Universitat Autònoma de Barcelona

Role of sigma 1 receptors on viscerosensitivity and inflammation of intestinal origin

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Role of sigma 1 receptors on viscerosensitivity and inflammation of intestinal origin

Presented by

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A dissertation in partial fulfilment of the

requirements for the degree of Doctor of Philosophy

Neuroscience Doctoral Program

Department of Cell Biology, Physiology and Immunology

Neuroscience Institute

Universitat Autònoma de Barcelona

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Bellaterra (Barcelona), September 2021

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I hereby declare that the Doctoral Thesis entitled “Role of sigma 1 receptors on viscerosensitivity and inflammation of intestinal origin”, submitted by Sergio López Estévez in fulfillment of the requirements for the degree of Doctor of Philosophy, has been carried out under my supervision and I authorize the submission to undertake its oral defense.

In witness whereof, I hereby sing this document.

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The research described in this thesis was conducted at the Department of Cell Biology, Physiology and Immunology, and the Institute of Neurosciences of the Universitat Autònoma de Barcelona. This work has been funded by the project Role of Esteve's new chemical entities on the modulation of visceral pain and inflammatory processes, from Laboratorios Esteve S.A.

Cover image: haematoxylin–eosin-stained colonic section from a control wild-type mouse.

"The most exciting phrase to hear in science,
the one that heralds new discoveries, is not 'Eureka!' but 'that's funny…' "

Isaac Asimov, 1920-1992
Biochemist, writer and scientific communicator

"I don't see the logic of rejecting data
just because they seem incredible."

Sir Fred Hoyle, 1915-2001
Astrophysicist and writer

CONTENTS

SUMMARY

Pain of visceral origin is a frequent finding in gastrointestinal disorders with an inflammatory component, such are inflammatory bowel disease or functional gastrointestinal disorders, with irritable bowel syndrome as the main representative. Despite this, there are no effective treatments for visceral pain. Sigma type 1 receptors (σ_1Rs) are ligand-regulated membrane proteins with neuromodulatory and immunodulatory activity. They have been implicated in somatic pain of different etiologies, including inflammatory pain, mediating pro-nociceptive responses and interacting with the analgesic effects of opioids. According to this, σ_1Rs are currently regarded a target for the pharmacological treatment of somatic pain.

In this work, we evaluate the implications of σ_1R as modulators of intestinal inflammation and inflammation-associated visceral pain. For this purpose, the intestinal inflammatory response, dextran sulfate sodium (DSS)-induced colitis model, and the development of visceral and referred somatic hypersensitivity have been assessed in both mice and rats. To assess intestinal hypersensitivity in rats, a long-term hypersensitivity model based on the induction of colitis with DSS has been set-up. The involvement of σ_1Rs has been assessed through its genetic (knockout mice constitutively lacking this receptor) or pharmacological blockade (use of the selective σ_1R antagonists BD1063 and E-52862). The interaction between μ-opiate receptors-mediated analgesia and σ_1Rs has been studied by the co-administration of morphine and BD13063 or the use of the dual μ-opioid receptor partial agonist and σ_1R antagonist EST3502. Somatic and visceral sensitivities have been determined using the von Frey test or through the assessment of viscerosomatic responses associated to colorectal distension. In parallel, changes in gene expression of different inflammation- and pain-related markers have been assessed, both in

peripheral (colon) and central tissues (lumbosacral spinal cord) (RT-qPCR and westtern Blot).

The results obtained with either the genetic (KO mice) or the pharmacological blockade (selective σ_1R antagonists) show that σ_1Rs do not modulate mechanical sensitivity under basal conditions. In mice, the constitutive absence of σ_1Rs or their pharmacological blockade attenuated the clinical development of inflammation, both acute and chronic, in the DSS-induced colitis model, without affecting the expected changes in inflammatory markers (up-regulation of inflammatory cytokines). During colitis, mice with functional σ_1Rs developed referred mechanical hypersensitivity, determined both in the abdominal wall (mixed visceral and somatic sensitivity) and in the paw (somatic sensitivity). In contrast, the lack of functional σ_1Rs was associated with the absence of referred mechanical hypersensitivity, eliciting responses essentially identical to those observed in animals without colitis. These changes could not be associated with a specific modulation of sensory-related markers implicated in pain processing, neither in the colon nor in the spinal cord. In rats, the DSS-induced colitis model results in a state of long-lasting hypersensitivity that appears to involve a combination of both peripheral (colon) and central (lumbosacral spinal cord) sensitization mechanisms. This state of hypersensitivity is not affected by the pharmacological blockade of σ_1Rs with BD1063, whereas it can be attenuated by de μ-opioid agonist morphine or the voltage gated calcium channels (α2δ subunit) ligand pregabalin. Compound EST73502 increased pain thresholds in rats during colorectal distention and showed anti-hyperalgesic activity during colitis-induced hypersensitivity.

Taken together, these results suggest that σ_1Rs exhibits anti-inflammatory and analgesic properties that make this receptor a potentially feasible target for the treatment of gastrointestinal disorders characterized by the presence of signs of inflammation and alterations in visceral sensitivity.

RESUM

El dolor d'origen visceral és una troballa freqüent en les alteracions gastrointestinals amb un component inflamatori, com són la malaltia inflamatòria intestinal, o les alteracions funcionals gastrointestinals, com la síndrome d'intestí irritable. Malgrat això, no existeixen tractaments efectius per al dolor visceral. Els receptors sigma de tipus 1 ($R\sigma_1$) són proteïnes de membrana regulades per lligand amb activitat neuromoduladora i immunoduladora. Aquests receptors s'han implicat en dolor somàtic de diferents etiologies, incloent l'inflamatori, intervenint respostes pronociceptives i interaccionant amb els efectes analgèsic dels opioides. Això ha fet que actualment els $R\sigma_1$ es considerin una diana per al tractament farmacològic del dolor somàtic.

En aquest treball s'avaluen les implicacions dels $R\sigma_1$ com a moduladors de la inflamació intestinal i del dolor visceral associat a aquesta. Amb aquesta finalitat, s'ha valorat la resposta inflamatòria intestinal, mitjançant el model de colitis induïda per dextrà sulfat de sodi (DSS), i el desenvolupament d'hipersensibilitat visceral i somàtica referida, tant en ratolins com en rates. Per valorar la hipersensibilitat intestinal en rates s'ha posat a punt un model d'hipersensibilitat a llarg termini que es basat en la inducció de colitis amb DSS. La implicació dels $R\sigma_1$ s'ha valorat mitjançant el seu bloqueig genètic (ratolins mancats de manera constitutiva d'aquest receptor) o farmacològic (ús dels antagonistes selectius BD1063 i E-52862). S'ha estudiat també la interacció entre mecanismes opioides dependents de receptors de tipus μ i els $R\sigma_1$ mitjançant la co-administració de morfina i BD13063 o l'ús de la molècula amb activitat dual EST73502 (agonista parcial μ-opioide i antagonista $R\sigma_1$). Les sensibilitats somàtica i visceral s'han determinat mitjançant el test de von Frey o la valoració de respostes viscerosomàtiques associades a la distensió colorectal. En paral·lel, s'han determinat canvis en l'expressió de marcadors relacionats amb la

inflamació i el dolor, tant a nivell perifèric (còlon) com a central (medul·la espinal lumbosacra) (RT-qPCR i westtern Blot).

Els resultats obtinguts, tant amb el bloqueig genètic com amb el farmacològic, mostren que els $R\sigma_1$ no modulen la sensibilitat mecànica en condicions basals. En el ratolí, l'absència constitutiva de $R\sigma_1$ o el seu bloqueig farmacològic va atenuar el desenvolupament clínic de la inflamació, tant aguda com crònica, en el model de colitis induïda per DSS, sense alterar els canvis esperats en marcadors inflamatoris (regulació a l'alça de citocines inflamatòries). Durant la colitis, els ratolins amb $R\sigma_1$ funcionals van desenvolupar hipersensibilitat mecànica referida, determinada tant a la paret abdominal (mescla de sensibilitat visceral i somàtica) com a la pota (sensibilitat somàtica). Per contra, l'absència de $R\sigma_1$ funcionals es va associar a una falta de desenvolupament d'hipersensibilitat mecànica referida, amb respostes bàsicament idèntiques a les observades en animals sense colitis. Aquests canvis no es van poder associar a una modulació específica de marcadors implicats en vies sensorials, ni en el còlon ni en la medul·la espinal. En la rata, el model de colitis induïda per DSS genera un estat d'hipersensibilitat a llarg termini que sembla implicar una combinació de mecanismes de sensibilització tant perifèrics (còlon) com a centrals (medul·la espinal lumbosacra). Aquest estat no s'altera pel bloqueig farmacològic dels $R\sigma_1$, tot i que és modulable per morfina o pregabalina. El compost EST73502 va augmentar els llindars de dolor en rates durant la distensió colorectal i va mostrar activitat antihiperalgèsica durant la hipersensibilitat còlica induïda per la inflamació.

En el seu conjunt, aquests resultats suggereixen que els $R\sigma_1$ presenten propietats antiinflamatòries i analgèsiques que fan del mateix una diana factible per al tractament de trastorns gastrointestinals caracteritzats per la presència de signes d'inflamació i alteracions de la sensibilitat visceral.

RESUMEN

El dolor de origen visceral es un hallazgo frecuente en las alteraciones gastrointestinales con un componente inflamatorio, como son la enfermedad inflamatoria intestinal, o las alteraciones funcionales gastrointestinales, como el síndrome de intestino irritable. A pesar de ello, no existen tratamientos efectivos para el dolor visceral. Los receptores sigma de tipo 1 ($R\sigma_1$) son proteínas de membrana reguladas por ligando con actividad neuromoduladora e inmunoduladora. Estos receptores se han implicado en dolor somático de diferentes etiologías, incluyendo el inflamatorio, mediando respuesta pronociceptivas e interaccionando con los efectos analgésicos de los opioides. Esto ha hecho que actualmente los $R\sigma_1$ se consideren una diana para el tratamiento farmacológico del dolor somático.

En este trabajo se evalúan las implicaciones de los $R\sigma_1$ como moduladores de la inflamación intestinal y del dolor visceral asociado a la misma. Para ello, se ha valorado la respuesta inflamatoria intestinal, mediante el modelo de colitis inducida por dextrano sulfato de sodio (DSS), y el desarrollo de hipersensibilidad visceral y somática referida en ratones y en ratas. Para valorar la hipersensibilidad intestinal en ratas se ha puesto a punto un modelo de hipersensibilidad a largo plazo basado en la inducción de colitis con DSS. La implicación de los $R\sigma_1$ se ha valorado mediante su bloqueo genético (ratones carentes de forma constitutiva de dicho receptor) o farmacológico (uso de los antagonistas selectivos BD1063 y E-52862). Se ha estudiado también la interacción entre mecanismos opioides dependientes de receptores de tipo μ y los $R\sigma_1$ mediante la co-administración de morfina y BD13063 o el empleo de la molécula con actividad dual EST3502 (agonista parcial μ-opioide y antagonista $R\sigma_1$). Las sensibilidades somática y visceral se han determinado mediante el test de von Frey o la valoración de respuestas viscerosomáticas asociadas a la

distensión colorrectal. En paralelo, se han determinado cambios en la expresión de marcadores relacionados con la inflamación y el dolor, tanto a nivel periférico (colon) como central (médula espinal lumbosacra) (RT-qPCR/westtern Blot).

Los resultados obtenidos, tanto con el bloqueo genético como con el farmacológico, muestran que los $R\sigma_1$ no modulan la sensibilidad mecánica en condiciones basales. En el ratón, la ausencia constitutiva de $R\sigma_1$ o su bloqueo farmacológico atenuó el desarrollo clínico de la inflamación, tanto aguda como crónica, en el modelo de colitis inducida por DSS, sin alterar los cambios esperados en marcadores inflamatorios (regulación al alza de citoquinas inflamatorias). Durante la colitis, los ratones con $R\sigma_1$ funcionales desarrollaron hipersensibilidad mecánica referida, determinada tanto en la pared abdominal (mezcla de sensibilidad visceral y somática) como en la pata (sensibilidad somática). Por el contrario, la ausencia de $R\sigma_1$ funcionales se asoció a una falta de desarrollo de hipersensibilidad mecánica referida, con respuestas básicamente idénticas a las observadas en animales sin colitis. Estos cambios no se pudieron asociar a una modulación específica de marcadores implicados en vías sensoriales, ni en el colon ni en la médula espinal. En la rata, el modelo de colitis inducida por DSS resulta en un estado de hipersensibilidad a largo plazo que parece implicar una combinación de mecanismos de sensibilización tanto periféricos (colon) como centrales (médula espinal lumbosacra). Este estado no se ve afectado por el bloqueo farmacológico de los $R\sigma_1$, mientras que es modulable por morfina o pregabalina. El compuesto EST73502 aumentó los umbrales de dolor en ratas durante la distensión colorrectal y mostró actividad antihiperalgésica durante la hipersensibilidad cólica inducida por la inflamación.

En su conjunto, estos resultados sugieren que los $R\sigma_1$ presentan propiedades antiinflamatorias y analgésicas que hacen del mismo una diana factible para el tratamiento de trastornos gastrointestinales caracterizados por la presencia de signos de inflamación y alteraciones de la sensibilidad visceral.

INTRODUCTION

1. THE GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract is a continuous semipermeable tubular structure with a primary purpose of breaking down ingested nutrients for absorption and metabolic use. These functions are accomplished through a series of organs, distributed from mouth to anus, with distinct roles. The largest region of the GI tract is the intestinal region and it is composed of the small (duodenum, jejunum and ileum) and the large intestine (cecum, colon, rectum and anal canal). Primary functions of the small intestine are digestion and absorption of nutrients. The large intestine is the last portion of the digestive tract, and is primarily concerned with water balance and waste compaction and excretion, with storage in the sigmoid colon and rectum prior to elimination.[1,2]

1.1. Structural organization

The different organs of the GI tract have different functions but a common structure. The esophagus, stomach, and intestine, either small or large, have a central luminal space with a wall organized in, mainly, four distinct concentric tissue layers (Fig. 1). From the innermost (luminal) to the outermost these layers are: the mucosa, the submucosa, the muscularis externa, and the adventitia or serosa (Fig. 1).[1,3]

- The mucosa includes the lining epithelium, lamina propria and a thin layer of smooth muscle, the muscularis mucosa. The mucosa of the large intestine consists of columnar epithelial cells, that allow the absorption of fluids and electrolytes, and mucus-secreting goblet cells, that offer lubrication to the mucosa. The lamina propria is composed mainly of connective tissue, in

addition to blood and lymphatic vessels. Moreover, it is infiltrated with lymphocytes and lymph nodes that protect the wall of the gastrointestinal tract from potentially harmful luminal contents, including the resident microbiota and ingested pathogens. The muscularis mucosa is a thin layer of smooth muscle that is the boundary between the mucosa and the submucosa.

- The submucosa is composed of a dense collagen stroma that supports the mucosa. It contains large blood and lymphatic vessels, and a nerve network known as the submucosal plexus.
- The muscularis externa has two layers of smooth muscle: an inner circular layer and an outer longitudinal layer. A nerve network, known as the myenteric plexus, lies between these two layers of smooth muscle.
- The adventitia or serosa is a thin layer of fibrovascular tissue. It is the outermost layer of the intestinal wall. The serosa is the site of entrance/exit of the extrinsic innervation and the vasculature in the GI tract.

All these layers present small structural and functional differences depending on the organ considered.[1,3]

The innervation of the GI tract has two main components: the extrinsic innervation and the intrinsic innervation. The extrinsic innervation depends upon de classical autonomic nervous system (sympathetic and parasympathetic -vagal innervation-). The intrinsic innervation is represented by the so-called enteric nervous system (ENS). The ENS is a component of the neural control system of the digestive tract, providing the basis for the local reflexes that control and coordinate GI functions: motility, secretion, absorption, blood supply and interaction with the immune and endocrine systems. Complex networks of intrinsic afferent

sensory neurons, interneurons and efferent motor and secretomotor neurons form the ENS. Moreover, the ENS is organized by two nervous plexuses that run along the entire length of the digestive tract (Fig. 1):[4,5]

- The submucosal plexus (Meissner) is localized within the submucosa. Its main function is the control of vascular and secretory reflexes within the epithelium.
- The myenteric plexus (Auerbach) is localized between the two layers of smooth muscle that form the muscularis externa. This network participates in the control of the motor patterns generated by the smooth muscle.

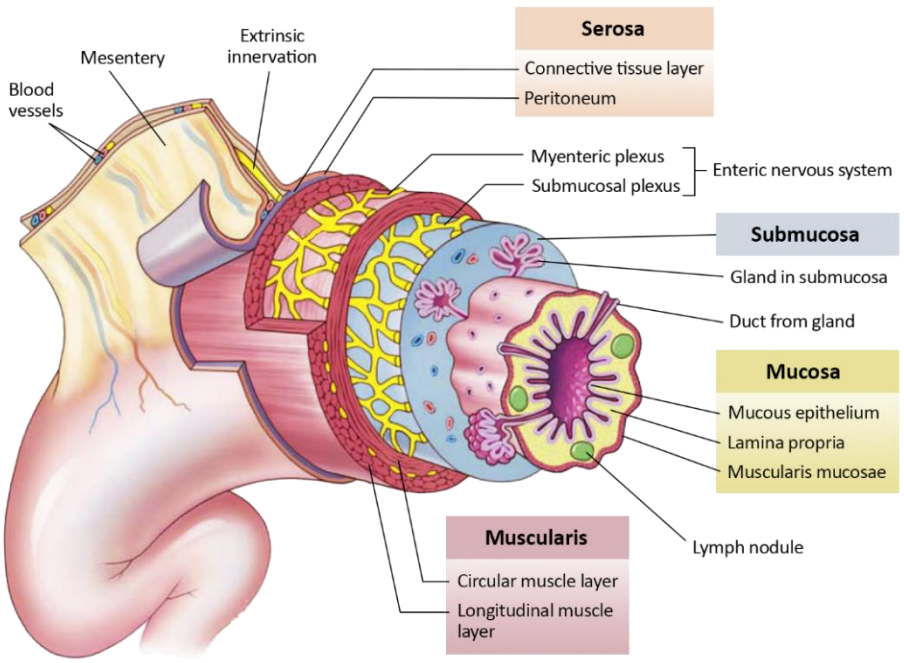


Figure 1. Diagram of the histological organization of the GI tract. Notice the organization of the enteric nervous system in two distinct plexuses: the submucosal plexus and the myenteric plexus. These plexuses are identified as discrete groups of neurons (enteric ganglia) and interganglionic connecting fibers. Adapted from Reed and Wickham (2009).[3]

1.2. Physiology

The functions of the GI tract differ according to the organ considered. Unlike the upper GI tract, the main functions of the large intestine are absorption of water and solidification of contents in feces, net forward propulsion of intestinal contents at an adequate rate, and temporary fecal storage by the descending colon prior to the eventual expulsion. In addition, the GI tract of mammals is home to a large community of microorganism that provide a variety of benefits to the host. Within the GI tract, the large intestine is the great reservoir of microorganisms and is where they reach their highest density.[6]

The intestinal epithelium capacity to function as a barrier between the external and internal environments is also essential for human health.[2,8] Accordingly, the colonic mucosa establishes a semi-permeable dynamic barrier that supports the active and passive transport of substances and excludes the entry of potentially harmful elements, a process strictly regulated by epithelial and immune components. The layer generated by epithelial cells united by tight junction complexes (intricate structures formed by the aggregation of several proteins, including claudins, occludins, zonula occludens and junction adhesion molecules) provides a physical barrier. This structure works in conjunction with stromal and immune cells to recognize pathogens and other luminal antigens and limit their direct contact with the epithelium (Fig. 2). In addition, the continuous and high turnover of intestinal epithelial cells helps to prevent the adhesion of pathogens to the intestinal wall and the abnormal colonization of the intestine.[7,9]

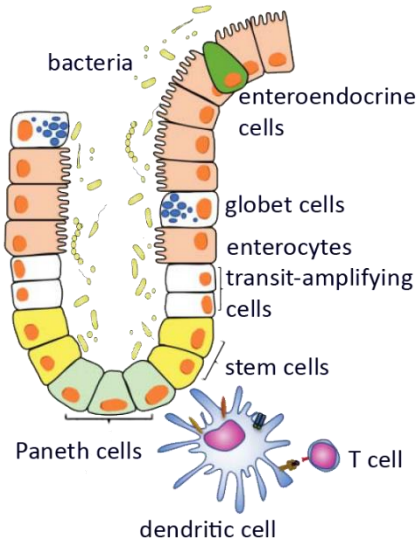


Figure 2. Schematic representation of the intestinal epithelium. The epithelium of a crypt and part of a villus are represented. Different epithelial cells can be identified: i) enterocytes, tall columnar absorptive cells with a 'brush-like border' on the apical surface, called microvilli; ii) goblet cells, which secrete mucin for lubrication of the intestinal contents and protection of the epithelium; iii) enteroendocrine cells that secrete different gut regulatory peptides; iv) stem cells that lie near the base of the crypt, allow renewal and give rise to the specialized epithelial cells; v) transit amplifying cells, located above stem cells; and vi) Paneth cells, which have a defensive function secreting antimicrobial molecules into the lumen The intestinal epithelium is tightly related with resident elements of the immune system, such as dendritic cells and T cells. Adapted from Takiishi et al. (2017).[7]

1.3. Functional and inflammatory gastrointestinal disorders

Clinical studies have shown that intestinal barrier function can be modulated by multiple factors, including diet, stress, infections, genetic predispositions or drug intake. These factors can lead to an increase in intestinal permeability, through mechanisms associated mainly, but not

exclusively, to the dysregulation of the expression of adhesion molecules.[7–9] The breakdown of the intestinal barrier leads to an induction of epithelial damage and the activation of intestinal and extraintestinal immune responses. In fact, a strong link has been established between abnormal intestinal permeability and several GI human diseases, such as inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS).[8,10]

1.3.1. Functional gastrointestinal disorders: irritable bowel syndrome

GI alterations affecting the mucosa, musculature, and innervation are common and manifest as ulceration, inflammation, obstruction, diarrhea, bloating, fullness, nausea, vomiting, and abdominal pain. Organic pathologies, such as GI cancers, IBD, celiac disease, peptic ulcer, and motility disorders, course with these symptoms. However, there are patients in whom no underlying structural abnormalities have been revealed to explain these symptoms. These pathologies are added within the group known as functional gastrointestinal disorders (FGD), with IBS, functional dyspepsia and functional constipation being the main representatives. FGD are still incompletely understood due to their heterogeneity and complex pathophysiology.[11] Among FGD, IBS represents the pathology with highest prevalence. IBS has recently been associated with states of systemic inflammation and altered microbiome diversity, which in turn perpetuates a cycle of chronic, low-grade, subclinical inflammation within the GI tract.

Apart from mucosal inflammation, neuroinflammation is probably involved in the pathophysiology of IBS via the gut-brain axis. Accordingly, FGD are recently more appropriately referred to as disorders of the gut-brain axis. A generally accepted hypothesis is that dysfunction of

bidirectional communication between the brain and the gut in response to several conditions, such as chronic stress, activates the hypothalamic-pituitary axis and the autonomic nervous system and plays a role in the symptomatology.[11,12] Furthermore, genetic factors, infections and alterations in the intestinal microbiota, low-grade mucosal inflammation, immune activation or alteration of intestinal permeability can also affect the pathophysiology of FGD, particularly as it relates to IBS.[13]

1.3.2. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a group of inflammatory disorders affecting the GI tract. IBD occurs worldwide but its incidence and prevalence vary widely among geographic regions. A recent retrospective study carried out by Lancet in 195 countries during the years 1990-2017 estimates that currently more than 3 million people in the USA and Europe have IBD, and its prevalence is estimated to exceed 0.3% in North America, Oceania, and many countries in Europe.[14] These data might indicate that there are common environmental pressures across these regions that act as important risk factors for IBD. Actually, this disease is believed to develop as a result of interactions between environmental, microbial, and immune-mediated factors in genetically susceptible individuals (Fig. 3).[15–17]

IBD is characterized by a chronic idiopathic inflammation of the intestine, with two main forms: ulcerative colitis (UC) and Crohn's disease (CD).[18,19] Differential epidemiological, histopathological and immunological characteristics allow the differentiation between UC and CD (Table 1).

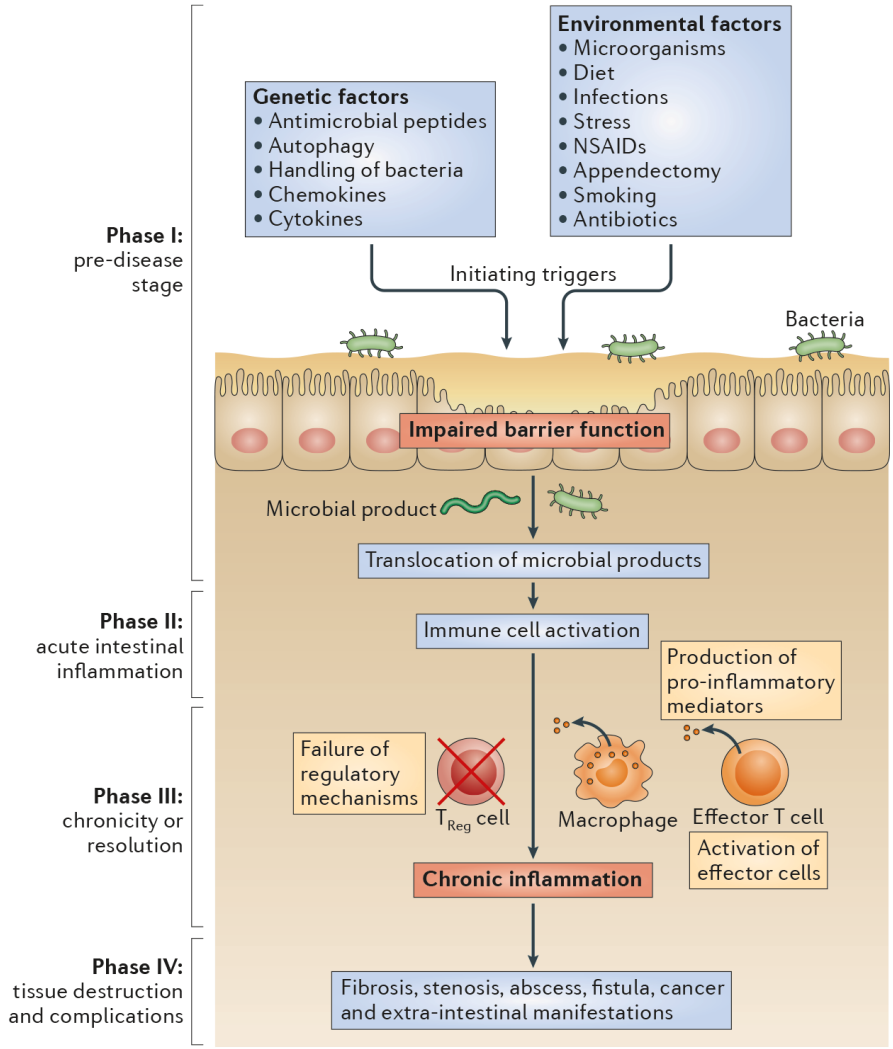


Figure 3. Conceptual framework for the pathogenesis of IBD. Genetic and environmental factors induce impaired barrier function in the intestinal mucosa. Altered barrier function subsequently induces the translocation of commensal bacteria and microbial products which leads to immune cell activation and cytokine production. If acute mucosal inflammation cannot be resolved by anti-inflammatory mechanisms and the suppression of pro-inflammatory immune responses, chronic intestinal inflammation develops. Similar mechanisms might operate during IBS. However, in this case, a state of low immune activation and low inflammation will result. NSAIDs: non-steroidal anti-inflammatory drugs; T_{Reg}cell: regulatory T cell. From Neurath (2014).[20]

Table 1. Epidemiological, histopathological and immunological features of Crohn's disease and ulcerative colitis.

	Crohn's Disease (CD)	Ulcerative Colitis (UC)
Incidence	More common in women than men 3.1 to 20.2 cases per 100,000 individuals per year	Equal rates in men and women 2.2 to 19.2 cases per 100,000 individuals per year
Prevalence	Higher prevalence than UC in developed countries	More prevalent in still-developing countries
Tissue distribution	Entire GI tract Patchy discontinuous inflammation	Colon, with potential backwash ileitis Continuous inflammation in the affected area
Histopathology	Transmural inflammation Thickened wall with granulomas, fissures and fibrosis	Inflammation restricted to mucosa and submucosa Altered crypt architecture, erosions and ulcers, loss of goblet cells
Inflammatory response	All immune cell types involved. High infiltration on lymphocytes and macrophages	Predominance of lymphocytes and neutrophils
Immunologic pattern	Th1/Th17: INF-γ, IL-17, TNFα, IL-6, IL-1β, IL-22	Th2/Th17: IL-5, LI-13, TNFα, IL-6, IL-1β, IL-17

From: Mayer (2010);[21] Khor et al. (2011);[22] Neurath (2014);[20] de Lange and Barrett (2015).[18]

1.4. Colonic inflammation

States of colonic inflammation, such as during IBD, or a low degree of intestinal inflammation, such as during IBS, involve structural changes and an immune response with common characteristics, as well as functional alterations.

Histopathological alterations

In the colon, inflammation is associated to the presence of histopathological abnormalities on the epithelial surface that could affect the architecture of the villi and crypts. Epithelial abnormalities include superficial epithelial damage, focal cell loss, erosions, alteration of mucosal components, and ulcers that reflect disease activity. The inflammatory infiltrate in the lamina propria is also characteristic of the presence of inflammation. The presence of neutrophils, above the ulcers, is considered the hallmark to differentiate an active inflammatory state from a remission or resting state.[23,24]

Inflammatory response

The GI tract has a complex immune system that is able to distinguish those innocuous and necessary elements of the host's luminal content from pathogens and potentially noxious components. Maintaining the balance between tolerance and activation of the immune system is a key element in intestinal health and it is disrupted during intestinal inflammation. In diseases with a significant inflammatory response, such are IBD and IBS, the recruitment of immune cells and the release of different pro-inflammatory cytokines promote the inflammation of the colon (Fig. 3). Overall, the main pro-inflammatory cytokines involved are interleukins-(IL-) 1β, 6, 12 and interferon-(IFN-) γ. Furthermore, the anti-inflammatory cytokine IL-10 also plays an important role in this process.[2,7,25]

- IL-1β is a pleiotropic cytokine produced by neutrophils, macrophages and dendritic cells. Its function is critical for the pathology of several chronic conditions. An increased activation of the IL-1 system in IBD has been described by several authors. Moreover, high levels of IL-1β correlated well with disease

activity and with the presence of active lesions in IBD tissue. However, IL-1β has a prominent role in the initiation, rather than in the perpetuation, of colonic inflammation.[20,26]

- IL-6 is synthesized by macrophages, fibroblasts, and T cells. IL-6 has essential immunoregulatory functions, and its over-production during experimental colitis and in patients with IBD suggests a key role in the development of intestinal inflammation. By one hand, it contributes significantly to the pathogenesis of the disease since it can exert pro-inflammatory functions by activating multiple target cells, including antigen-presenting cells and T cells. In addition, it prevents programmed cell death (apoptosis) of mucosal T cells and activates the production of pro-inflammatory cytokines. On the other hand, it also induces the proliferation of epithelial cells, therefore having a potential protective/anti-inflammatory role.[20,27]
- INF-γ is a multifunctional cytokine secreted by T cells and innate lymphoid cells. Its expression is increased in CD but not in UC. INF-γ has a high macrophage activation capacity, leading to intracellular pathogen clearance, and is typically classified as a pro-inflammatory cytokine. Furthermore, it also antagonizes the expression of suppressor cytokines, such as IL-10. Moreover, INF-γ alters tight junctions and induces apoptosis of intestinal epithelial cells. The loss of epithelial barrier function caused by IFN-γ could exaggerate intestinal diseases.[20,28,29]
- Members of the IL-12 family are heterodimeric cytokines (among which are IL-12, IL-23, IL-27, or IL-35) produced by macrophages and dendritic cells during intestinal inflammation. IL-12 has long been suggested to be involved in the pathogenesis of CD by triggering T-cell responses. Similarly, IL-23 (composed of the IL-

12p40 subunit and a p19 subunit) suppresses regulatory T cells in CD patients.[20,30,31]

- IL-10 is considered as the prototypical anti-inflammatory cytokine. IL-10 suppresses the production of pro-inflammatory cytokines by antigen-presenting cells and T cells, and induces Treg (regulatory) cells. Treg cells are crucially involved in maintaining intestinal mucosal homeostasis by suppressing abnormal immune responses against commensal flora or dietary antigens, and prevention of both activation and effector function of T cells that escaped other tolerance mechanisms. Both T cells and Treg cells are themselves important producers and targets of IL-10.[20,31]

In addition to these, other cytokines, such as TNFα, IL-17, IL-18 or IL-22, have been implicated in the pathogenesis of intestinal inflammation with different activities depending upon the location and type of inflammation. Overall, this heterogeneity highlight the complexity of the mucosal cytokine network and make it difficult to study adaptive immune responses in the pathogenesis of colonic inflammation.

2. PAIN

Since 1979, The International Association for the Study of Pain (IASP) defines pain as: "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage."[32] Indeed, pain is a complex entity formed by sensory, affective, motivational and cognitive dimensions, with individual connotations and influenced by previous experiences. Pain is a complex

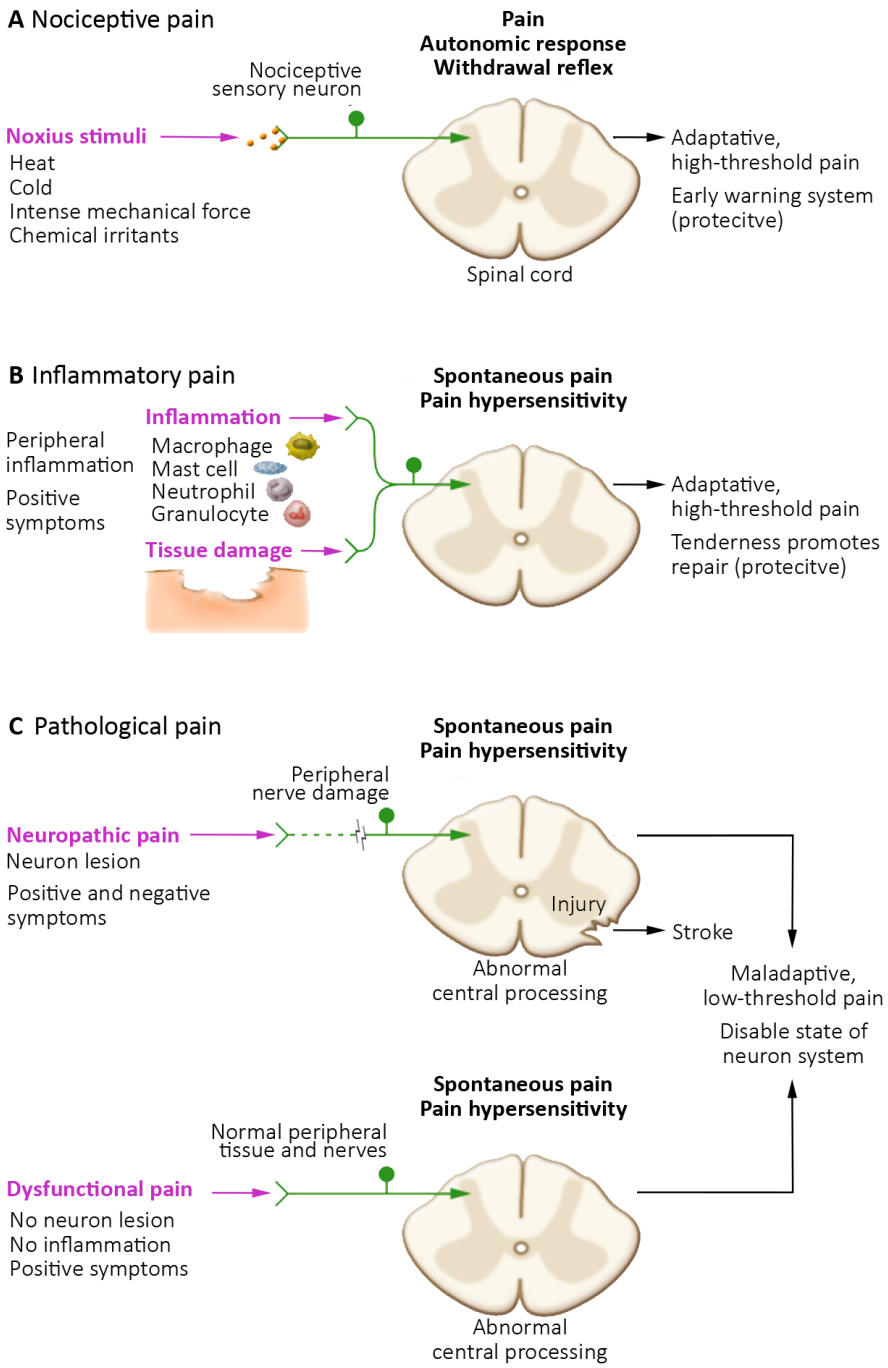
protection and alarm mechanism that is activated against actual or potential tissue damage, by allowing withdrawal from and avoidance of noxious stimuli, thus making possible the body to protect itself.

Traditionally, four components of pain have been determined:[33]

1. Nociception: the detection of tissue damage by specialized receptors.
2. Pain perception: the cognitive process that is triggered by a harmful stimulus, such as an injury or illness. However, pain can also occur without nociception, without an injury present.
3. Suffering: a negative and subjective response induced by pain in which psychological factors such as fear, anxiety or stress also intervene.
4. Pain-related behaviors: the behavioral repertoire associated to pain and suffering.

Despite its components, pain can be broadly divided into three classes according to the pathophysiological mechanisms involved (Fig. 4): nociceptive, inflammatory and pathological pain.[34,35] In all cases, a similar sensation is generated but the cause is different. Nociceptive pain is an early warning physiological protective system, a high threshold pain that is only activated in the presence of intense noxious stimuli; its protective function requires immediate attention and action. Inflammatory pain assists in the healing of the injured body part by creating a situation that discourages physical contact and movement after unavoidable tissue damage. This pain is caused by the activation of the immune system and is one of the main characteristics of inflammation. Finally, pathological pain does not correspond to any disorder but is related to the reduction of the receptor thresholds due to alterations of the nervous system; it can occur

after damage to the nervous system (neuropathic pain), but also in conditions without damage or inflammation (dysfunctional pain).



Moreover, the presence of pain over time can vary. The pain can be acute if it is limited in time and disappears with the resolution of the original damage or pathological process. In contrast, the pain sensation can persist over time, although the resolution of the originating process, leading to a state of chronic pain. Chronic pain may arise from an underlying irreversible disease or injury, but frequently is a relapsing, remitting and maladaptive condition characterized by sensitization to nociceptors in the absence of tissue damage (or after the resolution of the original damage).[36,37]

According to its anatomical origin, pain is divided also in two subcategories: somatic and visceral. Somatic pain occurs when pain receptors in somatic structures (including the skin, muscles, skeleton, joints, and connective tissues) are activated. On the other hand, visceral pain arises from, in, or around internal organs, mostly hollow viscera.

2.1. Physiology of visceral pain

A large number of patients with inflammatory and FGD present abdominal pain as the main reason for requiring medical attention. Indeed, the presence of visceral (abdominal) pain is considered a hallmark of FGD and IBD.

Figure 4. Pain classification. (A) Nociceptive pain represents the sensation associated with the detection of potentially damaging noxious stimuli and has a protective role. (B) Inflammatory pain is associated with tissue damage and immune response and generates pain hypersensitivity as an adaptive measure. (C) Pathological pain is a disease state caused by damage to the nervous system (neuropathic) or by its abnormal function (dysfunctional). From Woolf (2010).[34]

Besides differences in their anatomical origin, visceral and somatic pain also differ in the neurobiological mechanisms that mediate the sensory process.

Five clinical features of visceral pain have been identified:[38–40]

- Not all viscera evoke visceral pain. Actually, visceral pain often comes from hollow visceral organs, such as the urinary bladder or the intestine.
- It is not linked to actual visceral injury
- It is characterized by a vague and diffuse location.
- It is accompanied by motor and autonomic reflexes such as nausea and vomiting or peristalsis.
- It is able to generate referred pain in other locations, often remote somatic areas.

Unlike other types of pain, such as skin pain, the perception of visceral pain, and particularly that originating within the GI tract, is significantly limited. This is due to the unique thermal, chemical, biological, and mechanical environments within the GI tract. The temperature in the GI tract usually remains constant and variations in it do not evoke visceral pain. Similarly, regular exposure to chemicals, such as bile, fatty acids or peptides, generates certain tolerance in concrete areas where its presence is habitual, such us the stomach or the small intestine. Hence, chemicals or acids recognized for their ability to generate somatic pain may be less efficient in triggering visceral pain. Conversely, mechanical stimuli, such as deformation, distension or torsion, are highly effective stimuli generating intestinal pain.[39,41–43]

2.1.1. Neuroanatomical basis of gastrointestinal nociception

Here, we will focus on visceral pain that arises from the colon and rectum (colorectum).

Sensory innervation of the GI tract depends on both the enteric nervous system (ENS, see section *1.1 Structural Organization*) and extrinsic pathways (which can be of vagal or spinal origin) (Fig. 5).

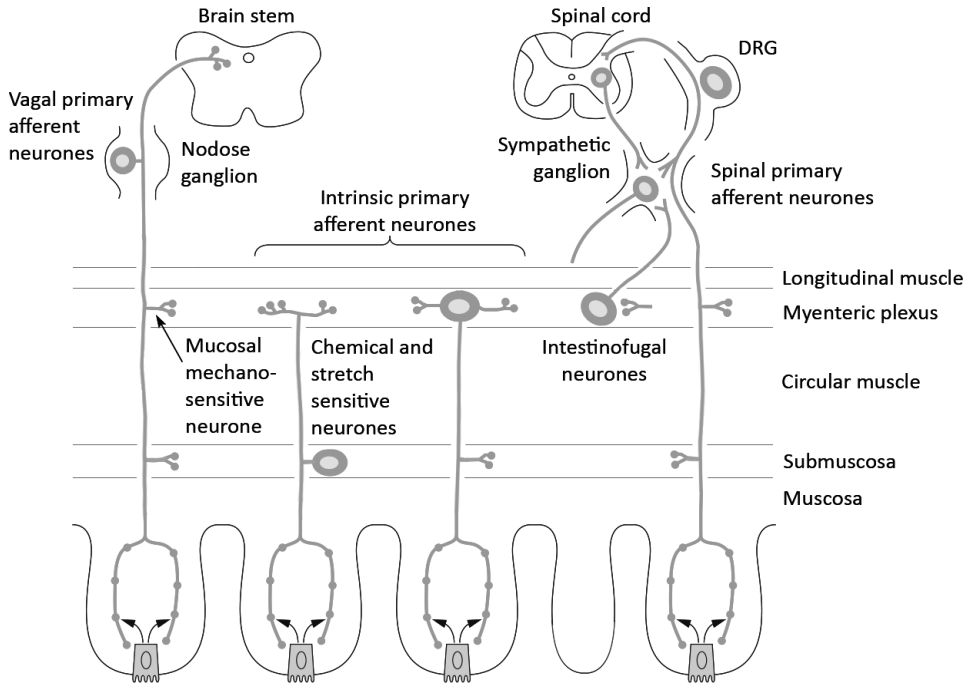


Figure 5. Schematic representation of the organization of the primary afferent neurons within the intestine and their relationship with the enteric nervous system and the extrinsic innervation of the GI tract. DRG: dorsal root ganglion. Adapted from Grundy (2002).[44]

Intrinsic innervation

Along with the rest of the GI tract, the ENS is also housed in the colon and rectum. The set of intrinsic sensory afferent neurons that are part of the ENS, do not project beyond the intestinal wall, so their activation *per se* is not enough to contribute to the conscious visceral sensations involved in visceral pain. For this reason, and to avoid confusion, sensory neurons in the ENS are known to as intrinsic primary afferent neurons rather than intrinsic "sensory" neurons.[4,5,45] Because of this functional organization, the intrinsic innervation of the GI tract plays a minor role in nociceptive processes.

Extrinsic innervation

The extrinsic nervous system, through vagal and spinal afferent signaling, allows the central nervous system (CNS) to consciously or unconsciously perceive different stimuli from the colon and rectum. Extrinsic sensory afferents take care of colorectal mechanotransduction to inform the CNS of mechanical stimuli within this section of the GI tract and drive conscious perceptions arising from this area, including discomfort and pain.

Vagal afferent endings are primarily concentrated in the upper GI tract (particularly, in the stomach and esophagus) and are sparse in the colon.[46] These sensory fibers are mainly unmyelinated C fibers, the axons of which project directly into the brainstem and their cell bodies are found in the nodose or the jugular ganglia. The terminals of the vagal afferents establish elaborate structures within the gastrointestinal wall, both in the circular and longitudinal muscle layers, and in the lamina propria. Vagal afferents have low activation thresholds and reach maximum responses within physiological levels, so they can evoke graded and innocuous sensations. For this reason, its predominant function is the conduction of physiological

sensory information associated with upper gastrointestinal sensations, such as satiety, nausea, and vomiting, with a minor role in the direct generation of reflex responses or pain-related sensitivity.[44,46,47] Indeed, the signaling of sensation of pain to the CNS by the activation of vagal afferences is uncertain. However, they may have a secondary role in the central inhibitory modulation of pain, activating regions of the brainstem that have a role in the descending modulation of pain.[48]

Spinal afferents project within the splanchnic nerves to the spinal cord. They represent 10-20% of the nerve fibers of the splanchnic nerves and are found in all layers of the intestinal wall. Combined tracer and electrophysiologic studies have described these fibers as the main sources of visceral nociception. Their endings in the mucosa participate in chemonociception, but they are also mechanosensitive. They can be classified into three groups according to their neurophysiological properties:[44–46]

1. Tonic mechanoreceptors. They have a tonic level of resting activity and are activated with low thresholds of distension. Its activation generates both physiological and painful sensations.
2. High threshold mechanoreceptors. They respond only to high intensity mechanical stimuli and, therefore, are considered mechanonociceptors. They can also be activated through inflammatory or ischemic mediators, in response to tissue damage.
3. Silent nociceptors. They are only activated in response to inflammatory mediators through a process of sensitization, mediating nociceptive responses.

Despite this classification, most visceral primary afferents are polymodal.[48]

Spinal afferents are unmyelinated C fibers and finely myelinated Aδ fibers. Their cell bodies are contained within a wide range of dorsal root ganglia (DRGs) with various distributions for different organs. The proportion of cell bodies assigned to viscera is small and this fact generates a sensation of poor localization of the pain, one of the main characteristics of visceral pain. The central projections of the spinal afferent neurons enter into spinal cord and establish a synaptic connection with second-order neurons, which distribute visceral information through the central neural structures. Finally, ascending spinal pathways project to the thalamic nuclei where the affective, emotional, and cognitive components of pain are generated. Prior to this, a viscerosomatic convergence of the cell bodies of spinal afferent fibers occurs at the dorsal horn level of the spinal cord. This convergence in the spinal cord is responsible for the phenomenon of referred pain, another of the main differential characteristic of visceral pain.[44,45,47]

2.1.2. Molecular basis of gastrointestinal nociception

Spinal visceral afferents, the main source of GI nociception, have a basic morphology and an ontogeny similar to somatic neurons. This suggests that the molecular events involved in the activation and signal transduction of neurons that are part of visceral nociception are not very different from their somatic counterparts. In general, peripheral terminals of nociceptors generate potentials by opening voltage-gated sodium channels (Na_V) in response to a depolarizing stimulus determined by an initial receptor-mediated effect. These potentials are terminated by a combination of temporary and voltage-dependent inactivation of these channels and the opening of a voltage-sensitive outward potassium line. Voltage-gated sodium channels are essential for the propagation of action potentials and the regulation of cellular excitability. Among the nine mammalian sodium

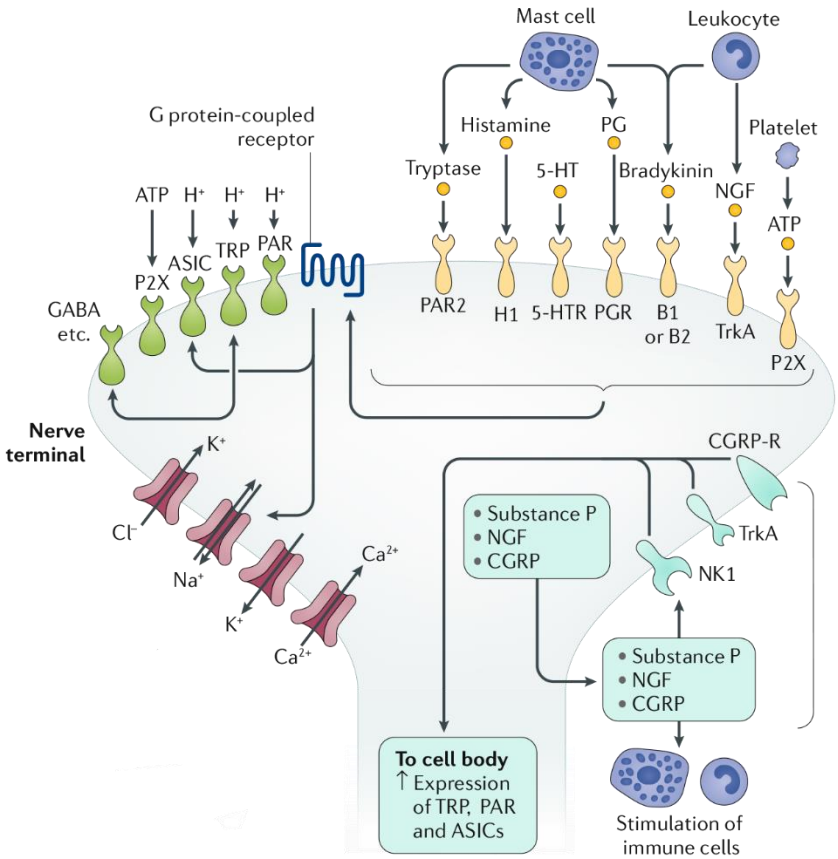
channels, $Na_V1.1$-$Na_V1.9$, $Na_V1.7$ are believed to be the ones that have a greater impact in mechanonociceptive responses associated with visceral sensitivity.[4,45,49]

In addition, several ion channels implicated in nociceptive mechanisms have been identified in sensory afferents arising from the GI tract. The most important group is the transient receptor potential channels (TRP). This family comprises more than 30 highly conserved structurally-related ion channels that serve various sensory functions. There is robust experimental evidence to suggest that TRPV1, TRPV4 and TRPA1 have roles, to varying degrees, in GI chemo-, thermo- and mechanonociception. Specially related to the GI tract, many of these channels can be activated with a variety of potentially harmful food products.[45,50,51] Furthermore, acid-sensing ion channels (ASIC 1–3)[45,50,52] and $P2_X$ purinoreceptors ($P2_X$ 1–9)[4,45,46,53] have also a direct role in transduction of GI mechanosensation and chemoception.

Central endings of the GI tract nociceptive pathways employ glutamate and others neuropeptides, such as substance P, serotonin (5-HT), and neurotrophic factors, as neurotransmitters and synaptic modulators. These mediators are particularly important in the transmission of information from the viscera. As in the somatic nervous system, neurotransmitters release depends on voltage-gated calcium channels located in the membrane of nerve endings. Receptors for these mediators (N-methyl-d-aspartic acid -NMDA-, AMPA, and receptors for tachykinins and 5-HT) have been located in postsynaptic neurons of the spinal cord (second-order neurons), and numerous pharmacological studies have demonstrated their importance in pain signaling.[45,54]

2.2. Pain sensitization in inflammatory pathologies

As previously mentioned, several GI tract pathologies evoke visceral pain.[4,55,56] Recruited immune cells induced by tissue injury or loss of the epithelial barrier, among other causes, lead to the secretion of different inflammatory mediators that have substantial effects on enteric and extrinsic afferent neuronal function through complex neuro-immune interactions. Among other changes, this generates alterations in visceral sensory perception and abdominal pain due to both peripheral and central sensitization processes.



