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Urinary bladder sigma-1 receptors: A new target for cystitis treatment

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ABSTRACT

No adequate treatment is available for painful urinary bladder disorders such as interstitial cystitis/bladder pain syndrome, and the identification of new urological therapeutic targets is an unmet need. The sigma-1 receptor (σ_1-R) modulates somatic pain, but its role in painful urological disorders is unexplored. The urothelium expresses many receptors typical of primary sensory neurons (e.g. TRPV1, TRPA1 and P2X3) and high levels of σ_1-R have been found in these neurons; we therefore hypothesized that σ_1-R may also be expressed in the urothelium and may have functional relevance in this tissue. With western blotting and immunohistochemical methods, we detected σ_1-R in the urinary bladder in wild-type (WT) but not in σ_1-R-knockout (σ_1-KO) mice. Interestingly, σ_1-R was located in the bladder urothelium not only in mouse, but also in human bladder sections. The severity of histopathological (edema, hemorrhage and urothelial desquamation) and biochemical alterations (enhanced myeloperoxidase activity and phosphorylation of extracellular regulated kinases 1/2 [pERK1/2]) that characterize cyclophosphamide-induced cystitis was lower in σ_1-KO than in WT mice. Moreover, cyclophosphamide-induced pain behaviors and referred mechanical hyperalgesia were dose-dependently reduced by σ_1-R antagonists (BD-1063, NE-100 and S1RA) in WT but not in σ1-KO mice. In contrast, the analgesic effect of morphine was greater in σ_1-KO than in WT mice. Together these findings suggest that σ_1-R plays a functional role in the mechanisms underlying cyclophosphamide-induced cystitis, and modulates morphine analgesia against urological pain. Therefore, σ_1-R may represent a new drug target for urinary bladder disorders.

1. Introduction

Different bladder pathologies such as interstitial cystitis/bladder pain syndrome (IC/BPS) and bladder cancer can cause severe pain and markedly affect patients' quality of life [1,2]. In particular, IC/BPS courses with intense suprapubic/pelvic pain together with increased urinary frequency and urgency, and can be manifested by cystoscopic abnormalities including petechial hemorrhages or ulcers and urothelial loss [3]. No effective treatment has been found to date, and achieving acceptable control of the symptoms generally requires trials with multiple therapeutic options [4]. The identification of new drug targets of potential use in the treatment of bladder pain may thus provide new treatment opportunities for IC/BPS and other painful urologic pathologies.

The sigma-1 receptor (σ_1-R) is a ligand-operated chaperone [5]. It has been cloned; its sequence is 90 % identical between humans and mice [6], and it does not resemble any other known mammalian protein – a feature that underscores its uniqueness [7]. σ_1-R modulates different types of somatic pain, including inflammatory and neuropathic pain [8–11], but its role in painful urological disorders remains entirely unexplored. The urothelium expresses many receptors typical of primary sensory neurons (e.g. TRPV1, TRPA1, P2X3, etc.) [12], and high levels of σ_1-R are detected in these neurons [13–15]; consequently, we hypothesized that σ_1-R may also be expressed in the urothelium. To test this hypothesis, we carried out immunohistochemical studies in human and mouse urinary bladder sections. We found σ_1-R in the urothelium of both species (see Results), and therefore decided to test its functional

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relevance in a mouse model of cystitis.

Among the many animal models of cystitis that have been developed [16,17], we choose the cyclophosphamide-induced cystitis model because it is one of the better known and most frequently used models of bladder inflammation and pain. Cyclophosphamide-induced cystitis produces several histopathological alterations in the urinary bladder, including edema, hemorrhage and urothelial desquamation [18,19]. Moreover, it results in the activation (phosphorylation) of extracellular signal-regulated kinases 1 and 2 (pERK1/2) and in neutrophil infiltration, which is manifested by an increase in myeloperoxidase (MPO) activity [18–20]. Therefore, to test the functional relevance of bladder σ_1-R, we first compared cyclophosphamide-induced bladder alterations in wild-type (WT) and σ_1-R knockout (σ_1-KO) mice. In addition, because cyclophosphamide produces spontaneous pain behaviors and mechanical hyperalgesia referred to the abdominal wall [21–25], we compared these pain manifestations in animals of both genotypes. Finally, we tested whether σ_1-R might offer a new drug target for the treatment of painful urinary bladder disorders by evaluating the effects of several selective σ_1-R antagonists (BD-1063, NE-100 and S1RA) [7] on the spontaneous pain and referred mechanical hyperalgesia induced by cyclophosphamide in mice, and their effects on one of the biochemical alterations induced in the bladder by cyclophosphamide, i.e. the increase in MPO activity. In all cases, to confirm that σ_1-R antagonists were acting on their target, we tested them in both WT and σ_1-KO mice; furthermore, we compared the effect of σ_1-R antagonists with that of morphine as an analgesic control drug.

2. Methods

2.1. Animals and drugs

Experiments were done in WT (Charles River, Barcelona, Spain) and homozygous σ_1-KO (Esteve Pharmaceuticals, Barcelona, Spain) CD-1 mice weighing 25–30 g. The σ_1-KO mice were generated on a CD-1 background as described previously [26]. Because IC/BPS is more common in women than in men [27], we choose female mice to perform the experiments. Animals were acclimated in our animal facilities for at least 1 week before testing, and were housed in colony cages in temperature- and light-controlled rooms (22 ± 1 °C, lights on at 8:00 AM and off at 8:00 PM, air replacement every 20 min). A standard laboratory diet (Harlan Teklad Research Diets, Madison, WI, USA) and tap water were available *ad libitum* until the beginning of the experiments. Animals were tested at random times throughout the estrous cycle. Testing took place during the light phase (from 9:00 AM to 3:00 PM). Mice were handled in accordance with the European Communities Council directive 2010/63, and all procedures were approved by the Research Ethics Committee of the University of Granada. In order to minimize the number of animals used in the study, when possible, the same animals were used for behavioral studies and to obtain urinary bladders for *in vitro* studies.

We used the following selective σ_1-R antagonists: BD-1063 (1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine dihydrochloride), supplied by Tocris Cookson Ltd. (Bristol, UK); NE-100 (*N,N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]ethylamine hydrochloride), synthesized as reported previously [28]; and S1RA (4-[2-[[5-methyl-1-(2-naphthalenyl)-1H-pyrazol-3-yl]oxy]ethyl] morpholine hydrochloride) [29], supplied by Esteve Pharmaceuticals. Morphine hydrochloride (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health, Madrid, Spain) was used as the opioid agonist drug. Each drug was dissolved in sterile physiological saline. Drug solutions were prepared immediately before the start of the experiments, and 5 ml/kg of the drug solution or its solvent was injected subcutaneously (s.c.) into the interscapular area.

Cyclophosphamide (Sigma-Aldrich, Madrid, Spain), which was used to induce cystitis, was dissolved in saline and injected intraperitoneally (i.p.) at a volume of 10 ml/kg. The same volume of solvent was injected in control animals. Different doses of cyclophosphamide were used during the study (10, 30, 100, 200 and 300 mg/kg).

In all cases the researchers who performed the experiments were unaware of the mouse genotype and were blinded to the treatment received by each animal.

2.2. Human and mouse urinary bladder immunohistochemistry

Formalin-fixed, paraffin-embedded adult human normal bladder sections (5 μm thick) mounted on glass slides were obtained from BioChain Institute (Newark, CA, USA, lot number B507075). Mouse urinary bladder sections were obtained from control and cyclophosphamide-treated WT and σ_1-KO mice, and were processed according to standard procedures [18,19]. Animals were anesthetized with isofluorane (IsoVet®, B. Braun, Barcelona, Spain) and perfused intracardially with 20 ml saline followed by 30 ml 3.7–4.0 % formaldehyde buffered to pH 7.0 (Panreac Quimica SLU, Barcelona, Spain). After perfusion, the mouse bladder was removed and the whole organ was post-fixed in 3.7–4.0 % formaldehyde for 24 h. Then the bladders were dehydrated in solutions of increasing ethanol concentrations, followed by immersion in xylene, and were then embedded in paraffin. Tissue sections (5 μm thick) were obtained with a microtome (Microm HM325, Thermo Scientific, Watham, MA, USA) and mounted on poly-L-lisine-coated glass slides.

