico de los opioides en los trastornos gastrointestinales.

Acronyms

β-MSH	β-melanocyte stimulating hormone
σ1R	sigma-1 receptor
σ1R-KO	σ1 receptor Knockout
5-HT	5- hydroxytryptamine
ACC	anterior cingulate cortex
ACs	adenylcyclases
ACTH	adrenocorticotropin hormone
ADH	antidiuretic hormone
ANOVA	analysis of variance
ASICs	acid sensing ion channels
ATP	adenosine triphosphate
BD-1047	N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino) ethylamine dihydrobromide
BD-1063	1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride
BPS	bladder pain syndrome
Ca^{2+}/CaM	Ca^{2+}/ CalModulin
cAMP	cyclic adenosine monophosphate
Capsaicin	8-methyl-N-vanillyl 6-nonamide
CGRP	calcitonin gene related peptide
CNS	central nervous system
CRH	corticotropin-releasing hormone
DAMGO	[D-Ala^2, N-$MePhe^4$, Gly-Ol] - enkephalin
DH	dorsal horn
DOR	δ-opioid receptor
DRG	dorsal root ganglia
DSS	dextran sulfate sodium
ENS	enteric nervous system
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase

FGIDs	functional gastrointestinal disorders
FSH	folicle-stimulating hormone
GABA	gamma amino butyric acid
GDNF	glial cell line-derived neurotrophic factor
GDP	guanosine diphosphate
GI	gastrointestinal
GIRK	G-protein coupled inwardly rectifying K^+
GPCRs	G-protein coupled receptors
GTP	guanosine triphosphate
HINT1	histidine triad nucleotide-binding protein 1
i.cl.	intracolonic
i.p.	intraperitoneal
i.pl.	intraplantar
i.v.	intravenously
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IGLEs	intraganglionic laminar endings
IMAs	intramuscular arrays
IP_3Rs	inositol 1,4,5- trisphosphate receptors
IPANs	intrinsic primary afferent neurons
KOR	κ-opioid receptor
LH	luteinizing hormone
MAM	mitochondrion associated ER membrane
MAPKs	mitogen activated protein kinases
MOR	μ-opioid receptor
$Na_v1.7$-KO	conditional $Na_v1.7$ Knockout
NaOH	sodium hydroxide
NE-100	N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl] ethylamine
NG	nodose ganglia
NGF	nerve growth factor
NMDA	N-methyl- D aspartate
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drugs
PAG	periaqueductal gray
PGRMC1	progesterone receptor membrane component 1
PKA/C	protein kinase A/C

PNS	peripheral nervous system
PRE-084	[2-(4-morpholinethyl)1]-phenyl cyclohexane carboxylate hydrochloride
ROS	reactive oxygen species
RVM	rostral ventral medulla
s.c.	subcutaneous
S1RA	4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine
SEM	standard errors of the mean
SK	small calcium activated K^+ channel
SKF-10047	N- allylnormetazocine
SNRIs	serotonin and norepinephrine reuptake inhibitors
SP	substance P
SSC	somatosensory cortex
SSRIs	selective serotonin reuptake inhibitors
STT	spinothalamic tract
TCAs	tricyclic antidepressants
TMEM97	endoplasmic reticulum-resident transmembrane protein-97
TNBS	2,4,6-trinitrobenzene-sulfonic acid
TRPA1	transient receptor potential ankyrin subtype 1
TRPM8	transient receptor potential melastatin 8
TRPV1	transient receptor potential ion channel for vanilloid 1
TSH	thyroid-stimulating hormone
TTX	tetrodotoxin
TTX-R	tetrodotoxin resistant
TTX-S	tetrodotoxin sensitive
VGCC	voltage-gated Ca^{2+} channel
VGKC	voltage-gated K^+ channel
VGSC	voltage-gated Na^{2+} channel
WDR	wide dynamic range
WT	wild-type

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Appendix A.