2.2.1. Peripheral sensitization

In afferent neurons, a local sensitization process occurs (generally in cases of injury or prolonged inflammation) due to persistent changes in the chemical environment that allow nociceptors activation at thresholds lower than the usual range (hypersensitivity). This primary hypersensitivity occurs at the site of injury (peripheral tissues, i.e. GI tract) and is the consequence of a greater presence of nociceptors sensitized by the original stimulus.[36,38]

In general, inflammatory mediators (such as bradykinin, histamine, 5-HT, tryptase, prostaglandin (PG) E, and ATP) are able to bind to specific receptors at neuronal termini and lower the neuronal activation threshold through the activation of G protein-coupled receptors (GPCRs). GPCRs activation leads to phosphorylation of ion channels and transducers,

Figure 6. Potential mechanism driving sensitization in peripheral sensory nerve endings. Ischaemia, inflammation and tissue damage can lead to release of mediators such as bradykinin, histamine, serotonin (5-HT), tryptase, prostaglandin (PG) E and ATP. These molecules can bind to specific receptors at neuronal endings and reduce the threshold for neuronal activation. Activation of G protein-coupled receptors (GPCRs) lead to the phosphorylation of ion channels and transducer channels, thus enhancing their activity. Moreover, chemokines, cytokines and neuropeptides, such as substance P, calcitonin gene-related peptide (CGRP) and nerve growth factor (NGF) contribute to this process. In addition, receptor activation leads to increased gene transcription and up-regulation of other receptors such as transient receptor potential (TRP), protease-activated receptors (PAR) and acid-sensing ion channels (ASICs), as well as neuronal production and release of more neuropeptides, which can stimulate the immune cells in a positive feedback loop. 5-HTR: 5-HT receptor; B1 and B2: bradykinin receptors; GABA: γ-aminobutyric acid; H1: histamine receptor 1; NK1: neurokinin 1; P2X: purinoceptor; PGR: prostaglandin receptor; TK1: tachykinin receptor 1; TrkA: tropomyosin receptor kinase A. Adapted from Drewes et al. (2020).[48]

rendering them even more sensitive to external activation. Moreover, mast cells, leukocytes, and platelets interact with visceral afferents also through chemokines, cytokines, and neuropeptides, such as substance P, calcitonin gene-related peptide (CGRP), and nerve growth factor (NGF). Activation of these receptors induces an up-regulation of TRP channels, protease-activated receptors (PAR), and ASIC channels. All together these changes result in increased sensitivity of nerve fibers, a process known as peripheral sensitization (Fig. 6).[4,45,48,57]

It is worth mentioning that in some GI tract afferents, GPCRs associated with somatostatin, opioids and cannabinoids have been identified as players that modulate sensitization by inhibition, thus evoking compensatory anti-nociceptive responses.[45]

2.2.2. Central sensitization

Increased discharge of sensory afferents within the CNS can lead to central sensitization, a phenomenon similar as that described above in the periphery. The process of central sensitization implies neuroplastic changes, including neuronal excitation and opening of latent pathways that do not normally mediate pain, leading to states of hypersensitivity characterized by the amplification of responses to both noxious (hyperalgesia) and innocuous (allodynia) stimuli.

During sensitization, stimulation of afferent nociceptive pathways triggers a greater transmitter release response in nerve terminals through a process that depends on voltage-gated calcium channels. In addition to glutamate, characteristic of somatic pain, several neuropeptides (such as substance P, 5-HT and neurotrophic factors) function as neurotransmitters and synaptic modulators of the pathways originated in the GI tract. Existent evidences suggest that other mediators such as NMDA, AMPA, and

tachykinins could play an important role in the transmission of visceral pain, although it has not yet been demonstrated. The repeated action of these mediators on second order neurons increases their activity, generating a state of central sensitization. Viscerosomatic convergence process takes place at this point and hypersensitivity responses appear in both somatic and visceral areas far from the point where the signal originated (referred pain).[45,54]

2.2.3. Modulatory influences on GI nociception

Visceral pain can be modulated by extra-nociceptive neuronal and non-neuronal influences. Both stress and psychologic factors, such as affective co-morbidities, have important roles in chronic visceral pain conditions. Responses to stress are modulated through an effector system called the "emotional motor system", whose main output components are the descending spinal pathways, the autonomic nervous system and the hypothalamic-pituitary axis:[45]

- Descending pathways of the supraspinal centers can inhibit or facilitate pain signals by selectively modulating nociceptive transmission to the terminals of primary afferent nociceptors and dorsal horn neurons that respond to noxious stimulation.[58,59]
- The autonomic nervous system integrates afferent information from the periphery and central signals related to emotional processes to generate homeostatic responses. Several studies show that the sympathetic system has pro-nociceptive actions while the parasympathetic (vagus) would have anti-nociceptive effects. Mechanisms involved in these effects are not fully understood.[54]

- Animal studies have shown that responsiveness of the hypothalamic-pituitary axis can be altered by adverse early life events, thus altering the individual ability to adapt and respond to the negative effects of stress in later life.[45,60]

2.3. Current treatment strategies

So far, gastrointestinal pain lacks a specific treatment and the same medications recommended for other types of pain are used in the clinical setting. The first treatment option for visceral pain is to act directly on the source producing the nociceptive response[61]. In any case, the more frequently used drugs to manage visceral pain are nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids.[4,49,55,62]

NSAIDs mechanism of action is based on the inhibition of prostaglandin production by cyclooxygenase (COX) enzymes to achieve an analgesic and anti-inflammatory effect. However, the use of NSAIDs in cases of IBD is not recommended due to the risk of NSAIDs of producing mucosal lesions as side effect associated with their long-term use.[49,55]

Opioids offer an effective alternative for the treatment of visceral pain,[62,63] even though their prolonged use may have the opposite effect in some patients: development of analgesic tolerance and nociceptive sensitization (opioid-induced hyperalgesia).[49,55,62] Opioids have an effect on the whole CNS and ENS. Activation of opioid receptors induces inhibition of adenylyl cyclase, downstream inhibition of CaV2.2 (voltage-gated Ca^{2+}) channels, and activation of inward-rectifying potassium channels, resulting in decreased neuronal excitability. In the GI tract, intestinal motility, and sphincter tone can be affected as well, leading to constipation, luminal stretch, nausea and vomiting. The combination of these factors leads to

opioid-induced intestinal dysfunction syndrome, which often has a negative impact on visceral pain *per se*.[64,65]

In addition, other targets for the treatment of visceral pain have been explored, such us blockers of NMDA receptors, the endocannabinoid system[66] or the use of the hormone oxytocin[4]. Special mention deserves the γ-aminobutyric acid (GABA) analogues pregabalin and gabapentin.[55,62] These GABAergic agents are α2δ ligands, an auxiliary subunit of voltage gated calcium channels, that reduce depolarization-induced calcium influx at nerve terminals, thus inhibiting the release of several excitatory neurotransmitters involved in pain mechanisms.[67] Pregabalin has shown analgesic effects on visceral pain in animal models[68] and recent clinical trials have shown that it may be beneficial for IBS abdominal pain, bloating and diarrhea.[69]

New approaches based on microbiota[70] or in epigenetics[71,72] are currently under study for the management of GI pain and might be clinical options in the future. However, the results are still inconclusive.

3. SIGMA RECEPTORS

Sigma receptors (σRs) are protein cell membrane receptors identified in the 1970s as a subgroup within the opioid receptors, due to the cross-reactivity of some ligands.[73] However, subsequent studies refuted this hypothesis by showing that σRs were not antagonized by neither naloxone nor naltrexone, classic opioid receptor antagonists.[74,75] Later, it was concluded that the binding sites for the σR and NMDA were identical,[76] although this hypothesis was also rejected. Nowadays, σRs are considered as a receptor class on their own.

Based on the pharmacological profile, function and molecular weight, two subtypes of σRs have been identified: σ_1R and σ_2R.[77,78] Both subtypes can co-localize although they are present in different proportions,[79] and are found in the nervous system and also in high density in many other tissues. Therefore, it is likely that σRs have important functions beyond the classically assigned role in neurotransmission.[80] The σ_2R has been identified as the progesterone receptor membrane component 1 and it has been involved in different cellular processes, such as regulation of cell proliferation and maintenance of cell viability. In 2017, the gene that encodes the σ_2R was identified.[81] This fact will allow a further study of this receptor. On the other hand, The σ_1R plays a key role in human physiology, and has been implicated in a variety of physiological processes, such as pain, certain endocrine and immune responses, and in memory, emotions, and sensory and motor functions[82–84] as well as in a variety of diseases of the cardiovascular and nervous systems. In agreement with these effects, the receptor is present in areas of the central nervous system involved in memory, emotion, sensory and motor function and in key areas implicated in pain control.[82–85] Because of their potential implication in pain processing, here we will focus on σ_1Rs.

3.1. The sigma 1 receptor

The σ_1R is a neuromodulatory, ligand-regulated membrane protein chaperone that exerts its functions through multiprotein complex assembly.[82,86]

3.1.1. Molecular structure

The σ_1R is encoded by the *SIGMAR1* gene, previously known as opioid sigma receptor 1 (*OPRS1*). The *SIGMAR1* gene is located at the human

chromosome 9 (9p13.3, band p13) and is approximately 7 kbp long. It contains four exons interrupted by three introns.[87] The gene was cloned for the first time from guinea pig liver,[88] but thereafter it has been cloned from several tissues, allowing the study of its molecular biology. Identification of the *SIGMAR1* gene enabled a series of expression studies and allowed the generation of a constitutive knockout (KO) mouse.[89] The viability of σ_1R KO mice suggests that σ_1Rs are not determinant at the embryogenic stages, thus allowing mutant mice to grow and behave, apparently, as their wild-type (WT) counterparts.

The *SIGMAR1* gene encodes a 24-kDA molecular weight protein of 223 amino acids highly conserved among vertebrates, especially in mammals (>90%), but with no similarity to any other mammalian protein. However, its amino acid sequence shared homology with fungal proteins involved in sterol synthesis, consistent with the known ability of sigma to interact with steroids, such as progesterone. However, the σ_1R by itself does not have sterol isomerase activity and does not affect cholesterol metabolism in mammalian cells.[85,88,90] For a long time, different representations of the Sigma 1 receptor structure have been made based on immunocytochemistry and protease protection assays.[82,91,92] In 2016, and corroborated by subsequent studies, the molecular structure of the receptor was obtained by crystallography.[86,93,94] According to these studies, the σ_1R is composed of three tightly associated protomers, each with a single transmembrane domain (Fig. 7A). The C-terminal ends of the protomers, located adjacent to the membrane on the cytosolic side, determine the trimeric structure of the receptor. The C-terminal ends present a highly conserved sequence between species, particularly at the ligand-binding domain (Fig. 7B) and the intermolecular interface between the three promoters (Fig. 7C).[86,93,94]

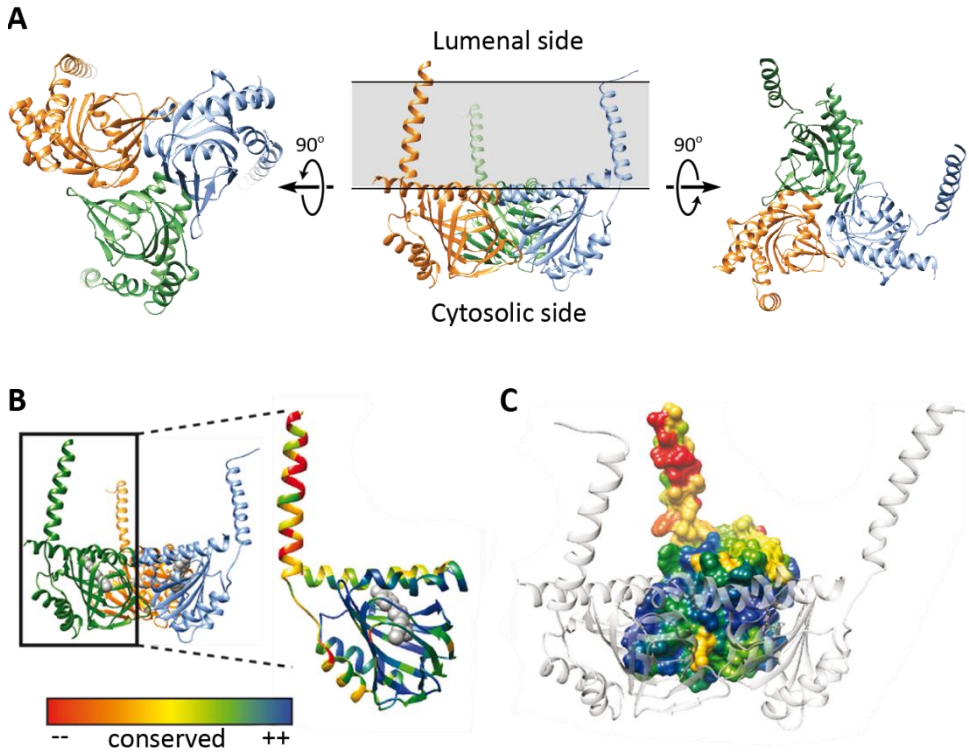


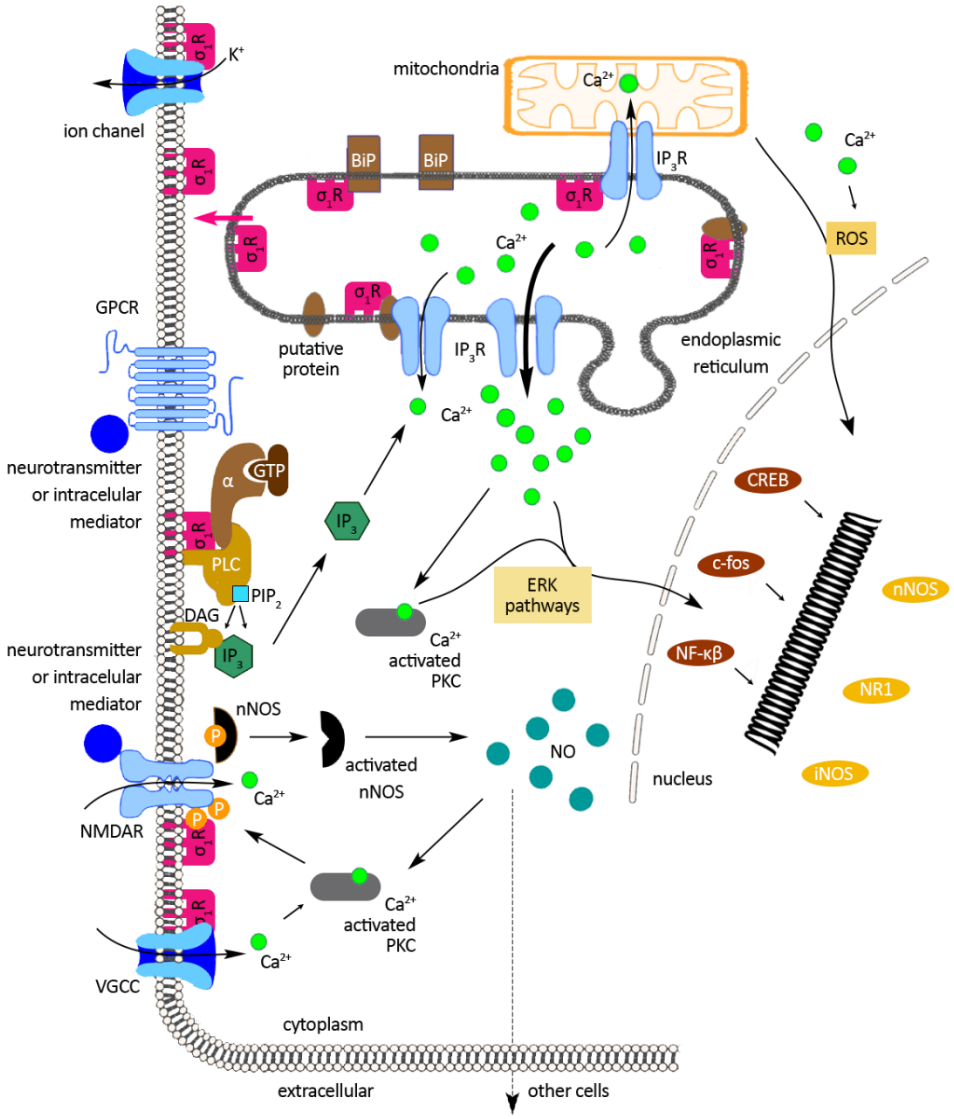
Figure 7. Molecular biology of σ_1R. A: overall structure of the σ_1R. Perpendicular view to the membrane plane, the σ_1R shows a triangular structure comprising three tightly associated protomers, each with a single transmembrane domain. From the side, the receptor reveals a flat membrane-associated surface. The location of the membrane plane is shown in grey. B: Structure of the σ_1 protomer. The receptor shows a cupin- like β-barrel fold flanked by four α-helices with the ligand (grey) bound at the center of the cupin domain. The receptor is colored by sequence conservation, revealing a high degree of conservation in the ligand- binding domain. C: The intermolecular interface among protomers of the receptor trimer is likewise highly conserved. Adapted from Schmidt et al. (2016).[86]

3.1.2. Mechanism of action

σ_1Rs are usually found anchored to the endoplasmic reticulum (ER) membrane, but they can also be found in nuclear and plasma membranes. In the ER, they are mainly located in the mitochondrion-associated ER membrane (called MAM).[92,95,96] From there, they can travel to other areas of the cell where they may interact with various membrane targets to modulate their function.[91,96]

Nowadays, a clearly defined molecular mechanism of action for the σ_1R remains elusive and it is considered a unique pharmacologically regulated integral membrane chaperone or scaffolding protein. In general, the σ_1R is considered a modulator of other signaling pathways, associated particularly to GPCRs and ion channels.[97] In the MAM, σ_1R forms a complex with a chaperone called binding immunoglobulin protein (BiP), which plays a central role in protein folding and quality control. The union of σ_1R and BiP generates a latent state of both proteins that minimizes the total activity of their respective chaperone functions. This association is Ca^{2+} dependent in a way that decreasing Ca^{2+} in the ER causes fast disassembly of the complex.[92,98] Under pathological or stressful conditions, the high presence of cytosolic inositol-1,4,5-trisphosphate (IP_3) generates a drop of Ca^{2+} concentration in the ER and induces the activation of the σ_1R through the disassembly of BiP. σ_1Rs prevent the degradation of IP_3 receptors, ensuring adequate Ca^{2+} entry into the mitochondria and increasing ATP production in the cell. The disassembly of the σ_1R-BiP complex also promotes the redistribution of the σ_1Rs located in the MAM to peripheral endoplasmic membranes, where they can bind directly or indirectly to various ion channels (mainly sodium and potassium), kinases and receptors, including NMDA and some GPCRs, such as dopamine D_1 and μ-opioid receptors.[82,85,91] At the plasma membrane, activation of the σ_1R stimulates phospholipase C

to produce IP_3 from phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis. Then, IP_3 binds to IP_3 receptors in the ER to promote Ca^{2+} efflux into the cytoplasm. All together, these actions, allow the amplification of intracellular signal transduction mechanisms that increase Ca^{2+} concentrations through NMDA receptors and voltage-gated Ca^{2+} channels (VGCCs) (Fig. 8).[85,99,100]



An increase in the production of nitric oxide (NO) is another consequence of σ_1R activation. σ_1R activation-associated increase of intracellular Ca^{2+} reduces the phosphorylation of neuronal NO synthase (nNOS) increasing its activity and leading to the synthesis of cytoplasmic NO. Moreover, activation of σ_1Rs transcriptionally modulates gene

Figure 8. Signal transduction pathways modulated by σ_1R activation. When activated, σ_1Rs located in the endoplasmic reticulum are released from the σ_1R-BiP complex, revealing its chaperone activity. In addition, σ_1R activation also promotes its redistribution from MAM to peripheral endoplasmic membranes, where it can bind ion channels, receptors or protein kinases. At the endoplasmic reticulum, σ_1Rs bind IP_3 receptor to enhance Ca^{2+} signaling from the endoplasmic reticulum into mitochondria to increase ATP production. The IP_3 receptor interaction could be facilitated or inhibited by coupling to other proteins modulating the Ca^{2+} efflux from the endoplasmic reticulum. At the plasma membrane, σ_1R regulates the activity of other signal transduction pathways, such as phospholipase C and PKC, and modulates the activity of neurotransmitter receptors and ion channels, including K+ and Ca^{2+} channels and NMDA receptors. At the cytoplasm, increased cytosolic Ca^{2+} reduces the phosphorylation of nNOS, leading to the synthesis of NO, which in turn stimulates PKC activity (activating NR1 subunit and ERK). In addition, NO can contribute to the pain facilitatory effect by diffusion to other cells. At the nucleus, σ_1Rs transcriptionally modulate gene expression of several proteins and transcription factors, such as nNOS, interleukins or c-Fos. α: α subunit of activated G protein phospholipase; BiP: binding immunoglobulin protein; CREB: cAMP response element binding protein; DAG: diacyl glycerol; ERK: extracellular signal-regulated kinase; GPCR: G protein-coupled receptor; GTP: guanosine triphosphate; IP_3: inositol-1,4,5-trisphosphate; NF: nuclear factor; NMDAR: N-Methyl-d-aspartic acid or N-Methyl-d-aspartate receptor; NO: nitric oxide; i/nNOS: NO synthase (inducible/neuronal); P: phosphate; PKC: protein kinase C; PIP_2: phosphatidylinositol- 4,5-bisphosphate; PLC: phospholipase C; ROS: reactive oxygen species; VGCC: voltage-gated calcium channel. Adapted from Zamanillo et al. (2013).[85]

expression of several proteins related to inflammatory, nociceptive and neuronal regulatory pathways. Of special interest is the modulation of nNOS and inducible NO synthase (iNOS). Overall, the resulting increase in NO promotes the activation of the NR1 subunit of the NMDA receptor and ERK through PKC-dependent phosphorylation (Fig. 8).[85,101]

As to date, no specific endogenous ligand for the σ_1R has been identified. Nevertheless, a large number of proteins, highly divergent in sequence and structure, bind to the receptor, although the mechanisms of interaction are not clear.[97,102]

3.2. Modulation by ligands

The high affinity of σ_1Rs for different proteins is consistent with the possibility that the σ_1R is indeed a ligand-operated chaperone. This model was first proposed by Hayashi and Su (2007)[92] and has since been widely accepted and validated. Therefore, actually, the σ_1R is considered a ligand-regulated molecular chaperone.[82,96,103,104] Despite this fact, no specific endogenous ligand for the σ_1R has been identified, although few molecules have been proposed as receptor modulators.

Recent crystallographic structure reveals how σ_1R is able to bind with high affinity to many structurally diverse ligands.[86,93,94] The process is mediated through a single electrostatic interaction between the residue Glu172 and a basic nitrogen present in most σ_1R ligands.[86] Due to the mechanism of action of the σ_1R, its ligands are considered ideal therapeutic drugs effective only under pathological conditions, but inactive under normal resting conditions.[96,105] Furthermore, it has been shown that σ_1R ligands regulate receptor activity in a clear agonist/antagonist manner.[82,96,103,104] However, it is difficult to determine the functional

agonist or antagonist nature of the σ_1R ligands due to: i) their modulatory action, vs. a classical direct effect;[82,104] ii) the conditions of the assay used and the readings assessed, which notably influence the results;[82] and iii) the pharmacological behavior of some σ_1R ligands, which do not show the classic linear dose-response curves.[82,104]

Taking into account the previous limitations, the classification of σ_1R ligands as agonists or antagonists has been largely based on *in vivo* comparative studies.[106–108] Currently, molecular and biochemical methods[86,94] are expected to better categorize ligands, which may require going beyond canonical agonist/antagonist definitions.

Table 2 summarizes the main characteristics of the best characterized ligands of σ_1Rs. It is worth mentioning that the number of newly reported σ_1R ligands is increasing rapidly.[109–115] In this work three σ_1R ligands have been used, namely BD1063, E-52862 and the dual compound EST73502 (Fig. 9). Therefore, we will focus on the characteristics of these compounds.

Table 2. Main sigma ligands and their characteristics.

Compound	Subtype Selectivity	Affinity for σ_1R	Function on σ_1R	Other Activities
Benzomorphans				
(–)-Pentazocine	σ_1/σ_2	++	Agonist	κ_1 agonist μ_1, μ_2, ligand low affinity δ κ_3 opioid ligand
(+)-Pentazocine	σ_1	+++	Agonist	NMDA receptor ligand
(+)-SKF-10,047	σ_1	+++	Agonist	NMDA receptor ligand
Antipsychotics				
Chlorpromazine	σ_1/σ_2	++	Undefined	Dopamine D_2 antagonist

Compound	Subtype Selectivity	Affinity for σ_1R	Function on σ_1R	Other Activities
Antipsychotics				
Haloperidol	σ_1/σ_2	+++	Antagonist	Dopamine D_2 and D_3 antagonist
Nemonapride	σ_1/σ_2	+++	Undefined	Dopamine D_2 antagonist
Antidepressants				
Clorgyline	σ_1	+++	Agonist	Irreversible monoamine oxidase A inhibitor
Fluoxetine	σ_1	+	Agonist	Selective 5-HT reuptake inhibitor
Fluvoxamine	σ_1	+++	Agonist	Selective 5-HT reuptake inhibitor
Imipramine	σ_1	++	Agonist	Monoamine reuptake inhibitor
Sertraline	σ_1	++	Agonist	Selective 5-HT reuptake inhibitor
Antitussives				
Carbetapentane	σ_1/σ_2	+++	Agonist	Muscarinic antagonist
Dextrome-thorphan	σ_1	++	Agonist	NMDA receptor allosteric antagonist
Dimemorfan	σ_1/σ_2	++	Agonist	
Parkinson's and/or Alzheimer's disease				
Amantadine	?	+	Agonist	NMDA antagonist, antiviral properties
Donepezil	σ_1/σ_2	+++	Agonist	Cholinesterase inhibitor
Memantine	+	+	Agonist	NMDA antagonist, antiviral properties
Drugs of abuse				
Cocaine	σ_1/σ_2	+	Agonist	Monoamine transporters inhibitor, amongst other actions
MDMA	σ_1/σ_2	+	Undefined	Preferential SERT inhibitor, among other actions
Metamphe-tamine	σ_1/σ_2	+	Undefined	Preferential DAT inhibitor, amongst other actions

Compound	Subtype Selectivity	Affinity for σ_1R	Function on σ_1R	Other Activities
Putative endogenous ligands (neurosteroids)				
DHEAS	σ_1	+	Agonist	$GABA_A$ negative modulator
PB-008	σ1	+	Agonist	
Pregnenolone sulfate	σ_1	+	Agonist	NMDA positive/$GABA_A$ negative modulator
Progesterone	σ_1	+++/++	Antagonist	
Testosterone	σ_1	+++/++	Undefined	
Anticonvulsants				
Phenytoin (DPH)	σ_1	n.a.	Allosteric modulator	Delayed rectifier K^+ channel blocker T- type Ca^{2+} current inhibitor Na^+ current inhibitor
Ropizine	σ_1	n.a.	Allosteric modulator	
Other σ drugs				
(+)-3-PPP	σ_1/σ_2	++	Agonist	σ_2 agonist NMDA receptor ligand Dopaminergic agonist
(+)-MR 200	σ_1/σ_2	+++	Antagonist	
(±)–PPCC [131]	σ_1/σ_2	+++	Agonist	
4-IBP	σ_1/σ_2	+++	Agonist	Dopamine D_2 ligand
BD 1008	σ_1/σ_2	+++	Antagonist	
BD 1047	σ_1	+++	Antagonist	β adrenoceptor ligand
BD 1063	σ_1	+++	Antagonist	
BD 737	σ_1/σ_2	+++	Agonist	
BD-1008	σ_1/σ_2	+++	Agonist	
BMS-181100 (BMY-14802)	σ_1	++	Agonist	
BMY 14802	σ_1/σ_2	++	Antagonist	5-HT_{1A} agonist
Dipeptide Phe–Phe	σ_1	n.d.	Antagonist	
DTG	σ_1/σ_2	+++	Agonist	
Dup 734	σ_1	+++	Antagonist	5-HT_2 antagonist
E–52862 (S1RA)	σ_1	+++	Antagonist	

Compound	Subtype Selectivity	Affinity for σ_1R	Function on σ_1R	Other Activities
Other σ drugs				
E-5842	σ_1	+++	Antagonist	Low to moderate affinity for dopamine, 5-HT and glutamate receptors
Eliprodil (SL-82.0715)	σ_1/σ_2	++	Undefined	NMDA antagonist, α_1 adrenoceptor ligand
EST73502	σ_1	++	Antagonist	
Haloperidol Metabolite I	σ_1	++	Antagonist	
Haloperidol Metabolite II	σ_1/σ_2	+++	Irreversible antagonist	Dopamine D_2 and D_3 ligand
Igmesine (JO-1784)	σ_1	+++	Agonist	
JO-1784 (Igmesine)	σ_1	+++	Agonist	
Metaphit	σ_1/σ_2	++	Irreversible antagonist	Acylator of phencyclidine (PCP) and σ_2 binding sites
MS-377	σ_1	+++	Antagonist	
NE-100	σ_1	+++	Antagonist	
NMIN	σ_1	n.d.	Antagonist	
OPC-14523	σ_1/σ_2	+++	Agonist	Agonist of pre- and post-synaptic 5-HT_{1A} receptors SERT inhibitor
Panamesine (EMD 57445)	σ_1/σ_2	+++	Antagonist	One of its metabolites is a dopaminergic antagonist
PRE 084	σ_1	+++	Agonist	
Rimcazole (BW-234U)	σ_1/σ_2	+	Antagonist	Dopamine transporter (DAT) inhibitor
SA4503	σ_1	+++	Agonist	
SR 31742A	?	+++	Undefined	High affinity for C8-C7 sterol isomerase

* Inhibition constant (Ki) values. +++: <50 nmol/L; ++: <500 nmol/L; +: <10 nmol/L; n.a.: not applicable; n.d.: not determined.

Data from: Hayashi and Su (2004);[108] Cobos et al. (2008);[95] García-Martínez et al. (2016);[114] García et al. (2020).[110]

3.2.1. BD1063

BD1063 (1- [2- (3,4-dichlorophenyl) ethyl] -4-methylpiperazine dihydrochloride; Fig 9A) is a selective σ_1R antagonist first characterized in 1995 using binding assays and behavioral studies in rats.[116] BD1063 shows a high affinity for σ_1Rs (K_i = 9.15 ± 1.28 nM, in guinea pig) although it also had some affinity for σ_2R (K_i = 449 ± 11 nM, in rat).[116] This dual activity is important because the final effects observed could result in apparent discrepancies due to the activation of one receptor subtype at low doses and the activation of other subtype at higher doses. BD1063 is inactive at dopamine, opiate, GABA and NMDA receptors, and it has a low micromolar affinity for 5-HT receptors.[116,117]

BD1063 has been shown to have anti-dystonic effects,[116] to reduce cocaine-induced gene activation in reward-related neural circuits[118] and to inhibit nociception and hypersensitivity in models of mechanical, thermal and neuropathic pain.[119–121] Moreover, evidences suggest that BD1063 potentiates opioid-mediated anti-nociception.[122–124]

3.2.2. E-52862

E-52862 (also known as S1RA or MR309; 4- [2 - [[5-methyl-1- (2-naphthalenyl) -1H-pyrazol-3-yl] oxy] ethyl] morpholine; Fig. 9) is a selective σ_1R antagonist developed by Laboratorios Esteve S.A. (Barcelona, Spain) as a result of a medical chemistry program aimed at the development of novel and selective σ_1R ligands. E-52862 has high affinity for the σ_1R in humans (K_i = 17 ±7 nM) without affinity for σ_2Rs (K_i > 1000 nM, in guinea pig).[109] E-52862 has not been significantly active in 170 other receptors, transporters, ion channels and enzymes, thus confirming its selectivity for σ_1Rs.[85,109,119]

E-52862 has positive effects in cardiovascular pathologies[125] and exhibits anti-nociceptive properties in several preclinical models of inflammatory pain.[83] In addition, E-52862 penetrates the blood-brain barrier and binds to σ_1R in the CNS, with a significant correlation between the degree of occupancy of σ_1Rs in the CNS and the anti-nociceptive effects observed.[100,119]

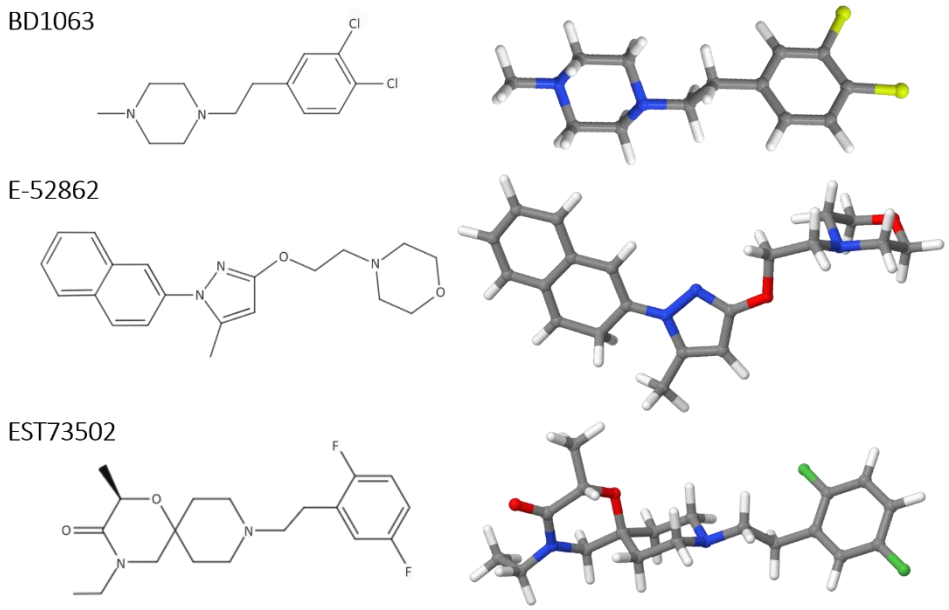


Figure 9. Molecular structure of the selective σ_1R antagonist BD1063 (A), E-52862 (B) and the dual compound EST73502 (C) in 2D and 3D layout. Nitrogen atoms are represented in blue, oxygen atoms are represented in red, chlorine atoms are represented in yellow, and fluorine atoms are represented in green. Images obtained from Jmol©.

3.2.3. EST73502

Since their discovery, several studies have highlighted the existence of positive interactions between opioid compounds and sigma ligands.

Specifically, a synergistic anti-nociceptive effect has been demonstrated with the simultaneous administration of σ_1R antagonists and opioid agonists.[122,126–128] Taking into account this phenomenon, there has been a great interest in the development of dual molecules combining σ_1R antagonistic and opioid agonistic properties. Compared to the standard clinical practice of using cocktails of drugs, dual agents offer the advantages of a broader spectrum of action, better treatment compliance, lowering the risk of drug–drug interactions, simpler pharmacokinetics, and less variability among patients. In this context, EST73502 [(R) -9- (2,5-difluorophenethyl) -4-ethyl-2-methyl-1-oxa-4,9-diazaspiro [5.5] undecan-3-one; Fig. 9] has recently been characterized as a dual μ-opioid receptor (MOR) partial agonist and σ_1R antagonist (K_i = 64 ±5 nM and K_i = 118 ±7 nM, for σ_1Rs and MORs, respectively, in human cells) without any significant affinity for another 180 molecular targets.[110]

EST73502 has shown positive effects in potentiating opioid analgesia in animal models of acute (paw mechanosensitivity) and chronic (partial sciatic nerve ligation) somatic pain. Furthermore, EST73502 decreased opioid-related side effects at the GI level.[110] Following these results, preliminary *in vitro* drug-drug interaction studies have also been performed and EST73502 is being tested in phase I adaptive clinical study (Study EudraCT Number 2018-000258-23).[129,130]

3.3. σ_1Rs and pain

From the perspective of a potential clinical use, a therapeutic role of σ_1Rs have been explored in pathologies related to addiction, neuroprotection and neurodegenerative diseases, psychiatric disorders, and pain.[132]

Particular interest has the application in the treatment of diverse pain states. Indeed, the σ1R is currently one of the most promising drug targets for the management of pain.

3.3.1. σ_1Rs during normal pain responses and during sensitization

Several pharmacological studies with σ_1R antagonists[100,119,133–137] and σ_1R KO mice[126,138–143] have largely corroborated the role of σ_1Rs in pain modulation.

The generation of a σ_1R KO mouse[89] has been a useful tool to study the participation of σ_1R in various types of pain. In fact, the notion that σ_1Rs may play a role in modulating pain in the absence of opioids came from studies using these animals. The constitutive absence of σ_1Rs in KO mice does not interfere with the perception of sensory stimuli during thermal or mechanical pain-related responses.[100,120,122,123,142] This is consistent with the observation that σ_1R ligands do not normally exert any effect by themselves in physiological conditions,[96,99] nor are they able to modify pain in classical models of acute thermal and mechanical nociception.[100,122,144] Altogether, these data strongly support de view that σ_1Rs do not participate in pain regulation in normal conditions. However, evidences indicate that σ_1Rs play a key role in modulating pain behavior during pain sensitization (states of hypersensitivity) and chronic pain conditions.

First evidences came from the constitutive absence of σ_1Rs in KO mice. In these animals, formalin-induced somatic pain sensitization was completely absent.[140] Follow-up studies using selective σ_1R antagonists, such as E-52862 and BD-1047, corroborated these findings and pointed to the spinal cord and supraspinal CNS regions as sites for σ_1R-dependent modulation of formalin-induced sensitization.[145,146] Subsequently, it has

been shown that both genetic (σ_1R KO animals) and pharmacological (BD1063, BD1047, NE-100 and E-52862) blockade of σ_1Rs inhibited somatic mechanical allodynia induced by intraplantar capsaicin.[119,147,148]

σ_1Rs have also shown modulatory effects in other models of neuropathic pain. For instance, strong attenuation of cold and mechanical hypersensitivity has been observed in σ_1R KO mice with paclitaxel-induced neuropathic pain[120] and also in mice exposed to partial sciatic nerve ligation.[100] More recently, a significant attenuation of both mechanical and thermal hypersensitivity has been reported in σ_1R KO mice following spinal cord injury. Accordingly, treatment of WT mice with the σ_1R antagonist E-52862 after spinal cord injury exerted anti-nociceptive effects.[136,138] Moreover, the acute administration of E-52862 also attenuated cold, mechanical, and heat hypersensitivity after sciatic nerve transection-induced hypersensitivity in WT mice.[126]

The role of σ_1Rs has also been assessed in states of inflammation-associated somatic pain sensitization, such as during the administration of carrageenan (acute inflammatory state) or Freund's complete adjuvant (CFA, chronic inflammatory pain model).[83] In these models, mechanical (paw pressure) and thermal (radiant heat) hyperalgesia was reversed in a dose-dependent manner by the selective σ_1R antagonists BD-1063 and E-52862 in WT animals.[143] These results are consistent with those obtained after subcutaneous administration of the selective σ_1R antagonists (-)-MRV3 and (+)-MR200 during intraplantar carrageenan-induced hyperalgesia.[149,150] Similar analgesic effects were also observed after local application of selective σ_1R antagonists in the inflamed tissue, thus suggesting a role for σ_1Rs in peripherally mediated analgesia.[143] Al together, these data suggest that both central and peripheral pharmacological blockade of σ_1Rs could be an effective option to treat inflammatory pain.

Finally, few studies have addressed the potential implication of σ_1Rs in visceral pain. In a model of intracolonic capsaicin-induced visceral pain and in a model of cyclophosphamide-induced cystitis, σ_1R KO mice showed a reduction in the number of pain behaviors compared to WT mice.[134,139] However, referred mechanical hyperalgesia of the abdominal wall during cyclophosphamide-induced cystitis was similar in WT and σ_1R KO animals.[139] Supporting this view, the selective σ_1R antagonists BD1063, E-52862 and NE-100 also inhibited the responses related to intracolonic capsaicin-induced visceral pain[134] and to by cyclophosphamide-induced cystitis in WT mice.[139]

Overall, these observations indicate that under sensitizing conditions, σ_1Rs are involved in pain sensitization and that their blockade might have anti-allodynic and anti-hyperalgesic effects, thus preventing states of hypersensitivity in different etiologies of pain.

3.3.2. Modulation of opioid-induced anti-nociception

A role for σ_1Rs modulating opioid analgesia was initially described in studies showing a tonic inhibitory control of σ_1Rs over opioid receptor-mediated anti-nociception.[151,152] Subsequently, several studies support a modulatory role for σ_1Rs on μ-, δ- and κ-opioid-mediated analgesia: σ_1R agonists diminish, whereas σ_1R antagonists and antisense treatments enhance, opioid-mediated anti-nociception.[124,133,153–155] These observations lead to the suggestion that the sigmaergic system might represent an anti-opioid system in which σ_1Rs exert a tonic inhibitory control on opioid-mediated signaling pathways.

Different studies have been conducted to characterize the interaction between opioid and sigma receptors in various animal models and in response to different stimuli. Blockade of σ_1Rs with E-52862 has been

shown to result in improved opioid anti-nociception in models of acute thermal pain (tail-flick test).[141] Likewise, in a model of inflammatory pain, the association of E-52862 with morphine, at doses inducing little or no analgesic-like effects when administered alone, resulted in a marked anti-nociceptive effect to heat stimuli and complete reversion of inflammatory tactile allodynia.[127] However, E-52862 did not increase the effect of morphine on grip strength deficits induced by joint inflammation.[127] Moreover, in a neuropathic model, enhancement effects on spared nerve injury (SNI)-induced hypersensitivity in mice have also been described for mechanical and thermal stimuli but not for cold stimuli after co-administration of E-52862 and morphine.[126] Regarding visceral pain, there are no pharmacological studies evaluating the co-administration of σ_1R antagonists and opioids. Nevertheless, morphine showed higher potency inhibiting visceral pain-related responses elicited by intracolonic capsaicin or during cyclophosphamide-induced cystitis in σ_1R KO mice than in WT animals.[134,139] Thus suggesting that, as described above for somatic pain, modulatory sigmaergic/opioidergic interactions are also present as it relates to visceral pain.

Sigmaergic/opioidergic interactions modulating pain seem to be site-specific. In fact, the interaction between the two systems is present when σ_1R ligands are administered systemically or supraspinally (intracerebral), but not after administration at the spinal level.[128,153,154] On the other hand, although opioids essentially lack anti-nociceptive activity when they act only in the periphery,[156] peripheral opioid-induced anti-nociception was improved by the local co-administration of the selective σ_1R antagonist BD1063.[122,123]

It is important to note that potentiation of anti-nociception was not accompanied by potentiation of opioid-induced side effects, such as the development of morphine analgesic tolerance, physical dependence,

inhibition of gastrointestinal transit, or mydriasis.[122,123,128,141,151] Therefore, all together, these data support the use of σ_1R antagonists as adjunctive therapy to improve opioid analgesia. These promising results have led to the development of new dual molecules with both agonist opioid and antagonist sigma components, as mentioned previously. Recently, EST73502 has been developed and characterized as a dual MOR agonist and σ_1R antagonist.[110] In preliminary studies, EST73502 potentiated opioid analgesia in animal models of acute (paw mechanosensitivity) and chronic pain (partial sciatic nerve ligation) with decreased opioid-related side effects at GI level.[110]

Altogether, these results suggest that σ_1 ligands, by themselves or in combination with opioids, may serve as an attractive starting point to develop potent and specific therapeutic agents for the treatment of visceral sensitivity, particularly in states of hypersensitivity.

4. ANIMAL MODELS FOR THE STUDY OF PAIN

Animal models represent a basic tool in the study of visceral pain and have been key dissecting the basic mechanisms and pathways implicated as well as in the in the identification and validation of potential therapeutic targets or the validation of compounds with potential clinical application. In general terms, an animal pain model is the procedure by which the reaction of an animal to a noxious (real or potential tissue damage producing) stimulus or to induced pathological situations is assessed. It should be noted that, in humans, pain is described as a complex sensation with a significant subjective emotional/psychological component. These characteristics make pain a process difficult to evaluate in animals, due to

the difficulty in determining its emotional/psychological substrata. Therefore, when assessing pain in animals, alternative surrogate markers have to be used. This is usually solved by assessing any of the multiple neuroendocrine, behavioral and/or autonomic responses constituting, together with the emotional/psychological component, the complete physiological response to a noxious situation. Furthermore, ideal pain models should also consider de generation of sensory deficits in pain responses, mainly states of hypersensitivity (characterized by the presence of increased pain-related responses). In any case, all animal pain models must minimize the suffering of the animals as much as possible.

In the context of the present work, we will focus on the characteristics of animal models for the study of visceral pain, in particular pain arising from the GI tract.

4.1. Visceral pain and hypersensitivity models

Animal models of visceral pain attempt to replicate the physiological/pathophysiological conditions that induce pain in internal organs, mainly hollow viscera. As it relates to pain originating within the GI tract, and taking into consideration the pathophysiological importance of hypersensitivity for GI pathologies,[4,55,56] most of the available models are directed towards the induction of hypersensitivity. Taking this into account, visceral pain models have traditionally been classified into two broad categories: models based on pain triggered by direct mechanical stimuli (traction pain models) and models based on chemically-induced inflammation (inflammatory and irritative pain models).[157–159] More recently, animal models based on genetic or microbiome alterations or in stress-associated sensitivity changes represent new approaches for the study of visceral pain.[158–161]

4.1.1. Colonic inflammatory pain models

Inflammatory models mimic some the immunological and histopathological features of IBD and FGD in humans and attempt to recreate the state of hypersensitivity detected in these patients. These models are based on the administration of compounds that generate a direct inflammatory response or elicit irritative responses that imply an activation of the immune system similar to that observed during inflammation. In any case, the procedures lead to the generation of pain responses and a state of sensitization, similarly to that observed in IBD or FGD.[158–160,162,163] Models based on irritation or inflammation have the advantage of being simple to generate and, therefore, can be used for high throughput studies. However, they present poor reproducibility and a questionable relationship with human pathology.[160] In addition, in many cases, the presence of long-lasting hypersensitivity, as observed in humans, is not evident. A summary of the main inflammatory/irritative models currently used is included in Table 3.

Dextran sodium sulfate-induced inflammation

One of the most widely used experimental model of intestinal inflammation is based on the use of dextran sodium sulfate (DSS). Administration of DSS in the drinking water (<10%, depending upon the species and strains considered) induces a selective inflammation of the colon (colitis) in mice, rats and hamsters.[164] As today, the DSS-induced colitis murine model is a validated and well-accepted model of IBD, characterized by its simplicity, reproducibility, and controllability.[165,166]

Oral administration of DSS induces severe colitis characterized by weight loss, bloody diarrhea, shortening of the colon, ulceration of the mucosa, loss of epithelial cells, and neutrophilic infiltration in the colon. The

mechanisms through which DSS induces inflammation of the colon are not completely understood. Accumulation of DSS in the colonic lumen seems to promote direct toxic effects of the compound on the epithelial cells of the basal crypts, thus affecting the integrity of the epithelial barrier.[195] Following epithelial injury, mucosal and submucosal immune cells are exposed to luminal antigens, resulting in a rapid and profound inflammatory immune response. The inflammation generated is characterized by innate immune mechanisms and is associated with increased production of various cytokines and chemokines, including IL-6, IL-10, IL-1β, IL-17, TNF-α, among others,[163,166] although species/stain-specific profiles are described.[196–198] DSS exposure induces acute colitis (with a peak inflammatory reaction occurring between 6-10 day, approximately after starting DSS exposure) which might progress in a strain-dependent manner, at least in mice, to a chronic colitis.[198,199]

Table 3. Rodent inflammatory models for the study of visceral pain from intestinal origin.

Inducing agent	Administration site	Species	References
Acetic acid	Intra-colonic	Mice, rats	167–170
Acetic acid	Intraperitoneal	Mice	167,168
Butyrate	Intra-colonic	Rats	171,172
Capsaicin	Intra-colonic	Mice, rats	134,167,173,174
Carrageenan	Oral	Mice, rats	175,176
DSS	Oral	Mice, rats	177–180
LPS	Intraperitoneal	Rats	181–183
Mustard oil	Intra-colonic	Mice, rats	184–187
TNBS/DNBS	Intra-colonic	Mice, Rat	188–192
Zymosan	Intra-colonic	Mice, rats	53,193,194

CRD: Colorectal distension; DSS: Dextran sodium sulfate; LPS: lipopolysaccharides; DNBS: 2,4-dinitrobenzenesulfonic acid; TNBS: 2,4,6-trinitrobenzenesulfonic acid.

Visceral (intestinal) pain responses during DSS-induced colitis have been studied in mice. Although with some contradictory data, studies consistently indicate the presence of intestinal (colonic) hypersensitivity during both the acute and the chronic phases of DSS-induced inflammation.[179,190,200–204] Nevertheless, viscerosensitivity associated to DSS-induced colitis, particularly as it relates to the presence of long-lasting hypersensitivity associated to chronic inflammation, has not been properly characterized and deserves further consideration as a model of visceral pain and hypersensitivity.

4.1.2. Colonic traction pain models

Accumulating preclinical and clinical evidence suggests that colorectal mechanical stimuli are effective evoking visceral pain.[42] Indeed, the mechanical stimulation, based on volume- or pressure-controlled distensions, of the colorectal area is used in humans, both clinical and experimentally, to assess pain of this region of the GI tract.[205]

Colorectal distension model

Colorectal distention (CRD) is a visceral pain induction method that produces aversive behaviors and measurable, reliable and reproducible cardiovascular and visceromotor responses useful for intra- and inter-animal studies.[41] The procedure has been extensively used to study visceral pain arising from the gut in rodents, particularly in rats. During CRD, a balloon is inserted via the anus in the distal colon of the rodent, under sedation, and then the balloon is inflated to elicit a distension of the viscera and the subsequent stimulation of local mechanoreceptors, thus eliciting visceral pain-related sensory responses. Distensions can be applied in a volume- or pressure-controlled manner. To compensate for the potential

accommodation of the smooth muscle to volume, pressure-controlled isobaric distensions, generated with a barostat system, are commonly used during CRD. In general, the CRD technique reproduces in rodents the quality, location and intensity of the human experience of visceral pain generated with the same procedure.[206–210]

Protocols applied during CRD allow to modulate the intensity and duration of the mechanical stimuli applied. Pressure pulses exerted elicit pressure-related measurable responses that include, among others, behavioral changes, contraction of the abdominal and pelvic muscles (the so-called viscerosomatic response) or autonomic cardiovascular changes (blood pressure and heart rate),[42] among others (Fig. 10). Interestingly, it has been observed that repeated phasic CRD at a noxious pressure produces a temporary mechanical hyperalgesic response, thus representing an acute mechanical sensitization model.[211–213]

4.2. Pain assessment in animal models

In animal models, quantitative assessment of pain (normal or in states of hypersensitivity) is complex. However, advances in neuroscience and pain physiology allow pain measurement to be more consistent, automated, and accurate. Physiological responses to painful conditions, such as increases in heart rate, respiration and blood pressure can be assessed using objective techniques such as electrophysiology, telemetry, electromyography, colonic manometry or other pain biomarkers (Fig. 10).[158,214] Moreover, behavioral responses of animals can also be assessed. These reactions, sometimes not very obvious, usually manifest as simple behaviors or nocifensive withdrawal of the whole or part of the body.[215,216] Behavioral methods used for nociception measurements in rodents are divided into stimulus-evoked and non-stimulus evoked (Table 4).[214] The detailed

description of these methods is out of the scope of the present work and can be found elsewhere.[214]

Table 4. Methods used to evaluate pain behaviors in rodents.

Stimulus-evoked pain-like behaviors	
Mechanical Stimuli	Manual von Frey
	Electronic von Frey
	Randall-Selitto test
	Colorectal distension
Heat Stimuli	Tail flick test
	Hot plate test
	Hargreaves test
	Thermal probe test
Cold Stimuli	Cold plate test
	Acetone evaporation test
	Cold plantar assay
Temperature Preference Test	
Non-stimulus evoked nociception	
Grimace scales	
Burrowing	
Weight bearing and gait analysis	
Automated behavioral analysis	

Adapted from Deuis et al. (2017)[214]

The method chosen to quantify pain depends on the pain model and the primary question that is being asked. Moreover, more than one test will likely be needed to fully translate data between basic animal studies and clinical conditions.[217] The present work, uses two methods to assess visceral pain originating within the GI tract (colon), assessment of visceromotor responses (VMRs) to CRD and the von Frey test.