Human and mouse urinary bladder sections were deparaffinized and hydrated in preparation for antigenic unmasking by heat treatment (steam at 95 °C for 20 min) in sodium citrate 10 mM buffer at pH 8.0 (Master Diagnóstica, Granada, Spain), followed by cooling in distilled water at room temperature for 10 min. Then endogenous peroxidase activity was blocked with 3 % (vol/vol) H_2O_2 in methanol for 15 min, followed by successive washes (5 min each) in distilled water and TBS-Tween 20 buffer (Tris buffered saline, pH 7.4, Thermo Scientific) with 0.1 % Tween 20.

For σ_1-R detection, the Ultra Vision Quanto peroxidase kit (TL-060-QHD, Thermo Scientific) was used. Nonspecific staining was blocked with Quanto Ultra V Block for 8 min at room temperature. Then samples were incubated for 30 min at room temperature with a σ_1-R mouse monoclonal primary antibody (sc-137075, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:400 dilution in TBS-Tween 20 buffer. After three washes (10 min each) with TBS-Tween 20 buffer, samples were incubated with Quanto amplifier for 10 min, rinsed in TBS-Tween 20 (3 washes of 5 min each), and then incubated with Quanto HRP (horseradish peroxidase) polymer for 10 min at room temperature. After 3 washes (5 min each) with TBS-Tween 20, the peroxidase reaction was visualized using 3, 3′-diaminobenzidine with the ImmPACT DAB Peroxidase Substrate Kit (SK-4105, Vector Laboratories, Burlingame, CA, USA). Briefly, bladder sections were incubated for 20 s at room temperature with the reagent under dim light conditions, followed immediately by two washes of 5 min each with distilled water. Then samples were counterstained (2 min) in Mayer's hematoxylin, and were washed with slightly alkalinized water for 5 min, followed by dehydration by rapid immersion (seconds) in solutions of increasing ethanol concentrations and 3 rapid washes in xylene. Finally, a drop of Vitro-Clud mounting medium (Deltalab, Barcelona, Spain) and a coverslip were place over the sample. Negative controls for σ_1-R detection were obtained by omitting the primary antibody, and all control assays indicated the absence of immunoreactivity. Images were acquired with a Nikon Eclipse 50i microscope equipped with a DS-Ri1 camera (Nikon Instruments Europe BV, Amsterdam, Netherlands).

2.3. Bladder histopathology

For histopathological studies three different histological alterations (urinary bladder edema, hemorrhage and urothelial desquamation) indicative of cyclophosphamide-induced cystitis were analyzed, according to previously described methods [18,19], in bladder sections

obtained from WT and σ_1-KO mice.

A morphometric analysis of each hematoxylin–eosin-stained section was performed with ImageJ software (http://rsb.info.nih.gov/ij/index.html), and the edematous area located in the lamina propria (between the urothelium and muscularis) was normalized with respect to the total area of the bladder section. The numbers of hemorrhagic foci and areas of urothelial desquamation were also counted in each section. All images were acquired with an Olympus BX51 microscope.

2.4. Western blotting

Experiments were performed as previously described in detail [9,15], with some modifications. The bladders were carefully removed from control and cyclophosphamide-treated WT and σ_1-KO mice (at 1, 3 or 5 h after the injection of cyclophosphamide 300 mg/kg i.p.), frozen immediately in liquid nitrogen, and stored at −80 °C. Bladders were thawed, finely minced with spring scissors, and homogenized in buffer (RIPA buffer with 0.5 % protease inhibitor cocktail and 1 % phosphatase inhibitor cocktail-2, all from Sigma-Aldrich). Then homogenates from three bladders were mixed and centrifuged at 1000 *g* for 5 min to decant large remains. Protein concentration in the supernatants was measured with the Bradford assay, and these supernatants were used for western blot analyses.

Equal amounts of protein (24 μg for ERK and 30 μg for σ_1-R) were fractionated on 12 % (wt/vol) SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Madrid, Spain). The membranes were incubated for 1 h at room temperature in blocking buffer containing 5 % dry skim milk in TBS-Tween 20, and were incubated overnight at 4 °C with a rabbit polyclonal antibody recognizing total ERK1/2 (M-5670, Sigma-Aldrich) at 1:40 000 dilution, a rabbit monoclonal antibody recognizing diphosphorylated ERKs 1 and 2 (pERK1/2) (4370S, Cell Signaling Technology, Danvers, MA, USA) at 1:2000, or with a goat polyclonal antibody recognizing σ_1-R (sc-22948, Santa Cruz Biotechnology, Heidelberg, Germany) at 1:250. To control for equal protein loading, rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used at 1:40000 or 1:80000 depending on the experiment (G9545, Sigma-Aldrich). All primary antibodies were diluted in TBS-Tween 20 buffer containing 0.5 % dry skim milk. When the primary antibody incubation period ended, the blots were washed 3 times for 10 min each with TBS-Tween 20 buffer and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG or donkey anti-goat IgG (both from Sigma-Aldrich), diluted at 1:2000 and 1:2500, respectively, in TBS-Tween 20 buffer containing 0.5 % dry skim milk. Then the membranes were washed with TBS-Tween 20 buffer 6 times for 10 min each, and the peroxidase reaction was revealed with an enhanced chemiluminescence method (ECL Prime Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. Chemiluminescence was detected with a LAS 3000 Image Analyzer System (Fujifilm, Tokyo, Japan). Densitometric analysis of the immunoreactive bands was done with Quantity One software (Bio-Rad) and normalized with respect to the intensity of the corresponding GAPDH immunoreactive bands.

2.5. Myeloperoxidase activity

Changes in MPO activity represent a reliable index of polymorphonuclear leukocyte infiltration [30]. Therefore, 5 h after the injection of cyclophosphamide, the urinary bladder of WT and σ_1-KO mice was dissected out, finely minced with spring scissors, and processed as previously described to measure MPO activity [30]. Briefly, minced urinary bladder samples were homogenized in 0.4 ml phosphate buffer (50 mM, pH 6.0) containing 0.5 % hexadecyltrimethylammonium bromide (HTAB, Sigma-Aldrich). The homogenates were freeze-thawed three times and centrifuged (6000 *g*, 10 min) to collect the supernatant, which was used for MPO activity assays adapted to a 96-well plate format. Briefly, 50 μl of supernatant or human neutrophil MPO standard (Sigma-Aldrich) was added to a 96-well plate. The reaction was initiated by adding 150 μl phosphate buffer containing 0.167 mg/ml *o*-dianisidine (Sigma-Aldrich) and 0.0005 % hydrogen peroxide (Sigma-Aldrich), and absorption was measured 5 min later at 450 nm (Microplate Spectrophotometer PowerWave X, BioTek Instruments, Winooski, VT, USA).

In some experiments, WT and σ_1-KO mice were treated with cyclophosphamide (300 mg/kg i.p.), and 2 h later were given a dose (s.c.) of a σ_1-R antagonist (BD-1063, NE-100 or S1RA) or morphine. After 3 h (i.e., 5 h after cyclophosphamide injection) the urinary bladder was dissected out and MPO activity was measured according to the procedures described above.