1 Drugs for the treatment of visceral pain

Table 1: Peripheral Neuromodulators

Peripheral neuromodulators	Action mode	Actions on GI function	Drugs
Antispasmodics	Smooth muscle relaxation	Antagonize the binding of acetylcholine to the muscarinic receptor at the neuromuscular junction, with smooth muscle relaxation as a consequence	Peppermint-oil
			Mebeverine
			Otilonium
			Pinaverium-bromide
			Dicyclomine
			Hyoscine
			Hyoscyamine
			Cimetropium
			Papaverine
			Trimebutine

Table 1 (continued)

Peripheral neuromodulators	Action mode	Actions on GI function	Drugs
Guanylate cyclase-C receptor agonists	Secretagoges	↑ intraluminal fluid secretion and analgesic effect via modulation of colonic nociceptors	Linaclotide
			Plecanatide
			Lubiprostone
			Tenapanor
Peripheral opioid receptor ligands	Antidiarrhoeals	↓ intestinal motility and affect water and electrolyte movement through the bowel	Eluxadoline
			Loperamide
			Asimadoline
			Diphenoxylate
			Naloxegol
SS receptor ligands	↑ GI symptoms	Modulate serotonin-sensitive GI processes	Alosetron
			Ramosetron
			Ondansetron
			Prucalopride
			Tegaserod
			Cilasentron
			Renzapride
			Mosapride
			Naronapride
			Velusetrag
			YKP10811

Table 1 (continued)

Peripheral neuromodulators	Action mode	Actions on GI function	Drugs
NSAID	Anti-inflammatory	Inhibit the activity of cyclooxygenase enzymes	Ibuprofen
			Diclofenac
			Paracetamol
Antibiotics	-	Modulate the gut microbiota	Metronidazole
			Ciprofloxacin
			Rifaximin

Table 1: Gastrointestinal (GI); serotonin (SS); non-steroidal anti-inflammatory drugs (NSAID). Collected from [17, 20, 26, 84, 87, 118, 119, 123, 328, 329, 330, 331].

Table 2: Central Neuromodulators

Central neuromodulators	Action mode	Actions on GI function	Drugs
TCAs	Presynaptic serotonin reuptake inhibition and noradrenaline reuptake inhibition	↓ the intensity of pain signals going from gut to brain	Amitriptyline
			Nortriptyline
			Trimipramine
			Imipramine
			Desipramine

Table 2 (continued)

Central neuromodulators	Action mode	Actions on GI function	Drugs
SSRIs	Presynaptic SS reuptake inhibition	↓ the intensity of pain signals going from gut to brain	Paroxetine
			Fluoxetine
			Sertraline
			Citalopram
			Escitalopram
Tetracyclic antidepressants	NA and SS activity through α-2 antagonism on NA and 5-HT neurons	↓ the intensity of pain signals going from gut to brain	Mirtazapine
			Mianserin
			Trazodone
SNRIs	SS and NA reuptake inhibitors	Presynaptic serotonin reuptake inhibition and noradrenaline reuptake inhibition	Duloxetine
			Venlafaxine
			Milnacipran
Atypical antidepressants	Dopamine and norepinephrine reuptake	Inhibit dopamine and norepinephrine reuptake at the presynaptic cleft	Bupropion
Atypical antipsychotics	Dopamine and 5-HT_{2A} neurotransmission	Their exact mechanism is unknown, but they have lower affinity for the dopamine receptor and a higher degree of 5-HT_{2A} occupancy than typical antipsychotic drugs	Quetiapine
			Olanzapine
			Sulpiride
			Apriprazole
			Aripiprazole
			Brexpiprazole

Table 2 (continued)

Central neuromodulators	Action mode	Actions on GI function	Drugs
Azapirones	Partial pre- and post-synaptic 5-HT_1 agonists	Anxiolytics, but the exact mechanism of them is unknown	Buspirone
			Tandospirone
Central opioids	Activate opioid receptors on nerve cells	↓ the sending of pain messages to the brain	Morphine
			Oxycodone
			Fentanyl
			Tramadol
			Ketorolac

Table 2: Noradrenalin (NA); serotonin (SS); tricyclic antidepressants (TCAs); selective serotonin reuptake inhibitors (SSRIs); serotonin and noradrenalin reuptake inhibitors (SNRIs); 5- hydroxytryptamine (5-HT). Collected from [17, 20, 26, 84, 87, 118, 119, 123, 328, 329, 330, 331].