4.2.1. The von Frey test

The von Frey test is a nociceptive test used for the assessment of aversive behaviors in responses to mechanical stimuli. This test is considered the gold standard for determining mechanical thresholds and assesses somatic mechanical hipersensitivity in rats and mice.[214,216]

During a von Frey test, animals are placed on a mesh with a pierceable bottom through which a monofilament (von Fey filament) is applied perpendicular to the surface of the animal until it bends, delivering a constant predetermined force. Although there are different methods to determine pain threshold and sensitivity all of them are based on the sequential application of different von Fey filaments.[216,218,219] Responses to the filaments are considered positive if the animal shows any nocifensive behavior during the application of the stimulus or immediately after removing the filament. This behavior is frequently a retirement (withdrawal) or the body part stimulated, Thus, the behavior assessed is commonly recognized as a withdrawal response.[214,216]

The von Frey test is commonly used to assess somatic sensitivity and it is usually based on the probing of the plantar surface, but can also be used in other areas of the body.[100,120,142] For instance, in visceral pain assessment, filaments are applied to the abdominal surface.[134,139] However, mechanical responses associated with visceral sensitivity measured in the abdomen are likely to include a somatic components, associated to the stimulation of the abdominal wall per se (peritoneal pain), in addition to the visceral component, associated to the stimulation of the underlying viscera (intestine).[220] Therefore, it is considered to reflect a combination of visceral and referred somatic hyperalgesia.[134,139]

4.2.2. Colorectal distension

As previously mentioned, the CRD technique is a visceral pain induction method in which a controlled pressure is applied to the distal colon and rectum, using a barostat system, with the aim of generating the stimulation of local mechanorreceptors.[41,42] Traditionally, reflex abdominal contractions to the distension applied, the so-called VMRs, have been used as a surrogate marker of visceral pain in this model (Fig. 10). the magnitude of the VMRs has been measured by electromyographic (EMG) activity of the abdominal muscle (invasive method) or by counting the number of contractions, as visually determined by the researcher. Tammpere et al. (2005) established an alternative non-invasive manometric method to quantify the magnitude of the VMRs associated to CRD in rats.[211] Abdominal contractions generate small changes in balloon pressure that can be quantified as a measure of the VMRs and are directly related to the EMG activity of the abdominal musculature.[68,211] Subsequently, this manometric approach has also been validated in mice[213] and it has been extensively used for pharmacological studies.[68,221–224]

As today, the CRD technique is the recommend method to assess GI pain, since: i) mimics a natural stimulus eliciting pain within the GI tract;[41,42] ii) is restricted to the viscera, so accurately assesses visceral sensitivity;[170,209,225] and iii) is equivalent to the method used in humans in a clinical setting to evaluate visceral (intestinal) pain.[226,227]

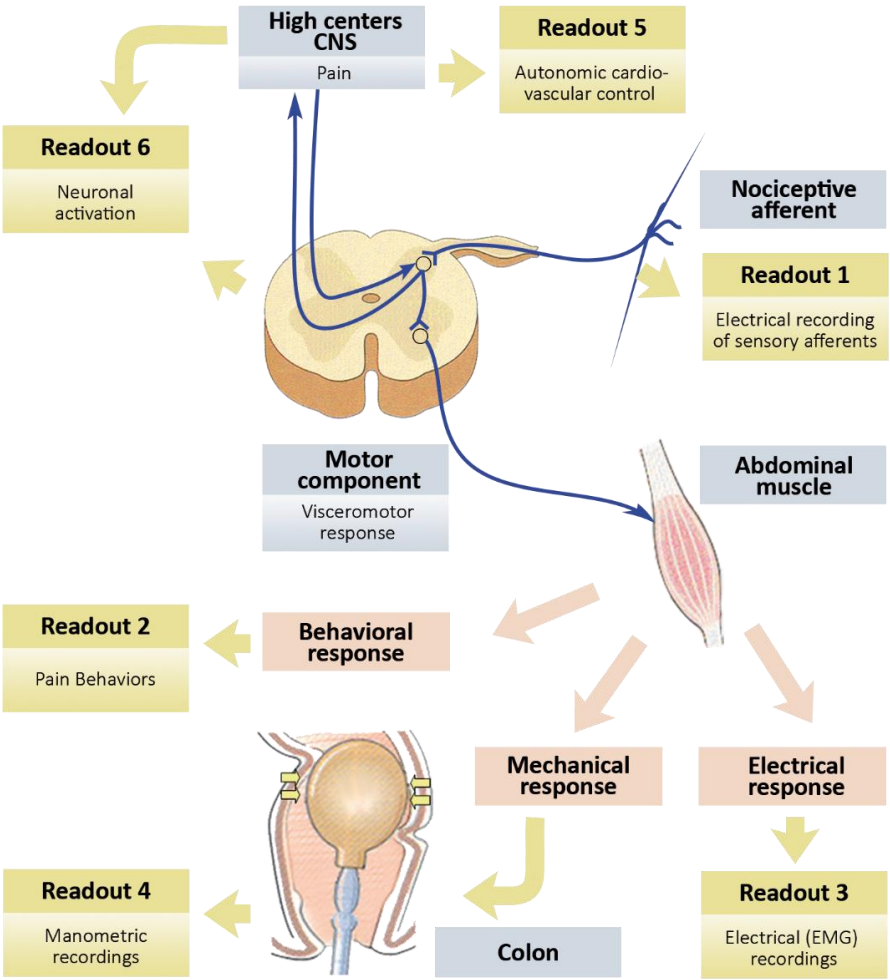


Figure 10. Readouts (surrogate markers) used for the assessment of visceral pain responses associated to colorectal distension in rodents. CNS: central nervous system.

HYPOTHESIS AND AIMS

Taking into account the previous background, σ_1Rs are currently a promising target for the treatment of somatic pain. Indeed, numerous evidences suggest that the selective pharmacological blockade of σ_1Rs could be a promising new therapy for the management of pain and pain sensitization of different etiologies such as neuropathic, inflammatory and postoperative pain. Moreover, potential σ_1R-mediated immunomodulatory effects might contribute to these positive actions. However, although these evidences, potential benefits in visceral pain, including states of sensitization, remain largely unexplored.

Therefore, this work is based on the **HYPOTHESIS** that:

σ_1Rs are implicated in the development of intestinal inflammation and inflammation-associated changes in visceral sensitivity, particularly as it relates to the development of sensitization, thus representing a feasible target for the pharmacological treatment of inflammation and visceral pain arising from the GI tract.

Considering this, the specific **AIMS** of this work are as follows:

1. To determine the role of σ_1Rs in the development on acute and chronic colonic inflammation.
2. To set up a model of long-lasting colonic hypersensitivity in rats, mimicking the state of hypersensitivity observed in humans with inflammatory and functional gastrointestinal disorders.
3. To determine the role of σ_1Rs in inflammation-associated changes in viscerosensitivity during intestinal inflammation.
4. To determine the potential modulatory role of σ_1Rs in central and peripheral sensory-related markers during intestinal inflammation.

To achieve these objectives, we characterized intestinal inflammation and the development of inflammation-associated changes in viscerosensitivity under the genetic and pharmacological blockade of σ_1Rs in mice and rats. Functional tests (von Frey and colorectal distension) were used to assess changes in sensitivity during the induction of intestinal inflammation or in healthy animals. Furthermore, peripheral and central inflammatory- and sensory-related markers potentially involved in these responses were also characterized.

CHAPTER 1

Intestinal inflammation-associated hypersensitivity is attenuated in a DSS model of colitis in σ_1 knockout C57BL/6 mice

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ABSTRACT

Sigma-1 receptors (σ_1R) have been implicated in several pain pathways. We assessed the implication of σ_1Rs in the development of intestinal inflammation and inflammation-associated referred hypersensitivity in a model of colitis in σ_1R knockout (KO) mice.

Colitis was induced with dextran sulfate sodium (DSS) in wild type (WT) and σ_1R KO mice. The development of referred mechanical hypersensitivity (von Frey test) was assessed. Colonic and spinal changes in expression of immune- and sensory-related markers were also investigated (RT-qPCR/Western blot).

Absence of σ_1Rs had little impact in colitis generation and progression, although during the chronic phase a reduction in edema and a down-regulation of iNOS gene expression was observed. In σ_1R KO mice, inflammation-associated hypersensitivity was significantly attenuated (paw) or completely prevented (abdomen). During colitis, in WT mice, changes in the colonic expression of nociceptive markers were observed during the acute and chronic phases of inflammation. Although σ_1R KO mice showed similar regulation in the acute phase, an attenuated response was observed during the chronic phase of colitis. These differences were especially relevant for CB2 and TRPV1 receptors, which could play an important role in σ_1-mediated regulation of sensitivity. No changes were detected on ERK phosphorylation at the level of the lumbosacral spinal cord.

In summary, intestinal inflammation-associated referred hyperalgesia was reduced (paw) or absent (abdomen) in σ_1R KO mice, thus confirming an important role for σ_1R in the development of colitis-associated hypersensitivity. These results identify σ_1Rs as a possible therapeutic target for the treatment of hypersensitivity associated to intestinal inflammation.

INTRODUCTION

Evidences suggest that inflammation within the gastrointestinal tract, even at a low degree, and the presence of visceral and somatic hypersensitivity are associated phenomena. Indeed, inflammatory conditions of the gastrointestinal tract, such as inflammatory bowel disease (IBD), are associated to both somatic and visceral hypersensitivity.[1,2] Moreover, irritable bowel syndrome (IBS), the main functional gastrointestinal disorder, has been associated to a low degree of intestinal inflammation and has altered colonic sensitivity with increased perception as key manifestations.[3,4] Additionally, changes in somatic sensitivity, characterized by referred hypersensitivity, have also been observed in states of intestinal inflammation.[5,6] In this context, intestinal inflammation and the associated state of hypersensitivity are still areas of medical needs, without fully effective therapeutic approaches.

During the last years, several targets have been explored as potential treatments of visceral pain arising from the gut. However, at the current time there are no effective treatments for this type of pain. Some efforts have been directed towards the validation of analgesic treatment positively validated against somatic pain, although the differences between these pain modalities might be relevant in this respect. In recent years, sigma-1 receptors (σ_1Rs) have been implicated in pain mechanisms and suggested as potential pharmacological target for the treatment of somatic pain. σ_1R is a neuromodulatory, ligand-regulated membrane protein chaperone that exerts its functions through multiprotein complex assembly.[7,8] The relation of σ_1 Rs and pain was first suggested by studies showing a relationship between σR systems and opioid-mediated analgesia.[9,10] Evidences indicate that σ_1R ligands fail to modify normal pain responses by themselves, as

demonstrated in classical models of thermal and mechanical acute nociception.[11–16] However, σ_1R ligands seem to play a key role in modulating pain behavior in states of sensitization and chronic pain conditions.[17–20] In this respect, recent studies show that both central and peripheral pharmacological blockade of σ_1Rs could be an effective option to treat inflammatory pain.[13,21–24] As it relates to visceral pain responses arising from the gut, evidences indicate a potential modulatory role for σ_1Rs. In this sense, σ_1Rs selective antagonists were effective preventing visceral pain-related responses elicited by intracolonic capsaicin in mice.[20]

Taking into account these considerations, the aims of the present study were to assess the potential modulatory role of σ_1Rs on intestinal inflammation and the development of inflammation-associated hypersensitivity in a murine model of dextran sulfate sodium (DSS)-induced colitis. With this objective, we assessed the development of colitis and inflammation-associated visceral and somatic hypersensitivity in σ_1R knockout (KO) mice compared to wild-type (WT) animals. Moreover, colitis-associated changes in peripheral (colon) and central (spinal cord) sensory-related markers implicated in pain processing and sensitization were also assessed.

MATERIALS AND METHODS

Animals

Male WT C57BL/6J mice (n=28, Charles River Laboratories, Lyon, France) and σ_1R KO C57BL/6J mice (n=31, Laboratorios Esteve S.A.., Barcelona, Spain), both aged 6-weeks at the time of starting the studies, were used.

Mice were group-housed in standard cages (four-six mice per cage) and maintained under standard condition of photoperiod (12:12 h light-dark cycle) and climate (20-22 ºC, 40-70% humidity), with *ad libitum* access to a standard diet and tap water, except when receiving DSS. Mice were allowed to acclimatize to the animal facility for at least 1 week before starting the studies. All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocols 3039 and 3957) and the Generalitat de Catalunya (protocols 8823 and 9915).

Colitis induction

A solution of DSS (45 kDa; 2% concentration in water; TdB Consultancy AB, Uppsala, Sweden) was used to induce colitis. Fresh DSS solutions were prepared daily during the 5-day treatment period (from experimental day 0 to day 5). Following this protocol animals develop an acute colitis (7-8 days after starting DSS exposure) that progresses to chronicity. Similar protocols have been used in previous studies in mice to induce colitis.[25–29] Normal tap water was used as the control treatment.

Evaluation of referred mechanical hypersensitivity: von Frey test

Animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which von Frey monofilaments (bending force range from 0.04 to 2 g; North Coast Medical, Inc.; Gilroy, Ca, USA) were applied for pain assessment. Pain sensitivity was evaluated after a 30 min habituation period to the testing environment. Referred pain was assessed in two separate regions, the abdominal wall and the hind paw. When assessing sensitivity of the abdominal wall the perianal and external genitalia areas were avoided, concentrating the mechanical stimulation on

the lower and mid abdomen, as previously reported.[20,30] Paw sensitivity was quantified by measuring the hind paw withdrawal response to punctate mechanical stimulation, as previously described.[18,24] In all cases, pain thresholds were determined using the up-down method paradigm and represent the mechanical stimulus that produces 50% of maximal response.[31] Data obtained were normalized to a baseline measurement (taken as 1), taken 24 h before starting the experimental procedures (Fig. 1). All measurements were performed twice, with a 30-min recovery period in between, by two independent investigators. The mean values of the two observations were taken, for each animal, as the measure of pain sensitivity.

Experimental protocol

WT and σ_1R KO mice were randomly divided into 2 experimental groups per genotype (n=12-19 per group). In a random assignment, the experimental groups received tap water or a solution of 2% DSS during a 5-day period (days 0–5). After DSS/water exposure, all animals received normal water and were allowed to recover for a 2-day (acute inflammatory phase) or a 16-day period (chronic inflammatory phase) before euthanasia. Individual body weight, general state and the presence of clinical signs were assessed on a daily basis throughout the study. Von Frey test was performed 4 times during the experimental procedure: the day before starting the administration of DSS (taken as a basal measure of sensitivity, day -1), at approximately half of time of DSS exposure (day 3), at the end of acute inflammatory phase (day 7) and at the end of the experiment (chronic inflammatory phase, day 21). Animals were euthanized for the collection of samples immediately after the last von Frey test evaluation (see below). See Fig. 1 for details of the experimental protocol.

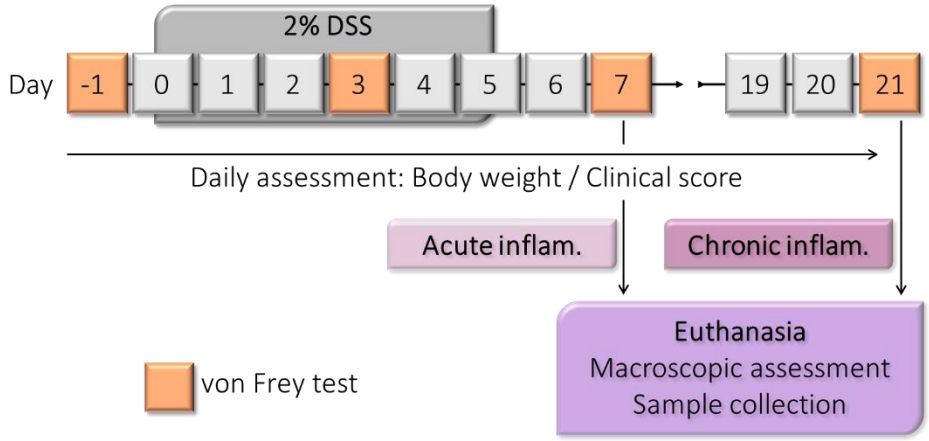

Figure 1. Schematic representation of the experimental protocols followed in the study.

Samples collection

Immediately after the last von Frey test (days 7 or 21, for the acute and chronic phase, respectively), mice were deeply anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and euthanatized by exsanguination through intracardiac puncture followed by cervical dislocation. Thereafter, a medial laparotomy was performed, the ceco-colonic region localized and the cecum and colon dissected. Afterward, two tissue samples from the proximal-middle colon (about 1.5 cm each) were collected. A sample was frozen immediately in liquid nitrogen and a second sample was fixed in 4% paraformaldehyde. After an overnight fixing, tissues were paraffin embedded and 5-µm-thick sections were obtained. The lumbar enlargement (L3-S2) of spinal cord was dissected and frozen immediately in liquid nitrogen. Frozen samples were stored at -80 ºC until analysis. In addition, the liver, the adrenal glands, the thymus, and the spleen were dissected and weighed. Serum was obtained by centrifugation of blood samples (15 min, 10000 g, 4 ºC) and maintained at -80 ºC until analysis.

Clinical and macroscopic assessment of inflammation

Clinical assessment of inflammation included daily monitoring of body weight, appearance of feces and general health condition.[26] A score (0–8) was assigned for health condition (including hunch posture, piloerection, fecal consistency and aspect of the anus); where 0 indicates normal activity/fur/normal feces/normal anus, 1 indicates abnormal gait/bristly fur/wet/watery feces/wet anus and 2 indicates prostrated animal/dirty fur/watery diarrhea/bloody rest on anus. At necropsy, the macroscopic appearance of the colon was scored following previously published procedures.[26] Briefly, the presence of inflammatory signs (inflammatory score): consistency of fecal contents (score 0–3); presence of visible fecal blood (score 0–3); evidence and extent of edema (0–3); wall thickness (0–3); tissue stiffness (0–2) and presence of ulcerations (0–1) were assessed; resulting in a maximum total score of 15.

Histological studies

For histological examination, hematoxylin-eosin-stained sections from the colon were obtained following standard procedures. A histopathological score (ranging from 0, normal, to 12, maximal alterations) was assigned to each animal.[32] Specifically, parameters scored included: epithelial structure (0: normal; 1: mild alterations of the villi; 2: local villi destruction and/or fusion; 3: generalized villi destruction and/or fusion), structure of the crypts (0: normal; 1: mild alterations of the crypts; 2: local destruction of the crypts; 3: generalized destruction of the crypts), presence of edema (0: normal; 1: mild local edema in submucosa and/or lamina propria; 2: moderate diffuse edema in submucosa and/or lamina propria; 3: severe generalized edema in submucosa and/or lamina propria), and presence of inflammatory infiltrate (0: normal; 1: mild localized

infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate). Scoring was performed on coded slides by two independent researchers and the mean value of the two scores was taken as the final score per animal.

Serum haptoglobin

Serum concentrations of haptoglobin were determined using a commercial ELISA kit, following manufacturer's instructions (sensitivity; 0.005 mg/ml; intraassay variability: 5.3–6.3%; interassay variability: 4.1–5.7%; "PHASE" TM Haptoglobin Assay; Tridelta Development Limited, Maynooth, County Kildare, Ireland).

Gene expression using Quantitative Reverse Transcription-PCR

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit (Ambion/Applied biosystems, Foster City, CA, USA). RNA was purified by via precipitation with lithium chloride.[33] Later, a two-step quantitative real-time PCR (RT-qPCR) was performed. RNA samples were converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The PCR reaction mixture was incubated on the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad). All samples were assayed in triplicate. The cycle thresholds for each sample were obtained and data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with the WT vehicle group serving as the calibrator.[34] TaqMan® gene expression assays (hydrolysis probes, Applied Biosystems) used included: cannabinoid receptors 1 (CB1) (Mm01212171_s1) and 2 (CB2) (Mm00438286_m1), interferon γ (INF-γ) (Mm01168134_m1), interleukin 1β (IL-1β) (Mm00434228_m1), 6 (IL-6) (Mm00446190_m1), 10 (IL-10)

(Mm00439614_m1) and 12 (IL-12p40) (Mm00434174_m1), μ-opioid receptor (MOR) (Mm01188089_m1), nerve growth factor (NGF) (Mm00443039_m1), nitric oxide synthase 2 (inducible, iNOS) (Mm00440502_m1), prostaglandin-endoperoxide synthase (Cyclooxygenase 2 (COX-2) (Mm00478374_m1), protease-activated receptor 2 (PAR2) (Mm00433160_m1), serotonin transporter (SERT) (Mm00439391_m1), transient receptor potential vanilloid 1 (TRPV1) (Mm01246302_m1) and 3 (TRPV3) (Mm00455003_m1), tryptophan hydroxylase 1 (TPH1) (Mm00493794_m1) and σ_1 receptor (σ_1R) (Mm00448086_m1). β-2-microglobulin (β2m) (Mm00437762_m1) was used as endogenous reference gene.

Protein expression using Western blot

Dissected spinal cord samples were homogenized by sonication in radioimmunoprecipitation assay (RIPA) buffer and the supernatant was obtained. Equal amounts of protein (30 μg) were fractionated by 10% (w/v) SDS–PAGE and transferred onto a polyvinylidene difluoride membrane, blocked with 5% non-fat dry milk in Tris–Tween 20-buffered Saline (T–TBS) for 1 h. Membranes were then incubated overnight at 4 ºC in 1% non-fat dry milk in T–TBS with rabbit primary polyclonal antibodies recognizing the mitogen-activated protein kinase (MAPK, total ERK ½, 1:30000) or mouse monoclonal antibodies recognizing the activated MAPK (diphosphorylated MAPK, pERK ½, 1:1000). Rabbit polyclonal anti-GAPDH antibody (1:20000) or mouse monoclonal anti-GAPDH antibody (1:80000) were used as a loading control, respectively. After washing with T–TBS, the blots were incubated for 1 h with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:4000) or goat anti-mouse IgG (1:2000). The immunoreactive bands were detected by a peroxidase reaction using an enhanced chemiluminescence method (WesternSure® PREMIUM Chemiluminescent

Substrate, Li-cor) and CDiGit ® Blot Scanner (Li-cor). All antibodies were obtained from Sigma–Aldrich Co. (Madrid, Spain). Quantification was realized with Image Studio™ Lite Software.

Statistical analysis

Data are expressed as mean ± SEM. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Data were analyzed by one-, two or three-way ANOVA, as appropriate, followed, when necessary, by a Bonferroni's multiple comparisons test. Data were considered statistically significant when $P<0.05$. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA) or SPSS program (version 17 for Windows, IBM, Madrid, Spain).

RESULTS

Colitis development in WT and σ_1R KO mice

Regardless the genotype considered, mice receiving water showed a steady and linear increase in body weight, without clinical signs throughout the experimental period (Fig. 2). Conversely, in animals exposed to DSS, body weight loss was observed from experimental day 6, with a peak reduction between days 9 and 10, and a progressive recovery up to day 21, although without reaching the body weight of control animals not exposed to DSS. No differences in this pattern were observed between σ_1R KO and WT mice. In animals exposed to DSS, clinical signs (mainly bristly fur, wet/watery feces and wet anus) consistent with the development of a colitic state appeared with similar temporal pattern to that described for

body weight changes, reaching a maximum at day 9 and disappearing completely by the end of the experimental time (Fig. 2). No genotype-related differences were observed in the incidence and severity of clinical signs. Water intake was similar across experimental groups (data not shown).

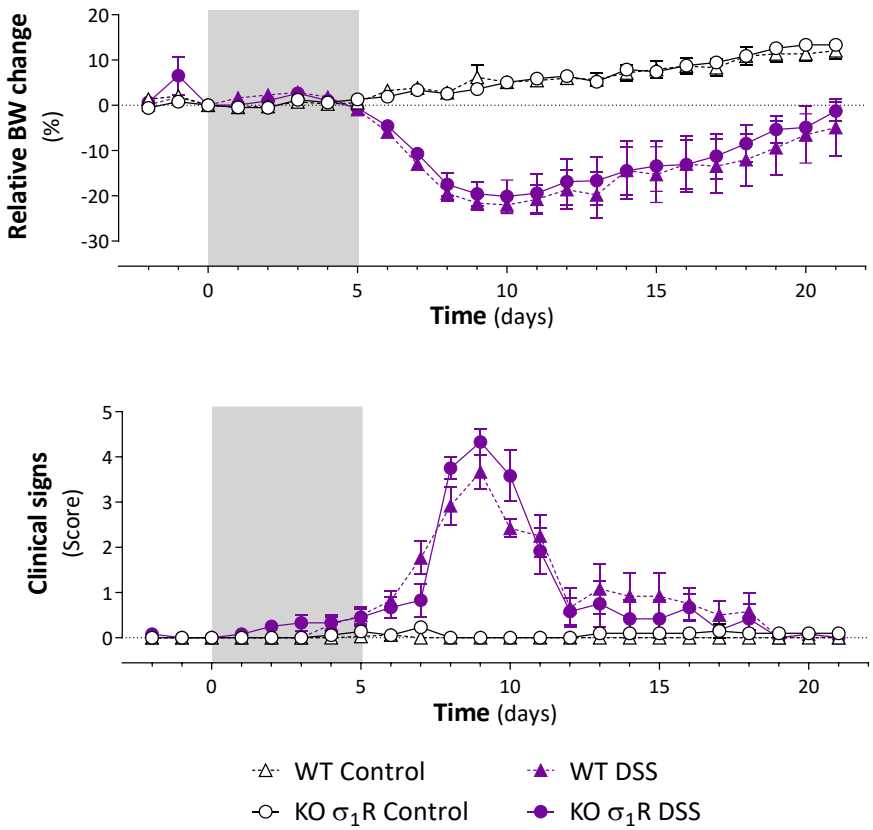

Figure 2. Time-related changes in relative body weight (% change from day 0, taken as 100%, upper panel) and clinical signs (lower panel) in the different experimental groups. Data are mean ± SEM (n=12-18). The DSS-treatment period is indicated by the grey area. BW: body weight.

At necropsy, WT mice receiving DSS showed macroscopic signs of colonic inflammation, both at the acute and chronic phase, characterized by shortening in length and an increase in its relative weight ($P<0.05$ vs. WT mice receiving water; Fig. 3A). Similar changes were observed in σ_1R KO mice. A slight attenuation in inflammation-related parameters was observed in σ_1R KO mice when compared to WT, although statistical significance was not achieved (Fig. 3A).

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Microscopic analysis of colonic tissue samples showed a normal histological structure in control animals. Regardless the genotype considered, exposure to DSS led to a similar significant increase in histopathological scores (Fig. 3B). Colonic alterations were attenuated during the chronic phase, however statistical significance was only achieved for σ_1R KO mice ($P<0.05$ vs. σ_1R KO mice during the acute phase). The improvement observed in σ_1R KO mice was mainly associated to a reduction in the presence of edema (statistically significant interaction by genotype and time on the presence of edema -$P<0.05$-, Fig. 3B). Regardless of the phenotype considered, no significant changes were observed for the relative weight of body organs (data not shown).

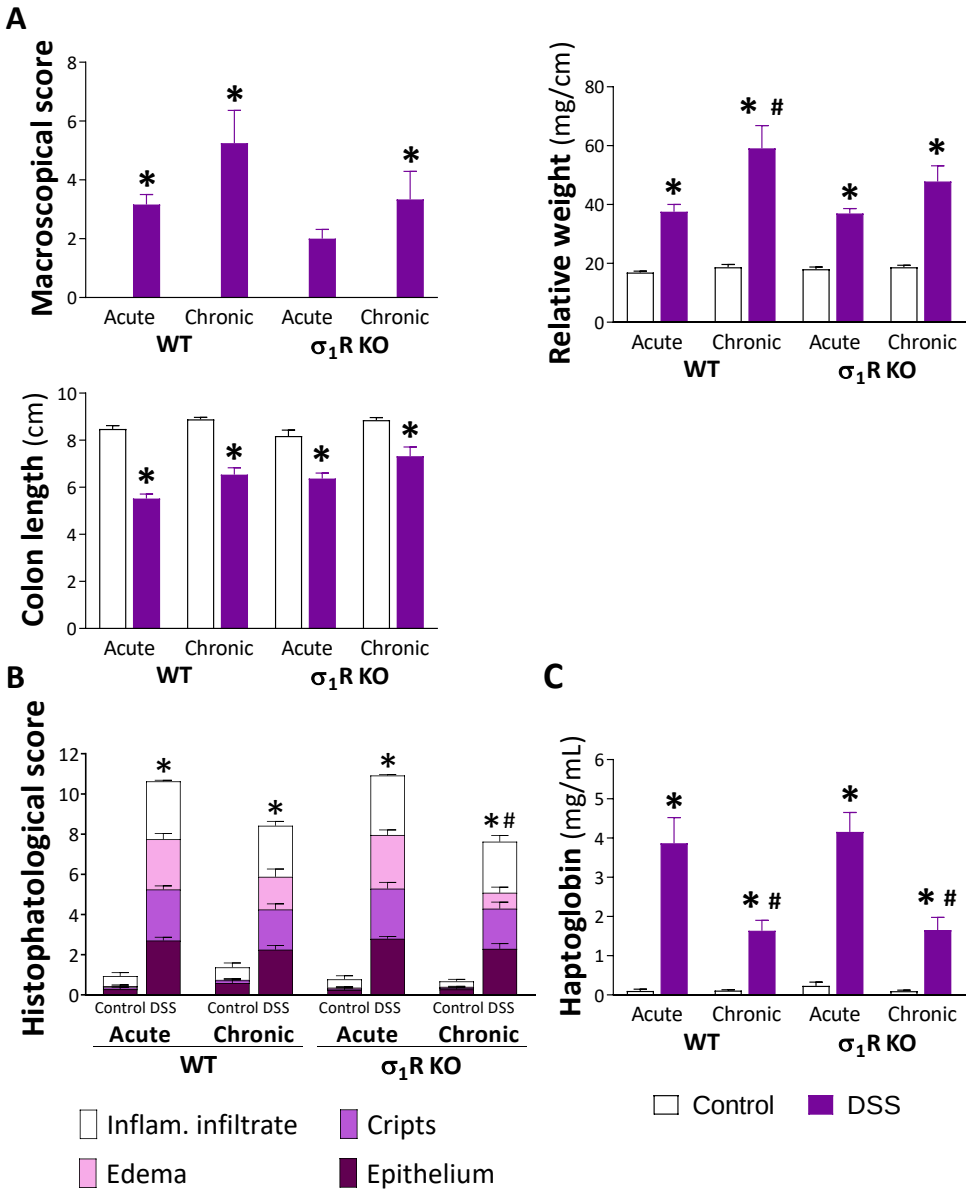


Figure 3. Assessment of colonic inflammation at the time of necropsy in the different experimental groups. A: Macroscopic scores. B: Histopathological scores: inflammatory infiltrate, edema, state of the crypts and epithelial structure. C: Plasma concentrations of the acute phase protein haptoglobin. Data are mean ± SEM of 6-10 animals per group. *: P<0.05 vs. respective control group; #: P<0.05 vs. respective acute DSS-treated group.

The acute phase protein haptoglobin showed a similar increase in WT and σ_1R KO animals during acute inflammation ($P<0.05$ vs. respective control). Haptoglobin levels showed a tendency towards normalization during the chronic phase, although they remained significantly increased when compared to non-inflamed animals (Fig. 3C).

Inflammatory markers are differentially regulated in WT and σ_1R KO mice during colitis

In control conditions, independently of the genotype and time of measurement, expression of the cytokines assessed was detectable in all colonic samples. In WT mice, colitis was associated to an up-regulation of the expression of the pro-inflammatory cytokines INF-γ, IL-1β and IL-6, which was particularly evident during the acute phase of inflammation and persisted during the chronic phase, although relatively attenuated (in all cases $P<0.05$ vs WT control mice, Fig. 4). No significant changes were observed in the local expression of IL-12p40 and IL-10.

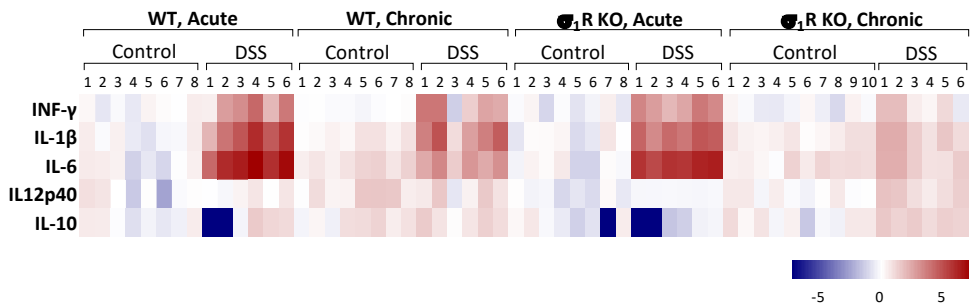


Figure 4. Heat map of the relative mRNA expression of colonic pro- (INF-γ, IL-1β, IL-6 and IL-12p40) and anti-inflammatory cytokines (IL-10) in the different experimental groups. Each vertical line, with a number, corresponds to an individual animal within the corresponding experimental group.

In σ_1R KO mice an up-regulation of INF-γ, IL-1β and IL-6 was observed during the acute phase of inflammation (all P<0.05 vs. non-inflamed animals), but expression levels were basically normalized during the chronic phase (all P>0.05 vs. non-inflamed animals, Fig. 4). A significant up-regulation of IL-10 expression was detected in σ_1R KO mice during the chronic phase (P<0.05 vs. other experimental groups). Similarly to that observed in WT animals, no changes were observed in the expression of IL12p-40.

Colonic expression of iNOS was up-regulated in acute colitic WT mice, although with relatively high variability (P<0.05 vs control WT group). This up-regulation persisted during the chronic phase, although with some attenuation, (Fig. 5). In σ_1R KO mice, iNOS expression was up-regulated during acute colitis, while returning during the chronic phase to the levels detected in non-inflamed animals (Fig. 5).

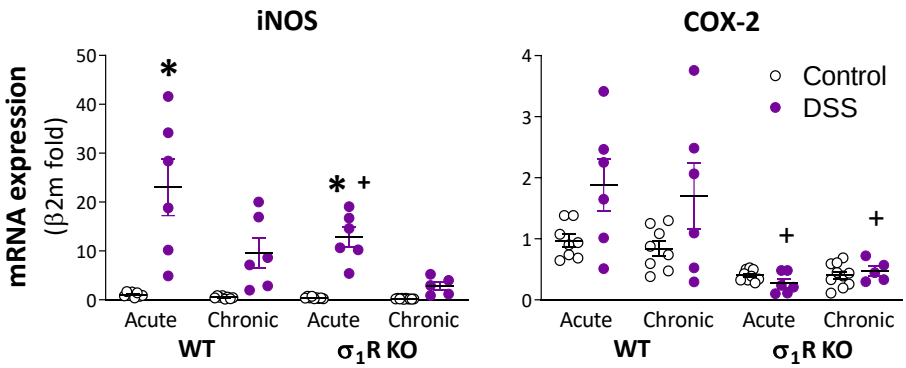


Figure 5. Colonic expression of inflammatory markers: iNOS, and COX-2. Each point represents an individual animal; the horizontal bar with errors represents the mean ± SEM. *: P<0.05 vs. respective control group; +: P<0.05 vs. respective WT DSS-treated group.

In colitic WT mice, 3 out of 5 animals showed COX-2 expression levels above the mean expression of the control group, during either the acute or the chronic phase, thus suggesting a tendency for COX-2 up-regulation during colitis. However, no statistical significance was achieved, probably because of the high interindividual variability observed. No expression changes were observed in σ_1R KO mice, regardless the inflammatory phase considered (Fig. 5).

Colitis-associated mechanical hypersensitivity is attenuated in σ_1R KO mice

Baseline (experimental day -1) abdominal and paw withdrawal thresholds during the von Frey test were similar in WT and σ_1R KO mice (Fig. 6).

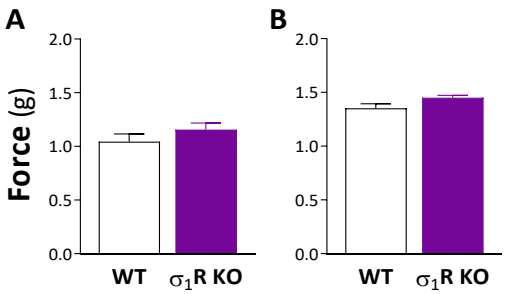


Figure 6. Baseline abdominal (A) and paw (B) withdrawal thresholds in the von Frey test in WT and σ_1R KO mice. Data are mean ± SEM of n=6-10 mice per group.

In healthy WT mice, abdominal and somatic mechanical sensitivity was stable throughout the experimental time (Fig. 7). However, in WT animals receiving DSS a reduction in the withdrawal thresholds was observed from experimental day 3 throughout experimental day 21, indicating the development of mechanical hypersensitivity (in all cases $P<0.05$ vs. control group).

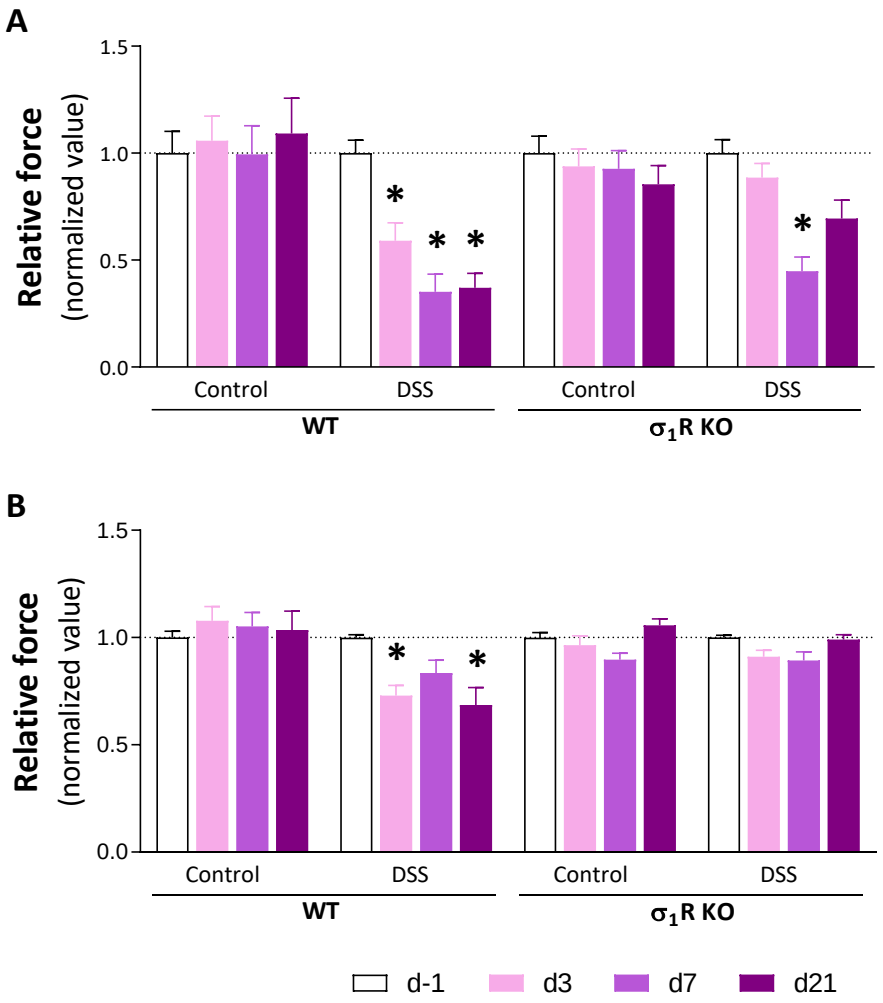


Figure 7. Sensitivity thresholds to mechanical stimulation during DSS-induced colitis in WT and σ_1R KO mice. (A) abdominal and (B) paw withdrawal thresholds in WT and σ_1R KO mice. In all cases, mechanical sensitivity was determined in basal conditions (day -1, d-1) and at experimental days 3 (d3), 7 (d7) and 21 (d21). Data show normalized values (relative force) with respect to basal measurements at experimental day -1, taken as a relative force of 1. Reductions in relative force indicate the development of hypersensitivity. Data are mean ± SEM of n=6-10 mice per group. *: P<0.05 vs. d-1 of respective experimental group.

As it relates to σ_1R KO mice, mechanical thresholds were stable in colitic animals and showed only a transitory reduction in experimental day 7 for abdominal sensitivity ($P<0.05$ vs. control group), with a return towards basal sensitivity on experimental day 21; whereas no changes were observed for somatic sensitivity (Fig. 7).

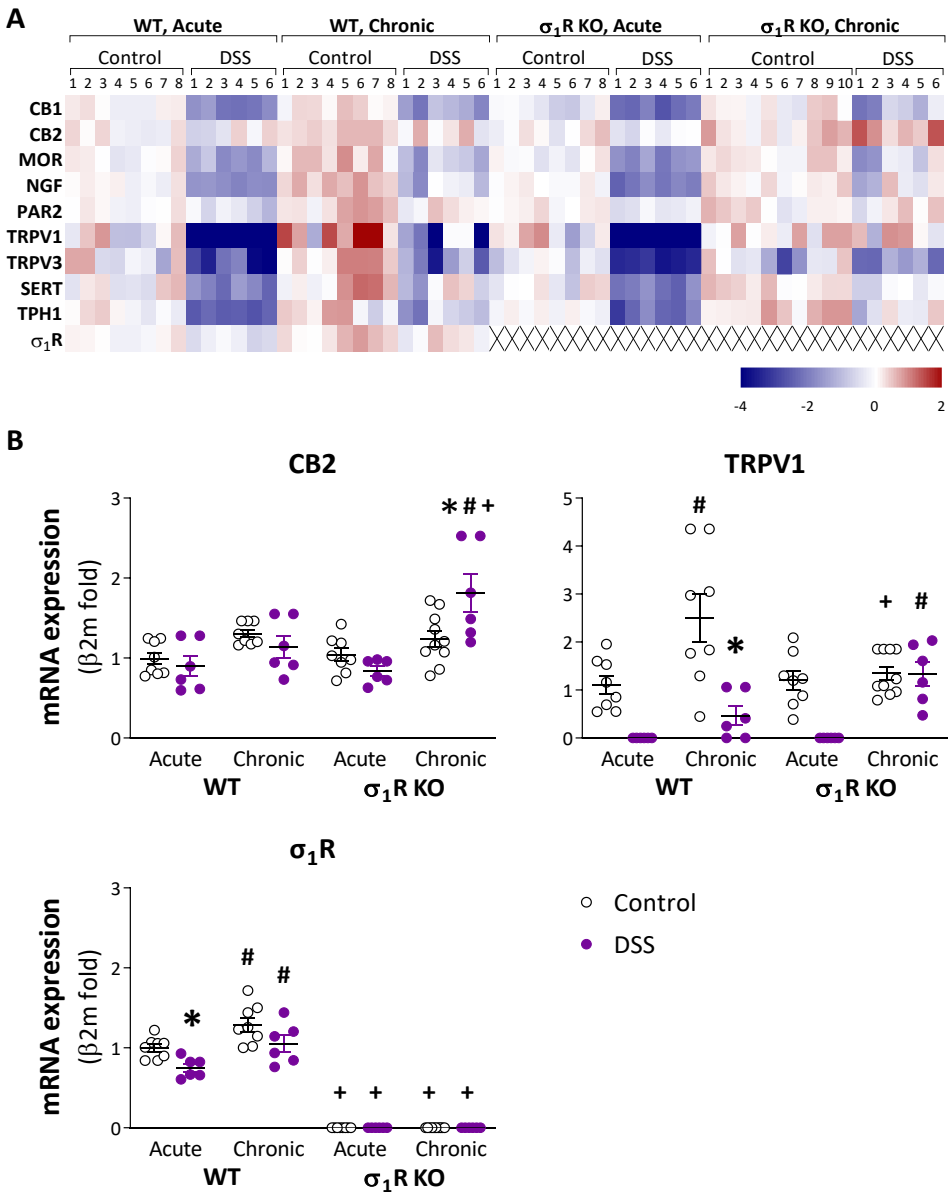
Expression of colonic sensory-related markers is modulated during DSS-induced colitis

Expression of the sensory-related markers assessed was detected in all colonic samples, except for σ_1R, that, as expected, was only found in WT animals. In healthy animals, expression levels of the markers assessed were comparable, regardless the genotype considered.

During colitis in WT mice, there was an overall down-regulation of all sensory markers analyzed when compared with non-inflamed animals. This was particularly evident during the acute phase, with a tendency towards normalization during the chronic phase (Fig. 8). In particular, except for CB2 and PAR-2 which showed no changes, all analyzed markers were down-regulated during both the acute and chronic phases of colitis in WT mice (in all cases $P<0.05$ vs. respective control group). In these animals, expression of σ1R was down-regulated during acute colitis ($P<0.05$ vs. control group), returning to basal expression levels during the chronic phase.

Regarding σ_1R KO mice, an overall down regulation was also observed during the acute phase, but a trend towards baseline levels was observed in the chronic phase of colitis. Nevertheless, CB1, MOR and TPH1 showed a persistent down-regulation during both the acute and the chronic phase of inflammation (in all cases $P<0.05$ vs. respective control group). On the other hand, expression of NGF, SERT, TRPV1 and TRPV3 in σ_1R KO mice was down-regulated only during the acute phase of colitis (in all cases $P<0.05$ vs.

respective control group), while during the chronic phase a slight down-regulation was detected, although statistical significance was not achieved (Fig. 8). In σ_1R KO mice, expression of CB2 was significantly up-regulated in the chronic phase of colitis.



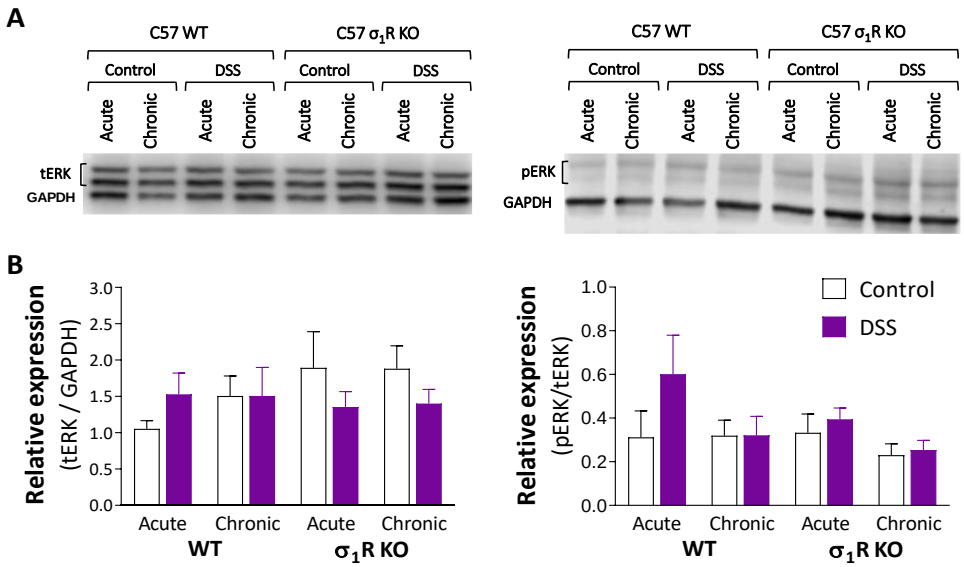


Figure 9. A: Representative Western blots showing tERK (left) and pERK (right) in the different experimental groups. B: Relative spinal expression of tERK (left) and pERK (right). Data are mean ± SEM of n=6 animals per group.

Figure 8. A: Heat map of the relative mRNA expression of sensory-related markers in the different experimental groups. Note that, as expected, no σ_1R expression was detected in σ_1R KO animals. Each vertical line, with a number, corresponds to an individual animal within the corresponding experimental group. "X" denotes samples with no expression detected. B: Detail of the expression changes for the mean genes modified, according to A. Each point represents an individual animal; the horizontal bar with errors represents the mean ± SEM. *: P<0.05 vs. respective control group; #: P<0.05 vs. respective acute control group; +: P<0.05 vs. respective WT group.

ERK expression within the spinal cord is not affected by colitis.

ERK protein was detected in all spinal cord samples, regardless the experimental group considered. Similar levels of tERK and pERK were detected in WT and σ_1R KO mice in control conditions. During colitis, regardless the phase considered, no changes were observed in tERK or pERK content or the ratio pERK/tERK (Fig. 9).

DISCUSSION

In the present study, we assessed the potential role of σ_1Rs in the development of colitis and inflammation-associated changes in referred mechanical sensitivity using a murine model KO for σ_1Rs. Results obtained indicate that σ_1Rs only marginally affected the development of intestinal (colonic) inflammation or its progression from acute to chronic, but they seem to play an important role in the development of inflammation-associated hypersensitivity.

In WT animals, exposure to DSS led to the development of colitis with similar clinical, histopathological and molecular alterations to those previously described.[25,26,35] Moreover, we also observed that the inflammatory condition showed a chronification, characterized by the persistence over time, although with some attenuation, of the structural, molecular and biochemical alterations observed during the acute phase and a remission of the clinical signs. This chronification process coincides with the evolution of DSS-induced colitis previously described for the same strain

of mice[25] and shares similarities with the quiescent phases of inflammatory bowel disease in humans.[35]

In σ_1R KO animals, exposure to DSS led to the induction of colitis with, essentially, the same characteristics as those discussed above for WT mice. Overall, these observations suggest that σ_1Rs play a minor role in the development of intestinal inflammation. Nevertheless, some differences were observed between WT and σ_1R mice, particularly as it relates to structural and molecular parameters during the chronic phase of colitis. Firstly, the presence of submucosal edema was significantly reduced in σ_1R KO vs. WT mice during chronic colitis. This finding agrees with previous data showing a reduction in subepithelial edema in σ_1R KO mice in a model of cyclophosphamide-induce cystitis[36] or the reduction in paw edema, elicited by the intraplantar injection of carrageenan, associated to the blockade of σ_1Rs with specific antagonists.[21,22] Moreover, the attenuation of paw edema might implicate NOS-dependent mechanisms affecting vascular permeability and extravasation.[21,22] Interestingly, in σ_1R KO mice treated with DSS, iNOS expression, which was moderately up-regulated in WT animals with colitis, showed similar expression levels as those detected in non-inflamed controls. This suggests that σ_1Rs might regulate vascular permeability and extravasation, at least partially throughout NO-dependent mechanisms, and thus, exert some modulatory effects on inflammation. Additionally, in σ_1R KO mice, expression of pro-inflammatory cytokines, as well as iNOS and COX-2, was normalized during the chronic phase of colitis, while remaining up-regulated in WT animals. Altogether, these data suggest that σ_1Rs might exert a positive immunomodulatory action, as previously suggested in other models,[21,36,37] likely facilitating the recovery in chronic conditions, at least as it relates to intestinal inflammation.

Compelling evidences implicate σ_1Rs in pain mechanisms.[38] In our experimental conditions, σ_1R KO and WT mice showed similar

mechanosensitivity, as determined by assessing withdrawal thresholds to the mechanical stimulation of the lower abdominal wall, likely reflecting responses associated to the mechanical stimulation of the abdominal wall and the underlying viscera (mainly intestine), or the hind limbs. These observations agree with previous data showing that σ_1R KO mice perceived and responded normally to acute somatic mechanical nociceptive stimuli[18,20] and with pharmacological observations showing that σ_1R ligands, either agonists or antagonists, have no effects by themselves on somatic mechanosensitivity in basal conditions.[13–15] Altogether, these results support the view that σ_1Rs are not involved in normal pain responses. Alternatively, we cannot discard that compensatory mechanisms, associated to the constitutive absence of σ_1Rs, lead to normal basal pain responses in these animals.

Intestinal inflammation has been associated to the development of visceral hypersensitivity as well as referred hyperalgesia in several body regions, including the abdominal wall, tail and hind paws.[1,5,6,39] In agreement with this, results obtained here show that colitic WT mice showed mechanical hypersensitivity, manifested as a persistent reduction in the withdrawal threshold to the mechanical stimulation of the abdominal wall (likely reflecting a combination of somatic hypersensitivity -from the abdominal wall per se- and visceral hypersensitivity of the underlying viscera -intestine-) and the hind limbs. Interestingly, the sensitizing effects of inflammation were significantly attenuated in σ_1R KO mice. Indeed, in σ_1R KO animals, paw sensitivity was not affected during colitis, while at the abdominal level only a transitory state of hypersensitivity was observed during the acute phase of colitis, with a clear tendency towards normalization during the chronic phase. These observations are in agreement with previous data showing a reduction in behavioral responses to visceral pain in σ_1R KO mice after intracolonic administration of

capsaicin[20] or during cystitis[36] and the reduction of nociceptive responses associated to neuropathic pain.[18,40,41] Moreover, these data in σ_1R KO mice further confirm pharmacological observations showing blockade of somatic pain responses by selective σ_1R antagonists.[13,21,22,24,42] Altogether, these data strongly suggest a key involvement of σ_1Rs in the development of inflammation-dependent referred hypersensitivity, consistent with previous reports. Moreover, taking into account the fact that the stimulation of the abdominal wall is likely to implicate somatic and visceral pain-related responses, our observations also support an implication of σ_1Rs in visceral sensitivity.

To further understand the mechanisms implicated in these changes we assessed the local (colon) expression of different sensory-related markers implicated in viscerosensitivity. During colitis, a general down-regulation of sensory markers was observed, regardless the genotype considered. Thus, supporting the development of nociceptive alterations, at least at a molecular level, during inflammation. It is difficult to establish a direct correlation between gene expression changes of pain-related markers and pain-related responses since a down-regulation was detected for both pro- and anti-nociceptive markers. Therefore, the final functional outcome is likely to depend upon the balance between changes in expression that favor or counteract pain-related mechanisms, as previously suggested for other experimental conditions related to intestinal sensitivity.[43,44] Despite this, distinctive changes in some pain-related markers were observed in σ_1R KO animals. Specifically, the up-regulation detected for CB2 during chronic colitis might be of particular significance. Given the anti-nociceptive effects associated to the activation of CB2,[45–47] the up-regulation observed might explain, at least in part, the attenuation of pain-related responses observed in these animals during colitis.

The TRPV family of receptors has been related to inflammation and pain. In particular, TRPV1 expression has been positively correlated with pain severity in patients with quiescent IBD[48] and an up-regulation has also been observed during colonic inflammation,[49] likely contributing to the visceral hypersensitivity observed after colitis.[50] In the present studies, a down regulation of TRPV1 associated with inflammation, either acute or chronic, was observed in WT mice, even though the presence of mechanical hypersensitivity. Moreover, these apparently contradictory observations reinforce the importance of the balance between pro- and anti-nociceptive mechanisms in the final outcome as it relates to pain, as discussed above. Alternatively, and given the pro-nociceptive effects of TRPV1, a down-regulation of the receptor might be interpreted as a compensatory mechanism developed under some conditions (such as acute inflammation) to avoid abnormal excessive pain. Furthermore, recent studies have described interactions between σ_1R and TRPV1 receptors,[51,52] thus indicating that both receptors might interact during states of hypersensitivity facilitating pain. Indeed, σ_1R antagonism results in the negative regulation of the protein expression of TRPV1 in the plasma membrane of sensory neurons and, consequently, a decrease in nociceptive responses.[51] Therefore, the lack of functional σ_1Rs, leading to an altered TRPV1-σ_1R interaction might contribute to de underlying mechanisms explaining the absence of hypersensitivity in σ_1R KO mice.