2.6. Cyclophosphamide-evoked visceral pain and referred hyperalgesia

Spontaneous pain-related behaviors and referred mechanical hyperalgesia induced by cyclophosphamide were tested with a previously described protocol [23–25], with small modifications [21,22]. Mice were housed in individual transparent plastic boxes (7 × 7 × 13 cm) on an elevated platform with a wire mesh floor, and with small mirrors behind and below the chambers to facilitate observation. After a 40-min habituation period, the animals were removed from the compartments and the cyclophosphamide solution (or its solvent) was injected. The animals were immediately returned to the compartment, where they were observed for 2 min every half-hour during a 4-h observation period after cyclophosphamide injection. The recorded pain-related behaviors were coded according to the following scale: 0 = normal, 1 = piloerection, 2 = marked piloerection, 3 = labored breathing, 4 = licking the abdomen, and 5 = stretching and contracting the abdomen [24]. If more than one of these behaviors were noted in a given observation period, the sum of the points for each different type of behavior was recorded; i.e., if one abdominal licking (4 points) and two stretching and contraction (5 points each) episodes occurred during a given observation period, the final score was 9 instead of 14 points. An overall score was obtained by summing the scores for each time point. At the end of the 4-h observation period, referred hyperalgesia was determined by measuring the withdrawal response to a punctate mechanical stimulus on the abdomen. Forces ranging from 0.02 to 2 g (0.19–19.6 mN) were applied to the abdomen with a series of calibrated von Frey filaments (Touch-Test Sensory Evaluators; North Coast Medical Inc., Gilroy, CA, USA) using the up–down paradigm [31]. The perianal area and external genitalia were avoided, and stimulation was applied to the lower and mid-abdomen as reported previously [32]. The filaments were applied three times for 2–3 s each, with between-application intervals of 5 s. Testing was initiated with the 0.4 g (3.92 mN) von Frey filament (i.e., the middle of the range). In each consecutive test, if there was no response to the filament, a stronger stimulus was then selected; if there was a positive response, a weaker one was then used. The response to the filament was considered positive if immediate licking or scratching at the application site, sharp retraction of the abdomen, or jumping was observed.

To evaluate the effect of the drugs on cyclophosphamide-induced visceral pain, we tested the effects of σ_1-R antagonists and morphine, as control drug, on the pain behavioral score and referred hyperalgesia. Different doses of BD-1063 (16–64 mg/kg), S1RA (32–128 mg/kg), NE-100 (16–64 mg/kg), morphine (1–8 mg/kg) or their solvent were administered s.c. 2 h after the i.p. injection of cyclophosphamide, and the pain behavioral score was recorded for 2 min every 30 min during 2 h as described above. To test the effects of the drugs on pain-related behaviors, a dose of 300 mg/kg cyclophosphamide was administered. This dose of cyclophosphamide was selected because it was the lowest one that produced maximum pain scores in WT mice (see Results, Fig. 6A), and therefore offered an optimal window to observe any reductions in this response.

In separate experiments we tested the effect of the same doses of σ_1-R antagonists and the control drug on mechanical hyperalgesia induced

by cyclophosphamide and referred to the abdominal wall. A cyclophosphamide dose of 100 mg/kg was selected for these experiments because it produced maximal reductions in the mechanical threshold for referred hyperalgesia in both WT and σ_1-KO mice (see Results, Fig. 8). In these experiments, the drug under study or its solvent was injected s.c. 2 h after the i.p. administration of cyclophosphamide, and 2 h later (i.e., 4 h after cyclophosphamide injection) the responses to abdominal stimulation were tested with von Frey filaments (up–down method).

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

2.7. Statistical analysis

The degree of referred hyperalgesia, expressed as the mechanical threshold that produced 50 % of the responses, was calculated with Up-Down Reader software [33]. When several means were compared, statistical analysis was done with one-way or two-way analysis of variance (ANOVA) depending on the experiment, followed by Bonferroni's post-hoc test. When two means were compared, Student's *t* test for unpaired values was used. The differences between values after either ANOVA, Bonferroni's or Student's *t* test were considered significant when the *p* value was below 0.05. All statistical analyses were done with the SigmaPlot 12.0 program (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Expression of σ_1-R in human and mouse urinary bladder

To determine the presence and localization of σ_1-R in the urinary bladder, we used immunostaining for σ_1-R in bladder sections of mouse and human origin. Immunohistochemical experiments in WT mouse urinary bladder sections detected σ_1-R immunoreactivity in the urothelium. Immunostaining was distributed throughout the cytoplasm of the different layers of urothelial cells (Fig. 1A). The specificity of immunostaining was demonstrated in two ways. First, we performed the same experiments in urinary bladder sections from σ_1-KO mice and found no immunostaining (Fig. 1B), which indicated that the σ_1-R antibody used here reacted exclusively with σ_1-R. Moreover, no σ_1-R immunoreactivity was found when urinary bladder sections from WT mice were incubated with only the secondary antibody (i.e. the primary σ_1-R antibody was omitted) (data not shown).

Experiments in human urinary bladder sections showed σ_1-R immunostaining in the same types of urothelial cells as in mouse bladder sections (compare Figs. 1C and 1A). No immunostaining was detected in human urinary bladder sections when the σ_1-R primary antibody was omitted (Fig. 1D).

To study whether cyclophosphamide treatment modified the expression of urinary bladder σ_1-R, we performed western blot experiments in bladder homogenates obtained from control mice and mice treated with a high dose of cyclophosphamide (300 mg/kg i.p.) before the bladders were removed. Western blot experiments with the σ_1-R antibody identified a band with a molecular weight slightly higher than 25 kDa in the urinary bladder of control WT mice (Fig. 2A). The bladders of cyclophosphamide-treated WT mice also showed similar σ_1-R bands (Fig. 2A). When σ_1-R band intensity was measured, it was found that cyclophosphamide treatment did not affect σ_1-R expression in the urinary bladder of WT mice at any time point evaluated (Fig. 2B).

Similar experiments were performed in σ_1-KO mouse urinary bladder homogenates, but no σ_1-R bands were found in either naïve or cyclophosphamide-treated animals (Figs. 2A and 2B). As expected, the loading control band (GAPDH) was expressed similarly in WT and σ_1-KO mice and in both experimental conditions (control and cyclophosphamide-treated animals) (Fig. 2A), which indicated that the differences in σ_1-R expression between WT and σ_1-KO mice were not due to experimental artefact.

In summary, we found that σ_1-R was expressed in the urothelium of both the human and mouse urinary bladder, and that cyclophosphamide treatment in mice did not modify σ_1-R expression in this tissue.

3.2. Cyclophosphamide-induced enhancement of myeloperoxidase activity and pERK1/2 expression in WT and σ_1-KO mice

To identify the functional relevance of urinary bladder σ_1-R, we compared the characteristics of two different biochemical alterations induced by cyclophosphamide in the bladder of WT and σ_1-KO mice. Cyclophosphamide treatment (10–300 mg/kg i.p.) produced a dose-dependent increase in MPO activity in the urinary bladder of WT mice (Fig. 3). Cyclophosphamide treatment also increases MPO activity in the bladder of σ_1-KO animals, but the increase was significantly lower in these animals than in WT mice (Fig. 3). The maximum difference in the effect of cyclophosphamide between WT and σ_1-KO mice was observed at 300 mg/kg (Fig. 3); therefore, this dose of cyclophosphamide was chosen for subsequent experiments.

We also compared cyclophosphamide-induced pERK1/2 expression in WT and σ_1-KO mice as an additional marker of the functional role of urinary bladder σ_1-R. As expected, western blot experiments showed that cyclophosphamide administration did not change bladder ERK1/2 levels in either WT or σ_1-KO mice (Fig. 4A), but produced a time-dependent increase in pERK1/2 expression in the bladder, particularly in WT mice (Fig. 4B). When pERK1/2 levels were quantified, it was found that the cyclophosphamide-induced increase in pERK1/2 levels was significantly higher in WT than in σ_1-KO animals at 3 and 5 h after drug administration (Fig. 4C).