Table 3: Novel agents

Pharmacological Target	Therapeutic effects	Experimental drugs	References
Immunological and inflammatory pathways	↓ inflammation and pain perception.	Mesalazine	[332, 333]
	↓ chronic stress-induced VH.	TAK-242	[334]

Table 3 (continued)

Pharmacological Target	Therapeutic effects	Experimental drugs	References
Histamine-1 receptor	↓ stress associated VH.	Ebastine	[335]
	↓ stress associated VH.	Fexofenadine	[335]
	↓ hyperalgesia, discomfort and abdominal pain.	Ketotifen	[336]
Serotonin receptor	↑ volume thresholds. ↓ colonic compliance, emotional motor system of brain activity and modulates gut sensitivity.	Alosetron	[337]
	↓ sensitivity to rectal distension, improves visceral sensation.	Tegaserod	[338]
TRPV1	↓ thermal hyperalgesia.	SB366791	[339]
	↓ number of abdominal contractions.	APHC1	[340]
	↓ number of abdominal contractions.	APHC3	[340]

Table 3 (continued)

Pharmacological Target	Therapeutic effects	Experimental drugs	References
NK receptor	↓ inflammatory associated hyperalgesia.	SR-140333	[341]
	↓ inflammatory associated hyperalgesia.	MEN-10930	[342]
	Modulation the colorectal hypersensitivity to distension.	Nepadutant	[343]
	Modulation the colorectal hypersensitivity to distension.	Saredutant	[344]
	↓ rectocolonic inhibitory reflex and abdominal contractions.	SR-142801	[345]
Opioid receptor	↓ pain sensation and volume or pressure stimuli perception.	Fedotozine	[346]
Tyrosine kinase receptor	↓ VH in colorectal distension.	k252A	[347]
Adrenergic receptor	↓ hetero-typical intermittent stress-induced VH.	Propanolol	[348, 349]
Glutamate receptor	↓ visceral perception induced by substance P.	CNQX	[350]

Table 3 (continued)

Pharmacological Target	Therapeutic effects	Experimental drugs	References
Cannabinoid receptor	↓ visceral hyperalgesia.	RQ-00202730	[351]
	↓ visceral hyperalgesia.	PF-03550096	[352]
Protease-activated receptor	↓ visceromotor response to colorectal distension.	AYPGKFNH	[353, 354]
Voltage-gated channel	↓ butyrate-induced VH.	Mibefradil	[355]
	↓ butyrate-induced VH.	Ethosuximide	[355]
	↓ butyrate-induced VH.	NP078585	[355]
	↓ visceral pain in TNBS induced inflammatory VH.	Nimodipine	[356]
	↓ visceral pain in TNBS induced inflammatory VH.	SNX482	[356]
	↓ in CSF glutamate release and ROS levels and visceral nociception.	MVIIA	[357]
	↓ in CSF glutamate release and ROS levels and visceral nociception.	Phα1β	[357]
	Inhibition of colonic nociceptors and ↓ pain responses to noxious colorectal distension.	α-conotoxin-cVc1.1	[358]
	↑ pain threshold and ↓ rectal hypersensitivity.	Lidocaine	[359]

Table 3 (continued)

Pharmacological Target	Therapeutic effects	Experimental drugs	References
	↓ MA and TH.	A-803467	[360]
	↓ inflammatory pain.	Ambroxol	[361]
	Reverse hyperalgesia after induction of intestinal inflammation.	TRTX-Hhn1b	[362]
	↓ pain-related behaviors and RMH.	TTX	[363]
	Analgesic properties and induces anaesthesia long-acting pain blocker in bladder pain syndrome.	STX	[364]
	↓ pain-related responses.	NeoSTX	[147]
	-	APETx2	[365]

Table 3: Visceral hypersensitivity (VH); transient receptor potential ion channel for vanilloid 1 (TRPV1); cerebrospinal fluid (CSF); 2,4,6-trinitrobenzene-sulfonic acid (TNBS); reactive oxygen species (ROS); mechanical allodynia (MA); thermal hyperalgesia (TH); referred mechanical hyperalgesia (RMH); Tetrodotoxin (TTX); Saxitoxin (STX). Modified from [26, 328].

2 Sigma-1 receptor interacting protein

Table 4: ER-MAM-mitochondria σ1R Partners

σ1R Partner	**Features of the interaction**
IP_3R_3	In addition to the regulation of Ca^{2+} mobilization from endoplasmic stores, the σ1R is also involved in the modulation of Ca^{2+} flow from ER to mitochondria through the interaction and consequent stabilization of the IP_3R_3 on the MAM, preventing unstable IP_3R_3 from being degraded [172]. Among others, this may also contribute to cell death in stress-induced damage [366]. Regarding the regulation of Ca^{2+} signalling in order to modulate action potential, the function of σ1R through IP_3R_3 exits at the MAM, the ER reticular network and the plasma membrane [204].
VDAC	Under normal conditions, VDAC localizes at the mitochondrial membrane but forms a complex with IP_3R from the ER to facilitate the Ca^{2+} efflux from ER to the mitochondria. The association with σ1R has been demonstrated in studies of cholesterol metabolism [367] but has been speculated σ1R function for maintaining the cross-talk ER-mitochondria [368]. Linked to this, [369] associates σ1R with VDAC2 via StAR protein that facilities the transport of cholesterol to the site of steroidogenesis into the mitochondria.
Ankyrin B	The cytoskeletal protein adaptor ankyrin is influenced by σ1R by the dissociation of ankyrin from the IP_3R, opening the Ca^{2+} efflux from ER to the cytosol [204].