Sensitization of pain mechanisms can occur at either peripheral and/or central levels. Our results, as discussed above, suggest that peripheral (colonic) changes might contribute to the sensitization processes associated to inflammation. Nevertheless, to assess the potential participation of central (spinal) sensitization, we also assessed ERK phosphorylation at the level of the lumbosacral spinal cord. Lower lumbar and upper sacral segments of the spinal cord represent the main site of

entry to the central nervous system for sensory afferents arising from the colon implicated in pain responses in rodents.[53] Within the spinal cord, ERK phosphorylation is regarded as a key process involved in pain processing and sensitization. Indeed, an increase in spinal phosphorylated ERK (pERK) has been described in several models of somatic[18] and visceral pain.[54] Moreover, σ_1Rs might be implicated in this process since phosphorylation of spinal ERK was attenuated in σ_1R KO mice in a model of neuropathic pain where somatic hypersensitivity was induced by peripheral nerve injury.[18,55] Although these evidences, in the present experimental conditions we did not detect changes in ERK phosphorylation neither at the acute nor the chronic phase of colitis, regardless the genotype considered. This might suggest the involvement of different mechanisms, with different involvement of ERK and/or different kinetics in the phosphorylation process, as it relates to the development of sensitization during inflammatory and neuropathic pain.

In summary, the present data show that σ_1Rs play a minor role in modulating intestinal (colonic) inflammation. Although some molecular markers of inflammation were attenuated in σ_1R KO mice, these changes did not translate in an evident clinical improvement and only correlated with a moderate reduction in submucosal edema. As expected, inflammation was associated to the development of hypersensitivity, likely of both somatic and visceral origin. These pain-related alterations were attenuated in σ_1R KO mice, thus confirming a role of σ_1Rs in the development of hypersensitivity. Overall, these observations suggest that σ_1Rs might represent a feasible target for the treatment hypersensitivity associated to intestinal inflammation.

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CHAPTER 2

Genetic and pharmacological blockade of σ_1Rs attenuate inflammation-associated hypersensitivity during acute colitis in CD1 mice

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ABSTRACT

Sigma-1 receptors (σ_1Rs) are implicated in nociception, including pain sensitization, and inflammation. We assessed the role of σ_1Rs on acute colitis-associated hypersensitivity using both genetic (constitutive knockout) and pharmacological blockade of the receptor.

Colitis was induced by exposure to dextran sodium sulfate (DSS, 5%) in wild-type (WT) and σ_1R KO mice. A von Frey test was used to assess referred mechanosensitivity (abdominal and plantar withdrawal responses). Mechanosensitivity was determined before, during and after DSS exposure. Effects of the selective σ_1R antagonists BD1063 and E-52862 were also assessed in WT animals. Expression of immune and sensory-related markers (RT-qPCR, Western blot) was assessed in colon and lumbosacral spinal cord.

Genetic ablation or pharmacological blockade of σ_1Rs attenuated acute colonic inflammation in a similar manner. Mechanosensitivity was similar in WT and σ_1R KO mice before colitis. In WT mice, but not in σ_1R KO, acute colitis was associated to the development of referred mechanical hypersensitivity, manifested as a reduction of the withdrawal thresholds to mechanical probing (paw and abdominal wall). In WT mice, BD1063 and E-52862 blocked acute colitis-associated hypersensitivity. A genotype- and treatment-related differential regulation of sensory related markers was detected locally (colon) and within the lumbosacral spinal cord.

These data suggest that σ_1Rs are involved in development of acute intestinal inflammation and the associated referred mechanical hypersensitivity. Selective modulation of sensory-related pathways within the colon and spinal cord might be part of the underlying mechanisms. These observations support the pharmacological use of σ_1R antagonists for the treatment of intestinal inflammation-induced hypersensitivity.

INTRODUCTION

Visceral pain is a common symptom in inflammatory and functional gastrointestinal disorders (FGDs).[1–3] In these conditions, multiple factors, including the release of inflammatory mediators combined with a disturbed epithelial barrier function, contribute to the sensitization of peripheral nerve endings within the gut wall, thus resulting in altered visceral sensory perception and abdominal pain.[3,4] Moreover, visceral pain typically refers to non-visceral somatic structures due to the convergence of visceral and somatic nerve fibers in the same second order neurons within the dorsal horn of the spinal cord.[5,6] Therefore, states of visceral pain/hypersensitivity are frequently associated to referred somatic hypersensitivity. Although this phenomena, visceral and somatic pain are different entities. So far, specific pharmacological treatments against pain arising from the gastrointestinal tract have not been approved, and clinicians often use the same medications as for somatic pain.[1]

In this respect, several studies suggest sigma 1 receptors (σ_1Rs) as an effective pharmacological target for pain treatment, including visceral pain. The σ_1R is a ligand-regulated molecular chaperone which has been implicated in a variety of physiological and pathological conditions.[7,8] σ_1Rs exert a series of neuromodulatory effects related to the modulation of pain mechanisms. Interestingly, several evidences suggest that pharmacological agonism or antagonism of σ_1R does not interfere with the perception of several stimuli in basal conditions.[9,10] Therefore, σ_1Rs will not interfere with normal pain responses. However, under pathological conditions with states of altered pain signaling, such as in some chemical and neuropathic somatic pain models, genetic or pharmacologic blockade σ_1Rs could have a positive impact on sensory mechanisms leading to the modulation of pain behavior

and hypersensitivity.[9,11,12] Following these observations, further studies have validated the pharmacological blockade of σ_1R as an effective option to treat central and peripheral inflammatory pain.[8,13,14] Although these evidences, similar studies related to visceral pain are scarce. In a model of visceral pain induced by intracolonic injection of capsaicin and in a model of cyclophosphamide-induced cystitis, the constitutive absence of σ_1Rs (σ_1R knockout -KO- mice) or the blockade of the receptor with selective antagonisms resulted in a reduction in the number of pain-related behaviors,[15,16] thus indicating a role in visceral pain mechanism.

Taking into account these considerations, this work aims to assess the potential role of σ_1Rs in the development of acute intestinal inflammation and inflammation-related hypersensitivity in a murine model of dextran sodium sulfate (DSS)-induced colitis. For this purpose, two approaches were followed: i) the genetic blockade of σ_1Rs, based on the constitutive absence of the receptor, by using KO mice; and ii) the pharmacological blockade of σ_1Rs, by the use of the selective σ_1R antagonist BD1063 and E-52862. Moreover, to gain insight into the underlying mechanisms associated to σ_1R-mediated effects, changes (gene and protein expression) in peripheral (colon) and central (spinal cord) sensory-related markers involved in pain processing and sensitization mechanisms were also characterized.

MATERIALS AND METHODS

Animals

Adult male CD1 mice (n=90; Crl:CD1(ICR); Charles River, France) and σ_1 receptor knockout CD1 mice (n=16; Esteve Pharmaceuticals S.A., Barcelona,

Spain), 6-7 week-old at the beginning of the studies, were used. Animals were group-housed in standard cages (4-6 animals per cage) and maintained in conventional conditions in an environmentally controlled room (20–22 ºC, 12 h light:dark cycle), with food and water ad libitum, except when receiving DSS. Mice were allowed to acclimatize to the animal facility for at least 1 week before starting the studies. All experiments were performed in accordance with EU and local regulations and were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocols 3039 and 3957) and the Generalitat de Catalunya (protocols 8823 and 9915).

Colitis induction

A solution of DSS (45 kDa; 3% concentration in water; TdB Consultancy AB, Uppsala, Sweden) was used to induce colitis. Fresh DSS solutions were prepared daily during the 5-day treatment period. Following this protocol, CD1 mice develop a flare of acute colitis that peaks 7-8 days after starting the exposure to DSS. Similar protocols have been used in previous studies in mice to induce colitis.[17,18] Control mice received normal tap water.

Drugs

The selective σ_1R antagonists BD1063 (1-[2-(3,4-dichlorophenyl) ethyl]-4-methylpiperazine dihydrochloride)[19] and E-52862 (also named S1RA or MR309; 4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine)[20] were used (Laboratorios Dr. Esteve S.A., Barcelona, Spain). 6-Thioguanine (6-TG) (Sigma-Aldrich, St Louis, MO, USA) and 5-aminosalicylic acid (mesalazine, 5-ASA) (Cayman Chemical, Ann Arbor, MI, USA) were used as positive controls, given their demonstrated anti-inflammatory activity within the gastrointestinal tract.[21,22] All drugs were dissolved immediately

before use in a 0.5% solution of hydroxypropylmethyl cellulose (HPMC; Sigma-Aldrich) in distilled water. Doses were selected based on previously published data and/or pilot studies in our experimental conditions

Evaluation of referred mechanical hypersensitivity: von Frey test

Mechanical sensitivity was determined using the classical von Frey test, following, with minor modifications, previously published protocols. Animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which von Frey monofilaments (bending force range from 0.04 to 2 g, North Coast Medical, Inc.; Gilroy, Ca, USA) were applied. Pain sensitivity was assessed after a 30 min habituation period to the testing environment. Referred pain was determined in two separate body regions, the hind paws and the abdominal wall. When testing sensitivity of the abdominal wall, the perianal and external genitalia areas were avoided, concentrating the stimulation on the lower and mid abdomen, as commonly reported in the literature.[15,16,23] Paw sensitivity was quantified by measuring the hind paw withdrawal response to punctate mechanical stimulation, as escribed elsewhere.[9,10] Pain thresholds were determined using the up-down method paradigm and represent the mechanical stimulus that produces 50% of the maximal response.[24,25] Data were normalized to a baseline measurement (taken as 1), obtained 24 h before starting the experimental procedures (Fig. 1). All measurements were performed twice, with a 30-40 min recovery period in between, by two, treatments-blinded, independent investigators. For each animal, the mean value of the two observations was taken as the measure of pain sensitivity.

Experimental protocol

In a first study, wild-type (WT) and σ_1R KO mice were randomly divided into 2 experimental groups per genotype, receiving, in a random assignment, tap water or a solution of 3% DSS during a 5-day period (days 0–5) (Table 1). Animals were euthanized after a 2-day recovery period following DSS exposure (experimental day 7) for the assessment of colitis and the obtention of samples (Fig. 1), coinciding with the peak of inflammation.[18,26,27] Throughout the study, individual body weight, the general state and the presence of clinical signs associated to de development of colitis were assessed on a daily basis. Mechanical sensitivity (von Frey test) was assessed at the beginning of the studies (experimental day -1, as a baseline measure of sensitivity), during DSS exposure (experimental day 3) and at the peak of inflammation (immediately before euthanasia, experimental day 7) (Fig.1).

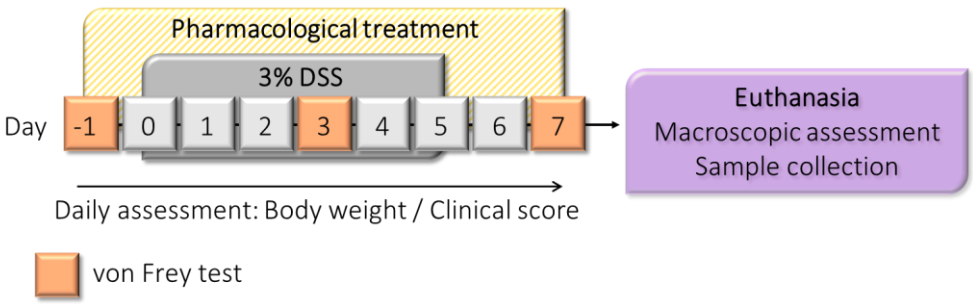


Figure 1. Details of the experimental protocols followed. See also Table 1 for details on the treatments applied. VF: von Frey test.

In a second study, WT mice were randomly divided in five experimental groups according to the treatment received (Table 1): vehicle (0.5% HPMC in distilled water, 5 ml/kg, po), BD1063 (20 mg/Kg, po, BID), E-52862 (20

mg/Kg, po, BID), 6-TG (2 mg/kg, po, SID) or 5-ASA (50 mg/kg, po, BID). Treatments were applied, in a preventive manner, starting 1 day before the initiation of the colitis induction protocol and were administered orally once (9:00-10:00 a.m.) or twice daily (9:00-10:00 a.m. and 18:00-19:00 p.m.), as indicated. Doses and treatment protocols were based on pilot studies or previous reports showing efficacy in similar experimental conditions. For each treatment group, animals were randomly divided in 2 subgroups, receiving either tap water or 3% DSS during a 5-day period (experimental days 0–5) for the induction of colitis (Fig. 1; Table 1). As in the previous study, animals were euthanized after a 2-day recovery period following DSS exposure (experimental day 7) for the assessment of colitis and the obtention of samples (see below). Throughout the study, individual body weight, the general state and the presence of clinical signs associated to de development of colitis were assessed on a daily basis. Mechanical sensitivity (von Frey test) was assessed at the beginning of the studies (experimental day -1, as a baseline measure of sensitivity), during DSS exposure (experimental day 3) and at the peak of inflammation (immediately before euthanasia, experimental day 7) (Fig.1).

Samples collection

Immediately after the last von Frey test (experimental day 7), mice were deeply anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and euthanatized by exsanguination through intracardiac puncture followed by cervical dislocation. Thereafter, a medial laparotomy was performed, the ceco-colonic region localized and the cecum and colon dissected. Two tissue samples from the proximal-middle colon (about 1.5 cm each) were collected. A sample was frozen immediately in liquid nitrogen. A second sample was fixed in 4% paraformaldehyde. After an overnight fixing, tissues were paraffin embedded and 5-µm-thick sections were obtained.

Lumbosacral (L3-S2) spinal cord samples were also collected and frozen immediately in liquid nitrogen. Frozen samples were stored at -80 ºC until analysis. During the necropsy, the liver, the adrenal glands, the thymus, and the spleen were dissected and weighed.

Table 1. Summary of experimental groups.

	Genotype	Colitis induction	Treatment	n
Study 1	Wild-type	No (Tap water)	-	6
		Yes (3% DSS)	-	6
	σ_1R knockout	No (Tap water)	-	8
		Yes (3% DSS)	-	8
Study 2	Wild-type	No (Tap water)	Vehicle (5 ml/kg, po, DIB)	15
			BD1063 (20 mg/Kg, po, BID)	6
			E-52862 (20 mg/Kg, po, BID)	9
			6-TG (2 mg/Kg, po, SID)	6
			5-ASA (50 mg/Kg, po, BID)	6
		Yes (3% DSS)	Vehicle (5 ml/kg, po, DIB)	15
			BD1063 (20 mg/Kg, po, BID)	6
			E-52862 (20 mg/Kg, po, BID)	9
			6-TG (2 mg/Kg, po, SID)	6
			5-ASA (50 mg/Kg, po, BID)	6

Clinical and macroscopic assessment of inflammation

Clinical assessment of inflammation included daily monitoring of body weight, appearance of faeces and general health condition.[18] A score (0–8) was assigned to the health condition (including hunch posture, piloerection, faecal consistency and anal inflammation); where 0 indicates normal activity/fur/faecal content/no anal inflammation, 1 indicates abnormal gait/bristly fur/wet anus/loose faecal content and 2 indicates prostrated animal/dirty fur/watery or bloody rest on anus/watery diarrhoea. At

necropsy, the macroscopic appearance of the colon was scored (macroscopic inflammatory score, 0-15) according to procedures established elsewhere.[18] In brief, consistency of fecal contents (score 0–3); presence of visible fecal blood (score 0–3); evidence and extent of edema (0–3); wall thickness (0–3); tissue stiffness (0–2) and presence of ulcerations (0–1) were assessed.

Histological studies

For histological examinations, hematoxylin-eosin-stained sections from the colon were obtained following standard procedures. A histopathological score (ranging from 0, normal, to 12, maximal alterations) was assigned to each animal.[28] Parameters scored included: epithelial structure (0: normal; 1: mild alterations of the villi; 2: local villi destruction and/or fusion; 3: generalized villi destruction and/or fusion), structure of the crypts (0: normal; 1: mild alterations of the crypts; 2: local destruction of the crypts; 3: generalized destruction of the crypts), presence of edema (0: normal; 1: mild local edema in submucosa and/or lamina propria; 2: moderate diffuse edema in submucosa and/or lamina propria; 3: severe generalized edema in submucosa and/or lamina propria), and presence of inflammatory infiltrate (0: normal; 1: mild localized infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate). Scoring was performed on coded slides by two independent researchers, the mean value of the two scores was taken as the final score per animal.

Gene expression: Quantitative Reverse Transcription-PCR

Total RNA was extracted from frozen tissue of colon and spinal cord samples using TRI reagent with Ribopure Kit (Ambion/Applied biosystems, Foster City, CA, USA). Later, a two-step quantitative real-time PCR (RT-

qPCR) was performed. RNA samples were converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The PCR reaction mixture was incubated on the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad). All samples were assayed in triplicate. The cycle thresholds for each sample were obtained and data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with the WT control (for the analysis of samples originated in the first experiment, including σ_1R KO mice) or the vehicle group (for the analysis of samples generated in the second experiment, pharmacological treatments in WT mice) serving as the calibrator.[29] TaqMan® gene expression assays (hydrolysis probes, Applied Biosystems) for interferon γ (INF-γ) (Mm01168134_m1), interleukin 1β (IL-1β) (Mm00434228_m1) interleukin 6 (IL-6) (Mm00446190_m1), interleukin 10 (IL-10) (Mm00439614_m1), interleukin 12 (IL-12p40) (Mm00434174_m1), cannabinoid receptor 1 (CB1) (Mm01212171_s1) and 2 (CB2) (Mm00438286_m1), μ-opioid receptor (MOR) (Mm01188089_m1), tryptophan hydroxylase 1 (TPH1) (Mm00493794_m1), transient receptor potential vanilloid 1 (TRPV1) (Mm01246302_m1), nerve growth factor (NGF) (Mm00443039_m1) and σ_1 receptor (σ_1R) (Mm00448086_m1) were used. β-2-micro-globulin (Mm00437762_m1) was used as endogenous reference gene.

Protein expression: Western Blot

Lumbosacral spinal cord samples were homogenized by sonication in radioimmunoprecipitation assay (RIPA) buffer and the supernatant was obtained. Equal amounts of protein (30 μg) were fractionated by 10% (w/v) SDS–PAGE and transferred onto a polyvinylidene difluoride membrane, blocked with 5% non-fat dry milk in Tris–Tween 20-buffered Saline (T–TBS) for 1 h. Membranes were then incubated in 1% non-fat dry milk in T–TBS overnight at 4 ºC with primary antibodies against the protein targets of

interest (Table 2). Mouse or rabbit anti-GAPDH antibody or anti-β-tubulin antibody were used as a loading control depending upon the protein of interest and the origin of the primary antibody used. After washing with T–TBS, the blots were incubated for 1 h with secondary peroxidase–conjugated antibodies (see Table 2). Immunoreactive bands were detected by a peroxidase reaction using an enhanced chemiluminescence method (WesternSure® PREMIUM Chemiluminescent Substrate, Li-cor) and CDiGit ® Blot Scanner (Li-cor). Quantification of Western blots was done with Image Studio™ Lite Software.

Table 2. Details of antibodies used for Western blot.

Type	Reactivity	Host	Dilution	Source
Primary	β-tubulin	Goat polyclonal	1:1000	Santa Cruz Biotech. INC. (#sc-9935)
Primary	CaMKII	Mouse monoclonal	1:2000	Invitrogen (#MA1-048)
Primary	pCaMKII	Mouse monoclonal	1:1000	Invitrogen (#MA1-047)
Primary	GFAP	Mouse monoclonal	1:10000	Cell signaling (#3670)
Primary	GAPDH	Mouse monoclonal	1:80000	Sigma-Aldrich (#G8795)
Primary	GAPDH	Rabbit polyclonal	1:20000	Sigma-Aldrich (#G9545)
Primary	tERK	Rabbit polyclonal	1:30000	Sigma-Aldrich (#M5670)
Primary	pERK	Mouse monoclonal	1:1000	Sigma-Aldrich (#M8159)
Primary	p38	Rabbit polyclonal	1:1000	Invitrogen (#AHO1202)
Primary	pp38	Rabbit monoclonal	1:1000	Invitrogen (#MA5-15177)
Secondary	Anti-Mouse IgG	Goat polyclonal	1:2000	Sigma-Aldrich (#A5278)
Secondary	Anti-Rabbit IgG	Goat polyclonal	1:4000	Sigma-Aldrich (#A9169)
Secondary	Anti-Goat IgG	Donkey polyclonal	1:2000	Abcam (#ab97110)

Statistical analysis

Data are expressed as mean±SEM. A robust analysis (one iteration) was used to obtain mean±SEM for RT-qPCR data. Data were analyzed by one-, two- or three-way ANOVA, as appropriate, followed, when necessary, by a Bonferroni's multiple comparisons test. Data were considered statistically significant when $P<0.05$. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) or SPSS program (version 17 for Windows, IBM, Madrid, Spain).

RESULTS

σ_1R KO CD1 mice develop an attenuated acute colitis

WT CD1 mice exposed to 3% DSS for 5 days showed clinical signs consistent with the development of acute colitis, including body weight loss, piloerection, loose feces/watery diarrhea, and presence fecal blood. These changes were particularly evident at experimental days 6 and 7, during the wash out period after DSS exposure. Clinical signs and body weight loss were attenuated in σ_1R KO animals exposed to DSS (Fig. 2). A Three-way ANOVA revealed significant effects for time ($P<0.05$), genotype ($P<0.05$) and DSS exposure ($P<0.05$) and significant effects interactions between the three factors ($P<0.05$), thus indicating a different response to DSS over time in both genotypes. Water intake was similar across experimental groups (data not shown).

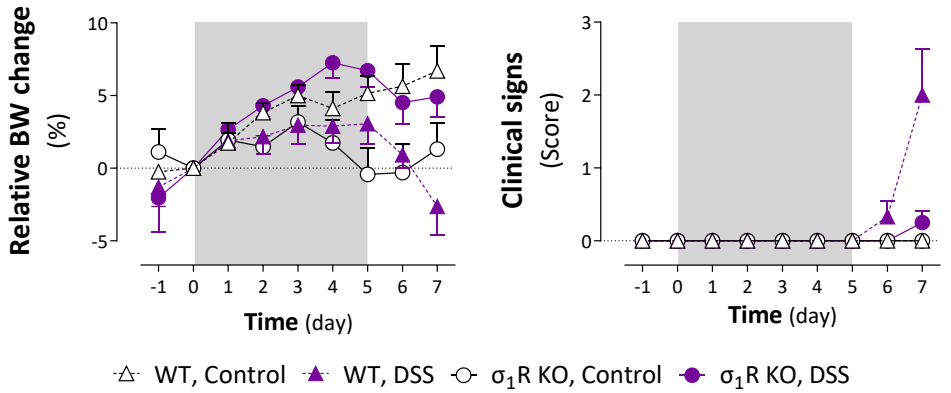


Figure 2. Changes in relative body weight (% change from day 0, taken as 100%) and clinical signs in WT and σ_1R KO mice. Data are mean±SEM (n=6-8). The DSS-treatment period is indicated by the grey area.

At necropsy, macroscopic signs of inflammation, together with shortening and increased relative weight of the colon were observed in WT mice receiving DSS (all $P<0.05$ vs. non-colitic animals; Fig. 3). These inflammation-related parameters were significantly attenuated in σ_1R KO mice ($P<0.05$ vs. DSS-exposed WT mice for the three parameters assessed; Fig. 3).

Microscopic analysis of colonic tissue samples showed a normal histological structure in control animals. Significant histopathological alterations were observed in WT mice exposed to DSS, reaching a total score of 10.04±1.44 ($P<0.05$ vs. non-colitic WT mice: 0.63±0.21; Fig. 4). In healthy σ_1R KO mice an essentially normal histological structure was observed. Significant histopathological alterations were observed in σ_1R KO mice exposed to DSS (histopathological score; 5.91±0.92; $P<0.05$ vs healthy σ_1R KO mice: 1.57±0.34), although a significant attenuation was observed vs. WT animals exposed to DSS ($P<0.05$, Fig. 4). This attenuation was

particularly evident as it relates to the presence of submucosal edema (σ_1R KO mice: 0.5±0.16; P<0.05 vs. WT mice: 2.54±0.37).

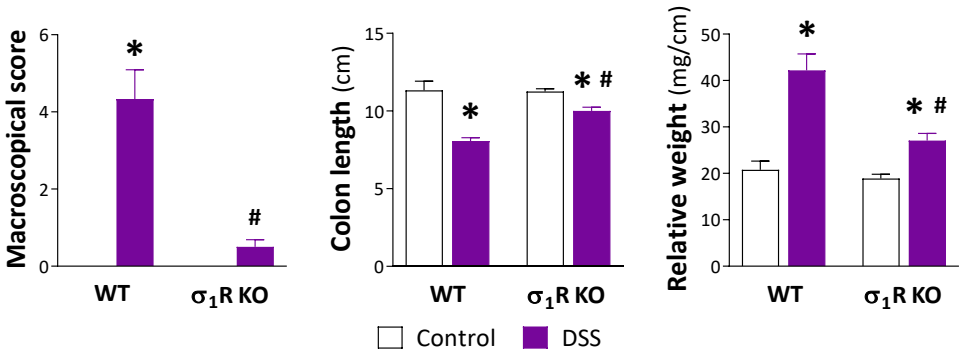


Figure 3. Assessment of DDS-induced colitis in WT and σ1R KO mice. Inflammation-related parameters (macroscopic scores, colonic length and colon relative weight) as determined during necropsy. Data are mean±SEM of 6-8 animals per group. *: P<0.05 vs. respective control group; #: P<0.05 vs. WT mice exposed to DSS.

mRNA of all the analyzed cytokines were detectable and quantifiable by RT-qPCR in the colonic tissues analyzed. Basal expression was similar in WT and σ_1R KO mice, except for the pro-inflammatory cytokine IL-12p40, which was up-regulated by 4-fold in σ_1R KO animals (P<0.05 vs. WT animals; Fig. 5). Regardless the genotype considered, expression of the pro-inflammatory cytokines INF-γ, IL-1β, and IL-6 was similarly up-regulated in DSS-treated mice (P<0.05 vs. control WT or σ_1R KO mice; Fig. 5). The pro-inflammatory cytokine IL-12p40 was up-regulated only in colitic WT mice (P<0.05 vs. control WT mice). No changes were observed in the expression of the anti-inflammatory cytokine IL-10, regardless the genotype considered (Fig. 5).

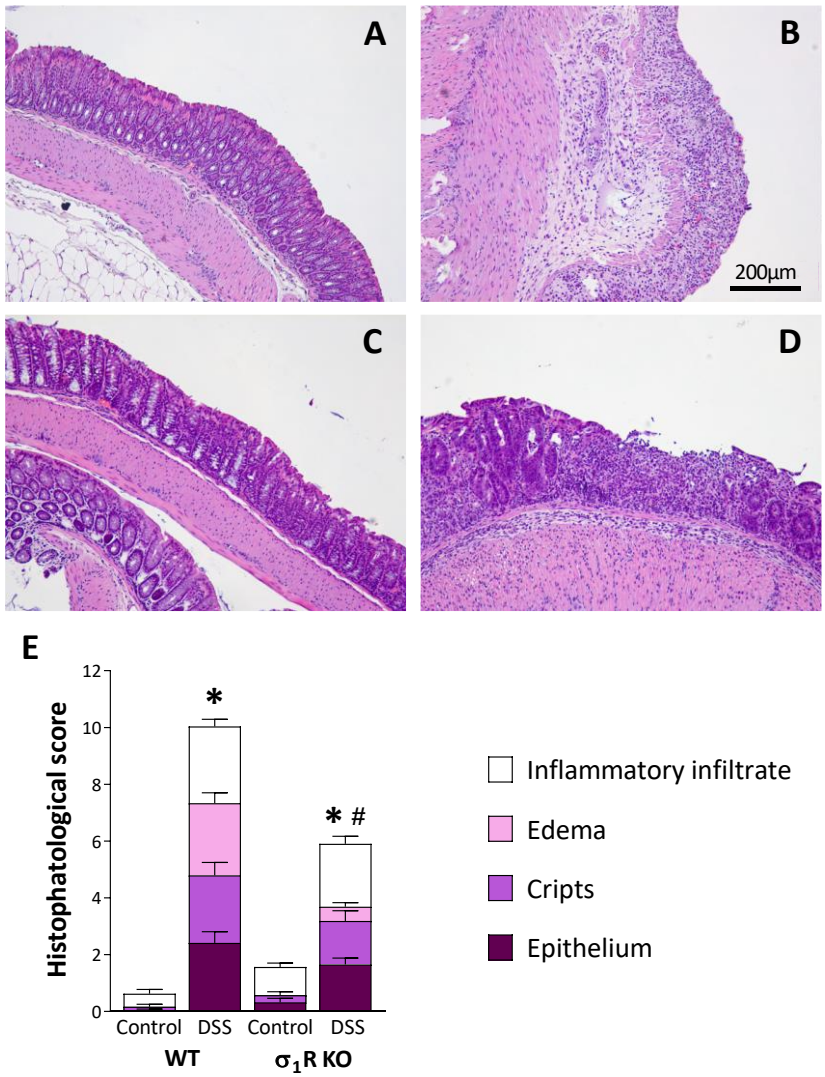


Figure 4. Histopathological assessment of the colon. A-D: Representative microphotographs showing hematoxylin and eosin-stained colonic slices from WT vehicle-treated mice (A), DSS-treated WT mice (B), σ_1R KO vehicle-treated mice (C) and DSS-treated σ_1R KO mice (D). Notice the submucosal edema observed in DSS-treated WT mice (B), while it was markedly reduced in DSS-treated σ_1R KO mice (D). Scale bar: 200 µm. E: Histopathological scores of the colon for the different experimental groups. Data are mean±SEM of 6-8 animals per group. *: P<0.05 vs. respective control group; #: P<0.05 vs. WT mice exposed to DSS.

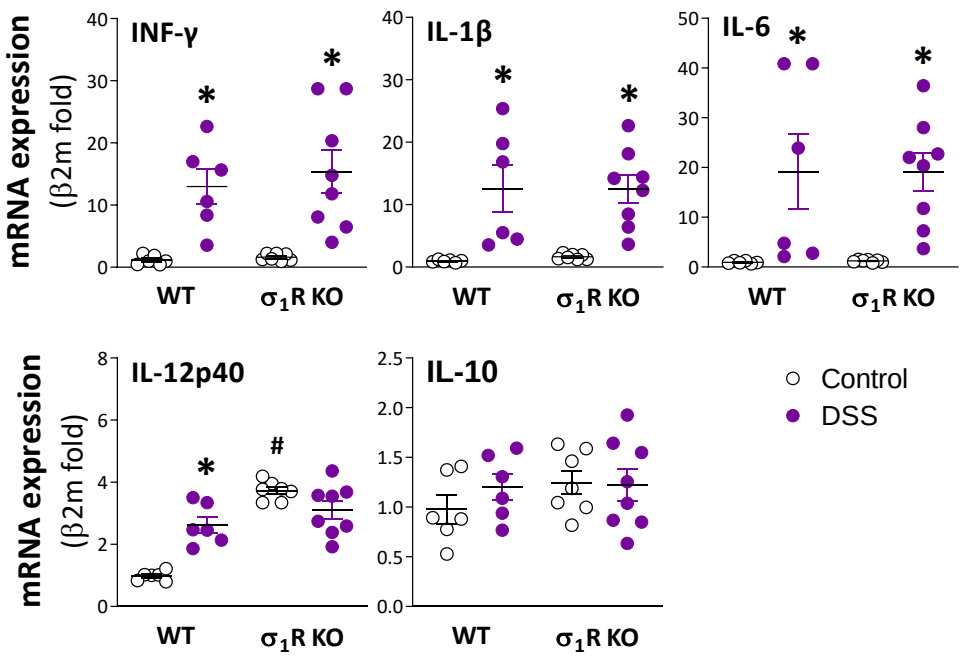


Figure 5. Colonic expression of pro- (interferon-γ (INF-γ), IL-1β, IL-6 and IL-12p40) and anti-inflammatory cytokines (IL-10) in WT and σ_1R KO mice. Each point represents an individual animal; the horizontal bar with errors represents the mean±SEM. *: P<0.05 vs. respective control group; #: P<0.05 vs. control WT mice.

σ_1R KO mice do not develop acute colitis-associated hypersensitivity

WT and σ_1R KO mice showed similar baseline abdominal (WT: 1.48±0.02 g; σ_1R KO: 1.43±0.04 g; P>0.05) and paw withdrawal thresholds (WT: 1.19±0.08 g; σ_1R KO: 1.21±0.09 g; P>0.05), as assessed with the von Frey test immediately before colitis induction (experimental day -1). During the development of acute colitis WT mice developed referred hyperalgesia, manifested as a time-related, progressive reduction in the paw and

abdominal withdrawal thresholds (both P<0.05 vs. respective sensitivity thresholds in basal conditions). In control animals not exposed to DSS paw and abdominal withdrawal thresholds remained stable over time (Fig. 6).

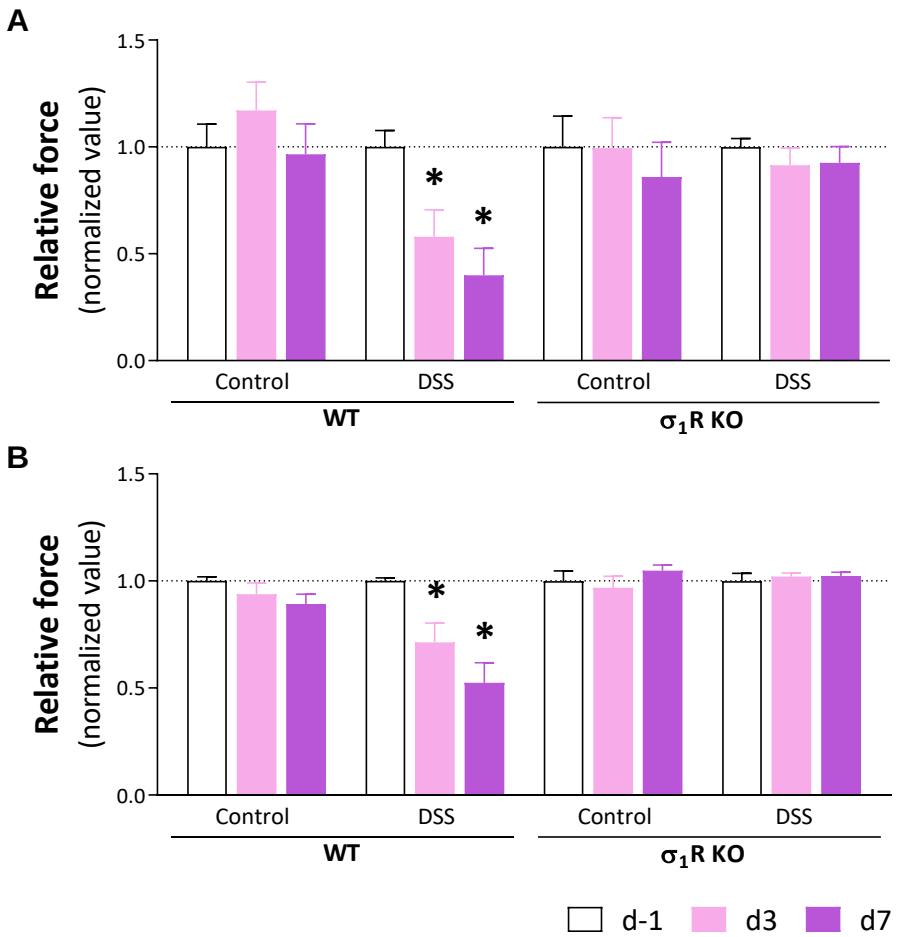


Figure 6. Time-course changes of sensitivity thresholds to mechanical probing during DSS-induced colitis in WT and σ_1R KO mice. The data represent abdominal withdrawal (A) and paw withdrawal thresholds (B) in WT and σ_1R KO mice [normalized for measurements at experimental day -1 (d-1) in each experimental group]. Data are mean±SEM of n=6 animals per group. d3: experimental day 3; d7: experimental day 7; *: P<0.05 vs. d-1 of respective group (basal response) or the same time-point in the corresponding control group.

Similar to that observed in WT animals, σ_1R KO mice not exposed to DSS showed a stable pain sensitivity throughout the experimental time (revealed by the absence of changes in the withdrawal thresholds). Moreover, no changes in pain sensitivity were detected in σ_1R KO mice exposed to DSS. In these animals, paw and abdominal withdrawal thresholds remained stable over time and were similar to basal values (determined in the same animals before colitis induction) or to the withdrawal thresholds determined in animals without colitis (Fig. 6).

mRNA for the sensory-related markers assessed was detected in all colonic samples, with the exception of σ_1Rs, which, as expected, was not detected in KO mice. A differential gene expression regulation was observed during colitis. Basal expression of anti-nociceptive (CB1 and MOR) and pro-nociceptive markers (NGF, TPH1) was similar in WT and σ_1R KO animals. However, a significant up-regulation for CB2 receptors (anti-nociceptive) and a significant down-regulation for TRPV1 (pro-nociceptive) was detected in σ_1R KO mice (both $P<0.05$ vs. expression levels in WT animals; Fig. 7).

During colitis, similar modulation in the expression of sensory-related markers was observed in both genotypes. Anti-nociceptive markers showed a general tendency towards a down-regulation, although statistical significance was only achieved for CB1 and MOR in σ_1R KO animals. Similarly, pro-inflammatory markers showed, in all cases, down-regulatory responses (Fig. 7).

Colitis did not affect the expression of σ_1R in WT mice (Fig. 8).

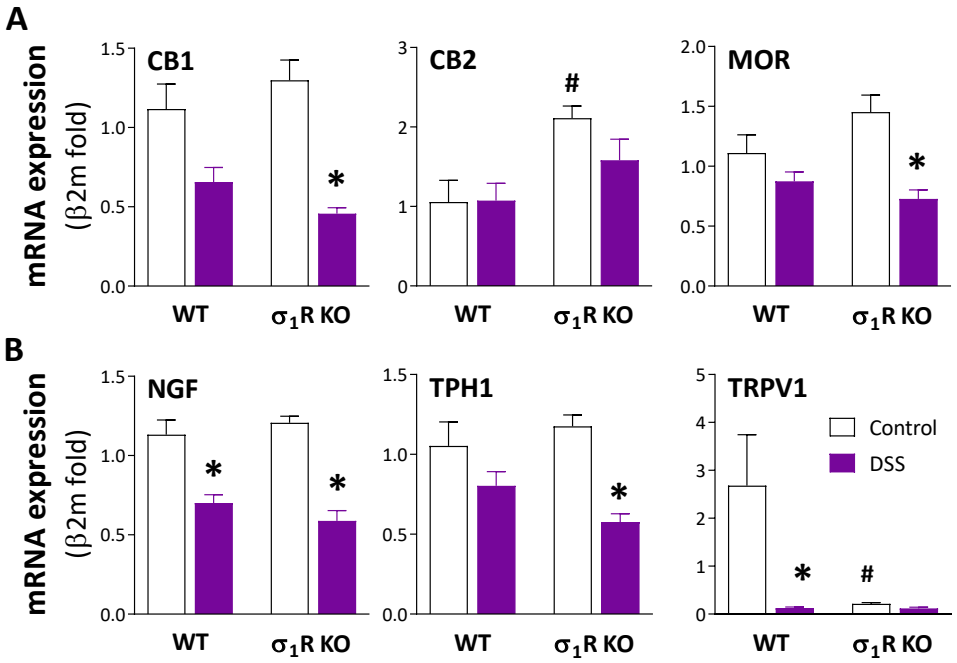


Figure 7. Colonic gene expression of sensory-related markers with anti-nociceptive (A: CB1, CB2 and MOR), and pro-nociceptive activity (B: NGF, TPH1 and TRPV1) in the different experimental groups. Data are mean±SEM, n=6-8 per group. *: P<0.05 vs. respective non DSS-exposed control group; #: P<0.05 vs. WT mice receiving DSS.

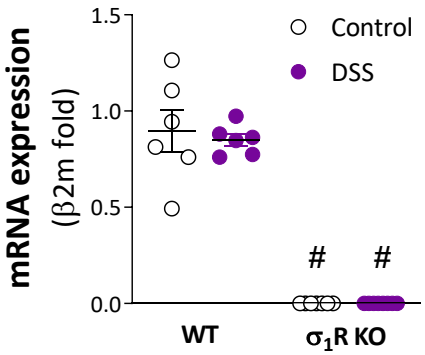


Figure 8. Colonic gene expression of σ_1Rs in WT and σ_1R KO mice. Each point represents an individual animal; the horizontal bar with errors represents the mean±SEM. #: P<0.05 vs. respective WT group.

At the spinal level, mRNA of the sensory pathways-related markers assessed was detected in all samples. Overall, neither genotype- nor inflammation-related changes were detected, with the exception of nNOS and σ_1Rs. As it relates to nNOS, a constitutive down-regulation was detected inσ_1R KO mice ($P<0.05$ vs. WT mice; Fig. 9). As expected, expression of σ_1Rs was not detected in the spinal cord of KO mice. Moreover, during colitisσ_1R expression was down-regulated by 30% in WT mice ($P<0.05$ vs. healthy WT mice; Fig. 9

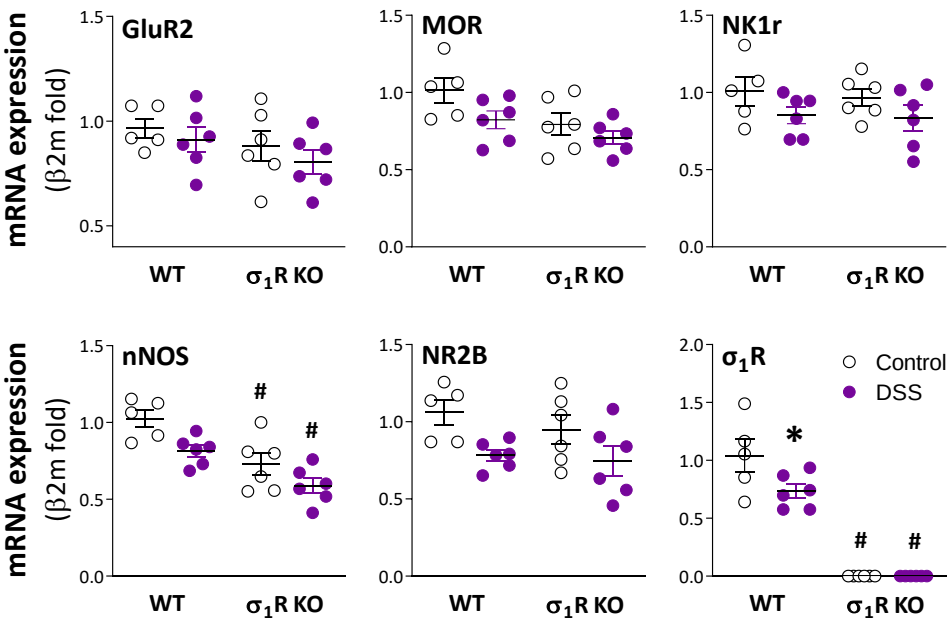


Figure 9. Gene expression of sensory pathways-related markers in the lumbosacral spinal cord of WT and σ_1R KO mice with or without colitis. Each point represents an individual animal; the horizontal bar with errors represents the mean±SEM. *: $P<0.05$ vs. respective control group; #: $P<0.05$ vs. respective WT mice group.

Presence of proteins implicated in sensitization mechanisms, namely pERK, pCaMKII, pp38 and GFAP, was detected in the lumbosacral spinal cord in all samples analyzed. Although with relatively large variability in some cases, overall, no consistent genotype- or inflammation-related changes were detected (Fig. 10).

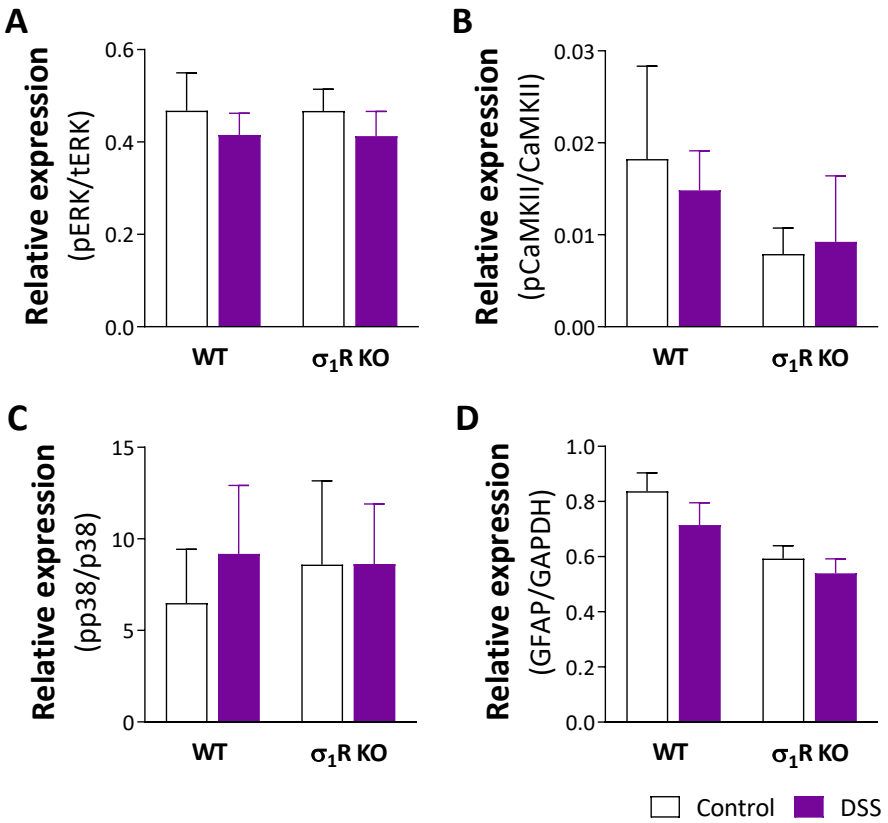


Figure 10. Relative spinal cord levels of pERK (A), pCaMKII (B), pp38 (C) and GFAP (D). Data are mean±SEM; n=6-8 animals per group.

Antagonism of σ_1Rs with BD1063 or E-52862 attenuates DSS-induced colitis

Vehicle-treated WT mice receiving DSS showed weight loss and clinical signs consistent with the induction of a colitic state. Treatment with BD1063 or E-52862 resulted in a similar attenuation of body weight loss and clinical signs, although statistical significance was not achieved, probably because of the relatively large variability observed in some cases.

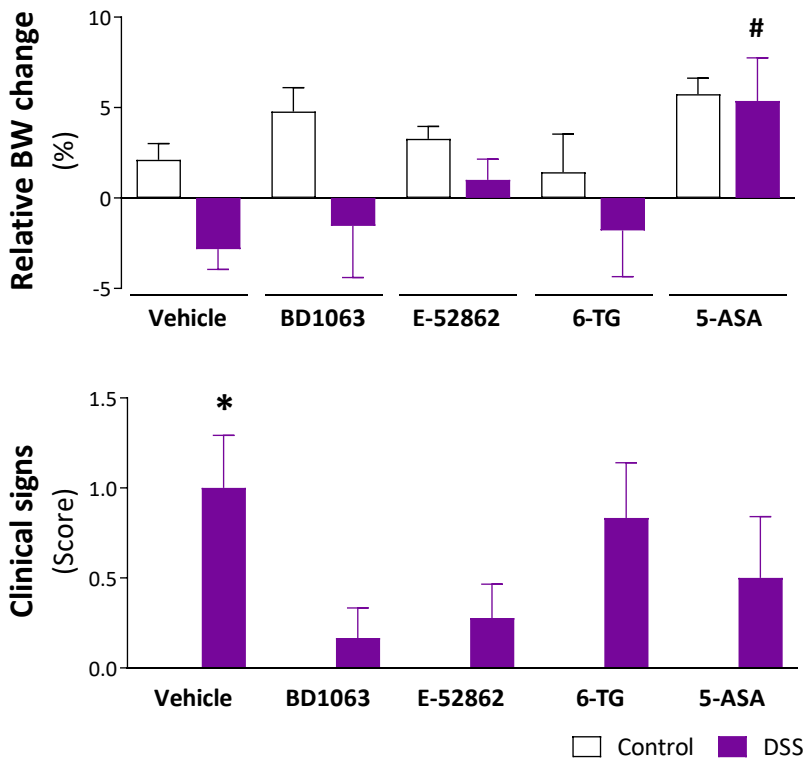
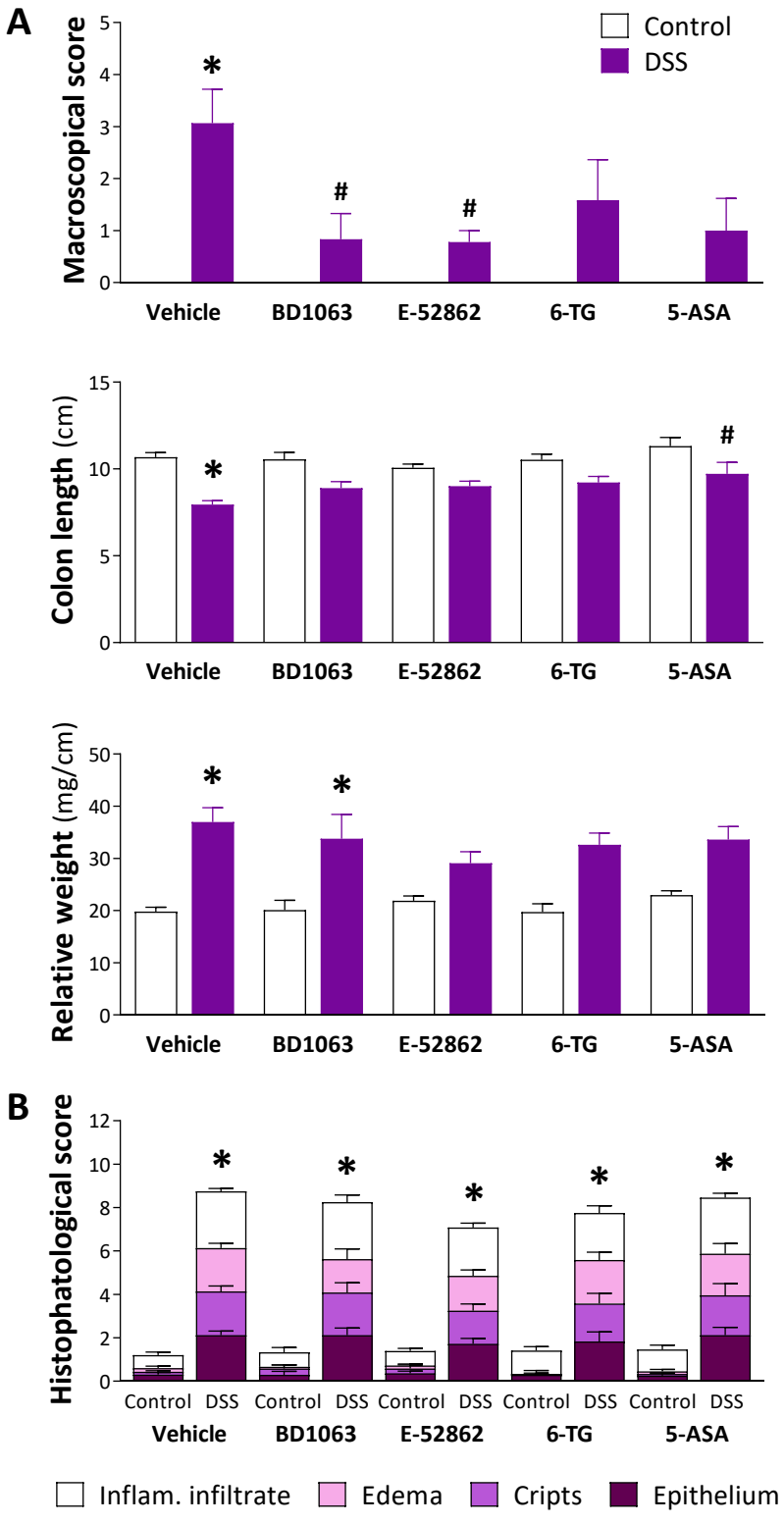


Figure 11. Changes in relative body weight (% change from day 0, taken as 100%) and clinical signs associated to exposure to DSS and pharmacological treatments (of σ_1R antagonists BD1063 or E-52862, 6-TG or 5-ASA) at experimental day 7. Data are mean±SEM (n=6-15 per group). *: P<0.05 vs. respective control group; #: P<0.05 vs. DSS-vehicle-treated group.


