



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

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ABSTRACT

Sigma-1 receptors (σ_1 R) have been implicated in several pain pathways. We assessed the implication of σ_1 R in the development of intestinal inflammation and inflammation-associated referred hypersensitivity in a model of colitis in σ_1 R knockout (KO) mice. Colitis was induced with dextran sulfate sodium (DSS) in wild type (WT) and σ_1 R 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_1 R 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_1 R 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_1 R 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_1 R KO mice, thus confirming an important role for σ_1 R in the development of colitis-associated hypersensitivity. These results identify σ_1 R 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: β_2 m, β -2-microglobulin; σ_1 Rs, 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-1 β , Interleukin 1 β ; 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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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_1 Rs) have been implicated in pain mechanisms and suggested as potential pharmacological target for the treatment of somatic pain. σ_1 R 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 σ R systems and opioid-mediated analgesia [9,10]. Evidences indicate that σ_1 R ligands fail to modify normal pain responses by themselves, as demonstrated in classical models of thermal and mechanical acute nociception [11,12]. However, σ_1 R 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_1 Rs 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_1 Rs. In this sense, σ_1 Rs 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_1 Rs 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_1 R 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_1 R 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_1 R 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 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- μ m-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 \times 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

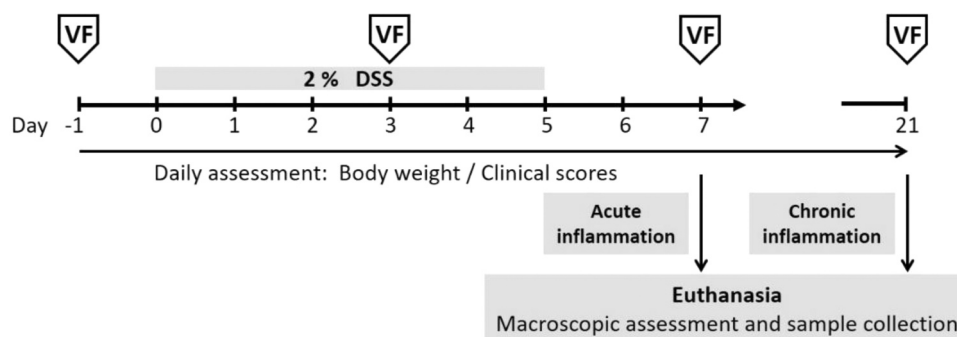


Fig. 1. Schematic representation of the experimental protocols followed in the study. VF: von Frey test.

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"™ 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 ($2^{-\Delta\Delta C_t}$) 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 1 β (IL-1 β) (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 (β 2m) (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 μ g) 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 $\frac{1}{2}$, 1:30000) or mouse monoclonal antibodies recognizing the activated MAPK (diphosphorylated MAPK, pERK $\frac{1}{2}$, 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 \pm SEM. A robust analysis (one iteration) was used to obtain mean \pm 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-1 β 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-1 β 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

