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Pharmacological activation of 5-HT$_7$ receptors reduces nerve injury-induced mechanical and thermal hypersensitivity

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ABSTRACT

The involvement of the 5-HT$_7$ receptor in nociception and pain, particularly chronic pain (i.e., neuropathic pain), has been poorly investigated. In the present study, we examined whether the 5-HT$_7$ receptor participates in some modulatory control of nerve injury-evoked mechanical hypersensitivity and thermal (heat) hyperalgesia in mice. Activation of 5-HT$_7$ receptors by systemic administration of the selective 5-HT$_7$ receptor agonist AS-19 (1 and 10 mg/kg) exerted a clear-cut reduction of mechanical and thermal hypersensitivities that were reversed by co-administering the selective 5-HT$_7$ receptor antagonist SB-258719. Interestingly, blocking of 5-HT$_7$ receptors with SB-258719 (2.5 and 10 mg/kg) enhanced mechanical (but not thermal) hypersensitivity in nerve-injured mice and induced mechanical hypersensitivity in sham-operated mice. Effectiveness of the treatment with a 5-HT$_7$ receptor agonist was maintained after repeated systemic administration: no tolerance to the antiallodynic and antihyperalgesic effects was developed following treatment with the selective 5-HT$_7$ receptor agonist E-57431 (10 mg/kg) twice daily for 11 days. The 5-HT$_7$ receptor co-localized with GABAergic cells in the dorsal horn of the spinal cord, suggesting that the activation of spinal inhibitory GABAergic interneurons could contribute to the analgesic effects of 5-HT$_7$ receptor agonists. In addition, a significant increase of 5-HT$_7$ receptors was found by immunohistochemistry in the ipsilateral dorsal horn of the spinal cord after nerve injury, suggesting a "pain"-triggered regulation of receptor expression. These results support the idea that the 5-HT$_7$ receptor subtype is involved in the control of pain and point to a new potential use of 5-HT$_7$ receptor agonists for the treatment of neuropathic pain.

1. Introduction

The serotonin (5-hydroxytryptamine [5-HT]) system has been recognized as one of the main neurotransmitter systems participating in pain transmission, processing and controlling. Both pronociceptive and antinociceptive effects have been attributed to 5-HT depending on the site and the receptor subtype it acts on [12,19,30,38,47]. Among the seven 5-HT receptor families identified so far, much of the pain research has focused on 5-HT$_{1A}$, 5-HT$_{1B/1D}$, 5-HT$_{2A/2C}$ and 5-HT$_3$ receptors [1,7,12,18,19,32,33], but the role played by other 5-HT receptors in nociception has been poorly or not thoroughly investigated. This is the case of the last identified 5-HT receptor, the 5-HT$_7$ receptor [25,44].

At the periphery, 5-HT$_7$ receptors have been found in dorsal root ganglia (DRG) [11,29,36,37]. Interestingly, increased levels of 5-HT$_7$ receptor messenger RNA have been reported in rat DRG after bee venom- [23] and complete Freund's adjuvant-induced inflammation [49]. Regarding its role, the only two studies addressing this issue suggest a pronociceptive role of peripheral 5-HT$_7$ receptors: (1) intraplantar injection of the 5-HT$_7$ receptor antagonist SB-269970 reduced formalin-induced nociception whereas intraplantar administration of non-selective 5-HT$_7$ receptor agonists such as 5-HT itself and 5-carboxamidotryptamine (5-CT) increased formalin-induced nociceptive behavior [39]; (2) intra-articular injection of the mixed 5-HT$_{1A}$/5-HT$_7$ receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), induced c-Fos expression in the dorsal horn of the rat spinal cord and this effect was prevented by intra-articular administration of the non-selective 5-HT$_7$ receptor antagonist methiothepin [29].

In the CNS, the presence of 5-HT$_7$ receptors in the spinal cord and supraspinal areas has also been reported [11,28,29,31,46]. In the spinal cord, the 5-HT$_7$ receptor was mainly found in the superficial laminae of the dorsal horn, postsynaptically in local interneurons, presynaptically in peptidergic fibers (including presumably primary afferents) and in astrocytes [11,29]. Four studies support

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an antinociceptive role of spinal and supraspinal 5-HT_7 receptors: (1) intrathecal administration of the 5-HT_7 receptor antagonist SB-269970 inhibited the antinociceptive effect of systemic morphine in the tail-flick test [10]; (2) spinal administration of SB-269970 also blocked the antinociceptive effects of morphine when the opioid was microinjected into the rostroventromedial medulla (RVM) [9]; (3) intracerebroventricular administration of methiothepin blocked the antinociceptive effect of the non-steroidal anti-inflammatory *S*-(+)-ketoprofen [8]; (4) microinjection of 8-OH-DPAT into the medial thalamus exerted antinociceptive effects in the tailshock test that were reversed by intrathalamic administration of SB-269970 [16].

In summary, data supporting a role for 5-HT_7 receptors in nociception are scarce and come mostly from studies using non-selective ligands. Particularly remarkable is the absence of data in chronic pain (i.e., neuropathic pain). We showed previously that subcutaneous administration of 5-HT_7 receptor agonists crossing the blood–brain barrier (BBB) dose-dependently inhibited capsaicin-induced mechanical hypersensitivity in mice [3]. It was thus argued that, in sensitizing conditions, the overall effect of activating 5-HT_7 receptors is antinociceptive. In the present study, the effect of systemically administered selective, BBB-penetrant 5-HT_7 receptor ligands were investigated in mice subjected to sensitizing neuropathic pain conditions. The cellular localization of spinal 5-HT_7 receptors and possible changes in 5-HT_7 receptor expression in the spinal dorsal horn following nerve injury were also investigated.

2. Methods

2.1. Animals

Male, 6- to 8-week-old, CD1 mice (Harlan Iberica, Spain) were used in these studies. Animals were housed in groups of five, provided with food and water *ad libitum* and kept in controlled laboratory conditions with the temperature maintained at 21 ± 1 °C and light in 12 h cycles (on at 07:00 h and off at 19:00 h). Experimental behavioral testing was carried out in a soundproof and air-regulated experimental room and was done in blind respect to treatment and surgical procedure. All experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals [51] and to ethical guidelines of the European Community on the Care and Use of Laboratory Animals (European Communities Council Directive of 24 November 1986, 86/609/ECC) and received approval by the local Ethical Committee.