In summary, the two biochemical alterations (increases in MPO and pERK1/2) induced by cyclophosphamide treatment in the urinary bladder were attenuated in σ_1-KO mice compared to WT mice.

3.3. Cyclophosphamide-induced changes in urinary bladder histology in WT and σ_1-KO mice

Hematoxylin–eosin staining in urinary bladder sections from control WT mice showed a normal appearance, with the different layers (muscularis, lamina propria and urothelium) being easily identifiable (Fig. 5A, left panel). The appearance of control WT mice sections was indistinguishable from that of control σ_1-KO mice.

Cyclophosphamide treatment produced marked histological alterations in the bladder of WT mice (at 5 h), with edema localized in the lamina propria (between the urothelium and muscularis), which was accompanied by areas of urothelial desquamation and hemorrhagic foci (Figs. 5A, central panel and 5C, left panel). Cyclophosphamide-induced histological alterations were attenuated in σ_1-KO mice (Fig. 5A, right panel). In particular, when the main manifestations of cystitis were quantified, it was found that σ_1-KO mouse urinary bladders showed a reduction in the area affected by edema (Fig. 5B), as well as fewer areas of urothelial desquamation and fewer hemorrhagic foci (Fig. 5C, right panel).

3.4. Effect of genetic and pharmacological blockade of σ_1-R on cyclophosphamide-induced pain and referred mechanical hyperalgesia. Comparison with morphine

Cyclophosphamide treatment produced pain behaviors in a dose-dependent manner in both WT and σ_1-KO animals (Fig. 6A). However, the pain behavioral score was higher in WT than in σ_1-KO animals, and

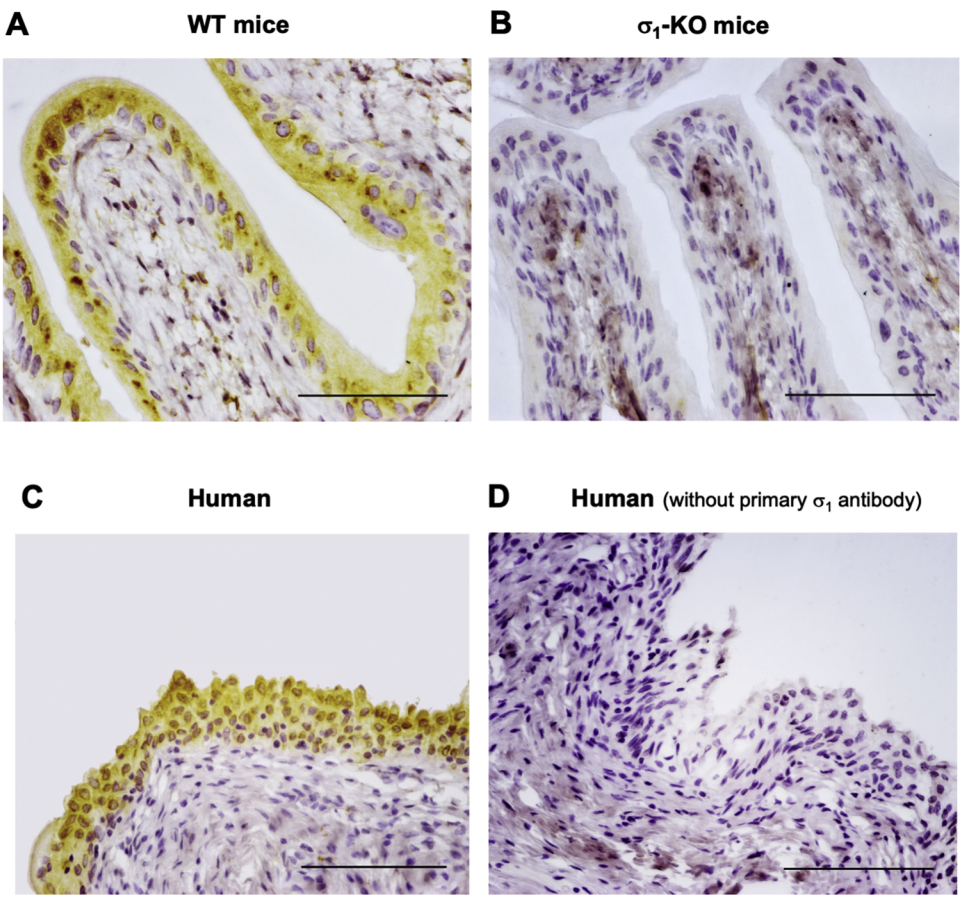

Fig. 1. Expression of σ_1 receptor in the human and mouse urinary bladder. Immunostaining for σ_1 receptors (brown) was found in the urothelium of wild-type (WT) mice (**A**), but not in σ_1 receptor knockout (σ_1-KO) mice (**B**). Immunostaining was also found in human urinary bladder sections incubated with the σ_1 receptor antibody (**C**), but not when the primary σ_1 antibody was omitted (**D**). The σ_1 receptor antibody was identified with the Ultra Vision Quanto™ peroxidase kit after heat-induced antigenic unmasking and endogenous peroxidase blockade. Sections were counterstained with Mayer's hematoxylin. Scale bar 100 μm.

was significantly higher in WT animals at doses of 30 mg/kg and greater. In both groups of animals the highest pain behavioral scores were seen at the highest dose of cyclophosphamide tested (300 mg/kg), but the score was significantly lower in σ_1-KO (20.33 ± 0.59) than in WT (34.83 ± 2.56) mice (Fig. 6A).

When the time course of pain induced by the highest dose of cyclophosphamide tested (300 mg/kg i.p.) was analyzed, it was found that cyclophosphamide injection produced a progressive increase in the pain behavioral score after longer times, and the differences with respect to solvent-treated animals became significant after 90 min (Fig. 6B). Cyclophosphamide-induced pain also occurred in σ_1-KO mice, and reached a peak at 120 min. After this time the behavioral score in σ_1-KO mice showed no further increase, and remained significantly lower than in WT animals (Fig. 6B).

Because the maximum pain behavioral score was seen in animals treated with 300 mg/kg cyclophosphamide i.p. (Fig. 6A), this dose was chosen to test the effects of σ_1 antagonists on cyclophosphamide-induced pain. All σ_1 antagonists tested (BD-1063, S1RA and NE-100), injected 120 min after cyclophosphamide (i.e. when pain behavior was clearly established (Fig. 6B), produced a dose-dependent decrease in the pain behavioral score in WT animals, but no change in σ_1-KO mice (Fig. 7A). None of the σ_1 antagonists abolished pain-related behaviors in WT mice; however, at the highest doses tested, all of them reduced the number of behaviors in WT mice to the same number observed in cyclophosphamide-treated σ_1-KO mice (Fig. 7A). In contrast, the s.c. administration of morphine (1–8 mg/kg) dose-dependently reduced the pain behavioral score elicited by cyclophosphamide (300 mg/kg i.p.), and almost completely inhibited these behaviors at the highest dose tested in both WT and σ_1-KO mice (Fig. 7B).

Cyclophosphamide treatment (10–300 mg/kg) also produced dose-dependent mechanical hyperalgesia referred to the abdominal wall in both WT and σ_1-KO mice (Fig. 8). The reduction in the mechanical pain threshold induced by cyclophosphamide was nonsignificantly different between WT and σ_1-KO mice, and the lowest threshold was seen at a dose of 100 mg/kg in both genotypes (Fig. 8); we therefore used this dose for subsequent pharmacological experiments.