A
Macroscopical score
Control
DSS
Vehicle
BD1063
E-52862
6-TG
5-ASA
Colon length (cm)
Relative weight (mg/cm)
B
Histophatological score
Control
DSS
Inflam. infiltrate
Edema
Cripts
Epithelium

At necropsy, colonic alterations consistent with the development of colitis, increased inflammatory scores, colon shortening and increase in relative weight, were also observed in vehicle-treated mice exposed to DSS, with (Fig. 12A). Antagonism of σ_1 Rs attenuated some of these parameters, but different effects were observed for BD1063 (reduction of inflammatory scores; $P<0.05$ vs. control) and E-52862 (reduction of inflammatory scores and relative weight; both $P<0.05$ vs. control). However, histopathological alterations were not affected by neither BD1063 nor E-52862. Indeed, animals receiving BD1063 or E-52862 showed essentially the same structural alterations and histopathological scores as those of vehicle-treated animals also exposed to DSS (Fig. 12B).

The reference compounds, 6-TG and 5-ASA, attenuated macroscopical scores of inflammation (although statistical significance was not achieved) and the increase of relative colonic weight ($P>0.5$ vs. relative weight in non-colitic animals), without improving the histopathological alterations (Fig. 12B).

mRNA of all the analyzed cytokines were detectable and quantifiable by RT-qPCR in all samples. In vehicle-treated animals, DSS exposure led to expression changes equal to those observed in the previous study comparing WT and σ_1 R KO animals. However, due to the variability observed, statistical significance was only detected for the E-52862-DSS group, which exhibited in all cases high inter-individual variability with high

Figure 12. Assessment of colonic inflammation at the time of necropsy in the different experimental groups. A: Macroscopic assessment of colonic inflammation (inflammatory score, length and relative weight). B: Histopathological scores. Data are mean±SEM of 6-15 animals per group. *: $P<0.05$ vs. respective non DSS-exposed control group; #: $P<0.05$ vs. vehicle-treated WT mice receiving DSS.

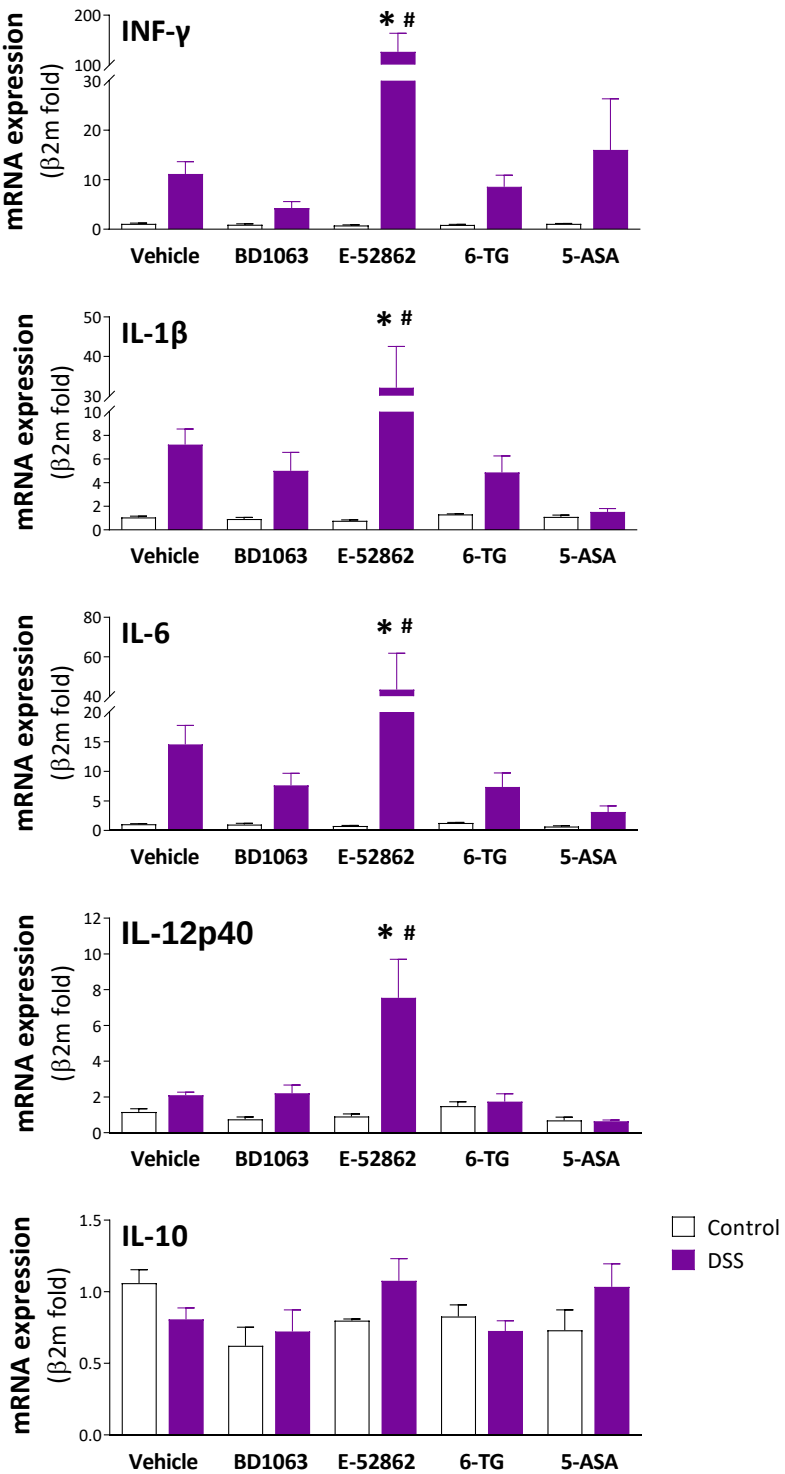
levels of expression. In any case, BD1063 showed a tendency to normalize the expression of the pro-inflammatory cytokines INF-γ, IL-1β and IL-6, without affecting the expression of IL-12p40. Surprisingly, as mentioned above, treatment with E-52862 resulted in a significant increase in the expression of all of the pro-inflammatory cytokines assessed, although with high inter-individual variability (Fig. 13). 6-TG and 5-ASA showed also a tendency to reduce the expression of pro-inflammatory cytokines, with the exception of INF-γ which in 5-ASA-DSS-treaetd animals showed an up-regulation associated to high interindividual variability (Fig. 13). No changes were observed in the expression of the anti-inflammatory cytokine IL-10, regardless the treatment considered.

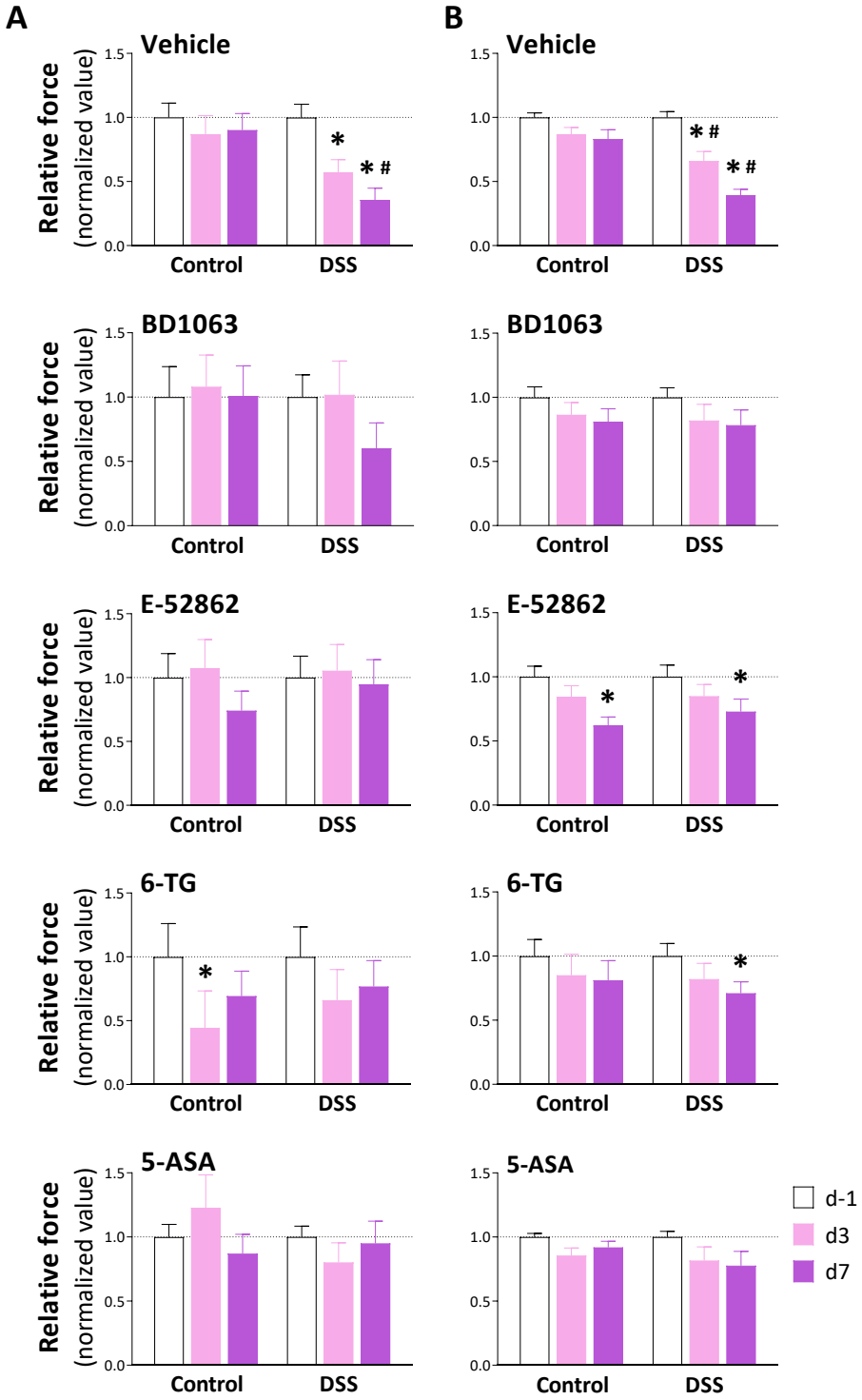
Antagonism of σ_1Rs with BD1063 or E-52862 attenuated colitis-associated hypersensitivity

As expected, vehicle-treated mice exposed to DSS developed referred hyperalgesia, manifested as a time-related, progressive reduction in the withdrawal thresholds determined in the paw and the abdominal wall (both $P<0.05$ vs. respective sensitivity thresholds in non-inflamed animals). In control animals not exposed to DSS paw and abdominal withdrawal thresholds remained stable over time (Fig. 14).

Treatment with BD1063 completely prevented the development of hypersensitivity, as assessed either in the paw or in the abdominal wall. On the other hand, treatment with E-52862, while completely preventing the

Figure 13. Expression of pro-(interferon-γ (INF-γ), IL-1β, IL-6 and IL-12p40) and anti-inflammatory cytokines (IL-10) in animals with DSS-induced colitis treated with the selective σ_1R antagonists BD1063 or E-52862, 6-TG or 5-ASA. . Data are mean±SEM of 6-15 animals per group. *: $P<0.05$ vs. respective non DSS-exposed control group; #: $P<0.05$ vs. vehicle-treated mice receiving DSS.


INF-γ
IL-1β
IL-6
IL-12p40
IL-10
mRNA expression
(β2m fold)
* #
Vehicle
BD1063
E-52862
6-TG
5-ASA
Control
DSS


A
B
Vehicle
BD1063
E-52862
6-TG
5-ASA
Relative force
(normalized value)
1.5
1.0
0.5
0.0
Control
DSS
*
*#
d-1
d3
d7

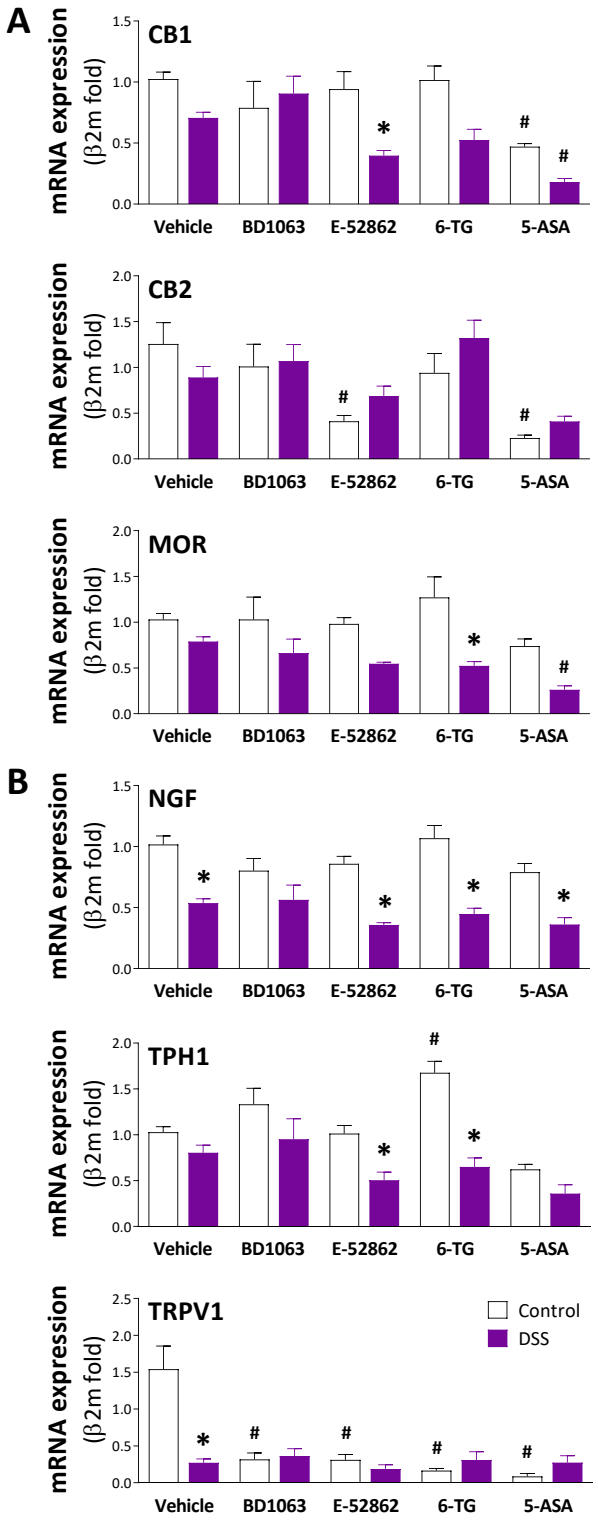
development of hyperalgesia as assessed at the abdominal wall, only partially prevented the sensitivity changes observed at the paw (Fig. 14).

Similar inhibitory effects were observed for 6-TG and 5-ASA. Treatment with 5-ASA completely prevented colitis-associated hypersensitivity while in 6-TG-treated animals paw hyperalgesia was detected only at experimental day 7 (Fig. 14).

mRNA for all sensory-related markers assessed was detected in all colonic samples. Overall, gene expression changes detected in vehicle-treated mice exposed to DSS were similar to those reported in the previous experiment, with a tendency for a general down-regulation during colitis, although in this case statistical significance was only reached for NGF and TRPV1 (Fig. 15).

Treatment with BD1063 had no significant effects on gene expression (healthy or colitic animals), with the exception of TRPV1, which was significantly down-regulated in healthy animal (Fig. 15). Treatment with E-52862 resulted in a down-regulation of the expression of CB2 and TRPV1 in healthy animals. During colitis, E-52862-treated animals presented an overall down-regulation in the expression of sensory markers; similar to that observed in vehicle-treated animals, but in this case statistical significance was achieved for the expression of CB1, NGF and TPH1 (all $P<0.05$ vs. E-52862-treated animals not exposed to DSS; Fig. 15).

Figure 14. Effects of BD1063, E-52862, 6-TG and 5-ASA on acute colitis-associated hypersensitivity. The data represent abdominal (A) and paw withdrawal thresholds (B) assessed at experimental days -1 (control, d-1), 3 (d3) and 7 (d7). In all cases data are normalized for measurements at experimental day -1. . Data are mean±SEM of n=6-15 animals per group. *: $P<0.05$ vs. d-1 of respective group (basal response); #: $P<0.05$ vs. respective control group.


A
CB1
CB2
MOR
B
NGF
TPH1
TRPV1
mRNA expression
(β2m fold)
Vehicle
BD1063
E-52862
6-TG
5-ASA
Control
DSS

6-TG, per se, had minor effects on gene expression, with the more relevant effect being the down-regulation of TRPV1 expression (P<0.05 vs. vehicle-treated healthy mice; Fig. 15). In animals with colitis receiving 6-TG changes in gene expression were comparable to those observed in vehicle-treated colitic mice. In healthy animals, 5-ASA lead to de down-regulation of CB1 and CB2 receptors in addition to that of TRPV1 (all P<0.05 vs. vehicle-treated healthy mice; Fig. 15). During colitis, a similar general down-regulation of sensory markers described in vehicle-treated animals was detected in 5-ASA-treated animals (Fig. 15).

Regardless the treatment considered or the presence or not of inflammation, no changes were detected for the colonic expression of σ_1Rs (Fig. 16).

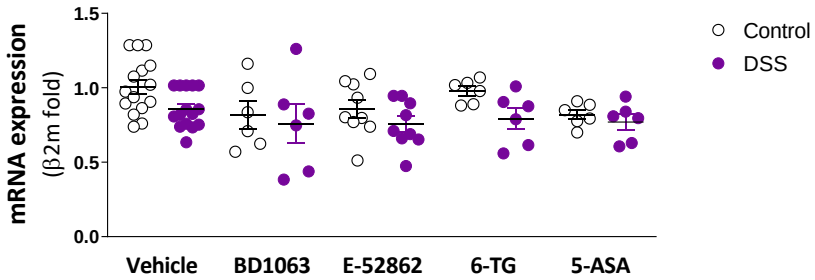


Figure 16. Effect of σ_1R antagonist on the colonic expression of σ_1Rs. Each point represents an individual animal; the horizontal bar with errors represents the mean±SEM.

Figure 15. Colonic gene expression of sensory-related markers with anti-nociceptive (A: CB1, CB2 and MOR), and pro-nociceptive activity (B: NGF, TPH1 and TRPV1) in the different experimental groups. Data are mean±SEM, n=6-15 per group. *: P<0.05 vs. respective non DSS-exposed control group; #: P<0.05 vs. vehicle-treated mice receiving DSS.

DISCUSSION

Previous studies have demonstrated the involvement of σRs in different pain models.[30,31] This study expands this knowledge by assessing the implication of σ_1Rs in the development of hypersensitivity associated to intestinal inflammation. The present results show that σ_1Rs are implicated in the development of acute inflammation within the gastrointestinal tract and the associated changes in referred sensitivity at the paw (somatic) and abdominal wall (likely implicating somatic and visceral perception). Two different approaches were used to characterize the role of σ_1Rs; the genetic, constitutive absence of the receptor using KO mice for the receptor and the pharmacological blockade with the selective σ_1R antagonists BD1063 and E-52862.

Intestinal inflammation associated to sensorial alterations, characterized by changes in somatic and visceral sensitivity (somatic and visceral hyperalgesia), are common finding in several gastrointestinal (GI) pathologies, mainly in inflammatory bowel disease and FGDs (with irritable bowel syndrome as main representative).[1–3] Several animal models have been designed to mimic inflammatory conditions of the gut. Among them, DSS-induced colitis is a well validated and accepted model of intestinal inflammation. Upon DSS exposure, mice develop a state of acute colitis reminiscent of the flares observed in humans with inflammatory bowel disease. Responses observed are strain-related.[17,18] In CD1 mice, this response occurs 7-9 days after starting DSS exposure, with the presence of a flare of acute colitis, and resolves by day 14.[26,27] Consistent with these observations, in the present experimental conditions, CD1 mice exposed during a 5-days period to 3% DSS developed an acute state of colitis, as assessed at experimental day 7, characterized by presence of clinical signs,

including body weight loss, histopathological alterations and up-regulation of pro-inflammatory cytokines. Overall, these alterations agree with those described in comparable experimental conditions[26,27] and are consistent with an acute inflammatory response.

First evidence of a potential involvement of the σ_1R in the development of intestinal inflammation comes from the attenuation of clinical signs and changes in body weight observed in σ_1R KO animals exposed to DSS. The apparent resistance to inflammation in σ_1R KO mice is further supported by the macroscopic assessment of the colon at the time of necropsy, showing an attenuation of inflammation-related parameters vs. WT animals. This was further confirmed by the attenuation of colonic histopathological alterations. The histopathological improvement was associated mainly to a reduction in submucosal edema. This agrees with previous results showing a reduction in subepithelial edema in σ_1R KO mice in a model of cyclophosphamide-induce cystitis (González-Cano et al., 2020) or the reduction in paw edema, elicited by the intraplantar injection of carrageenan, associated to the blockade of σ_1Rs with specific antagonists.[32,33] Although the mechanisms mediating these effects have not been fully elucidated, they might implicate a NOS-dependent modulation of vascular permeability and extravasation.[32,33] Pharmacological blockade of σ_1Rs in WT mice partially reproduced the responses observed in σ_1R KO mice. Both BD1063 and E-52862 attenuated clinical signs of colitis and improved colonic macroscopic signs of inflammation. However, at the histopathological level, no signs of improvement were observed vs. the alterations observed in vehicle-treated animals with colitis, particularly when considering submucosal edema. This agrees with previous studies showing that the pharmacological blockade with the same antagonists used here failed to reduce paw edema in inflammatory pain models.[10,13,14] Overall, this might represent limits of the

pharmacological blockade of σ_1Rs vs. their constitutive absence in KO animals. 6-TG and 5-ASA modulated inflammation in a similar manner, with an improvement in clinical and macroscopic signs of inflammation, but without amelioration of the histopathological scores.

The clinical attenuation of colitis observed in σ_1R KO mice or during the treatment with selective σ_1R antagonists did not correlate with gene expression of pro-inflammatory cytokines, which were up-regulated in similar proportion to that detected in control conditions. Overall, colonic expression of pro- (INF-γ, IL-1β, and LI-6) and anti-inflammatory cytokines (IL-10) was similar in non-inflamed WT and σ_1R KO mice. However, IL-12p40 was constitutively up-regulated on KO mice, thus suggesting that these animals might be prone to develop immune-mediated responses. Indeed, previous evidences suggest an immunomodulatory role for σ_1Rs pointing towards a potential anti-inflammatory activity.[34] Accordingly, lack of functional σ_1Rs, as in σ_1R KO mice, should translate into enhanced inflammatory responses. However, and according to that observed experimentally, compensatory mechanisms in these animals might lead to a state in which inflammatory response are constitutively attenuated.[35,present observations] Moreover, in animals exposed to DSS, treatment with E-52862 led to an unexpected significant up-regulation of all the pro-inflammatory cytokines assessed. This reinforces the potential immunomodulatory role of σ_1Rs in states of inflammation and highlights differences in the pharmacological profile of E-52862 and BD1063. As expected, 6-TG and 5-ASA showed a similar modulatory action on cytokines expression, with an overall tendency towards normalization.

Baseline mechanical sensitivity, as assessed in the hind limbs or the abdominal wall, was similar in WT and σ_1R KO mice. These results are in agreement with previous data indicating that naive σ_1R KO mice perceive mechanical and thermal stimuli normally.[9,10] Moreover, pharmacological

blockade of σ_1Rs in healthy animals did not affect, per se, mechanical sensitivity, with the exception of a punctual somatic hyperalgesia observed in E-52862-treated mice when assessing paw sensitivity. Overall, this agrees with studies with σ antagonists showing that σ_1R ligands have no effects by themselves, but are able to modulate signaling pathways under pathological conditions.[9,11,12] The reduction in somatic sensitivity thresholds observed in E-52862-treated animals, either with or without acute colitis, might reflect a compound-related hyperalgesic effect. However, to the best of our knowledge, similar effects have not been described before for E-52862.

Intestinal hypersensitivity has been associated to the peripheral sensitization of primary colonic sensory afferents, resulting in visceral hypersensitivity and referred hyperalgesia in several body regions, including the abdominal wall, hind paws and tail.[2,4,6,36] In the DSS-induced colitis model, persistent harmful stimulation of visceral nociceptors through inflammatory mediators would be responsible for inducing acute visceral and somatic referred hyperalgesia.[4,37] Accordingly, WT control mice exposed to DSS developed referred hypersensitivity, as determined by reductions in pain thresholds to the mechanical probing of the hind paws and the abdominal wall (likely reflecting mixed somatic and visceral -intestinal- pain responses). In contrast, no changes in mechanical sensitivity were observed in σ_1R KO mice. Likewise, the selective σ_1R antagonists BD1063 and E-52862 effectively reduced inflammation-induced hypersensitivity, leading to, essentially, the same responses observed in control conditions or in σ_1R KO mice. This is in agreement with the reported analgesic effects of BD1063 on a models of intracolonic capsaicin- and cystitis-induced visceral pain.[15,16] Altogether, these results confirm a role for σ_1Rs in the development of visceral inflammation-associated hypersensitivity, as previously reported from other models of inflammatory

pain.[10,13,14,32,33] Altogether, these data support the view that antagonism of σ_1Rs might represent a feasible approach for the treatment of pain arising from the gastrointestinal tract.

To gain insight in the mechanisms implicated in the nociceptive responses described above we assessed the expression of different sensory-related markers, both at the level of the colon and within the lumbosacral spinal cord. Except for CB2 receptors and TRPV1, basal expression of sensory-related markers was similar in WT and σ_1R KO mice. Overexpression of CB2 receptors in σ_1R KO mice might be related to the potential immunomodulatory effects described for σ_1Rs,[34] and also observed here through the up-regulation of IL-12p40, since CB2 receptors are largely associated to immune cells.[38] On the other hand, down-regulation of TRPV1 suggest a direct interaction between σ_1Rs and the activity of sensory afferents, since within the colon TRPV1 is primarily expressed on primary sensory afferents mediating pro-algesic responses.[39,40] Interestingly, treatment with BD1063 or E-52862 resulted also in a significant down-regulation of the colonic expression of TRPV1. These data are in line with observations indicating that antagonism of σ_1Rs is able to decrease nociceptive responses through negative regulation of TRPV1 protein expression in plasma and in the membranes of sensory neurons.[41,42] Therefore, an altered TRPV1-σ_1R interaction might contribute to de underlying mechanisms explaining the absence of hypersensitivity in the absence of functional σ_1Rs (KO mice or pharmacological blockade). All together, these changes (up-regulation of CB2-mediated analgesic affects and inhibition of TRPV1-mediated pro-algesic effects) suggest a basal analgesic state in these animals. However, as described above, σ_1R KO mice exhibited normal mechanical sensitivity,[9,10,present observations] thus suggesting the presence of additional compensatory mechanisms, as discussed above.

In any case, these observations further reinforce the implication of σ_1Rs on pain mechanisms.

During colitis, a general down regulation of colonic sensory-related markers, either with analgesic or pro-algesic activity was observed. Again, this was particularly evident for TRPV1. Colonic expression of TRPV1 might be highly variable with experimental model- and species-related variations. Indeed, ulcerative colitis patients show reduced expression of TRPV1 while during DSS-induced intestinal inflammation no changes (acute inflammation) or increased expression (chronic inflammation) has been reported.[43,44] Given the pro-nociceptive effects of TRPV1, its down-regulation during acute colitis might be interpreted as a compensatory mechanism developed during acute inflammation to avoid abnormal excessive pain. Overall, it is difficult to establish a direct correlation between gene expression of sensory-related markers and functional outcomes as it relates to pain sensitivity since a trend towards a down-regulation was observed for anti- and pro-nociceptive markers. In this situation, the final functional responses will depend upon the balance between anti- and pro-nociceptive mechanisms, as previously suggested for other states of altered intestinal sensitivity.[45–47]

Besides peripheral locations, pain sensitization can occur also at central level. To assess the potential participation of central (spinal) sensitization in the responses observed during acute colitis, we also assessed the lumbosacral expression (gene or protein levels) of several markers related to spinal processing of sensory-related signals and the sensitization process. Overall, with the exception of the expression of σ_1Rs and nNOS, no differences between WT and KO or treated and untreated animals were detected. nNOS expression was constitutively down-regulated in the lumbosacral spinal cord of σ_1R KO mice. Recent evidences indicate that during peripheral neuropathy spinal σ_1R-induced pain hypersensitivity is

mediated by nNOS activation.[48,49] Moreover, spinal activation of the σ_1R by agonists increased nNOS activity and nitric oxide (NO) production, which leads to the development of hypersensitivity.[32] Therefore, it is feasible to assume that the constitutive lack of σ_1Rs might have consequences in the activity and/or expression of components of associated signaling processes involved in pain sensitization, such as nNOS. Alterations of this pathway might be part of the underlying mechanisms explaining the absence of sensitization during acute colitis in σ_1R KO mice. In this context, it is noteworthy that, in WT animals, colitis induced a σ_1R down-regulation within the lumbosacral spinal cord. This could act as a compensatory mechanism, regulating sensitization pathways in a negative manner, such as the nNOS pathway discussed above, with the objective of avoiding aberrant/excessive sensitization. These observations warrant further studies addressed to dissect in details these pathways.

Since only changes associated to acute inflammation were assessed in this work, we cannot discard the implication of these mechanisms in a process of inflammation-related long-term vs. early acute sensitization. For instance, in models of inflammatory/neuropathic pain, modulation of some of these pathways (i.e. ERK phosphorylation) was observed in the spinal cord up to 14 days after the application of the sensitizing stimuli.[9,50] However, acute modulation of the same pathways during inflammation or neuropathic damage has also been described.[51,52] Therefore, we cannot discard that multiple factors, including experimental model-, species- and time of testing-related, might account for these apparent discrepancies. Alternatively, the implication of other, specific, mechanisms acting in visceral sensitization cannot be discarded.

In summary, the present results, based on the genetic ablation or the pharmacological blockade of σ_1Rs in a model of acute intestinal inflammation, show that σ_1Rs has positive modulatory effects on intestinal

inflammation, particularly at the clinical level, as well as on the development of inflammation-associated pain, likely preventing both somatic and visceral hypersensitivity. These observations, together with previous evidences in similar models, support the pharmacological interest of σ_1R antagonists for the treatment of intestinal inflammation and inflammation-associated hypersensitivity, having potential for their clinical use in inflammatory and functional gastrointestinal disorders.

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CHAPTER 3

Long-lasting visceral hypersensitivity in a model of DSS-induced colitis in rats

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ABSTRACT

Persistent visceral hypersensitivity is a key component of functional and inflammatory gastrointestinal diseases. Current animal models fail to fully reproduce the characteristics of visceral pain in humans, particularly as it relates to persistent hypersensitivity. This work explores the validity of DSS-induced colitis in rats as a model to mimic chronic intestinal hypersensitivity.

Exposure to DSS (5% for 7 days) was used to induce colitis in rats. Thereafter, changes in viscerosensitivity (visceromotor responses to colorectal distension -CRD-), the presence of somatic referred pain (mechanosensitivity of the hind paws, von Frey test) and the expression (qRT-PCR) of sensory-related marker (colon, lumbosacral DRGs and lumbosacral spinal cord) were assessed at different times during the 35-day period after colitis induction.

Following colitis, a sustained increase in visceromotor responses to CRD were observed, indicative of the presence of visceral hypersensitivity. Responses in animals without colitis remained stable over time. In colitic animals, somatic referred hypersensitivity was also detected. DSS-induced colitis was associated to a differential expression of sensory-related markers (with both pro- and anti-nociceptive action) in the colon, lumbosacral DRGs and lumbosacral spinal cord; indicating the presence of peripheral and central sensitization.

DSS-induced colitis in rats is associated to the generation of a long-lasting state of visceral (colonic) hypersensitivity. This model reproduces the changes in intestinal sensitivity characteristics of inflammatory and functional gastrointestinal disorders in humans and can be used in the characterization of new pharmacological treatments against visceral pain.

INTRODUCTION

Visceral hypersensitivity is a common component of functional and inflammatory gastrointestinal (GI) disorders in humans. Indeed, persistent alterations in GI sensitivity with altered pain thresholds and signs of hypersensitivity are considered key findings in functional GI disorders, particularly in irritable bowel syndrome (IBS).[1–3] Similar long-lasting alterations in sensitivity, although with some inconsistencies, are also reported during the active and inactive phases of inflammatory bowel disease (IBD).[4,5] Although the clinical importance of visceral pain, the development of effective treatments directed towards the specific control of this pain modality has been hampered by: i) the complex physiology of visceral pain; ii) the differences in the neural and immune substrata undelaying visceral vs. somatic pain; and iii) the difficulties in developing animal models with high translational validity, particularly as it relates to persistent/chronic hypersensitivity.[6]

Taking this into account, numerous animal models have been developed for the assessment of visceral sensitivity in normal conditions or in states of sensitization. A systematic review of these models is out of the scope of the present work and can be found elsewhere (see as examples: Regmi and Shah, 2020;[7] Johnson et al., 2020;[8] West and McVey Neufeld, 2021;[9] Accarie and Vanuytsel, 2020).[10] Several animal models of intestinal hypersensitivity have been developed based on the evidence that inflammation seems to be a significant pathophysiological component of inflammatory and functional GI disorders, as mentioned above. Overall, postinflammatory models have been based on the local administration (intracolonic enemas) of different active compounds (such as acetic acid, capsaicin, mustard oil, zymosan, trinitrobenzene sulfonic acid -TNBS- or

dinitrobenzene sulfonic acid -DNBS-) or the experimental infection with biological agents (such as *Trichinella spiralis*, *Nippostrongylus brasiliensis* or *Campylobacter* species).[11–13] From the available models, a recent report recommends the use of the intracolonic TNBS postinflammatory model of colonic hypersensitivity as the primary model for screening novel therapeutics for visceral pain of GI origin.[8] This recommendation was based on four characteristics of the model: simplicity, characterization in rodent species, reproducibility across laboratories and construct validity and translational relevance. However, data available on the TNBS model is limited and can be considered insufficient as to endorse its use over similar models.

One of the most commonly used models of intestinal inflammation is based on the dextran sulfate sodium (DSS) exposure in rodents. This model has strong similarities with the characteristic histopathology of intestinal inflammation in humans and it is regarded as a valid model of human intestinal inflammation. The DSS-induced colitis model has been extensively validated in both rats[14–20] and mice.[21–25] Some reports suggest that the DSS model elicits an inflammatory response more similar to human IBD than the TNBS model.[26] In fact, exposure to DSS elicits a colitic response that, according to its histopathological features and immunologic profile, resembles human ulcerative colitis, while TNBS-induced colitis is more similar to Crohn's disease.[27] Visceral pain responses during DSS-induced colitis have been studied mainly in mice. Although with some contradictory data, several studies consistently indicate the presence of intestinal (colonic) hypersensitivity during both the acute and the chronic phases of DSS-induced inflammation.[28–32]

With the objective of broaden the available models to assess visceral pain, particularly as it related to the presence of chronic hypersensitivity, the present work evaluates the validity of DSS-induced colitis in rats as a

model to assess intestinal (colonic) sensitivity, with special emphasis in the present of long-term sensitization. Moreover, we also assessed changes in the expression of sensory-related markers at the periphery (colon and dorsal root ganglion) or the spinal cord level, as a way to gain insight into the mechanisms underlying inflammation-associated changes in viscerosensitivity following DSS exposure.

MATERIALS AND METHODS

Animals

Female adult Sprague Dawley rats (Crl:OFA(SD); 10-12 week–old at the beginning of the study; Charles River, France) were used. Female rats were used because of the higher prevalence of GI dysfunctions associated with visceral pain, such as IBS, in women.[33,34] Given the inconsistent observations related to the potential effect of the estrous cycle in sensitivity, the phase of the cycle was not taken into consideration in the present studies.[35–40] Animals were group-housed (3-4 animals per cage) in standard cages and were maintained in conventional conditions in an environmentally controlled room (20–22 ºC, 12 h light:dark cycle), with food and water ad libitum, except when receiving DSS. Animals were allowed to acclimatize to the animal facility for at least 1 week prior the start of the studies. All experiments were performed in accordance with EU regulations and were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocols 3958 and 3961) and the Generalitat de Catalunya (protocol 9915).

DSS-induced colitis

Rats received a solution of 5% DSS (45 kDa; TdB Consultancy AB, Uppsala, Sweden) in their drinking water for seven days to induce colitis. The same protocol has been previously used for the induction of colitis in rats.[14–18] Fresh DSS solutions were prepared daily and control rats received normal tap water. Individual body weight, the general state and the presence of clinical signs of inflammation were assessed on a daily basis throughout the study.

Assessment of colonic sensitivity: Colorectal distension

For the assessment of colonic sensitivity, the colorectal distension (CRD) method was used, following, with minor modifications, previously published procedures.[41] Before starting experiments, rats were gradually habituated (45 to 75 minutes per day, at 15 minutes steps) to Bollmann cages to reduce motion artefacts and minimize the effects of stress-related responses. On the day of the experiment, rats were anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain). A plastic balloon (made in-house; length: 2.5 cm; diameter: 1.5 cm) with connecting catheter was inserted in the distal colon (2 cm from the base of the balloon to the anus). The catheter was fixed with tape to the tail of the animal. Animals were then placed in Bollmann cages and allowed to recover from anesthesia for at least 20-30 min before starting the CRD protocol.

The balloons were connected to pressure transducers (P-602, CFM-k33, 100 mmHg, Bronkhorst HI-TEC, Veenendal, The Netherlands) to control intraballoon pressure during the CRD procedure. A customized barostat system was used to manage balloon inflation and to measure pressure changes during CRD. The pressure was monitored and kept constant by a custom computer software (Pharmlab 5.0 online, AstraZeneca R&D,

Mölndal, Sweden) running on a standard computer. The same system served also for data acquisition.

For CRD, a protocol consisting of repetitive phasic distensions at 80 mmHg, with a pulse duration of 30 s at 5 min intervals was used. This protocol served to induce acute mechanical hypersensitivity and has been extensively used to assess colonic mechanosensitvity.[41–44] Rapid pressure changes in the distending balloon, reflecting contractions of the abdominal muscles, were used to assess visceromotor responses (VMRs). The analogue input channels were sampled with individual sampling rates, and digital filtering was performed on the signals. The balloon pressure signals were sampled at 50 Hz. A highpass filter at 1 Hz was used to separate the contraction-induced pressure changes from the slow varying pressure generated by the barostat. A resistance in the airflow between the pressure generator and the pressure transducer further enhanced the pressure variations induced by the abdominal contractions of the animal. A customized software (CDR Analytics v2.0, J4 Style) was used to quantify the magnitude of the highpass-filtered balloon pressure signals. The average rectified value of the highpass-filtered balloon pressure signals was calculated for 30 s before the pulse (i.e. baseline activity) and for the duration of the pulse. This measure allows the contractions of the abdominal muscles to be distinguished from the relaxations or compensations of the barostat. When calculating the magnitude of the highpass-filtered balloon pressure signals, the first and last 4.5 seconds of each pulse were excluded since these reflect artifact signals produced by the barostat during inflation and deflation and do not originate from the animal. The total response to a CRD protocol was calculated as the area under curve (AUC) of the corresponding VMRs registered during the distension time, corrected for the baseline activity.

Evaluation of referred mechanical somatic hyperalgesia: von Frey test

Animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor, through which von Frey monofilaments were applied for pain assessment (bending force range from 1 to 26 g; North Coast Medical, Inc.; Gilroy, CA, USA). Pain sensitivity was evaluated after a 30-40 min habituation period to the testing environment. Somatic referred pain was quantified by measuring in the hind paws the withdrawal response to punctate mechanical stimulation, as previously described.[45,46] For each animal, the mean measure of the right and left hid paw was taken as the measure of sensitivity. Pain thresholds were determined using the up-down method paradigm and represent the mechanical threshold that produces 50% of the maximal response.[47] The percentage of DSS-induced algesic effect was calculated as: % = [(PWB - PWI) / PWD] * 100, where PWB and PWI are the basal paw threshold (g) and paw threshold on the day of interest, respectively.

Experimental protocols

Animals were weighted and randomly distributed in two experimental groups (control and colitis; experimental day -1). Thereafter they received 5% DSS during 7 consecutive days to induce colitis (experimental days 0 to 7). The control group received normal tap water during the same period. After finishing the induction of colitis, a resting period of 9 days (experimental days 7 to 16) was allowed and thereafter somatic (von Frey test) and visceral sensitivity was assessed at regular intervals throughout the following 20-day period (experimental days 16 to 35), with 3-4 days between consecutive tests. Basal somatic and visceral sensitivity were assessed at the start of the DSS exposure (experimental days -1 and 0 for

somatic and visceral sensitivity, respectively). In addition, at experimental days 0 (basal), 21 and 35 some animals were euthanized for the assessment of inflammation (see below). A schematic representation of the experimental protocols is included in Fig. 1.

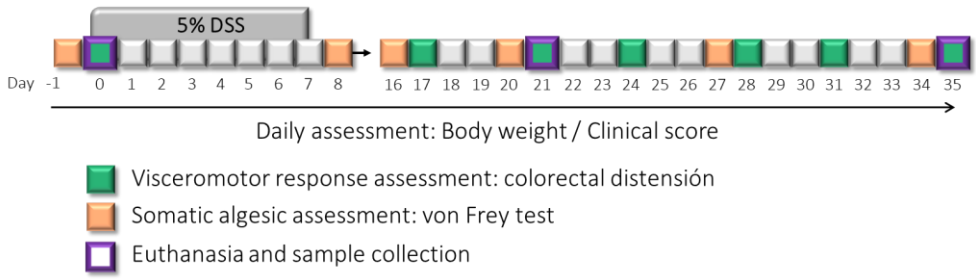

Figure 1. Schematic representation of the experimental protocols followed in the study.

Samples collection

At experimental days 0, 21 and 35, control and DSS-treated rats were deeply anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and euthanatized by exsanguination through intracardiac puncture. Thereafter, a medial laparotomy was performed, the ceco-colonic region localized and the colon dissected. Afterward, a tissue sample from the proximal colon were collected and frozen immediately in liquid nitrogen. In addition, dorsal root ganglia (DRGs) corresponding to the lumbosacral region of the spinal cord and the lumbosacral region of the spinal cord (L3-S2) were also collected and frozen immediately. Frozen samples were stored at -80 ºC until analysis. At the same time, tissue samples of the middle colon (about 3 cm) were collected and fixed overnight in 4% paraformaldehyde. Afterwards, tissues were paraffin embedded and 5-µm-thick sections obtained.

Clinical and macroscopic assessment of inflammation

Clinical assessment of inflammation included daily monitoring of body weight, appearance of faeces and general health condition. A scores (0–8) were assigned for health condition (including hunch posture, piloerection, faecal consistency and anal inflammation); where 0 indicates normal activity/fur/faecal content/no anal inflammation, 1 indicates abnormal gait/bristly fur/wet anus/loose faecal content and 2 indicates prostrated animal/dirty fur/watery or bloody rest on anus/watery diarrhea. At necropsy, the macroscopic appearance of the colon (inflammatory score) was scored based on: consistency of faecal content (score 0–3) and presence of visible faecal blood (score 0–3). The score was also based on the extent of oedema (0–3), thickness (0–3), stiffness (0–2) and presence of ulcerations (0–1); resulting in a maximum total score of 15.[22]

Histological studies

Hematoxylin-eosin-stained sections from the colon were obtained, following standard procedures, for their histological examination. A histopathological score (ranging from 0, normal, to 12, maximal alterations) was assigned to each animal. Specifically, parameters scored included: epithelial structure (0: normal; 1: mild alterations of the villi; 2: local villi destruction and/or fusion; 3: generalized villi destruction and/or fusion), structure of the crypts (0: normal; 1: mild alterations of the crypts; 2: local destruction of the crypts; 3: generalized destruction of the crypts), presence of edema (0: normal; 1: mild local edema in submucosa and/or lamina propria; 2: moderate diffuse edema in submucosa and/or lamina propria; 3: severe generalized edema in submucosa and/or lamina propria), and presence of inflammatory infiltrate (0: normal; 1: mild localized

infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate). Scoring was performed on coded slides by two independent researchers.

Gene expression using Quantitative Reverse Transcription-PCR

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit (Ambion/Applied biosystems, Foster City, CA, USA). RNA was purified by via precipitation with lithium chloride.[48] Later, a two-step quantitative real-time PCR (RT-qPCR) was performed. RNA samples were converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR (iTaq Universal™ SYBR® Green Supermix, Bio-Rad) was incubated on the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad). All samples were assayed in triplicate. The cycle thresholds for each sample were obtained and data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with non-inflamed vehicle-treated animals serving as the calibrator.[49] PrimePCR™ SYBR® Green Assay (Bio-Rad) for interferon γ (INF-γ; qRnoCID0006848), interleukin 1β (IL-1β; qRnoCID0004680) and interleukin 10 (IL-10; qRnoCID0005930) were used for inflammatory response. Cannabinoid receptor 1 (CB1; qRnoCED0008430) and 2 (CB2; qRnoCED0008595), μ-opioid receptor (MOR; qRnoCED0003071), transient receptor potential vanilloid 1 (TRPV1; qRnoCID0003147) and 3 (TRPV3; qRnoCID0007727), nerve growth factor (NGF; qRnoCID0003911), receptor activity modifying protein 1 (Ramp1; qRnoCED0001653), and σ_1 receptor (σ_1R; qRnoCED0005919) were used for assessment of neuronal/sensory activity. Actin β (Actb; qRnoCID0056984) and Hypoxanthine-guanine phosphoribosyltransferase 1 (Hprt1; qRnoCED0057020) were used as endogenous reference genes.

Statistical analysis

Data are expressed as mean±SEM. A robust analysis (one iteration) was used to obtain mean±SEM for RT-qPCR data. Data were analyzed by one- or two-way ANOVA, with or without repeated measures, as appropriate, followed, when necessary, by a Bonferroni's multiple comparisons test. Data were considered statistically significant when $P<0.05$. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) or SPSS program (version 17 for Windows, IBM, Madrid, Spain).

RESULTS

In non-inflamed animals, none of the parameters assessed showed time-related changes. Therefore, control data obtained at experimental days 0, 21 and 35 has been pooled as a single control group.

Colitis development and follow-up

Rats not exposed to DSS showed a linear body weight increase throughout the experimental time, with no clinical signs of inflammation. In contrast, animals exposed to DSS showed changes in body weight and presented clinical signs, consistent with the development of colitis, in a time-related manner (Fig. 2). Body weight showed a decline from experimental day 2 ($P<0.05$ vs. body weight at day 0; $P<0.05$ vs. body weight in animals without colitis at the corresponding days), with a peak reduction of 7.0±2.9% by day 17. Thereafter, body weight increased slowly but without recovering the growth ratio observed in control conditions (Fig.

2A). In the same animals, clinical signs of colitis, such as piloerection, loose feces/watery diarrhea, and fecal blood were observed, with a peak between experimental days 7 and 10 ($P<0.05$ vs. control group; Fig. 2B). Thereafter, clinical signs subsided and were absent from experimental day 21 day onwards.

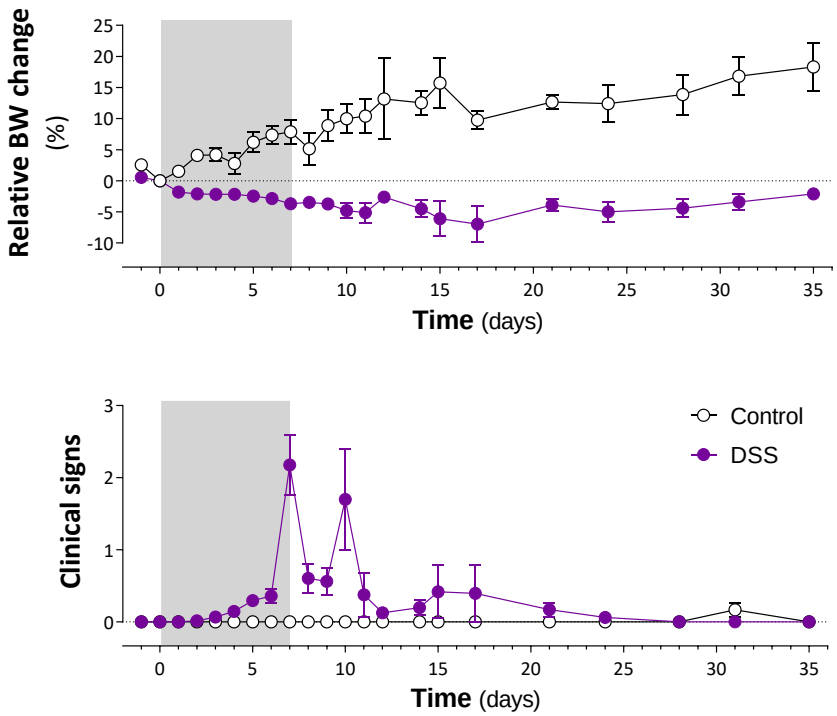

Figure 2. Clinical characterization of DSS-induced colitis in rats: Changes in relative body weight (% change from day 0, taken as 100%) and clinical signs. Data are mean±SEM (n=5-16). The grey square denotes the period of DSS exposure.

At experimental days 21 and 35, the colon of animals exposed to DSS showed some macroscopic signs of inflammation (Fig. 3A-C) and

histopathological alterations, particularly as it relates to the presence of inflammatory infiltrate through the colonic wall (Fig. 3D).

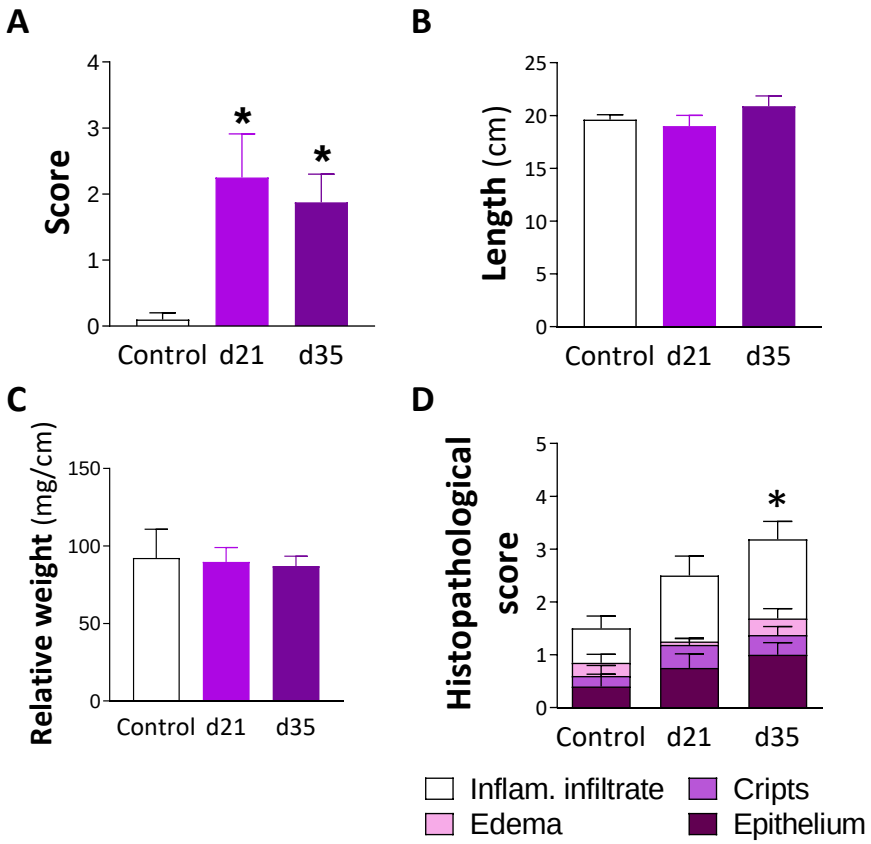

Figure 3. Macro and microscopic assessment of the colon at the time of necropsy in healthy controls and animals with DSS-induced colitis at experimental days 21 (d21) and 35 (d35). A: macroscopic assessment of the colon; B: colon length; C: colonic relative weight; D: colonic histopathological scores. Data are mean±SEM of 4-5 animals per group. d21: experimental day 21; d35: experimental day 35: *: $P<0.05$ vs. control group.

In all cases, expression (RT-qPCR) of INF-γ, IL-1β and IL-10 was detectable and quantifiable in colon, lumbosacral DRGs and lumbosacral spinal cord. In animals with colitis, time-related changes in the expression of colonic

pro-inflammatory cytokines, INF-γ and IL-1B, with an up-regulation by experimental day 21 (IL-1B: P <0.05 vs. control; Fig. 4A) and a normalization in the expression by day 35 were observed. Similar changes, although delayed in time, were detected in DRGs and spinal cord. In this case, up-regulation of pro-inflammatory cytokines increased with time, reaching statistically significant values at experimental day 35 (Fig. 4B-C).