2.2. Drugs

Drugs used for treatments were AS-19 (dimethyl-[5-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)-1,2,3,4-tetrahydro-naphthalen-2(*S*)-yl]-amine) [17,41], SB-258719 (*N*,3-dimethyl-*N*-[1(*R*)-methyl-3-(4-methyl-1-piperidinyl)propyl]benzenesulfonamide hydrochloride) [12,22,35], and E-57431 (2-(2-(dimethylamino)ethyl)-4-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)phenol). Table 1 summarizes the affinity and selectivity of these drugs. AS-19 is a potent selective 5-HT_7 receptor agonist [3], SB-258719 is a potent selective 5-HT_7 receptor antagonist [13,26,40], and E-57431 is a new potent selective 5-HT_7 receptor agonist developed by Laboratorios Esteve and described herein for the first time (Table 1). AS-19 was purchased from Tocris Bioscience (UK) and SB-258719 and E-57431 were synthesized for the purpose of this study at Esteve. AS-19 was dissolved in 1% DMSO and E-57431 and SB-258719 in aqueous solutions (0.5% hydroxypropyl methyl cellulose, HPMC; and physiological saline, respectively). Compounds and vehicles were administered through the subcutaneous (s.c.) or intraperitoneal (i.p.) routes, in a volume of 5 or 10 ml/kg, respectively. When two compounds were co-administered, they were sequentially injected s.c. in opposite flanks of the mice, immediately one after the other.

Table 1
Binding profile of the 5-HT_7 receptor ligands AS-19, SB-258719 and E-57431.

Receptor	Affinity [K_i (nM)]		
	AS-19	SB-258719	E-57431
h5-HT_{1A}	89.7 (149.5×)	n.s.	n.s.
r5-HT_{1B}	490 (816.6×)	n.s.	n.s.
h5-HT_{1D}	6.6 (11×)	n.s.	53 (112.7×)
h5-HT_{2A}	n.s.	n.s.	560 (1191.5×)
h5-HT_{2B}	n.s.	n.s.	n.s.
h5-HT_{2C}	n.s.	n.s.	n.s.
h5-HT_3	n.s.	–	n.s.
h5-HT_{4e}	–	–	n.s.
gp5-HT_4	n.s.	n.s.	n.s.
h5-HT_{5A}	98.5 (164.2×)	–	n.s.
h5-HT_6	n.s.	n.s.	n.s.
h5-HT_7	0.60	31.6	0.47
h5-HT transporter (SERT)	n.s.	–	n.s
Other receptors	n.s.[a]	n.s.[b]	n.s.[c]

n.s., not significant (K_i > 1 μM or % inhibition at 1 μM < 50%); –, data not available. gp, Guinea pig; h, human; ha, hamster; m, mouse; r, rat; rb, rabbit.
Data obtained from CEREP and MDS Pharma Services (E-57431); Brenchat et al. [3] (AS-19); and Forbes et al. [13] (SB-258719).
Data in brackets following K_i values represent the affinity ratio vs. 5-HT7 receptors calculated as K_i for the tested receptor/K_i for 5-HT_7 receptor. It is expressed as number-fold higher (×) for 5-HT_7 than for the tested receptor.
[a] See in Brenchat et al. [3] the panel of other receptors assayed.
[b] See in Forbes et al. [13] the panel of other receptors assayed.
[c] The following panel of other receptors was assayed (MDS Pharma Services): A_1 (h), A_{2A} (h), A_3 (h), α_{1A} (r), α_{1B} (r), α_{1D} (h), α_{2A} (h), α_{2C} (h), β_1 (h), β_2 (h), β_3 (h), AM_1 (h), AM_2 (h), Aldosterone (r), Anaphylatoxin C5a (h), Androgen (Testosterone) AR (r), AT_1 (h), AT_2 (h), APJ (h), ANF (gp), BB_1 (h), BB_2 (h), BB_3 (h), B_1 (h), B_2 (h), Calcitonin (h), $CGRP_1$ (h), Ca^{2+} channel (L-Type benzothiazepine) (r), Ca^{2+} channel (L-Type dihydropyridine) (r), Ca^{2+} channel (L-Type phenylalkylamine) (r), Ca^{2+} channel (N-Type) (r), CB_1 (h), CCR2B (h), CCR4 (h), CCR5 (h), CX3CR1 (h), CXCR2 (IL-$8R_B$) (h), CCK_1 (h), CCK_2 (h), Colchicine (r), CRF_1 (h), D_1 (h), D_{2S} (h), D_3 (h), $D_{4.2}$ (h), D_5 (h), ET_A (h), ET_B (h), EGF (h), EPOR (h), ERα (h), ERβ (h), GPR103 (h), GPR8 (h), $GABA_A$ (chloride channel, TBOB) (r), $GABA_A$ (flunitrazepam, central) (r), $GABA_A$ (muscimol, central) (r), $GABA_{B1A}$ (h), $GABA_{B1B}$ (h), Gabapentin (r), GAL1 (h), GAL2 (h), Glucocorticoid (h), Glutamate (AMPA) (r), Glutamate (Kainate) (r), Glutamate (NMDA, agonism) (r), Glutamate (NMDA, glycine) (r), Glutamate (NMDA, phencyclidine) (r), Glutamate (NMDA, polyamine) (r), Glycine, strychnine-sensitive (r), GHS, Ghrelin (h), H_1 (h), H_2 (h), H_3 (h), H_4 (h), I_2 (r), IP_3 (r), Insulin (r), IL-2 (m), IL-6 (h), Leptin (m), Leucotriene (LTB_4) (h), Leucotriene ($CysLT_1$) (h), Leucotriene ($CysLT_2$) (h), MC_1 (h), MC_3 (h), MC_4 (h), MC_5 (h), MT_1 (h), MT_2 (h), Motilin (h), M_1 (h), M_2 (h), M_3 (h), M_4 (h), M_5 (h), NMU_1 (h), NMU_2 (h), NPY_1 (h), NPY_2 (h), NT_1 (h), FPR1 (h), FPRL1 (h), Ach. Nic. (h), Ach. Nic. α_1 (h), Ach. Nic. α7(r), δ opiate (OP1, DOP) (h), κ opiate (OP2, KOP) (h), μ opiate (OP3, MOP) (h), ORL_1 (h), Phorbol ester (m), PAF (h) PDGF (m), K^+ channel (K_A) (r), K^+ channel (K_{ATP}) (ha), K^+ channel (SK_{CA}) (r), K^+ channel HERG (h), PR-B (h), CRTH2 (h), DP (h), EP_2 (h), EP_4 (h), Prostanoid, Thromboxane A_2 (TP) (h), P_{2X} (rb), P_{2Y} (r), RXRα (h), Rolipram (r), RyR3 (r), σ1 (h), σ2 (r), Na^+ channel (site 2) (r), SST1 (h), SST2 (h), SST3 (h), SST4 (h), SST5 (h), NK_1 (h), NK_2 (h), NK_3 (h), Thyroid Hormone (r), TRH (r), TGF-β (m), Adenosine transporter (gp), Choline transporter (r), Dopamine (DAT) transporter (h), GABA transporter (r), Monoamine transporter (rb), Norepinephrine transporter (NET) (h), TNF (non-selective) (h), Urotensin II (h), Vanilloid (r), VIP_1 (h), V_{1A} (h), V_{1B} (h), V_2 (h), Vitamin D_3 (h).

