Role of Sigma-1 Receptors in Visceral Pain

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A mi abuelo Manuel

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"Es costumbre tener por agradecido al que manifiesta los beneficios de que fue objeto; pero el más agradecido de todos es quién no olvida el beneficio para recordar al bienhechor"

Ludwig Börne

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INTRODUCTION

1. VISCERAL PAIN: GENERAL OVERVIEW

Although it may at first appear that the information coming from viscera is scarce, studies of viscera-brain crosstalk have revealed a complex, bidirectional communication system that not only ensures the proper maintenance of internal homeostasis but is likely to have multiple effects on affect, motivation and higher cognitive functions, including intuitive decision-making (Mayer, 2011). Most of the information that arises from viscera is non-conscious information; individuals do not usually perceive signals emanating from within their bodies, and visceral pain is one of the few sensations of which we are conscious.

According to the International Association of Pain, visceral pain is that which arises from internal organs of the body, such as heart, lungs, colon or urinary bladder, among others (IASP, 2012). Visceral pain results from the activation of nociceptors on the thoracic, pelvic, or abdominal viscera, and it may be produced by direct inflammation of a visceral organ or the occlusion of hollow viscera or visceral conduits (e.g. urinary colic, one of the most intense forms of pain that a human being can experience (Mayer and Wong, 2012).), or by functional visceral disorders such as irritable bowel syndrome (IBS), [a dysfunctional condition causing recurrent attacks of abdominal pain]. When angina pectoris, painful bladder syndrome, gastroesophageal reflux disease, endometriosis, and dyspepsia are added to this list, the widespread impact of visceral pain becomes clear (Robinson and Gebhart, 2008).

Visceral pain is the most common pain produced by disease, is difficult to manage and is the most frequent reason why patients seek medical attention (Cervero and Laird, 1999). Chronic pain and discomfort from the viscera affect up to 10% of the general population, and therefore represent a large unmet need for treatment and represents a major economic burden (Blackshaw et al., 2010). Abdominal pain is the most frequent symptom prompting an outpatient clinic visit. These patients require considerable health care resources, with an annual treatment cost of €28.4 billion across Europe (Hillilä et al., 2010). One of the most common causes of abdominal pain is IBS, which has been estimated to affect 25% of the population in numerous countries and accounts for 40-50% of all gastroenterologic

consultations worldwide (Chang, 2004). Among other causes of visceral pain, inflammatory bowel disease (IBD) (which includes ulcerative colitis and Crohn´s disease) also has a high prevalence, affecting 2.2 millions of patients in Europe alone (Loftus, 2004), and bladder pain syndrome (BPS) (which includes the heterogeneous spectrum of painful interstitial cystitis) has a variable prevalence that can reach up to 30% (Engeler et al., 2012).

Visceral pain is often described in terms of six clinical characteristics (Cervero and Laird, 1999; Chang, 2004; Giamberardino, 2009): (1) It may not be evoked from all visceral organs as not all viscera are innervated by sensory receptors or possibly because of the lack of an appropriate nociceptive stimulus. In the past, viscera were considered insensitive to pain, mostly because their responses had not been tested with adequate stimuli (e.g. cutting, crushing, burning are generally ineffective when applied to the viscera). (2) It is not always linked to injury, hence the non-structural or functional properties of visceral pain. (3) It is diffuse in character and is typically difficult to localize (the patient typically uses the whole palm of the hand to indicate the painful area, instead of the tip of the finger) unless adjacent structures, such as the parietal peritoneum or pleura, are involved (e.g. appendicitis). (4) It is often accompanied by accentuated motor and autonomic reflexes (e.g. nausea and vomiting, pallor, sweating), which are considered a reaction to a warning system, and strong emotional reactions. (5) It is referred to the body wall; the area that is affected is generally referred segmentally and superficially (i.e., muscle, skin or both), being innervated by the same spinal nerve as the affected viscera. In place of referred pain, hyperalgesia may also appear (Kanai, 2011; McMahon et al., 1995). Cutaneous referred pain is usually seen in almost every episode of visceral pain and is one of the most characteristic symptoms of many visceral diseases (e.g. left arm pain in myocardial infarction). What is called referred pain in the clinical literature appears to be two separate phenomena: (a) the sensation is transferred to another site (e.g. angina can be felt in the chest, neck, and arm), and/or (b) same-segmental sites as those of affected viscera become more sensitive to inputs applied directly to skin or somatic tissues (e.g., flank muscle becomes sensitive to palpation in the urinary colic pain produced by movement of a kidney stone). This latter phenomenon is also

described as secondary somatic hyperalgesia. (6) It is referred to another viscus, a phenomenon called viscero-visceral hyperalgesia. It consists of an enhancement of painful symptoms due to the interaction between two affected internal organs that have at least partially overlapping sensory projections (e.g. uterus and colon) (Giamberardino, 2009; Sengupta, 2009).

Pharmacotherapy is the main tool for the symptomatic treatment of visceral pain. Generally, treatment recommendations for visceral pain have been the same as for somatic pain, but frequently visceral pain responds poorly to standard analgesic drugs; moreover, drugs that are used with some efficacy to treat somatic pain often present unwanted effects on the viscera. For instance, NSAIDs are considered the treatment of choice for biliary colic (Colli et al., 2012) and are also very effective for renal colic treatment (Davis, 2012) but are not useful to treat IBS (Ruepert et al., 2011) and produce clinical deterioration in patients with IBD (Makharia, 2011). Short-term opioids are usually indicated for breakthrough or exacerbated pain and periodic flare-ups in visceral pathologies. Long-term opioids are considered after all other therapeutic options have been exhausted, because their analgesic action are accompanied by frequent side-effects such as constipation and sedation (Engeler et al., 2012; Joranson et al., 2000); moreover, the use of opioids is associated with increased mortality and morbidity in patients with Chrone's Disease and are not considered a treatment of choice in IBD (Lichtenstein et al., 2012; Mowat et al., 2011). In some visceral pathologies such as IBS, neither NSAIDs nor opioid agonists are useful for treatment of abdominal pain, and other drugs are used instead, such as spasmolytics or antidepressants (both tricyclic and selective serotonin reuptake inhibitors) (Ruepert et al., 2011). Tricyclic antidepressants are also useful as a second-line treatment of interstitial cystitis (Hanno et al., 2011).

Treatment of visceral inflammation is another way to ameliorate some forms of visceral pain. Corticosteroids are used in the treatment of diseases such as IBD and cystitis; however, despite a relatively good short-term effect, a continuous period of administration can produce very serious side effects (Munkholm et al., 1994), making it difficult to justify their selection for long-term treatment (Engeler et al., 2012). Aminosalicylates (sulfasalazine, mesalazine) are used as anti-inflammatory

drugs in IBD with some efficacy, but their anti-inflammatory activity is restricted to this visceral pathology (Katz, 2007; Wilkins et al., 2011).

Taking the above circumstances into account, there is a need for a better understanding of the neurophysiological mechanisms underlying visceral pain and for new drugs with different mechanisms of action to those already known (Mayer, 2011; Robinson and Gebhart, 2008).

2. VISCERAL INNERVATION

All viscera are dually innervated by parasympathetic and sympathetic efferents (Fig. I) that control the motility, gland secretion and vascular tone of viscera. There are also different types of primary afferent neurons that detect physical or chemical stimuli. All viscera have extrinsic afferent neurons that travel in the same nerves as parasympathetic (vagus or pelvic nerves) and sympathetic (splanchnic) efferents (Bielefeldt et al., 2005; Hillilä et al., 2010; Song et al., 2009) and mainly convey information about them to the central nervous system (CNS)(Fig. II). Moreover, in the digestive tract there are intrinsic primary afferent neurons located within the enteric nervous system and viscerofugal neurons that have cell bodies in the gut wall and project to prevertebral ganglia (Fig. I & III).

Many viscera are innervated by receptors that do not evoke conscious perception and are not sensory receptors in the strict sense (Cervero and Laird, 1999). The subset of afferents that give rise to conscious sensation are called “sensory neurons”. Splanchnic afferent cell bodies are in the spinal thoracolumbar dorsal root ganglia (DRG) and innervate some thoracic, abdominal and pelvic viscera (Fig. II). Visceral sensation that start in visceral sensory neurons travel along hypogastric, lumbar colonic and splanchnic nerves to terminate in thoracolumbar regions, traversing both prevertebral and paravertebral ganglia en route to the spinal cord (Fig. II).

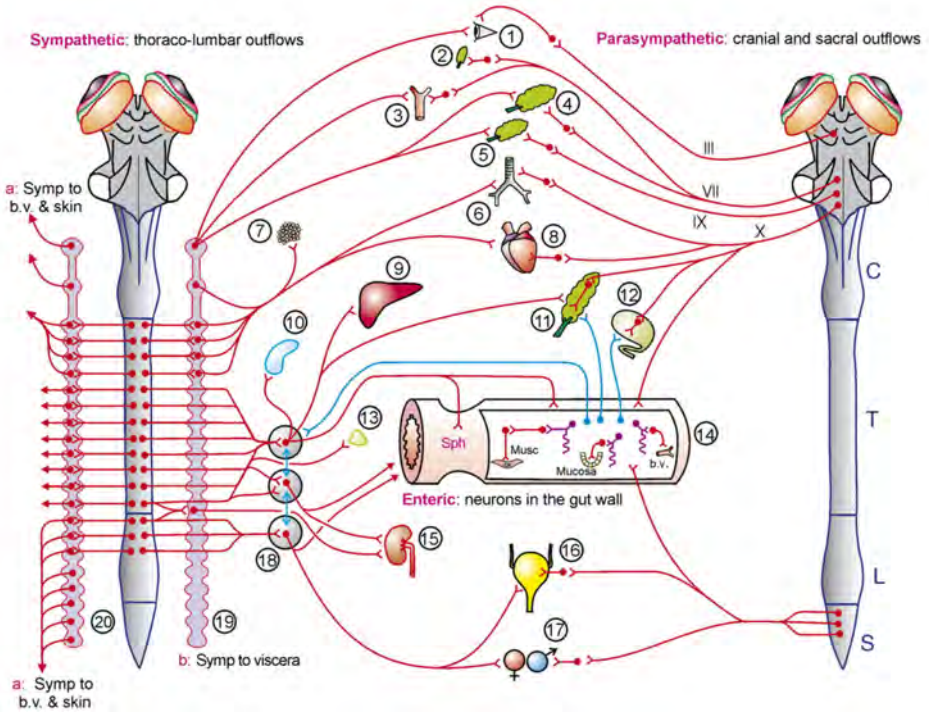

Fig. I. Schematic representation of the efferent pathways of the autonomic nervous system. The enteric division is represented by neurons within the gut wall (centre of diagram, 14). Sympathetic outflows. a: On the left side of the spinal cord, sympathetic chain (paravertebral) ganglia are represented; some neurons of these ganglia supply blood vessels (b.v.) throughout the body and effectors in the skin (sweat glands pilomotor muscles). These pathways have synapses in the paravertebral ganglia. For simplicity of illustration, pathways that run rostrally and caudally within the sympathetic chains are not illustrated. b: On the right side of the cord are the connections that pass first through the sympathetic chains and then through prevertebral ganglia and plexuses (18) to supply visceral organs. Synapses occur in either prevertebral or paravertebral ganglia. Parasympathetic outflows. These emerge from cranial and sacral levels and innervate structures in the head, neck, abdomen and pelvis but not in the limbs. Enteric neurons. These are represented within the outline of the intestine (14). The enteric reflex circuits contain intrinsic primary afferent neurons (purple), interneurons, and motor neurons (red). As illustrated, these control muscles (musc), secretory epithelium of the mucosa, and blood vessels (b.v.). Sph: this indicates the sphincter regions of the intestine, which are controlled by enteric and extrinsic neurons. Peripherally confined neural connections between organs. These are marked in blue. One of the neurons that contribute to these circuits is the viscerofugal neuron, which projects from the intestine to prevertebral ganglia. Target tissues and organs: (1) Eye, (2) Lacrimal glands, (3) Intracranial arteries, (4, 5) Salivary glands, (6) Airways, (7) Brown fat, (8) Heart, (9) Liver, (10) Spleen, (11) Pancreas, (12) Gallbladder, (13) Adrenal gland, (14) Tubular gastrointestinal tract, (15) Kidney, (16) Urinary bladder, (17) Genital organs, (18) Prevertebral ganglia and plexuses, (19, 20) Sympathetic chains (paravertebral ganglia and their interconnections). Spinal cord levels: C: Cervical, T: Thoracic, L: Lumbar, S: Sacral (modified from Furness, 2006).

In the prevertebral (sympathetic) ganglia, extrinsic afferents of visceral nerves often give rise to collateral synapse in the motor neurons (Fig. III) and may therefore influence the functioning of the affected viscera (particularly in the digestive system). Moreover, visceral extrinsic afferent fibres that enter the spinal cord *via* the paravertebral ganglia come in a wide form reaching distant cord segments.

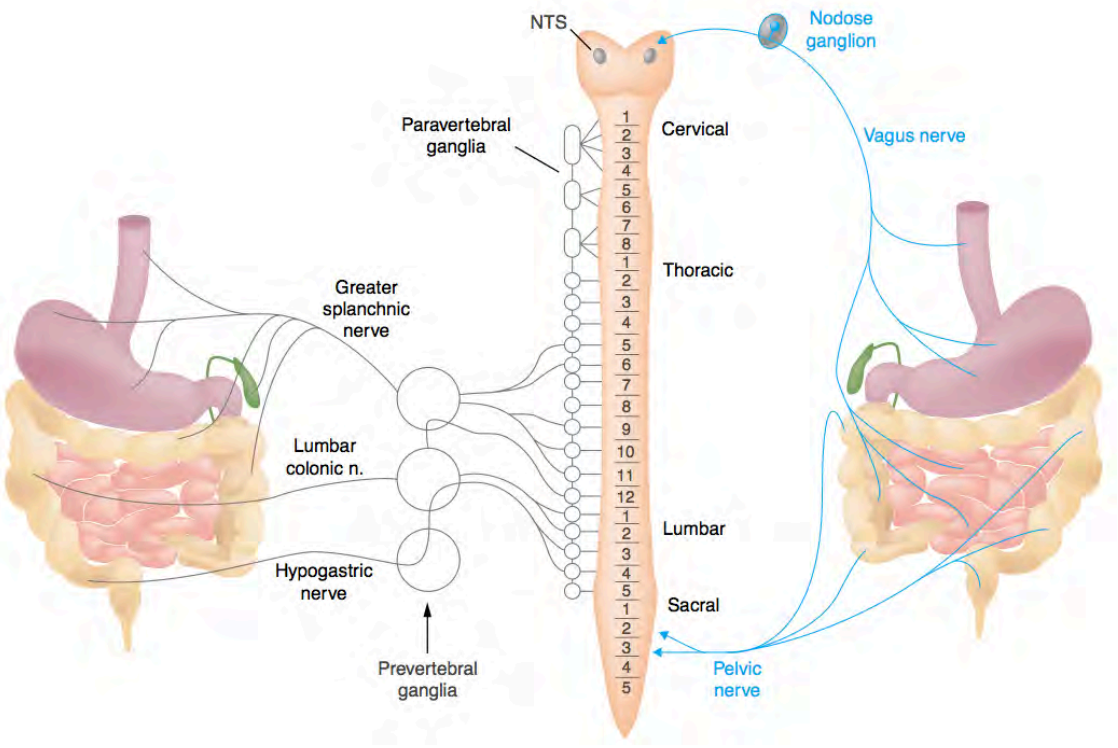

Fig. II. Sensory innervation of the gastrointestinal tract. Representation of visceral afferents pathways through sympathetic (left) and parasympathetic (right) innervation. NTS: Nucleus tractus solitary (modified from Blackshaw and Gebhart, 2002).

Cell bodies of vagal afferent fibres are in the nodose and jugular ganglia, innervate some thoracic and abdominal viscera and terminate in the brainstem (Fig. II). Although there is clear evidence that vagal afferents may influence the perception of visceral pain by interacting with spinal pathways centrally (Meller et al., 1990; Robinson and Gebhart, 2008; Wang et al., 2004), they do not themselves evoke pain when activated (Blackshaw et al., 2010; Grundy, 2005; Malin et al., 2009). Pelvic viscera that are not innervated by the vagus nerve (urinary bladder, descending colon and reproductive organs) are innervated by afferents in the

pelvic nerve, which project to the lumbosacral spinal cord and encode both innocuous and noxious stimuli. Functionally, this dual innervation contributes to differential processing of visceral stimuli by different regions of the neuraxis (Blackshaw et al., 2010; Caterina et al., 1997; Cortright et al., 2007; Wesselmann et al., 2009). Specifically, the majority of splanchnic fibres were shown to come from the mesentery or serosa, whereas pelvic afferents primarily innervate the muscular and mucosal layers of the distal colon and bladder (Brierley et al., 2004; Davis et al., 2000; Xu and Gebhart, 2008).

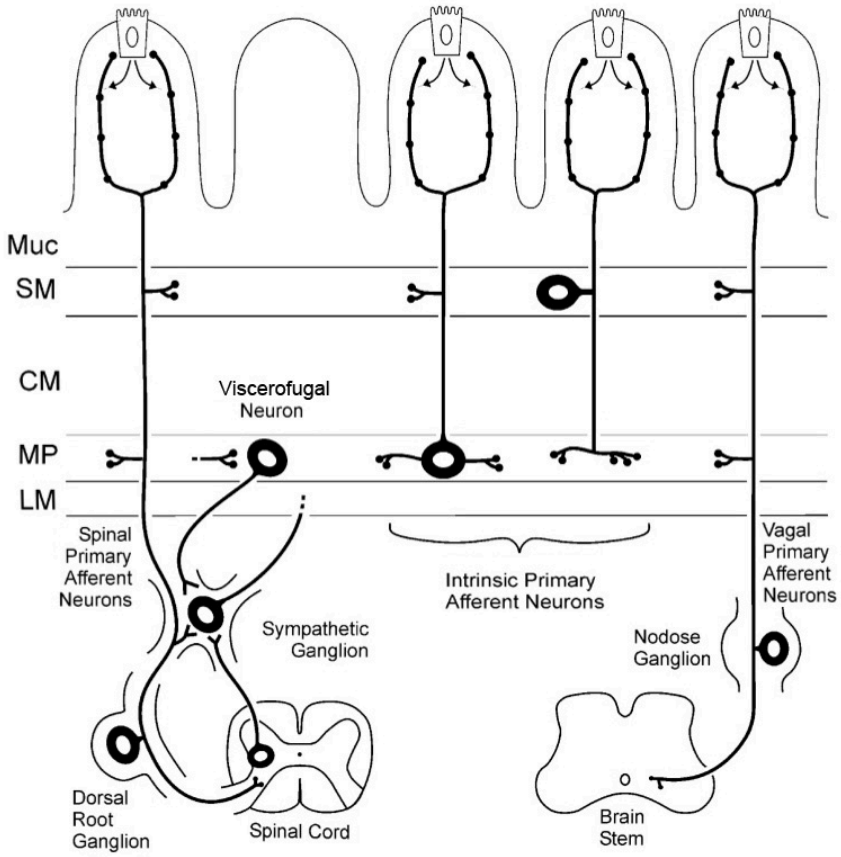

Fig. III. The afferent neurons of the digestive tract. Intrinsic primary afferent neurons and viscerofugal neurons have their cell bodies in the gut wall. Extrinsic primary afferent neurons have cell bodies in dorsal root ganglia (spinal primary afferent neurons) and vagal (nodose and jugular) ganglia. In the sympathetic ganglia (left side), spinal primary afferents often give rise to collateral synapse with sympathetic efferent neurons. LM: Longitudinal muscle, CM: Circular muscle, MP: Myenteric plexus, SM: Submucosa, Muc: Mucosa (adapted from Furness et al., 2004).

2.1 PERIPHERAL NERVOUS SYSTEM

Two of the most common origins of visceral pain in humans are the intestine and urinary bladder (see section 1). Therefore, experimental models of intestinal and urinary bladder pain were used in this Thesis. Moreover, although we review the basic mechanisms involved in pain arising from viscera in general, a more detailed analysis is given of the peripheral terminals of primary afferent neurons in the colon and urinary bladder, in order to improve understanding of the pathophysiology of pain in the two experimental models used.

2.1.1 COLON NERVE ENDINGS

There are just five basic types of sensory neurons innervating the gastrointestinal tract, each with specific anatomical endings in the gut wall (Fig. IV): type I: 'intraganglionic laminar' endings predominantly located in myenteric ganglia (myenteric plexus) within the gut wall, which primarily detect innocuous or possibly noxious mechanical distortion; type II: 'mucosal' afferents, with subepithelial endings, sensitive to enteroendocrine cell mediators and light mechanical distortion; type III: 'muscular–mucosal' afferents, with mechanosensitive endings between the muscularis mucosae and the mucosa, which detect both muscular activity and mucosal distortion; type IV: 'intramuscular' endings, with afferents primarily in the smooth muscle layers of the gut wall, which probably detect mechanical stimuli; and type V: 'vascular' (serosa) afferents, with sensitive endings primarily in blood vessels, which are sensitive to intense mechanical stimulation but modulated by a wide range of chemical mediators of damage and inflammation (Brookes et al., 2013; Robinson and Gebhart, 2008). Endings of this last type are sensitive to ischaemia, hypoxia and capsaicin (Cervero and Laird, 1999; Haupt et al., 1983; Longhurst et al., 1984) and are believed to comprise a major type of nociceptor.

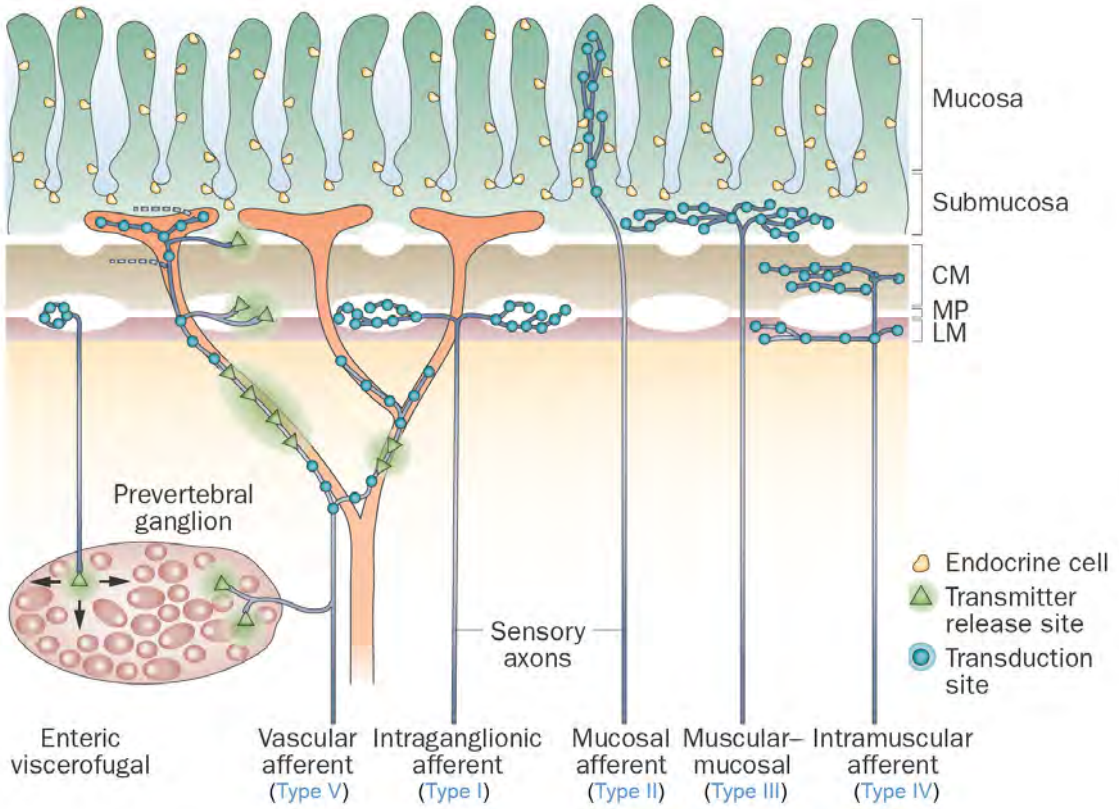

Fig. IV Morphological types of extrinsic sensory neurons in the gut. The five types of sensory endings in the gut wall are outlined, together with enteric viscerofugal neurons (for the sake of completeness). Transduction sites are shown as open circles; transmitter release sites are shown as open triangles. CM: Circular muscule, MP: Myenteric plexus, LM: Longitudinal muscle (image modified from Brookes et al., 2013).

The morphology of the peripheral endings of vascular afferent fibres has been described in detail. They give rise to fine-branching peri-arterial axons that are preferentially associated with arterial branch points. Importantly, they are not restricted to mesenteric vessels but continue into the gut wall, innervating the arteries and second-order arterioles in the submucosa but not finer branches or capillaries. The same afferent unit can have transduction sites on both mesenteric and submucosal vessels (Hillilä et al., 2010; Song et al., 2009). These afferents have high distension thresholds and slow firing rates and frequently contain calcitonin gene related peptide (CGRP) (Robinson and Gebhart, 2008; Wang et al., 2004) and TRPV1 receptors (Blackshaw et al., 2010; Malin et al., 2009).

TRPV1 belongs to the transient receptor potential (TRP) channel family and is activated by heat, protons and vanilloid ligands such as capsaicin (Caterina et al.,

1997; 2000; Joranson et al., 2000). These receptors have been strongly implicated in nociception and pain (Blackshaw et al., 2010; Caterina et al., 1997; Cortright et al., 2007; Wesselmann et al., 2009), including thermal nociception and inflammatory hyperalgesia and allodynia (Davis et al., 2000). However, TRPV1 is more highly expressed in visceral innervating afferents than in skin innervating afferents (Christianson et al., 2006b; Ward et al., 2003). The importance of these receptors in visceral pain appears to be established. Expression of TRPV1 is increased in colonic nerve fibres of patients with IBD (Yiangou et al., 2001), in patients with rectal hypersensitivity (Chan et al., 2003) and in patients with IBS, and this enhancement of TRPV1 expression is correlated with the intensity of abdominal pain (Akbar et al., 2008). Conversely, administration of TRPV1 antagonists can attenuate disease severity in dextran sulphate sodium-induced colitis in mice (Kimball et al., 2004).

Given the major importance of TRPV1 receptors in intestinal pain we have used a model of visceral pain (intracolonic instillation of capsaicin) that activates these receptors in the experimental part of this Thesis.

2.1.2 URINARY BLADDER NERVE ENDINGS

Four types of neuronal endings can be distinguished in the bladder depending on their location: (i) 'urothelial', which respond to gentle stroking of the bladder, (ii) 'muscular/urothelial', which respond to both urothelial stroking and graded stretch of the bladder, (iii) 'muscular', which have receptive fields in the bladder wall that are optimally activated by probing and stretch, but are not responsive to fine urothelial stroking, and (iv) 'serosal', which are located principally near the base of the bladder or near vascular elements in the bladder wall. This last type may be involved in chemosensitivity as well as in urinary frequency changes caused by chemical bladder inflammation (Xu and Gebhart, 2008).

Two main mechanisms of bladder afferent activation have been described, direct and indirect. The direct mechanism involves physical activation of afferent nerve

endings *via* stimulation of mechano-gated receptors, whereas the indirect one involves activation of receptors on nerve fibres *via* mediators released from surrounding non-neuronal cells (Birder, 2011). The urothelium plays a key role in this indirect mechanism.

The urothelium forms the interface between the urinary space and the underlying vasculature and connective, nervous, and muscular tissues (Fig. V). It is composed of at least three layers: a basal cell layer attached to a basement membrane, an intermediate layer, and a superficial or apical layer composed of large hexagonal cells (diameters of 25–250 mm) known as "umbrella cells" (Lewis, 2000). The apical surface of the urothelium is also covered with a sulphated polysaccharide glycosaminoglycan (GAG) or mucin layer that is thought to act as a nonspecific antiadherence factor and as a defence mechanism against infection (Parsons, 2007).