Table 4 (continued)

σ1R Partner	**Features of the interaction**
STIM1	When the extracellular Ca^{2+} is exhausted, the σ1R is shown to bind STIM1 at the ER and the result is a slowed down of the recruitment of STIM1 to the ER-plasma membrane junction where STIM1 binds Orai1, whatever inhibits the store-operated Ca^{2+} entry [208].
p35	σ1R interacts with p35 [217], whatever leads to axon elongation via the myristoylation of p25.
TRP	A molecular in vitro study [370] demonstrates the physical interactions of σ1R with several polymodal TRPs Ca^{2+} channels (thermo-channels in this case, TRPA1, TRPV1 and TRPM8), and the dependence of its binding on Ca^{2+} levels. Although TRPV channels are located mostly on the plasma membrane, some of them have also been shown to be located in the ER (see review [371]). Concerning TRPV1 activation, it has been demonstrated that σ1R can associate with TRPV1 in a direct protein-protein interaction [249] to promote cytotoxicity via activation of EIF2α K3, phosphorylation of EIF2α, and expression of GADD153 [372].
Stress sensors: PERK, IRE1, ATF6	In various models of oxidative stress (in retinal neurons and CHO cells) [198] and [197] respectively, showed that σ1R plays a role in the stress response. The group of S.B. Smith [198] revealed that in the presence of a σ1R agonist (pentazocine) the expression level of PERK, ATF4, ATF6 and IRE1 decreased, and Su et al., [197] found that under ER stress, IRE1 is stabilized by σ1Rs.

Table 4 (continued)

σ1R Partner	**Features of the interaction**
Insig	According to [373], σ1Rs are involved in the differentiation of oligodendrocytes by engaging in the degradation of specific sets of ER proteins involved in lipid homeostasis.
ELMOD	σ1R has been described to interact with ELMOD2, a protein of the ELMOD family of guanine nucleotide exchangers that function as GAP, inhibiting it. ELMOD2 in turn acts on several GTPases [374], including RAC1 and ARF6 (related to the induction of cellular response to viruses [375]), so σ1R could modulate the activity of small GTPases and this function could underlie its role in regulating the innate response [376].
Rac1-GTPase	There are also studies that indicate that σ1R could be physically associated with RAC1 in MAM and regulate its activity. Besides that, σ1R promotes dendritic spine formation and attenuate free radical formation interacting with Rac1-GTPase [377, 378].

Table 4: Inositol 1,4,5- trisphosphate receptor 3 (IP_3R_3); endoplasmic reticulum (ER); mitochondrion associated ER membrane (MAM); voltage-dependent anion channels (VDACs); steroidogenic acute regulatory (StAR); transient receptor potential ion channel for vanilloid 1 (TRPV1); stromal interaction molecule 1 (STIM1); Chinese hamster ovary (CHO); insulin-induced gene (Insig); engulfment and cell motility domain (ELMOD); GTPase activating protein (GAP); protein kinase RNA like ER-kinase (PERK); inositol requiring enzyme 1 (IRE1); activating transcription factor (ATF6); eukaryotic translation initiation factor 2α (eIF2α); transcription factor 4 (ATF4).

Table 5: Nucleus σ1R Partners

σ**1R Partner**	**Features of the interaction**
Emerin	Tsai et al., [222] found that σ1R recruits chromatin-remodelling molecules, including Lamin A/C, HDACs and BAF, through the integral nuclear envelope protein Emerin to control gene transcription.
Znf179	In relation to ROS accumulation and the consequent cytotoxic effects induced in pathological oxidative stress, the group of Chuang et al., [171] identified by protein-protein interaction assays in mouse neuroblastoma cells the brain protein Znf179 as a downstream target of σ1R regulation. That it could theoretically mediate the neuroprotective effects of the σ1R agonists DHEA/DHEAS, capable of reducing the activation of apoptotic pathways [379]. Moreover, Maurice et al., [380] has also reported the role of σ1R agonists under physiological conditions as inducers of moderate oxidative stress involving complex I activity, while under pathological conditions, in line with [171], σ1R activity may contribute to a rapid restoration of mitochondrial physiology.