2.3. Binding profile and cAMP measurements

Binding affinities of E-57431 were determined by commercial radioligand binding assays by MDS Pharma and CEREP (see Table 1), according to their standard assay protocols (for details of the experimental conditions see http://discovery.mdsps.com/Catalog/OnlineCatalog/Profiling/Assays/AssayList.aspx?id=5 and http://www.cerep.fr/Cerep/Users/pages/catalog/binding/catalog.asp).

For the determination of efficacy and potency of the 5-HT_7 receptor agonist, cAMP measurements were performed using a system based on homogeneous time-resolved fluorescence (HTRF)

applied to human embryonic kidney (HEK)-293F cells that stably express the human 5-$HT_{7(a)}$ receptor, as previously described [3]. The HTRF cAMP kit from CisBio (CisBio Int., France) was used according to the instructions of the manufacturer. Briefly, after overnight incubation in serum-free medium, cells were added to 96-well plates (20,000 cells/well) in Ham's F12 (Gibco, Invitrogen Co., Spain) incubation buffer (40 μl/well) containing 1 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma–Aldrich Co., Spain) and 20 μM pargyline (Sigma–Aldrich Co.). Then, 10 μl of different concentrations of E-57431 was added, and the plates were incubated for 30 min at 37 °C. The reaction was stopped by using a mixture of 25 μl of cryptate and 25 μl of XL-665 prepared in the lysis buffer supplied by the manufacturer. Plates were then incubated for an additional hour at room temperature, and cAMP contents were calculated from the 665 nm/620 nm ratio using a RubyStar Plate reader (BMG LabTech, Germany).

2.4. Neuropathic pain model: partial sciatic nerve ligation

The partial sciatic nerve ligation model was used to induce neuropathic pain, according to the method previously described [27,43]. This model consists of partial injury to the sciatic nerve at mid-thigh level. Surgery was performed under isoflurane (Iso-Flo®, Abbott-Laboratorios Esteve, Barcelona, Spain) anesthesia (induction: 3%; surgery: 2%). Briefly, mice were anaesthetized and the common sciatic nerve was exposed at the level of the mid-thigh of the right hindpaw. Partial nerve injury was produced at about 1 cm proximal to the nerve trifurcation by tying a tight ligature around approximately 33–50% of the diameter of the sciatic nerve using 9-0 non-absorbable virgin silk suture (Alcon surgical, USA). Ligature enclosed the outer 1/3–1/2 sciatic nerve whereas the rest of the nerve (inner 2/3–1/2) was leaved "uninjured". The muscle was then stitched with 6-0 silk suture and the skin incision was closed with wound clips. Control, sham-operated, mice underwent the same surgical procedure and the sciatic nerve was exposed, but not ligated.

2.5. Nociceptive behavioral tests

Mechanical hypersensitivity and thermal hyperalgesia were used as outcome measures of neuropathic pain and as indicators of the possible antinociceptive effect of treatments.

2.5.1. Evaluation of mechanical hypersensitivity

Acute administration. Mechanical hypersensitivity was quantified by determining the pressure threshold eliciting withdrawal of the ipsilateral hindpaw in response to stimulation with a von Frey filament applied onto the plantar surface (dynamic plantar aesthesiometer; Ugo Basile, Comerio, Italy) [20]. The electronic von Frey device applied a single non-flexible filament (0.5 mm in diameter) with increasing force (0.1 g/s; from 0 to 5 g) against the plantar surface over a 50-s period. The nocifensive paw withdrawal response automatically turned off the stimulus, and the pressure eliciting the response was recorded. For measurements, mice were placed individually into compartment enclosures in a test chamber with a framed metal mesh floor and allowed to acclimate for 1 h before testing. Paw withdrawal thresholds were measured in triplicate for each animal, allowing at least 30 s intervals between successive measurements.

Repeated administration. Mechanical hypersensitivity (allodynia) was quantified by measuring the hindpaw withdrawal response to manual von Frey filament stimulation [6]. Briefly, animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which the von Frey monofilaments (bending force range from 0.008 to 2 g) (North Coast Medical, Inc., San Jose, CA, USA) were applied and thresholds were measured using the up–down paradigm. The filament of 0.4 g was used at first. Then, the strength of the next filament was decreased when the animal responded or was increased when the animal did not respond. This up–down procedure was stopped four measures after the first change in animal responding. The threshold of response was calculated by using the up–down Excel spreadsheet generously provided by Basbaum's laboratory (UCSF, San Francisco, USA). Clear paw withdrawal, shaking or licking was considered as a nociceptive-like response.