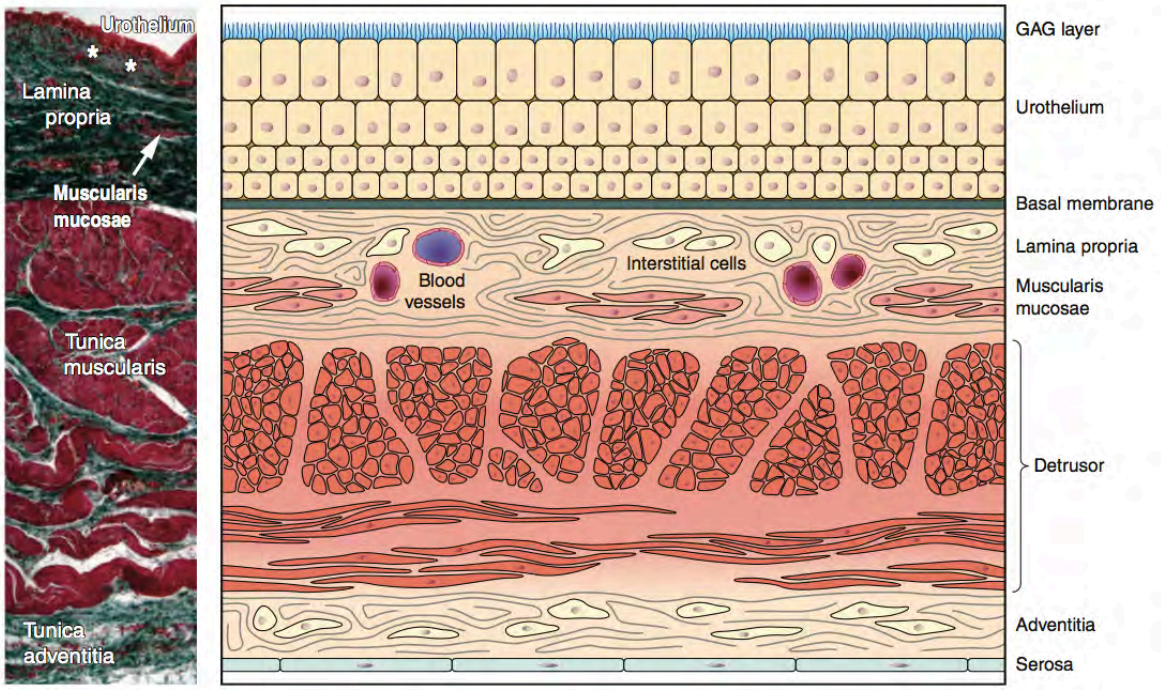

Fig. V. Structure of urinary bladder. Left: Counterstained transverse section through human urinary bladder. Right: Graphic representation of the different components. GAG: Glycosaminoglycan (modified from Birder and Andersson, 2013).

The epithelium of the urinary bladder not only acts as a simple barrier but also exhibits specialized sensory properties and plays a key role in the detection and transmission of nociceptive stimuli. Findings from a number of studies suggest that the urothelium exhibits the properties of both a "sensor" (expressing receptors/ion channels capable of responding to thermal, mechanical and chemical stimuli) and a "transducer" (able to release chemicals). Thus, urothelial cells have the capacity to sense changes in their extracellular environment, including the ability to respond to chemical, mechanical, and thermal stimuli that may communicate the state of the urothelial environment to the underlying nerve and muscle systems. (Birder, 2011)

The expression of numerous receptors/ion channels similar to those found in nociceptors and mechanoreceptors has been observed in the urothelium. Examples of neuronal "sensor molecules" (receptors/ion channels) identified in the urothelium include the receptors for various TRP channels, such as TRPA1 and TRPV1, which are also expressed in afferent nerves (Birder, 2005). The TRPV1 channels of the urinary bladder appear to have pathophysiological relevance in humans, given that patients with neurogenic detrusor overactivity exhibit significant increases in the number of TRPV1-expressing nerves and in TRPV1 expression in the urothelium (Apostolidis et al., 2005). Another member of the TRP family, TRPA1 (characterized as a thermoreceptor and chemoreceptor activated by noxious cold and irritants), is expressed in C-fibre afferents and in the urothelium, and TRPA1 agonists induce bladder hyperreflexia (Streng et al., 2008). Urothelial cells also secrete transmitters or mediators, including neurotrophins, peptides, ATP, acetylcholine, prostaglandins, prostacyclin, nitric oxide (NO) and cytokines, which are capable of modulating, activating or inhibiting sensory nerves (Birder and Andersson, 2013).

2.1.3 PRIMARY AFFERENT FIBRES AND NEURONS

Visceral extrinsic primary afferent neurons have almost exclusively thinly myelinated Aδ-fibres and unmyelinated C-fibres. The unmyelinated afferent fibres

(C-fibres) constitute the largest group of afferent fibres in visceral nerves. Up to eighty percent of visceral DRG somata can have C-fibres, whereas fewer than forty percent generally have Aδ-fibres (Cervero and Laird, 1999; Chang, 2004; Kuo et al., 1983; Robinson and Gebhart, 2008). Myelinated Aδ-fibres are mechanosensitive and are probably responsible for the sensation of fullness. They are activated by both low (non-nociceptive) and high (nociceptive) pressures. Unmyelinated C-fibres are activated by cold, heat or irritation of the mucosa. C-fibres have primarily nociceptive functions and do not usually respond to distension in a physiological situation (less than 10% of the unmyelinated afferents of the pelvic nerve can be activated by extreme noxious pressure stimuli) (Brookes et al., 2013; Jänig and Koltzenburg, 1990; Robinson and Gebhart, 2008), but may do so under pathological conditions (Blackshaw et al., 2010; Cervero, 1985; Cervero and Laird, 1999; Haupt et al., 1983; Häbler et al., 1990; Kanai, 2011; Longhurst et al., 1984).

Most visceral sensory neurons are peptidergic neurons that express CGRP and substance P (Perry and Lawson, 1998). Only some of them are non-peptidergic and express IB4 and $P2X_3$ (Bradbury et al., 1998). The first major fraction of peptidergic neurons further increases during inflammatory processes associated with pain (Vizzard, 2001).

In addition to TRPV1 and TRPA1, which are expressed not only in colon and urinary bladder but also in many other viscera, other important molecules are involved in the detection or transmission of nociceptive potential in visceral pain. These molecules include: other transient receptor potential ion channels (TRPs) such as TRPV3, TRPV4 and TRPM8 (Blackshaw et al., 2010; Davis, 2012); acid sensing ion channels (ASIC) (Davis, 2012; Holzer, 2011); voltage-gated sodium channels, such as Nav 1.8 (Laird et al., 2005; 2002; Robinson and Gebhart, 2008); voltage-gated calcium channels (Cervero and Laird, 1999; 2003; Davis, 2012); opioid receptors (Davis, 2012; Gebhart et al., 2000; Hillilä et al., 2010); 5-HT receptors (Wang et al., 2004; Wesselmann et al., 2009); gamma aminobutyric acid receptors (Blackshaw et al., 2010; Davis, 2012); N-methyl-D-aspartate receptors (Giamberardino, 1999; Joranson et al., 2000); ATP-gated channels (Burnstock, 2009; Davis, 2012; Wesselmann et al., 2009) and somatostatin and bradykinin

receptors (Davis, 2012; Mayer, 2011; Robinson and Gebhart, 2008), among others.

2.2 CENTRAL NERVOUS SYSTEM

2.2.1 SPINAL CORD AND ASCENDING PATHWAYS

Only 5-15% of DRG neurons are visceral (Giamberardino, 2009), but they have a terminal arborisation in spinal cord that extends rostrocaudally for 5 to 10 segments and covers the mediolateral and dorsoventral extent of the dorsal horn. Visceral afferents end in the superficial dorsal horn (laminae I and II), where somatic nociceptors also end, in the intermediolateral cell column and the sacral parasympathetic nucleus, where they influence the output of sympathetic and parasympathetic efferent fibres innervating the viscera, and around the central canal, in an area called lamina X, but they are absent in laminae IIi and III (Fig. VI) (Christianson et al., 2006b; Sugiura et al., 1993; Ward et al., 2003; Yiangou et al., 2001). This extreme terminal arborisation of visceral afferent fibres may explain the difficult localisation of visceral pain (Traub, 2006).

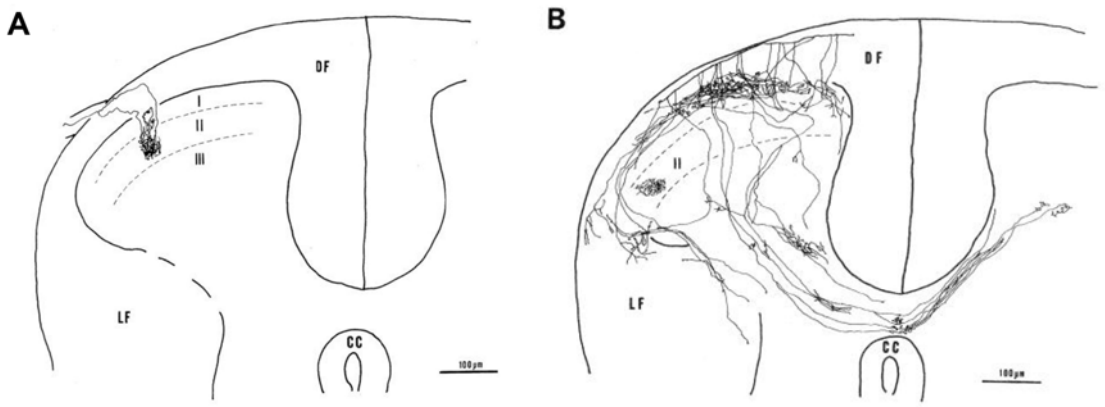

Fig.VI. Differences in central projections of a single somatic (A) or visceral (B) primary afferent C-fibre of guinea pig. DF: Dorsal funiculus, LF: Lateral funiculus, CC: Central canal (modified from Sugiura et al., 1989).

The convergence of afferent input pulses is a defining characteristic of spinal visceroceptive neurons. Practically all visceral spinal neurons in the spinal cord receive convergent somatic input pulses (Fig. VII). Anatomic and electrophysiological studies show viscerosomatic convergence in both dorsal horn and supraspinal centres, and it has been reported that 60-75% of all neurons in the thoracic spinal cord are viscerosomatic (Al-Chaer et al., 1996a; Alarcon and Cervero, 1990; Chan et al., 2003; Sugiura et al., 1993). This convergence was initially proposed by Ruch in 1946 (theory of convergence-projection whereby one spinal cord neuron is activated by both somatic and visceral neurons) to explain referred cutaneous pain phenomena (McMahon et al., 1995).

Viscero-visceral convergence of afferent information in the spinal cord is another characteristic phenomenon (Fig. VII). It enhances both direct and referred painful symptoms through interaction between two affected internal organs that have at least partially overlapping sensory projections (e.g. colon and urinary bladder) (Giamberardino, 2009; Giamberardino et al., 2010).

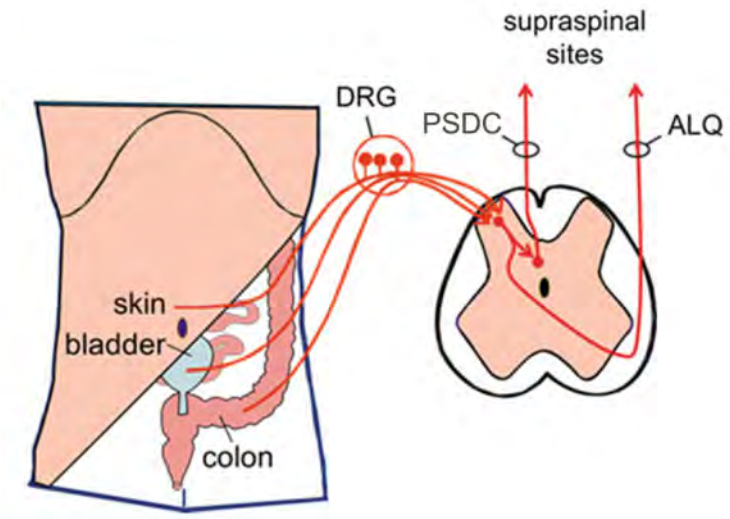

Fig.VII. Viscerosomatic and viscero-visceral convergence of primary sensory afferent from bladder, colon and skin to the same second-order neuron on the spinal dorsal horn. Visceral sensory information is transmitted rostrally in the contralateral anterolateral quadrant (ALQ) of the spinal cord or ipsilateral postsynaptic dorsal column (PSDC) to supraspinal sites. DRG: Dorsal root ganglia (modified from Schwartz and Gebhart, 2014).

Centrally, ascending pathways involved in the transmission of nociceptive information include the spinothalamic tract and other spinal tracts located in the anterolateral system. Furthermore, recent studies described a role for dorsal column in viscerosensorial processing (Palecek, 2004; Schwartz and Gebhart, 2014) (Fig. VII).

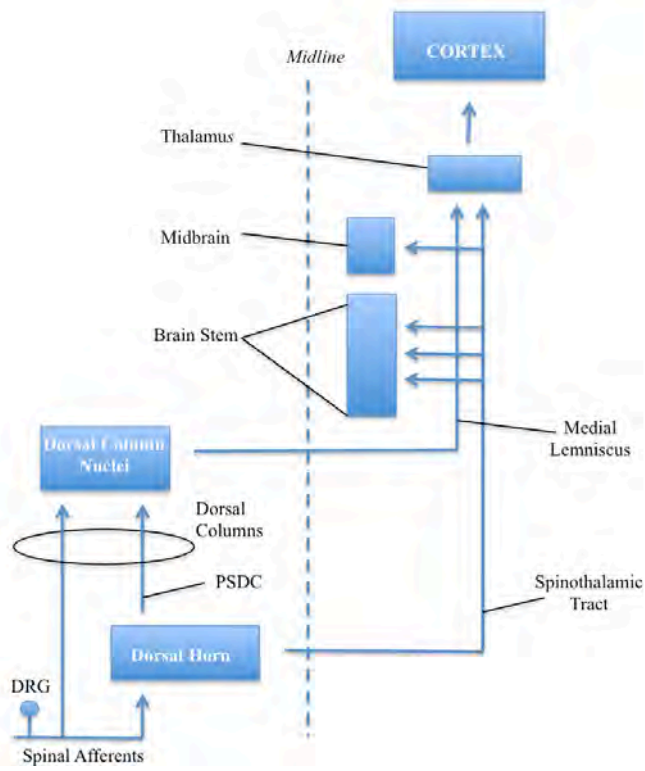

Fig. VIII. Schematic representation of the principal afferent pathways involved in pain. The most important in visceral pain are the postsynaptic dorsal columns (PSDCs) and spinothalamic tract (modified from Al-Chaer and Willis, 2006).

The spinothalamic pathway is the main transport circuit for nociceptive information from the skin, joints and muscles to supraspinal structures. Spinothalamic second-order neurons, which are synapsed by the primary afferents at the spinal level mainly at laminae I and V (Apkarian and Hodge, 1989; Lewis, 2000; Willis et al., 1979), decussate to the contralateral side of the spinal cord and ascend, carrying the sensation of pain to the ventral thalamus, directly (neospinochalamic tract) or with intermedial synapse in the reticular formation (spinoreticular tract), mesencephalon (spinomesencephalic tract) or hypothalamus (spinohypothalamic tract)(Fig. VIII). The spinothalamic tract is

important but not essential in visceral pain. In fact, bilateral sectioning of this pathway does not block behavioral responses to painful visceral stimulation in rats. In contrast, a much greater reduction in the behavioral response to visceral pain is obtained by blocking the dorsal column pathway (Parsons, 2007; Willis, 2007). This pathway is composed on one side by the axons of directly ascending first-order neurons and on the other side by axons of second-order neurons that synapse in split-level spinal laminae III and X and then ascend to the ventral thalamus (Bennett et al., 1983; Giesler et al., 1984; Kanai, 2011). The latter is called the postsynaptic dorsal column (PSDC) system and is more important in visceral pain. It has been demonstrated that these cells respond to both mechanical and chemical irritation of the viscera (Al-Chaer et al., 1999; 1996b; Birder, 2005). It is likely that this visceral information through the dorsal column system eventually converges with somatic information to reach the thalamus (Apostolidis et al., 2005; Willis and Westlund, 1997). Further support has been given to the notion that all of the complex information involved in pain perception is not transmitted through the same pathway in visceral nociceptive transmission. Thus, the dorsal column pathway lesion is effective to decrease behavioral responses, but bilateral lesions of the dorsal columns do not affect responses of the autonomic nervous system to painful visceral stimuli or the reflex responses mediated by the brainstem, indicating that the information contributing to the affective and discriminative dimensions and the autonomic nervous system response to visceral pain pass through different pathways within the spinal cord (Ness, 2000).

2.2.2 SUPRASPINAL STRUCTURES AND DESCENDING MODULATORY SYSTEM

Ascending noxious stimuli can activate several cortical areas besides the thalamus. These areas can differ according to the viscera stimulated, the site of stimulation and/or the type of stimulus (heat, distension or inflammation) (Blackshaw et al., 2010; Davis, 2012; Derbyshire, 2003), but the principal areas activated are the somatosensory, insular, prefrontal and anterior cingulate cortices (Fig. IX).

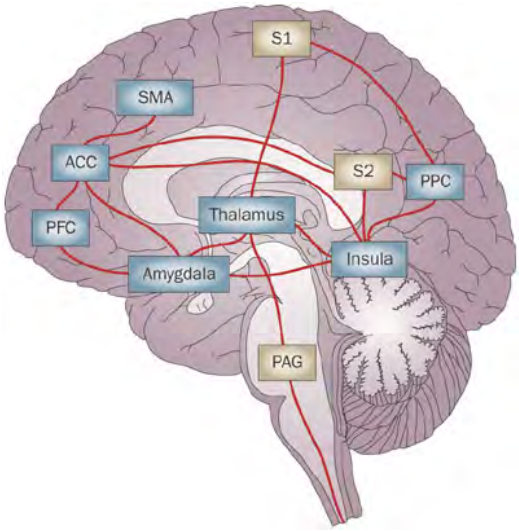

Fig. IX. Representation of the supraspinal structures involved in visceral pain processing. ACC: Anterior cingulate cortex, PAG: Periaqueductal grey matter, PFC: Prefrontal cortex, PPC: Posterior parietal cortex, S1: Primary somatosensory cortex, S2: Secondary somatosensory cortex, SMA: Supplementary motor area (modified from Bushnell et al., 2013).

The primary and secondary somatosensory cortices (S1/S2) receive the axons arising from neurons in the ventral thalamus that encode the intensity and localization of the stimulus. Visceral sensation is primarily represented in the secondary somatosensory cortex, whereas representation in S1 is vague (Aziz et al., 2000). The secondary somatosensory cortex (S2) sends projections to other structures such as the prefrontal cortex (which mainly plays a role in the cognitive influence of pain), the insula and the anterior cingulate cortex.

The insular cortex is the brain structure most consistently reported to be activated in pain studies (Mauguière, 2004). The insula integrates multimodal sensory and emotional information, has an important function in integrating visceral sensory and motor activity together with limbic integration and is particularly important in pain perception from the viscera (Augustine, 1996; Davis, 2012). It has connections with the amygdala and anterior cingulate cortex subregions. It is thought to have an interoceptive function, related to the image that we have of the state of our body.

The anterior cingulate cortex (ACC) is involved in the affective-motivational component and cognitive assessment. It would be related to the feeling of "disgust" that pain generates. This region also produces autonomic and emotional responses and descending modulatory responses (Zhuo, 2007).

The supplementary motor area (SMA) and posterior parietal cortex (PPC) are implicated in the body response to perceived pain, i.e., as defence to avoid the nocive stimulus causing the pain perception. These areas are less represented in visceral pain than in somatic pain (Mayer et al., 2009).

The vagal afferents project mainly to parabrachial nuclei, which in turn connect with the limbic and insula regions. There is no evidence of vagal projections connecting with S1 or S2. There is further evidence that vagal afferents transmit non-discriminative information such as satiety or nausea (Aziz et al., 2000).

The central nervous system in not a passive recipient of sensory input, and sensory processing is subject to complex modulation involving descending modulatory systems (Fig. X). The ability to inhibit or increase nociceptive signals to higher centres and thereby modulate pain perception is mediated by complex supraspinal circuits.

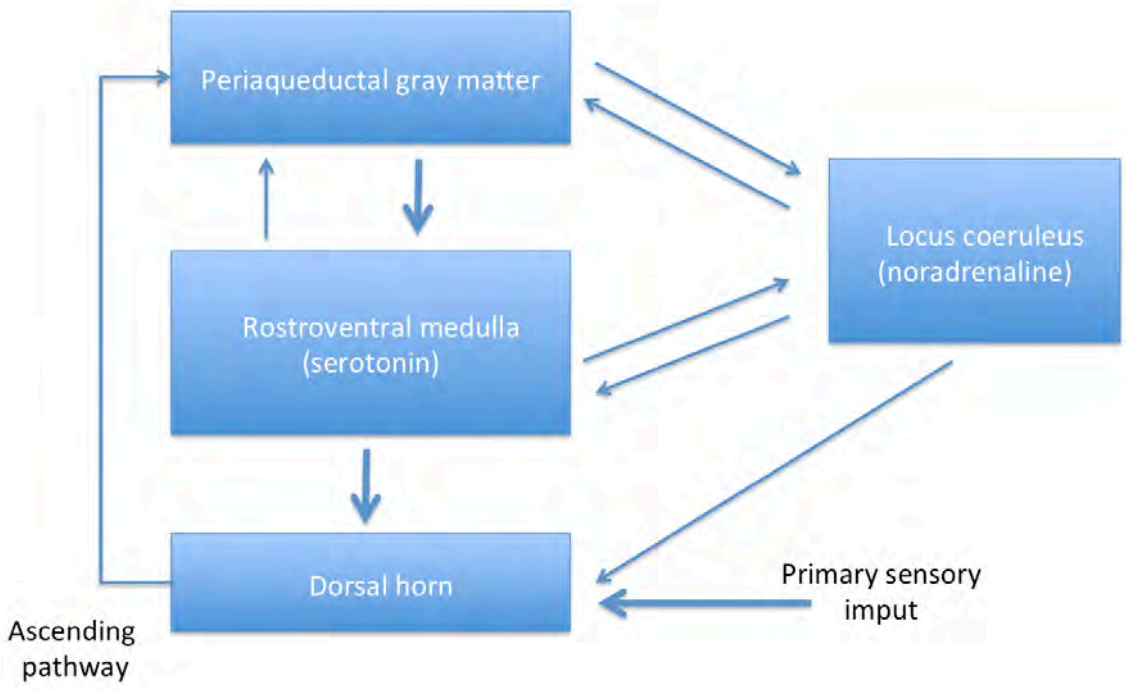

Fig. X. Spinal-medullary-spinal negative feedback loop underlying an endogenous analgesic system called into play by nociceptive stimuli (modified from Basbaum and Fields, 1984).

Descending inhibition and excitation are modulated by the spinal pathways of the dorsolateral funiculi and ventral/ventrolateral funiculi, respectively (Millan, 2002). Brainstem structures participate in the formation of descending pathways to the dorsal horn in a spinobulbar spinal loop that controls pain transmission by numerous monoaminergic and peptidergic (including endogenous opioids) transmitters in their spinal terminals (Tavares and Lima, 2002).

The anterior cingulate cortex is the central cortical region involved in modulating descending pain control with efferents to periaqueductal grey matter (PAG). Projections of laminae I and X also arrive in PAG, but direct connections of PAG with spinal cord have not been found. However, classical studies have shown that electrical stimulation of the PAG leads to suppression of pain (Reynolds, 1969). This is attributed to descending projections sent by the PAG to monoaminergic brainstem nuclei such as the locus coeruleus (which has a large number of noradrenergic neurons that descend to contact mainly with spinothalamic neurons in laminae I and V) and the rostroventral medulla (many raphe neurons are the origin of serotonergic projections that can modulate spinal nociceptive processing). Thus, locus coeruleus and rostroventral medulla send information downstream to the dorsal horn of the spinal cord and influence synaptic transmission of visceral sensory signals at this level (Giamberardino, 2009).

3. SIGMA-1 RECEPTORS

3.1. GENERAL OVERVIEW OF SIGMA RECEPTORS

The existence of sigma (σ) receptors was first proposed by Martin and coworkers in 1976. They were initially considered as a subtype of opioid receptor to account for the psychotomimetic actions of (±)-SKF-10,047 (*N*-allylnormetazocine) and other racemic benzomorphans. This initial confusion was due to the complex pharmacology of this racemic compound; later studies showed that whereas (–)-SKF-10,047 binds to μ and κ opioids, so that its effects are reversible by naloxone, the (+)-isomer lacks affinity for opioid receptors but binds to PCP (phencyclidine) binding sites with low affinity and to a different site with high affinity that still retains the designation of σ receptor (Cobos et al., 2008; Matsumoto et al., 2003; Zamanillo et al., 2013).

Nowadays, σ receptors are considered as an independent drug target entity and have been pharmacologically characterized in two subtypes, termed σ_1 and σ_2 (reviewed by (Cobos et al., 2008; Matsumoto et al., 2003). The σ_1 receptor was first cloned in guinea pig liver (Hanner et al., 1996) and later in other rodent and human tissues, including the brain of mice (Pan YX et al., 1998). σ_1 knockout mice (Langa et al., 2003) have been obtained and are helping to improve knowledge of the biochemical and functional characteristics of the σ_1 receptor. The σ_2 receptor has not been cloned, and its pharmacology and pathophysiological implications are less well studied than those of the σ_1 receptor. However, the σ_2 receptor was recently identified as progesterone receptor membrane component 1 (Xu et al., 2011) and appears to play an important role in the development of apoptosis and the regulation of intracellular calcium homeostasis (Crawford and Bowen, 2002; Vilner and Bowen, 2000).

3.2. MOLECULAR STRUCTURE AND LIGANDS OF σ_1 RECEPTORS

The locus coding for the σ_1 receptor is located in human chromosome 9 (9p13.3) and mouse chromosome 4. The gene (SIGMAR1) is 7kbp long and contains four exons and three introns (Prasad et al., 1998); it encodes a 29-kDa single polypeptide of 223 amino acids with a high sequence homology between several species (including human and mouse), but it is structurally unrelated to any other known protein in mammals. Aydar and coworkers presented evidence that the σ_1 receptor in the plasma membrane has two transmembrane segments: residues 11-29 and 80-100 (Aydar et al., 2002). Subsequent studies reported the existence of two additional hydrophobic segments (one of them partially overlapping the second transmembrane domain) corresponding to steroid binding domain-like sites (SBDL) and purportedly responsible for ligand binding (Brune et al., 2013)(Fig. XI).

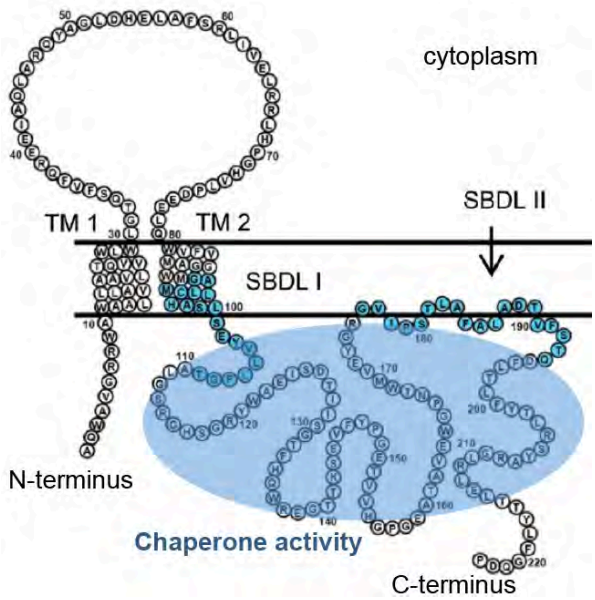

Fig. XI. Structural representation of σ_1 receptors. SBDL 1: Steroid binding domain-like 1, SBDL2: Steroid binding domain-like 2, TM1: Transmembrane domain 1, TM2: Transmembrane domain 2 (modified from Brune et al., 2013).