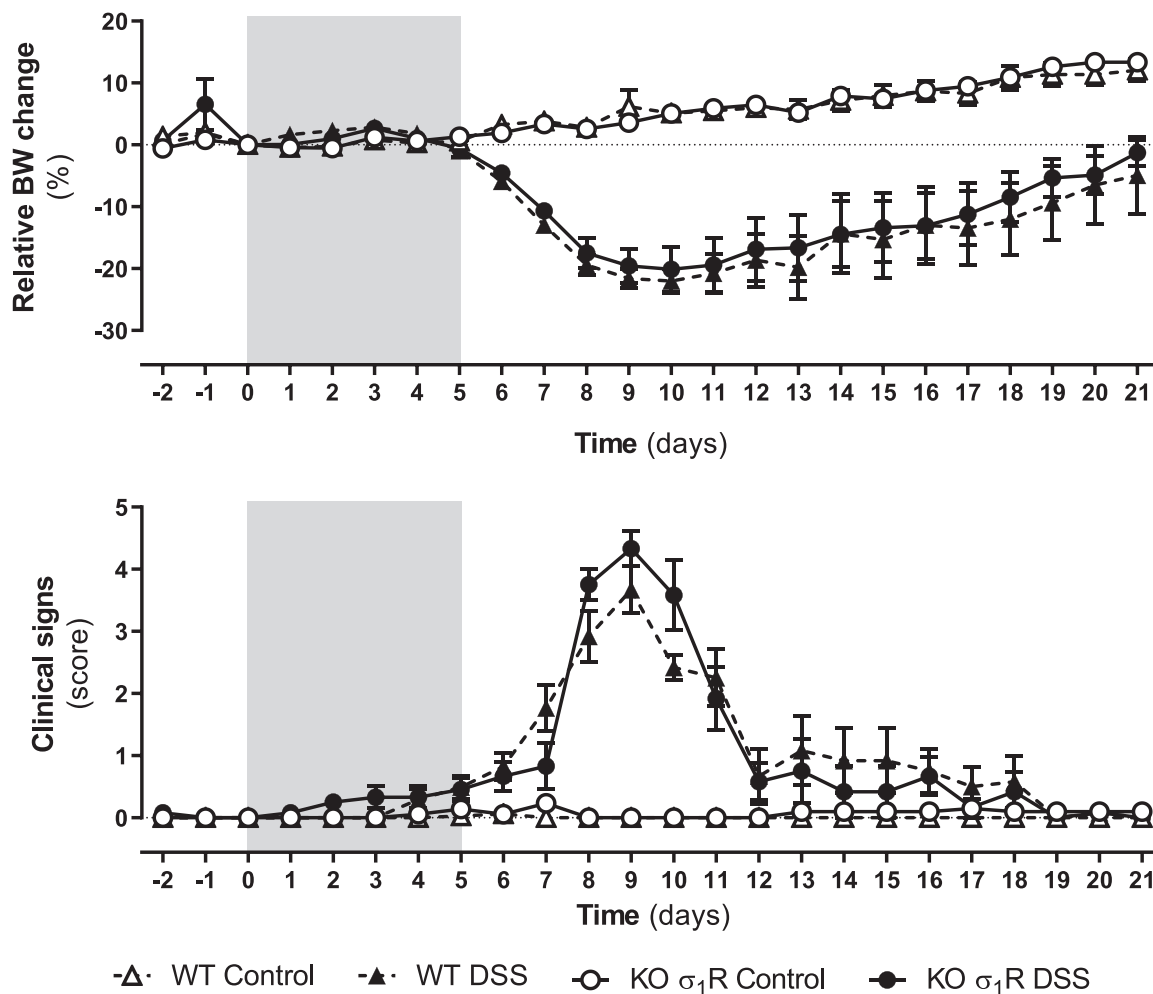


Fig. 2. Time-related changes in relative body weight (% change from day 0, taken as 100%, upper panel) and clinical signs (lower panel) in the different experimental groups. Data are mean \pm SEM ($n = 12$ –18). The DSS-treatment period is indicated by the grey area.