2.5.2. Evaluation of thermal hyperalgesia

Thermal hyperalgesia was assessed, in both acute and repeated administration experiments, using the plantar test by determination of the hindpaw withdrawal latency in response to a thermal stimulus (radiant heat) [15]. On the day of the test, mice were placed into plastic compartment enclosures on the glass surface of the plantar test device (Ugo Basile) and allowed to acclimate to their environment for 1 h before testing. The heat source, a mobile infrared photobeam, was then applied onto the plantar surface of the right hindpaw and latency time for withdrawal from the thermal stimulus was automatically determined. Response latency was defined as the time from the onset of exposure to the cessation of the photobeam when the photodiode motion sensor detected the withdrawal of the paw. The intensity of the infrared photobeam was adjusted based on previous studies to produce baseline response latencies ranging between 8 and 12 s in control untreated mice. A cut-off time of 20 s was imposed to prevent tissue damage in the absence of response. Paw withdrawal latencies were measured in triplicate for each animal, with at least one minute interval between successive measurements.

2.5.3. Experimental approach

Acute administration. The effectiveness of acute treatments with 5-HT_7 receptor ligands (AS-19 and SB-258719) on neuropathic pain-related behaviors as well as pharmacological reversion of the effects was investigated in independent groups of nerve-injured ($n = 12$) and sham-operated ($n = 12$) mice using either automatic von Frey or plantar test, depending on the experiment. Mice were habituated to the environment of each experimental test during 2 days. After the habituation period, responses in the test were established during 2 consecutive days to obtain pre-surgery baseline values. One day after baseline measurements, surgery to generate nerve injury or sham operation was carried out. Post-surgery responses of mice treated with vehicle were obtained on day 10 after surgery. On days 11–13 post-surgery, mice were treated s.c. with either three different doses (0.1, 1 and 10 mg/kg) of a 5-HT_7 receptor ligand (the agonist AS-19 or the antagonist SB-258719) following a *Latin square* design or with single compounds (day 11 the antagonist, and day 12 the agonist) followed by the combination of the two compounds (day 13) in reversion experiments. Finally, on day 14 after surgery, mice were administered with vehicle and responses (post-treatment values) were evaluated as an internal control to ensure that mechanical hypersensitivity and thermal hyperalgesia were not influenced by previous treatments. Behavioral evaluation was always performed 30 min after treatment with either vehicle or 5-HT_7 receptor ligands.

Repeated administration. The effectiveness of repeated administration of the 5-HT_7 receptor agonist E-57431 on the development of neuropathic pain-related behaviors was investigated in nerve-injured ($n = 24$; 12 receiving drug treatment and 12 vehicle) and sham-operated ($n = 24$; 12 receiving drug treatment and 12 vehicle) mice using manual von Frey and plantar tests. After the habituation period, baseline pre-surgery responses were established during two consecutive days for each test in the following sequence: von Frey test and plantar test (15 min interval

between each test). One day after baseline measurements, sciatic nerve injury or sham operation was induced. Treatment with vehicle or E-57431 (10 mg/kg) started the day of surgery (day 0) and was maintained for a period of 11 days. Treatments were administered by i.p. route twice daily (9:00 and 19:00 h). Animals were tested 30 min after the morning administration on days 3, 6 and 10 after the surgical procedure. On days 12, 15 and 20 after surgery all mice received vehicle and were tested using the same sequence (30 min von Frey test and 45 min plantar test) to know if post-treatment values of mechanical allodynia and thermal hyperalgesia were influenced by previous repeated treatments.

2.6. Rotarod motor coordination test

To investigate the possibility that treatments could affect motor coordination and thus the responsiveness of mice in the nociceptive behavioral tests, the motor performance of mice treated with 5-HT_7 receptor agonists (AS-19 or E-57431) or vehicle ($n = 10$ per group) was assessed by means of an automated rotarod (Panlab SL, Barcelona, Spain). Briefly, mice were required to walk against the motion of an elevated rotating drum at 10 rpm and the latency to fall down was recorded automatically. Before drug treatments, mice were trained and animals that were unable to stay moving on the rod for 240 s were discarded for the study. With the selected animals, rotarod latencies were measured 30, 60, 120 and 180 min after the i.p. administration of compounds or vehicle.

2.7. Immunohistochemistry

2.7.1. Antibodies

The antibody used to identify 5-HT_7 receptors is commercially available (formerly DiaSorin, Antony, France; now commercialized by ImmunoStar Inc., Hudson, USA, Cat. No. 24430). This antibody is an affinity-purified rabbit polyclonal antiserum specific for amino acids 8–23 of rat 5-HT_7 receptor, a sequence producing no significant alignments with other 5-HT receptors or non-related proteins (Protein Blast NCBI). The specificity of the antibody was tested by several methods, including Western blot and immunocytochemistry [4,31]. The antibody labeled cells transfected with the 5-HT_7 receptor gene but not untransfected ones. Western blot analysis has shown that a single band of 70 kDa, compatible with the size of the receptor, is labeled [4]. The immunolabeling was absent in transfected cells after preabsorption with the immunogen [31], and in tissue sections from rat spinal cord [11] and brain [31] pre-incubated with the immunogen. Finally, intraventricular injection of 8-OH-DPAT (mixed 5-HT_{1A}/5-HT_7 agonist) together with a specific 5-HT_{1A} antagonist induced c-fos expression only in cell bodies immunoreactive for this anti-5-HT_7 receptor antibody [31].

A commercially available mouse monoclonal antibody to GABA (clone GB-69; Sigma–Aldrich Co., Cat. No. A0310) was used for double 5-HT_7 receptor/GABA immunohistochemistry. The characterization and specificity of the monoclonal GABA antibody have been described by the provider (see product Datasheet). Cross-reactivity with glutamate was discarded based on the finding that immunolabeling of neurons was abolished by preabsorption of the antibody with a GABA–BSA conjugate, while pre-incubation with an L-glutamate-conjugate did not interfere with normal labeling [22]. In addition, comparable labeling was found when compared immunohistochemical localization of GABA with this antibody and the GABA-synthesizing enzyme glutamic acid decarboxylase [24].