Table 5: Histone deacetylases (HDACs); barrier-to-autointegration factor (BAF), reactive oxygen species (ROS); Zinc finger 179 (Znf179); dehydroepiandrosterone (DHEA); dehydroepiandrosterone sulfate (DHEAS).

Table 6: Plasma Membrane σ1R Partners

σ**1R Partner**	**Features of the interaction**
Na_v channel	It has been demonstrated, through AFM experiments, that σ1R interacts with cardiac $Na_v1.5$ channels [381]. A direct interaction σ1R-neuronal Na_v channels has not yet been described. σ1R agonists exert inhibitory action on the Na^+ current [205] and therefore of the action potential initiation and propagation [382].
K_v channel	σ1Rs regulate the trafficking of K^+ channel subunits from the ER to the plasma membrane [169] from $K_v1.2$ [252], $K_v1.3$ [383], $K_v1.4$ [202], $K_v1.5$ [384], and $K_v2.1$ [385] and alters their kinetics for returning the depolarized cell to a resting state during action potentials. It has been shown that translocation and maturation in the ER/Golgi space of human either-à-gogo related gene (hERG, a gene encoding the pore-forming subunit of the delayed rectifier with rapid activation of the K^+ channel) is enhanced by σ1R in the presence of ligands of the latter [386].
Ca_v channel	With reference to the main transducers of membrane potential changes, it has been published that σ1R activation by different synthetic agonists negatively influences on all Ca_v channels subtypes functions found on the cell body in order to modulate Ca^{2+} homeostasis [256]. The L-type (distributed at skeletal, cardiac and retinal synapses, mainly) and N-type ($Ca_v2.2$, in large part located at central and peripheral synapses) Ca^{2+} channels have been identified as a direct target for the σ1R in the nervous system, and the σ1R agonist SKF-10047 directly inhibited Ca^{2+} currents [255, 387, 388].

Table 6 (continued)

σ1R Partner	**Features of the interaction**
NMDA receptor	σ1R influences synaptic functions through the stimulation of the NMDA activity perhaps through altering responses to Ca^{2+} signals as well as stimulating the expression of NMDA receptors and its traffic to the plasma membrane [243].
ASIC	In cortical neurons, Herrera and co-workers [250] have shown that under acidic pH conditions (e.g., during ischaemia), those channels are activated in the plasma membrane, and the ligand agonist activation of σ1R causes inhibition of Ca^{2+} influx ASIC1a induced. Two years later it was demonstrated *in vitro*, the direct interaction between σ1Rs and ASIC1a in kidney cultured cells by atomic force microscopy imaging [203].
SK3 channel	Recently the physical interaction of σ1R with SK3, a Ca^{2+}-activated K^{+} channel (KCNN3), has been published. σ1R is required to increase Ca^{2+} influx by triggering the coupling between SK3 to Orai1 (a voltage-independent Ca^{2+} channel) which drives invasive process in colorectal cancer cells [389].
MOR	The σ1R interacts with GPCRs and this is implicated in the regulation of MOR activity, with the σ1R antagonists being able to potentiate opioid-induced cell signalling [206].
Dopamine receptor	The interaction of the σ1R with D1-2 through the development of heteroreceptor complexes in a cocaine exposure scenario has been established towards understanding the molecular basis of cocaine addiction [390, 391] and dopamine-adenosine-σ1R complexes in the context of the neurobiology of schizophrenia [392].
CB1 receptor	Physical σ1R-CB1R interaction has been described that controls the interaction of CB1 with NMDAR [245].

Table 6 (continued)

σ**1R Partner**	**Features of the interaction**
Serotonin 5-HT receptor	The σ1R associated with the 5-HT receptor promotes presynaptic glutamate released in the rat prelimbic cortex [239].

Table 6: Atomic force microscopy (AFM); human either-à-gogo related gene (hERG); N-methyl- D aspartate (NMDA); acid-sensing ion channels (ASICs); small calcium activated K^+ channel (SK); G-protein coupled receptors (GPCRs); μ-opioid receptor (MOR); dopamine receptor D1-2 (D1-2); cannabinoid receptor 1 (CB1R); 5- hydroxytryptamine (5-HT).