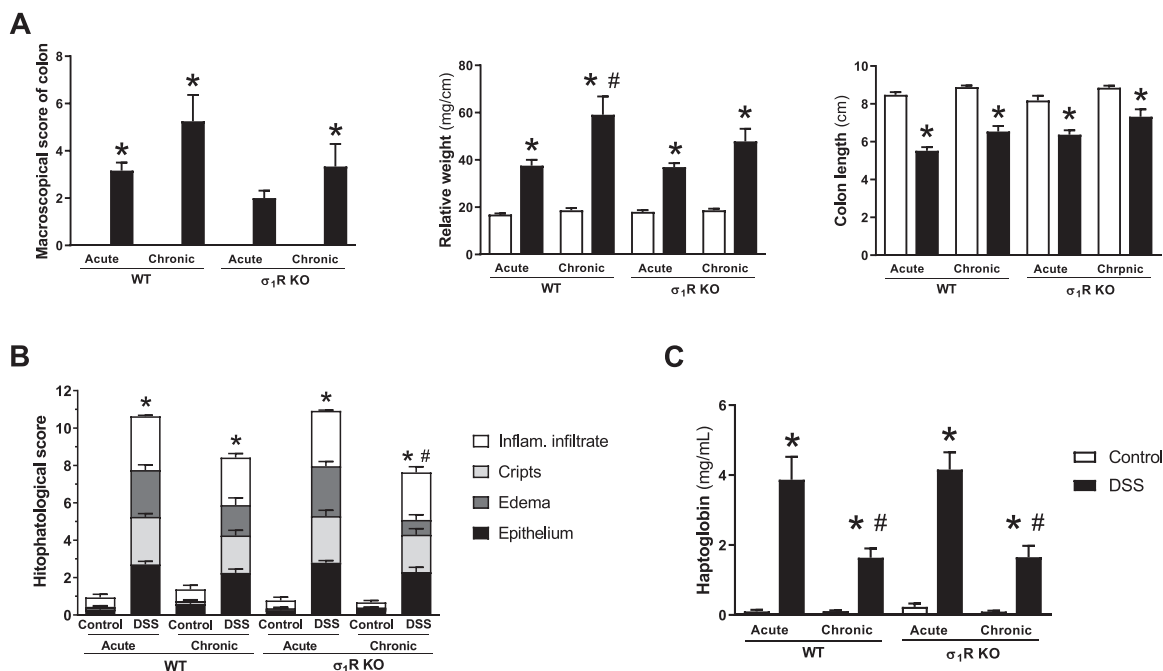


Fig. 3. Assessment of colonic inflammation at the time of necropsy in the different experimental groups. (A) Macroscopic scores. (B) Histopathological scores: inflammatory infiltrate, edema, state of the crypts and epithelial structure. (C) Plasma concentrations of the acute phase protein haptoglobin. Data are mean \pm 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).

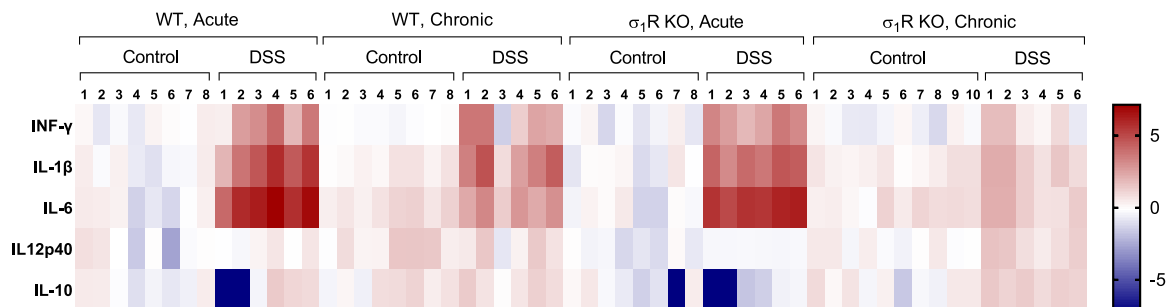


Fig. 4. Heat map of the relative mRNA expression of colonic pro- (INF- γ , IL-1 β , IL-6 and IL-12p40) and anti-inflammatory cytokines (IL-10) in the different experimental groups. Each vertical line, with a number, corresponds to an individual animal within the corresponding experimental group.