More recently, the existence of a chaperone domain (Hayashi and Su, 2007) within residues 112-223 of the σ_1 receptor was described (Ortega-Roldan et al., 2013). This domain confers to this receptor the ability to modify the function of a variety of target proteins, which underlies the modulatory actions of σ_1 receptors. There is evidence for splice variants of the σ_1 receptor that lead to at least two truncated

versions of the σ_1 receptor (Shioda et al., 2012). In addition, nascent data describe σ_1 receptor polymorphisms that have functional consequences in diseases such as schizophrenia or alcoholism (Ishiguro et al., 1998; Miyatake et al., 2004).The endogenous ligand of the σ_1 receptor remains unidentified. Neurosteroids (steroids locally synthesized in the central and peripheral nervous systems) such as pregnenolone, dehydroepiandrosterone (DHEA), their sulphate esters, progesterone and allopregnanolone are currently considered the most probable endogenous σ_1 ligands (Cobos et al., 2008; Maurice et al., 2001). Among the steroids tested, progesterone was the most potent inhibitor of σ_1-specific radioligand binding (Schwarz et al., 1989). Moreover, the existence of another putative endogenous ligand, *N*,*N*-dimethyltryptamine (DMT), a natural hallucinogen, has been reported (Fontanilla et al., 2009). However, whether neurosteroids or DMT are the endogenous ligands of the σ_1 receptor remains controversial, because the affinity of most neurosteroids or DMT for σ_1 receptors does not appear to be high enough for an endogenous ligand (Cobos et al., 2008; Fontanilla et al., 2009; Schwarz et al., 1989). Hence, the endogenous σ_1 ligand (if any) has not yet been unequivocally described.

Some selective and high-affinity σ_1 drugs have been developed and are considered prototypical σ_1 ligands. Examples of these ligands are the σ_1 agonists PRE-084 and (+)-pentazocine and the σ_1 antagonists BD 1063 and NE-100 (Cobos et al., 2008). The number of σ ligands is increasing rapidly with the development of new compounds (Collina et al., 2007; Marrazzo et al., 2002; Matsumoto et al., 2001; Ronsisvalle et al., 2000). The σ_1 antagonist S1RA deserves special mention. It has demonstrated an elevated selectivity for σ_1 receptors, lacking affinity for 170 additional targets (Romero et al., 2012). This drug is currently in Phase II clinical trials for pain treatment after completing a Phase I study with excellent kinetics and tolerability results (Abadias et al., 2013).

3.3. SUBCELLULAR AND ANATOMICAL DISTRIBUTION OF σ_1 RECEPTORS

σ_1 receptors are principally found in the endoplasmatic reticulum (ER), accumulate in regions characterized by a high cholesterol amount (Palmer et al., 2007) and are particularly enriched in the ER-mitochondrion interface called MAM (mitochondria-associated endoplasmic reticulum membrane), an ER subcomponent that physically interacts with the mitochondrial outer membrane and plays an important role in the transfer of Ca^{2+} from the reticulum to mitochondria, stimulating oxidative metabolism and regulating Ca^{2+} homeostasis (Pinton et al., 2008). Several studies have demonstrated that σ_1 receptors are highly mobile upon stimulation by ligands or under prolonged cellular stress and that they translocate to other areas of the cell, such as the extended ER reticular network or plasma membrane, under these conditions (Su et al., 2010).

Anatomically, σ_1 receptors are extensively distributed in different areas of the central nervous system, where they have been thoroughly studied. They are widely distributed in the brain, including important areas for pain control such as the PAG, locus coeruleus and rostroventral medulla (RVM) (Roh et al., 2011; Sánchez-Fernández et al., 2013; Zamanillo et al., 2013). Numerous σ_1 receptors are also present in the spinal cord, mainly in the superficial layers of the dorsal horn (Alonso et al., 2000). It was recently found that σ_1 receptors are also present in the peripheral nervous system in neuronal bodies of the DRG at a much higher density than in pain-related central nervous system areas (Bangaru et al., 2013; Sánchez-Fernández et al., 2014). σ_1 receptors have also been located in Schwann cells, specifically in their cytoplasm and in the paranodal region of Ranvier nodes (Palacios et al., 2004).

Most pain studies have focused on the importance of σ_1 receptor located in central nervous system; however, other locations of this receptor in peripheral organs, such as the gastrointestinal tract, liver, kidney, spleen, heart and bladder (e.g. (Bhuiyan and Fukunaga, 2011; Bowen, 2000; Stone et al., 2006; present study), can be of major functional importance.

3.4. SIGMA-1 RECEPTORS AND PAIN

Chien and Pasternak were the first to report the involvement of σ_1 receptors in analgesia (Chien and Pasternak, 1993). They demonstrated that σ_1 receptors play an important role in the modulation of opioid analgesia using the tail-flick test in mice. The systemic administration of σ_1 agonists, including the selective σ_1 agonist (+)-pentazocine, antagonized the antinociception induced by morphine in the tail-flick test (Chien and Pasternak, 1993; 1994; Mei and Pasternak, 2002). Conversely, haloperidol, an antipsychotic drug able to block σ_1 receptors, was found to potentiate opioid analgesia in humans (Maltbie et al., 1979) and rodents (Chien and Pasternak, 1994). Further experiments with other opioids and specific σ_1 antagonists confirmed the role of σ_1 receptors as modulators of opioid analgesia (Sánchez-Fernández et al., 2013; 2014; Vidal-Torres et al., 2014). These data suggest the presence of an anti-opioid σ system in which the σ_1 receptor exerts a tonic inhibitory control on opioid receptor-mediated signalling pathways. The direct molecular interaction between σ_1 and μ opioid receptors was recently reported (Kim et al., 2010). These receptors can be co-immunoprecipitated and have been shown to negatively modulate μ opioid signalling, given that σ_1 antagonism increases GTPγS in response to the μ opioid agonist DAMGO (Kim et al., 2010). Although the effect of σ_1 receptors was first thought to be exclusively centrally mediated (Mei and Pasternak, 2002), evidence was recently published of a peripheral effect on opioid modulation by σ_1 receptors (Sánchez-Fernández et al., 2013; 2014). Importantly, this modulation of opioid analgesia by σ_1 receptors does not modify the non-analgesic effects of opioids, such as constipation, hyperlocomotion, mydriasis or physical dependence (Chien and Pasternak, 1994; Sánchez-Fernández et al., 2013; Vidal-Torres et al., 2013), underlining the possible clinical relevance of the association of σ_1-receptor antagonists with μ-opioid agonists.

Modulation of opioid analgesia is not the only role of σ_1 receptors in pain perception, and they may also be involved in nociception in the absence of opioid drugs. The first evidence on the role of σ_1 receptors in pain control *per se* was obtained by our group in a formalin-induced pain model in mice. We found that σ_1

knockout mice showed less pain after intraplantar administration of formalin (Cendán et al., 2005a) and that σ_1 antagonists (haloperidol and some of its metabolites) produced dose-dependent analgesia in this pain model, showing a good correlation between their affinity for the σ_1 receptor and their analgesic potency (Cendán et al., 2005b). It was later reported that at least part of the analgesic effect of σ_1 receptor antagonists in the formalin model is exerted on the spinal cord by interfering with central sensitization mechanisms (Kim HW et al., 2006; Kim et al., 2008). In another model in which central sensitization mechanisms play a key role, we found that the σ_1 receptor antagonists BD-1063, BD-1047 and NE-100 antagonized mechanical allodynia induced by intraplantar capsaicin administration in the mouse (Entrena et al., 2009b) and that reduced haloperidol, an irreversible σ_1 receptor blocker (Cobos et al., 2007; Entrena et al., 2009a), produced a long-lasting antiallodynic effect (Entrena et al., 2009a).

It is noteworthy that the effects of σ_1 inhibition in either capsaicin-induced secondary mechanical hypersensitivity or in formalin-induced pain were not reversed by the opioid antagonist naloxone (Cendán et al., 2005b; Entrena et al., 2009a). This definitively indicates that these effects are independent of modulation of the opioidergic system and that σ_1 receptors can act *via* non-opioid mechanisms to decrease pain transmission.

σ_1 receptors are also able to modulate neuropathic pain. Thus, in the neuropathic pain produced by peripheral nerve trauma, systemic administration of the σ_1 antagonist S1RA fully reversed the hypersensitivity to mechanical and thermal painful stimuli (Bura et al., 2013; Díaz et al., 2012; Romero et al., 2012). Likewise, in the neuropathic pain produced by antineoplastic drugs, systemic treatment with σ_1 antagonists (BD-1063 and S1RA) not only abolished mechanical and cold allodynia once the neuropathy was fully developed (Nieto et al., 2012) but also completely prevented the hypersensitivity associated with the neuropathy, even after discontinuation of the treatment with σ_1 antagonists, suggesting that σ_1 inhibition has a protective role against the neuronal toxicity induced by paclitaxel (Nieto et al., 2012; 2014). These effects of σ_1 antagonists in neuropathic pain models appear to be related to the ability of the σ_1 receptor to facilitate central

sensitization mechanisms both electrophysiologically (wind-up phenomenon) and biochemically (enhancement of pERK in the spinal cord) (de la Puente et al., 2009; Nieto et al., 2012; Romero et al., 2012). In fact, intrathecal administration of σ_1 receptor antagonists inhibited neuropathic pain and some of its biochemical consequences in the spinal cord (Moon et al., 2013).

In contrast to neuropathic pain, inflammatory pain is characterized by a more pronounced enhancement of nociceptor responsiveness (peripheral sensitization) in response to the milieu of inflammatory mediators released at the inflammation site (Patapoutian et al., 2009). Using standard models of inflammatory pain (intraplantar administration of carrageenan or complete Freud´s adjuvant (CFA)) it was reported that the systemic administration of several σ_1 antagonists, including the selective σ_1 drugs S1RA and BD-1063, were able to ameliorate acute inflammatory pain hypersensitivity (Gris et al., 2014; Parenti et al., 2014a; 2014b; Tejada et al., 2014). Interestingly, we showed that peripheral σ_1 pharmacological antagonism (by BD-1063 and S1RA) in the inflamed site was sufficient to fully abolish inflammatory hyperalgesia (to both heat and mechanical stimuli) (Tejada et al., 2014), indicating that the activity of peripheral σ_1 receptors during inflammation is needed for the sensory alterations induced by a painful inflammation.

No data have previously been published on the effect of σ_1 receptors in visceral pain. As reported above, somatic and visceral pain differ at the level of nociceptor, ascending pathways and supraspinal areas activated by the different noxious stimuli and in other factors around nociceptive transmission. Therefore, although research in models of somatic pain has yielded vast amounts of data, they are not applicable to visceral pain. This lack of data prompted us to evaluate the role of σ_1 receptors in different models of visceral pain.

RATIONALE AND GOALS

1. RATIONALE

Visceral pain is one of the most common forms of pathological pain and one of the most frequent reasons for individuals to seek medical attention (Cervero and Laird, 1999; Robinson and Gebhart, 2008). The most common types of gastrointestinal and urological visceral pain in the clinical setting are irritable bowel syndrome (IBS) (representing up to 20% of consultations in gastroenterology), inflammatory bowel disease (IBD) and interstitial cystitis (Grundmann and Yoon, 2010; Marinkovic et al., 2009), which all have a substantial impact on the quality of life of the patients. The psychophysical characteristics of human visceral and somatic pain are very different (Strigo et al., 2002). Moreover, studies in experimental animals have evidenced differences in: a) the neurochemistry of primary afferent fibres innervating the viscera, skin or muscle (Lu et al., 2001; Perry and Lawson, 1998), b) the spinal neurons, ascending pathways and supraspinal nuclei projections involved in the transmission of cutaneous and visceral nociceptive information (Braz et al., 2005; Schwartz and Gebhart, 2014; Willis, 2007), and c) the neurobiological mechanisms activated by visceral pain and those activated by somatic pain (Al-Chaer and Traub, 2002; Foreman, 2004). Therefore, results obtained in models of cutaneous/somatic pain cannot be extrapolated to visceral pain.

Visceral pain is usually caused by an injury to hollow viscera and is very often associated with a cutaneous "referred pain" in a given skin area, whose afferent nerves project to the same spinal cord segment as the affected viscera; the development of cutaneous hyperalgesia in the area of referred pain is also frequent (Cervero and Laird, 1999; Robinson and Gebhart, 2008). Different models of visceral pain (which also generates cutaneous hyperalgesia) induced by activation of hollow viscera have been developed in rodents. Among these, capsaicin-induced stimulation of the colon and cyclophosphamide-induced cystitis are of particular interest (Bon et al., 2003; Laird et al., 2001; Olivar and Laird, 1999) because cystitis and intestinal pain are highly common types of visceral pain in humans, as mentioned above. Both animal models result in altered animal behavior indicative of visceral pain and cutaneous hyperalgesia to punctate mechanical stimuli

(equivalent to cutaneous hyperalgesia described in humans), with bladder inflammation in the cystitis model (Bon et al., 2003; Laird et al., 2001; Olivar and Laird, 1999), Furthermore, both animal models have been well validated in humans. Cyclophosphamide is an antineoplastic drug whose dose-limiting adverse effect in patients is a hemorrhagic cystitis with identical characteristics to that found in the mouse (Olivar and Laird, 1999). Similarly, intestinal application of capsaicin in humans produces visceral pain and cutaneous-referred pain (Arendt-Nielsen et al., 2008; Drewes et al., 2003).

Sigma (σ) receptors were first described in 1976 in studies with non-selective agonists of opioid receptors (SKF-10,047) (Martin et al., 1976) and were for some years considered incorrectly as a subtype of traditional opioid receptors. Subsequent studies showed them to be specific receptors that differ from other known receptors (Reviews: Cobos et al., 2008; Maurice and Su, 2009). Two subtypes have been described, σ_1 and σ_2, and both are widely distributed in the central nervous system, although their expression patterns differ among several areas (Bouchard and Quirion, 1997; Kitaichi et al., 2000; Sánchez-Fernández et al., 2014).

The σ_1 receptor was first cloned in guinea pig liver (Hanner et al., 1996) and later in other rodent and human tissues, including the brain of mice (Pan et al., 1998). It is a small (223 amino acids) protein that is structurally unrelated to any other known protein in mammals. It has two transmembrane domains and an endoplasmic reticulum retention signal in the N-terminal region (Cobos et al., 2008; Guitart et al., 2004). The presence of a chaperone domain within its structure (Hayashi and Su, 2007) was recently described, which may explain part of the action of the σ_1 receptor. This receptor has a rich pharmacology, and the best studied σ_1 receptor-specific antagonists are NE-100, S1RA and BD-1063 (Cobos et al., 2008; Maurice and Su, 2009; Romero et al., 2012). Moreover, σ_1-KO mice have been obtained, helping to improve our knowledge of the biochemical and functional characteristics of the σ_1 receptor (Cendán et al., 2005a; de la Puente et al., 2009; Entrena et al., 2009b; Langa et al., 2003).

There is a high density of σ_1 receptors in areas of the central nervous system with particular relevance for the transmission and modulation of nociceptive information, such as the surface layers (I and II) of spinal cord dorsal horn and the periaqueductal gray, among others (Alonso et al., 2000; Kitaichi et al., 2000). It was recently reported that the σ_1 receptor is also present in the peripheral nervous system in neuronal bodies of the DRG, where σ_1 receptors are found at a much higher density than in pain-related areas of the central nervous system (Bangaru et al., 2013; Sánchez-Fernández et al., 2014). The σ_1 receptors have also been located in Schwann cells, specifically in their cytoplasm and in the nodes of Ranvier paranodal region (Palacios et al., 2004).

Various functional data suggest involvement of the σ_1 receptor in cutaneous/somatic nociception. Thus, administration of σ_1 antagonists and antisense oligodeoxynucleotides against σ_1 receptor potentiate the analgesic effect induced by μ, δ, κ_1 and κ_3 opioid receptor agonists in the tail-flick test in mice, whereas the σ_1 receptor agonist (+)-pentazocine produces the opposite effect (Chien and Pasternak, 1994; King et al., 1997; Mei and Pasternak, 2002; 2007). Moreover, we have shown that σ_1-KO mice show less pain after intraplantar administration of formalin (Cendán et al., 2005a) and that σ_1 antagonists produce a dose-dependent analgesia in this model of pain, with a good relationship between their potency to block σ_1 receptors and their analgesic potency (Cendán et al., 2005b). Conversely, we have demonstrated that σ_1 receptor antagonists reverse the mechanical allodynia induced by intraplantar administration of capsaicin in mice (Entrena et al., 2009a; 2009b). Furthermore, σ_1-KO mice exhibit less allodynia after intraplantar administration of capsaicin (Entrena et al., 2009b) and in models of neuropathic pain (de la Puente et al., 2009; Nieto et al., 2012; Romero et al., 2012). Recently, various σ_1 antagonists, both systemically and peripherally (at the inflamed site) administered, have been found to completely abolish pain hypersensitivity in inflammatory models of pain such as intraplantar administration of carrageenan or complete Freud´s adjuvant (CFA) (Gris et al., 2014; Parenti et al., 2014a; 2014b; Tejada et al., 2014). Hence, there is wide evidence that σ_1 receptor inhibition reduces nociceptive, inflammatory and

neuropathic pain originated in the skin or somatic structures.

2. GOALS

The characteristics, pathophysiological mechanisms and response to drugs of visceral pain are different from those of cutaneous/somatic pain, as reported above, and results obtained in models of cutaneous/somatic pain cannot be extrapolated to visceral pain. The inhibition of σ_1 receptors is useful for the treatment of different types of cutaneous/somatic pain, but there has been no previous study of the role of σ_1 receptors in models of visceral pain. Therefore, our **global objective** was to evaluate the effect of pharmacological blockade and genetic inactivation of the σ_1 receptor in models of non-inflammatory and inflammatory visceral pain in mice (using models of pain that have been previously validated in humans to maximize the translational value of our results). To reach this goal, we performed different kinds of experiments with various specific objectives.

The **first specific objective** was to study the influence of the σ_1 receptor in a non-inflammatory model of visceral pain, the intracolonic capsaicin model. To meet this objective, we used two different experimental approaches. We first compared the responses of WT and σ_1-KO mice to intracolonic capsaicin and then studied the effect of several σ_1 antagonists (BD-1063, NE-100 and S1RA) in capsaicin-treated animals of both genotypes. In both cases we evaluate the behavioral manifestations indicative of spontaneous pain and the referred cutaneous mechanical hyperalgesia induced by the algogen.

The pathophysiological mechanisms involved in inflammatory and non-inflammatory pain are different. Therefore, the **second specific objective** of this Doctoral Thesis was to determine the role of the σ_1 receptor in a model of inflammatory visceral pain, the cyclophosphamide-induced cystitis model, and to identify whether σ_1 receptors are present in the urinary bladder and may be

involved in the results obtained. Several types of experiment were performed to meet this objective:

a. Given that the bladder urothelium expresses many receptors and channels that are expressed in DRG neurons and the σ_1 receptor is expressed in DRG neurons, we studied whether this receptor is also present in the bladder (performing western blot and immunohistochemistry experiments). Futhermore, we studied whether its expression in mouse bladder is similar to that in human bladder.
b. The functional role of urinary bladder σ_1 receptor was evaluated by studying the effect of its absence (in σ_1-KO animals) on cyclophosphamide-induced bladder inflammation, measuring both histological (oedema, haemorrhage, and desquamation) and biochemical (enhancement of MPO activity and pERK1/2 levels) parameters indicative of inflammation of this viscera.
c. The behavioral consequences of σ_1 receptor inhibition in cyclophosphamide-induced pain were determined by measuring the pain behavioral score and the referred mechanical hyperalgesia induced by the antineoplastic in WT and σ_1-KO mice, and by evaluating the effect on these pain parameters of the administration of σ_1 antagonists (BD-1063, NE-100 and S1RA) in animals of both genotypes.

The experiments performed to reach the two aforementioned specific objectives strongly suggested that the σ_1 antagonists may represent a new pharmacological tool for the treatment of visceral pain; therefore, the **third specific objective** was to compare the effect of the σ_1 antagonists with those of different control analgesic drugs, including opioid agonists (morphine) and NSAIDs (indomethacin and ketoprofen), in the two models of visceral pain studied. Furthermore, given that the σ_1 receptor modulates the analgesic effects of opioids in cutaneous/somatic pain models, we compared the effect of morphine between WT and σ_1-KO mice in the visceral pain models.

MATERIALS AND METHODS

1. ANIMALS AND DRUGS

1.1 ANIMALS

Experiments were performed in female WT (Charles River, Barcelona, Spain) and σ_1-KO (Laboratorios Esteve, Barcelona, Spain) CD-1 mice weighing 25–30 g. The σ_1-KO mice were generated on a CD-1 background as described previously (Entrena et al., 2009b). Animals were acclimated in our animal facilities for at least 1 week before testing, housed in colony cages in temperature- and light-controlled rooms (22 ± 1°C, lights on at 8:00 AM and off at 8:00 PM, air replacement every 20 min). A standard laboratory diet (Harlan Teklad Research Diets, Madison, WI) and tap water were available *ad libitum* until the beginning of the experiments. Testing took place during the light phase (from 9:00 AM to 3:00 PM). Mice were handled in accordance with the European communities council Directive of November 24, 1986 (86/609/Ecc), and the experimental protocols were approved by the University of Granada Research Ethics Committee.

1.2. DRUGS AND DRUG ADMINISTRATION

We used the selective σ_1 receptor antagonists BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride), supplied by Tocris Cookson Ltd. (Bristol, UK); NE-100 (N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine hydrochloride), synthesized as reported previously (Nakazato et al., 1999); and S1RA (4-[2-[[5-methyl-1-(2- naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride) (Romero et al., 2012), supplied by Laboratorios Esteve. Morphine hydrochloride (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health, Madrid, Spain) was used as opioid control drug, and ketoprofen (Sigma-Aldrich Química S.A., Madrid, Spain) and indomethacin (Sigma–Aldrich Química S.A., Madrid, Spain) were used as non-steroidal anti-inflammatory control drugs. All of these drugs were dissolved in sterile physiologic saline, with the exception of ketoprofen and indomethacin, which were dissolved in 10% absolute ethanol and 90% saline or 5% sodium

bicarbonate and 95% saline (Panreac Química S.L.U., Barcelona, Spain), respectively. Drug solutions were prepared immediately before the start of the experiments, and 5 ml/kg of the drug or its solvent was injected subcutaneously into the interscapular area.

Capsaicin (Sigma-Aldrich), which was used to induce intracolonic pain, was prepared to make up a 1% (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. The capsaicin solution was prepared once per week and stored at −20°C in aliquots, which were thawed and diluted at the appropriate concentrations on the day of the experiment. The capsaicin solution (50 µl) was instilled into the colon by introducing through the anus a fine round-tip cannula (external diameter, 0.61 mm; length, 4 cm) connected to a 1710 Tll Hamilton microsyringe (Teknokroma, Barcelona, Spain). Control animals were intracolonically instilled with the same volume of capsaicin solvent.

Cyclophosphamide (Sigma-Aldrich), which was used to induce cystitis, was dissolved in saline and injected intraperitoneally (i.p.) at the volume of 10 ml/kg. Control animals were injected with the same volume of solvent.

2. INTRACOLONIC CAPSAICIN MODEL

2.1. GENERAL PROCEDURES FOR EVALUATING INTRACOLONIC CAPSAICIN-EVOKED VISCERAL PAIN AND REFERRED HYPERALGESIA

Spontaneous pain-related behaviors and referred mechanical hyperalgesia induced by intracolonic capsaicin were tested following a previously described protocol (Laird et al., 2001), with small modifications. Mice were housed in individual transparent plastic boxes (7 × 7 × 13 cm) on an elevated platform with a wire mesh floor (small mirrors behind and below the chambers enhanced observation of the animals). After a 40 min habituation period, animals were removed from the compartments, and a capsaicin solution (or its solvent) was instilled intracolonically after application of petroleum jelly on the perianal area to

avoid stimulation of somatic areas through contact with the algogen. The animal was immediately returned to the compartment, where the number of pain-related behaviors (licking of the abdomen, stretching of the abdomen, and abdominal retractions) were counted by direct observation for 20 min.

The presence of capsaicin-induced referred hyperalgesia was determined by measuring the withdrawal response to a punctate mechanical stimulation of the abdomen at 20 min after the instillation of capsaicin (or its solvent). Forces ranging from 0.02 to 2 g (0.19–19.6 mN) were applied to the abdomen with a series of calibrated von Frey filaments (Touch-Test Sensory Evaluators; North Coast Medical Inc., Gilroy, CA) using the up–down paradigm (Chaplan et al., 1994). Perianal and external genitalia areas were avoided, concentrating the stimulation on the lower and mild abdomen, as reported previously (Laird et al., 2001). Filaments were applied three times for 2–3 s each one, with interapplication intervals of 5 s. Testing was initiated with the 0.4 g (3.92 mN) von Frey filament (i.e., the middle of the range). In each consecutive test, if there was no response to the filament, a stronger stimulus was then selected; if there was a positive response, a weaker one was then used. The response to the filament was considered positive if immediate licking/scratching of the application site, sharp retraction of the abdomen, or jumping was observed.

The experimenter who evaluated the behavioral responses was blinded to the treatment and genotype of experimental subjects. In all cases, experiments in WT or σ_1-KO groups, solvent- or capsaicin-treated groups, and saline- or drug-treatment groups were run in parallel. Each animal was used only once and received a single concentration of capsaicin (or its solvent) and a single dose of one drug (or its solvent).

2.2. COMPARISON OF THE EFFECT OF DIFFERENT CONCENTRATIONS OF INTRACOLONIC CAPSAICIN IN NAÏVE WT AND σ_1-KO MICE

WT and σ_1-KO mice were administered different capsaicin concentrations (0.01–

1%) intracolonically, and the pain-related behaviors and referred hyperalgesia to abdominal mechanical stimulation induced by each concentration were recorded consecutively in the same animal, following the procedure described above (see the first and second paragraphs of the section 2.1). This allowed the construction of concentration–response curves (concentration vs. number of behaviors or mechanical threshold) and identification of the optimal concentrations of capsaicin for the pharmacologic studies (see the next section for details).

2.3. COMPARISON OF DRUG EFFECTS ON VISCERAL PAIN INDUCED BY INTRACOLONIC ADMINISTRATION OF CAPSAICIN IN WT AND σ_1-KO MICE

Two experimental approaches were used to evaluate the effect of the drugs on capsaicin-induced visceral pain and referred hyperalgesia. First, we tested the effect of a fixed dose of a σ_1 receptor antagonist on the responses induced by different concentrations of capsaicin. Second, we tested the effects of several doses of various σ_1 receptor antagonists and control drugs on the pain-related behavior and referred hyperalgesia induced by a fixed concentration of capsaicin.

In the first experimental approach, BD-1063 (32 mg/kg) or its solvent was administered subcutaneously 30 min before the intracolonic instillation of different concentrations (0.01–1%) of capsaicin, and the number of pain-related behaviors was counted for 20 min after the capsaicin instillation. Immediately afterward (i.e., 50 min after injection of the drug), its effect on the capsaicin-induced referred hyperalgesia was assessed in the same animal by stimulation of the abdomen with von Frey filaments as described above in the section 2.1. Experiments were performed in both WT and σ_1-KO mice.