Appendix B. List of publications

Publications resulting from this thesis

Effects of tetrodotoxin in mouse models of visceral pain.
González-Cano R[1], Tejada MÁ[1], Artacho-Cordón A[1], Nieto FR, Entrena JM, Wood JN, Cendán CM. *Marine Drugs.* 15(6): 188, 2017. (D1, 10/116). Area: Pharmacology, Toxicology and Pharmaceutics. DOI: 10.3390/md15060188. IF (ISI): 4,379.

[1] These authors contributed equally to this work.

New strategy for treating visceral pain: improving opioid analgesia by blocking the Sigma-1 receptor. In preparation.

Publications related to this thesis

Dual Sigma-1 receptor antagonists and hydrogen sulfide-releasing compounds for pain treatment: Design, synthesis, and pharmacological evaluation.
Dichiara M, Artacho-Cordón A, Turnaturi R, Santos-Caballero M, González-Cano R, Pasquinucci L, Barbaraci C, Rodríguez-Gómez I, Gómez-Guzmán M, Marrazzo A, Cobos EJ, Amata E. *European Journal of Medicinal Chemistry.* 230: 114091, 2022. (D1, 19/297). Area: Pharmacology.
DOI: 10.1016/j.ejmech.2021.114091. IF (ISI): 6,514.

Discovery of a Sigma-1 receptor antagonist by combination of unbiased cell painting and thermal proteome profiling.
Wilke J, Kawamura T, Xu H, Brause A, Friese A, Metz M, Schepmann D, Wünsch B, Artacho-Cordón A, Nieto FR, Watanabe N, Osada H, Ziegler S, Waldmann H. *Cell Chem Biol.* S2451-9456(21)00009-X, 2021. (Q1, 30/297). Area: Biochemistry and molecular biology. DOI: 10.1016/j.chembiol.2021.01.009. IF (ISI): 7,739.

Urinary bladder Sigma-1 receptors: a new target for cystitis treatment.
González-Cano R, Artacho-Cordón A, Romero L, Tejada MA, Nieto FR, Merlos M, Cañizares FJ, Cendán CM, Fernández-Segura E, Baeyens, JM. *Pharmacol Res.* 155: 104724, 2020. (D1, 19/270). Area: Pharmacology and pharmacy. DOI: 10.1016/j.phrs.2020.104724. IF (ISI): 5,893.

Modulation of peripheral μ-opioid analgesia by Sigma-1 receptors.
Sánchez-Fernández C, Montilla-García Á, González-Cano R, Nieto FR, Romero L, Artacho-Cordón A, Montes R, Fernandez-Pastor B, Merlos M, Baeyens JM, Entrena JM, Cobos EJ. *J. Pharmacol Exp Ther.* 348(1): 32-45, 2014. (Q1, 41/255) Area: Pharmacology and pharmacy. DOI: 10.1124/jpet.113.208272. IF (ISI): 3,972.

Potentiation of morphine-induced mechanical antinociception by Sigma-1 receptor inhibition: role of peripheral Sigma-1 receptors.
Sánchez-Fernández C, Nieto FR, González-Cano R, Artacho-Cordón A, Romero L, Montilla-García Á, Zamanillo D, Baeyens JM, Entrena JM, Cobos EJ. *Neuropharmacology.* 70:348-58, 2013. (D1, 23/256). Area: Pharmacology and pharmacy. DOI: 10.1021/np300783a. IF (ISI): 4,819.

El receptor Sigma-1: un freno biológico a la analgesia opioide periférica.
Sánchez-Fernández C, Montilla-García Á, González-Cano R, Nieto FR, Romero L, Artacho-Cordón A, Montes R, Baeyens JM, Entrena JM, Cobos EJ. Actualidad en Farmacología y Terapéutica. 11(4): 282-284, 2013.

Other publications

Matrix metalloproteinases: potential therapy to prevent the development of second malignancies after breast radiotherapy.
Artacho-Cordón F; Ríos-Arrabal S; Lara PC; Artacho-Cordón A; Calvente I; Núñez MI. *Surgical Oncology.* E143-E151, 2012. (Q2, 54/199). Area: Surgery. DOI: 10.1016/j.suronc.2012.06.001. IF (ISI): 2,136.

Tumour microenvironment and breast cancer progression: a complex scenario.
Artacho-Cordón A; Artacho-Cordón F; Ríos-Arrabal S; Núñez MI. *Cancer Biology and Therapy.* 13(1): 14-24, 2012. (Q2, 75/203). Area: Oncology. DOI: 10.4161/cbt.13.1.18869. IF (ISI): 3,287.