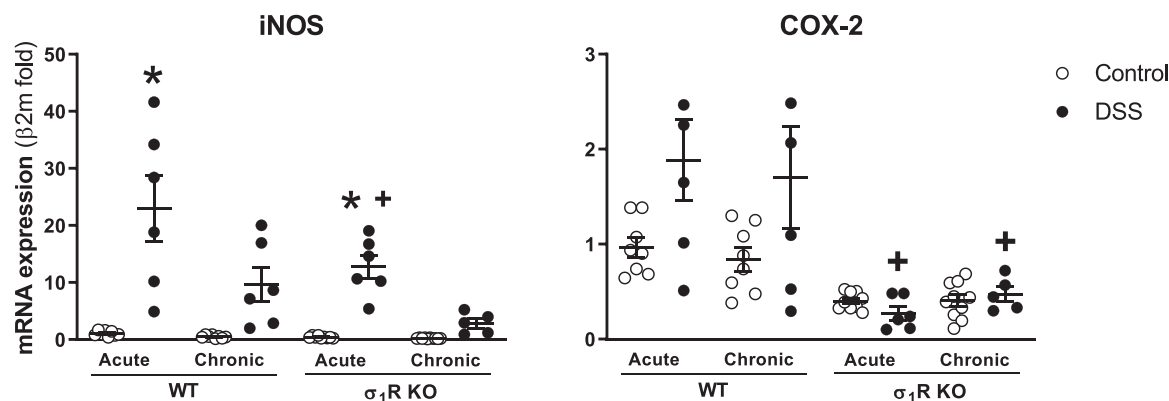


Fig. 5. Colonic expression of inflammatory markers: iNOS, and COX-2. Each point represents an individual animal; the horizontal bar with errors represents the mean \pm SEM. * $P < 0.05$ vs. respective control group; + $P < 0.05$ vs. respective WT DSS-treated group.

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_1R s in the development of colitis and inflammation-associated changes in referred mechanical sensitivity using a murine model KO for σ_1R s. Results obtained indicate that σ_1R s 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

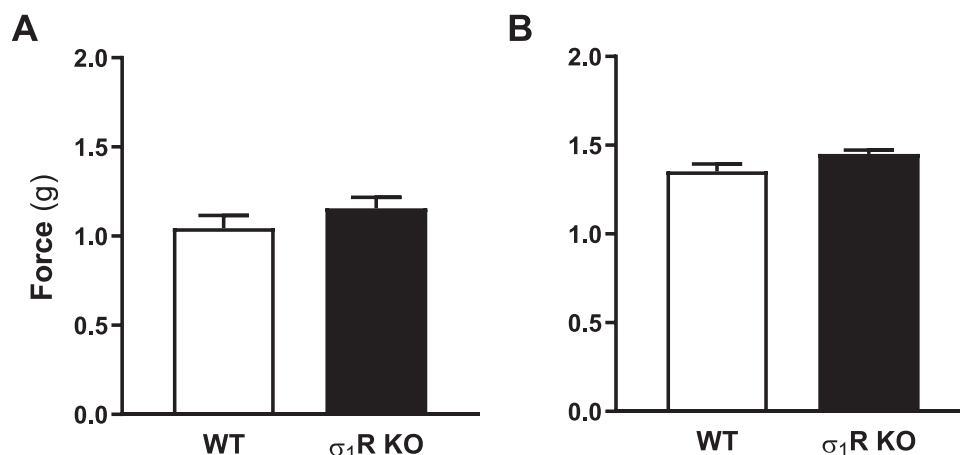


Fig. 6. Baseline abdominal (A) and paw (B) withdrawal thresholds in the von Frey test in WT and σ_1R KO mice. Data are mean \pm SEM of $n = 6-10$ mice per group.