In the second experimental approach, different doses of BD-1063 (16–64 mg/kg), S1RA (32–128 mg/kg), NE-100 (8–64 mg/kg), morphine (1–16 mg/kg), ketoprofen (32–128 mg/kg), or their solvents were administered subcutaneously at 30 min before the intracolonic instillation of 1% capsaicin, and the number of pain-related behaviors was counted for 20 min after administration of capsaicin.

This concentration of capsaicin was selected because it produces the maximum number of pain-related behaviors in WT and σ_1-KO mice (Fig. 1A) and therefore offers the maximum window for observing any reductions in this response. In separate experiments, we tested the effect of the same doses of the σ_1 receptor antagonists and the control drugs on the referred hyperalgesia induced by a fixed concentration of capsaicin. In these experiments, the drug under study or its solvent was injected subcutaneously at 30 min before the intracolonic instillation of 0.1% capsaicin and, 20 min after the instillation, the response of the animal to abdominal stimulation with von Frey filaments was tested using the up–down method, as described above (see section 2.1). A capsaicin concentration of 0.1% was selected for these experiments because it reaches the maximum reduction in the mechanical threshold for referred hyperalgesia in WT and σ_1-KO mice (Fig. 1B).

3. CYCLOPHOSPHAMIDE-INDUCED CYSTITIS MODEL

3.1. CYCLOPHOSPHAMIDE-EVOKED VISCERAL PAIN AND REFERRED HYPERALGESIA

3.1.1. GENERAL PROCEDURES

Spontaneous pain-related behaviors and referred mechanical hyperalgesia induced by cyclophosphamide were tested following a previously described protocol (Laird et al., 2002; Olivar and Laird, 1999; Wantuch et al., 2007), with small modifications. Mice were housed in individual transparent plastic boxes (7 × 7 × 13 cm) on an elevated platform with a wire mesh floor (small mirrors behind and below the chambers enhanced observation of the animals). After a 40 min habituation period, animals were removed from the compartments and injected with the cyclophosphamide solution (or its solvent). The animals were immediately returned to the compartment, where they were observed for 2 min every half-hour over a 4 h observation period after the cyclophosphamide injection. The recorded pain-related behaviors were coded according to the

following scale: 0 = normal, 1 = piloerection, 2 = strong piloerection, 3 = laboured breathing, 4 = licking of the abdomen and 5 = stretching and contractions of the abdomen (Olivar and Laird, 1999). If more than one of these behaviors was noted in one observation period, the sum of the corresponding points to the different types of behaviors was assigned; i.e., if two stretching and contractions (5 points each) and one abdominal licking (4 points) occurred during an observation period, the final score was 9 instead of 14 points. An overall score was obtained by summing the scores assigned at each time point. At the end of the 4 h observation period, referred hyperalgesia was determined by measuring the withdrawal response to a punctate mechanical stimulation of the abdomen using the up-down paradigm (Chaplan et al., 1994) as described for capsaicin-induced referred hyperalgesia (see section 2.1).

The experimenter who evaluated the behavioral responses was blinded to the treatment and genotype of experimental subjects. In all cases, experiments in WT or σ_1-KO groups, solvent- or cyclophosphamide-treated groups, and saline- or drug-treatment groups were run in parallel. Each animal was used only once and received a single concentration of cyclophosphamide (or its solvent) and a single dose of one drug (or its solvent).

3.1.2. COMPARISON OF THE EFFECT OF DIFFERENT CONCENTRATIONS OF CYCLOPHOSPHAMIDE IN NAÏVE WT AND σ_1-KO MICE

WT and σ_1-KO mice were administered with different doses of cyclophosphamide (10-300 mg/kg), and the pain-related behaviors and the referred hyperalgesia to abdominal mechanical stimulation induced by each concentration were recorded consecutively in the same animal, following the procedure described above (see section 3.1.1). This allowed the construction of dose-response curves (dose *vs.* pain score or mechanical threshold) and identification of the optimal doses of cyclophosphamide for the pharmacological studies (see below for details).

3.1.3. COMPARISON OF DRUG EFFECTS ON VISCERAL PAIN AND REFERRED HYPERALGESIA INDUCED BY CYCLOPHOSPHAMIDE IN WT AND σ_1-KO MICE

To evaluate the effect of the drugs on cyclophosphamide-induced visceral pain, we tested the effects of several doses of σ_1 receptor antagonists and control drugs on the pain behavioral score and the referred hyperalgesia. Thus, different doses of BD-1063 (16-64 mg/kg), S1RA (32-128 mg/kg), NE-100 (16-64 mg/kg), morphine (1-8 mg/kg), indomethacin (2-8 mg/kg), or their solvents were administered s.c. at 2 hours after the i.p. injection of cyclophosphamide and the pain behavioral score was recorded every 30 min during 2 hours as described in section 3.1.1. To test the effects of the drugs on the pain-related behaviors a concentration of 300 mg/kg of cyclophosphamide was administered. This concentration of cyclophosphamide was selected because it was the lowest dose that produces the maximum pain score in WT mice (see Fig. 12A) and therefore offers the maximum window for observing any reductions in this response. In separate experiments, we tested the effect of the same doses of the σ_1 receptor antagonists and the control drugs on the referred hyperalgesia induced by cyclophosphamide. A cyclophosphamide dose of 100 mg/kg was selected for these experiments because it produces the maximum reduction in the mechanical threshold for referred hyperalgesia in WT and σ_1-KO mice (see Fig. 13). In these experiments, the drug under study or its solvent was s.c. injected at 2 hours after the i.p. administration of cyclophosphamide, and 2 hours later (i.e., 4 hours after the cyclophosphamide injection) the response of the animal to abdominal stimulation with von Frey filaments was tested using the up-down method, as described in the general procedures (see section 3.1.1).

3.2. COMPARISON OF THE EFFECTS OF DIFFERENT CONCENTRATIONS OF CYCLOPHOSPHAMIDE AND SEVERAL DRUGS ON URINARY BLADDER MYELOPEROXIDASE ACTIVITY

Changes in myeloperoxidase (MPO) activity represent a reliable index of polymorphonuclear leukocyte infiltration (Rouleau et al., 2000). Therefore, five hours after the injection of cyclophosphamide, the urinary bladder was dissected

out and finely minced using spring scissors. Then it was homogenized in 0.4 ml of phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma-Aldrich). After that, homogenates were freeze thawed three times and centrifuged (6000 g, 10 min) to collect the supernatant that was used for MPO activity assay adapted to a 96-well plate format. Briefly, 50 µl of supernatants or human neutrophil MPO standards (Sigma-Aldrich) were added to a 96-well plate. The reaction was initiated by the addition of 150 µl of phosphate buffer containing 0.167 mg/ml o-dianisidine (Sigma-Aldrich) and 0.0005% hydrogen peroxide (Sigma-Aldrich) and absorption was measured 5 min later at 450 nm (Microplate Spectrophotometer PowerWave X, Bio-tek instruments. Inc).

3.3. IMMUNOHISTOCHEMISTRY OF HUMAN AND MICE URINARY BLADDER SAMPLES

3.3.1 GENERAL PROCEDURES

Naïve and cyclophosphamide-treated WT and σ_1-KO mice were anesthetized with isofluorane (IsoVet®, B. Braun, Barcelona, Spain) and perfused intracardially with 20 ml saline followed by 30 ml of 3.7-4.0% formaldehyde buffered to pH 7.0 (Panreac Quimica SLU, Barcelona, Spain). After perfusion, the mouse bladder was dissected and fixed as a whole in 3.7-4.0% formaldehyde for 24 h. Then the bladders were dehydrated in solutions of increasing ethanol concentration (50%, 70%, 80%, 90%, 95%, 100%, 100%, 30 min of immersion in each solution), followed by immersion during 10 min in xylene and were embedded in paraffin following standard procedures. Tissue sections (5 µm thick) were obtained with a microtome (Microm HM325, Thermo Scientific), mounted in poly-L-lisine coated glass slides, deparaffinized (three washes of xylene for 5 min each) and hydrated (two washes of 100% ethanol for 3 min each, followed by washes of 95% and 70% ethanol for 3 min each, and 5 min in distilled water). Then bladder slides were heat-treated, in sodium citrate 10 mM, pH 8.0, buffer (Master Diagnóstica, Granada, Spain), for antigenic unmasking in a vegetable steamer at 95°C for 20 min, after which were cooled in distilled water at room temperature for 10 min.

Formalin fixed paraffin embedded adult human bladder sections (5 μm thick) mounted in glass slides (BioChain Institute Inc, Newark, California, lot number B507075) were deparaffinized, hydrated and heat-treated for antigenic unmasking following the same procedures used for mice urinary bladder slices.

3.3.2. PROCEDURES FOR σ_1-RECEPTOR DETECTION IN URINARY BLADDERS

In all the experiments, the "General procedures" described above (see section 3.3.1) were followed, including antigenic unmasking. After this, endogenous peroxidase activity of urinary bladder slices was blocked with 3% (v/v) H_2O_2 in methanol for 15 min, followed by washes (5 min each) in distilled water and TBS-Tween 20 buffer (Tris buffered saline, pH 7.4, Fisher BioReagents, with 0.1% Tween 20), successively.

For σ_1-receptor detection, the UltraVision Quanto peroxidase kit (Thermo Fisher Scientific, Watham, MA) was used. Nonspecific staining was blocked with Quanto Ultra V Block for 8 min at room temperature. Then samples were incubated, for 30 min at room temperature, with a mouse monoclonal primary antibody (sc-137075, Santa Cruz Biotechnology Inc, Santa Cruz, CA) diluted 1/400 in TBS-Tween 20 buffer. After three washes (10 min each) with TBS-Tween 20 buffer, samples were incubated with Quanto amplifier for 10 min, rinsed in TBS-Tween 20 (3 washes of 5 min each), and then incubated with Quanto polymer for 10 min at room temperature. After 3 washes (5 min each) with TBS-Tween 20, the peroxidase reaction was visualized using 3, 3'-diaminobenzidine (DAB) with the ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA). Briefly, bladder slices were incubated during 20 s at room temperature with the reactive (under faint light conditions), followed immediately by two washes of 5 min each with distilled water. Then samples were counterstained (2 min) in Mayer's haematoxylin and were washed with slightly alkalinized water during 5 min, followed by dehydration by quick (seconds) immersion in solutions of increasing ethanol concentration (95%, 95%, 100%, 100%) and 3 quick washes in xylene. Finally, a drop of mounting media Vitro-Clud (Deltalab, Barcelona, Spain) and a

coverslip were put over the sample.

Negative controls of σ_1-receptor detection were obtained by omission of the primary antibody and resulted in absence of immunoreactivity. Images were acquired using a Nikon Eclipse 50i microscope equipped with a DS-Ri1 camera (Nikon Instruments Europe BV, Amsterdam, Netherlands).

3.4. URINARY BLADDER HISTOPATHOLOGY OF CYCLOPHOSPHAMIDE-TREATED WT AND σ_1-KO MICE

Formalin fixed paraffin embedded urinary bladder slices (5 μm thick) were obtained, from naïve and cyclophosphamide-treated WT and σ_1-KO mice, as described in the 'General procedures', Section 3.3.1. Then, samples were deparaffinized, hydrated (as described in "General procedures") and stained in haematoxylin eosin. Images were obtained with an Olympus BX51 microscope. Three different histological alterations (urinary bladder oedema, haemorrhage and urothelium desquamation) indicative of cyclophosphamide-induced cystitis were analyzed (Martins et al., 2012; Santos et al., 2010).

A morphometric analysis of each slice was performed, with the ImageJ software (http://rsb.info.nih.gov/ij/index.html), and the oedematous area located between lamina propria and muscularis was normalized with respect to the total area of the bladder section. The number of haemorrhagic foci and urothelium desquamation areas were also counted in each slice.

3.5. WESTERN BLOTTING FOR σ_1-RECEPTOR AND EXTRACELLULAR SIGNAL REGULATED-KINASE (ERK) DETECTION IN WT AND σ_1-KO MICE URINARY BLADDERS

Experiments were performed as previously described in detail (Nieto et al., 2012), with some modifications. The bladders were carefully removed from naïve and cyclophosphamide-treated (at 1, 3 or 5 hours after a cyclophosphamide 300

mg/kg, i.p. injection) WT and σ_1-KO mice, frozen immediately in liquid nitrogen, and stored at -80°C. Bladders were thaw out, finely minced using spring scissors and homogenized in buffer (RIPA buffer with 0.5% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail-2, all from Sigma-Aldrich Química S.A., Madrid, Spain). Then, homogenates from three bladders were mixed and centrifuged at 1,000 g for 5 min to decant large remains. The supernatant, whose protein concentration was measured using the Bradford assay, was used for Western blot analyses. Equal amounts of protein (24 μg for ERK and 30 μg for sigma-1) were fractionated by 12% (w/v) SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Madrid, Spain). Membranes were incubated for 1 hour at room temperature in blocking buffer containing 5% dry skim milk in TBS-Tween 20 (Tris buffered saline, pH 7.4, Fisher Bioreagents, with 0.1% Tween-20) and were incubated overnight at 4°C with rabbit polyclonal antibody recognizing the extracellular signal-regulated kinases 1 and 2 (total ERK1/2) (M-5670, Sigma, Saint Louis, MO) at 1:40,000 dilution, rabbit monoclonal antibody recognizing the diphosphorylated ERKs 1 and 2 (pERK1/2) (4370S, Cell Signalling Technology, Danvers, MA) at 1:2,000 or with goat polyclonal antibody recognizing the σ_1 receptor (sc-22948, Santa Cruz Biotechnology, Heidelberg, Germany) at 1:250. To control for equal protein loading, rabbit polyclonal anti-GAPDH antibody (1:40,000 or 1:80,000 respectively, depending on the experiment) (G9545, Sigma) was used. All primary antibodies were diluted in TBS-Tween 20 buffer containing 0.5% dry skim milk. When primary antibody incubation period ended, the blots were washed 3 times for 10 minutes each with TBS-Tween 20 buffer and then incubated for 1 hour at room temperature with horseradish peroxidase–conjugated goat anti-rabbit IgG or donkey anti-goat IgG (both from Sigma), diluted at 1:2,000 and 1:2,500, respectively, in TBS-Tween 20 buffer containing 0.5% dry skim milk. Then, the membranes were washed with TBS-Tween 20 buffer, 6 times for 10 minutes each, and the peroxidase reaction was revealed by an enhanced chemiluminescence method (ECL Prime Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, UK), according to manufacturer's instructions. Chemiluminescence was detected with an LAS 3000 Image Analyzer System (Fujifilm, Tokyo, Japan). The densitometric

analysis of immunoreactive bands was done using the Quantity One software (Bio-Rad) and normalized with respect to the intensity of the corresponding GAPDH immunoreactive bands.

4. STATISTICAL ANALYSIS

The degree of referred pain, expressed as the mechanical threshold that produces 50% of responses, was calculated using the formula of Dixon (Dixon, 1980): 50% mechanical threshold (g) = [(10 $^{(Xf + \kappa\delta)}$) /10,000], where Xf = value (in logarithmic units) of the final von Frey filament used, κ = tabular value for the pattern of positive/negative responses, and δ = mean difference (in log units) between stimuli. ED50 values (dose producing half of the maximal response) and their standard errors were calculated using nonlinear regression analysis of the equation for a sigmoid plot and were compared by means of Snedecor's F test using the GraphPad Prism 5.00 program (GraphPad Software Inc., San Diego, CA). A value of $P < 0.05$ was considered statistically significant.

When several means were compared, statistical analysis was carried out with one-way or two-way analysis of variance (ANOVA) depending on the experiment, followed by a Bonferroni post-hoc test. When two means were compared a Student's t test for non-paired values was used. The differences between values either after ANOVAs, Bonferroni's or Student's t tests were considered significant when the p value was below 0.05. Statistical analysis was performed with the SigmaPlot 12.0 program (Systat Software Inc., San Jose, CA, USA).

RESULTS

1. ROLE OF σ1 RECEPTORS ON INTRACOLONIC CAPSAICIN MODEL

1.1. COMPARISON OF CAPSAICIN-INDUCED VISCERAL PAIN BETWEEN WT AND σ1-KO MICE

In both WT and σ1-KO mice, intracolonic administration of the capsaicin solvent elicited a small number of abdominal licking behaviors that could be clearly differentiated from normal grooming activity (Fig. 1A), whereas intracolonic instillation of capsaicin induced multiple types of pain-related behavior (e.g., licking, stretching, and contraction of the abdomen). The number of pain-related behaviors induced by capsaicin (0.01–1%) increased in a concentration-dependent manner in both WT and σ1-KO mice (Fig. 1A), but the pattern of this response differed markedly between them.

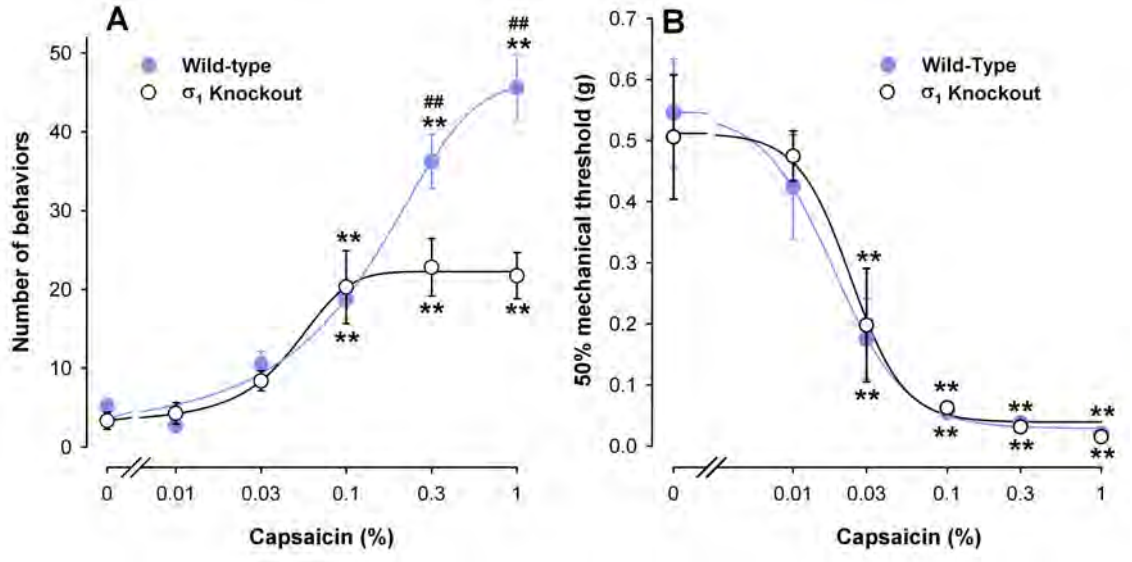

Fig. 1. Pain-related behaviors (**A**) and referred mechanical hyperalgesia (**B**) induced by intracolonic administration of different concentrations of capsaicin (0.01–1%) or its solvent (0) in wild-type (WT) and σ1 receptor knockout (σ1-KO) mice. The behavioral pain responses (licking of abdomen, stretching, abdominal retractions) were recorded during the first 20 min after instillation of capsaicin, and referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments) was measured at 20 min after instillation of capsaicin. Each point and vertical line represents the mean ± SEM of values obtained in 10-12 animals per group. Statistically significant differences between the values obtained in capsaicin- and solvent-treated animals: $^{**}p < 0.01$. Statistically significant differences between the values obtained in WT and σ1-KO animals at the same concentration of capsaicin: $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

In WT mice, the maximum number of pain responses (46 ± 4.2) was evoked at the highest concentration tested (1%); in σ_1-KO mice, the maximum number of pain responses, which was half that in WT mice, reached a plateau from a concentration of 0.1% (Fig. 1A). The difference in number of pain responses between WT and σ_1-KO animals was statistically significant at concentrations of 0.3% and 1% (Fig. 1A). In contrast, there were no significant differences between WT and σ_1-KO mice in the intracolonic capsaicin-induced referred mechanical hyperalgesia as evaluated with von Frey filaments (Fig. 1B). Instillation of capsaicin (0.01–1%) reduced the mechanical threshold in a concentration-dependent manner in both groups, which showed almost identical stimulus–response curves (Fig. 1B). The difference between these two groups and solvent-treated mice reached statistical significance at capsaicin concentrations of 0.03% and higher (Fig. 1B). No significant difference was found between the 50% mechanical thresholds in animals treated with capsaicin solvent (0.54 ± 0.08 and 0.52 ± 0.05 g in WT and σ_1-KO mice, respectively) and those observed in naïve animals without any instillation (0.53 ± 0.09 and 0.51 ± 0.10g in WT and σ_1-KO mice, respectively).

In summary, the intracolonic administration of capsaicin evoked concentration-dependent visceral pain-related behaviors and referred mechanical hyperalgesia in both WT and σ_1-KO mice. The number of pain-related behaviors was lower in the σ_1-KO mice, but the mechanical hyperalgesia did not differ between the mouse types.

1.2. EFFECTS OF σ_1 RECEPTOR ANTAGONISTS ON VISCERAL PAIN INDUCED BY INTRACOLONIC CAPSAICIN

The subcutaneous administration of the selective σ_1 antagonist BD-1063 (32 mg/kg) at 30 min before the intracolonic instillation of 0.1–1% capsaicin reduced the number of pain-related behaviors in WT mice (Fig. 2A) but not in σ_1-KO mice (Fig. 2B). The maximum number of 1% capsaicin–induced pain-related behaviors was highly similar between WT mice treated with BD-1063 (22 ± 3.9) and σ_1-KO mice treated with saline (22 ± 2.9) or with BD-1063 (20 ± 2.8). BD-1063 (32

mg/kg) also inhibited the referred mechanical hyperalgesia in WT mice, promoting a shift to the right of the concentration–response curve of capsaicin (Fig. 3A), but it had no effect on the referred hyperalgesia in σ1-KO mice (Fig. 3B).

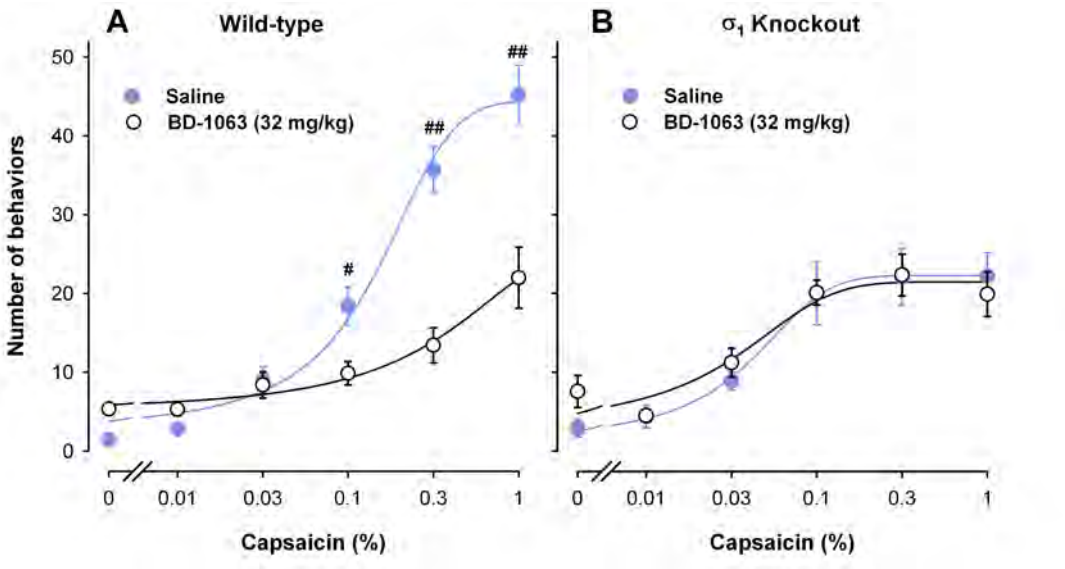

Fig. 2. Effects of the subcutaneous administration of BD-1063 (32 mg/kg) or saline on the pain-related behaviors evoked by intracolonic administration of different concentrations of capsaicin (0.01–1%) or its solvent (0) in wild-type (**A**) and σ1 receptor knockout (**B**) mice. BD- 1063 or saline was injected at 30 min before the administration of capsaicin or its solvent. The behavioral pain responses (licking of abdomen, stretching, abdominal retractions) were recorded during the first 20 min after instillation of capsaicin or its solvent. Each point and vertical line represents the mean ± SEM of values obtained in 10-12 animals per group. Statistically significant differences compared to BD-1063–injected mice: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

To determine whether the effects of BD-1063 were dose-dependent and shared by other σ1 antagonists, we administered various doses of the selective σ1 antagonists BD-1063, S1RA, and NE-100 at 30 min before instillation of the capsaicin concentrations found to induce the maximum number of pain-related behaviors and maximum referred hyperalgesia (1% and 0.1% capsaicin, respectively). The subcutaneous administration of BD-1063 (16–64 mg/ kg), S1RA (32–128 mg/kg), and NE-100 (8–64 mg/kg) induced a dose-dependent inhibition of capsaicin-induced pain responses in WT mice but produced no change in σ1-KO mice (Fig. 4). None of the σ1 antagonists abolished pain-related behaviors in WT mice; however,

at the highest doses tested, all of them reduced the number of behaviors in WT mice to the same number observed in capsaicin-treated σ_1-KO mice (fig. 4).

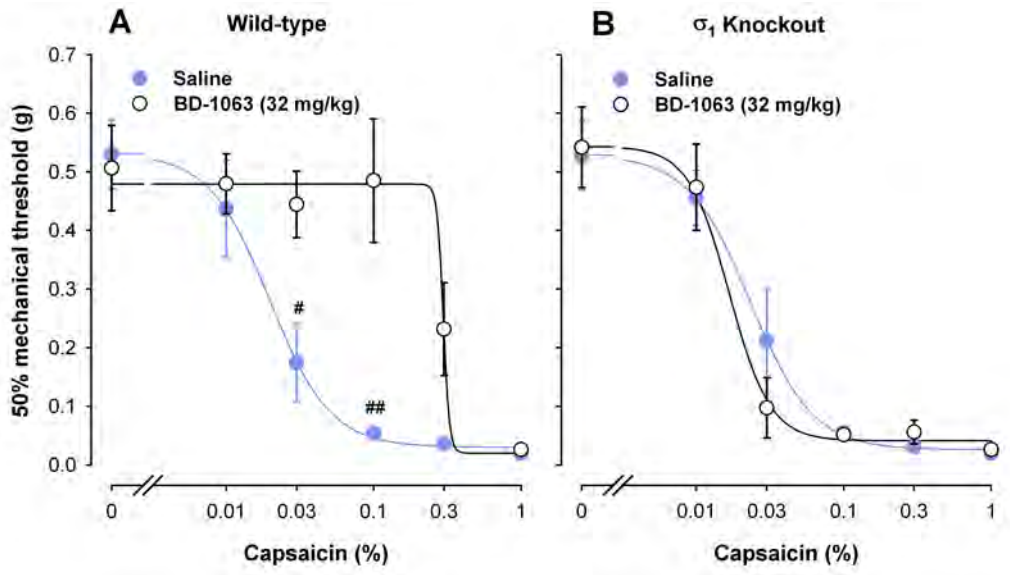

Fig. 3. Effects of the subcutaneous administration of BD-1063 (32 mg/kg) or saline on the referred mechanical hyperalgesia induced by intracolonic administration of different concentrations of capsaicin (0.01–1%) or its solvent (0) in wild-type (**A**) and σ_1 receptor knockout (**B**) mice. BD-1063 or saline was injected at 30 min before the administration of capsaicin or its solvent. The referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments) was measured at 20 min after instillation of capsaicin or its solvent. Each point and vertical line represents the mean ± SEM of values obtained in 10-12 animals per group. Statistically significant differences compared to BD-1063–injected mice: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

In WT mice, the referred mechanical hyperalgesia induced by 0.1% capsaicin was almost completely reversed by pretreatment with the highest doses of BD-1063 or S1RA (Fig. 5), yielding a 50% mechanical threshold (0.48 ± 0.1 g and 0.47 ± 0.07 g in BD-1063 32 mg/kg plus capsaicin and S1RA 128 mg/kg plus capsaicin groups, respectively) that was close to the threshold obtained in capsaicin solvent-treated animals (0.53 ± 0.05 g). The subcutaneous administration of NE-100 also dose-dependently reversed the capsaicin- induced mechanical hyperalgesia in WT mice (Fig. 5). However, NE-100 was less effective than BD-1063 and S1RA, and the 50% maximal mechanical threshold was lower in the capsaicin-treated mice

preadministered with the highest dose (64 mg/kg) of NE-100 (0.33 ± 0.06 g) than in the mice treated with capsaicin solvent (0.53 ± 0.05 g) (Fig. 5). NE-100 doses higher than 64 mg/kg produced locomotor alterations (ataxia and motor incoordination) that interfered with the mechanical threshold measurement (data not shown).