2.7.2. Immunohistochemical procedure

Single immunoperoxidase labeling (quantification of 5-HT_7 receptor immunostaining). On day 11 after surgery, independent groups of nerve-injured ($n = 6$) and sham-operated ($n = 3$) mice not exposed to pharmacological treatments were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused intracardially with cold saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Spinal cords were removed and the L4–L5 segments were dissected out and postfixed for 4 h in the same fixative. Then, spinal cord segments were washed in PB and serial coronal sections (40 μm thick) were obtained using a vibratome (vibrocut FTB, Germany) and collected in phosphate-buffered saline (PBS) to be processed immunohistochemically as free-floating sections. Sections were pre-incubated with 0.3% H_2O_2 in PBS for 30 min at room temperature (RT) to block endogenous peroxidase activity and then, after washing three times with PBS, with normal goat serum diluted 1:100 in PBS for 1 h at RT to prevent unspecific staining. Sections were then incubated for 24 h at 4 °C with the 5-HT_7 receptor rabbit antiserum (ImmunoStar, Cat. No. 24430) diluted 1:125 in PBS with 1% bovine serum albumin (PBS–BSA) [11,31]. The sections were washed three times for 10 min each in PBS–BSA and incubated with anti-rabbit biotinylated antiserum diluted 1:200 in PBS–BSA (Vectastain Vector, USA) for 1 h at RT. After washing the sections three times in PBS–BSA, an avidin–biotin–peroxidase complex was applied (diluted 1:100 in PBS, Vectastain Vector) for 1 h at RT. The sections were washed again in PBS, then immersed in a chromogen solution containing 0.05% 3,3′-diaminobenzidine-tetrahydrochloride and 0.01% H_2O_2 in PBS for 5 min at RT and reaction was stopped by several washes in PBS. The immunostained sections were placed on gelatin-coated slides, air dried and dehydrated before being mounted on DPX (Fluka, Spain) for microscopic observation and photography.

Sections from nerve-injured and sham-operated mice were simultaneously processed for immunohistochemistry in order to avoid methodological variations that would affect the intensity of staining. Three L4–L5 spinal cord sections per mouse were randomly selected and fields containing the ipsilateral dorsal horn were digitized using a video camera (Olympus DP70) connected to a microscope (Olympus BX61) and interfaced to a computer. The boundary of the dorsal horn laminae was traced and the mean density of immunostaining was quantified based on the inverse computer grayscale (from 0 = white to 255 = black) using the National Institutes of Health (NIH) Image J software. Individual immunodensity values were corrected by subtracting the background (labeling in the white matter) for each section.

Double immunofluorescence labeling of 5-HT_7 receptor and GABA. On day 15 after surgery, independent groups of nerve-injured ($n = 3$) and sham-operated ($n = 3$) mice were anesthetized with ketamine/xylacin (50/10 mg/kg) and then intracardially perfused with heparinized phosphate buffer, followed by 4% paraformaldehyde. The lumbar region of the spinal cord was removed, cryopreserved in 30% sucrose solution at 4 °C, embedded in O.C.T., sliced in 25 μm sections on a cryostat and mounted on silane-coated slides.

The slides were incubated in the blocking solution (3% of normal goat serum, 0,05% Triton 100 in PB 0.1 M) for 1 h and then in a mixture containing polyclonal anti-5-HT_7 receptor antibody (1:250, ImmunoStar Inc.) and monoclonal anti-GABA antibody (1:750, Sigma–Aldrich Co.) in blocking solution at 4 °C overnight. The sections were washed three times for 10 min each in PB followed by incubation for 1 h with CY3-conjugated anti-rabbit secondary antibody (1:500, Jackson Immunoresearch, Baltimore, PA, USA) and CY2-conjugated anti-mouse secondary antibody (1:500, Jackson Immunoresearch) in blocking solution.

Confocal images were obtained using a Leica SP2 confocal microscope, adapted to an inverted Leica DM IRBE microscope. Tissue sections of the lumbar dorsal horn were examined with a 40× 1.25 NA oil immersion in Leica Plan Apochromatic objective at 2× zoom. CY2 and CY3 were excited with the 488 nm line of an Argon laser and the 543 nm line of a Green Neon laser, respectively, and

double immunofluorescence images of three stained sections were taken for each animal in a sequential mode. From each section, images were always recorded from the ipsilateral and contralateral dorsal horns.

2.8. Data analysis

For neuropathic pain-related behaviors, statistical analysis to test the effect of treatment in nerve-injured and sham-operated mice was made using an initial ANOVA followed by Newman–Keuls (acute administration) or Bonferroni (repeated administration) multiple comparison tests. For the rotarod test, statistic analysis to test the effect of treatments on latency was made using ANOVA followed by Newman–Keuls's post hoc comparison.

For the histological study, the expression of 5-HT$_7$ receptor was estimated as the density of immunostaining using anti-5-HT$_7$ receptor antibodies. Immunodensity values in nerve-injured mice in laminae I–II and laminae III–V of the ipsilateral dorsal horn were compared with values obtained in sham-operated mice using one-way ANOVA followed by Newman–Keuls's post hoc comparison.

Values presented in graphs are the mean ± SEM. The level of significance was set at $p < 0.05$.

3. Results

3.1. Selectivity, efficacy and potency of E-57431

E-57431 is a new highly selective, potent 5-HT$_7$ receptor agonist. It showed high affinity for 5-HT$_7$ receptors (K_i = 0.47 nM), some affinity for 5-HT$_{1D}$ (K_i = 53 nM) and 5-HT$_{2A}$ (K_i = 560 nM) and no significant affinity (K_i > 1 μM or % inhibition at 1 μM < 50%) for other 5-HT receptor subtypes and 160 additional targets including receptors, transporters and ion channels included in the commercial binding screening package (Table 1). When tested in a functional assay, E-57431 concentration-dependently increased cAMP formation in HEK-293F/h5-HT$_7$ cells (data not shown) and behaved as a full agonist, with high efficacy (E_{max} = 94.5 ± 1%; E_{max} for 5-HT considered 100%) and potency (EC_{50} = 21.5 ± 1 nM) at 5-HT$_7$ receptors.

Information on the selectivity (binding profile) of the rest of pharmacological tools used in this study has also been compiled in Table 1.