The referred mechanical hyperalgesia induced by cyclophosphamide (100 mg/kg i.p.) was dose-dependently inhibited by the administration of σ_1 antagonists in WT mice (Fig. 9A). The highest doses of all σ_1 antagonists tested (BD-1063, S1RA and NE-100) in WT mice produced mechanical threshold values that were close to the thresholds obtained in cyclophosphamide solvent-treated animals (0.83 ± 0.03 g) (Fig. 9A). In contrast, all three σ_1-R antagonists were devoid of effect in σ_1-KO animals (Fig. 9A). The administration of morphine (0.5–4 mg/kg s.c.) dose-dependently reversed the referred mechanical hyperalgesia produced by cyclophosphamide (100 mg/kg i.p.) in WT animals (Fig. 9B). Interestingly, morphine treatment not only fully reversed mechanical hyperalgesia but also produced an analgesic effect (mechanical threshold values above those in cyclophosphamide solvent-treated animals) at doses greater than 4 mg/kg in WT mice (Fig. 9B). Morphine also dose-dependently inhibited cyclophosphamide-induced hyperalgesia and produced analgesia in σ_1-KO mice (Fig. 9B), although the dose-response curve was shifted to the left in these animals in comparison to the curve obtained in WT mice (Fig. 9B).

3.5. Effect of treatment with σ_1-R antagonists and morphine on cyclophosphamide-induced enhancement of myeloperoxidase activity

To determine whether drug treatment modified the enhancement of MPO activity induced by cyclophosphamide (300 mg/kg) in the urinary bladder, we tested the effect of σ_1-R antagonists and morphine on this variable. Treatment of WT animals with BD-1063, S1RA or NE-100 (64, 128 and 64 mg/kg, respectively) produced a significant reduction in the cyclophosphamide-induced increase in MPO activity in WT animals,

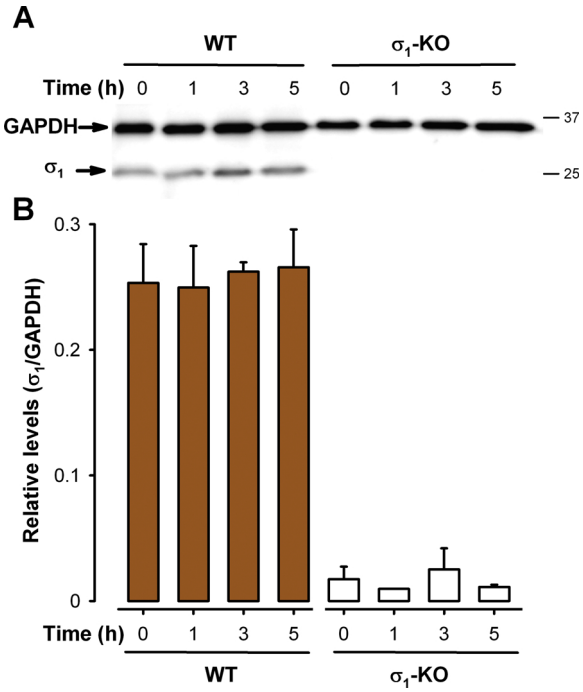

Fig. 2. Expression of σ_1 receptor in the urinary bladder of control and cyclophosphamide-treated wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. (**A**) Representative immunoblots for σ_1 receptor detected in the urinary bladder of control (0), WT and σ_1-KO mice, and at 1, 3 or 5 h (1, 3, 5) after treatment with cyclophosphamide (300 mg/kg i.p.) in both groups of experimental animals. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. The migration positions of molecular mass standards (in kDa) are shown at the right of the gels. (**B**) Quantification of immunoblotting for σ_1 receptor in WT and σ_1-KO mice. Each bar and vertical line represent the mean ± SEM (standard error of the mean) of the densitometric values obtained in 8 animals. The σ_1 receptor band intensities were relativized to those of their corresponding GAPDH loading control bands. Note that no σ_1 receptor expression was found in samples from σ_1-KO mice.

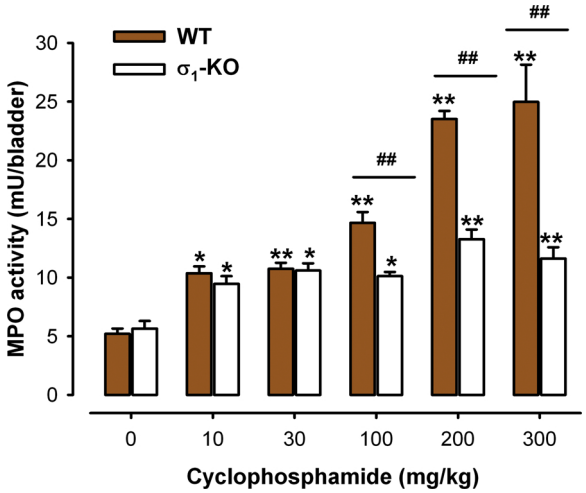

Fig. 3. Myeloperoxidase activity (MPO) in the urinary bladder of control and cyclophosphamide-treated wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. Urinary bladder MPO activity was measured 5 h after the i.p. injection of cyclophosphamide (10–300 mg/kg) or its solvent (0). Each bar and vertical line represent the mean ± SEM of the values obtained in 5 to 7 animals. Statistically significant differences between the values obtained in cyclophosphamide-treated and control animals: $^{*}p < 0.05$, $^{**}p < 0.01$; and between the values obtained in WT and σ_1-KO mice at the same dose of cyclophosphamide: $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

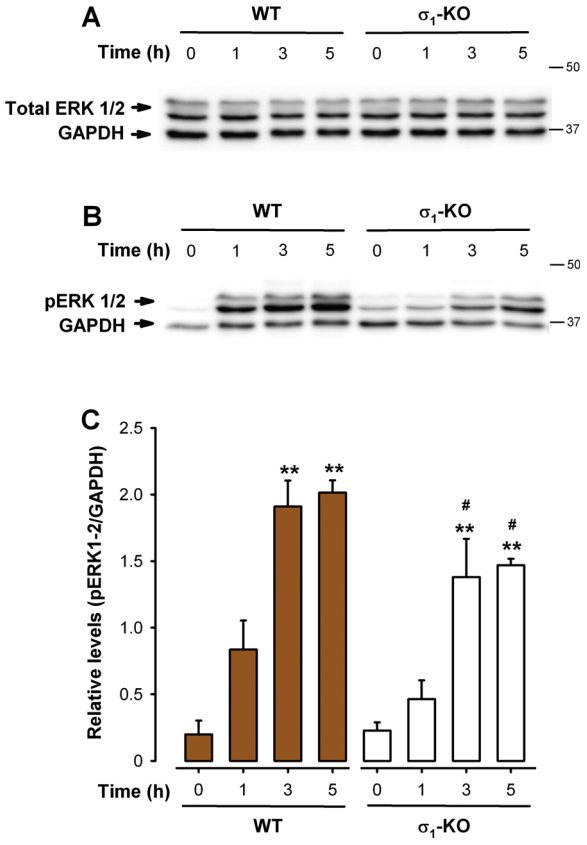

Fig. 4. Total extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphorylated ERK1/2 (pERK1/2) in the urinary bladder of control and cyclophosphamide-treated wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. (**A** and **B**) Representative immunoblots for urinary bladder total ERK1/2 and pERK1/2. In both cases, bladders were obtained from control animals (0) and at 1, 3 or 5 h (1, 3, 5) after treatment with cyclophosphamide (300 mg/kg i.p.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. The migration positions of molecular mass standards (in kDa) are shown at the right of the gels. (**C**) Quantification of immunoblotting for pERK1/2 in WT and σ_1-KO mice. The pERK1/2 band intensities were relativized to those of their corresponding loading control GAPDH bands. Each bar and vertical line represent the mean ± SEM of the values obtained in 5 to 7 animals. Statistically significant differences between the values obtained in cyclophosphamide-treated and control animals: $^{**}p < 0.01$; and between the values obtained in WT and σ_1-KO mice at different times after cyclophosphamide injection: $^{\#}p < 0.05$ (two-way ANOVA followed by Bonferroni test).

but was devoid of effect in σ_1-KO mice (Fig. 10A). In contrast, treatment with the dose of morphine (16 mg/kg s.c.) that produced maximum reversion of cyclophosphamide-induced pain behaviors and referred mechanical hyperalgesia did not significantly affect the cyclophosphamide-induced increase in MPO activity in either WT or σ_1-KO mice (Fig. 10B).