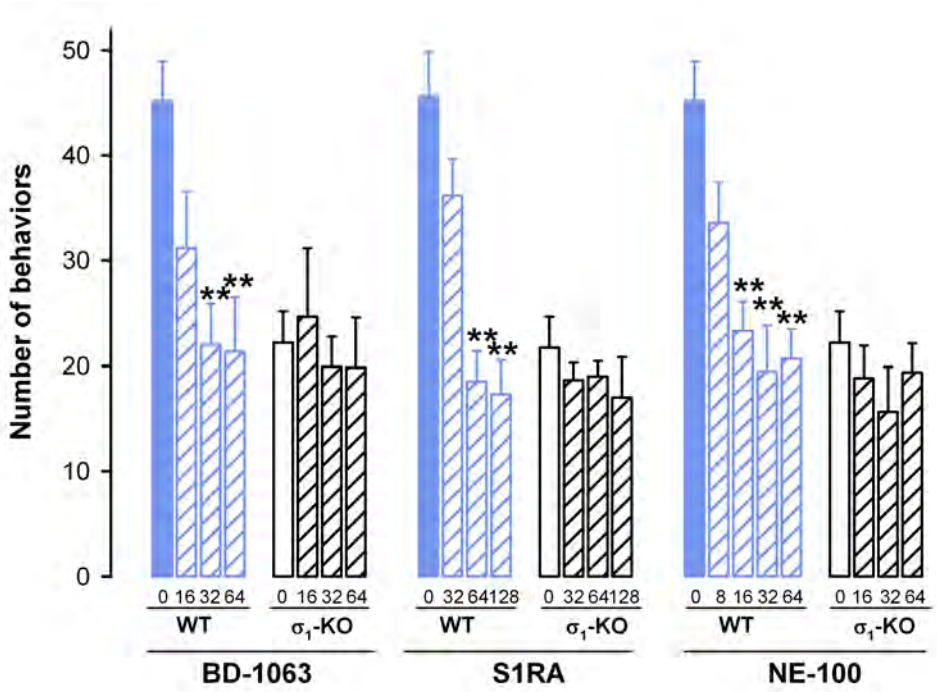

Fig. 4. Effects of the subcutaneous administration of BD-1063 (16–64 mg/kg), S1RA (32–128 mg/kg), NE-100 (8–64 mg/ kg), and saline (0) on the pain-related behaviors evoked by intracolonic administration of 1% capsaicin in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The drug or saline was injected at 30 min before instillation of capsaicin. Behavioral pain responses (licking of abdomen, stretching, abdominal retractions) were recorded during the first 20 min after instillation of capsaicin. Each bar and vertical line represents the mean ± SEM of values obtained in 10-12 animals per group. Statistically significant differences between the values obtained in drug- and saline-injected mice: $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

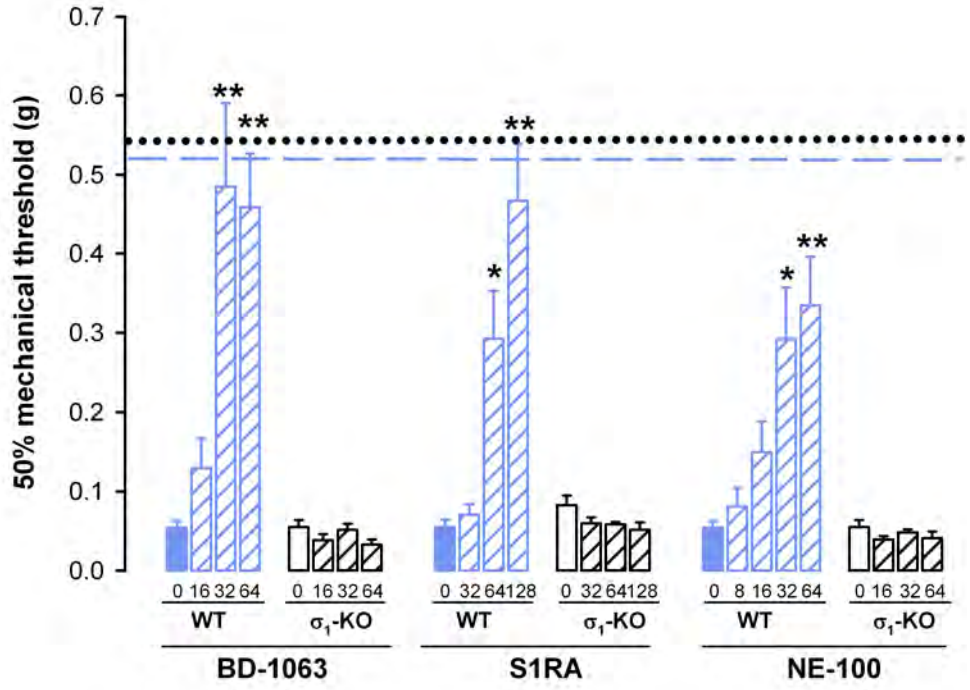

Fig. 5. Effects of the subcutaneous administration of BD-1063 (16–64 mg/kg), S1RA (32–128 mg/kg), NE-100 (8–64 mg/ kg), and saline (0) on the referred mechanical hyperalgesia induced by intracolonic administration of capsaicin 0.1% in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The drug or saline was injected at 30 min before the capsaicin instillation. The referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments) was measured at 20 min after the capsaicin instillation. Each bar and vertical line represents the mean ± SEM of values obtained in 10-12 animals per group. The dashed and dotted lines indicate the 50% threshold in capsaicin solvent-treated WT and σ_1-KO mice, respectively. Statistically significant differences between the values obtained in drug- and saline-injected mice: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

1.3. COMPARISON OF THE EFFECTS OF CONTROL DRUGS ON VISCERAL PAIN INDUCED BY INTRACOLONIC CAPSAICIN

As expected, the subcutaneous administration of morphine (1–16 mg/kg) produced a dose-dependent inhibition of capsaicin-induced pain-related behavioral responses and mechanical referred hyperalgesia in WT and σ_1-KO mice (Figs. 6 and 7). In both mouse types, the pain-related behaviors induced by intracolonic 1% capsaicin were significantly reduced by morphine at doses of greater than or equal to 3 mg/kg and completely abolished by a dose of 8 mg/kg (Fig. 6A).

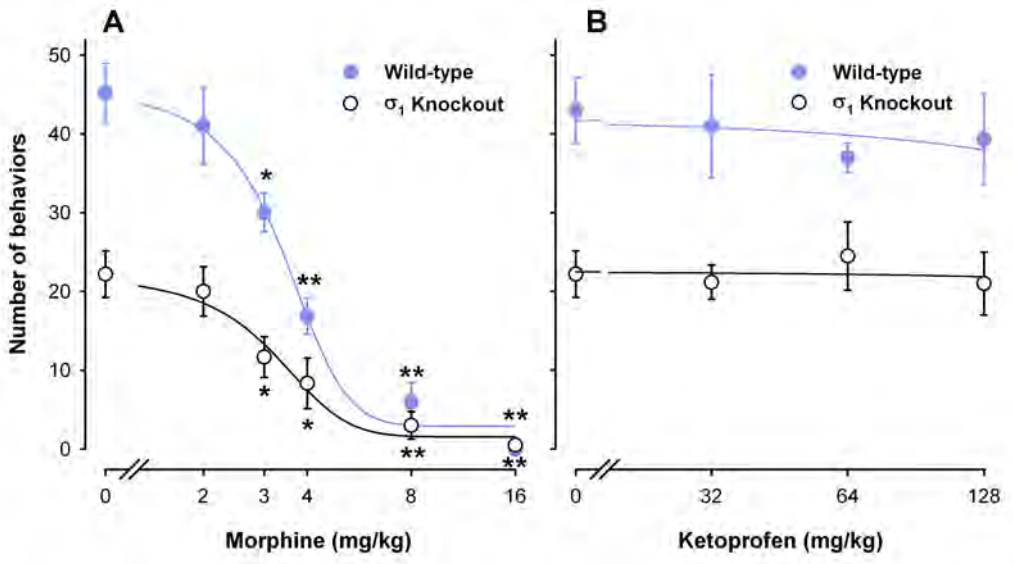

Fig. 6. Effects of the subcutaneous administration of morphine (2–16 mg/kg) (**A**) and ketoprofen (32–128 mg/kg) (**B**) on the pain-related behaviors evoked by intracolonic administration of capsaicin 1% in wild-type and σ_1 receptor knockout mice. The drug or its vehicle was injected at 30 min before the administration of capsaicin. Behavioral pain responses (licking of abdomen, stretching, abdominal retractions) were recorded during the first 20 min after the capsaicin instillation. Each point and vertical line represents the mean ± SEM of values obtained in 10-12 animals per group. Statistically significant differences between the values obtained in drug- and vehicle-injected mice: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

Morphine administration fully reversed the referred mechanical hyperalgesia and exerted a clear and robust analgesic action in both mouse types (Fig. 7A), yielding a higher mechanical threshold than that observed in mice treated with capsaicin solvent (0.53 ± 0.05 and 0.52 ± 0.06 g in WT and σ_1-KO mice, respectively). In fact, at the highest morphine doses tested (8 and 16 mg/kg), most of the mice made no response to the strongest stimulus (2 g). The overall effect of morphine on the referred mechanical hyperalgesia evoked by 0.1% capsaicin was statistically different in σ_1-KO compared with WT mice (Fig. 7A). In particular, the effect of morphine (1–4 mg/kg) was significantly greater in σ_1-KO mice than in WT mice ($p < 0.05$) (Fig. 7A). The subcutaneous administration of ketoprofen (32–128 mg/ kg) had no effect on the pain-related responses or referred mechanical hyperalgesia at any dose tested in either σ_1-KO or WT mice (Figs. 6B and 7B).

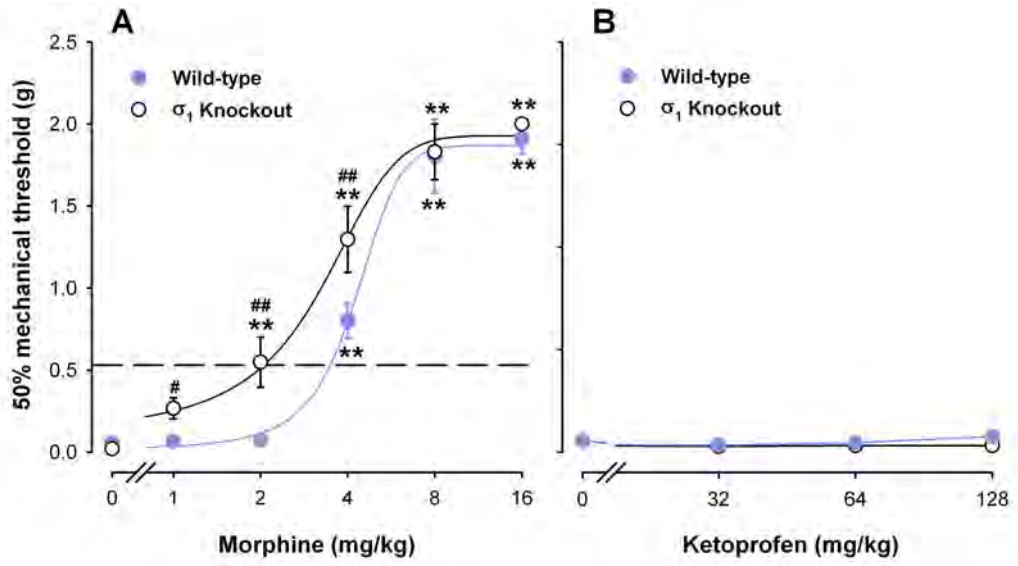

Fig. 7. Effects of the subcutaneous administration of morphine (1–16mg/kg) (**A**) and ketoprofen (32–128 mg/kg) (**B**) on the referred mechanical hyperalgesia induced by intracolonic administration of capsaicin 0.1% in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The drug or its vehicle was injected at 30 min before instillation of capsaicin. The referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments) was measured at 20 min after the capsaicin instillation. Each point and vertical line represents the mean ± SEM of values obtained in 10-12 animals per group. The dashed line indicates the 50% threshold in capsaicin solvent-treated WT and σ_1-KO mice. Note that the higher doses of morphine increase the mechanical threshold to above the control value (i.e., exert analgesic effects). Statistically significant differences between the values obtained in morphine- and vehicle-injected mice: $^{**}p < 0.01$. Statistically significant differences between the values obtained in WT and σ_1-KO animals at the same dose of morphine: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

2. ROLE OF σ_1 RECEPTORS ON CYCLOPHOSPHAMIDE INDUCED CYSTITIS MODEL

2.1. EXPRESSION OF σ_1 RECEPTORS IN HUMAN AND MICE URINARY BLADDER SLICES

To identify whether σ_1 receptors are present in urinary bladder and where are they localized within the bladder, we immunostained σ_1 receptors in urinary bladder slices from human and mouse origin.

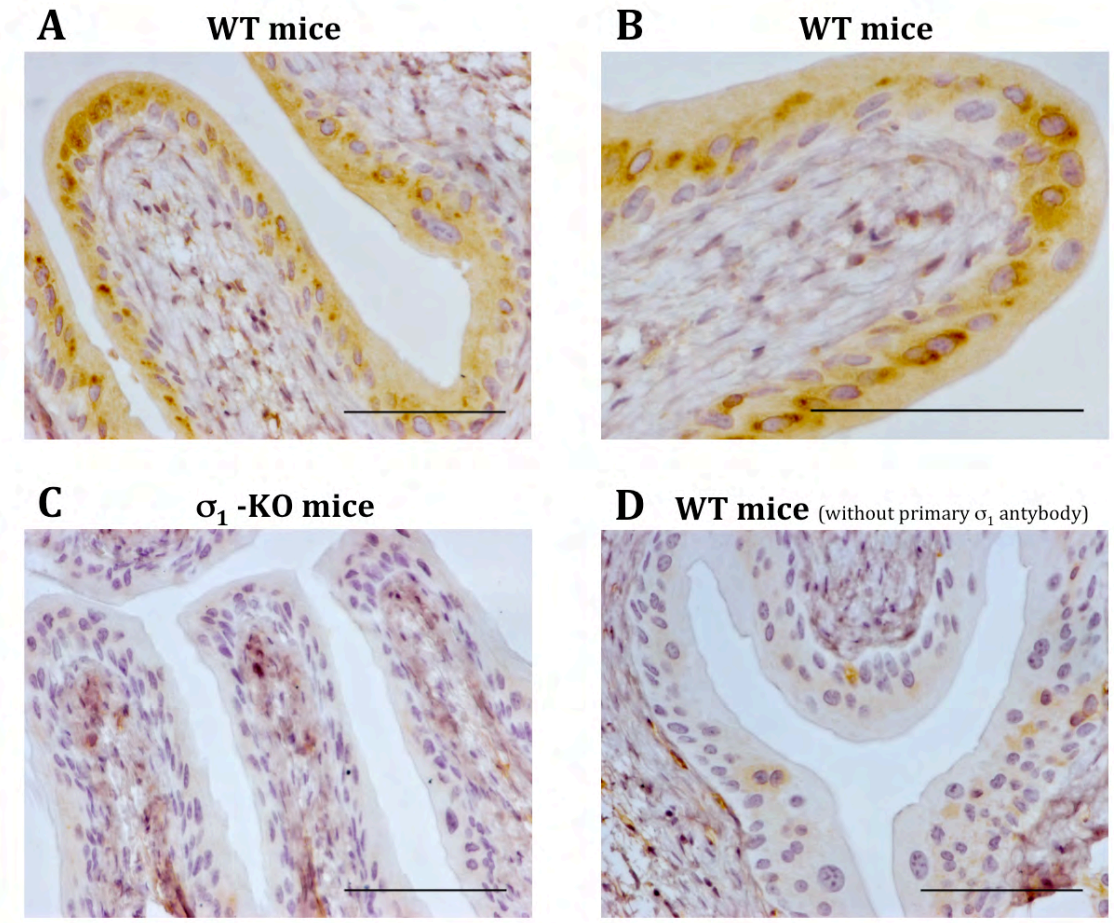

Fig. 8. Expression of σ_1 receptor in the mice urinary bladders. Immunostaining for σ_1 receptors was found in the urothelium of wild-type (WT) mice (**A** and **B**), but not in σ_1 receptor knockout (σ_1-KO) mice (**C**) or when the primary σ_1 antibody was omitted (**D**). The σ_1 receptor antibody (diluted 1/400) was identified with the UltraVision Quanto™ peroxidase kit, after heat-induced antigenic unmasking and endogenous peroxidase blockade. Sections were counterstained in Mayer's haematoxylin. Scale bar 100 μm.

Immunohistochemistry experiments in WT mice urinary bladder slices detected the σ_1 receptor immunoreactivity in the urothelium (Fig. 8A). The immunostaining was distributed throughout the cytoplasm of the different layers of urothelial cells and was particularly concentrated around the nucleus and within circular cytoplasmic structures (Fig. 8B). The specificity of the immunostaining was demonstrated by two ways. Firstly, we performed the same experiments in urinary bladder slices from σ_1-KO mice and we found no immunostaining (Fig 8C), which indicates that the σ_1 receptor antibody used was reacting exclusively with the σ_1 receptor. Moreover, no σ_1 receptor immunoreactivity was found when urinary bladder slices from WT-mice were incubated only with the secondary antibody (i.e. the primary σ_1 receptor antibody was omitted) (Fig. 8D).

Experiments performed in human urinary bladder slices found the σ_1 receptor immunostaining in the same types of urothelial cells than in mice bladder slices (compare Fig 8A and 9A). No immunostaining was detected in the human urinary bladder slices if the σ_1 receptor primary antibody was omitted (Fig. 9B).

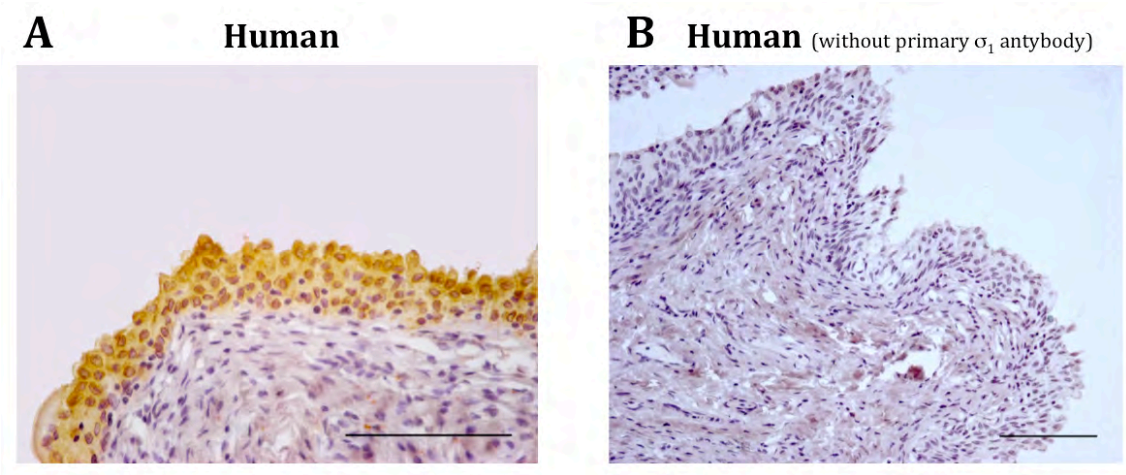

Fig. 9. Expression of σ_1 receptor in the human urinary bladders. Immunostaining for σ_1 receptors was found in the urothelium of human urinary bladder sections incubated with the σ_1 receptor antibody (**A**), but not when the primary σ_1 antibody was omitted (**B**). The σ_1 receptor antibody (diluted 1/400) was identified with the UltraVision Quanto™ peroxidase kit, after heat-induced antigenic unmasking and endogenous peroxidase blockade. Sections were counterstained in Mayer's haematoxylin. Scale bar 100 µm.

In summary, we found that σ_1 receptor is expressed in the urothelium of both human and mice urinary bladder and shows a similar intracellular distribution in both species.

2.2. EXPRESSION OF σ_1 RECEPTORS IN URINARY BLADDER HOMOGENATES OF NAÏVE AND CYCLOPHOSPHAMIDE-TREATED WT AND σ_1-KO MICE

To study whether cyclophosphamide-treatment modify the expression of urinary bladder σ_1 receptors, we performed western blot experiments in urinary bladder homogenates obtained from naïve mice and from mice treated with a high dose of cyclophosphamide (300 mg/kg, i.p.) before the bladders were dissected.

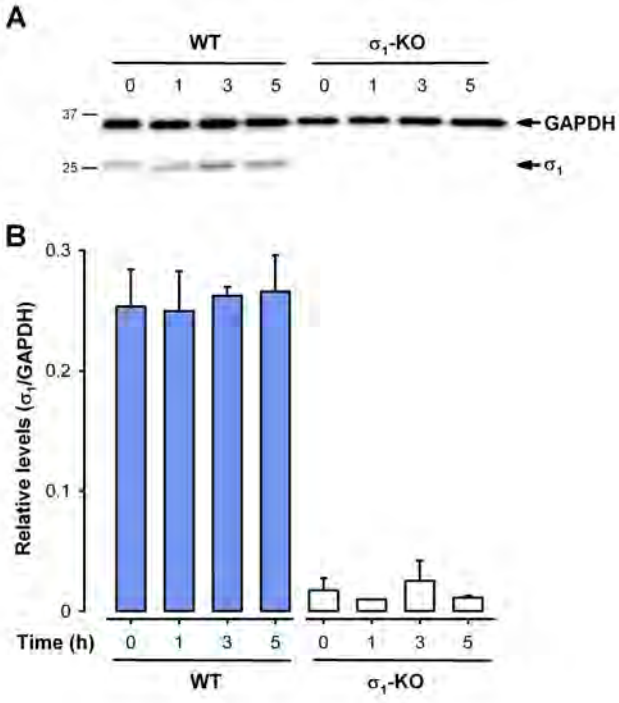

Fig. 10. Expression of σ_1 receptor in the urinary bladders of naïve and cyclophosphamide-treated wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. (**A**) Representative immunoblots for σ_1 receptors obtained in urinary bladder of naïve (0) WT and σ_1-KO mice, and at 1, 3 or 5 hours (1, 3, 5) after treatment with cyclophosphamide (300 mg/kg, i.p.) of both kind of animals. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. The σ_1 receptor antibody was diluted 1/250, whereas GAPDH antibody was diluted 1/80000. The migration positions of molecular mass standards (in kDa) are shown to the left of the gel. (**B**) Quantification of immunoblotting for the σ_1 receptor in WT and σ_1-KO mice urinary bladder. Each bar and vertical line represents the mean ± S.E.M. of the densitometric values obtained in eight animals. The σ_1-receptor band intensities were relativized to those of their corresponding GAPDH loading control bands. Note that no σ_1-receptor expression was found in samples from σ_1-KO mice.

Western blot experiments with the σ_1 receptor antibody identified a band of a molecular weight slightly higher than 25 kDa in the urinary bladder of control (naïve) WT mice (Fig. 10A). This band has a molecular weight similar to that previously found for σ_1 receptor in mice peripheral and central nervous system tissues (Sánchez-Fernández et al., 2014). The urinary bladders of cyclophosphamide-treated WT mice also showed similar σ_1 receptor bands (Fig. 10A). When σ_1 receptor bands intensity was measured it was found that cyclophosphamide-treatment did not affect σ_1 receptor expression in the urinary bladder of WT mice at any time evaluated (Fig. 10B).

Similar experiments performed in σ_1-KO mice urinary bladder homogenates did not find the σ_1 receptor band either in naïve or cyclophosphamide-treated animals (Fig. 10A & 10B). As expected, the loading control (GAPDH) band was similarly expressed in WT and σ_1-KO mice and in both experimental conditions (naïve and cyclophosphamide-treated animals) (Fig. 10A), which indicates that the differences in σ_1 receptor expression between WT and σ_1-KO mice was not due to any experimental artefact.

In summary, western blot experiments further support that σ_1 receptor is present in mice urinary bladder and indicate that cyclophosphamide-treatment did not modify σ_1 receptor expression in this tissue.

2.3. CYCLOPHOSPHAMIDE-INDUCED ENHANCEMENT OF MYELOPEROXIDASE ACTIVITY AND pERK1/2 EXPRESSION IN URINARY BLADDER HOMOGENATES OF WT AND σ_1-KO MICE

To identify the functional relevance of the urinary bladder σ_1 receptor we compared the characteristics of two different biochemical alterations induced by cyclophosphamide in the bladders of WT and σ_1-KO mice.

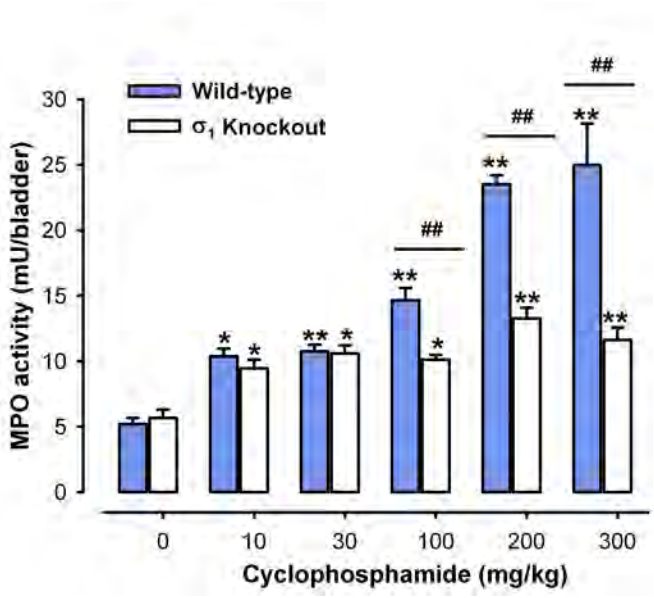

Fig. 11. Myeloperoxidase (MPO) activity in the urinary bladders of control and cyclophosphamide-treated wild-type (WT) and σ_1 receptor knockout mice. Values of urinary bladder MPO activity were measured five hours after the i.p. injection of cyclophosphamide (10–300 mg/kg) or its solvent (0). Statistically significant differences between the values obtained in cyclophosphamide-treated and control animals: $^{*}p < 0.05$,$^{**}p < 0.01$; and between the values obtained in WT and σ_1-KO mice at the same dose of cyclophosphamide: $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

Cyclophosphamide-treatment (10-300 mg/kg, i.p.) induced a dose-dependent enhancement of MPO activity in the urinary bladder of WT mice (Fig. 11), which indicates neutrophil infiltration of the tissue, and agrees with data previously published (Martins et al., 2012; Santos et al., 2010). Cyclophosphamide-treatment also increases MPO activity in the urinary bladder of σ_1-KO animals, but the enhancement was significantly lower in these animals than in WT mice (Fig. 11). The maximum difference between cyclophosphamide-effect in WT and σ_1-KO mice was observed at 300 mg/kg (Fig. 11); therefore, this dose of cyclophosphamide was chosen for the rest of experiments.