3.2. AS-19, a selective 5-HT$_7$ receptor agonist, inhibits mechanical hypersensitivity and thermal hyperalgesia secondary to nerve injury

Partial sciatic nerve ligation induced mechanical hypersensitivity and thermal hyperalgesia. Mechanical hypersensitivity was evidenced by a reduced pressure threshold evoking withdrawal of the ipsilateral hindpaw on day 10 post-surgery compared to baseline pre-surgery values (Fig. 1A). In turn, thermal hyperalgesia was evidenced by a decreased withdrawal latency of the ipsilateral hindpaw in response to a thermal stimulus on day 10 post-surgery compared to baseline pre-surgery values (Fig. 2A). Sham operation did not induce mechanical hypersensitivity (Fig. 1B) or thermal hyperalgesia (Fig. 2B) as no significant changes of the response were found in sham-operated mice 10 days after surgery compared to baseline pre-surgery values.

Systemic administration of the 5-HT$_7$ receptor agonist AS-19 on days 11–13 at doses of 1 and 10 mg/kg significantly inhibited mechanical hypersensitivity and thermal hyperalgesia (Figs. 1A and 2A). At these doses, AS-19 restored the withdrawal threshold in response to mechanical stimulation and the withdrawal latency in response to thermal stimulation of the nerve-injured hindpaw to baseline pre-surgery values.

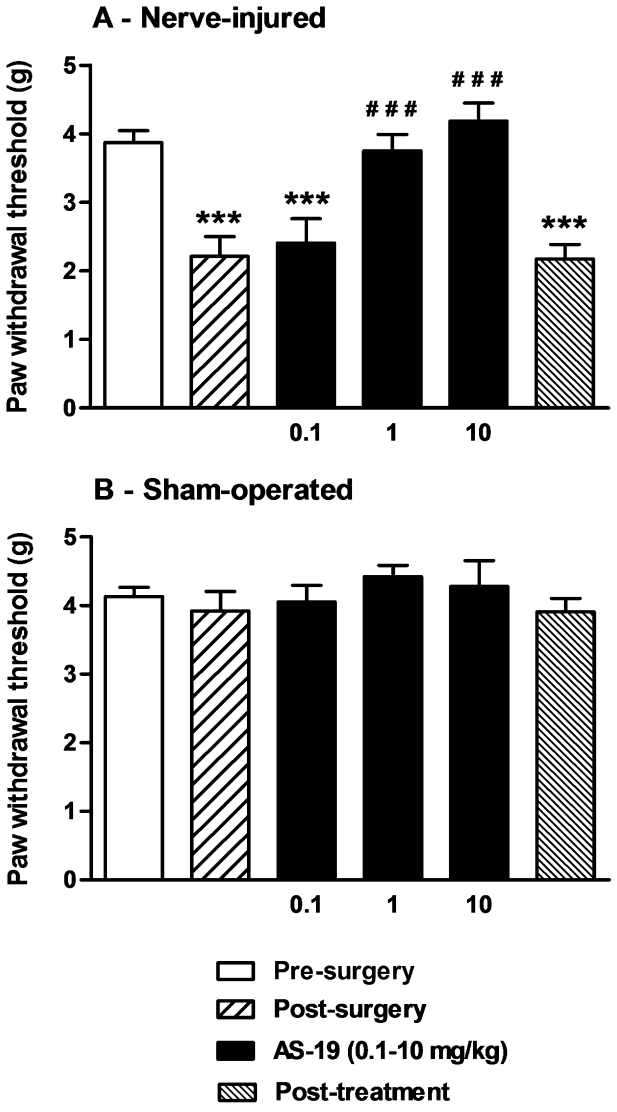

Fig. 1. Dose–response effect of AS-19 on mechanical hypersensitivity. Pressure threshold evoking withdrawal of the ipsilateral hindpaw in response to mechanical stimulation (electronic von Frey) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on days 11–13 post-surgery after treatment with three different doses of AS-19 in a Latin square design, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that mechanical hypersensitivity developed after partial sciatic nerve ligation (but not after sham operation) and that AS-19 at doses of 1 and 10 mg/kg inhibited nerve injury-induced mechanical hypersensitivity. $^{***}p < 0.001$ vs. pre-surgery; $^{###}p < 0.001$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

The reduction of mechanical hypersensitivity and thermal antihyperalgesic effects disappeared after the withdrawal of the AS-19 treatment (post-treatment values on day 14 were not significantly different from pre-treatment post-surgery values on day 10). Treatment with a lower dose (0.1 mg/kg) of AS-19 was ineffective as no modification of these behavioral manifestations of neuropathic pain was found compared to vehicle treatment (Figs. 1A and 2A). AS-19 did not produce significant effects at 0.1, 1 and 10 mg/kg in sham-operated mice (Figs. 1B and 2B).

3.3. SB-258719, a selective 5-HT$_7$ receptor antagonist, promotes mechanical hypersensitivity but not thermal hyperalgesia

Activation of 5-HT$_7$ receptors by the selective agonist AS-19 reduced mechanical and thermal hypersensitivities in nerve-injured mice, but does a selective 5-HT$_7$ receptor antagonist exert pronoci-