4. Discussion

This study reports, to the best of our knowledge, the first evidence of the presence of a new receptor (σ_1-R) in the human and mouse urinary bladder, and documents the functional role of this receptor in the biochemical, histopathological and behavioral manifestations of cyclophosphamide-induced cystitis by comparing responses in WT and σ_1-KO mice. Our findings also show that σ_1-R antagonists ameliorate the manifestations of cystitis, and therefore suggest that σ_1-R may represent a new drug target for the treatment of urological diseases.

We identified σ_1-R in the urinary bladder with western blotting and immunohistochemical techniques. The σ_1-R signal was absent in σ_1-KO

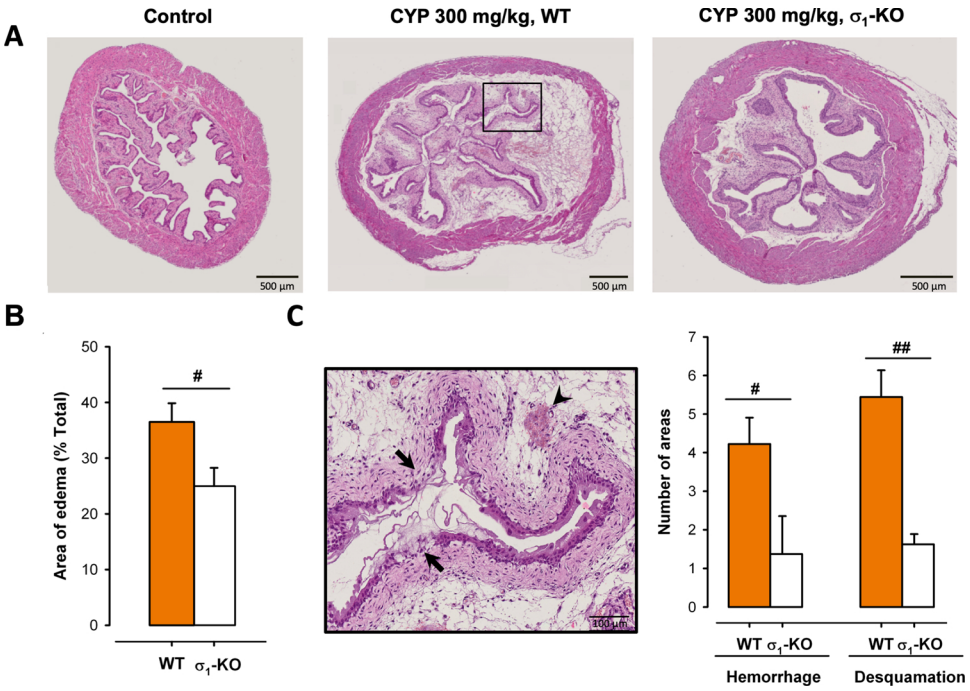

Fig. 5. Histological analysis of cyclophosphamide-induced urinary bladder alterations in wild-type (WT) and σ_1-knockout (σ_1-KO) mice. (**A**) Photomicrographs of hematoxylin–eosin-stained urinary bladders representative of solvent-treated (control) mice and cyclophosphamide-treated (300 mg/kg i.p., CYP 300) WT and σ_1-KO mice. Urinary bladders were obtained 5 h after treatment. Scale bar 500 µm. (**B**) Quantification of edema located in the lamina propria (relativized to the total area of the urinary bladder section) in cyclophosphamide-treated WT and σ_1-KO mice. (**C**) (Left) Detail from **A** (CYP 300, WT mice) showing an area of hemorrhage (arrowhead) and urothelial desquamation (arrow). Scale bar 100 µm. (Right) Number of areas of hemorrhage and urothelial desquamation in bladders of WT and σ_1-KO mice treated with cyclophosphamide. (**B** and **C**) Each bar and vertical line represent the mean ± SEM of values obtained in 8 animals. Statistically significant differences between the values obtained in WT and σ_1-KO animals: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ (Student's *t* test).

animals, which indicates that it was specifically due to antibody binding to the σ_1-R protein in both techniques. The western blot results are fully consistent with a previous study in nervous tissue [15], and identified a band with a molecular weight (25 kDa) that matches the expected molecular weight of the cloned σ_1-R [6]. This receptor was concentrated in the urothelium of the human and mouse urinary bladder. This tissue is important for bladder function, is affected in a number of bladder disorders, and expresses many receptors and channels similar to those in primary afferent sensory neurons [12]. Interestingly, σ_1-R is present at high densities in the dorsal root ganglia [13–15], which further supports this similarity.

Two previously identified biochemical markers of chemically induced cystitis are the enhancement of MPO activity [18,19,30,34] and of pERK1/2 expression [20,35] in the urinary bladder. We confirmed that cyclophosphamide treatment increased the levels of both markers in the WT mouse urinary bladder, and found that the increase was smaller in σ_1-KO mice, which suggests that σ_1-R is necessary for both biochemical changes. Previous studies reported that neuropathy-induced enhancement of spinal cord pERK1/2 expression was also reduced in σ_1-KO animals [9,36], which suggests that σ_1-R regulates ERK1/2 activation in several tissues and pathological pain models. The enhancement of urinary bladder MPO activity is an index of neutrophil infiltration [19,30,34], and this activity was reduced not only in σ_1-KO mice, but also in WT animals treated with σ_1-R antagonists. These results indirectly indicate that σ_1-R modulates neutrophil infiltration. This effect may be related to the ability of σ_1-R to regulate the expression of activated leucocyte cell adhesion molecule (ALCAM) [37], an immunoglobulin involved in the transendothelial migration of neutrophils [38]. Cyclophosphamide-induced cystitis increases the levels of cytokines TNFα and interleukin-1β in the urinary bladder [18]. Interestingly, previous studies in different models of non-visceral pain (bone cancer, spinal cord contusion and osteoarthritis) reported that σ_1-R antagonism inhibited TNFα and interleukin-1β production in the spinal cord [39–41]. These findings suggest the hypothesis that at least part of the effects of σ_1-R antagonism on cyclophosphamide-induced cystitis may also be related to the inhibition of cytokine production in the bladder. Further studies should be done to confirm or rule out this hypothesis.

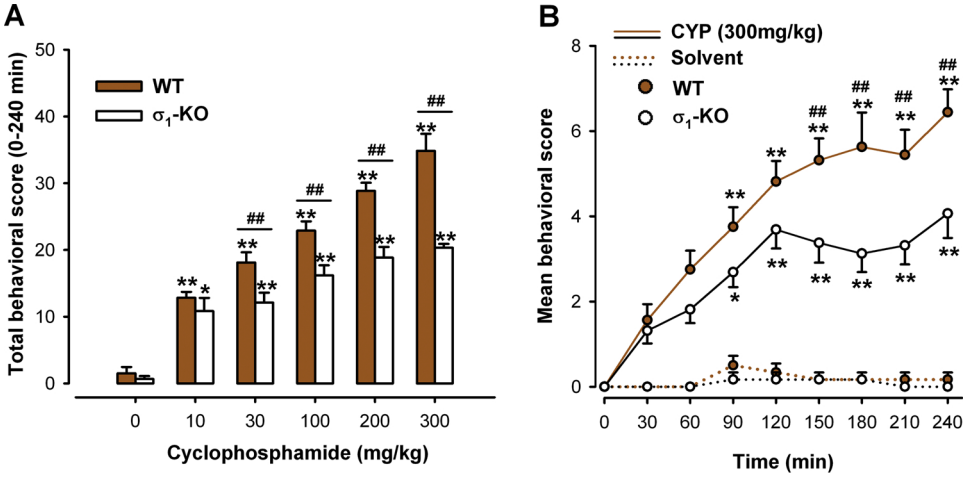

Fig. 6. Pain-related behaviors induced by cyclophosphamide in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. (**A**) Values of total pain behavioral scores at different doses of cyclophosphamide (10–300 mg/kg i.p.) or its solvent (0) in WT and σ_1-KO mice. (**B**) Time course of pain-related behavioral scores after the i.p. administration of cyclophosphamide (CYP 300 mg/kg) or its solvent. Pain-related behavioral responses were recorded at 30-min intervals during the 4-h observation period after the injection of cyclophosphamide or its solvent. (**A** and **B**) Each point or bar and vertical line represent the mean ± SEM of values obtained in 10 to 12 animals. Statistically significant differences between the values obtained in cyclophosphamide- and solvent-treated animals: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in WT and σ_1-KO animals: $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