Cyclophosphamide-treatment also induces activation (phosphorylation) of extracellular receptor kinase1/2 (ERK1/2) in the urinary bladder, which is manifested by an increase in the expression of pERK1/2 in this tissue (Corrow and Vizzard, 2007). Consequently, we compared the cyclophosphamide-induced pERK1/2 expression in WT and σ_1-KO mice as an additional marker of the

functional role of urinary bladder σ_1 receptors. As expected, western blot experiments showed that cyclophosphamide administration did not change bladder ERK1/2 levels either in WT or σ_1-KO mice (Fig. 12A), but produced a time-dependent enhancement of pERK1/2 expression in the urinary bladder, especially in the WT mice (Fig.12B). When pERK1/2 levels were quantified it was found that the cyclophosphamide-induced enhancement of pERK1/2 levels was significantly greater in WT than in σ_1-KO animals at 3 and 5 h after drug administration (Fig. 12C).

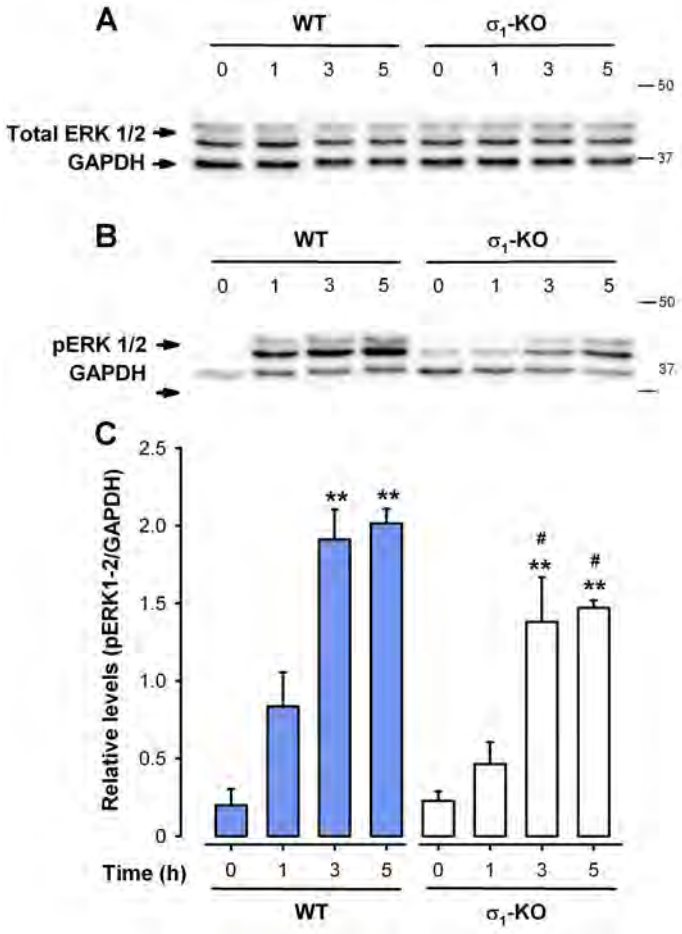

Fig. 12. Total extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphorylated ERK1/2 (pERK1/2) in the urinary bladders of control and cyclophosphamide-treated wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. (**A** and **B**) Representative immunoblots for urinary bladder total ERK1/2 and pERK1/2, respectively. In both cases, bladders were obtained from naïve animals (0) and at 1, 3 or 5 hours (1, 3, 5) after treatment with cyclophosphamide (300 mg/kg, i.p.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. Antibody dilutions were: total ERK1/2 at 1/40000, pERK1/2 at 1/2000 and GAPDH at 1/40000. The migration positions of molecular mass standards (in kDa) are shown to the left of the gels. (**C**) Quantification of immunoblotting for pERK1/2 in WT and σ_1-KO mice. The pERK1/2 bands intensities were relativized to those of their corresponding loading control GAPDH bands. (**C**) Each

bar and vertical line represents the mean ± SEM of the values obtained in 5-7 animals. Statistically significant differences between the values obtained in cyclophosphamide-treated and control animals: $^{**}p < 0.01$; and between the values obtained in WT and σ_1-KO mice at the same time after injection of cyclophosphamide: $^{\#}p < 0.05$ (two-way ANOVA followed by Bonferroni test).

In summary, the two biochemical alterations (enhancement of MPO and pERK) induced by cyclophosphamide treatment in urinary bladders were attenuated in σ_1-KO mice in comparision to WT mice.

2.4. CYCLOPHOSPHAMIDE-INDUCED CHANGES IN URINARY BLADDER HISTOLOGY OF WT AND σ_1-KO MICE

Haematoxylin-eosin stained urinary bladder sections obtained from naïve WT mice showed a normal appearance, being muscularis, lamina propria and urothelium easily identifiable (Fig. 13A, left panel). The appearance of naïve WT mice sections was indistinguishable from that of naïve σ_1-KO mice (not shown).

Five hours after cyclophosphamide-treatment numerous alterations were observed in WT mice urinary bladder sections: the bladders showed a marked oedema, localized between lamina propria and muscularis, which was accompanied by areas of urothelium desquamation and haemorrhagic foci (Fig. 13A, middle panel). These histological alterations are characteristics of cyclophosphamide-induced cystitis in rodents (Martins et al., 2012; Santos et al., 2010).

Cyclophosphamide-induced histological alterations were attenuated in σ_1-KO mice (Fig. 13A, right panel). In particular, when the main cystitis manifestations were quantified it was found that σ_1-KO mice urinary bladders showed a reduction in the oedematous area (Fig. 13B), as well as less areas of urothelium desquamation and haemorrhagic foci (Fig. 13C).

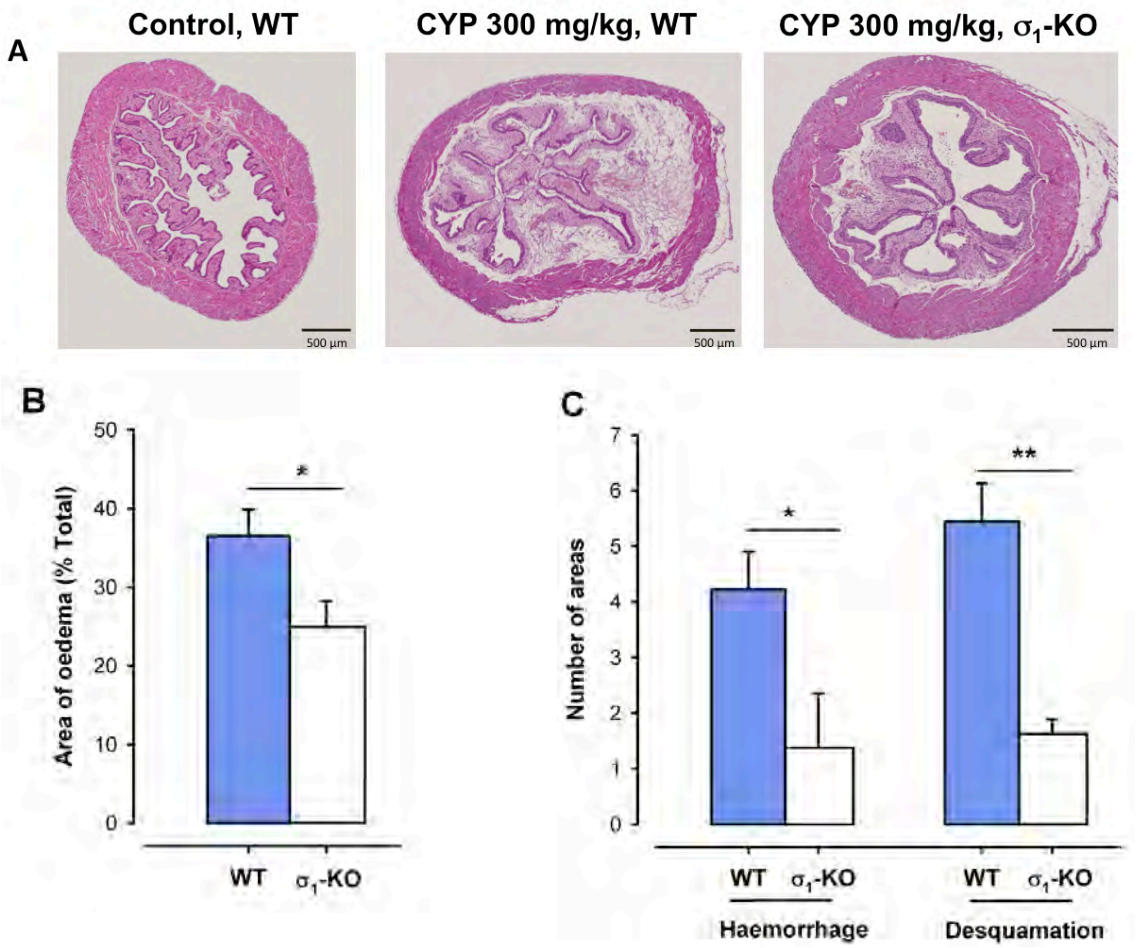

Fig. 13. Histological analysis of wild-type (WT) and σ_1-knockout (σ_1-KO) mice urinary bladders five hours after i.p. injection of cyclophosphamide (300 mg/kg, CYP 300) or its solvent. (**A**) Photomicrographs of representative haematoxylin/eosin stained urinary bladders representative of solvent-treated wild-type (Control, WT) and cyclophosphamide-treated wild-type (CYP 300 mg/kg, WT) and σ_1 knockout (CYP 300 mg/kg, σ_1-KO) mice. Scale bar = 500 µm. (**B**) Representation of the area of oedema located between lamina propria and mucularis (relativized to the total area of the urinary bladder section) in solvent- and cyclophosphamide-treated WT and σ_1-KO mice. (**C**) Number of areas of haemorrhage and desquamation in bladders of WT and σ_1-KO mice treated with cyclophosphamide (no areas of haemorrhage or urothelium desquamation were observed in saline-treated animals). (**B** and **C**) Each bar and vertical line represents the mean ± SEM of values obtained in 8 animals. Statistically significant differences between the values obtained in wild-type and σ_1 knockout animals: $^{*}p < 0.05$; $^{**}p < 0.01$ (Student´s *t* test).

In summary, the histological changes (oedema, haemorrhage and urothelium desquamation) produced in the urinary bladder by cyclophosphamide-treatment were reduced in σ_1-KO mice in comparison to WT mice.

2.5. COMPARISION OF CYCLOPHOSPHAMIDE-INDUCED PAIN AND REFERRED MECHANICAL HYPERALGESIA BETWEEN WT AND σ_1-KO MICE

Cyclophosphamide treatment produced a dose-dependent enhancement of the pain behavioral score in WT and σ_1-KO animals (Fig.14A). However, this increase was more pronounced in WT than in σ_1-KO animals, being the behavioral score significantly higher in WT animals at doses of 30 mg/kg and above. Both groups of animals reached their maximum behavioral score at the highest dose of cyclophosphamide tested (300 mg/kg), but the score was significantly lower in σ_1-KO (20.33 ± 0.59) than in WT (34.83 ± 2.56) mice (Fig. 14A).

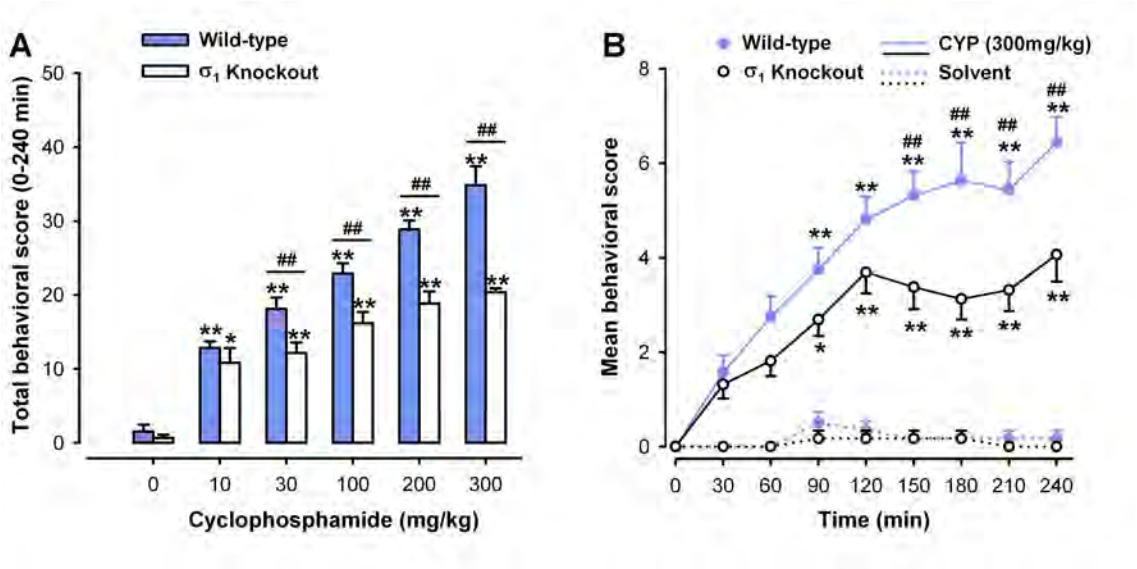

Fig. 14. Pain-related behaviors induced by cyclophosphamide in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. (**A**) Values of total behavioral scores induced by different doses of cyclophosphamide (10–300 mg/kg, i.p.) or its solvent (0) in WT and σ_1-KO mice. (**B**) Time-course of the pain behavioral scores after the i.p. administration of cyclophosphamide (CYP 300 mg/kg) or its solvent. The pain behavioral responses were recorded at 30 min intervals over the 4 h observation period after the cyclophosphamide or its solvent injection. (**A** and **B**) Each point or bar and vertical line represents the mean ± SEM of values obtained in 10–12 animals. Statistically significant differences between the values obtained in cyclophosphamide- and solvent-treated animals: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in WT and σ_1-KO animals at the same dose of cyclophosphamide: $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

When the time course of the pain induced by the highest dose of cyclophosphamide tested (300 mg/kg, i.p.) was analysed, it was found that cyclophosphamide injection produced a rise in the pain behavioral score which increased as evaluation time goes on, reaching significant differences with respect to the solvent-treated animals after 90 min (Fig. 14B). Cyclophosphamide-induced pain also occurred in σ_1-KO, and reach the peak at 120 min. After this time the behavioral score on the σ_1-KO mice does not continue to rise and remains significantly lower than that of the WT animals.

Cyclophosphamide treatment also produced a dose dependent mechanical hyperalgesia referred to the abdominal wall, which was non-significantly different between WT and σ_1-KO mice. Injection of cyclophosphamide (10-300 mg/kg) reduced the mechanical threshold in a concentration-dependent maner in both groups (Fig. 15). The lower mechanical threshold was reached from the dose of 100 mg/kg, thus we used this dose for the following pharmacological experiments.

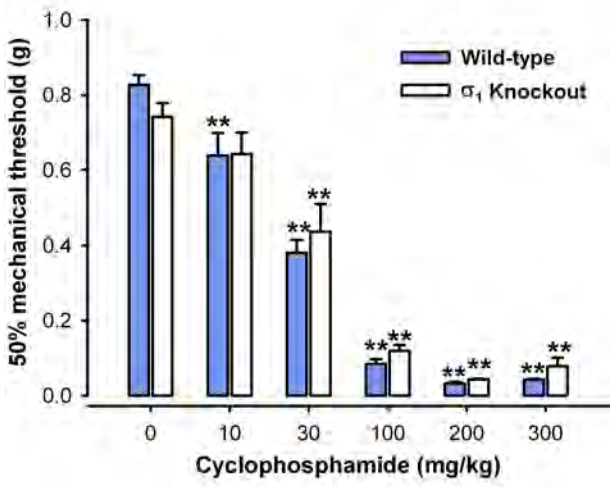

Fig. 15. Referred mechanical hyperalgesia induced by i.p. administration of different doses of cyclophosphamide (10–300 mg/kg) or its solvent (0) in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments) was measured at 240 min after the cyclophosphamide injection. Each bar and vertical line represents the mean ± SEM of values obtained in 10–12 animals. Statistically significant differences between the values obtained in cyclophosphamide- and solvent-treated animals: $^{**}p < 0.01$ (two-way ANOVA followed by Bonferroni test). No differences were found between the values obtained in WT and σ_1-KO mice.

2.6. EFFECTS OF σ1 ANTAGONIST ON CYCLOPHOSPHAMIDE INDUCED PAIN AND REFERRED MECHANICAL HYPERALGESIA

Since the maximun pain behavioral score was reached in animals treated with 300 mg/kg og i.p. cyclophosphamide (Fig. 14A), this dose was chosen to test the effects of σ1-antagonist on cyclophosphamide-induced pain. All σ1 antagonists (BD-1063, S1RA and NE-100), injected 120 min after cyclophosphamide (i.e. when pain was clearly established, Fig. 14B), produced a dose-dependent inhibition of the pain behavioral score in WT animals, but no change in σ1-KO mice (Fig. 16). None of the σ1 antagonists abolished pain-related behaviors; however, at the highest doses tested, all of them reduced the number of behaviors in WT mice to the same number observed in cyclophosphamide treated σ1-KO mice (Fig. 16).

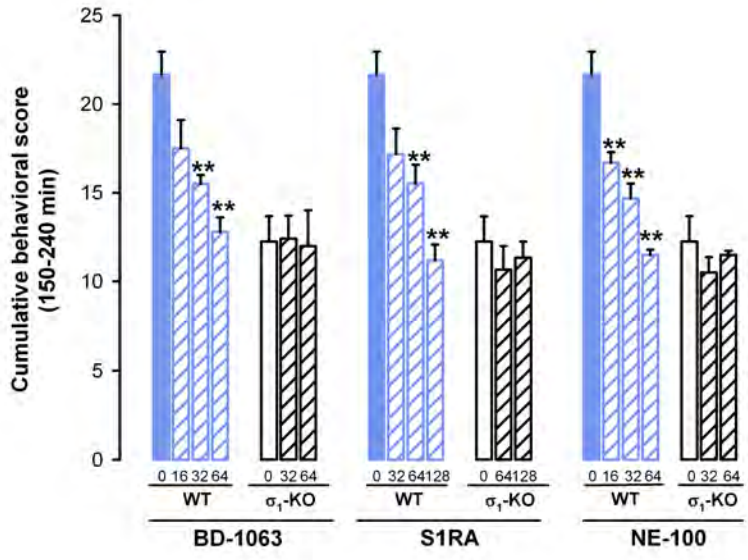

Fig. 16. Effects of the s.c. administration of BD-1063 (16–64 mg/kg), S1RA (32–128 mg/kg), NE-100 (16–64 mg/kg), or saline (0) on the pain-related behaviors evoked by i.p. administration of cyclophosphamide (300 mg/kg) in wild-type (WT) and σ1 receptor knockout (σ1-KO) mice. The drug or saline was injected at 120 min after the administration of cyclophosphamide. Behavioral pain responses were recorded at 30 min intervals over the 150-240 min observation period after the cyclophosphamide injection. Each bar and vertical line represents the mean ± SEM of values obtained in 10–12 animals. Statistically significant differences between the values obtained in drug- and saline-injected mice:$^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

The referred mechanical hyperalgesia induced by cyclophosphamide (100 mg/kg i.p.) was also dose-dependently inhibited by the administration of the σ_1 antagonist in WT mice (Fig.17). The highest doses of the σ_1 receptors antagonist tested (BD-1063, S1RA and NE-100) in WT mice, produced mechanical threshold values that were close to the threshold obtained in cyclophosphamide solvent-treated animals (0.83 ± 0.03 g) (Fig.17). By contrast, all the σ_1 receptor antagonits were devoid of effect in σ_1-KO animals (Fig.17).

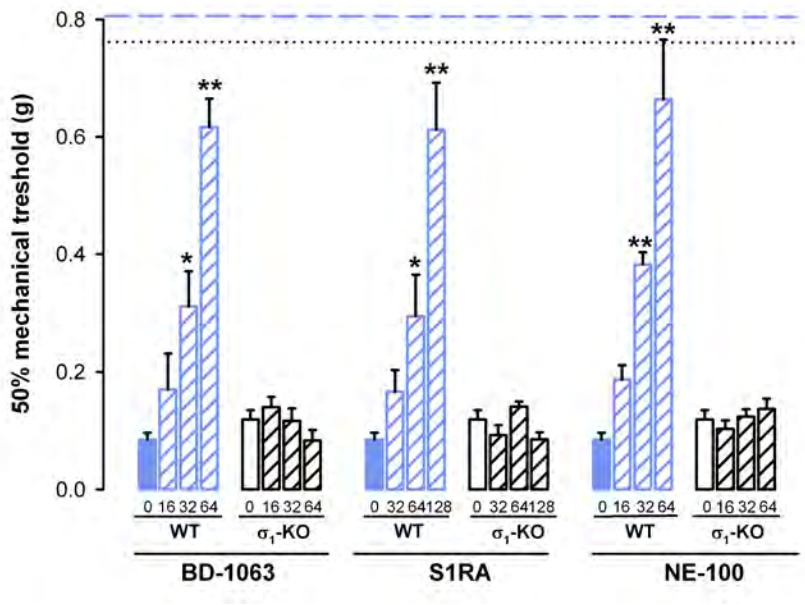

Fig. 17. Effects of the s.c. administration of BD-1063 (16–64 mg/kg), S1RA (32–128 mg/kg), NE-100 (16–64 mg/kg), or saline (0) on the referred mechanical hyperalgesia induced by i.p. administration of cyclophosphamide (100 mg/kg) in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The drug or saline was injected at 120 min after the administration of cyclophosphamide or its solvent. The referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments) was measured at 240 min after the cyclophosphamide injection. Each bar and vertical line represents the mean ± SEM of values obtained in 10–12 animals.The dashed and dotted lines indicate the 50% threshold force in cyclophosphamide solvent-treated WT and σ_1-KO mice, respectively. Statistically significant differences between the values obtained in drug- and saline-injected mice: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

2.7. COMPARISON OF THE EFFECT OF CONTROL DRUGS ON VISCERAL PAIN INDUCED BY CYCLOPHOSPHAMIDE

The subcutaneous administration of morphine (1-8 mg/kg) dose-dependently reduced the pain behavioral score elicited by cyclophosphamide (300 mg/kg i.p.) to almost completely inhibit it at the highest dose tested (Fig. 18). Morphine-teatment also dose-dependently inhibited the pain behavioral score in σ_1-KO mice, and abolished it at the dose of 8 mg/kg (Fig. 18).

Similarly, the subcutaneous administration of the anti-inflammatory drug indomethacin (2-8 mg/kg) produced a dose-dependent decrease of the painful behavioral score. This effect was observed in both WT as in σ_1-KO animals.

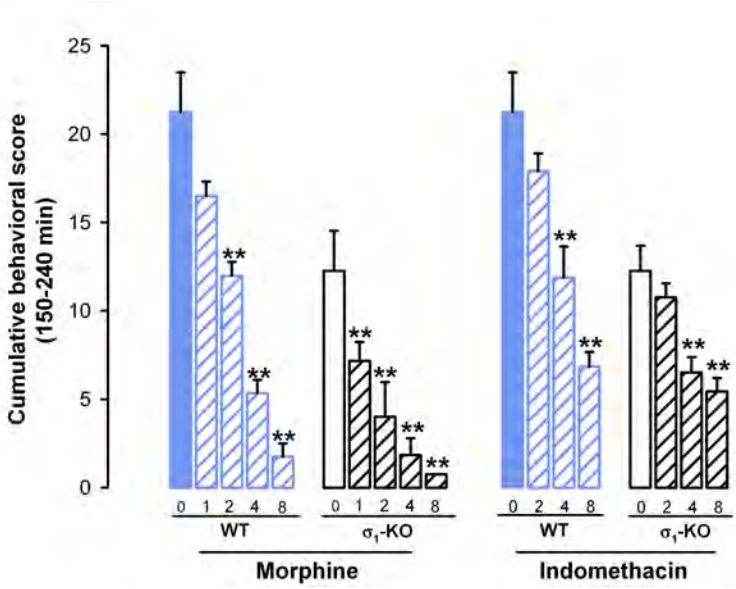

Fig. 18. Effects of the s.c. administration of morphine (1–8 mg/kg) or indomethacin (2–8 mg/kg) on the pain-related behaviors evoked by i.p. administration of cyclophosphamide (300 mg/kg) in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The drug or saline was injected at 120 min after the administration of cyclophosphamide. Behavioral pain responses were recorded at 30 min intervals over the 150-240 min observation period after the cyclophosphamide injection. Each point and vertical line represents the mean ± SEM of values obtained in 10–12 animals. Statistically significant differences between the values obtained in drug- and vehicle-injected mice: $^{**}p < 0.01$(one-way ANOVA followed by Bonferroni test).

The treatment with morphine (0.5-16 mg/kg s.c.), dose-dependently reversed the referred mechanical hyperalgesia produced by cyclophosphamide (100 mg/kg i.p.) in WT animals, producing a strong analgesic effect (Fig. 19A). Interestingly, morphine-treatment not only fully reversed mechanical hyperalgesia but also produced analgesic effect at doses greater than 4 mg/kg in WT mice (Fig. 19A). Morphine also dose-dependently inhibited cyclophosphamide-induced hyperalgesia and produced analgesia in σ_1-KO mice (Fig. 19A), althought the dose-response curve was shifted to the left in these animals in comparison to that obtained in WT mice (Fig. 19A)

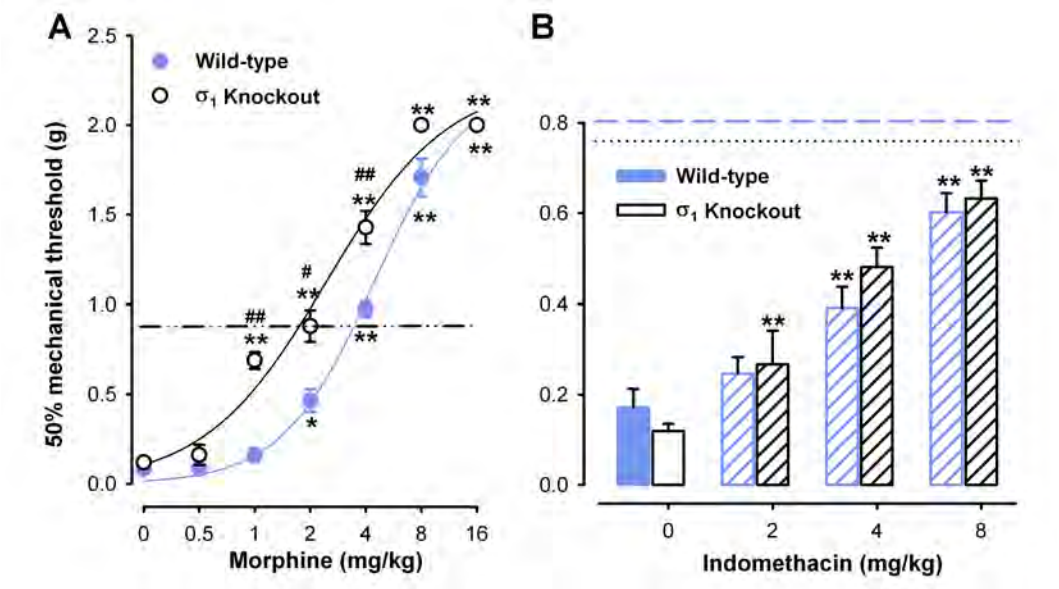

Fig. 19. Effects of the s.c. administration of morphine (0.5–16 mg/kg) (**A**) and indomethacin (2–8 mg/kg) (**B**) on the referred mechanical hyperalgesia evoked by i.p. administration of cyclophosphamide (100 mg/kg) in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The drug or saline was injected at 120 min after the administration of cyclophosphamide or its solvent. The referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments) was measured at 240 min after the cyclophosphamide injection. Each bar and vertical line represents the mean ± SEM of values obtained in 10–12 animals. The dashed and dotted lines indicate the 50% threshold force in cyclophosphamide solvent-treated WT and σ_1-KO mice, respectively. Note that the highest doses of morphine increased the mechanical threshold to above the control value (i.e., exerted analgesic effects). Statistically significant differences between the values obtained in drug- and vehicle-injected mice: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in WT and σ_1-KO animals at the same dose of drug: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

Indomethacin (2-8 mg/kg s.c.) dose-dependently decreased the referred mechanical hyperalgesia caused by cyclophosphamide (100 mg/kg i.p.) but it did not produce analgesic effects (Fig. 19B). Moreover, no differences between the effect of indomethacin on WT and σ_1-KO mice were found at any dose tested (Fig. 19B).