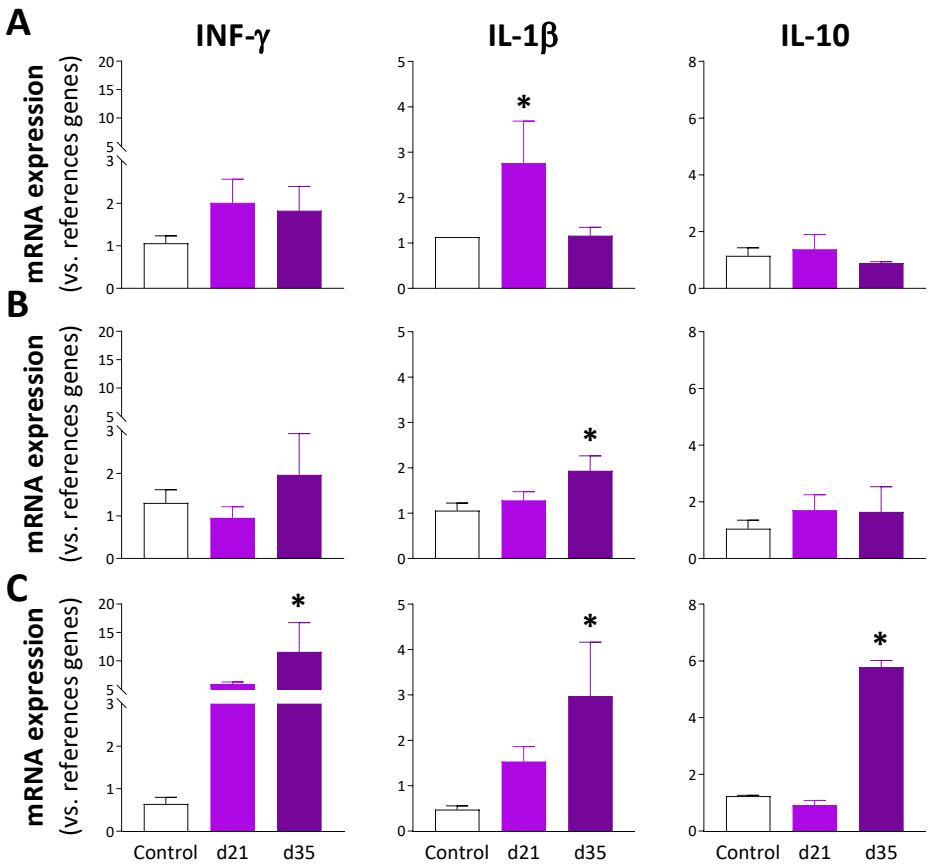

Figure 4. Effects of DSS-induced colitis on the expression of pro-(INF-γ) and anti-inflammatory cytokines (IL-10) in colon (A), lumbosacral DRGs (B) and lumbosacral spinal cord (C) as. Data are mean±SEM, n=4-5 per group. d21: experimental day 21; d35: experimental day 35: *: P<0.05 vs. control group.

The expression of the anti-inflammatory cytokine IL-10 was only modified at the lumbosacral spinal cord at experimental day 35, showing a significant up-regulation ($P<0.05$ vs. control; Fig. 4).

Effects of DSS-induced colitis on colonic sensitivity

In control conditions (experimental day 0), repetitive phasic CRD at 80 mmHg elicited VMRs similar to those described elsewhere when using this protocol. CRD evoked a significant increase in VMRs vs. basal activity (first distension: 0.21±0.04; $P<0.05$ vs. basal activity). Moreover, VMRs showed a distension-related increase along the CRD protocol (first distension: 0.21±0.04, $P<0.05$ vs. 12^{th} distension: 0.39±0.5), with an overall 201±27% increase in the response observed from the first to the last distension (Fig. 5A). Animals not exposed to DSS maintained this response to phasic CRD throughout the experimental time with only small, non-significant, fluctuations, as assessed in experimental days 17, 21, 24, 28, 31 and 35 ($P<0.05$ two-way ANOVA with time and inflammation as factors; Fig. 5). Animals exposed to DSS showed increased VMRs to phasic CRD from experimental day 17 when compared to healthy animals. Increased responses to CRD were maintained on time until the end of the experimental time ($P<0.05$ vs. control; Fig. 5C). As an example, at experimental day 35, in colitic animals VMRs to phasic CRD increased from the first (0.40±0.06) to the last distension (0.88±0.12; $P<0.05$ vs. VMR during the first distension), with an overall 255±33% increase from the first to the last distension (Fig. 5).

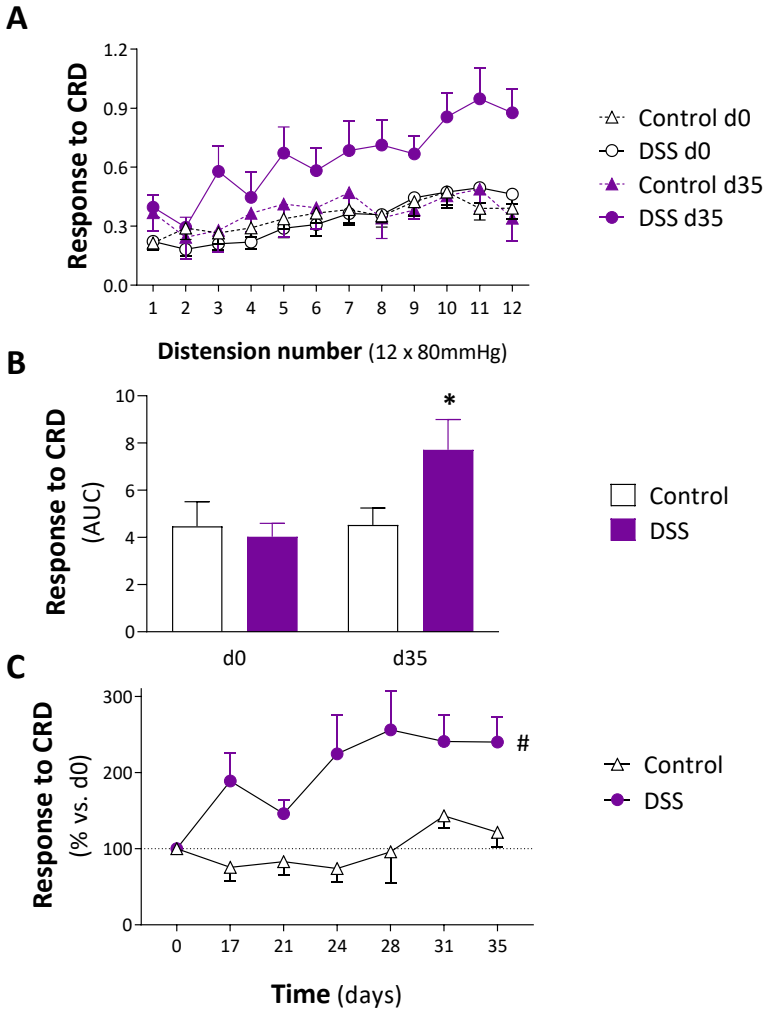

Figure 5. Effect of DSS-induced colitis on VMR responses to phasic repetitive CRD. A: VMRs to repetitive phasic CRD in healthy and DSS-induced colitic rats at experimental days 0 (d0) and 35 (d35). B: Cumulative responses to phasic CRD (AUC) of the experimental groups included in A. C: Time-course changes in the cumulative responses to phasic CRD (AUC) throughout the experimental time. Data represents % change from the VMRs determined in basal conditions at experimental day 0, taken as 100%. Data are mean±SEM (n=7-16). *: P<0.05 vs. control group; #: P<0.05 vs. control (P<0.05 two-way ANOVA with time and inflammation as factors).

Effects of DSS-induced colitis on referred somatic mechanical sensitivity

In colitic rats, small fluctuations in referred mechanical sensitivity, manifested as a reduction in the force needed to evoke a paw withdrawal reflex, was observed from experimental day 16 (vs. sensitivity measures determined at experimental day -1, taken as basal sensitivity). However, significance was only achieved at experimental days 16 and 27 (Fig. 6).

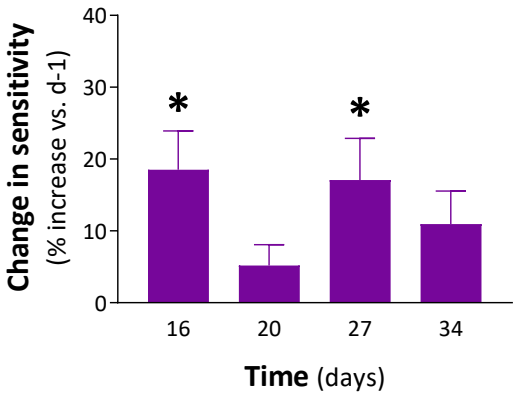

Figure 6. Time course changes (increase) in mechanical referred somatic sensitivity in animals with DSS-induced colitis as assessed in the hind paws [relative to experimental day -1, taken as basal sensitivity, 0% change]. Data are mean±SEM of n=8 animals per group. *: P<0.05 vs. basal sensitivity (experimental day -1).

Effects of DSS-induced colitis on sensory-related markers

mRNA for the sensory-related markers assessed was detected in all samples, except for Ramp1 which was not detected in two spinal cord samples. In control rats, expression levels remained stable over time. As it relates to the colon, in animals with DSS-induced colitis, only the expression

of TRPV1 and Ramp1, on experimental day 35, was affected, with a significant up-regulation (both $P<0.05$ vs. control; Fig. 7). On the other hand, colonic expression of σ_1Rs was significantly down-regulated ($P<0.05$ vs. control, for experimental days 21 and 35; Fig. 7).

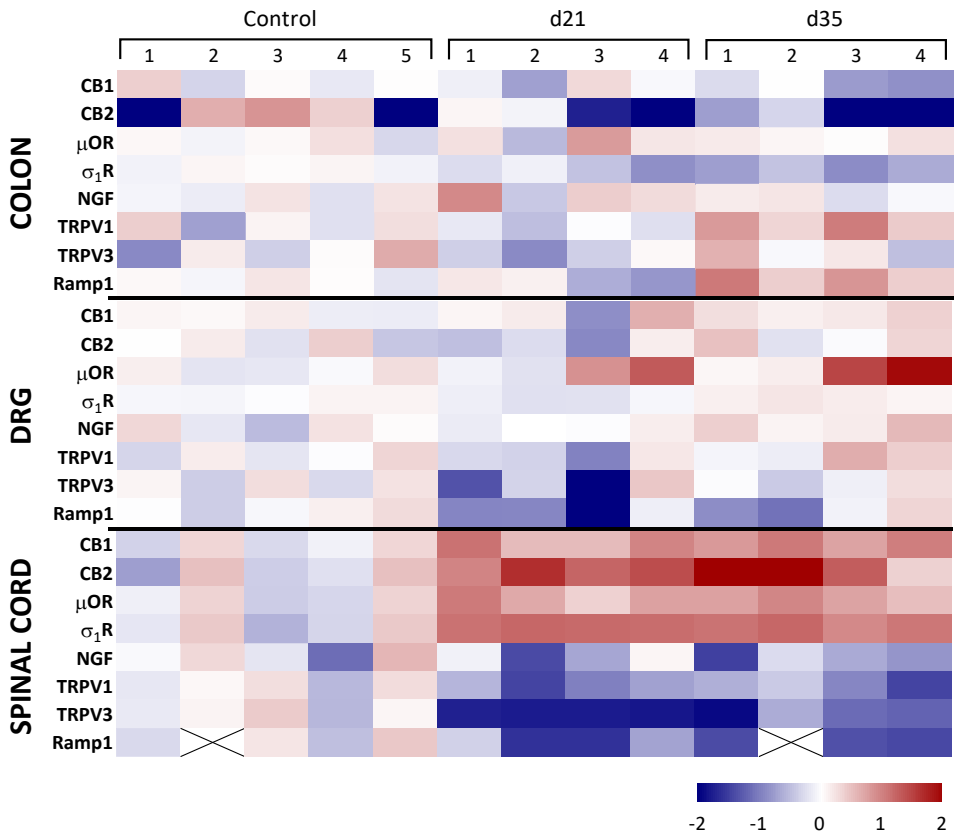

Figure 7. Heat map of the relative mRNA expression of sensory-related markers in colon, lumbosacral DRGs and lumbosacral spinal cord in control conditions and after DSS-induced colitis (experimental days 21 and 35). The cross (X) denotes the two spinal cord samples in which no expression of Ramp1 was detected.

Within the lumbosacral spinal cord, DSS-induced colitis was associated to an overall up-regulation of anti-nociceptive-related markers ($P<0.05$ for

CB1, CB2 and MOR vs. control; Fig. 7) and a down-regulation of pro-nociceptive-related markers ($P<0.05$ for TRPV1 and 3 and CGRP vs. control; Fig. 7). Spinal σ_1Rs were up-regulated during colitis ($P<0.05$ vs. controls, regardless the time considered; Fig. 7).

In lumbosacral DRGs, only σ_1R expression was affected, with a dual response including a down-regulation at early times (experimental day 21) ($P<0.05$ vs control) followed by an up-regulation at experimental day 35 ($P<0.05$ vs. control; Fig. 7).

DISCUSSION

Robust models for the study of visceral pain and the characterization of potential therapeutic targets are still a need. Taking into account the clinical characteristics of visceral pain of intestinal origin these models should, ideally, involve states of long-lasting hypersensitivity. In the present report, we present evidences supporting the potential value of DDS-induced colitis in rats as a way to induce long-lasting colonic hypersensitivity, thus providing an animal model to investigate visceral pain mechanisms, to characterize new therapeutic targets and for the validation/screening of new drugs.

It is well recognized that inflammation and pain are associated phenomena in multiple organic systems, including somatic and visceral structures. Within the GI tract, inflammatory and functional bowel disorders are characterized by the coexistence of inflammation and visceral pain, with chronic states of hypersensitivity as a distinctive feature.[1–3] Taking this into account, animal models to study visceral pain arising from the gut are largely based on the induction of inflammation or irritation (with

the consequent immune activation) of the GI tract.[7,8,10,50] In this context, DSS-induced colitis is a well-validated and accepted model of human colitis (in particular ulcerative colitis).[21–25] Compared to other inflammatory models, such as TNBS/DNBS-induced inflammation, the DSS model induces a less aggressive inflammatory response that, according to some authors, resembles in a more accurate manner the inflammation observed in humans.[26] In the present conditions, exposure to DSS led to the induction of colitis, as determined by the presence of clinical signs consistent with those previously reported for this model.[15–18] As previously characterized in mice, in rats DSS exposure leads to an acute state of colitis that partially resolves over time[15,20] generating a state of chronic inflammation, which was evident up to 4 weeks after the induction of inflammation. During this state, although no clinical signs were noticed and a gain of body weight was observed, histopathological and molecular alterations (moderate up-regulation of inflammatory cytokines) consistent with the present of inflammation were still detected. These long-term changes are reminiscent of the quiescent phases observed IBD patients when the flares of the disease resolve and to low degree on inflammation described in IBS patients.[1,2] Therefore, the model might bear utility reproducing the functional/structural/molecular alterations of IBS or latent IBD.

Interestingly, evidence of long-lasting immune activation outside the intestine, specifically in neural substrata important in sensory processing from the colon, i.e. lumbosacral DRGs and spinal cord was also observed. Moreover, expression changes of immune-related mediators at these locations increased over time, thus further supporting the presence of long-lasting molecular alteration, even outside the original site of inflammation, with potential significance in the processing of afferent sensory signals. These changes might suggest an inflammation-associated remodeling of central (spinal) mechanisms that might be related to alterations in pain

sensitivity, as previously suggested for other models of gastrointestinal inflammation, such as during *T. spiralis*-induced enteritis.[51] Altogether, these observations support the presence of changes in visceral sensitivity during DSS-induced colitis.

In animals without colitis, phasic CRD elicited VMRs indicative of visceral pain, with the presence of acute mechanical sensitization; similar to that previously described in similar experimental conditions using the same distension parading.[42] In animals with DSS-induced colitis, similar responses to phasic CRD were observed. However, during colitis, overall responses to CRD were increased when compared to those obtained in non colitic animals, thus suggesting the presence of inflammation-associated hypersensitivity. Moreover, this response persisted over time, as assessed in repeated occasions between days 17 and 35 after the induction of inflammation. This suggests that the hyperalgesic responses associated to inflammation were long-lasting, even though the resolution of colitis from a clinical point of view (see above). Indeed, we have observed a similar hyperalgesic state in animals with DSS-induced colitis up to 49 days after the induction of inflammation (data not shown). Altogether, these observations suggest that DSS-induced colitis results in a reproducible long-term state of colonic mechanical hyperalgesia, thus representing a feasible animal model to study this condition.

Visceral pain sensitization, either in humans or in relevant animal models, has been associated to peripheral and central sensitization mechanism implying changes in sensory pathways leading to an increase in pain transmission and, therefore, to exacerbated pain manifestations (hypersensitivity).[52,53] Inflammatory mediators, such as cytokines, released during inflammation have been directly implicated in peripheral sensitization. During DSS-induced colitis, there is a significant local (colon) up-regulation of pro-inflammatory cytokines (present observations).[20,22]

This might contribute to the increased responses to CRD observed in animals with colitis. Moreover, an up-regulation in pro-inflammatory cytokines was also observed at central sites related with the processing of sensory signals arising from the colon (lumbosacral DRGs and lumbosacral region of the spinal cord).[54,55] Therefore, it is feasible to assume that a similar cytokine-mediated sensitization process might take place at central (spinal relevant areas). Al together, these data suggest that a combination of peripheral and central (spinal) sensitization might occur during DDS-induced colitis as the underlying mechanism explaining the mechanical hypersensitivity observe in this model. Interestingly, this reproduces the sensitization mechanisms postulated in humans with visceral (intestinal) hypersensitivity, such as during IBS, which is supposed to include central and peripheral alterations in pain processing.[5,56,57]

To further understand the mechanisms mediating colitis-associated visceral hypersensitivity the peripheral (colon) and central (spinal) expression of different sensory-related markers, mediating both pro-nociceptive and anti-nociceptive responses, was also assessed. Interestingly, differential tissue (colon vs. spinal cord)- and sensory-related (pro-nociceptive vs. anti-nociceptive) modulatory responses were detected during inflammation. Within the colon, an up-regulation of the pro-nociceptive markers TRPV1 and Ramp1 was detected, thus suggesting a facilitation of nociceptive responses consistent with the state of hyperalgesia observed in the same animals. This, agrees with the described role of TRPV1 and its modulation during inflammation in other animal models as well as in humans.[16,58–60] On the other hand, the up-regulation of Ramp1 could lead to an increase of CGRP-mediated pro-nociceptive responses at peripheral sites.[61] Within the lumbosacral spinal cord, an overall down-regulation of pro-nociceptive markers and an up-regulation of anti-nociceptive markers was observed. These responses might seem

incongruent with the presence of hypersensitivity and central spinal sensitization (as discussed above), but, as a whole, they can be interpreted as the expression of compensatory mechanisms normalizing nociceptive mechanisms and thus avoiding excessive/aberrant pain responses in states of sensitization.

It is noteworthy that during colitis the pro-nociceptive σ_1R followed a reverse pattern of expression. It was down-regulated at the periphery (colon) and up-regulated at central levels (lumbosacral spinal cord). Activation of σ_1Rs in the spinal cord produce mechanical hypersensitivity and increased pain responses in different animal models.[62,63] Therefore, the changes observed might support a direct role for σ_1Rs on the development of inflammation-associated central sensitization.

One of the distinctive characteristics of visceral pain is its capacity to generate referred somatic pain, revealed as sensitivity changes (hypersensitivity) that can be detected in the paws, tail or even the abdominal wall.[6,64] To explore this possibility, we also assessed the presence of referred somatic pain during colitis-induced visceral hypersensitivity. For this, we evaluated time-related mechanical sensitivity (withdrawal responses) of the hind paws in the same animals with DSS-induced colitis used to assess viscerosensitivity. In the present conditions, moderate pro-algesic responses to the mechanical probing of the hind paws were detected, thus indicating a relatively poor association between DSS-induced colitis and the induction of referred somatic hyperalgesia.

In summary, we present evidences supporting the feasibility of DSS-induced colitis in rats as an experimental model of long-lasting visceral hypersensitivity, reminiscent of the hypersensitive state observed in humans with functional and inflammatory GI disorders. The changes in viscerosensitivity observed are likely to result from the interaction between

central (spinal cord) and peripheral (colon) sensitizing mechanisms implicating a site-specific modulation of both pro- and anti-nociceptive pathways and resulting in a persistent state of sensitization. The overall characteristics of the model makes feasible its use in the characterization of new therapeutic target and the validation of new drugs for the treatment of visceral pain arising from the GI tract. In any case, these results warrant further validation studies as to ensure the translational relevance of the model.

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CHAPTER 4

Modulatory role of σ_1Rs on normal viscerosensitivity and inflammation-induced hypersensitivity in a model of colorectal distension in rats

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ABSTRACT

In humans, visceral hypersensitivity is a key component of functional and inflammatory gastrointestinal (GI) disorders. Sigma-1 receptors (σ_1Rs) modulate nociception and pain sensitization and might be important in inflammatory states. Here, we assessed the potential involvement of the selective σ_1R antagonist BD1063 and the dual compound EST73502 (with antagonist sigma and agonist μ opioid components) in visceral sensitivity in basal conditions and during intestinal inflammation-induced hypersensitivity.

Adult, female SD rats were used. The DSS-induced colitis model was used to induce long-lasting inflammation-associated visceral (colonic) hypersensitivity. Colonic sensitivity was assessed by determining pain-related visceromotor responses (VMRs) to isobaric colorectal distension (CRD) with a barostat.

In normal conditions, BD1063 (80 or 120 mg/kg, sc) did not affect neither VMRs nor pain thresholds, while morphine (0.1-10 mg/kg, sc) and pregabalin (10-200 μmol/kg, po) reduced VMRs and increased pain thresholds in a dose-related manner. EST73502 significantly increased pain thresholds. Neither BD1063 nor EST73502 (0.1 or 1 mg/kg, sc) affected mechanical sensitization during CRD. BD1063 did not affect VMRs in animals with colitis-induced hypersensitivity. Moreover, no synergy was observed in the co-administration of BD1063 with morphine or pregabalin. On the other hand, EST73502 (1 or 3 mg/kg, sc) significantly attenuated colitis-associated hypersensitivity, showing a selective anti-hyperalgesic activity.

These results indicate that σ_1Rs play a minor role in the modulation of viscerosensitivity, either in normal conditions or during inflammation-induced hypersensitivity.

INTRODUCTION

Abdominal pain and visceral hypersensitivity are common findings in inflammatory and functional gastrointestinal (GI) disorders.[1–3] Indeed, overt states of inflammation, such as during inflammatory bowel disease, or a low degree of intestinal inflammation, such as during irritable bowel syndrome, are associated to altered colonic sensitivity during colorectal distension; with indications of the presence of both allodynia (painful sensation to stimuli that are not normally painful) and hyperalgesia (a nociceptive stimulus is perceived with a greater intensity than normal).[4,5]

Current treatments for visceral pain are usually not directed selectively to reduce visceral sensitivity and are characterized by having a systemic effect, thus reducing efficacy and/or increasing the possibility of undesired side effects.[2,6,7] Therefore, drugs acting on specific visceral pain-related therapeutic targets are needed.

During the last years, sigma 1 receptors (σ_1Rs) have been explored as a new target for the treatment of pain.[8,9] σ_1R-mediated analgesic effects have been described in different animal models of chemical and neuropathic pain, as deduced from studies on σ_1R knockout (KO) mice[10,11] and using selective σ_1R antagonists.[11–15] Moreover, the use of different selective σ_1R antagonists has shown positive effects in reducing inflammation-related hyperalgesia.[16–19] Regarding visceral pain, the antagonism of the σ_1R have also been tested. In particular, González-Cano et al. showed a reduction in visceral hyperalgesia, both in a model of intracolonic capsaicin-induced pain and in a model of cystitis. In these models, the constitutive knockout of the receptor or the use of selective σ_1R antagonists resulted in significant attenuation of visceral pain-related responses, thus suggesting a potential

value in the treatment of visceral pain.[20,21] Despite these works, the evidence of efficacy in visceral pain is still limited and additional studies are needed.

Since its discovery, σ_1R has been related to the opioid system. Initially it was considered a new type of opioid receptor. However, the absence of the canonical opioid G-protein coupled receptor structure leads to the consideration of σ_1Rs as a separate receptor class. Nevertheless, compelling evidences show a function interaction between the opioidergic and the sigmaergic systems. Indeed, σ_1Rs can modulate opioid analgesia *in vivo* and opioid receptor signaling mechanisms *in vitro.*[22–24] The combined use of opioids and sigma antagonists has been explored in several animal models of nociception. These studies indicate that analgesic effects of σ_1Rs are mediated by dual, opioid-dependent and opioid-independent, mechanisms. In general, a synergetic anti-nociceptive effect was revealed upon the simultaneous administration of both σ_1R antagonists and opioid agonists.[14,25–27] These observations led to the development of new dual molecules with both agonist opioid and antagonist sigma components. In this context, EST73502 has been recently characterized as a dual μ-opioid receptor agonist and σ_1R antagonist.[28] In preliminary studies, EST73502 potentiated opioid analgesia in animal models of acute (paw mechanosensitivity) and chronic pain (partial sciatic nerve ligation) with decreased opioid-related side effects at GI level.[28]

Following these considerations, this study aims to assess the potential modulatory role of σ_1Rs o visceral pain airing from the GI tract (colon). For this purpose, we assessed the effects of the selective σ_1R antagonist BD1063 on a model of colorectal distension (CRD)-induced visceral pain in rats.[29–32] To characterize potential interactions between the sigmaergic and the opioidergic systems we also assessed the effects of the co-administration of BD1063 and morphine as well as the effects of the dual

sigma-opioid molecule EST73502. Since the presence of sensitization is a common phenomenon associated to visceral pain[1–3] we assessed pain responses associated to CRD in healthy animals and in animals with inflammation-associated hypersensitivity. For this, we used a model of DSS-induced colitis, which results in the induction of long-lasting inflammatory hyperalgesia.[33–35]

MATERIALS AND METHODS

Animals

Female Sprague Dawley (SD) rats, 10-12 week–old at the beginning of the experiments, (Charles River, France) were used. All animals were maintained in conventional conditions in an environmentally controlled room (20–22 ºC, 12 h light:dark cycle), with food and water *ad libitum*, except when receiving DSS (see below). Rats were allowed to acclimatize to the animal facility for at least 1 week before starting the studies. Females were used because of the higher prevalence of GI dysfunctions associated with visceral pain, such as IBS, in women.[36] Given the inconclusive observations from previous studies as it relates to a possible relationship between the estrus cycle and visceral sensitivity,[32,37] the phase of the estrous cycle was not taken into consideration in the current study. All experiments were performed in accordance with EU regulations and were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocols 3958 and 3961) and the Generalitat de Catalunya (protocol 9915).

Induction of inflammation-associated colonic hypersensitivity

The model of DSS-induced mild colitis was used to generate long-lasting colonic hypersensitivity, according to previously published protocols.[34,35,38] For colitis induction, rats received 5% DSS (45 kDa; TdB Consultancy AB, Uppsala, Sweden) in their drinking water for 7 consecutive days. Fresh DSS solutions were prepared daily. Thereafter, after a 10-day recovery period, animals were tested for their state of colonic sensitivity during colorectal distension (see below). With this procedure, rats develop a long-lasting colonic sensitization state characterized by increased pain-related responses during colorectal distension.[34,35,38] Control rats received normal tap water.

Chemicals

BD1063 (1-[2-(3,4-dichlorophenyl) ethyl]-4-methylpiperazine dihydrochloride, 80 and 120 mg/kg, po), EST73502 [(R)-9-(2,5-difluorophenethyl)-4-ethyl-2-methyl-1-oxa-4,9-diazaspiro[5.5]undecan-3-one, 0.1, 1 and 3 mg/Kg, sc], morphine hydrochloride (0.02, 0.1, 0.3, 1, 3 and 10 mg/kg, sc) and pregabalin [(S)-(+)-3-(aminomethyl)-5-methylhexanoic acid, 10, 50 and 200 µmol/kg, po; equivalent to 1.59, 7.96 and 31.85 mg/kg, respectively] were used. All drugs were freshly dissolved in 0.9% physiological sterile saline solution (B-Braun; Melsungen, Germany) immediately before use. Doses were selected based on previously published data and/or pilot studies in our experimental conditions. All chemicals were provided by Laboratorios Esteve S.A. (Barcelona, Spain).

Colorectal distension

For colorectal distension (CRD), previously published procedures, with minor adaptations, were followed.[31] Prior to experiments, rats were habituated for three consecutive days (45 to 75 minutes per day, at 15 minutes steps between consecutive days) to Bollmann cages (Plexi-glass tubes, length 18 cm, diameter 6 cm), to reduce motion artefacts and confounding effects due to restraint stress. On the day of the experiment, rats were anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain). A plastic balloon (made in-house; length: 2.5cm; diameter: 1.5cm) with connecting catheter was inserted in the distal colon (2cm from the base of the balloon to the anus). The catheter was fixed with tape to the tail of the animal. Animals were then placed in Bollmann cages and allowed to recover from anesthesia for at least 20-30 min before starting the CRD protocol.

The balloons were connected to pressure transducers (P-602, CFM-k33, 100 mmHg, Bronkhorst HI-TEC, Veenendal, The Netherlands) to control intraballoon pressure during the CRD procedure. A customized barostat system (AstraZeneca, Mölndal, Sweden) was used to manage air inflation and balloon pressure changes during the procedure. A customized computer software (Pharmlab 5.0 on-line, AstraZeneca R&D, Mölndal, Sweden), running on a standard computer, was used to control de barostat and to collect data.

Two protocols of colorectal isobaric, phasic distension were used: i) increasing phasic distensions from 10 to 80mmHg, at 10 mmHg steps, with a pulse duration of 30 s at 5 min intervals, and ii) repetitive phasic, noxious distensions at 80 mmHg, 12 pulses with a pulse duration of 30 s at 5 min intervals. The protocol consisting on increasing phasic distensions served to assess pain thresholds. Pain threshold was defined as the pressure of the distending pulse at which the response evoked exceeded the main baseline

activity plus 3 times the standard deviation. The protocol consisting on repetitive noxious distensions served to assess the development of acute mechanical hypersensitivity. Animals were used on multiple occasions, with at least a 3- to 4-day interval between consecutive experiments. The same protocols have been used elsewhere to assess pain responses elicited by colorectal distension and its pharmacological modulation in rodents.[29,31,39–41]

Rapid pressure changes in the distending balloon, reflecting contractions of the abdominal muscles, were used to assess visceromotor responses (VMR). The analogue input channels were sampled with individual sampling rates, and digital filtering was performed on the signals. The balloon pressure signals were sampled at 50 Hz. A highpass filter at 1 Hz was used to separate the contraction-induced pressure changes from the slow varying pressure generated by the barostat. A resistance in the airflow between the pressure generator and the pressure transducer further enhanced the pressure variations induced by the abdominal contractions of the animal. A customized software (CRD Analytics v2.0, J4 Style) was used to quantify the magnitude of the highpass-filtered balloon pressure signals. The average rectified value of the highpass-filtered balloon pressure signals was calculated for 30 s before the pulse (i.e. baseline activity) and for the duration of the pulse. This measure allows the contractions of the abdominal muscles to be distinguished from the relaxations or compensations of the barostat. When calculating the magnitude of the highpass-filtered balloon pressure signals, the first 4.5 seconds of each pulse were excluded since these reflect artifact signals produced by the barostat during inflation and deflation and do not originate from the animal.

Experimental protocols

Solutions or homogenous suspensions of the different drugs were prepared at the time of administration. Doses were selected based on preliminary experiments and published data showing efficacy in different pain models[28,31,42] and were based on the average treated-group body weight on the respective day. Control animals received only the respective vehicle. Subcutaneous administrations (0.5 ml/kg) were performed 30 minutes before beginning the CRD protocol; oral administrations (5 ml/kg) were performed 1 hour before starting CRD.

Statistical analysis

Data are expressed as mean±SEM. Data were analyzed by one-, two- or three-way ANOVA, with or without repeated measures, as appropriate, followed, when necessary, by a Bonferroni's multiple comparisons test. A Student's t test, paired or unpaired as appropriate, was used for comparisons between two groups. Data were considered statistically significant when $P<0.05$. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) or SPSS program (version 17 for Windows, IBM, Madrid, Spain).

RESULTS

The σ_1R antagonist BD1063 did not affect basal viscerosensitivity during CRD

In vehicle-treated animals the increasing phasic distensions protocol caused o pressure-elated increase in VMRs, with main pain thresholds

oscillating between 30 and 40 mmHg (33.6±5.0 mmHg, n=8). BD1063, either at 80 or 120 mg/kg (n=4 and 8, respectively), did not affect neither VMR nor pain thresholds (Fig. 1). Morphine (0.1, 0.3, 1, 3 or 10 mg/kg; n=4-8 for each) reduced VMR in a dose-related manner, with a complete abolition of VMRs at the dose of 1mg/Kg or higher, although relatively high interindividual variability was observed. Morphine also increased pain thresholds in a dose-related manner. A clear increase in the pain threshold was already observed at the dose of 1 mg/kg (63.3±10.5 mmHg) however, statistical significance was only achieved at the dose of 10 mg/kg (77.5±1.6 mmHg; $P<0.05$ vs. vehicle: 35.0±8.7; Fig. 1). Similarly, pregabalin (10, 50 or 200 µmol/Kg; n=4-5 for each) reduced VMRs in a dose-related manner. Despite these effects, no significant changes were observed in pain thresholds, probably because of the relatively large interindividual variability, although a clear tendency for an increase was observed at the higher dose tested (62.0±12.0 mmHg; $P>0.05$ vs. vehicle: 35.0±15.5) (Fig. 1).

Acute mechanical sensitization is not affected by the blockade of σ_1Rs with BD1063

Mechanical sensitization induced by repetitive phasic distensions at 80 mmHg was observed in healthy rats, with similar characteristics to those previously described using the same sensitization protocol.29,31,39,41 Vehicle (0.5 ml/kg, sc)-treated rats showed a significant increase in the VMR when compared with their respective basal activities (VMR 1st distension: 0.27±0.05; $P<0.05$ vs. basal: 0.04±0.01). Moreover, VMRs increased by 138±40% from the first (0.27±0.05) to the 12th distension (0.53±0.05) (Fig. 2). Selective antagonism of σ1Rs with BD1063 (80 or 120 mg/kg, n=8 and 7, respectively) did not affect acute mechanical sensitization during repetitive CRD. At 80 mg/kg, VMRs increased by 130±13% from the first (0.31±0.06)

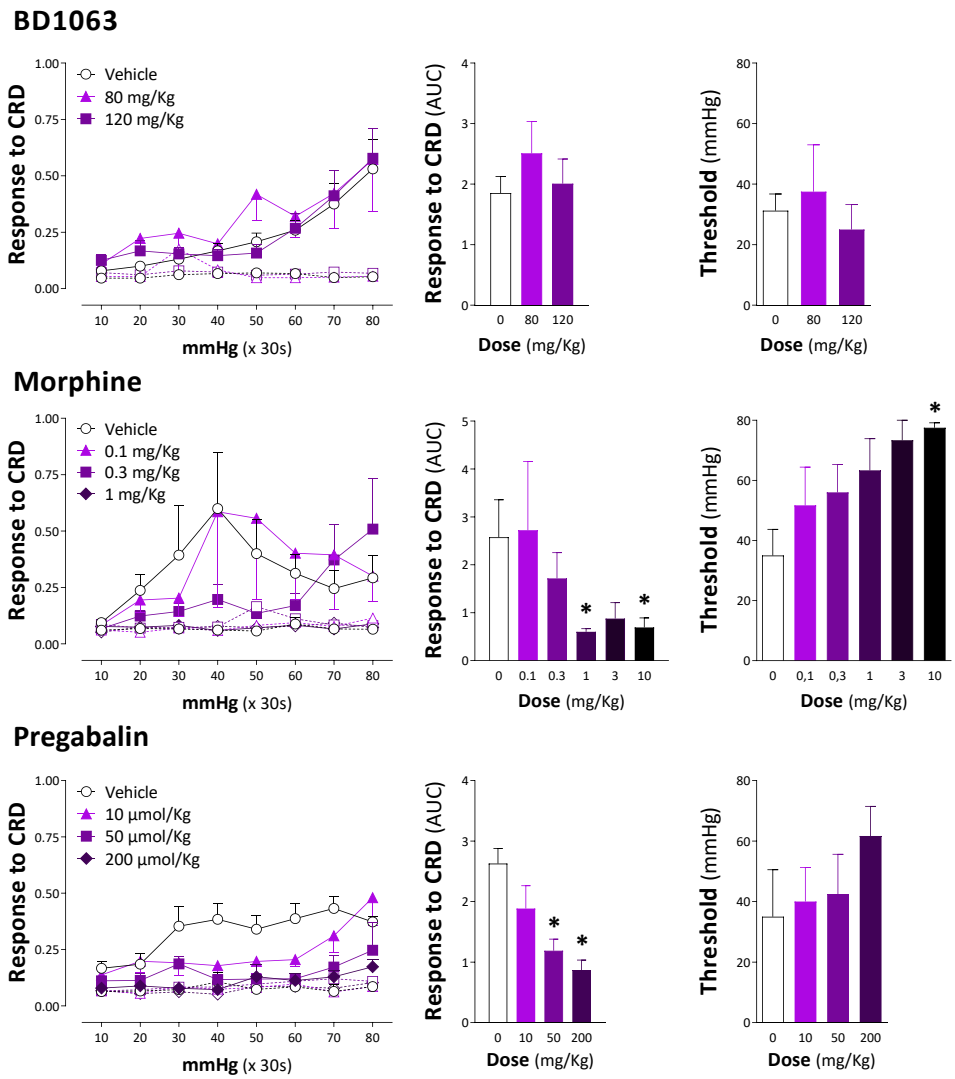


Figure 1. Effects of BD1063, morphine and pregabalin on the VMRs to increasing phasic colorectal distensions (10–80 mmHg. Left panels show VMRs for the different treatments. Solid symbols with continuous lines reflect the response to CRD. Empty symbols with broken lines reflect baseline activity prior to each distension. Central panels shows overall responses (AUC) to CRD for the different treatments. Right panels shows the corresponding pain thresholds during CDR. Data are mean±SEM, n=3-10 per group. *: P<0.05 vs. vehicle-treated group.

to the 12th distension (0.68±0.10), while at 120 mg/kg VMRs increased by 122±38% from the first (0.20±0.04) to the 12th distension (0.48±0.13) (in all cases P>0.05 vs. responses in vehicle-treated animals; Fig. 2).

Morphine (0.02, 0.1 and 1 mg/kg, n=5-7) inhibited in a dose-related manner the responses to repetitive CRD (Fig. 2). In vehicle-treated animals (n=8), VMRs increased by 85±22% from the first (0.34±0.06) to the 12^{th} distension (0.61±0.12). At 0.02 mg/kg morphine did not affect the sensitization process [182±25% increase in VMRs from the first (0.32±0.06) to the 12^{th} distension (0.92±0.18); P>0.05 vs. vehicle group]. At 0.1 mg/kg, morphine reduced by 48% the overall response to CRD; however, statistical significance was not achieved. At 1 mg/kg, morphine reduced by 66% the overall response to CRD (P<0.05 vs. vehicle; Fig. 2). At 1 mg/kg, the starting response (1^{st} distension) to CRD was completely prevented (0.08±0.02; P>0.05 vs. basal VMR: 0.07±0.02; P<0.05 vs. VMRs to first distension in vehicle-treated group: 0.34±0.06). VMRs remained at basal levels until the sixth distension, thereafter a relatively low distension-related increase in VMRs was observed, reaching a value of 0.24±0.08 during the 12^{th} distension (P<0.05 vs. VMR during the 12^{th} distension in vehicle-treated animals: 0.61±0.12).

Pregabalin-treated rats showed a similar response than vehicle (oral)-treated rats. During vehicle treatment (n=12), VMRs increased by 100±28% (first distension: 0.33±0.08, 12^{th} distension: 0.63±0.15; Fig. 2). No changes in VMRs were observed at the lowest dose of pregabalin (10 µmol/kg, po, n=6), with a 139±43% increase in VMRs from the first (0.40±0.11) to the 12^{th} distension (0.87±0.24) (P>0.05 vs. vehicle group. Fig. 2). At 50 and 200 µmol/kg (n=6, each) the overall response to CRD was reduced by 10% and 22%, respectively (P>0.05 vs. vehicle group). However, a clear sensitization process was still observed (increase in VMRs from the first to the 12^{th} distension: 79±26% at 50 µmol/kg; 98±25% at 200 µmol/kg; Fig. 2).

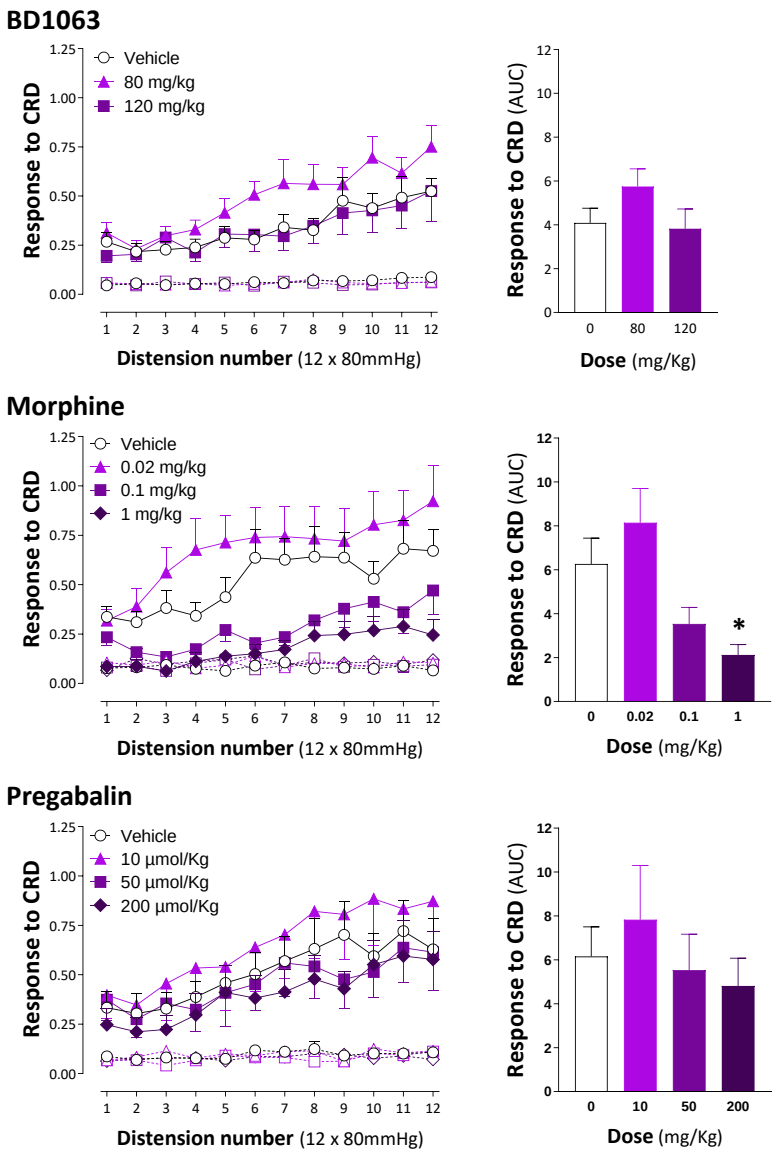


Figure 2. Effects of BD1063, morphine and pregabalin on VMRs to repetitive CRD. Left panels shows VMRs during phasic repetitive CRD for the different treatments applied. Solid symbols with continuous lines reflect the response to CRD. Empty symbols with broken lines reflect baseline activity prior to each distension. Graphs to the right show cumulative responses (AUC) during the whole repetitive phasic CRD procedure. Data are mean±SEM of 5-12 animals per group. *: P<0.05 vs. vehicle-treated group.

EST73502 attenuated VMRs during increasing phasic CRD but did not affect acute mechanical sensitization

Treatment with the dual compound EST73502 (1mg/kg, sc) totally blocked VMRs to increasing phasic CRD up to 50 mmHg. At distension pressures above 50 mmHg VMRs responses were similar to those recorded in vehicle-treated animals. The overall response to CRD was reduced by 32% (n= 7-10; Fig. 3). This translated in a significant increase in pain thresholds (58.0±8.3 mmHg; P<0.05 vs. vehicle: 22.9±36 mmHg; n= 7-10; Fig. 3).

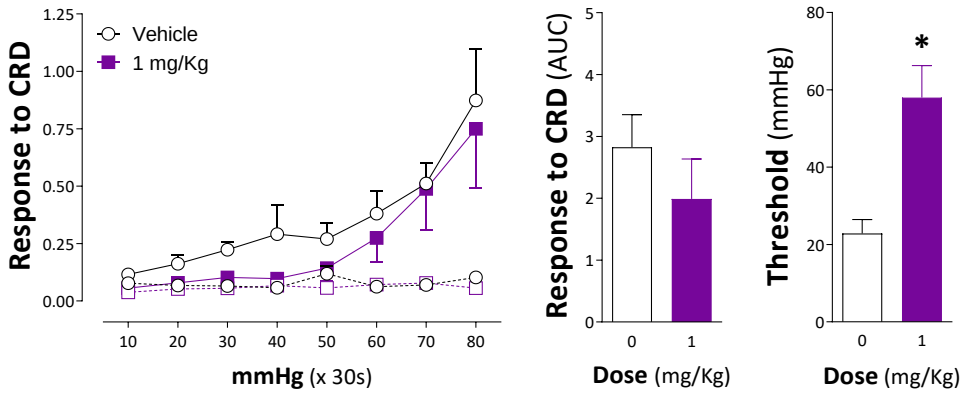


Figure 3. Effect of EST73502 on VMRs and pain thresholds. The left panel shows VMRs during phasic ascending CRD. Solid symbols with continuous lines reflect the response to the CRD. Empty symbols with broken lines reflect baseline activity prior to each distension. The central and right panels show cumulative VMRs (AUC) during the whole CRD protocol and pain thresholds, respectively. Data are mean±SEM of 7 (vehicle) and 10 (EST73502) animals. *: P<0.05 vs. vehicle-treated group.

In vehicle-treated animals (n=6), repetitive CRD increased VMRs by 221±55%, from the first (0.49±0.14) to the 12th pulse (1.30±0.23 Fig. 4). EST73502, regardless the dose considered, did not affect VMRs during

repetitive phasic CRD, which resulted in VMRs and a sensitization of similar magnitude to those observed in vehicle-treated animals (285±68%, n=7, and 149±38%, n=12, for 0.1 and 1 mg/kg, respectively; Fig. 4).

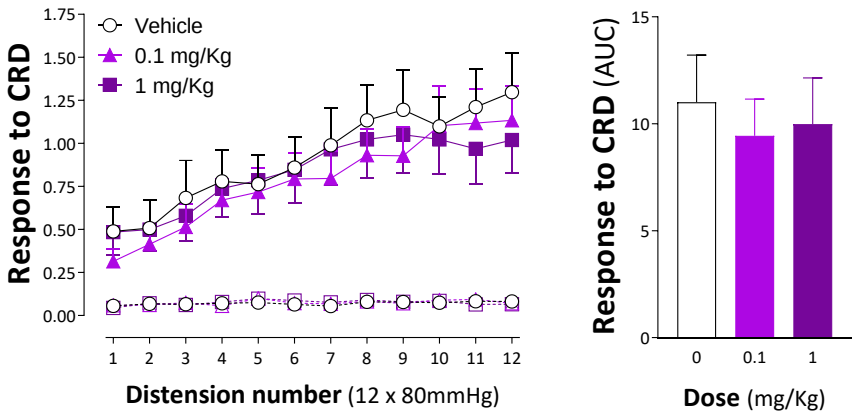


Figure 4. Effects of EST73502 on VMRs to repetitive CRD. The left panel shows VMRs during repetitive phasic CRD in the different experimental groups. Solid symbols with continuous lines reflect the response to CRD. Empty symbols with broken lines reflect baseline activity prior to each distension. The right panel show cumulative responses (AUC) during the whole CRD procedure. Data are mean±SEM of 6-12 animals per group.

BD1063 did not affect VMRs after colitis-induced hypersensitivity

After colitis, and as expected, animals developed a long-lasting mechanical hypersensitivity, manifested as increased responses to CRD. As a reference, Fig. 5 shows VMRs to phasic repetitive CRD, in the same animals, before and at day 49 after colitis induction. Before colitis animals showed an acute mechanical sensitization, similar to that described above

[VMRs to CRD increased by 81±23% from the first (0.34±0.06) to the 12th distension (0.50±0.07)]. After colitis, the initial response to CRD was higher than that observed before inflammation (0.72±0.22; P<0.05 vs. response before colitis: 0.34±0.06) and increased throughout the CRD protocol reaching a value of 0.88±0.25 during the last distension. Overall, this represented a 68±31% increase in the VMRs from the first to the 12th distension and an increase in the total response to the CRD protocol of 102±39% vs. the response observed before colitis (P<0.05; Fig. 5).

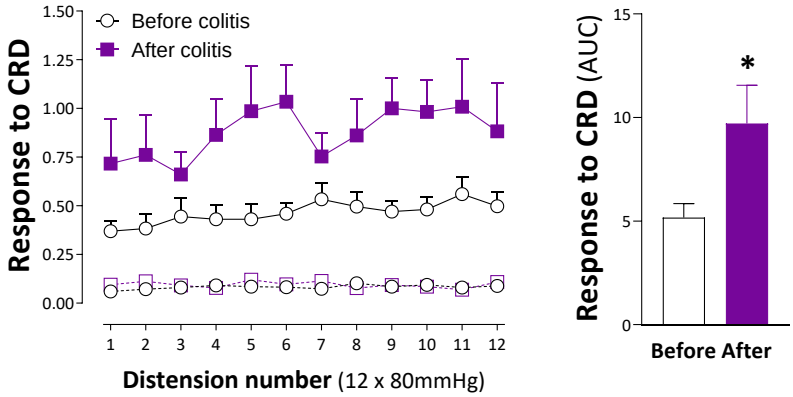


Figure 5. Inflammation (colitis)-induced hypersensitivity in rats. The left panel shows VMRs to phasic repetitive CRD, in the same animals, before and 49 days after the induction of colitis. Solid symbols with continuous lines reflect the response to CRD. Empty symbols with broken lines reflect baseline activity prior to each distension. The graph to the right represents the overall response to the CRD procedure (AUC). Data are mean±SEM of 7 animals per group.

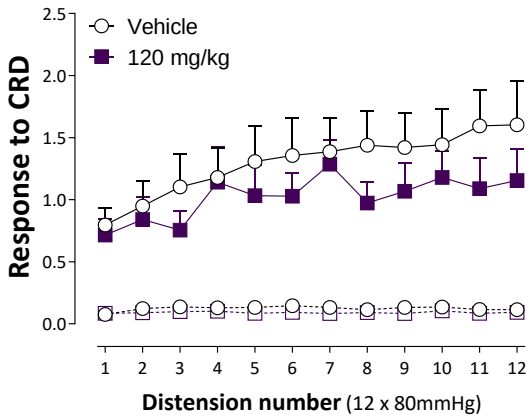
In animals with colitis-induced visceral hypersensitivity, BD1063, at 120 mg/Kg (n=8), attenuated VMRs during repetitive phasic CRD, but statistical significance was not achieved. In vehicle-treated animals, VMRs increased by 89±37%, from the first response (0.85±0.14) to the 12th distension

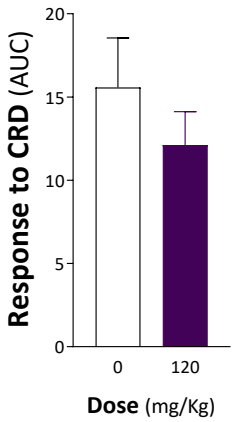
(1.52±0.40). In BD1063-treated animals, VMRs increased by 51±17% from the first (0.71±0.13) to the 12th distension (0.99±0.23). BD1063 reduced by 22% the overall response to CRD (P=0.0971 vs. vehicle-treated group). Higher doses were not tested because of the appearance of side-effects.

In sensitized animals, morphine reduced VMRs to repetitive phasic CRD in a dose-related manner (Fig. 6). Responses to repetitive CRD were not affected by the dose of 0.3 mg/kg (n=7), but at 1 and 3 mg/kg (n=7 for each) a similar attenuation of VMRs was observed. At 1 and 3 mg/kg morphine attenuated the hyperalgesic state associated to inflammation resulting in a response to repetitive CRD similar to that observed in healthy animals, with the development of similar acute mechanical sensitization. Starting (first distension) responses to CRD were 0.32±0.05 and 0.34±0.09, for 1 and 3 mg/kg, respectively (both P<0.05 vs. vehicle: 0.68±0.11) and increased throughout the protocol up to 0.90±0.20 and 0.84±0.30 during the last distension, for 1 and 3 mg/kg respectively (both P<0.05 vs. vehicle group: 1.52±0.26; Fig. 6). Accordingly, the overall response to CRD was reduced by 44% and 43% (1 and 3 mg/kg, respectively) (both P<0.05 vs response in the vehicle group).