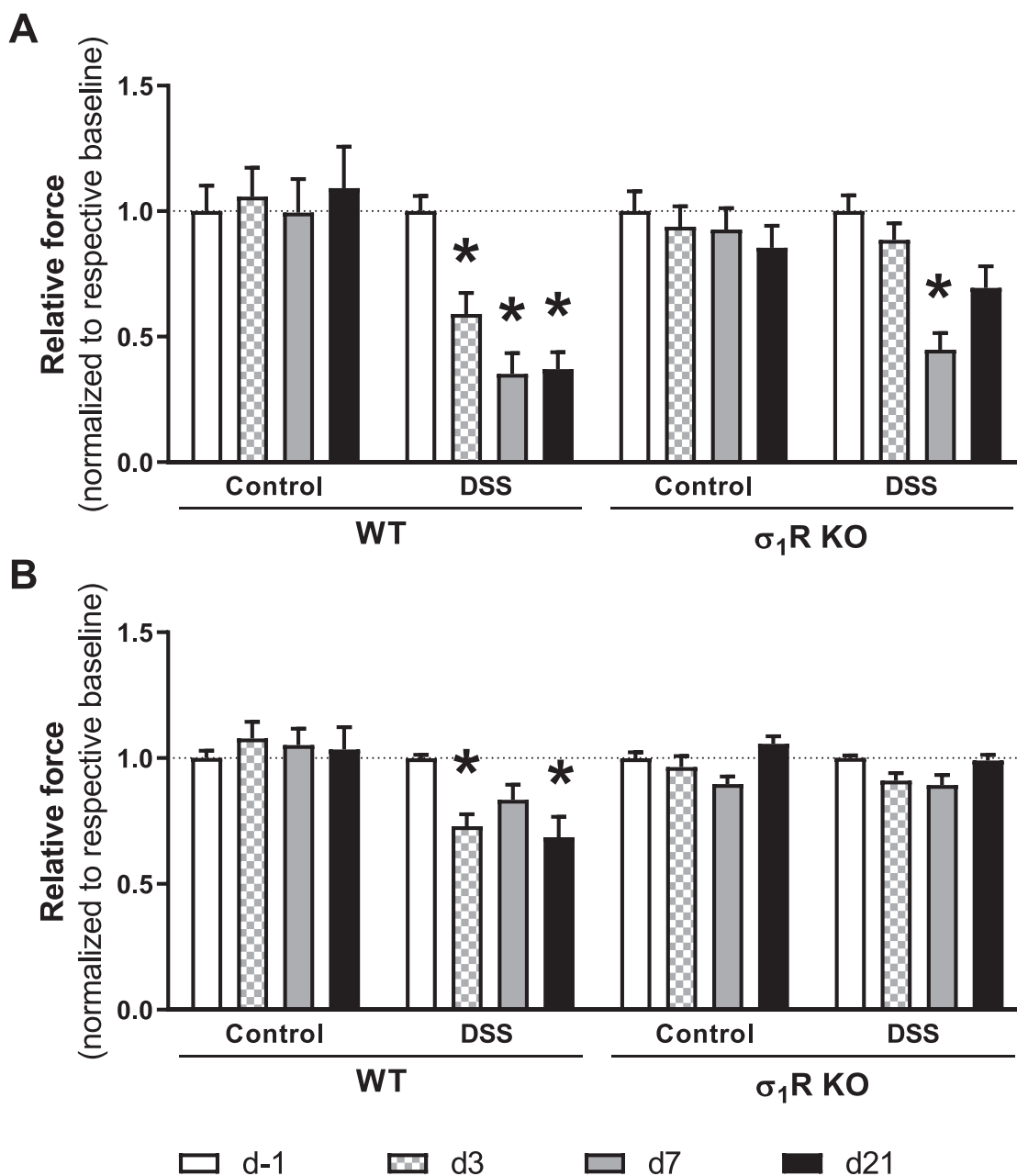


Fig. 7. Sensitivity thresholds to mechanical stimulation during DSS-induced colitis in WT and σ_1 R KO mice. (A) abdominal and (B) paw withdrawal thresholds in WT and σ_1 R KO mice. In all cases, mechanical sensitivity was determined in basal conditions (day -1, d-1) and at experimental days 3 (d3), 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 \pm 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_1 Rs play a minor role in the development of intestinal inflammation. Nevertheless, some differences were observed between WT and σ_1 R 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_1 R KO vs. WT mice during chronic colitis. This finding agrees with previous data showing a reduction in subepithelial edema in σ_1 R KO mice in a model of cyclophosphamide-induced cystitis [31] or the reduction in paw edema, elicited by the intraplantar injection of carrageenan, associated to the blockade of σ_1 Rs 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_1 R 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_1 Rs might regulate vascular permeability and extravasation, at least partially throughout NO-dependent mechanisms, and thus, exert some modulatory effects on inflammation. Additionally, in σ_1 R 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_1 Rs 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_1 Rs in pain mechanisms [12]. In our experimental conditions, σ_1 R KO and WT mice showed similar mechanosensitivity, as determined by assessing withdrawal thresholds