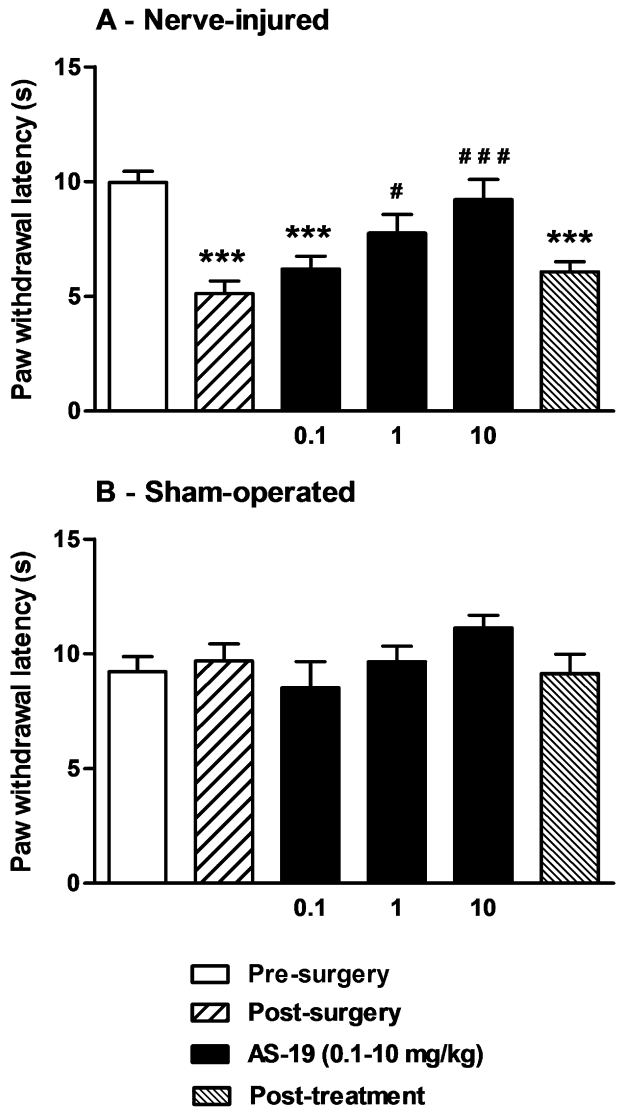

Fig. 2. Dose–response effect of AS-19 on thermal hyperalgesia. Latency of withdrawal of the ipsilateral hindpaw in response to thermal stimulus (plantar test) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on days 11–13 post-surgery after treatment with three different doses of AS-19 in a Latin square design, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that thermal hyperalgesia developed after partial sciatic nerve ligation (but not after sham operation) and that AS-19 at doses of 1 and 10 mg/kg exerted antihyperalgesic effects. $^{***}p < 0.001$ vs. pre-surgery; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

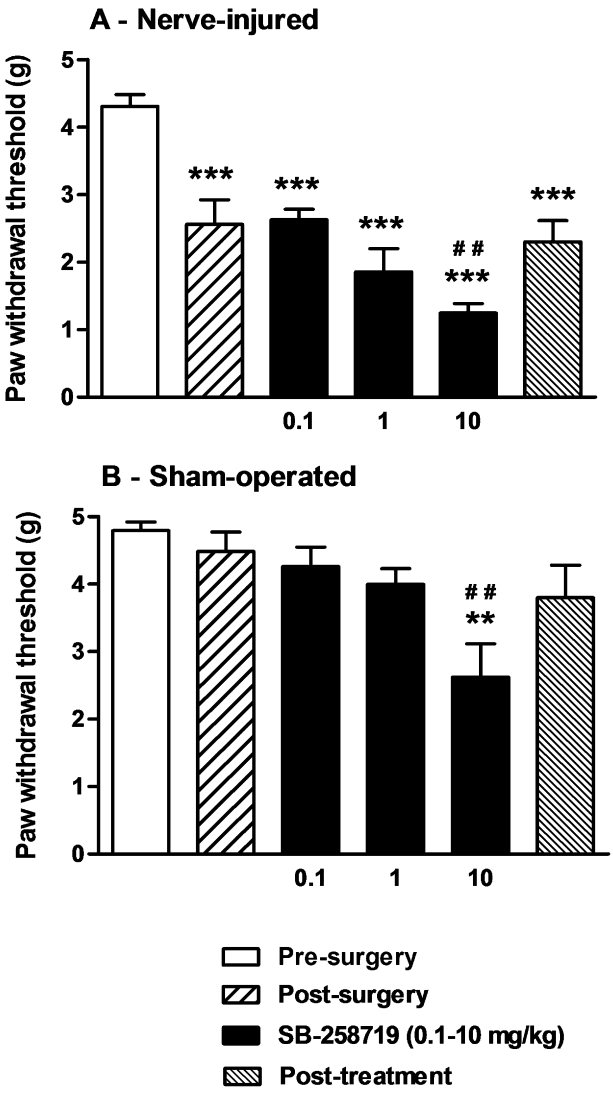

Fig. 3. Dose–response effect of SB-258719 on mechanical hypersensitivity. Pressure threshold evoking withdrawal of the ipsilateral hindpaw in response to mechanical stimulation (electronic von Frey) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on days 11–13 post-surgery after treatment with three different doses of SB-258719 in a Latin square design, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that mechanical hypersensitivity developed after partial sciatic nerve ligation (but not after sham operation) and that SB-258719 at the dose of 10 mg/kg significantly promoted mechanical hypersensitivity in both nerve-injured and sham-operated mice. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pre-surgery; $^{\#\#}p < 0.01$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

ceptive effects? To investigate this possibility, we administered the 5-HT_7 receptor antagonist SB-258719 to nerve-injured and sham-operated mice.

We observed that subcutaneous treatment with SB-258719 at doses of 2.5 and 10 mg/kg significantly decreased the mechanical threshold evoking withdrawal of the ipsilateral, nerve-injured hindpaw below post-surgery values (Figs. 3A and 5A). In addition, SB-258719 was able to induce mechanical hypersensitivity in sham-operated mice when subcutaneously administered at doses of 2.5 and 10 mg/kg (Figs. 3B and 5B). Promotion of mechanical hypersensitivity did not occur at lower doses (0.1 and 1 mg/kg) (Fig. 3A).

Contrary to mechanical hypersensitivity, thermal hyperalgesia was not promoted by the 5-HT_7 receptor antagonist SB-258719 at any tested dose in mice exposed either to sciatic nerve injury or to sham operation (Fig. 4).

3.4. Reversion of the inhibitory effects of AS-19 on mechanical and thermal hypersensitivity by SB-258719

In order to confirm that the activation of 5-HT_7 receptors was unambiguously responsible for the inhibition of nerve injury-induced mechanical and thermal hypersensitivities, we used the 5-HT_7 receptor antagonist SB-258719 to pharmacologically reverse the inhibitory effects exerted by the 5-HT_7 receptor agonist AS-19. As shown in Figs. 5A and 6A, the effects on mechanical and thermal hypersensitivity elicited by AS-19 (1 mg/kg) in nerve-injured mice were significantly reduced when the agonist was co-administered with SB-258719 (2.5 mg/kg). Similarly, the promotion of mechanical hypersensitivity by SB-258719 (2.5 mg/kg) in sham-operated mice was blocked by co-administration of AS-19 (1 mg/kg) (Fig. 5B).