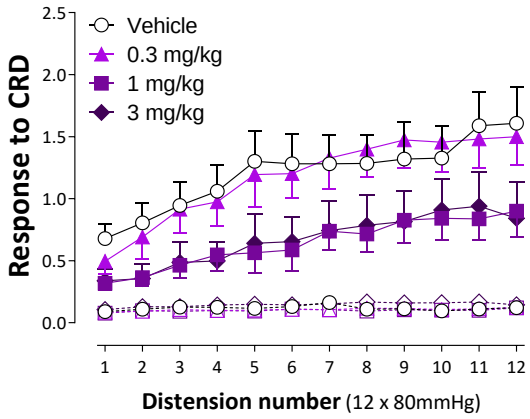
Pregabalin administered at 200 µmol/Kg reduced the overall response to CRD by 53% (P<0.05 vs respective vehicle-treated group, n=7 for each; Fig. 6). No changes were observed at 80 µmol/Kg (n=7). At 200 µmol/kg, pregabalin attenuated the hyperalgesic state associated to inflammation resulting in a response to repetitive CRD similar to that observed in non-sensitized animals, with the development of similar acute mechanical sensitization. Starting (first distension) response to CRD was 0.35±0.06 (P<0.05 vs. vehicle: 0.70±0.13) and increased throughout the protocol up to 0.77±0.11 during the last distension (P<0.05 vs. vehicle: 1.72±0.32; Fig. 6).

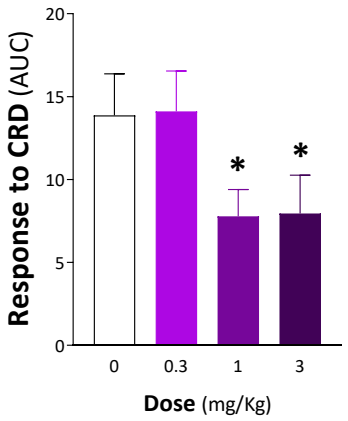
BD1063



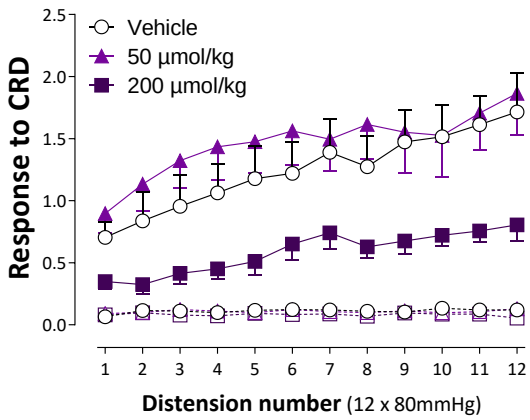


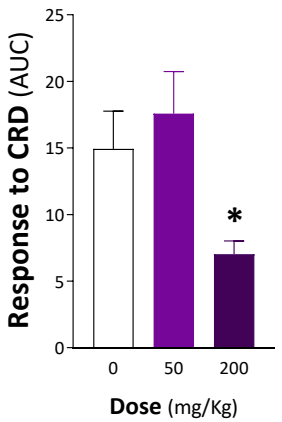
Morphine





Pregabalin





To assess potential interactions and synergies in states of hypersensitivity we also tested the effects of the co-administration of BD1063 with morphine or pregabalin. Co-administration of BD1063 and morphine (80 mg/kg and 0.3 mg/kg, respectively) did not modify VMRs during repetitive phasic CRD and the overall response (AUC) was similar in vehicle- (12.74±3.05, n=6) and morphine/BD1063-treated animals (13.74±3.51, n=6; P>0.05; Fig. 7). Co-administration of BD1063 and pregabalin (80 or 120 mg/kg and 200 µmol/Kg, n= 3 and 7, respectively) evoked VMR responses similar to those observed in vehicle-treated animals. This combination resulted in a loss of pregabalin anti-hyperalgesic effects (as observed when pregabalin was administered alone) (Figs. 6 and 7).

EST73502 attenuated hyperalgesic responses after colitis-induced hypersensitivity

In animals with colitis-induced hypersensitivity, EST73502 at 1 and 3 mg/kg (n=8 for each) reduced VMRs during repetitive phasic CRD by 39% and 47%, respectively (both P<0.05 vs. vehicle-treated group; Fig. 8). EST73502 attenuated the hyperalgesic state associated to inflammation, resulting in a response to repetitive CRD similar to that observed in non-

Figure 6. Effects of BD1063, morphine and pregabalin on VMRs to CRD in rats with colitis-induced hypersensitivity. Left panels shows VMRs to phasic repetitive CRD for the different treatments applied. Solid symbols with continuous lines reflect the response to CRD. Empty symbols with broken lines reflect baseline activity prior to each distension. Right panels show overall responses to the CRD procedure (AUC). Data are mean±SEM, 7-8 animals per group. *: P<0.05 vs. vehicle-treated group.

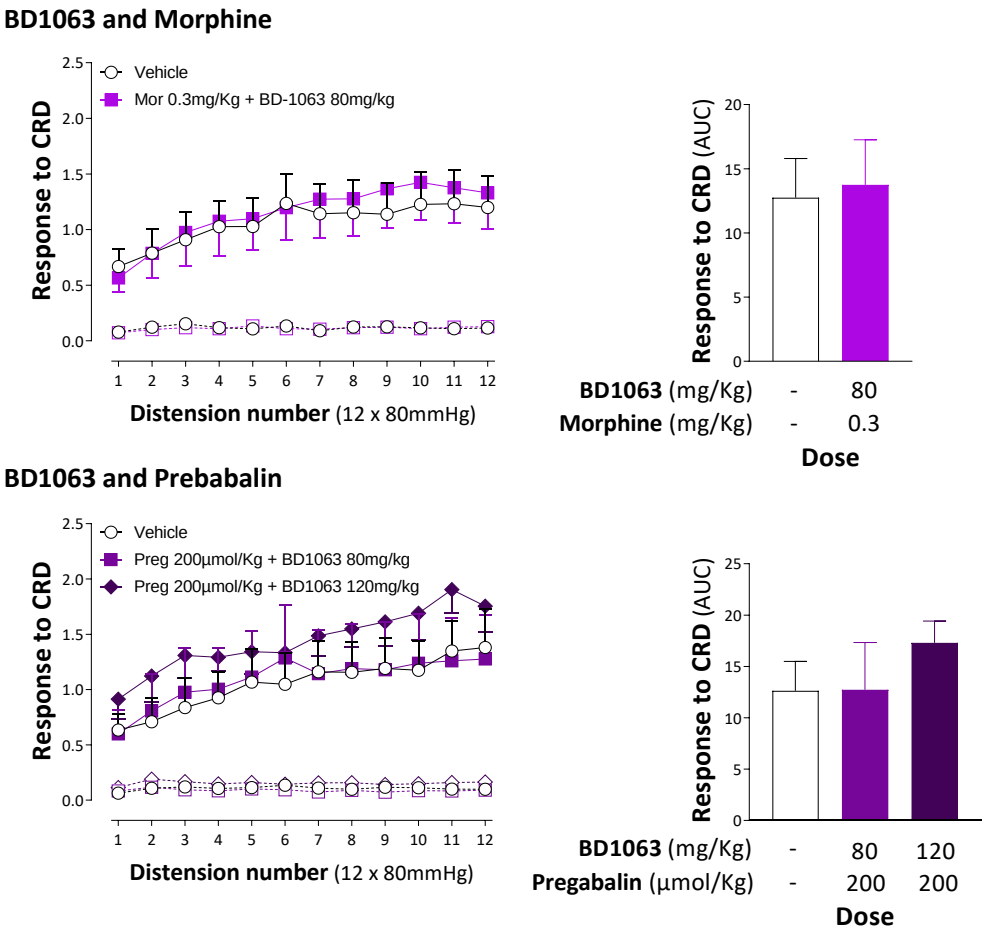


Figure 7. Effects of BD1063 combined with morphine or pregabalin on VMRs to repetitive phasic CRD in animals with colitis-induced hypersensitivity (left panel), and cumulative VMR (AUC) during the whole CRD protocol (right panel). Solid symbols with continuous lines reflect the response to CRD. Empty symbols with broken lines reflect baseline activity prior to each distension. Data are mean±SEM, 3-7 animals per group.

sensitized animals during repetitive phasic CRD, with the development of similar acute mechanical sensitization. Starting (first distension) responses sensitized animals during repetitive phasic CRD, with the development of

similar acute mechanical sensitization. Starting (first distension) responses to CRD were 0.0.30±0.08 and 0.31±0.06, for 1 and 3 mg/kg, respectively (both P<0.05 vs. vehicle: 0.65±0.11) and increased throughout the protocol up to 0.84±0.24 and 0.79±0.15 during the last distension, for 1 and 3 mg/kg respectively (both P<0.05 vs. vehicle group: 1.48±0.23; Fig. 6). Overall, VMRs observed after EST73502 were similar to those obtained in animals without colitis-associated hypersensitivity.

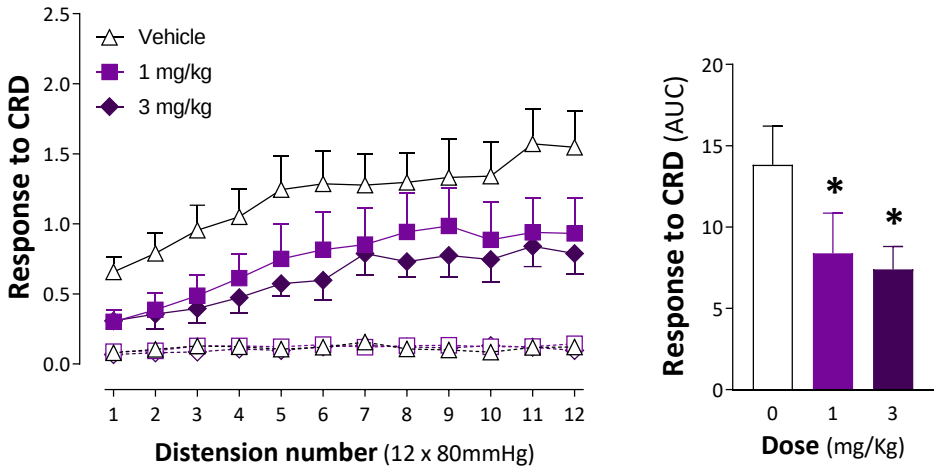


Figure 8. Effects of EST73502 on the VMRs to CRD in animals with colitis-induced hypersensitivity. The left panel shows VMRs to phasic repetitive CRD in the different experimental groups. Solid symbols with continuous lines reflect the response to CRD. Empty symbols with broken lines reflect baseline activity prior to each distension. The graph to the right represents the overall response to the CRD procedure (AUC). Data are mean±SEM, n=8 animals per group. *: P<0.05 vs. vehicle-treated group.

DISCUSSION

The present study aimed to evaluate the role of σ_1Rs on visceral pain arising from the GI tract, including normal pain responses as well as states of hypersensitivity and, therefore, to determine their potential as a therapeutic target for the treatment of this pain modality. Our findings indicate that the dual compound EST73502 (with both agonist opioid and antagonist sigma components) increases visceral pain threshold in healthy animals and prevents hyperalgesic responses during sates of inflammation-induced hypersensitivity.

VMRs to increasing phasic colorectal distensions or to repetitive phasic distensions have been used to determine basal visceral sensitivity, including pain thresholds, and acute mechanical hyperalgesia in several studies.[29–32] Our results are in agreement with previous works showing that VMRs increased in a pressure-related manner during ascending phasic distension and that repetitive phasic, noxious distensions (80 mmHg) are able to elicit acute mechanical hyperalgesia.[29,31,41,43] Moreover, pain thresholds in the present experimental conditions are similar to those described in the literature using similar approaches to assess visceral pain,[29,44] and can be defined, in normal conditions, at around an intracolonic pressures of 30-40 mmHg.

In normal conditions, acute treatment with the selective σ_1R antagonist BD1063 did not affect VMRs associated to increasing phasic distensions. These negative findings are not associated to the procedures used to assess pain. Similar CRD procedures to those used here have been extensively used to assess the pharmacological modulation of visceral pain arising from the GI tract and have shown that both analgesic and proalgesic effects can

be detected in a reproducible manner using the same technique.[29,39,44–46] Accordingly, morphine and pregabalin exerted similar analgesic affects as those described in previous reports in comparable experimental conditions.[29,31,47–50] Moreover, lack of effects of BD1063 are likely not related to the use of ineffective doses of the compound. Indeed, BD1063 was administered at relatively high doses, which have been effective treating both visceral pain arising from the urinary bladder, and acute inflammatory somatic hyperalgesia in mice.[19,21] Further supporting the present findings, similar negative results have been described in a murine model of visceral pain induced by intracolonic administration of capsaicin in which BD1063 was unable to induce analgesic-like responses.[20] Moreover, the present observations related to visceral pain agree with previous data showing that sigma ligands fail, for themselves, to modify somatic pain in classical models of thermal[19,22] or mechanical nociception[19,25,51,52] in basal conditions. The lack of involvement of σ_1Rs on pain responses in normal conditions is further supported by observations in σ_1R KO mice. In these animals, the constitutive absence of the receptor did not interfere with the perception of different kinds of nociceptive stimuli.[10,16,19,25,26,53] Altogether, these observations support the view that σ_1Rs play a minor role in the regulation of visceral pain in basal conditions, but do not rule out a potential modulatory role in states of sensitization.

Pain sensitization leading to visceral hypersensitivity is a common component of functional and inflammatory GI disorders.[1–3] To assess the potential implication of σ_1Rs on states of sensitization we used two models associated to the development of visceral (colonic) hyperalgesia: acute mechanical hyperalgesia induced by repetitive noxious CRD and long-lasting inflammatory hyperalgesia associated to a state of colitis. Models based on mechanical, inflammatory (colitis) and irritative (leading to inflammation-like responses) sensitization of the colon have been previously validated

and used to assess potential pharmacological treatments against visceral pain/hypersensitivity.[29,31,40,43,47,54,55] Responses obtained here in control conditions are similar to those previously reported for the same or similar models, indicating the development of visceral (colonic) hyperalgesia, as shown by the consistent increase in VMRs during CRD. Moreover, we also show that animals with inflammation-induced hyperalgesia are able to develop, in addition, acute mechanical hyperalgesia during repetitive phasic CRD.

Antagonism of σ_1Rs with BD1063 did not affect the development of acute mechanical hyperalgesia during repetitive noxious phasic CRD, despite the administration of relatively high doses of the compound, which have been effective attenuating acute somatic mechanical inflammatory hyperalgesia in mice.[19] In the same experimental conditions, consistent with previous reports, morphine prevented the development of acute hyperalgesia.[29,44,49] However, against to that previously reported, pregabalin induced only an slight attenuation of the hyperalgesic response, although, according to previous reports, effective doses of the compound were used.[31] Moreover, BD1063 also failed to reduce inflammation (colitis)-induced hyperalgesia. Similarly to that described for the acute mechanical sensitization, morphine and pregabalin modulated in a positive manner VMRs during inflammation-induced hypersensitivity. Overall, these negative results contrast with previous data showing that BD1063 was able to reduce referred mechanical hypersensitivity in a murine model of intracolonic capsaicin-induced pain[20] and to completely prevent referred mechanical hyperalgesia (abdominal well) in a murine model of cystitis;[21] thus suggesting a role for σ_1Rs modulating hypersensitivity associated to visceral inflammation. Similarly, σ_1Rs have been implicated in somatic sensitization in several animal models of inflammatory, chemical and neuropathic pain.[11,12,14–16,19] In any case, our observations agree with

previous data showing the absence of interaction between opioids (at least as it relates to the κ-opioid system) and σ_1Rs modulating visceral pain responses in the acetic acid-induced writhing test in mice.[56] At this time, we cannot give a feasible explanation for these apparent discrepancies, but they might suggest that visceral pain arising from the gut might have unique characteristics vs. other pain modalities (i.e. somatic pain) o pain airing from other viscera.

Despite not showing effects by itself, the σ_1R antagonist BD1063 could potentiate the effect of opioid-induced analgesia.[22–24] Indeed, a synergistic interaction between μ-opioid-mediated analgesia and σ_1Rs has been described in several models of somatic pain.[14,25–27] Here, we present preliminary data with a combination of BD1063 and an ineffective dose of morphine exploring a potential analgesic synergistic interaction during inflammation-induced visceral hypersensitivity. Results obtained suggest the absence of positive interaction between σ_1Rs and the opioid system, which might reflect differences between somatic and visceral pain mechanisms. Nevertheless, the present observations warrant further extensive studies, out of the original scope of this work, exploring wider dose ranges and/or other opioids besides morphine. We also explored the potential analgesic interaction between BD1063 and pregabalin. Surprisingly, the co-administration of BD1063 and an effective dose of pregabalin resulted in a loss of the pregabalin-dependent analgesic effects observed when de compound was administered alone. These observations suggest that sigma receptors might represent a promiscuous system interacting with multiple pathways leading to the inhibition or the facilitation of pain mechanisms depending upon de signaling routes activated and/or the origin of type of hypersensitivity developed (i.e. mechanical vs. inflammatory). As in the previous case, it would be necessary to study other mechanisms of modulation of σ_1R in addition to the protein-

protein interactions known so far.[57] In any case, further studies should explore in more detail the effects described here.

Taking into consideration the synergistic interaction between μ-opioid-mediated analgesia and σ_1Rs observed in some models[14,25–27] an alternative approach to de combined use of opioids and sigma ligands has been to design molecules with dual σ_1 and opioid activity, such as EST73502. EST73502 is a recently characterized compound with dual μ-opioid agonistic (K_i: 64±5 nM) and σ_1 antagonistic activity (K_i: 118±7 nM) that exhibits analgesic activity (somatic) with reduced opioid-induced relevant adverse events.[28] In agreement with the observed analgesic effects on somatic pain, EST73502 reduced VMRs in normal conditions, particularly at distension pressures below 50 mmHg, and increased pain thresholds (from about 40 mmHg to about 70 -mmHg), thus further supporting its analgesic properties. However, the analgesic effect disappeared at high distension pressures and was absent when inducing acute mechanical sensitization (based in the application of repetitive phasic distension at 80 mmHg, above the threshold for pain observed during the treatment with EST73502). Future studies should dissect how the opioidergic and the sigmaergic components of EST73502 contribute to these effects. The limited data available so far indicate that EST73502 has analgesic effects on basal sensitivity and during acute mechanical nociception (paw pressure), mainly due to a potentiation of the opioid analgesia.[28] In our conditions, although EST3502 modulated viscerosensitivity in basal conditions it was devoid of any effect during acute mechanical sensitization associated to repetitive phasic CRD, as mentioned above. On the other hand, in animals with colitis-induced hypersensitivity EST73502 significantly attenuated VMRs. Interestingly, responses observed in EST73502-treated rats with inflammation-induced hypersensitivity were similar to those observed during the acute mechanical sensitization in normal (without inflammation)

animals. This might suggest that: i) different mechanisms mediate inflammation-induced sensitization vs. acute mechanical sensitization; ii) EST73502 is acting selectively on sensitization mechanisms operating during inflammation but not in those mediating the mechanical stimulation-dependent acute sensitization. Therefore, during inflammation, EST73502 could act as a selective anti-hyperlagesic agent, without affecting normal pain responses. These effects are reminiscent to those described for TRPV1 channels, which selective blockade resulted in visceral anti-hyperalgesic effects, without hypoalgesic activity, during mechanical colonic sensitization in rats.[39] Further studies should evaluate in detail de anti-hyperalgesic effects of EST73502 and its potential therapeutic use for the treatment of visceral pain in pathologies characterized by the presence of inflammation-associated GI hypersensitivity, such are inflammatory and functional GI disorders.

In summary, the present results, derived from a model of visceral mechanosensitivity, either in normal conditions or in states of inflammation-associated hyperalgesia, suggest that σ_1Rs plays a minor role modulating viscerosensitivity. In any case, σ_1Rs would facilitate visceral nociceptive responses, since their antagonism with the selective receptor antagonist BD1063, increased pain thresholds during CRD without affecting the overall response to pain, either in normal conditions or in states of visceral sensitization. Moreover, against to that described using other pain models, a functional interaction between σ_1Rs and μ opioid receptors, based on the combination BD1063/morphine or the dual molecule EST73502, could not be demonstrated in the present experimental conditions. Nevertheless, EST73502 seems to exhibit selective anti-hyperalgesic properties associated to inflammation-dependent hypersensitivity, thus warranting further studies assessing this analgesic property.

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GENERAL DISCUSSION

Visceral pain is one of the most common symptoms in patients with inflammatory and FGD, thus considering this symptom a hallmark of these diseases. Although its high incidence, visceral pain lacks a specific treatment. In this context, accepted treatments for somatic pain are also used for the treatment of visceral pain,[4,62] despite the fact that these two pain modalities share only some characteristics.[38–40] This fact, together with the high number of adverse effects of nonspecific treatments, makes visceral pain an area of medical need still to be covered. Recent evidences have drawn significant attention on σ_1Rs as a potential target for pain management. Compelling data indicate that σ_1Rs play a significant role in pain and pain sensitization processes and can have also immunomodulatory actions, as demonstrated from different models of somatic pain.[85,156]

Taking into account these evidences, this work explores the hypothesis that σ_1Rs might be a feasible target for the treatment of visceral pain arising from the GI tract, including beneficial immunomodulatory activities during states of intestinal inflammation, frequently associated with pain.

σ_1Rs do not modulate baseline mechanosensitivity

Mice lacking σ1Rs showed similar mechanosensitivity to WT animals, as determined by assessing withdrawal thresholds to the mechanical stimulation of the lower abdominal wall, likely reflecting sensitivity of the abdominal wall *per se* and the underlying viscera (mainly intestine), or the hind limbs. Furthermore, the pharmacological blockade of σ1Rs in healthy animals did not affect mechanical sensitivity, as assessed by the von Frey test in mice or during CDR in rats. These results confirm previous observations indicating that naive σ1R KO mice perceive mechanical and thermal stimuli normally[100,134,142] and agree with pharmacological observations showing that σ1R ligands, either agonists or antagonists, have

no effects by themselves on somatic mechanosensitivity under baseline conditions.[122,123,136,143] Taken together, these observations support the view that σ1Rs play a minor role in the regulation of visceral pain in baseline conditions, at least as it relates to mechanosensitivity. Nevertheless, these data do not rule out a potential modulatory role for σ1Rs in states of altered sensitivity, particularly during pain sensitization.

Sensory-related markers are altered in σ_1Rs KO mice

Despite the absence of alterations in baseline nociceptive responses, σ_1R KO mice showed changes in the constitutive expression of several sensory-related markers implicated in visceral sensitivity. In particular, colonic expression of CB2 (mediator of analgesic effects[228–230]) and TRPV1 (mediator of pro-nociceptive responses[179,231]) were up- and down-regulated, respectively. Moreover, a down-regulation in the constitutive expression of nNOS, implicated in the local modulation of nociceptive pathways[232–234] was also detected in the lumbosacral spinal cord of σ_1R KO mice. σ_1Further suggesting a σ_1R-dependent modulation of sensory pathways, the pharmacological blockade of σ_1Rs in WT mice also affected the expression of sensory-related markers. In this case, antagonism of σ_1Rs consistently reduced the colonic expression of TRPV1, with minor or inconsistent effects in other sensory markers, particularly CB2. This probably reflects differences in efficacy between the constitutive absence of the receptor and its pharmacological blockade.

In any case, since the changes observed affect both pro- and anti-nociceptive pathways at relevant sites for pain processing it is feasible to assume that the resulting functional responses are likely to depend upon the balance between changes that favor or counteract pain-related mechanisms, as previously suggested for other experimental conditions

related to intestinal sensitivity.[167,173,235] In this case, the overall modulation of sensory mechanisms in the absence of functional σ_1Rs results in a state of normosensitivity, at least as it relates to mechanosensitivity.

Blockade of σ_1Rs attenuates DSS-induced colitis in mice

In the present experimental conditions, exposure to DSS led to the development of a colitic state characterized by the presence of clinical signs, colonic macroscopic and microscopic alterations and molecular changes similar to those previously described for this model.[164,198,236,237] During the constitutive absence of σ_1Rs, DSS-induced colitis, either in its acute o chronic phase, was attenuated. This attenuation, with some strain-related differences, was particularly evident as it related to clinical and colonic macroscopic signs of inflammation. Interestingly, a reduction of submucosal edema was observed in colitic σ_1R KO mice. This, together with the attenuation in the colonic expression of iNOS, suggests that σ_1Rs could modulate intestinal inflammation and fluid extravasation through NO-dependent mechanisms, as previously suggested.[149,150] Moreover, these effects are consistent with previous observations in a model of cyclophosphamide-induced cystitis pointing towards a role for σ_1Rs in the generation of edema.[139] Although the amelioration of clinical signs of colitis, inflammation-associated changes in inflammatory markers, particularly the up-regulation of pro-inflammatory cytokines and the increase in circulating haptoglobin, was still present in σ_1R KO mice. Overall, these data indicate a potential modulatory activity σ_1Rs, despite not limiting the full expression of the repertoire of immune-related mediators of inflammation, at least as to the production of cytokines and haptoglobin relates.

Pharmacological blockade of σ_1R with the antagonists BD1063 and E-52862 during DSS exposure in WT mice only partially reproduced the

attenuated inflammatory response observed in KO animals. This is consistent with previous studies showing that the pharmacological blockade with the same antagonists used here were less effective that the constitutive absence of the receptor modulating inflammation in several models of inflammatory somatic pain.[127,142,143] In general, as mentioned previously, this could be related to pharmacokinetic/pharmacodynamic properties of the antagonists, with practical limits vs. the constitutive lack of σ_1Rs in KO animals.

Blockade of σ_1Rs prevents the development of colitis-associated hypersensitivity

Intestinal inflammation has been associated with the development of visceral hypersensitivity and referred hyperalgesia in diverse regions of the body, including the abdominal wall, tail, and hind legs.[45,62,238–240] Consistent with this, during DSS-induced colitis WT mice showed a reduction in the withdrawal threshold to mechanical stimulation of the hind paws and the abdominal wall, reflecting the development of referred hypersensitivity. Interestingly, this response was not observed in σ_1R KO animals or in σ_1R antagonist-treated WT mice. These observations are in agreement with previous data showing a reduction in visceral pain-related behavioral responses in σ_1R KO mice after intracolonic administration of capsaicin[134] or during cyclophosphamide-induced cystitis.[139]. Altogether, these results support a key role for σ_1Rs in the development of visceral inflammation-associated hypersensitivity, similarly to that previously demonstrated for other somatic inflammatory pain models.[127,142,143,149,150]

The development of inflammation-associated intestinal hypersensitivity is mediated by peripheral sensitization of primary sensory afferents from the intestine, mainly induced by inflammatory mediators, and/or central

sensitization at spinal and/or supraspinal levels.[4,57,62] It is expected that sensitization will occur together with specific modulation of sensory-related mediators (receptors and/or ligands associated to the activity of sensory pathways).[48] However, in the present conditions, regardless the approach considered (genetic vs. pharmacological), no consistent changes were detected in the expression of different sensory-related markers associated to pro- and anti-nociceptive processes, neither at the level of the colon nor within the lumbosacral spinal cord (main site of entry of colonic sensory afferents into the central nervous system)[5]. Therefore, it is difficult to establish a correlation between the functional changes observed and a modulatory role of σ_1Rs on sensory-related pathways. Interestingly, during the genetic or pharmacological blockade of σ_1Rs the pattern of inflammatory cytokines was similar to that observed in WT animals, thus suggesting that the primary stimulus mediating peripheral sensitization was present. Despite this, no hypersensitivity was observed in the absence of functional σ_1Rs. This suggest that the absence of hypersensitivity should not be just regarded as a consequence of the attenuated clinical inflammation, but rather as a σ_1R-mediated modulation of sensory signals. In any case, these observations warrant further studies to dissect the underlying mechanisms and the site of action (peripheral vs. central) for the modulatory effects of σ_1Rs.

Peripheral and central sensitization mediates visceral hypersensitivity during DSS-induced colitis in rats

In rats with DSS-induced colitis, VMRs to CRD were increased compared to non-colitic rats, thus indicating the presence of inflammation-associated mechanical hypersensitivity. Furthermore, this state of hypersensitivity persisted over time, as assessed between days 17 and 35 after the induction of inflammation. Overall, these characteristics validate the rat DSS-induced

colitis model as a long-lasting model of colonic hypersensitivity to study the mechanisms of visceral pain. On the other hand, rats with DSS-induced colitis also showed changes in hind paw withdrawal responses to punctate mechanical stimulation, indicative of the presence of referred somatic hypersensitivity, likely reflecting a state of central sensitization.

As mentioned, visceral pain hypersensitivity has been associated with peripheral and central sensitization mechanisms, involving changes in sensory pathways that lead to an increase in pain transmission.[45,62,238–240] In addition to the positive regulation of colonic pro-inflammatory cytokines observed after exposure to DSS, similar up-regulation was also detected in central sites related to the processing of sensory signals from the colon, namely lumbosacral DRGs and lumbosacral spinal cord. Given the demonstrated role of inflammatory mediators on pain sensitization[4,57,62] it if feasible to assume that this expression changes might be associated to alterations in sensory pathways (including the structural and/or molecular levels) as underlying cause of the hypersensitivity observed at the functional level. During inflammation, differential tissue (colon, DRGs, and spinal cord) and sensory (pro-nociceptive vs. anti-nociceptive) modulatory responses were detected. Indeed, changes in the expression of both pro-nociceptive- and anti-nociceptive-related markers were evidenced (with either up-regulation or down-regulation). Therefore, as previously discussed, it is difficult to establish a direct correlation between functional responses and gene expression and it should be assumed that the final functional outcome would depend upon the balance between pro- and anti-nociceptive signals. Moreover, the possibility that specific up-regulation of anti-nociceptive pathways are favored, as a compensatory response to avoid excessive pain in states of sensitization, should be considered.

In any case, these data suggest that a combination of peripheral and central sensitization could occur during DSS-induced colitis as part of the underlying mechanisms that explain the mechanical hypersensitivity observed in this model. Indeed, a similar sensitization process have been suggested in humans during IBD, which is assumed to include alterations in the central and peripheral processing of pain.[63,241,242]

The σ_1R antagonist BD1063 fails to modulate VMRs to CRD during colitis-associated sensitization

Against that expected from the previous findings, the σ_1R antagonist BD1063 failed to modulate inflammation-induced mechanical hyperalgesia during repetitive CRD in rats. These negative results contrast with previous data showing that BD1063 was able to prevent or reduce referred mechanical hypersensitivity in the murine model of DSS-induced colitis (present work) or intracolonic capsaicin-induced pain[134] and in a murine model of cystitis.[139] At this time, we cannot provide a workable explanation for these apparent discrepancies, but they might suggest that visceral pain arising from the intestine may have unique characteristics compared to other pain modalities (i.e., somatic pain) or pain originating from others viscera.

Despite showing no effects by itself, the σ_1R antagonist BD1063 could potentiate the effect of opioid-induced analgesia.[152,154,243] However, in the present conditions no interaction with μ-opioid receptors was evidenced when BD1063 and morphine were co-administered. This negative finding is difficult to explain and might suggest, again fundamental differences between visceral and somatic pain. In addition, co-administration of BD1063 and the voltage-gated calcium channels blocker pregabalin resulted in a negative interaction with the loss of pregabalin-mediated

analgesia. These observations suggest that sigma receptors could represent a promiscuous system acting on different mechanisms related to pain modulation, beyond the recognized modulation of opioidergic analgesia, favoring or disfavoring pain perception.

The dual opioid-sigma 1 molecule EST73502 elicits analgesia during CRD in rats

The recently designed molecule EST73502 has been characterized as a dual MOR partial agonist and a σ_1R antagonist[110] with analgesic effects in animal models of acute somatic pain (mechanosensitivity of the paw).[110] In the present studies, EST73502 reduced VMRs and increased pain thresholds during CRD under normal conditions, consisting with its analgesic properties on somatic pain. The existing data so far relate the synergy between the opioid and the sigma components of the molecule as the underlying mechanism of this effect.[110] Future studies should examine how the opioidergic and sigmaergic components of EST73502 contribute to its analgesic effects during CDR.

In rats with colitis-induced hypersensitivity, EST73502 significantly attenuated VMRs during repetitive CRD, eliciting responses similar to those observed during acute mechanical sensitization in healthy animals. This could imply the existence of different mechanisms mediating inflammation-induced sensitization and acute mechanical sensitization. Furthermore, EST73502 seems to selectively act on the mechanisms of inflammation-induced sensitization, but not on those that mediate acute mechanical sensitization in non-inflamed conditions. These effects are reminiscent of those described for TRPV1 channels, whose selective blockade resulted in visceral anti-hyperalgesic effects, without hypoalgesic activity, during mechanical sensitization of the colon in rats.[244] This suggests that EST73502

could also act as a selective anti-hyperlagesic agent, without affecting normal pain responses. Although these promising results, further studies are necessary in order to conclude the anti-hyperalgesic effects of EST73502 and its potential therapeutic use on inflammation-associated visceral hypersensitivity.

Overall, the present results show that σ_1Rs have anti-inflammatory properties, being able to attenuate the clinical signs of intestinal inflammation. Furthermore, σ_1R blockade shows modulatory, analgesic-like, properties on hyperalgesic states associated to intestinal inflammation, likely reflecting a modulation of inflammation-associated central and peripheral sensitization of pain mechanisms. Therefore, the results presented here indicate that σ_1R are a potentially feasible target for the treatment of GI disorders characterized by the presence of signs of inflammation and alterations in visceral sensitivity.

CONCLUSIONS

1. Sigma 1 receptors are not involved in basal mechanosensitivity, as determined by the mechanical probing of the abdominal wall and the paw in sigma 1 receptor knockout mice and during the pharmacological blockade of the receptor with the selective antagonists BD1063 or E-52862 in mice or during colorectal distension in rats. Although this apparent normosensitive state, changes in colonic and lumbosacral spinal cord expression of the sensory-related markers transient receptor potential cation channel subfamily V member 1 (TRPV1), cannabinoid receptor type 2 (CB2) and neuronal nitric oxide synthase (nNOS) are detected during the genetic and pharmacological blockade of sigma 1 receptors in mice.

2. In mice, blockade of sigma 1 receptors attenuates acute and chronic colitis in the dextran sulfate sodium (DSS) model, as indicated by results obtained during the constitutive absence of the receptor in knockout animals or during the selective antagonism with BD1063 or E-52862. Nevertheless, neither the up-regulated expression of cytokines nor the increased serum levels of the acute phase protein haptoglobin are affected by the blockade of sigma 1 receptors during colitis.

3. In mice, sigma 1 receptors are implicated in the development of referred mechanical hypersensitivity during dextran sulfate sodium (DSS)-induced colitis; as shown by the lack of changes in abdominal and paw withdrawal responses in animals with constitutive absence of functional receptors or during the pharmacological blockade with the selective antagonists BD1063 or E-52862.

4. In mice, no consistent genotype- and treatment-related modulation of sensory-related makers is detected in the colon during dextran sulfate

sodium (DSS)-induced inflammation. Similarly, within the lumbosacral spinal cord, regardless the genotype or the treatment considered, no evidences of the modulation of sensory-related pathways are found during dextran sulfate sodium (DSS)-induced colitis.

5. In rats, exposure to dextran sulfate sodium (DSS) induces a colitis that leads to a state of long-lasting colonic mechanical hypersensitivity, as determined in the colorectal distension model, with limited somatic referred hyperalgesia. Changes in the expression pattern of immune- and sensory-related makers in colon and lumbosacral spinal cord indicate the implication of peripheral and central sensitization processes as part of the underlying mechanisms explaining the observed hypersensitivity.

6. In rats with colitis-associated colonic hypersensitivity, pharmacological blockade of sigma 1 receptors with the selective antagonist BD1063 does not affect visceral pain responses, as assessed during colorectal distension. No evidences of synergy with opioids or voltage gated calcium channels, as revealed through the co-administration of BD1063 and the μ-opioid agonist morphine or the α2δ antagonist pregabalin, respectively, are detected.

7. In healthy rats, the dual μ-opioid receptor partial agonist and sigma 1 receptor antagonist EST73502 increases sensory thresholds during mechanical stimulation of the colon.

8. In rats, the dual μ-opioid receptor partial agonist and σ1R antagonist EST73502 does not affect acute mechanical sensitization associated to repetitive CRD, while exhibiting anti-hyperalgesic activity during colitis-associated colonic hypersensitivity.

Final conclusion:

Sigma 1 receptors exhibit anti-inflammatory and analgesic properties that make this receptor a feasible target for the management of gastrointestinal disorders characterized by the presence of inflammatory signs and alterations in visceral sensitivity.

CONCLUSIONS

1. Els receptors sigma de tipus 1 no estan implicats en la regulació de la mecanosensibilitat en condicions basals, tal com s'observa a partir de l'estimulació mecànica tant de la paret abdominal com de la pota en ratolins mancats d'aquest receptor i a partir del bloqueig farmacològic del mateix amb els antagonistes selectius BD1063 o E-52862 en ratolins o durant la distensió colorectal en rates. Malgrat aquest aparent estat de normosensibilitat, al colon i la medul·la espinal de ratolí, es detecten canvis en l'expressió dels marcadors sensorials receptor de potencial transitori V1 (TRPV1), receptor cannabinoid de tipus 2 (CB2) i òxid nítric sintasa neuronal (nNOS) tant durant el bloqueig genètic com el farmacològic del receptor sigma de tipus 1.

2. En el ratolí, el bloqueig dels receptors sigma de tipus 1 atenua la colitis aguda i crònica en el model de dextrà sulfat de sodi (DSS), com indiquen els resultat en animals amb absència constitutiva del receptor o els obtinguts durant el seu antagonisme selectiu amb BD1063 o E-52862. No obstant això, el bloqueig dels receptors sigma de tipus 1 no afecta la regulació a l'alça de l'expressió gènica de citocines ni els nivells sèrics de la proteïna de fase aguda haptoglobina durant la colitis.

3. En el ratolí, els receptors sigma de tipus 1 estan implicats en el desenvolupament d'hipersensibilitat mecànica referida durant la colitis induïda per dextrà sulfat de sodi (DSS); com demostra l'absència de canvis en les respostes motores abdominals i en el reflex de retirada de la pota en els animals amb absència constitutiva de receptors funcionals o durant el bloqueig farmacològic dels mateixos amb els antagonistes selectius BD1063 o E-52862.

4. En el còlon de ratolins amb colitis induïda per dextrà sulfat de sodi (DSS), no es detecta modulació dels marcadors sensorials consistent amb el genotip i el tractament aplicat. De la mateixa manera, en la medul·la espinal lumbosacra, amb independència del genotip i del tractament considerat, no es troben evidències de la modulació de vies sensorials durant la colitis induïda pel dextrà sulfat de sodi (DSS).

5. En la rata, l'exposició al dextrà sulfat de sodi (DSS) indueix una colitis que condueix a un estat d'hipersensibilitat mecànica còlica de llarga durada, tal i com es determina en el model de distensió colorectal, amb hiperalgèsia somàtica referida limitada. Els canvis en el patró d'expressió dels marcadors relacionats amb la immunitat i la sensibilitat en el còlon i la medul·la espinal lumbosacra indiquen la implicació de processos de sensibilització, tant a nivell perifèric com central, com a part dels mecanismes subjacents que expliquen aquesta hipersensibilitat.

6. En rates amb hipersensibilitat còlica associada a un estat de colitis, el bloqueig farmacològic dels receptors sigma de tipus 1 amb l'antagonista selectiu BD1063 no afecta les respostes de dolor visceral avaluades durant la distensió colorectal. No es detecten evidències de sinergia amb els opioides o amb els canals de calci activats per voltatge, com es posa de manifest mitjançant la co-administració de BD1063 i l'agonista μ-opioide morfina o l'antagonista α2δ pregabalina, respectivament.

7. En rates sanes, el compost EST73502, amb activitat agonista parcial en el receptor μ-opioide i activitat antagonista en el receptor sigma de tipus 1, augmenta els llindars sensorials durant l'estimulació mecànica del còlon.

8. En rates, el compost EST73502, amb activitat agonista parcial en el receptor μ-opioide i activitat antagonista en el receptor sigma de tipus 1, no afecta la sensibilització mecànica aguda associada a la distensió colorrectal repetitiva, mentre que mostra activitat antihiperalgèsica durant la hipersensibilitat còlica associada a un estat de colitis.

Conclusió final:

Els receptors sigma de tipus 1 presenten propietats antiinflamatòries i analgèsiques que fan d'aquest receptor una diana factible per al tractament de trastorns gastrointestinals caracteritzats per la presència de signes d'inflamació i alteracions de la sensibilitat visceral.

CONCLUSIONES

1. Los receptores sigma de tipo 1 no están implicados en la regulación de la mecanosensibilidad en condiciones basales, tal y como se observa a partir de la estimulación mecánica, tanto de la pared abdominal como de la pata, en ratones carentes de dicho receptor y a partir del bloqueo farmacológico del mismo con los antagonistas selectivos BD1063 o E-52862 en ratones o durante la distensión colorrectal en ratas. A pesar de este aparente estado de normosensibilidad, en el colon y la medula espinal de ratón, se detectan cambios en la expresión de los marcadores sensoriales receptor de potencial transitorio V1 (TRPV1), receptor cannabinoide de tipo 2 (CB2) y óxido nítrico sintasa neuronal (nNOS), tanto durante el bloqueo genético como el farmacológico del receptor sigma de tipo 1.

2. En el ratón, el bloqueo de los receptores sigma de tipo 1 atenúa la colitis aguda y crónica en el modelo de dextrano sulfato de sodio (DSS), como indican los resultados en animales con ausencia constitutiva del receptor o los obtenidos durante su antagonismo selectivo con BD1063 o E-52862. Sin embargo, el bloqueo de los receptores sigma de tipo 1 no afecta la regulación al alza de la expresión génica de citoquinas ni los niveles séricos de la proteína de fase aguda haptoglobina durante la colitis.

3. En el ratón, los receptores sigma de tipo 1 están implicados en el desarrollo de hipersensibilidad mecánica referida durante la colitis inducida por dextrano sulfato de sodio (DSS); como demuestra la ausencia de cambios en las respuestas motoras abdominales y en el reflejo de retirada de la pata en los animales con falta constitutiva de receptores funcionales o durante el bloqueo farmacológico de los mismos con los antagonistas selectivos BD1063 o E-52862.

4. En el colon de ratones con colitis inducida por dextrano sulfato de sodio (DSS), no se detecta una modulación de los marcadores sensoriales consistente con el genotipo y el tratamiento aplicado. De forma similar, en la médula espinal lumbosacra, con independencia del genotipo y del tratamiento considerado, no se encuentran evidencias de la modulación de vías sensoriales durante la colitis inducida por dextrano sulfato de sodio (DSS).

5. En la rata, la exposición al dextrano sulfato de sodio (DSS) induce una colitis que lleva a un estado de hipersensibilidad mecánica cólica de larga duración, tal y como se determina en el modelo de distensión colorrectal, con hiperalgesia somática referida limitada. Los cambios en el patrón de expresión de los marcadores relacionados con la inmunidad y la sensibilidad en el colon y la médula espinal lumbosacra indican la implicación de procesos de sensibilización, tanto a nivel periférico como central, como parte de los mecanismos subyacentes que explican esta hipersensibilidad.

6. En ratas con hipersensibilidad cólica asociada a un estado de colitis, el bloqueo farmacológico de los receptores sigma de tipo 1 con el antagonista selectivo BD1063 no afecta las respuestas de dolor visceral evaluadas durante la distensión colorrectal. No se detectan evidencias de sinergia con los opioides o con los canales de calcio activados por voltaje, como se pone de manifiesto mediante la co-administración de BD1063 y el agonista μ-opioide morfina o el antagonista α2δ pregabalina, respectivamente.

7. En ratas sanas, el compuesto EST73502, con actividad agonista parcial sobre el receptor μ-opioide y actividad antagonista sobre el receptor

sigma de tipo 1, aumenta los umbrales sensoriales durante la estimulación mecánica del colon.

8. En ratas, el compuesto EST73502, con actividad agonista parcial sobre el receptor μ-opioide y actividad antagonista sobre el receptor sigma de tipo 1, no afecta la sensibilización mecánica aguda asociada a la distensión colorrectal repetitiva, mientras que muestra actividad antihiperalgésica durante la hipersensibilidad cólica asociada a un estado de colitis.

Conclusión final:

Los receptores sigma de tipo 1 presentan propiedades antiinflamatorias y analgésicas que hacen de este receptor una diana factible para el tratamiento de trastornos gastrointestinales caracterizados por la presencia de signos de inflamación y alteraciones de la sensibilidad visceral.

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ABBREVIATIONS

5-ASA:	5-aminosalicylic acid
5-HT:	serotonin, 5-hydroxytryptamine
6-TG:	6-thioguanine
Actb:	Actin β
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA:	analysis of variance
ASIC:	acid-sensing ion channel
ATP:	adenosine triphosphate
AUC:	area under the curve
BID:	bis in die, twice daily
BiP:	binding immunoglobulin protein
BW:	body weight
CamKII:	calcium/calmodulin-dependent protein kinase type II
CB1/2:	cannabinoid receptor type 1/2
CD:	Crohn's disease
cDNA:	complementary deoxyribonucleic acid
CFA:	Freund's complete adjuvant
CGRP:	calcitonin gene-related peptide
CNS	central nervous system
COX:	cyclooxygenase
CRD:	colorectal distension
CREB:	cAMP response element binding protein
DAG:	diacyl glycerol
DNBS	2,4-dinitrobenzenesulfonic acid
DRG:	dorsal root ganglion
DSS:	dextran sodium sulfate
EMG:	electromyographic
ENS:	enteric nervous system
ER:	endoplasmic reticulum
ERK:	extracellular signal-regulated kinase
FGD:	functional gastrointestinal disorder
GABA:	γ-aminobutyric acid analogues
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GFAP:	glial fibrillary acidic protein

GI:	gastrointestinal
GluR2:	glutamate ionotropic receptor AMPA type subunit 2
GPCR:	G protein-coupled receptor
GTP:	guanosine triphosphate
HPMC:	(hydroxypropyl)methyl cellulose
Hprt1:	Hypoxanthine-guanine phosphoribosyltransferase 1
IBD:	inflammatory bowel disease
IBS:	irritable bowel syndrome
IL:	interleukin
INF-γ:	interferon-γ
iNOS:	nitric oxide synthase 2 (inducible)
IP_3:	inositol-1,4,5-trisphosphate
KO:	knockout
LPS	lipopolysaccharide
MAM:	mitochondrion-associated endoplasmic reticulum membrane
MAPK:	mitogen-activated protein kinase
mRNA:	messenger RNA
MOR:	μ-opioid receptor
Na_V:	voltage-gated sodium channels
NF:	nuclear factor
NGF:	nerve growth factor
NK1r:	neurokinin type 1 receptor
NMDA:	N-Methyl-d-aspartic acid or N-Methyl-d-aspartate
nNOS:	neuronal nitric oxide synthase
NO:	nitric oxide
NOS:	nitric oxide synthase
NR2B:	glutamate [NMDA] receptor subunit epsilon-2
NSAID:	nonsteroidal anti-inflammatory drug
$P2_X$:	purinoreceptor
PAR:	protease-activated receptor
PCR:	polymerase chain reaction
pCamKII:	phosphorylated CamKII
pERK:	phosphorylated ERK
PG:	prostaglandin

PIP2:	phosphatidylinositol- 4,5-bisphosphate
PKC:	protein kinase C
PLC:	phospholipase C
po:	per os, orally
pp38:	phosphorylated p38
Ramp1:	receptor activity modifying protein 1
RIPA:	radioimmunoprecipitation assay
RNA:	ribonucleic acid
ROS:	reactive oxygen species
RT-qPCR:	reverse transcription quantitative polymerase chain reaction
sc:	subcutaneous
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM:	standard error of the mean
SERT:	serotonin transporter
SID:	once daily
TK1:	tachykinin receptor 1
TNBS:	2,4,6-trinitrobenzenesulfonic acid
TNF-α:	tumor necrosis factor α
TPH1:	tryptophan hydroxylase isoform 1
TrkA:	tropomyosin receptor kinase A
TRP:	transient receptor potential channel
TRPA1:	transient receptor potential cation channel, subfamily A 1
TRPV1/3/4:	transient receptor potential cation channel subfamily V member 1/3/4
T-TBS:	tris–tween 20-buffered saline
UC:	ulcerative colitis
VGCC:	voltage-gated calcium channel
VMR:	visceromotor response
WT:	wild-type
β2m:	β-2-microglobulin
σR:	sigma receptor
$\sigma_{1/2}$R:	sigma 1/2 receptor

APPENDICES

PUBLICATIONS DERIVED FROM THIS THESIS

Papers

López-Estévez S, Gris G, de la Puente B, Carceller A, Martínez V. Intestinal inflammation-associated hypersensitivity is attenuated in a DSS model of colitis in Sigma-1 knockout C57BL/6 mice. *Biomed Pharmacother* 2021; 143: 112126.

DOI: 10.1016/j.biopha.2021.112126

IF: 6.529; Q1 (Medicine, Research & Experimental)

López-Estévez S, López-Torrellardona JM, Parera M, Martínez V. Long-lasting visceral hypersensitivity in a model of DSS-induced colitis in rats. *J Neurogastroenterol Motil* (Submitted).

López-Estévez S, Aguilera M, Gris G, de la Puente B, Carceller A, Martínez V. Genetic and pharmacological blockade of σ_1Rs attenuate inflammation-associated hypersensitivity during acute colitis in CD1 mice.

(Manuscript in preparation).

López-Estévez S, Codony X, Zamanillo D, Merlos M, Martínez V. Modulatory role of σ_1Rs on normal viscerosensitivity and inflammation-induced hypersensitivity in a model of colorectal distension in rats.

(Manuscript in preparation).

Abstracts

López-Estévez S, Martínez V. La colitis inducida por DSS genera hipersensibilidad visceral crónica en ratas.

XXVII Reunión del Grupo Español de Motilidad Digestiva (*GEMD*). Barcelona (Spain), 2019. Oral communication.

López-Estévez S, Martínez V. DSS-induced colitis generates chronic visceral hypersensitivity in rats. *Acta Physiol (Oxf)* 2019; 227/S718;82-83.

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López-Estévez S, Gris G, de la Puente B, Zamanillo D, Codony X, Merlos M, Martínez V. Receptores Sigma 1 y desarrollo de sensibilidad visceral en un modelo murino de colitis. *Rev Soc Esp del Dolo*r 2018; 25/S2:12.

XV Congreso Nacional de la Sociedad Española de dolor (*SED*). Palma (Spain), 2018. Poster.

López-Estévez S, Aguilera M, Gris G, de la Puente B, Codony X, Zamanillo D, Martínez V. Ratones carentes del receptor Sigma 1 presentan una inflamación intestinal atenuada.

XXV Reunión del Grupo Español de Motilidad Digestiva (*GEMD*). Valencia (Spain), 2017. Oral communication.

Gris G, López-Estévez S, de la Puente B, Zamanillo D, Codony X, Merlos M, Martínez V. Sigma-1 receptor knockout mice show attenuated inflammation and lack of visceral and referred somatic mechanical hypersensisivity during colitis.

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12th Congress of European Crohn's and Colitis Organisation (ECCO). Barcelona (Spain), 2017. Poster.

Intestinal inflammation-associated hypersensitivity is attenuated in a DSS model of colitis in sigma-1 knockout C57BL/6 mice

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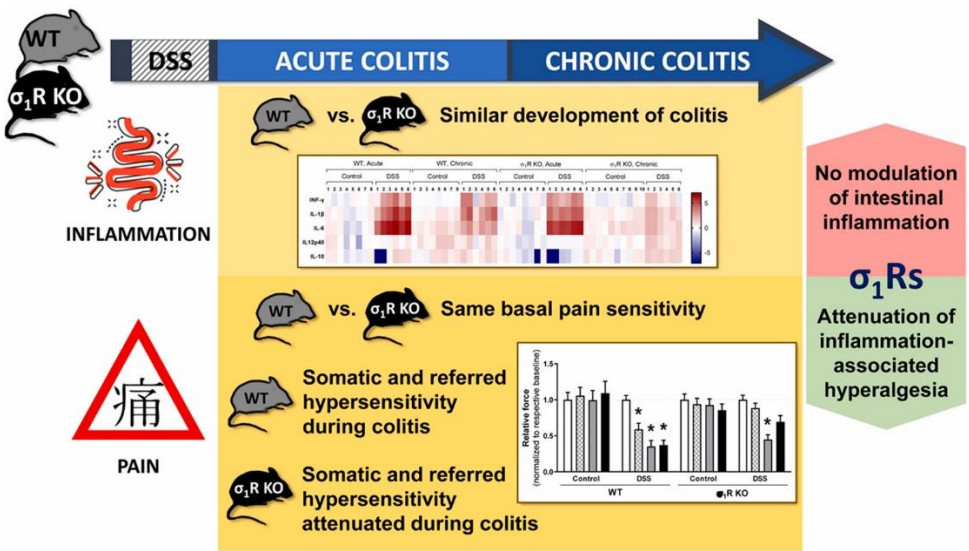
Biomed Pharmacother 2021; 143: 112126.