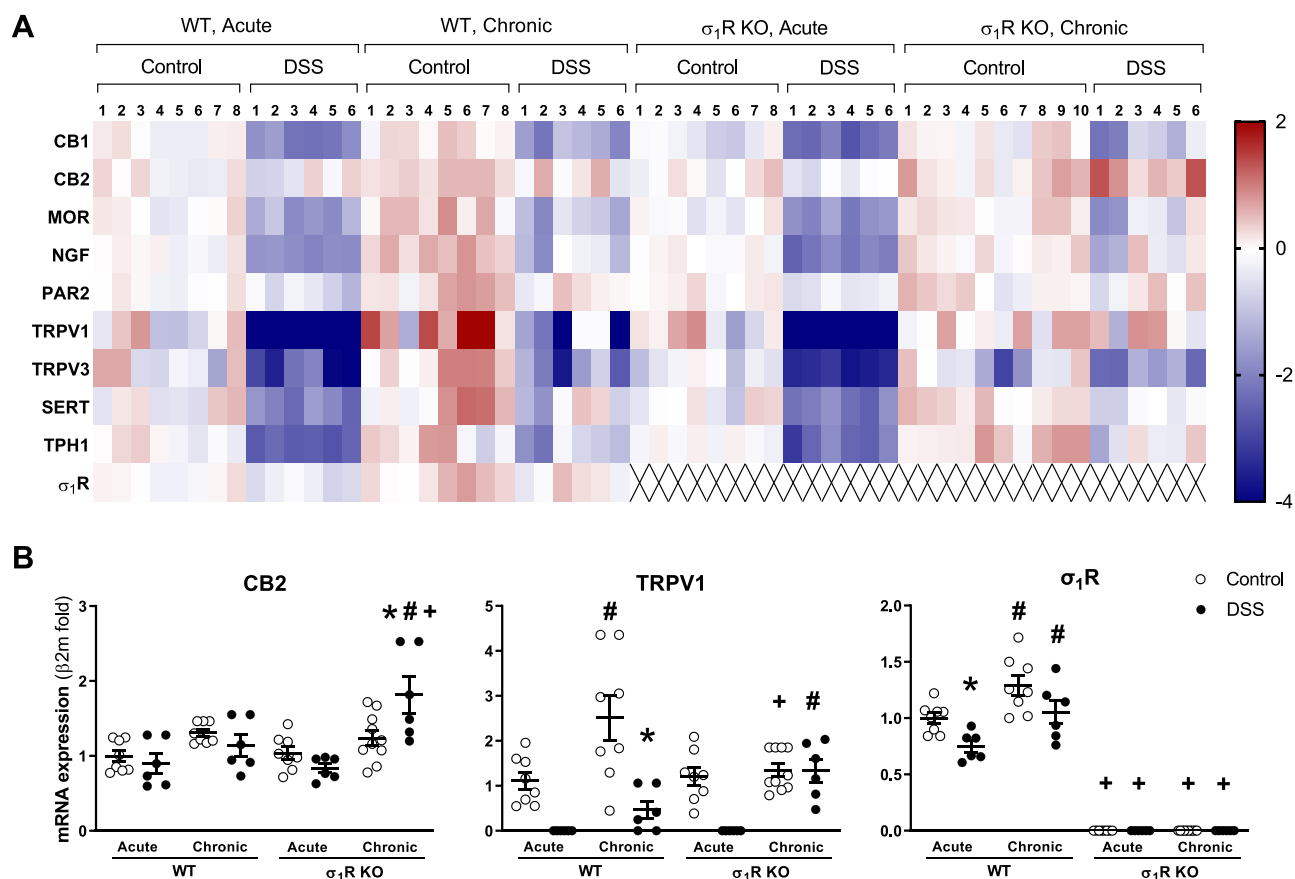


Fig. 8. (A) Heat map of the relative mRNA expression of sensory-related markers in the different experimental groups. Note that, as expected, no σ_1R expression was detected in σ_1R KO animals. Each vertical line, with a number, corresponds to an individual animal within the corresponding experimental 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 \pm 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_1R s are not involved in normal pain responses. Alternatively, we cannot discard that compensatory mechanisms, associated to the constitutive absence of σ_1R s, 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_1R s 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_1R s 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