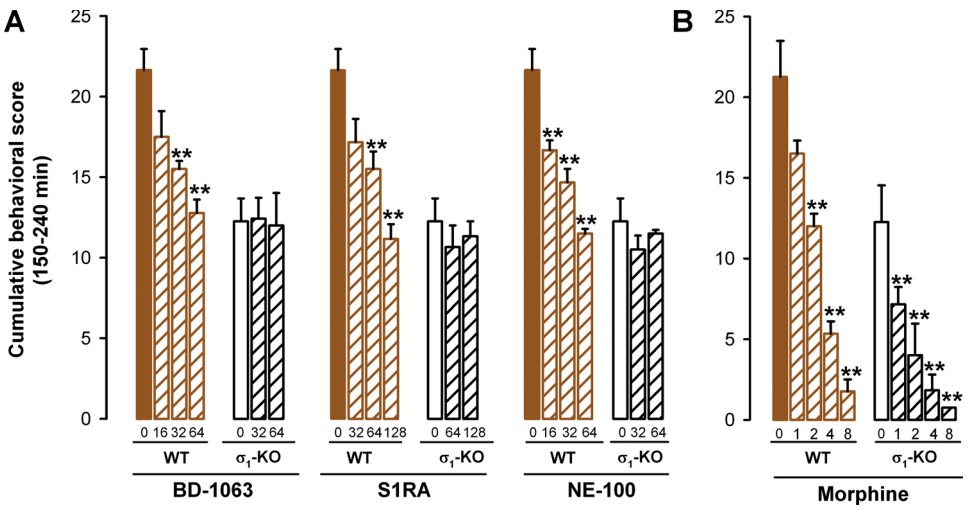

Fig. 7. Effects of treatment with σ_1 receptor antagonists (BD-1063, S1RA or NE-100) or morphine on pain-related behaviors evoked by cyclophosphamide in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The σ_1 receptor antagonist (**A**), morphine (**B**), or their solvent (saline, 0) was injected s.c. 120 min after the i.p. administration of cyclophosphamide (300 mg/kg), i.e. when the pain behavior was clearly established. Pain-related behavioral responses were recorded at 30-min intervals during the 150- to 240-min observation period after cyclophosphamide injection. Each bar and vertical line represent the mean ± SEM of values obtained in 10 to 12 animals. Statistically significant differences between the values obtained in mice treated with the drug or saline: $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test). Note that σ_1 receptor antagonists had no effect in σ_1-KO mice, whereas morphine reduced pain-related behaviors in both WT and σ_1-KO mice.

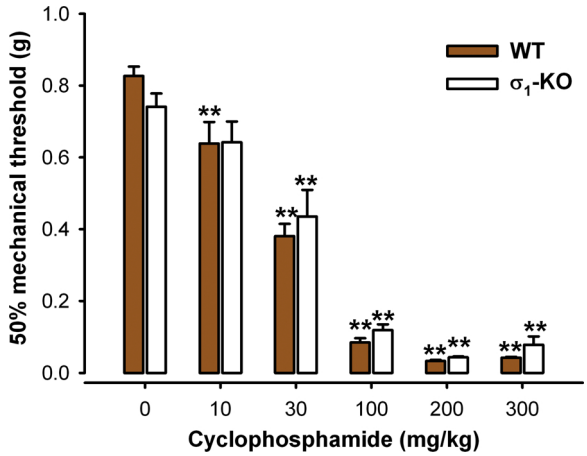

Fig. 8. Referred mechanical hyperalgesia induced in the abdominal wall by cyclophosphamide treatment in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments and expressed as the threshold force that produced 50 % of the responses) was measured 240 min after the i.p. administration of different doses of cyclophosphamide (10–300 mg/kg) or its solvent (0). Each bar and vertical line represent the mean ± SEM of values obtained in 10 to 12 animals. Statistically significant differences between the values obtained in cyclophosphamide- and solvent-treated animals: $^{**}p < 0.01$. No significant differences were found between the values obtained in WT and σ_1-KO mice (two-way ANOVA followed by Bonferroni test).

Cyclophosphamide-induced cystitis also produces profound changes in urinary bladder histology, with edema, hemorrhage and urothelial desquamation [18,19,34]. All of these changes were observed in WT mice but were attenuated in σ_1-KO animals, which further supports the functional relevance of urinary bladder σ_1-R in the modulation of cystitis manifestations. These results also offer an alternative explanation for the lower MPO increase produced by cyclophosphamide in σ_1-KO animals, which may be indirectly due to the less severe bladder lesions induced by cyclophosphamide in these animals, and the consequently lower neutrophil infiltration.

The inhibition of σ_1-R function by σ_1-R antagonists in WT mice or by σ_1-R genetic deletion in σ_1-KO mice reduced the behavioral manifestations of pain induced by cyclophosphamide. However, σ_1-R antagonists did not reverse the effect of cyclophosphamide in σ_1-KO mice, which indicates that the effect of these drugs was due to their specific interaction with σ_1-R. Previous studies found similar results with the pharmacological and genetic inhibition of σ_1-R function in models of chemically-induced visceral (intracolonic capsaicin) [32] and somatic (intraplantar formalin) pain [8,42]; however, the inhibition of σ_1-R function did not modify acute nociceptive pain induced by thermal or mechanical stimuli [26,36,43]. These results point to differences between the underlying mechanisms of pain depending on the type of painful stimulus, and support the need for additional studies to evaluate the role of σ_1-R function in models of urological pain induced by non-chemical stimuli. The acute administration of σ_1-R antagonists dose-dependently inhibited referred mechanical hyperalgesia in WT mice, but not in σ_1-KO animals. These results are consistent with those of previous studies of mechanical hypersensitivity in models of somatic [9–11,26,36,44] and visceral (intestinal) [32] pain, and suggest that σ_1-R plays a key role in pain-induced mechanical hypersensitivity. Cyclophosphamide-induced referred mechanical hyperalgesia is related to astrocytic activation in the spinal cord [45], and σ_1-R antagonists are known to inhibit this activation in models of neuropathic pain [44,46]. These findings suggest the hypothesis that the antihyperalgesic effect of σ_1-R antagonists reported here may be related to this phenomenon.

Interestingly, cyclophosphamide-induced referred hyperalgesia was similar in WT and σ_1-KO mice, which suggests that σ_1-KO animals develop compensatory mechanisms to express mechanical hypersensitivity. Previous studies in another model of visceral pain reported similar results [32]; however, the mechanical hypersensitivity observed in several models of somatic pain was reported to be markedly reduced or abolished in σ_1-KO mice [9,11,26,36,47], which suggests that the mechanisms underlying somatic and visceral mechanical hypersensitivity are different.

Pain and mechanical hypersensitivity are relevant in several human urinary bladder disorders [2,3,48,49]. Because σ_1-R antagonists inhibit the manifestations of cystitis in mice, and because σ_1-R are distributed similarly in the human and mouse urinary bladder, σ_1-R may offer a new drug target for the treatment of cystitis in humans. In particular, S1RA is a highly selective σ_1-R antagonist [29], is well tolerated in humans [50], and is able to reduce neuropathic pain induced by oxaliplatin in patients with colorectal cancer [51]. Therefore, it may be a potentially attractive drug for testing in human urinary bladder disorders for which no adequate treatment is currently available.