DISCUSSION

1. ROLE OF σ_1 RECEPTORS ON INTRACOLONIC CAPSAICIN MODEL

The main findings of this section of the Thesis were that the pain-related behaviors induced by intracolonic capsaicin were reduced markedly in σ_1-KO mice and that the pharmacological blockade of σ_1 receptors inhibited the capsaicin-induced pain-related behaviors and mechanical referred hyperalgesia in WT mice but not in σ_1-KO animals. This is the first report to present evidence of a role for the σ_1 receptor in a pure visceral pain model.

In agreement with previous studies (Kawao et al., 2004; Laird et al., 2001; 2002), we found that intracolonic capsaicin evoked concentration-dependent visceral pain-related behaviors and referred mechanical hyperalgesia. The number of capsaicin-induced pain-related behaviors was significantly lower in σ_1-KO mice than in WT mice. It has been postulated that the acute visceral pain evoked by intracolonic capsaicin is attributable to the direct activation of nociceptors in the colon (Cervero and Laird, 2009). In this regard, our group previously reported that the response in the first phase of intraplantar formalin-induced pain, also attributed to the direct activation of nociceptors, was attenuated by 61% in σ_1-KO mice (Cendán et al., 2005a), similar to the reduction in the number of pain-related behaviors (52%) observed in the present σ_1-KO mice.

The mechanical threshold was similar between naïve WT and σ_1-KO animals (0.53 ± 0.09 and 0.51 ± 0.1 g, respectively), indicating that σ_1-KO animals perceive and respond normally to a punctate mechanical stimulus applied to the abdomen. This finding is in agreement with various reports showing that the absence of σ_1 receptors in naïve σ_1-KO animals does not interfere with the perception of mechanical and thermal stimuli applied to the hind paw or with the motor response to these stimuli (Cendán et al., 2005b; de la Puente et al., 2009; Entrena et al., 2009b;Nieto et al., 2012; Romero et al., 2012).

Capsaicin-induced referred mechanical hyperalgesia did not differ between WT and σ_1-KO mice, in strong contrast to a previous finding by our group that intraplantar capsaicin was completely unable to induce mechanical hypersensitivity to a punctate stimulus in σ_1-KO mice (Entrena et al., 2009b).

Although somatic- and visceral-induced hyperalgesic states are both mediated by an enhanced sensitivity of nociceptive neurons in the central nervous system (central sensitization), they differ widely in the neurobiological mechanisms that mediate the sensory process (Cervero and Laird, 2009; Robinson and Gebhart, 2008). Thus, mouse colonic spinal primary afferent neurons are located in T8–L1 and L6–S1 dorsal root ganglia, and are mostly peptidergic CGRP- and TRPV1-positive neurons (Christianson et al., 2006b; 2006a; Robinson and Gebhart, 2008; Robinson et al., 2004), whereas neurons innervating the hind paw are found at L3-L5 dorsal root ganglia, and a higher percentage of them are nonpeptidergic IB4-positive than are peptidergic CGRP- or TRPV1-positive (Christianson et al., 2006a; Lu et al., 2001). Thus, this apparent discrepancy may be related to the distinct neurochemistries of the primary afferents activated after the intraplantar (somatic) and intracolonic (visceral) administration of capsaicin. In this context, a greater abdominal referred hyperalgesia was reported in α_{2A}-adrenoceptor knockout mice than in WT mice after intracolonic capsaicin, whereas paw mechanical hyperalgesia after intraplantar capsaicin was similar in the two mouse types (Mansikka et al., 2004). Furthermore, the differences between somatic and visceral mechanical hypersensitivity induced by capsaicin are confirmed by the fact that, in our laboratory, the maximum mechanical pain hypersensitivity in WT mice was obtained after the intraplantar administration of 1 μg of capsaicin (20 μl of a 0.005% capsaicin solution) (Entrena et al., 2009b), whereas 50 μg of intracolonic capsaicin (50 μl of a 0.1% capsaicin solution) was required to reach the maximum referred mechanical hyperalgesia in the present study. Moreover, the duration of acute pain induced by intraplantar capsaicin (5 min) is much shorter than that of intracolonic capsaicin (20 min). Therefore, it could be possible that after intracolonic administration of capsaicin, the painful stimulus duration and intensity may be too strong to permit a suppression of referred mechanical hyperalgesia in σ_1-KO mice in comparison with σ_1-KO mice treated with capsaicin intraplantarly.

In the present Thesis, subcutaneous administration of the σ_1 receptor antagonists BD-1063, S1RA, and NE-100 dose-dependently inhibited the number of pain-related behaviors and the referred mechanical hyperalgesia induced by

intracolonic capsaicin in WT mice but not in σ_1-KO mice. According to these results, the effects of the σ_1 antagonists are specifically mediated by their interaction with σ_1 receptors. The highest doses of all of the σ_1 receptor antagonists tested reduced the number of capsaicin-induced pain-related behaviors in WT mice to the same number observed in σ_1-KO animals, indicating that these doses of BD-1063, S1RA, and NE-100 are sufficient to totally block the fraction of σ_1 receptors required to fully express the pain-related behaviors. The remaining response to capsaicin in σ_1-KO and WT animals treated with the highest doses of σ_1 receptor antagonists indicates that mechanisms other than σ_1 receptor activation are also implicated in the acute visceral pain induced by capsaicin in both mouse types. It was previously reported that σ_1 receptor antagonists had no effect on acute pain induced by thermal or mechanical stimuli to the paw skin (Cendán et al., 2005b; Díaz et al., 2009; Entrena et al., 2009a; 2009b) but almost completely abolished the first phase of formalin-induced pain (Cendán et al., 2005b; Romero et al., 2012) and, in the present study, partially reduced capsaicin-induced visceral pain-related behaviors. These data indicate that the analgesic actions of σ_1 receptor antagonists may depend on the type of pain (cutaneous, somatic, or visceral) and on the nociceptive stimulus applied (thermal, mechanical, or chemical).

The referred mechanical hyperalgesia induced by intracolonic capsaicin in WT mice was almost totally reversed by pretreatment with BD-1063 and S1RA but only partially reversed by NE-100. The lesser effect of NE-100 was not attributable to a pharmacokinetic difference (shorter duration of action), because the same reversion was obtained when NE-100 (64 mg/kg) was administered at 10 or 30 min before intracolonic capsaicin (50% mechanical thresholds: 0.32 ± 0.02 and 0.33 ± 0.06 g, respectively). However, we cannot rule out a pharmacodynamic difference between NE-100 and the other two σ_1 antagonists. At any rate, the antihyperalgesic effect of BD-1063, S1RA, and NE-100 in WT mice was attributable to their interaction with σ_1 receptors, given their complete lack of effect in σ_1-KO mice. The role of σ_1 receptors in pain hypersensitivity in somatic pain models in WT mice has been documented previously. It has been reported that σ_1 receptor

antagonists reduce the pain responses in the second phase of the formalin test (Cendán et al., 2005b; Kim HW et al., 2006; Romero et al., 2012), the mechanical hypersensitivity induced by capsaicin (Entrena et al., 2009a; 2009b; Romero et al., 2012), and the neuropathic pain responses in various models (Nieto et al., 2012; Romero et al., 2012; Son and Kwon, 2010).

There is an apparent discrepancy between the normal occurrence of capsaicin-induced referred hyperalgesia in σ_1-KO mice and the inhibition of this hyperalgesia in WT mice treated with σ_1 receptor antagonists. This mismatch may be attributable to the development of compensatory mechanisms in σ_1-KO mice versus WT animals, a general fact in genetic knockout experiments (Gingrich and Hen, 2000). Thus, there have been reports of compensatory effects and conflicting results between knockout animals and pharmacological experiments in different areas such as GABAergic modulation of seizure activity (Voss et al., 2010), endocannabinoid signaling (Min et al., 2010), the role of 5-HT_7 receptors in depression (Guscott et al., 2005), and the role of δ-subunit-containing $GABA_A$ receptors in nociception (Bonin et al., 2011), among others.

Ketoprofen proved ineffective against the visceral pain induced by intracolonic capsaicin, despite administration of doses that were up to 90-fold and 2.5-fold higher than those shown to be effective in the writhing test (ED50 = 1.41 mg/kg) and in the second inflammatory phase of the formalin test (ED50 = 49.56 mg/kg) in mice (Girard et al., 2008). Thus, the lack of effect of ketoprofen in our study cannot be attributed to the use of low doses of the drug and appears to indicate that prostaglandins are not implicated in capsaicin-induced visceral pain. By contrast, morphine totally abolished the pain-related behaviors and the mechanical referred hyperalgesia induced by intracolonic capsaicin in both WT and σ_1-KO mice. These results agree with previous reports that morphine significantly inhibited intracolonic capsaicin-evoked c-Fos activation in the spinal cord (Mitrovic et al., 2010) and the visceral pain induced by intracolonic mustard oil (Laird et al., 2001; Shin et al., 2006). Interestingly, we found that morphine produced a similar reduction in the number of pain-related behaviors in both types of mouse (ED50 = 3.12±0.35 and 3.47±0.22 mg/kg in σ_1-KO and WT mice,

respectively; $p > 0.05$) but was significantly ($p < 0.01$, Snedecor's F test) more potent against pain induced by mechanical stimulation of the abdominal wall in σ_1-KO mice (ED50 = 3.25±0.40 mg/kg) than in WT mice (ED50 = 4.34 ± 0.21 mg/kg). Studies in models of cutaneous pain induced by thermal stimuli previously demonstrated an enhancement of morphine analgesia by σ_1 receptor antagonists or by antisense oligodeoxynucleotides against σ_1 receptors (Chien and Pasternak, 1993; 1994; Mei and Pasternak, 2002). However, the present study reports the first evidence that σ_1 receptors modulate morphine-induced analgesia in a visceral pain model.

An increased number of capsaicin receptor TRPV1-expressing nerve fibres were reported in rectosigmoid mucosa biopsy specimens from patients with irritable bowel syndrome, one of the most prevalent human gastroenterological pain disorders, and the level of TRPV1 expression correlated with the intensity of abdominal pain (Akbar et al., 2008). Our data may therefore have clinical relevance, because the visceral pain induced by intracolonic instillation of capsaicin to mice can mimic this painful condition through the activation of TRPV1 receptors, suggesting a possible role for σ_1 receptor antagonists in irritable bowel syndrome treatment.

In conclusion, these findings demonstrate that σ_1 receptors play a key role in enteric visceral pain, an important and prevalent pain condition, suggesting that σ_1 receptor blockade may possibly represent a novel therapeutic strategy for treating this pain condition.

2. ROLE OF σ_1 RECEPTORS ON CYCLOPHOSPHAMIDE INDUCED CYSTITIS MODEL

In this section of the Thesis, we identified for the first time a new receptor (σ_1 receptor) in the human and mouse urinary bladder and demonstrated its functional relevance in facilitating the biochemical, histopathological and behavioral manifestations of cyclophosphamide-induced cystitis. The study also showed that σ_1 receptor antagonists ameliorate cystitis manifestations, suggesting that the σ_1 receptor may represent a new target for the treatment of urological diseases.

We identified σ_1 receptors in the urinary bladder by using western blotting and immunohistochemical techniques. The σ_1 receptor signal was lost in σ_1-KO animals, indicating that it was specifically due to identification of the σ_1 receptor protein by the antibody in both techniques. The western blotting results fully agree with previous findings in nervous tissue (Sánchez-Fernández et al., 2014) and identify a band with a molecular weight consonant with the expected molecular weight (25,22 KDa) of the cloned σ_1 receptor (Pan et al., 1998). The σ_1 receptor was concentrated in the urothelium of the human and mouse urinary bladder. This structure is very important for urinary bladder function; it is affected in various bladder disorders, and expresses numerous receptors and channels similar to those of sensory neurons (Birder and Andersson, 2013). Interestingly, the σ_1 receptor is present in a high density in dorsal root ganglia (Bangaru et al., 2013; Sánchez-Fernández et al., 2014), which further supports this similarity.

Two previously identified biochemical markers of cyclophosphamide-induced cystitis are the enhancement of MPO activity (Martins et al., 2012; Rouleau et al., 2000; Santos et al., 2010) and of pERK1/2 expression (Corrow and Vizzard, 2007; Qiao and Gulick, 2007) in the urinary bladder. We confirmed that cyclophosphamide treatment enhanced the levels of both markers in urinary bladder of WT mice and showed that the enhancement was lower in σ_1-KO mice, suggesting that the σ_1 receptor is necessary for both biochemical changes. Previous studies have reported that neuropathy-induced enhancement of spinal

cord pERK1/2 expression was also reduced in σ_1-KO animals (de la Puente et al., 2009; Nieto et al., 2012), suggesting that the σ_1 receptor regulates ERK1/2 activation independently of the tissue considered and the pathology that induces the activation. Enhancement of urinary bladder MPO activity is an index of neutrophil infiltration (Rouleau et al., 2000; Santos et al., 2010) and is reduced not only in σ_1-KO mice but also in WT animals treated with σ_1 receptor antagonists (Supplementary Figure S1), which indirectly indicates that σ_1 receptors modulate neutrophil infiltration. This effect might be related to the ability of the σ_1 receptor to regulate the expression of activated leukocyte cell adhesion molecule (ALCAM) (Yao et al., 2011), which is involved in the transendothelial migration of neutrophils (Weidle et al., 2010).

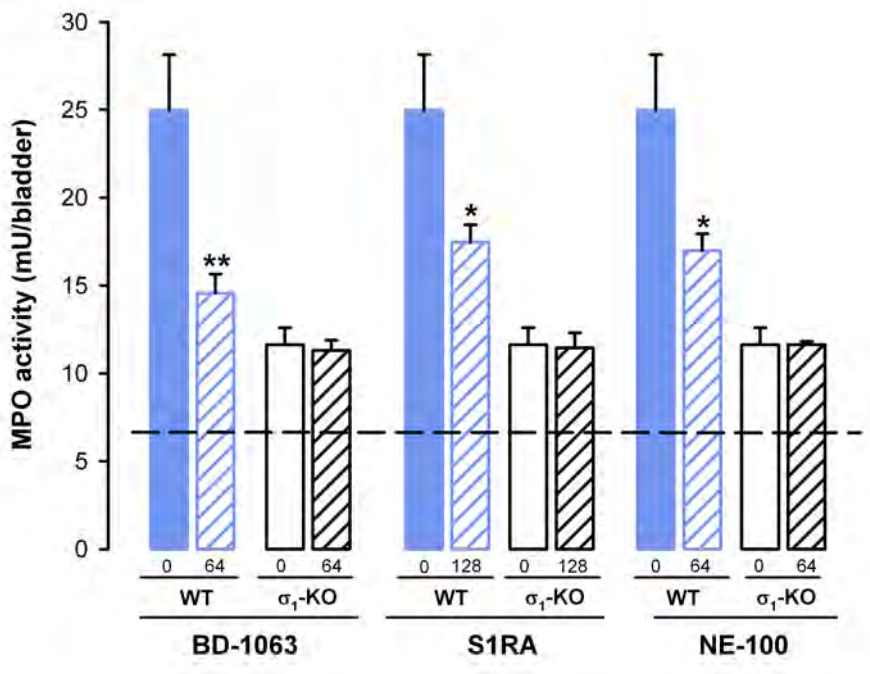

Fig. S1. Effects of treatment with σ_1 receptor antagonists (BD-1063, S1RA or NE-100) or saline on myeloperoxidase (MPO) activity induced by cyclophosphamide in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The s.c. administration of the σ_1 receptor antagonist or its solvent (saline, 0) was performed at 120 min after the i.p. administration of cyclophosphamide (300 mg/kg). The bladder was removed five hours after the injection of cyclophosphamide. Each bar and vertical line represents the mean ± SEM of values obtained in 5–7 animals. The dashed line indicates the MPO activity in naïve animals without any injection. Statistically significant differences between the values obtained in drug- and saline-injected mice: $^{*}p < 0.05$; $^{**}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

Cyclophosphamide-induced cystitis also produces profound changes in urinary bladder histology, with oedema, haemorrhage and urothelium desquamation (Martins et al., 2012; Santos et al., 2010). All of these changes were observed in WT mice but were attenuated in σ_1-KO animals, further supporting the functional relevance of urinary bladder σ_1 receptors in controlling cystitis manifestations.

Inhibition of σ_1 receptor function (by σ_1 receptor antagonists in WT mice or by σ_1 receptor genetic deletion in σ_1-KO mice) reduces the behavioral manifestations of pain induced by cyclophosphamide. The effect of σ_1 receptor antagonists was lost in σ_1-KO mice, which indicates that it was due to their specific interaction with σ_1 receptors. These results agree with previous data of this Thesis obtained in the model of chemically-induced visceral pain (intracolonic capsaicin) (See section 1 of the discussion) and with previous data of our group in a model of chemically-induced somatic pain (intraplantar formalin) (Cendán et al., 2005a); however, inhibition of σ_1 receptor function does not modify somatic acute pain induced by thermal or mechanical stimuli (Cendán et al., 2005b; de la Puente et al., 2009; Entrena et al., 2009b; Sánchez-Fernández et al., 2013). These results again highlight differences between the underlying mechanisms of pain depending on the type of nociceptive stimulus and support the interest of evaluating the role of σ_1 receptor function in models of urological pain induced by non-chemical stimuli. Acute administration of σ_1 receptor antagonists dose-dependently inhibited the referred mechanical hyperalgesia in WT mice and their effects were lost in σ_1-KO animals. These results agree with those of previous studies of mechanical hypersensitivity/allodynia in models of somatic (de la Puente et al., 2009; Entrena et al., 2009b; Moon et al., 2013; Nieto et al., 2012; Roh et al., 2008) and visceral (See section 1 of discussion) pain and suggest that the σ_1 receptor is an important mechanism for pain-induced mechanical hypersensitivity. Interestingly, cyclophosphamide-induced referred hyperalgesia was similar in WT and σ_1-KO mice, suggesting that σ_1-KO animals develop compensatory mechanisms to express mechanical hypersensitivity, similarly to observations in the intracolonic capsaicin model (as described in the previous section of the discussion). However, the mechanical hypersensitivity observed in several models of somatic pain is

abolished in σ_1-KO mice (de la Puente et al., 2009; Entrena et al., 2009b; Nieto et al., 2012), which indicates that the mechanisms underlying somatic and visceral mechanical hypersensitivity are different.

Pain and mechanical hypersensitivity are relevant in several human urinary bladder disorders (Keay et al., 2014; Lai et al., 2014; Offiah et al., 2013). Given that σ_1 receptor antagonists inhibit these cystitis manifestations in mice and σ_1 receptors are similarly distributed in human and mouse urinary bladder, the σ_1 receptor might represent a new drug target for cystitis treatment in humans. In particular, S1RA is a very selective σ_1 receptor antagonist (Romero et al., 2012) that is well tolerated in humans (Abadias et al., 2012) and might represent an attractive drug for testing in human urinary bladder disorders for which no adequate treatment is available.

In contrast to the intracolonic capsaicin model discussed in the previous section (Section 1), which is mainly due to the direct activation of nociceptive terminals, the cyclophosphamide cystitis model involves a patent visceral inflammation (Martins et al., 2012; Santos et al., 2010). We found that indomethacin equally reduced ($p > 0.05$, Snedecor's F test) behavioral responses in WT (ED50 = 3.79 ± 0.28 mg/kg) and σ_1-KO (ED50 = 2.56 ± 0.69 mg/kg) mice as well as the referred hyperalgesia (ED50 = 2.12 ± 0.16 and 2.13 ± 0.17 mg/kg in WT and σ_1-KO, respectively). The activity of indomethacin in this model of visceral inflammation is consistent with the results of other studies demonstrating the efficacy of NSAIDs in somatic (Gris et al., 2014) and visceral inflammatory models (Augé et al., 2013).

Morphine reduced cyclophosphamide-induced pain (ED50 = 1.97 ± 0.28 and 1.04 ± 0.32 mg/kg in WT and σ_1-KO, respectively) and mechanical hypersensitivity (ED50 = 5.97 ± 0.15 and 3.37 ± 0.68 mg/kg in WT and σ_1-KO, respectively), and its effects on these responses were greater in σ_1-KO than in WT mice ($p < 0.05$, Snedecor's F test). These results agree with the findings of previous studies in models of somatic pain (Sánchez-Fernández et al., 2013; 2014; Vidal-Torres et al., 2013) and with those obtained in the intracolonic capsaicin model (see section 1 of the discussion). Interestingly, inhibition of σ_1 receptor function increases the analgesic effects of morphine and other opioid agonists but not their side effects (Mei and

Pasternak, 2002; Sánchez-Fernández et al., 2013; 2014; Vidal-Torres et al., 2013). Opioid agonists are used to treat several urological disorders (Borda et al., 2014; Hanno et al., 2011); therefore, the association of σ_1 receptor antagonists to opioid agonists might be an interesting possibility for their treatment. In fact, unpublished results obtained by our group show that σ_1 receptor antagonists increase morphine-induced analgesia in the cyclophosphamide-induced cystitis model.

In summary, this section of the Thesis shows that σ_1 receptors are present in the human and mice urothelium and may play a functional role in the mechanisms underlying cyclophosphamide-induced cystitis, suggesting that σ_1 receptors might represent a new drug target for the treatment of urological disorders.

CONCLUSIONS

SPECIFIC CONCLUSIONS

1. The intracolonic administration of capsaicin produces a dose-dependent increase in pain behaviors and cutaneous-referred mechanical hyperalgesia in wild-type mice.

2. The intraperitoneal administration of cyclophosphamide to wild-type mice also produces dose-dependent increases in pain score and cutaneous-referred mechanical hyperalgesia. The behavioral alterations are accompanied by urinary bladder inflammation, which is manifested by both histological (oedema, desquamation and haemorrhage) and biochemical (increases in MPO activity and pERK1/2 levels) changes.

3. Sigma-1 receptors are expressed in human and mice bladder urothelium, and their genetic inactivation results in a reduction of the cyclophosphamide-induced histological (oedema, desquamation and haemorrhage) and biochemical (increase of MPO and pERK1/2) inflammatory markers in the urinary bladder, which suggests that these peripherally located σ_1-receptors are functionally relevant.

4. The genetic inactivation of σ_1 receptors, in σ_1-KO animals, reduces the number of pain behaviors in both models of visceral pain evaluated but does not modify the referred mechanical hyperalgesia in either model.

5. The subcutaneous administration to wild-type mice of the selective σ_1 receptor antagonists tested (BD-1063, NE-100 and S1RA) dose-dependently reduces pain responses and referred hyperalgesia in both models (intracolonic capsaicin and cyclophosphamide cystitis). These effects appear to be mediated through their interaction with σ_1 receptors, because none of these drugs has any effect in σ_1-KO mice.

6. The subcutaneous administration of morphine dose-dependently reduces the pain behaviors and the referred mechanical hyperalgesia in the two models of visceral pain evaluated and in both types of mice (wild-type and σ_1-KO). The effects of morphine are greater in σ_1-KO than in wild-type mice (particularly when mechanical hyperalgesia was considered), which suggests that σ_1 receptors modulate opioid-induced visceral antinociception.

7. The subcutaneous administration of ketoprofen shows no effect in the intracolonic capsaicin (non-inflammatory) model, whereas indomethacin produces a dose-dependent reduction of both the behavioral pain score and the mechanical referred hyperalgesia in the cyclophosphamide-induced cystitis (inflammatory) model. The effect of the NSAID in this model is of a similar magnitude in wild-type and σ_1-KO mice.

GENERAL CONCLUSION

The sigma-1 receptor plays an important functional role in different models of visceral pain (with or without inflammation) and may therefore be a novel drug target for the treatment of this type of pain.

ABBREVIATIONS

LIST OF ABBREVIATIONS

ACC	Anterior cingulate cortex
ALCAM	Activated leucocyte cell adhesion molecule
ALQ	Anterolateral quadrant
ANOVA	Analysis of variance
ASIC	Acid-sensing ion channels
ATP	Adenosin triphosphate
BD-1063	1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride
BPS	Bladder pain syndrome
CFA	Complete Freud´s adjuvant
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
DAB	3, 3'-diaminobenzidine
DHEA	Dehydroepiandrosterone
DMT	*N*,*N*-dimethyltryptamine

DRG	Dorsal root ganglia
ED50	Effective dose 50
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
Fig.	Figure
GABA	Gamma aminobutyric acid
GAG	Glycosaminoglican
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GTPγS	Guanosine 5'-O-(γ-thio)triphosphate
HTAB	Hexadecyltrimethylammonium bromide
i.p.	Intraperitoneal
IASP	International association for the study of pain
IB4	Isolectin B4
IBD	Inflamatory bowel disease
IBS	Irritable bowel syndrome
kDa	Kilodalton

kg	Kilogram
KO	Knockout
MAM	Mitochondria-associated endoplasmic reticulum membrane
mg	Milligram
mM	Millimolar
MPO	Myeloperoxidase
Nav	Voltage-gated sodium channel
NE-100	N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine hydrochloride
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drug
NTS	Nucleous tractus solitary
°C	Celsius degrees
$P2X_3$	Purinergic receptor P2X, ligand-gated ion channel, 3
PAG	Periaqueductal grey matter
PCP	Phencyclidine
pERK 1/2	Diphosphorylated ERKs 1 and 2

PFC	Prefontral cortex
PPC	Posterior parietal cortex
PSDC	Postsynaptic dorsal column
RIPA buffer	Radioimmunoprecipitation assay buffer
RVM	Rostroventral medulla
S1 cortex	Primary somatosensory cortex
S1RA	4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride
S2 cortex	Secondary somatosensory cortex
SBDL site	Steroid binding domain-like site
SMA	Supplementary motor area
σ receptor	Sigma receptor
σ_1 receptor	Sigma-1 receptor
σ_2 receptor	Sigma-2 receptor
TRPA1	Transient receptor potential type A1
TRPM8	Transient receptor potential type M8
TRPV1	Transient receptor potential vanilloid type 1

TRPV3	Transient receptor potential vanilloid type 3
TRPV4	Transient receptor potential vanilloid type 4
w/v	Weight per volume
WT	Wild-type

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RESUMEN

1. ANTECEDENTES Y OBJETIVOS

1.1. ANTECEDENTES

El dolor visceral es una de las formas más comunes de dolor patológico y una de las principales razones por las que los pacientes buscan atención médica (Cervero and Laird, 1999; Robinson and Gebhart, 2008). El dolor urológico y gastrointestinal es muy frecuente en clínica, particularmente en pacientes con síndrome del intestino irritable, enfermedad inflamatoria intestinal o cistitis intersticial (Grundmann and Yoon, 2010; Marinkovic et al., 2009); todas ellas patologías con un impacto sustancial en la calidad de vida de los pacientes. Las características fisiológicas del dolor somático y visceral en humanos son muy diferentes (Strigo et al., 2002). Además, estudios en animales de experimentación han demostrado que: a) la neuroquímica de las aferentes primarias que inervan las vísceras, la piel o el músculo es diferente (Lu et al., 2001; Perry and Lawson, 1998), b) las neuronas espinales, las vías ascendentes y los núcleos de proyección supraespinal implicados en la transmisión de la información nociceptiva somática y visceral son distintos (Braz et al., 2005; Schwartz and Gebhart, 2014; Willis, 2007), y c) los mecanismos neurobiológicos activados por el dolor visceral no son los mismos que los activados por el dolor somático (Al-Chaer and Traub, 2002; Foreman, 2004). Por todo ello, los resultados obtenidos en modelos de dolor cutáneo/somático no pueden ser meramente extrapolados al dolor visceral.