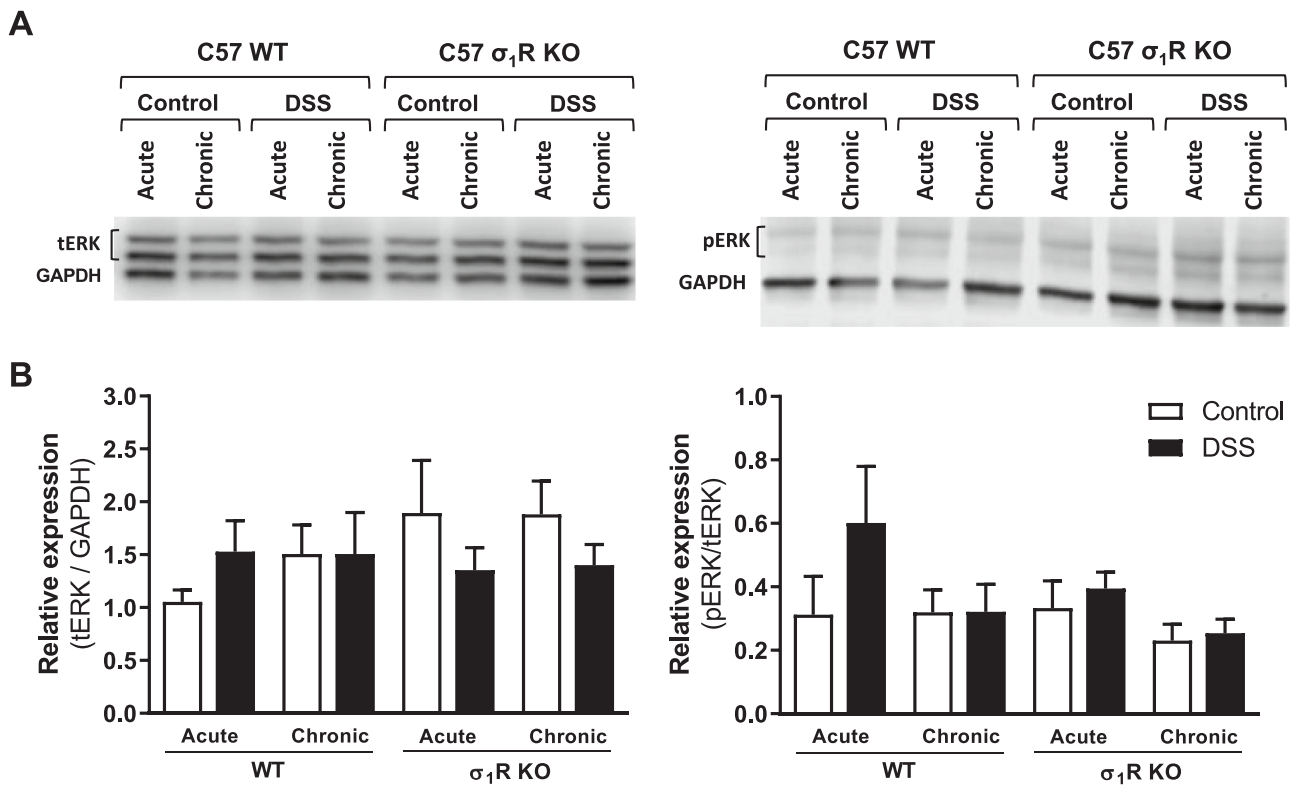


Fig. 9. (A) Representative Western blots showing tERK (left) and pERK (right) in the different experimental groups. (B) Relative spinal expression of tERK (left) and pERK (right). Data are mean \pm SEM of $n = 6$ animals per group.

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_1 R 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_1 R and TRPV1 receptors [47,48], thus indicating that both receptors might interact during states of hypersensitivity facilitating pain. Indeed, σ_1 R 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_1 Rs, leading to an altered TRPV1- σ_1 R interaction might contribute to de underlying mechanisms explaining the absence of hypersensitivity in σ_1 R 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_1 Rs might be implicated in this process since phosphorylation of spinal ERK was attenuated in σ_1 R 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_1 Rs play a minor role in modulating intestinal (colonic) inflammation. Although some molecular markers of inflammation were attenuated in σ_1 R 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_1 R KO mice, thus confirming a role of σ_1 Rs in the development of hypersensitivity. Overall, these observations suggest that σ_1 Rs might represent a feasible target for the treatment of hypersensitivity associated to intestinal inflammation.

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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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