Biomedicine & Pharmacotherapy 143 (2021) 112126

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Intestinal inflammation-associated hypersensitivity is attenuated in a DSS model of colitis in Sigma-1 knockout C57BL/6 mice

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ARTICLE INFO

Keywords:
Colitis
Hyperalgesia
Hypersensitivity
Intestinal inflammation
Pain
Sigma 1 receptor

ABSTRACT

Sigma-1 receptors (σ_1R) have been implicated in several pain pathways. We assessed the implication of σ_1Rs in the development of intestinal inflammation and inflammation-associated referred hypersensitivity in a model of colitis in σ_1R knockout (KO) mice. Colitis was induced with dextran sulfate sodium (DSS) in wild type (WT) and σ_1R KO mice. The development of referred mechanical hypersensitivity (von Frey test) was assessed. Colonic and spinal changes in expression of immune- and sensory-related markers were also investigated (RT-qPCR/Western blot). Absence of σ_1Rs had little impact in colitis generation and progression, although during the chronic phase a reduction in edema and a down-regulation of iNOS gene expression was observed. In σ_1R KO mice, inflammation-associated hypersensitivity was significantly attenuated (paw) or completely prevented (abdomen). During colitis, in WT mice, changes in the colonic expression of nociceptive markers were observed during the acute and chronic phases of inflammation. Although σ_1R KO mice showed similar regulation in the acute phase, an attenuated response was observed during the chronic phase of colitis. These differences were especially relevant for CB2 and TRPV1 receptors, which could play an important role in σ_1-mediated regulation of sensitivity. No changes were detected on ERK phosphorylation at the level of the lumbosacral spinal cord. In summary, intestinal inflammation-associated referred hyperalgesia was reduced (paw) or absent (abdomen) in σ_1R KO mice, thus confirming an important role for σ_1R in the development of colitis-associated hypersensitivity. These results identify σ_1Rs as a possible therapeutic target for the treatment of hypersensitivity associated to intestinal inflammation.

1. Introduction

Evidences suggest that inflammation within the gastrointestinal tract, even at a low degree, and the presence of visceral and somatic hypersensitivity are associated phenomena. Indeed, inflammatory conditions of the gastrointestinal tract, such as inflammatory bowel disease (IBD), are associated to both somatic and visceral hypersensitivity [1,2]. Moreover, irritable bowel syndrome (IBS), the main functional gastrointestinal disorder, has been associated to a low degree of intestinal inflammation and has altered colonic sensitivity with increased perception as key manifestations [3,4]. Additionally, changes in somatic sensitivity, characterized by referred hypersensitivity, have also been observed in states of intestinal inflammation [5,6]. In this context, intestinal inflammation and the associated state of hypersensitivity are still areas of medical needs, without fully effective therapeutic approaches.

During the last years, several targets have been explored as potential treatments of visceral pain arising from the gut. However, at the current

Abbreviations: β2m, β-2-microglobulin; σ_1Rs, sigma-1 receptors; CB1, Cannabinoid receptor 1; CB2, Cannabinoid receptor 2; COX-2, Cyclooxygenase 2; DSS, Dextran sulfate sodium; IBD, Inflammatory bowel disease; IBS, Irritable bowel syndrome; INF-γ, Interferon γ; IL-1β, Interleukin 1β; IL-6, Interleukin 6; IL-10, Interleukin 10; IL-12p40, Interleukin 12 subunit p40; iNOS, Nitric oxide synthase 2 (inducible); MOR, μ-Opioid receptor; NGF, Nerve growth factor; PAR2, Protease activated receptor 2; SERT, Serotonin transporter; TPH1, Tryptophan hydroxylase 1; TRPV1/3, Transient receptor potential vanilloid 1 and 3; VF, von Frey.

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https://doi.org/10.1016/j.biopha.2021.112126
Received 9 July 2021; Received in revised form 20 August 2021; Accepted 24 August 2021

time there are no effective treatments for this type of pain. Some efforts have been directed towards the validation of analgesic treatment positively validated against somatic pain, although the differences between these pain modalities might be relevant in this respect. In recent years, sigma-1 receptors (σ_1Rs) have been implicated in pain mechanisms and suggested as potential pharmacological target for the treatment of somatic pain. σ_1R is a neuromodulatory, ligand-regulated membrane protein chaperone that exerts its functions through multiprotein complex assembly [7,8]. The relation of σ_1 Rs and pain was first suggested by studies showing a relationship between σR systems and opioid-mediated analgesia [9,10]. Evidences indicate that σ_1R ligands fail to modify normal pain responses by themselves, as demonstrated in classical models of thermal and mechanical acute nociception [11,12]. However, σ_1R ligands seem to play a key role in modulating pain behavior in states of sensitization and chronic pain conditions [13]. In this respect, recent studies show that both central and peripheral pharmacological blockade of σ_1Rs could be an effective option to treat inflammatory pain [13–16]. As it relates to visceral pain responses arising from the gut, evidences indicate a potential modulatory role for σ_1Rs. In this sense, σ_1Rs selective antagonists were effective preventing visceral pain-related responses elicited by intracolonic capsaicin in mice [17].

Taking into account these considerations, the aims of the present study were to assess the potential modulatory role of σ_1Rs on intestinal inflammation and the development of inflammation-associated hypersensitivity in a murine model of dextran sulfate sodium (DSS)-induced colitis. With this objective, we assessed the development of colitis and inflammation-associated visceral and somatic hypersensitivity in σ_1R knockout (KO) mice compared to wild-type (WT) animals. Moreover, colitis-associated changes in peripheral (colon) and central (spinal cord) sensory-related markers implicated in pain processing and sensitization were also assessed.

2. Materials and methods

2.1. Animals

Male WT C57BL/6J mice (n = 28, Charles River Laboratories, Lyon, France) and σ_1R KO C57BL/6J mice (n = 31, Esteve Pharmaceuticals S. A., Barcelona, Spain), both aged 6-weeks at the time of starting the studies, were used. Mice were group-housed in standard cages (four-six mice per cage) and maintained under standard condition of photoperiod (12:12 h light-dark cycle) and climate (20–22 °C, 40–70% humidity), with *ad libitum* access to a standard diet and tap water, except when receiving DSS. Mice were allowed to acclimatize to the animal facility for at least 1 week before starting the studies. All procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona (protocols 3039 and 3957) and the Generalitat de Catalunya (protocols 8823 and 9915).

2.2. Colitis induction

A solution of DSS (45 kDa; 2% concentration in water; TdB Consultancy AB, Uppsala, Sweden) was used to induce colitis. Fresh DSS solutions were prepared daily during the 5-day treatment period (from experimental day 0 to day 5). Following this protocol animals develop an acute colitis (7–8 days after starting DSS exposure) that progresses to chronicity. Similar protocols have been used in previous studies in mice to induce colitis [18–22]. Normal tap water was used as the control treatment.

2.3. Evaluation of referred mechanical hypersensitivity: von Frey test

Animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which von Frey monofilaments (bending force range from 0.04 to 2 g; North Coast Medical, Inc.; Gilroy, CA, USA) were applied for pain assessment. Pain sensitivity was evaluated after a 30 min habituation period to the testing environment. Referred pain was assessed in two separate regions, the abdominal wall and the hind paw. When assessing sensitivity of the abdominal wall the perianal and external genitalia areas were avoided, concentrating the mechanical stimulation on the lower and mid abdomen, as previously reported [17,23]. Paw sensitivity was quantified by measuring the hind paw withdrawal response to punctate mechanical stimulation, as previously described [24,25]. In all cases, pain thresholds were determined using the up-down method paradigm and represent the mechanical stimulus that produces 50% of maximal response [26]. Data obtained were normalized to a baseline measurement (taken as 1), taken 24 h before starting the experimental procedures (Fig. 1). All measurements were performed twice, with a 30-min recovery period in between, by two independent investigators. The mean values of the two observations were taken, for each animal, as the measure of pain sensitivity.

2.4. Experimental protocol

WT and σ_1R KO mice were randomly divided into 2 experimental groups per genotype (n = 12–19 per group). In a random assignment, the experimental groups received tap water or a solution of 2% DSS during a 5-day period (days 0–5). After DSS/water exposure, all animals received normal water and were allowed to recover for a 2-day (acute inflammatory phase) or a 16-day period (chronic inflammatory phase) before euthanasia. Individual body weight, general state and the presence of clinical signs were assessed on a daily basis throughout the study. Von Frey test was performed 4 times during the experimental procedure: the day before starting the administration of DSS (taken as a basal measure of sensitivity, day −1), at approximately half of time of DSS exposure (day 3), at the end of acute inflammatory phase (day 7) and at the end of the experiment (chronic inflammatory phase, day 21). Animals were euthanized for the collection of samples immediately after the last von Frey test evaluation (see below). See Fig. 1 for details of the experimental protocol.

2.5. Samples collection

Immediately after the last von Frey test (days 7 or 21, for the acute and chronic phase, respectively), mice were deeply anesthetized with isoflurane (Isoflo; Esteve, Barcelona, Spain) and euthanatized by exsanguination through intracardiac puncture followed by cervical dislocation. Thereafter, a medial laparotomy was performed, the cecocolonic region localized and the cecum and colon dissected. Afterward, two tissue samples from the proximal-middle colon (about 1.5 cm each) were collected. A sample was frozen immediately in liquid nitrogen and a second sample was fixed in 4% paraformaldehyde. After an overnight fixing, tissues were paraffin embedded and 5-μm-thick sections were obtained. The lumbar enlargement (L3-S2) of spinal cord was dissected and frozen immediately in liquid nitrogen. Frozen samples were stored at −80 °C until analysis. In addition, the liver, the adrenal glands, the thymus, and the spleen were dissected and weighed. Serum was obtained by centrifugation of blood samples (15 min, 10,000×*g*, 4 °C) and maintained at −80 °C until analysis.

2.6. Clinical and macroscopic assessment of inflammation

Clinical assessment of inflammation included daily monitoring of body weight, appearance of feces and general health condition [19]. A score (0−8) was assigned for health condition (including hunch posture, piloerection, fecal consistency and aspect of the anus); where 0 indicates normal activity/fur/normal feces/normal anus, 1 indicates abnormal gait/bristly fur/wet/watery feces/wet anus and 2 indicates prostrated animal/dirty fur/watery diarrhea/bloody rest on anus. At necropsy, the macroscopic appearance of the colon was scored following previously published procedures [19]. Briefly, the presence of inflammatory signs (inflammatory score): consistency of fecal contents (score 0–3); presence

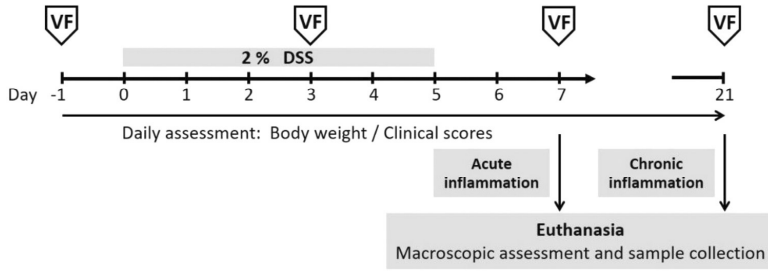

Fig. 1. Schematic representation of the experimental protocols followed in the study. VF: von Frey test.

of visible fecal blood (score 0–3); evidence and extent of edema (0−3); wall thickness (0−3); tissue stiffness (0−2) and presence of ulcerations (0−1) were assessed; resulting in a maximum total score of 15.

2.7. Histological studies

For histological examination, hematoxylin-eosin-stained sections from the colon were obtained following standard procedures. A histopathological score (ranging from 0, normal, to 12, maximal alterations) was assigned to each animal [27]. Specifically, parameters scored included: epithelial structure (0: normal; 1: mild alterations of the villi; 2: local villi destruction and/or fusion; 3: generalized villi destruction and/or fusion), structure of the crypts (0: normal; 1: mild alterations of the crypts; 2: local destruction of the crypts; 3: generalized destruction of the crypts), presence of edema (0: normal; 1: mild local edema in submucosa and/or lamina propria; 2: moderate diffuse edema in submucosa and/or lamina propria; 3: severe generalized edema in submucosa and/or lamina propria), and presence of inflammatory infiltrate (0: normal; 1: mild localized infiltrate; 2: mild generalized infiltrate; 3: severe generalized infiltrate). Scoring was performed on coded slides by two independent researchers and the mean value of the two scores was taken as the final score per animal.

2.8. Serum haptoglobin

Serum concentrations of haptoglobin were determined using a commercial ELISA kit, following manufacturer's instructions (sensitivity; 0.005 mg/ml; intraassay variability: 5.3–6.3%; interassay variability: 4.1–5.7%; "PHASE" TM Haptoglobin Assay; Tridelta Development Limited, Maynooth, County Kildare, Ireland).

2.9. Gene expression using Quantitative Reverse Transcription-PCR

Total RNA was extracted from frozen tissue samples using TRI reagent with Ribopure Kit (Ambion/Applied biosystems, Foster City, CA, USA). RNA was purified by via precipitation with lithium chloride [28]. Later, a two-step quantitative real-time PCR (RT-qPCR) was performed. RNA samples were converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The PCR reaction mixture was incubated on the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad). All samples were assayed in triplicate. The cycle thresholds for each sample were obtained and data were analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with the WT vehicle group serving as the calibrator [29]. TaqMan® gene expression assays (hydrolysis probes, Applied Biosystems) used included: cannabinoid receptors 1 (CB1) (Mm01212171_s1) and 2 (CB2) (Mm00438286_m1), interferon γ (INF-γ) (Mm01168134_m1), interleukin 1β (IL-1β) (Mm00434228_m1), 6 (IL-6) (Mm00446190_m1), 10 (IL-10) (Mm00439614_m1) and 12 (IL-12p40) (Mm00434174_m1), μ-opioid receptor (MOR) (Mm01188089_m1), nerve growth factor (NGF) (Mm00443039_m1), nitric oxide synthase 2 (inducible, iNOS) (Mm00440502_m1), prostaglandin-endoperoxide synthase (Cyclo-oxygenase 2, COX-2) (Mm00478374_m1), protease activated receptor 2 (PAR2) (Mm00433160_m1), serotonin transporter (SERT) (Mm00439391_m1), transient receptor potential vanilloid 1 (TRPV1) (Mm01246302_m1) and 3 (TRPV3) (Mm00455003_m1), tryptophan hydroxylase 1 (TPH1) (Mm00493794_m1) and σ_1 receptor (σ_1R) (Mm00448086_m1). β-2-microglobulin (β2m) (Mm00437762_m1) was used as endogenous reference gene.

2.10. Protein expression using Western blot

Dissected spinal cord samples were homogenized by sonication in radioimmunoprecipitation assay (RIPA) buffer and the supernatant was obtained. Equal amounts of protein (30 μg) were fractionated by 10% (w/v) SDS–PAGE and transferred onto a polyvinylidene difluoride membrane, blocked with 5% non-fat dry milk in Tris–Tween 20-buffered Saline (T–TBS) for 1 h. Membranes were then incubated overnight at 4 °C in 1% non-fat dry milk in T–TBS with rabbit primary polyclonal antibodies recognizing the mitogen-activated protein kinase (MAPK, total ERK ½, 1:30000) or mouse monoclonal antibodies recognizing the activated MAPK (diphosphorylated MAPK, pERK ½, 1:1000). Rabbit polyclonal anti-GAPDH antibody (1:20000) or mouse monoclonal anti-GAPDH antibody (1:80000) were used as a loading control, respectively. After washing with T–TBS, the blots were incubated for 1 h with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:4000) or goat anti-mouse IgG (1:2000). The immunoreactive bands were detected by a peroxidase reaction using an enhanced chemiluminescence method (WesternSure® PREMIUM Chemiluminescent Substrate, Li-cor) and CDiGit ® Blot Scanner (Li-cor). All antibodies were obtained from Sigma–Aldrich Co. (Madrid, Spain). Quantification was realized with Image Studio™ Lite Software.

2.11. Statistical analysis

Data are expressed as mean ± SEM. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Data were analyzed by one-, two or three-way ANOVA, as appropriate, followed, when necessary, by a Bonferroni's multiple comparisons test. Data were considered statistically significant when $P < 0.05$. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA) or SPSS program (version 17 for Windows, IBM, Madrid, Spain).

3. Results

3.1. Colitis development in WT and σ_1R KO mice

Regardless the genotype considered, mice receiving water showed a steady and linear increase in body weight, without clinical signs

throughout the experimental period (Fig. 2). Conversely, in animals exposed to DSS, body weight loss was observed from experimental day 6, with a peak reduction between days 9 and 10, and a progressive recovery up to day 21, although without reaching the body weight of control animals not exposed to DSS. No differences in this pattern were observed between σ_1R KO and WT mice. In animals exposed to DSS, clinical signs (mainly bristly fur, wet/watery feces and wet anus) consistent with the development of a colitic state appeared with similar temporal pattern to that described for body weight changes, reaching a maximum at day 9 and disappearing completely by the end of the experimental time (Fig. 2). No genotype-related differences were observed in the incidence and severity of clinical signs. Water intake was similar across experimental groups (data not shown).

At necropsy, WT mice receiving DSS showed macroscopic signs of colonic inflammation, both at the acute and chronic phase, characterized by shortening in length and an increase in its relative weight ($P < 0.05$ vs. WT mice receiving water; Fig. 3A). Similar changes were observed in σ_1R KO mice. A slight attenuation in inflammation-related parameters was observed in σ_1R KO mice when compared to WT, although statistical significance was not achieved (Fig. 3A).

Microscopic analysis of colonic tissue samples showed a normal histological structure in control animals. Regardless the genotype considered, exposure to DSS led to a similar significant increase in histopathological scores (Fig. 3B). Colonic alterations were attenuated during the chronic phase, however statistical significance was only achieved for σ_1R KO mice ($P < 0.05$ vs. σ_1R KO mice during the acute phase). The improvement observed in σ_1R KO mice was mainly associated to a reduction in the presence of edema (statistically significant interaction by genotype and time on the presence of edema -$P < 0.05$-, Fig. 3B). Regardless of the phenotype considered, no significant changes were observed for the relative weight of body organs (data not shown).

The acute phase protein haptoglobin showed a similar increase in WT and σ_1R KO animals during acute inflammation ($P < 0.05$ vs. respective control). Haptoglobin levels showed a tendency towards normalization during the chronic phase, although they remained significantly increased when compared to non-inflamed animals (Fig. 3C).

3.2. *Inflammatory markers are differentially regulated in WT and σ_1R KO mice during colitis*

In control conditions, independently of the genotype and time of measurement, expression of the cytokines assessed was detectable in all colonic samples. In WT mice, colitis was associated to an up-regulation of the expression of the pro-inflammatory cytokines INF-γ, IL-1β and IL-6, which was particularly evident during the acute phase of inflammation and persisted during the chronic phase, although relatively attenuated (in all cases $P < 0.05$ vs WT control mice, Fig. 4). No significant changes were observed in the local expression of IL-12p40 and IL-10.

In σ_1R KO mice an up-regulation of INF-γ, IL-1β and IL-6 was observed during the acute phase of inflammation (all $P < 0.05$ vs. non-inflamed animals), but expression levels were basically normalized

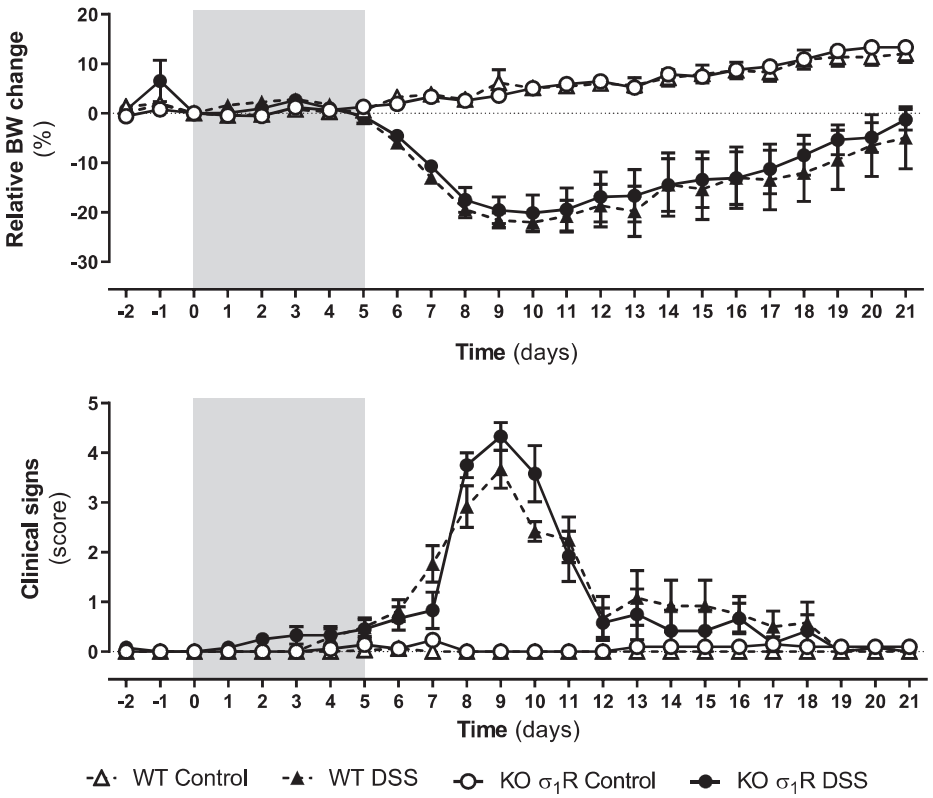

Fig. 2. Time-related changes in relative body weight (% change from day 0, taken as 100%, upper panel) and clinical signs (lower panel) in the different experimental groups. Data are mean ± SEM (n = 12–18). The DSS-treatment period is indicated by the grey area.

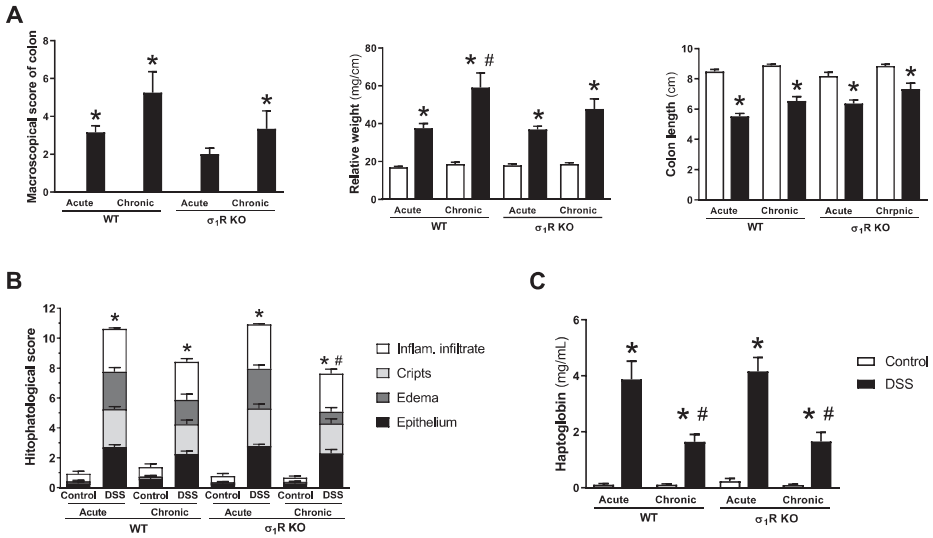

Fig. 3. Assessment of colonic inflammation at the time of necropsy in the different experimental groups. (A) Macroscopic scores. (B) Histopathological scores: inflammatory infiltrate, edema, state of the crypts and epithelial structure. (C) Plasma concentrations of the acute phase protein haptoglobin. Data are mean ± SEM of 6–10 animals per group. *P < 0.05 vs. respective control group; #P < 0.05 vs. respective acute DSS-treated group.

during the chronic phase (all P > 0.05 vs. non-inflamed animals, Fig. 4). A significant up-regulation of IL-10 expression was detected in σ_1R KO mice during the chronic phase (P < 0.05 vs. other experimental groups). Similarly to that observed in WT animals, no changes were observed in the expression of IL12p-40.

Colonic expression of iNOS was up-regulated in acute colitic WT mice, although with relatively high variability (P < 0.05 vs control WT group). This up-regulation persisted during the chronic phase, although with some attenuation, (Fig. 5). In σ_1R KO mice, iNOS expression was up-regulated during acute colitis, while returning during the chronic phase to the levels detected in non-inflamed animals (Fig. 5).

In colitic WT mice, 3 out of 5 animals showed COX-2 expression levels above the mean expression of the control group, during either the acute or the chronic phase, thus suggesting a tendency for COX-2 up-regulation during colitis. However, no statistical significance was achieved, probably because of the high interindividual variability observed. No expression changes were observed in σ_1R KO mice, regardless the inflammatory phase considered (Fig. 5).

3.3. *Colitis-associated mechanical hypersensitivity is attenuated in σ_1R KO mice*

Baseline (experimental day −1) abdominal and paw withdrawal thresholds during the von Frey test were similar in WT and σ_1R KO mice (Fig. 6).

In healthy WT mice, abdominal and somatic mechanical sensitivity was stable throughout the experimental time (Fig. 7). However, in WT animals receiving DSS a reduction in the withdrawal thresholds was observed from experimental day 3 throughout experimental day 21, indicating the development of mechanical hypersensitivity (in all cases P < 0.05 vs. control group).

As it relates to σ_1R KO mice, mechanical thresholds were stable in colitic animals and showed only a transitory reduction in experimental day 7 for abdominal sensitivity (P < 0.05 vs. control group), with a return towards basal sensitivity on experimental day 21; whereas no changes were observed for somatic sensitivity (Fig. 7).

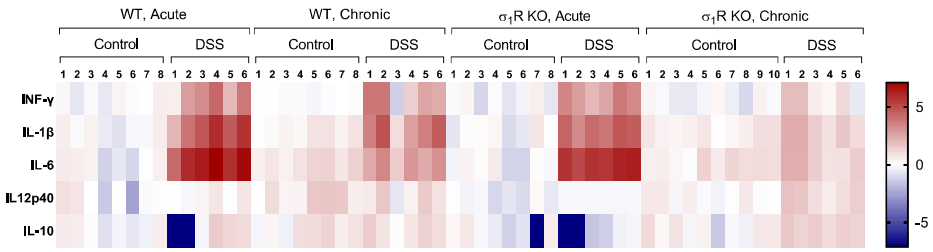

Fig. 4. Heat map of the relative mRNA expression of colonic pro- (INF-γ, IL-1β, IL-6 and IL-12p40) and anti-inflammatory cytokines (IL-10) in the different experimental groups. Each vertical line, with a number, corresponds to an individual animal within the corresponding experimental group.

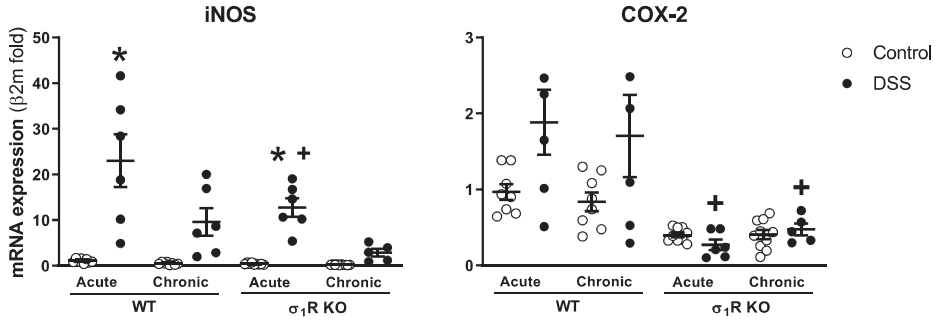

Fig. 5. Colonic expression of inflammatory markers: iNOS, and COX-2. Each point represents an individual animal; the horizontal bar with errors represents the mean ± SEM. *P < 0.05 vs. respective control group; $^{+}$P < 0.05 vs. respective WT DSS-treated group.

3.4. Expression of colonic sensory-related markers is modulated during DSS-induced colitis

Expression of the sensory-related markers assessed was detected in all colonic samples, except for σ_1R, that, as expected, was only found in WT animals. In healthy animals, expression levels of the markers assessed were comparable, regardless the genotype considered.

During colitis in WT mice, there was an overall down-regulation of all sensory markers analyzed when compared with non-inflamed animals. This was particularly evident during the acute phase, with a tendency towards normalization during the chronic phase (Fig. 8). In particular, except for CB2 and PAR-2 which showed no changes, all analyzed markers were down-regulated during both the acute and chronic phases of colitis in WT mice (in all cases P < 0.05 vs. respective control group). In these animals, expression of σ_1R was down-regulated during acute colitis (P < 0.05 vs. control group), returning to basal expression levels during the chronic phase.

Regarding σ_1R KO mice, an overall down regulation was also observed during the acute phase, but a trend towards baseline levels was observed in the chronic phase of colitis. Nevertheless, CB1, MOR and TPH1 showed a persistent down-regulation during both the acute and the chronic phase of inflammation (in all cases P < 0.05 vs. respective control group). On the other hand, expression of NGF, SERT, TRPV1 and TRPV3 in σ_1R KO mice was down-regulated only during the acute phase of colitis (in all cases P < 0.05 vs. respective control group), while during the chronic phase a slight down-regulation was detected, although statistical significance was not achieved (Fig. 8). In σ_1R KO mice, expression of CB2 was significantly up-regulated in the chronic phase of colitis.

3.5. ERK expression within the spinal cord is not affected by colitis

ERK protein was detected in all spinal cord samples, regardless the experimental group considered. Similar levels of tERK and pERK were detected in WT and σ_1R KO mice in control conditions. During colitis, regardless the phase considered, no changes were observed in tERK or pERK content or the ratio pERK/tERK (Fig. 9).

4. Discussion

In the present study, we assessed the potential role of σ_1Rs in the development of colitis and inflammation-associated changes in referred mechanical sensitivity using a murine model KO for σ_1Rs. Results obtained indicate that σ_1Rs only marginally affected the development of intestinal (colonic) inflammation or its progression from acute to chronic, but they seem to play an important role in the development of inflammation-associated hypersensitivity.

In WT animals, exposure to DSS led to the development of colitis with similar clinical, histopathological and molecular alterations to those previously described [18,19,30]. Moreover, we also observed that the inflammatory condition showed a chronification, characterized by the persistence over time, although with some attenuation, of the structural, molecular and biochemical alterations observed during the acute phase and a remission of the clinical signs. This chronification process coincides with the evolution of DSS-induced colitis previously described for the same strain of mice [18] and shares similarities with the quiescent phases of inflammatory bowel disease in humans [30].

In σ_1R KO animals, exposure to DSS led to the induction of colitis with, essentially, the same characteristics as those discussed above for

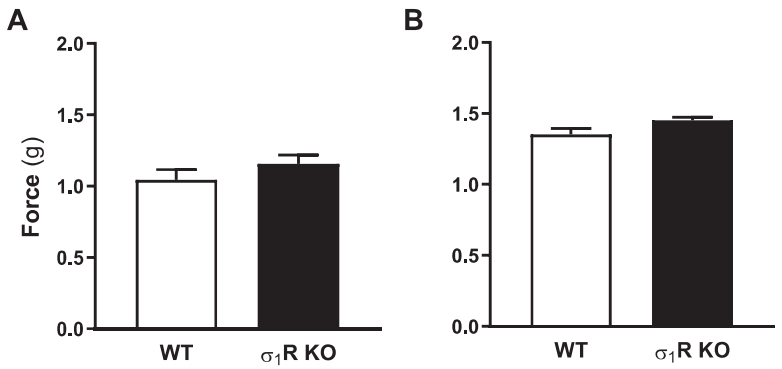

Fig. 6. Baseline abdominal (A) and paw (B) withdrawal thresholds in the von Frey test in WT and σ_1R KO mice. Data are mean ± SEM of n = 6–10 mice per group.

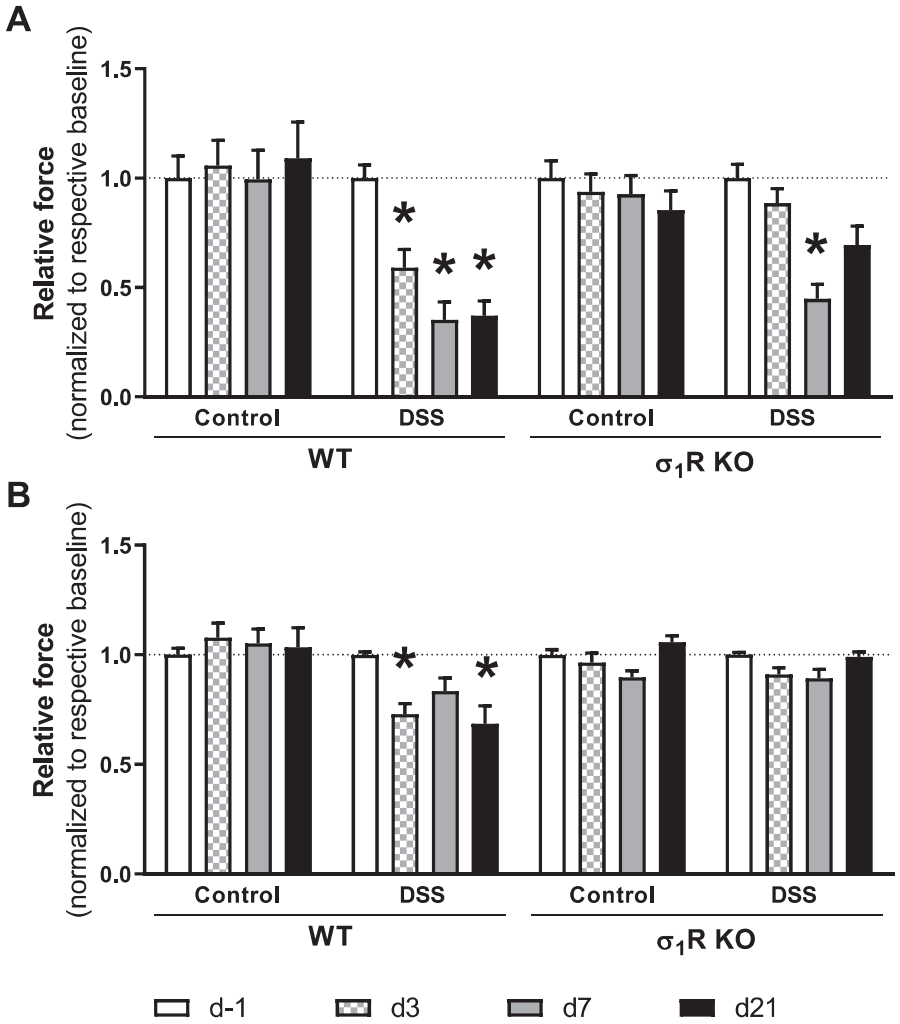

Fig. 7. Sensitivity thresholds to mechanical stimulation during DSS-induced colitis in WT and σ_1R KO mice. (A) abdominal and (B) paw withdrawal thresholds in WT and σ_1R KO mice. In all cases, mechanical sensitivity was determined in basal conditions (day −1, d-1) and at experimental days 3 (d3),7 (d7) and 21 (d21). Data show normalized values (relative force) with respect to basal measurements at experimental day −1, taken as a relative force of 1. Reductions in relative force indicate the development of hypersensitivity. Data are mean ± SEM of n = 6–10 mice per group. *P < 0.05 vs. d-1 of respective experimental group.

WT mice. Overall, these observations suggest that σ_1Rs play a minor role in the development of intestinal inflammation. Nevertheless, some differences were observed between WT and σ_1R mice, particularly as it relates to structural and molecular parameters during the chronic phase of colitis. Firstly, the presence of submucosal edema was significantly reduced in σ_1R KO vs. WT mice during chronic colitis. This finding agrees with previous data showing a reduction in subepithelial edema in σ_1R KO mice in a model of cyclophosphamide-induce cystitis [31] or the reduction in paw edema, elicited by the intraplantar injection of carrageenan, associated to the blockade of σ_1Rs with specific antagonists [14,15]. Moreover, the attenuation of paw edema might implicate NOS-dependent mechanisms affecting vascular permeability and extravasation [14,15]. Interestingly, in σ_1R KO mice treated with DSS, iNOS expression, which was moderately up-regulated in WT animals with colitis, showed similar expression levels as those detected in non-inflamed controls. This suggests that σ_1Rs might regulate vascular permeability and extravasation, at least partially throughout NO-dependent mechanisms, and thus, exert some modulatory effects on inflammation. Additionally, in σ_1R KO mice, expression of pro-inflammatory cytokines, as well as iNOS and COX-2, was normalized during the chronic phase of colitis, while remaining up-regulated in WT animals. Altogether, these data suggest that σ_1Rs might exert a positive immunomodulatory action, as previously suggested in other models [14,31,32], likely facilitating the recovery in chronic conditions, at least as it relates to intestinal inflammation.

Compelling evidences implicate σ_1Rs in pain mechanisms [12]. In our experimental conditions, σ_1R KO and WT mice showed similar mechanosensitivity, as determined by assessing withdrawal thresholds

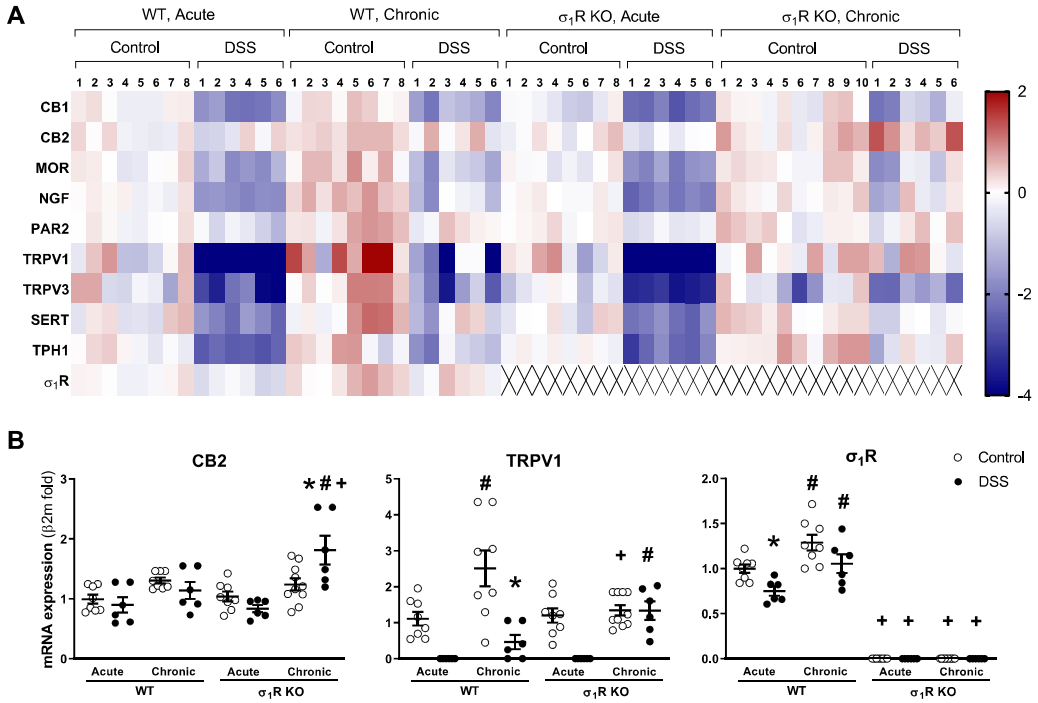

Fig. 8. (A) Heat map of the relative mRNA expression of sensory-related markers in the different experimental groups. Note that, as expected, no σ_1R expression was detected in σ_1R KO animals. Each vertical line, with a number, corresponds to an individual animal within the corresponding experimental group. "X" denotes samples with no expression detected. (B) Detail of the expression changes for the mean genes modified, according to A. Each point represents an individual animal; the horizontal bar with errors represents the mean ± SEM. $^{*}P < 0.05$ vs. respective control group; $^{\#}P < 0.05$ vs. respective acute control group; $^{+}P < 0.05$ vs. respective WT group.

to the mechanical stimulation of the lower abdominal wall, likely reflecting responses associated to the mechanical stimulation of the abdominal wall and the underlying viscera (mainly intestine), or the hind limbs. These observations agree with previous data showing that σ_1R KO mice perceived and responded normally to acute somatic mechanical nociceptive stimuli [17,24] and with pharmacological observations showing that σ_1R ligands, either agonists or antagonists, have no effects by themselves on somatic mechanosensitivity in basal conditions [11,33,34]. Altogether, these results support the view that σ_1Rs are not involved in normal pain responses. Alternatively, we cannot discard that compensatory mechanisms, associated to the constitutive absence of σ_1Rs, lead to normal basal pain responses in these animals.

Intestinal inflammation has been associated to the development of visceral hypersensitivity as well as referred hyperalgesia in several body regions, including the abdominal wall, tail and hind paws [1,5,6,35]. In agreement with this, results obtained here show that colitic WT mice showed mechanical hypersensitivity, manifested as a persistent reduction in the withdrawal threshold to the mechanical stimulation of the abdominal wall (likely reflecting a combination of somatic hypersensitivity -from the abdominal wall per se- and visceral hypersensitivity of the underlying viscera -intestine-) and the hind limbs. Interestingly, the sensitizing effects of inflammation were significantly attenuated in σ_1R KO mice. Indeed, in σ_1R KO animals, paw sensitivity was not affected during colitis, while at the abdominal level only a transitory state of hypersensitivity was observed during the acute phase of colitis, with a clear tendency towards normalization during the chronic phase. These observations are in agreement with previous data showing a reduction in behavioral responses to visceral pain in σ_1R KO mice after intracolonic administration of capsaicin [17] or during cystitis [31] and the reduction of nociceptive responses associated to neuropathic pain [24,36,37]. Moreover, these data in σ_1R KO mice further confirm pharmacological observations showing blockade of somatic pain responses by selective σ_1R antagonists [14,15,25,34,38]. Altogether, these data strongly suggest a key involvement of σ_1Rs in the development of inflammation-dependent referred hypersensitivity, consistent with previous reports. Moreover, taking into account the fact that the stimulation of the abdominal wall is likely to implicate somatic and visceral pain-related responses, our observations also support an implication of σ_1Rs in visceral sensitivity.

To further understand the mechanisms implicated in these changes we assessed the local (colon) expression of different sensory-related markers implicated in viscerosensitivity. During colitis, a general down-regulation of sensory markers was observed, regardless the genotype considered. Thus, supporting the development of nociceptive alterations, at least at a molecular level, during inflammation. It is difficult to establish a direct correlation between gene expression changes of pain-related markers and pain-related responses since a

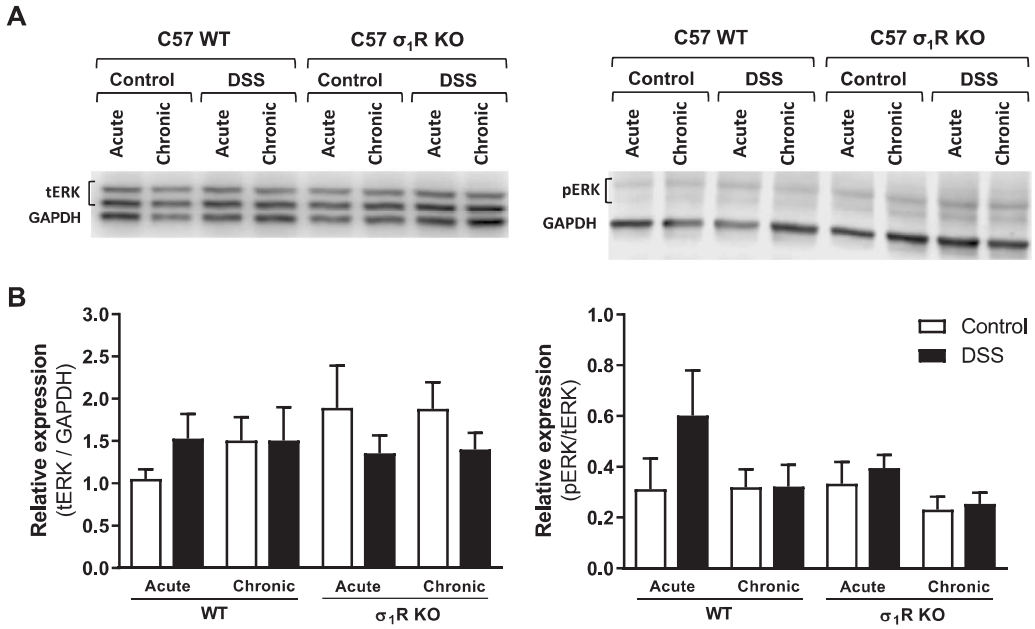

Fig. 9. (A) Representative Western blots showing tERK (left) and pERK (right) in the different experimental groups. (B) Relative spinal expression of tERK (left) and pERK (right). Data are mean ± SEM of n = 6 animals per group.

down-regulation was detected for both pro- and anti-nociceptive markers. Therefore, the final functional outcome is likely to depend upon the balance between changes in expression that favor or counteract pain-related mechanisms, as previously suggested for other experimental conditions related to intestinal sensitivity [39,40]. Despite this, distinctive changes in some pain-related markers were observed in σ_1R KO animals. Specifically, the up-regulation detected for CB2 during chronic colitis might be of particular significance. Given the anti-nociceptive effects associated to the activation of CB2 [41–43], the up-regulation observed might explain, at least in part, the attenuation of pain-related responses observed in these animals during colitis.

The TRPV family of receptors has been related to inflammation and pain. In particular, TRPV1 expression has been positively correlated with pain severity in patients with quiescent IBD [44] and an up-regulation has also been observed during colonic inflammation [45], likely contributing to the visceral hypersensitivity observed after colitis [46]. In the present studies, a down regulation of TRPV1 associated with inflammation, either acute or chronic, was observed in WT mice, even though the presence of mechanical hypersensitivity. These apparently contradictory observations reinforce the importance of the balance between pro- and anti-nociceptive mechanisms in the final outcome as it relates to pain, as discussed above. Alternatively, and given the pro-nociceptive effects of TRPV1, a down-regulation of the receptor might be interpreted as a compensatory mechanism developed under some conditions (such as acute inflammation) to avoid abnormal excessive pain. Furthermore, recent studies have described interactions between σ_1R and TRPV1 receptors [47,48], thus indicating that both receptors might interact during states of hypersensitivity facilitating pain. Indeed, σ_1R antagonism results in the negative regulation of the protein expression of TRPV1 in the plasma membrane of sensory neurons and, consequently, a decrease in nociceptive responses [47]. Therefore, the lack of functional σ_1Rs, leading to an altered TRPV1-σ_1R interaction might contribute to de underlying mechanisms explaining the absence of hypersensitivity in σ_1R KO mice.

Sensitization of pain mechanisms can occur at either peripheral and/or central levels. Our results, as discussed above, suggest that peripheral (colonic) changes might contribute to the sensitization processes associated to inflammation. Nevertheless, to assess the potential participation of central (spinal) sensitization, we also assessed ERK phosphorylation at the level of the lumbosacral spinal cord. Lower lumbar and upper sacral segments of the spinal cord represent the main site of entry to the central nervous system for sensory afferents arising from the colon implicated in pain responses in rodents [49]. Within the spinal cord, ERK phosphorylation is regarded as a key process involved in pain processing and sensitization. Indeed, an increase in spinal phosphorylated ERK (pERK) has been described in several models of somatic [24] and visceral pain [50]. Moreover, σ_1Rs might be implicated in this process since phosphorylation of spinal ERK was attenuated in σ_1R KO mice in a model of neuropathic pain where somatic hypersensitivity was induced by peripheral nerve injury [24,51]. Although these evidences, in the present experimental conditions we did not detect changes in ERK phosphorylation neither at the acute nor the chronic phase of colitis, regardless the genotype considered. This might suggest the involvement of different mechanisms, with different involvement of ERK and/or different kinetics in the phosphorylation process, as it relates to the development of sensitization during inflammatory and neuropathic pain.

In summary, the present data show that σ_1Rs play a minor role in modulating intestinal (colonic) inflammation. Although some molecular markers of inflammation were attenuated in σ_1R KO mice, these changes did not translate in an evident clinical improvement and only correlated with a moderate reduction in submucosal edema. As expected, inflammation was associated to the development of hypersensitivity, likely of both somatic and visceral origin. These pain-related alterations were attenuated in σ_1R KO mice, thus confirming a role of σ_1Rs in the development of hypersensitivity. Overall, these observations suggest that σ_1Rs might represent a feasible target for the treatment of hypersensitivity associated to intestinal inflammation.

Funding

The present work was funded by Laboratories ESTEVE, Barcelona, Spain.

CRediT authorship contribution statement

Vicente Martínez: Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision. **Sergio López-Estévez:** Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Georgia Gris:** Investigation, Writing - review & editing. **Beatriz de la Puente:** Investigation, Writing - review & editing. **Alicia Carceller:** Investigation.

Conflict of interest statement

The authors GG, BdlP and AC were full-time employees of ESTEVE when this work was performed. These authors have no other relevant affiliation or financial involvement, have received no payment in preparation of this manuscript or have any conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. The rest of authors declare no competing interests.

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Laboratory technician, 2014-2016.

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Centre d'experimentació animal. Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol (IGTP).

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Organizing committee, XI Conference of the Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona (UAB).

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Publications

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Grants and awards

Travel award to the best oral presentation. XXVII Reunión Anual del Grupo Español de Motilidad Digestiva (GEMD). Barcelona (Spain), 2019

Travel award for PhD students. Institut de Neurociències, Universitat Autònoma de Barcelona (INc UAB), 2019.

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Travel award for young scientists. Toll 2018 Editing Innate Immunity congress, Porto (Portugal), 2018.

Award to the best poster. VIII Conference of the Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona (UAB), 2016.

Participation in conferences and meetings

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ACKNOWLEDGEMENTS

Diuen que tot va bé si acaba bé. I en aquest camí, malgrat no haver estat fàcil, he tingut la sort de poder comptar amb grans acompanyants sense els quals tot aquest treball no hagués estat possible.

M'agradaria agrair a en Vicente Martínez, qui em va empènyer i convèncer per portar a terme aquesta tasca, la confiança dipositada en mi des d'un principi. Ambos sabemos las conversaciones y discusiones que hemos tenido, y de las cuales yo he aprendido mucho y seguro que tú también. Aunque creo que también en eso discutiríamos. Por todo ello, y por tu atención hasta en el más mínimo detalle, gracias.

Gràcies a tot l'equip d'Esteve, per la seva ajuda i la seva col·laboració sempre que m'ha calgut. I en especial, a la Georgia, l'Alicia i la Bea, amb qui vaig passar molt bones estones tant al laboratori com fora d'ell, i a les que considero les meves post-docs de referència.

També m'agradaria agrair al personal del departament de fisiologia de la facultat de veterinària la seva implicació durant la meva estada. Sou molts, però podeu estar segurs que no m'oblido de vosaltres.

Vull agrair a tot l'equip de Peptomyc i també al grup de Modelització de teràpies anti-tumorals la seva confiança i el fet de poder compaginar la finalització de la tesi amb la meva tasca al laboratori. Sílvia, gràcies per pensar en mi, i gràcies a tota la resta per fer-me sentir tan còmode.

No vull oblidar-me de tots els animals que han col·laborat, de forma inesperada i involuntària, en la realització d'aquesta tesi. La major implicació és la seva.

No menys importants, sinó tot el contrari, sou tots aquells que heu estat lluny de les pipetes i els microscopis, però molt a prop en tot aquest camí. A tots vosaltres us voldria donar les gràcies.

A la Colla, los amigos de siempre que desde el primer momento habéis tenido más confianza que yo en todo esto, y con los que toca celebrarlo como se merece ahora que acaba. Gracias a todos, y gracias a Josep por partida doble, por dejarse enredar y perder miles de horas diseñando programas de anàlisis.

Gràcies a les meves tres veterinàries de capçalera: Mònica, Marina i Sara. Vosaltres, més que ningú, sabeu el què han estat aquests anys. Em sembla que heu patit aquesta tesi casi tant com jo però ho heu sabut dissimular prou bé. No cal dir-ho però sort que hi éreu i sort que hi sou.

Maries, a les dues, gràcies per les escapades, per les xerrades (o rajades), pels sopars infinitament post-posats, i fins i tot per les continues preguntes de com va la tesi?

Ana, Paula y Thomas, sabéis ya a qué huele la luna? Tengo que decir que, ahora que se acaba, el día es raro y hoy mismo podría tocarle a cualquiera diez boleros. Pero no pasa nada, nos vamos de aquí! Y los tres sabéis dónde. Gracias!

Martí, has estat un gran recolzament durant tota la tesi, des de ben al principi, fins al final. Especialment en la redacció de tots els annexos que mai veuran la llum però que han ajudat a que tota la resta tirés endavant. Gràcies per tot plegat.

Desde Barcelona, Madrid o Boston, gracias por el apoyo, Juan Carlos, por la empatía y la complicidad. Porque tengo la sensación de haber hecho esta tesis contigo y porque creo que parte de ella también es tuya.

Finalmente, me gustaría agradecer el apoyo de aquellos que están más cerca: mi familia.

A Laura, Dani y las sobris. Por el cariño, los besos y vuestra contagiosa alegría. Ahora sí, padel, piscina, cellers y lo que queráis!

A ti, Fran, por tu apoyo, a veces difícil de entender, pero siempre reconfortante. Por la paciencia infinita y la ilusión de cada día. Y por lo más importante de todo, por compartir la vida conmigo.

Y, en especial, a mis padres, Francisca y José Ramón, porque gracias a vuestro esfuerzo y valores he podido llegar hasta aquí, y quienes, a pesar de andar tan perdidos como yo, siempre me habéis apoyado.

A todos vosotros, muchas gracias.