El dolor visceral está generalmente causado por lesiones en las vísceras huecas y, muy a menudo, se asocia a un “dolor referido” en una determinada zona cutánea según la víscera afectada, siendo muy frecuente que se produzca hiperalgesia cutánea en la zona del dolor referido (Cervero and Laird, 1999; Robinson and Gebhart, 2008). Se han desarrollado distintos modelos animales de dolor visceral que provocan una activación de vísceras huecas y generan hiperalgesia cutánea. Entre ellos son particularmente interesantes los modelos de estimulación química del colon mediante capsaicina y de cistitis inducida por ciclofosfamida (Bon et al., 2003; Laird et al., 2001; Olivar and Laird, 1999), ya que, como hemos comentado la cistitis y el dolor intestinal son tipos de dolor visceral muy frecuentes en humanos. Ambos modelos producen una alteración del comportamiento del animal

indicativa de dolor visceral, una hiperalgesia cutánea a estímulos mecánicos puntiformes (equivalente a la hiperalgesia cutánea descrita por los humanos) y en el modelo de la cistitis, una inflamación de la vejiga (Bon et al., 2003; Laird et al., 2001; Olivar and Laird, 1999). Además, ambos modelos animales han sido validados en humanos, ya que la ciclofosfamida es un fármaco antitumoral entre cuyos efectos indeseables dosis-limitantes se encuentra una cistitis hemorrágica de características idénticas a la que aparece en el ratón (Olivar and Laird, 1999). De igual modo la aplicación intestinal de capsaicina en humanos produce dolor visceral y dolor cutáneo referido (Arendt-Nielsen et al., 2008; Drewes et al., 2003).

Los receptores sigma (σ) fueron descritos por primera vez en 1976 en estudios con agonistas no selectivos de los receptores opioides (SKF-10.047) (Martin et al., 1976) y durante algunos años fueron considerados erróneamente como un subtipo de receptores opioides. Estudios posteriores demostraron que son unos receptores específicos y diferentes del resto de receptores conocidos y que se pueden distinguir dos subtipos, denominados σ_1 y σ_2 (Revisiones: Cobos et al., 2008; Maurice and Su, 2009). Ambos subtipos están ampliamente distribuidos en el sistema nervioso central, aunque su patrón de expresión en distintas áreas es diferente (Bouchard and Quirion, 1997; Kitaichi et al., 2000; Sánchez-Fernández et al., 2014).

El receptor σ_1 fue clonado en primer lugar en hígado de cobaya (Hanner et al., 1996) y posteriormente en otros tejidos humanos y de roedores, incluyendo el cerebro de ratón (Pan et al., 1998). Es una proteína pequeña (223 aminoácidos) que no guarda relación estructural con ninguna otra proteína conocida de mamíferos. Posee dos dominios transmembrana y una señal de retención al retículo endoplasmático en la región N-terminal (Cobos et al., 2008; Guitart et al., 2004). La presencia de un dominio chaperona en su estructura ha sido descrita recientemente (Hayashi and Su, 2007), y puede explicar parte de las acciónes de este receptor. El receptor σ_1 tiene una rica farmacología. En la actualidad los antagonistas específicos mejor estudiados son NE-100, S1RA y BD-1063 (Cobos et al., 2008; Maurice and Su, 2009; Romero et al., 2012). Se han generado ratones knockout σ_1 que están ayudando a definir mejor las características bioquímicas y

funcionales del receptor σ_1 (Cendán et al., 2005a; de la Puente et al., 2009; Entrena et al., 2009b; Langa et al., 2003).

Existe una alta densidad de receptores σ_1 en zonas del sistema nervioso con especial relevancia en la transmisión y modulación de la información nociceptiva, como las láminas superficiales (I y II) del asta dorsal de la sustancia gris de la médula espinal y la sustancia gris periacueductal, entre otras (Alonso et al., 2000; Kitaichi et al., 2000). Recientemente, se ha descrito la presencia de receptores σ_1 en el sistema nervioso periférico, concretamente, en los cuerpos neuronales de los ganglios de la raíz dorsal, siendo la concentración de receptores σ_1 mayor en estos ganglios que en las áreas del sistema nervioso central implicadas en la nocicepción (Bangaru et al., 2013; Sánchez-Fernández et al., 2014). Los receptores σ_1 también se encuentran en las células de Schwann, específicamente en su citoplasma y en la región paranodal de los nódulos de Ranvier.

Diversos datos sugieren una implicación funcional de los receptores σ_1 en la nocicepción cutánea/somática. Así, la administración de antagonistas σ_1 y de oligonucleótidos antisentido frente al receptor σ_1 potencia la analgesia inducida por agonistas de receptores opioides μ, δ, κ_1 y κ_3, en los modelos de retirada de la cola de un foco calorífico y placa caliente en el ratón, mientras que la (+)pentazocina (un agonista σ_1) produce efectos opuestos (Chien and Pasternak, 1994; King et al., 1997; Mei and Pasternak, 2002; 2007). Por otra parte, se ha demostrado que los ratones knockout σ_1 muestran menos dolor tras la administración intraplantar de formalina (Cendán et al., 2005a) y que antagonistas σ_1 producen una analgesia dosis-dependiente en este modelo de dolor, existiendo una buena correlación entre su potencia para bloquear los receptores σ_1 y su potencia analgésica (Cendán et al., 2005b). Por otro lado, se ha demostrado que los antagonistas σ_1 revierten la alodínia mecánica inducida por la administración intraplantar de capsaicina en el ratón (Entrena et al., 2009a; 2009b). Además, los ratones knockout σ_1 muestran una menor alodínia tras la administración intraplantar de capsaicina (Entrena et al., 2009b) y en modelos de dolor neuropático (de la Puente et al., 2009; Nieto et al., 2012; Romero et al., 2012). Recientemente, se ha descrito que varios antagonistas σ_1, administrados de

manera sistémica o local (en el foco de inflamación) son capaces de abolir la hipersensibilidad dolorosa en modelos de dolor inflamatorio como la administración intraplantar de carragenina o de adyuvante completo de Freud (CFA) (Gris et al., 2014; Parenti et al., 2014a; 2014b; Tejada et al., 2014). Por tanto, hay una amplia evidencia de que la inhibición de los receptores σ_1 reduce el dolor nociceptivo, inflamatorio o neuropático originado en la piel o en estructuras somáticas.

1.2. OBJETIVOS

Las características, los mecanismos fisiopatológicos y la respuesta a los fármacos del dolor visceral son distintos de los del dolor cutáneo; por tanto, los resultados obtenidos en modelos de dolor cutáneo/somático no pueden ser extrapolados al dolor visceral. La inhibición de los receptores σ_1 es útil para el tratamiento de distintos tipos de dolor cutáneo/somático, pero no hay datos previos del papel de los receptores σ_1 en el dolor visceral. Por ello, nuestro **objetivo principal** fue evaluar el efecto del bloqueo farmacológico y de la inactivación genética de los receptores σ_1 en modelos de dolor visceral inflamatorio y no inflamatorio (utilizando modelos de dolor que han sido previamente validados en humanos para maximizar la validez translacional de nuestros resultados). Para alcanzar este objetivo, realizamos diferentes tipos de experimentos con varios objetivos específicos.

El **primer objetivo específico** fue estudiar la influencia del receptor σ_1 en un modelo de dolor visceral no inflamatorio, el modelo de la capsaicina intracolónica. Para alcanzar este objetivo, usamos dos aproximaciones experimentales. Primero, comparamos las respuestas de ratones salvajes y knockout σ_1 a la administración intracolónica de capsaicina. Posteriormente, estudiamos el efecto de diferentes antagonistas σ_1 (BD-1063, NE-100 y S1RA) en animales de ambos genotipos tratados con capsaicina intracolónica. En ambos casos, evaluamos las manifestaciones comportamentales indicativas de dolor espontáneo y la hiperalgesia cutánea referida producidas por el algógeno.

Los mecanismos fisiopatológicos implicados en el dolor inflamatorio y no inflamatorio son distintos. Por ello, el **segundo objetivo específico** de esta Tesis Doctoral fue determinar el papel de los receptores σ_1 en un modelo de dolor visceral inflamatorio, el modelo de la cistitis inducida por ciclofosfamida, e identificar si los receptores σ_1 están presentes en la vejiga urinaria y son funcionalmente relevantes. Para alcanzar este objetivo se realizaron varios tipos de experimentos:

a. Puesto que el urotelio de la vejiga expresa diferentes canales y receptores que también se expresan en neuronas del ganglio de la raíz dorsal y que los receptores σ_1 se expresan en el ganglio de la raíz dorsal, quisimos estudiar si este receptor está presente también en la vejiga (realizando experimentos de "western blotting" e inmunohistoquímica). Además, estudiamos si la expresión del receptor σ_1 en la vejiga de ratón es similar a la expresión en la vejiga humana.
b. El papel funcional de los receptores σ_1 en la vejiga urinaria fue evaluado estudiando el efecto de su ausencia (con animales knockout σ_1) en el modelo de la cistitis por ciclofosfamida, midiendo tanto parámetros histológicos (edema, hemorragia y descamación del urotelio) como bioquímicos (aumento de actividad mieloperoxidasa y niveles de pERK) indicativos de inflamación en la vejiga.
c. Las consecuencias comportamentales de la inhibición de los receptores σ_1 en la cistitis por ciclofosfamida fueron determinadas: a) comparando las alteraciones comportamentales indicativas de dolor y la hiperalgesia mecánica referida producidas por el antineoplásico en ratones salvajes y knockout σ_1, y b) evaluando el efecto en esos parámetros de la administración de antagonistas σ_1 (BD-1063, NE-100 y S1RA) en animales de ambos genotipos.

Los experimentos realizados para alcanzar los dos objetivos específicos anteriormente mencionados sugirieron que los antagonistas σ_1 podrían representar una nueva herramienta farmacológica para el tratamiento del dolor visceral; por ello, el **tercer objetivo específico** fue comparar el efecto de los

antagonistas σ_1 con el de diferentes fármacos analgésicos control, incluyendo agonistas opioides (morfina) y anti-inflamatorios no esteroideos (AINEs) (indometacina y ketoprofeno), en los dos modelos de dolor visceral estudiados. Además, dado que los receptores σ_1 modulan el efecto analgésico de los opioides en modelos de dolor cutáneo/somático, comparamos el efecto de la morfina en ratones salvajes y knockout σ_1 en los modelos de dolor visceral.

2. MÉTODOS

2.1. ANIMALES Y FÁRMACOS

2.1.1 ANIMALES

Los experimentos fueron realizados en ratones hembra de la cepa CD-1 (Charles River, Barcelona, España) y en ratones knockout para el receptor σ_1 (Esteve, Barcelona, España). Los ratones knockout σ_1 se generaron con un origen genético CD-1, como se describió previamente (Entrena et al., 2009a). Los animales fueron manipulados de acuerdo con la Directiva del Consejo de Comunidades Europeas de 24 de Noviembre de 1986 (86/609/Ecc). El protocolo experimental fue aprobado por el Comité de Ética de Experimentación Animal de la Universidad de Granada.

El experimentador que evaluó las respuestas comportamentales en los ratones, no conocía el tratamiento ni el genotipo de los animales. En todos los casos, los experimentos entre los diferentes grupos se realizaron en paralelo. Cada animal fue utilizado una única vez, con una única concentración de algógeno (o solvente) y fármaco (o solvente).

2.1.2 FÁRMACOS

Se usaron los antagonistas selectivos para el receptor σ_1 BD-1063, NE-100 y S1RA. Morfina fue usada como control opioide, y ketoprofeno e indometacina fueron usados como controles de fármacos AINEs. Todos los fármacos fueron disueltos en

suero fisiológico salino esteril, con la excepción de ketoprofeno e indometacina, los cuales se disolvieron en 10% de etanol absoluto y 90% de salino o en 5% de bicarbonato sódico y 95% de salino, respectivamente. Los fármacos fueron preparados inmediatamente antes de comenzar los experimentos, inyectándose subcutáneamente 5 ml/kg de la solución del fármaco o su solvente en el área subescapular.

La capsaicina, que fue usada para producir dolor intracolónico, fue disuelta en 10% de Tween 80, 10% de etanol absoluto y 80% de salino. Esta solución de capsaicina (50 µl) fue instilada en el colon introduciendo una fina cánula a través del ano. Los animales control fueron instilados intracolónicamente con el mismo volumen del solvente de capsaicina.

La ciclofosfamida (usada para inducir cistitis) fue disuelta en salino e inyectada intraperitonealmente en un volumen de 10 ml/kg. Los animales control fueron inyectados con el mismo volumen de solvente.

2.2. MODELO DE CAPSAICINA INTRACOLÓNICA

En este modelo, se evaluaron los comportamientos indicativos de dolor espontáneo y la hiperalgesia mecánica referida producidos por la capsaicina intracolónica siguiendo el protocolo descrito previamente (Laird et al., 2001), con pequeñas modificaciones.

Tras 40 minutos de habituación, los animales fueron instilados con la solución de capsaicina (0,01%-1%) (o su solvente) y fueron observados durante 20 minutos, registrándose el número de comportamientos relacionados con el dolor visceral (lamidos, estiramientos y contracciones del abdomen).

La presencia de hiperalgesia referida fue determinada midiendo el umbral de respuesta a una estimulación mecánica puntiforme del abdomen, 20 minutos después de la instilación de la solución de capsaicina (o su solvente). Para ello, se aplicaron filamentos de von Frey en un rango de fuerzas de 0,02 a 2 g (0,19-19,6 mN) usando el “método del arriba y abajo” (Chaplan et al., 1994). Cada filamento se

aplicó tres veces durante 2-3 segundos cada una, con un tiempo de descanso de 5 segundos entre cada aplicación. Las mediciones se iniciaron con el filamento de 0,4 g (3,92 mN) y se aumentaba la fuerza aplicada en caso de no existir respuesta o se disminuía si aparecía una respuesta positiva. La respuestas eran consideradas positivas cuando se observaba salto, lamido o retracción del abdomen tras la aplicación del filamento.

Los fármacos fueron administrados de manera subcutánea 30 minutos antes de la instilación de la solución de capsaicina.

2.3. MODELO DE CISTITIS POR CICLOFOSFAMIDA

2.3.1. DOLOR VISCERAL E HIPERALGESIA REFERIDA

Los comportamientos indicativos de dolor espontáneo y la hiperalgesia mecánica referida producidos por la ciclofosfamida fueron evaluados siguiendo el protocolo descrito previamente (Laird et al., 2002; Olivar y Laird, 1999; Wantuch et al., 2007), con pequeñas modificaciones.

Tras 40 minutos de habituación los animales fueron inyectados con ciclofosfamida (10-300 mg/kg) (o su solvente) y observados 2 minutos cada media hora durante 4 horas. Los comportamientos dolorosos fueron puntuados de acuerdo con la siguiente escala: 0 = normal, 1 = piloerección, 2 = fuerte piloerección, 3 = dificultad respiratoria, 4 = lamido del abdomen, 5 = contracción del abdomen o contorsión (Olivar y Laird, 1999). Si ocurría mas de una respuesta diferente en un periodo de observación, se le otorgaba la suma de las correspondientes respuestas, y si aparecían varias respuestas iguales, únicamente se consideraba la puntuación de una de las respuestas.

Al final de las 4 horas de observación se determinó la hiperalgesia referida mediante la medición del umbral de respuesta ante una estimulación mecánica puntiforme del abdomen, mediante el "método del arriba y abajo" (Chaplan et al., 1994) descrito anteriormente (sección 2.2 del resumen).

Los fármacos fueron administrados por vía subcutánea 2 horas después de la inyección de ciclofosfamida.

2.3.2. ACTIVIDAD MIELOPEROXIDASA

Cinco horas después de la inyección de ciclofosfamida las vejigas urinarias fueron extraídas, homogeneizadas en tampón fosfato (50 mM, pH 6,0) con 0,5% de bromuro de hexadeciltrimetilamonio y centrifugadas (6000 g 10 minutos) para recoger el sobrenadante, el cual se usó para medir la actividad mieloperoxidasa siguiendo el método previamente descrito (Rouleau et al., 2000).

2.3.3. INMUNOHISTOQUÍMICA E HISTOPATOLOGÍA

Secciones de vejigas urinarias (5 µm de grosor) humanas (adquiridas a BioChain Institute Inc, Newark, California, número de lote B507075) o de ratón fijadas en formalina se desparafinaron, hidrataron y calentaron (95°C, 20 min) en tampón citrato (pH 8,0) para desenmascaramiento antigénico. La actividad peroxidasa endógena se bloqueó con 3% de H_2O_2 en metanol durante 15 minutos. Las muestras fueron incubadas (30 min) con un anticuerpo monoclonal con afinidad por los receptores σ_1, el cual fue visualizado con el kit UltraVision Quanto. Las muestras fueron finalmente teñidas con hematoxilina de Mayer.

Para el estudio histopatológico, se realizó un análisis morfométrico para cada corte (teñidos con hematoxilina-eosina) mediante el programa ImageJ, y el área edematosa localizada entre la lámina propia y la muscular se normalizó con respecto al área total de la sección de vejiga. El número de zonas de descamación urotelial y de focos hemorrágicos también fueron contabilizados en cada corte.

2.3.4. WESTERN BLOTTING

Los experimentos se realizaron de manera similar al protocolo previamente descrito (Nieto et al., 2012), con algunas modificaciones. Las vejigas urinarias se homogeneizaron y centrifugaron (1000 g, 5 min) y las proteínas de la fracción sobrenadante (20 µg) fueron fraccionadas en gel al 12% SDS-poliacrilamida y transferidas a una membrana de nitrocelulosa. Dichas membranas fueron incubadas durante una hora en tampón de bloqueo (5% leche en polvo con Tween 20 en TBS, pH 7.5) y durante toda la noche a 4°C con anticuerpos primarios capaces de reconocer los receptores σ_1, la ERK1/2 total, la pERK1/2 o el GAPDH (el cual se usó como control de carga). Como anticuerpos secundarios se usaron anticuerpos frente a IgGs de cabra o de burro conjugados con peroxidasa de rábano. Por último, la peroxidasa fue puesta de manifiesto con el reactivo de detección ECL Prime Western Blotting.

3. RESULTADOS

3.1. MODELO DE CAPSAICINA INTRACOLÓNICA

La instilación de la solución de capsaicina produjo diferentes tipos de comportamientos dolorosos (lamidos, estiramientos y contracciones del abdomen). El número de estas respuestas aumentó dosis-dependientemente conforme se aumentó la concentración de capsaicina tanto en ratones salvajes como en ratones knockout σ_1, pero en los ratones knockout el aumento fue significativamente menor.

Por el contrario, no se encontraron diferencias significativas en la hiperalgesia mecánica referida entre ratones salvajes y knockout σ_1. La solución de capsaicina redujo el umbral mecánico de manera dosis-dependiente en ambos grupos.

El bloqueo farmacológico de los receptores σ_1 mediante los antagonistas selectivos BD-1063, NE-100 y S1RA, administrados por vía subcutánea 30 minutos antes de la instilación de la solución de capsaicina (1%), produjo una inhibición dosis-

dependiente del número de respuestas comportamentales dolorosas. Sin embargo, ninguno de los antagonistas σ_1 probados produjo ningún efecto en los ratones knockout σ_1. Los antagonistas σ_1 no fueron capaces de eliminar completamente las manifestaciones dolorosas provocadas por la capsaicina, sin embargo, a la dosis mayor probada todos redujeron el número de respuestas a valores similares a los obtenidos en los ratones knockout σ_1.

En los ratones salvajes, la hiperalgesia mecánica referida producida por la capsaicina (0,1%) fue revertida casi completamente con la dosis mayor de los antagonistas σ_1. Sin embargo, dichos antagonistas σ_1 no modificaron la hiperalgesia mecánica referida en los ratones knockout σ_1, lo cual pone de manifiesto la especificidad de los fármacos.

La administración subcutánea de morfina produjo una inhibición dosis-dependiente de los comportamientos dolorosos producidos por la capsaicina tanto en los ratones salvajes como en los knockout σ_1, así como una reversión de la hiperalgesia mecánica referida (llegando a superarse el umbral mecánico de animales tratados con el solvente de capsaicina). El efecto de la morfina fue significativamente mayor en los animales knockout σ_1 que en los salvajes. La administración de ketoprofeno no produjo ningún efecto en las respuestas comportamentales ni en la hiperalgesia mecánica referida en ninguno de los dos genotipos.

3.2. MODELO DE CISTITIS POR CICLOFOSFAMIDA

3.2.1. EXPRESIÓN DE RECEPTORES σ_1 EN VEJIGAS HUMANAS Y DE RATÓN

Los experimentos inmunohistoquímicos en ratones salvajes detectaron la presencia del receptor σ_1 en el citoplasma de diferentes capas de células del urotelio, estando particularmente concentrado alrededor del núcleo y junto a estructuras citoplasmáticas circulares. No se encontraron receptores σ_1 en ratones knockout σ_1, apoyando la especificidad del anticuerpo. En las vejigas humanas, se

encontraron también receptores σ_1 en el urotelio, con un patrón similar a las vejigas de ratón.

Los experimentos de "western blot" con anticuerpos frente a los receptores σ_1 identificaron una banda con un peso molecular ligeramente superior de 25 kDa en la vejiga de los ratones salvajes pero no en la de los ratones knockout σ_1 y mostraron que el tratamiento con ciclofosfamida no altera la densidad de dicha banda.

3.2.2. ACTIVIDAD MIELOPEROXIDASA Y EXPRESIÓN DE pERK1/2

El tratamiento con ciclofosfamida produjo un aumento dosis-dependiente de la actividad mieloperoxidasa en las vejigas de los ratones salvajes, pero un aumento significativamente menor en las animales knockout para el receptor σ_1. La mayor diferencia en el efecto de la ciclofosfamida entre ratones salvajes y knockout σ_1 se observó a la dosis de 300 mg/kg. Esta dosis de ciclofosfamida no modificó la expresión de ERK1/2 total, pero produjo un aumento tiempo-dependiente de la expresión de pERK1/2 que fue significativamente superior en ratones salvajes que en ratones knockout σ_1 a las 3 y 5 horas.

3.2.3. ANÁLISIS HISTOLÓGICO

Las secciones de vejiga urinaria de ratones salvajes teñidas con hematoxilina-eosina mostraron una apariencia normal, que no presentó diferencias con las vejigas control de ratones knockout σ_1. El tratamiento con ciclofosfamida en ratones salvajes produjo (a las 5 horas) una marcada cistitis con edema, descamaciones del urotelio y focos hemorrágicos. Esta cistitis inducida por la ciclofosfamida fue menos marcada en los ratones knockout σ_1, los cuales mostraron una significativa reducción en el área de edema, así como un menor número de focos hemorrágicos y de descamación.

3.2.4. DOLOR VISCERAL E HIPERALGESIA REFERIDA

Puesto que los resultados bioquímicos e histopatológicos sugirieron que los receptores σ_1 modulan las alteraciones en la vejiga urinaria inducidas por la ciclofosfamida, evaluamos el efecto de la inhibición de la función de los receptores σ_1 en la respuesta comportamental de la cistitis.

El tratamiento con ciclofosfamida produjo un aumento dosis-dependiente en la puntuación comportamental dolorosa en los animales salvajes. Este efecto de la ciclofosfamida fue significativamente menor en los animales knockout σ_1, encontrándose las máximas diferencias entre ambos genotipos con la dosis de 300 mg/kg. La puntuación comportamental para esta dosis de ciclofosfamida mostró un curso temporal con marcadas diferencias entre animales salvajes y knockout σ_1 a partir de los 150 minutos.

Todos los antagonistas σ_1 (BD-1063, S1RA y NE-100), inyectados 120 minutos después de la ciclofosfamida, produjeron una inhibición dosis-dependiente de la puntuación comportamental dolorosa en los ratones salvajes. Sin embargo, estos fármacos carecieron de efecto en los ratones knockout σ_1, indicando la especificidad de su efecto. Por otro lado, tanto morfina como indometacina inhibieron el dolor inducido por ciclofosfamida tanto en ratones salvajes como knockout σ_1.

La administración de ciclofosfamida también produjo una hiperalgesia dosis-dependiente referida a la pared abdominal, que no fue significativamente diferente entre ratones salvajes y knockout σ_1. Cuando los receptores σ_1 fueron bloqueados mediante fármacos, todos los antagonistas σ_1 inhibieron dosis-dependientemente la hiperalgesia mecánica producida por la ciclofosfamida en ratones salvajes, pero carecieron de efecto en ratones knockout σ_1. Sin embargo, indometacina redujo la hiperalgesia mecánica producida por ciclofosfamida en ambos genotipos mientras que morfina inhibió la hiperalgesia mecánica y produjo analgesia (en dosis superiores a 4 mg/kg) en ratones salvajes, teniendo un efecto aún mayor en ratones knockout σ_1.

4. CONCLUSIONES

4.1. CONCLUSIONES ESPECÍFICAS

1. La administración intracolónica de capsaicina incrementa de forma dosis-dependiente el comportamiento indicativo de dolor y la hiperalgesia mecánica referida en los animales salvajes.

2. La administración intraperitoneal de ciclofosfamida en los animales salvajes produce un incremento dosis-dependiente en la puntuación comportamental indicativa de dolor y en la hiperalgesia mecánica referida. Las alteraciones comportamentales son acompañadas de inflamación de la vejiga urinaria, la cual se manifiesta mediante cambios histológicos (edema, descamación del urotelio y hemorragia) y bioquímicos (incremento de la actividad de mieloperoxidasa y de los niveles de pERK1/2).

3. Los receptores sigma-1 se expresan en el urotelio de la vejiga humana y del ratón, y su inactivación genética reduce las alteraciones histológicas (edema, descamación del urotelio y hemorrágia) y bioquímicas (incremento de la actividad de mieloperoxidasa y pERK1/2) inducidas por la administración de ciclofosfamida, lo que pone de manifiesto la relevancia funcional de los receptores sigma-1 en la vejiga.

4. La inactivación genética de los receptores sigma-1, en animales knockout σ_1, reduce el número de comportamientos dolorosos en ambos modelos de dolor visceral evaluados, pero no modifica la hiperalgesia mecánica referida en ninguno de ellos.

5. La administración subcutánea de los antagonistas selectivos sigma-1, BD-1063, NE-100 y S1RA, disminuye dosis-dependientemente las respuestas dolorosas así como la hiperalgesia mecánica referida en ambos modelos (capsaicina intracolónica y cistitis por ciclofosfamida) en animales salvajes. Este efecto parece estar mediado por la interacción con los receptores sigma-1, ya que ninguno de estos fármacos tiene efecto en los animales knockout σ_1.

6. La administración subcutánea de morfina reduce dosis-dependientemente los comportamientos dolorosos y la hiperalgesia mecánica referida en los dos modelos de dolor visceral evaluados y en ambos genotipos. El efecto de la morfina es superior en ratones knockout σ_1 que en ratones salvajes (particularmente si consideramos la hiperalgesia mecánica), lo que sugiere que los receptores sigma-1 modulan la antinocicepción visceral inducida por opioides.

7. La administración subcutánea de ketoprofeno no produce ningún efecto en el modelo de capsaicina intracolónica (no-inflamatorio), mientras que la indometacina produce una reducción dosis-dependiente tanto de los comportamientos dolorosos como de la hiperalgesia mecánica referida en el modelo de cistitis inducida por ciclofosfamida (inflamatorio). Los efectos de la indometacina en este modelo son de una magnitud similar en ratones salvajes y knockout σ_1.

4.2. CONCLUSIÓN GENERAL

Los receptores sigma-1 juegan un importante papel funcional en diferentes modelos de dolor visceral (con o sin inflamación) y por ello pueden ser una nueva diana farmacológica para el tratamiento de este tipo de dolor.