It was reported previously that morphine inhibits spontaneous pain-related behaviors and referred mechanical hyperalgesia, but does not reduce the inflammatory response in the urinary bladder induced by cyclophosphamide in rats [52,53]. Our results are consistent with these findings: we found a dose-dependent reduction in pain-related behaviors and referred mechanical hyperalgesia, but no effect on the

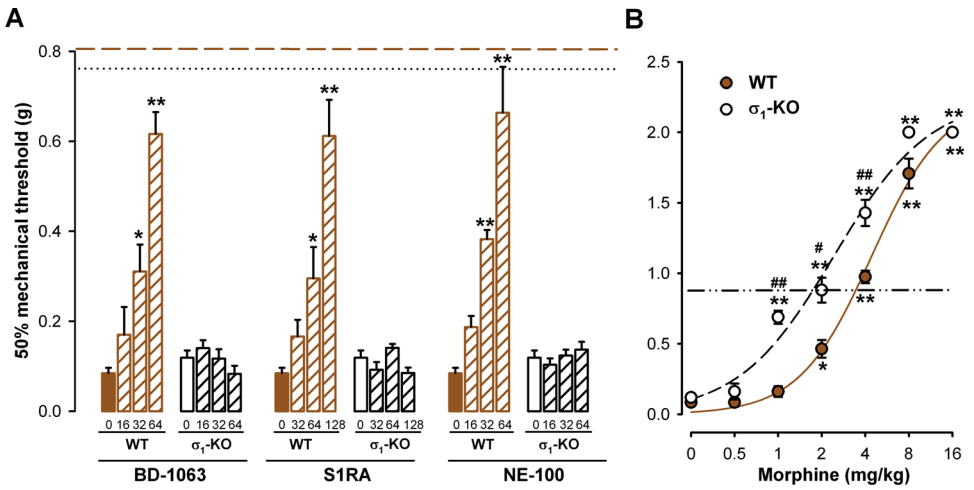

Fig. 9. Effects of treatment with σ_1 receptor antagonists (BD-1063, S1RA or NE-100) or morphine on referred mechanical hyperalgesia induced by cyclophosphamide in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The σ_1 receptor antagonist (**A**), morphine (**B**), or their solvent (saline, 0) was injected s.c. 120 min after the i.p. administration of cyclophosphamide (100 mg/kg) or its solvent. Referred mechanical hyperalgesia (evaluated by stimulation of the abdomen with von Frey filaments and expressed as the threshold force that produced 50 % of the responses) was measured 240 min after cyclophosphamide injection. Each bar and vertical line represent the mean ± SEM of values obtained in 10 to 12 animals. The dashed and dotted lines indicate the 50 % threshold force in solvent-treated WT and σ_1-KO mice, respectively. Statistically significant differences between the values obtained in mice treated with the drug or saline: $^{*}p < 0.05$; $^{**}p < 0.01$; and between the values obtained in WT and σ_1-KO animals at the same dose of morphine: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ (two-way ANOVA followed by Bonferroni test).

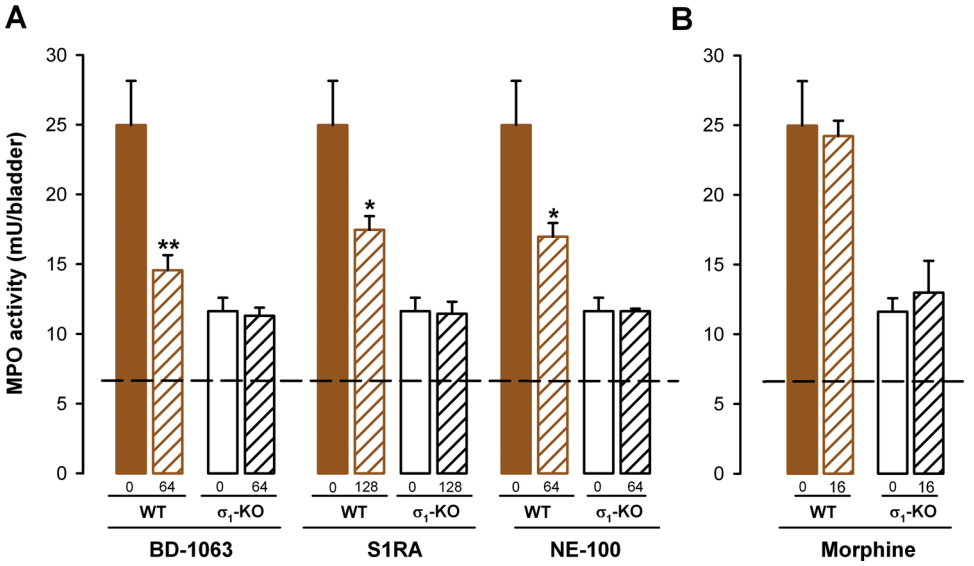

Fig. 10. Effects of treatment with σ_1 receptor antagonists (BD-1063, S1RA or NE-100) or morphine on myeloperoxidase activity (MPO) induced by cyclophosphamide in wild-type (WT) and σ_1 receptor knockout (σ_1-KO) mice. The σ_1 receptor antagonist (**A**), morphine (**B**), or their solvent (saline, 0) was injected s.c. 2 h after the i.p. administration of cyclophosphamide (300 mg/kg). The bladder was removed 5 h after cyclophosphamide injection. Each bar and vertical line represent the mean ± SEM of values obtained in 5 to 7 animals. The dashed line indicates the MPO activity in naïve animals (no injection). Statistically significant differences between the values obtained in mice treated with the drug or saline: $^{*}p < 0.05$; $^{**}p < 0.01$ (one-way ANOVA followed by Bonferroni test).

increase in urinary bladder MPO induced by cyclophosphamide in mice. Taken together, these results suggest that morphine produces an analgesic effect without reducing urinary bladder inflammation in rodent models of cystitis. The inhibition by morphine of cyclophosphamide-induced pain and mechanical hypersensitivity was greater in σ_1-KO than in WT mice. These results are consistent with those of previous studies in models of somatic and visceral pain [14,15,32,42,54]. Interestingly, it has been shown that the intravesical administration of morphine, at doses that do not produce a relevant concentration of this opioid in blood, induces analgesia after bladder surgery in humans [55], which suggest that it exerts this effect through peripheral opioid receptors located in the urinary bladder. We previously demonstrated that peripheral σ_1-R modulates the peripheral analgesic effects of μ-opioid agonists in models of somatic pain [14,15,43]; therefore it could be hypothesized that a similar mechanism may be involved in the potentiation of morphine analgesia in the model of cyclophosphamide-induced cystitis tested here. Further studies are necessary to test this hypothesis. The inhibition of σ_1-R function increases the analgesic effects of morphine and other opioid agonists without increasing their side effects [15,42,54,56]. Because opioid agonists are used to treat several urological disorders [57–59], it is tempting to hypothesize that the association of σ_1-R antagonists to opioid agonists might offer a potentially interesting approach for the treatment of these disorders. In this connection, unpublished results from our group showed that σ_1-R antagonists increased the analgesia induced not only by morphine but also by other opioids in cyclophosphamide-induced cystitis in mice (manuscript in preparation).

In summary, this study shows that σ_1-R is present in the human and mouse urinary bladder urothelium, and demonstrates that σ_1-R plays a functional role in the mechanisms underlying cyclophosphamide-induced cystitis in mice. We suggest that σ_1-R may represent a new drug target for the treatment of urological disorders.

Declaration of Competing Interest

M.M. is an employee of Esteve Pharmaceuticals. None of the authors have conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.104724.

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