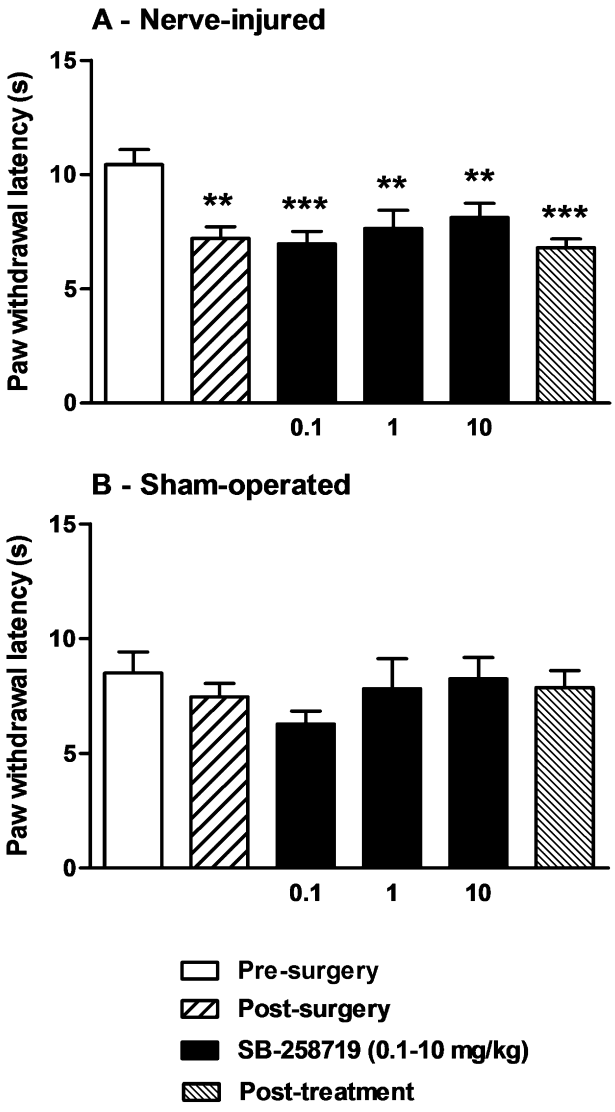

Fig. 4. Dose–response effect of SB-258719 on thermal hyperalgesia. Latency of withdrawal of the ipsilateral hindpaw in response to thermal stimulus (plantar test) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on days 11–13 post-surgery after treatment with three different doses of SB-258719 in a Latin square design, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that thermal hyperalgesia developed after partial sciatic nerve ligation (but not after sham operation) and that SB-258719 did not exert any significant effect. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pre-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

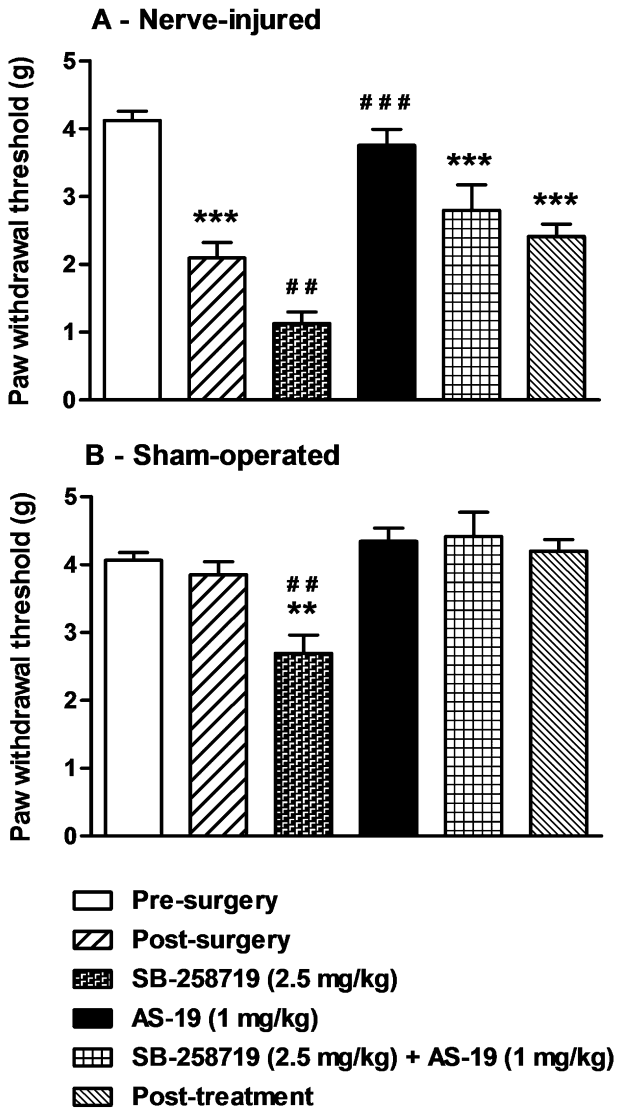

Fig. 5. Reversion of the effects of AS-19 and SB-258719 on mechanical hypersensitivity. Pressure threshold evoking withdrawal of the ipsilateral hindpaw in response to mechanical stimulation (electronic von Frey) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on day 11–13 post-surgery after treatment with SB-258719, AS-19 or their combination, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that SB-258719 (2.5 mg/kg) promoted mechanical hypersensitivity in both nerve-injured and sham-operated mice. In contrast, AS-19 (1 mg/kg) reduced hypersensitivity only in nerve-injured mice. Combination of SB-258719 and AS-19 resulted in the blockade of their respective, opposite effects. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pre-surgery; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

3.5. Effectiveness of the treatment with the 5-HT$_7$ receptor agonist E-57431 was maintained after repeated administration

The effectiveness of repeated administration of the 5-HT$_7$ receptor agonist E-57431 on the development of neuropathic pain-related behaviors was investigated in nerve-injured and sham-operated mice. Mice were administered i.p. twice daily with vehicle or E-57431 (10 mg/kg) for a period of 11 days after the surgery. On days 3, 6 and 10 of treatment mice were sequentially assessed for mechanical hypersensitivity (allodynia) (evaluated by manual von Frey stimulation 30 min after the morning administration) and thermal (heat) hyperalgesia (evaluated by the plantar test 45 min after the morning administration).

As expected, mechanical allodynia and thermal hyperalgesia developed in vehicle-treated mice exposed to sciatic nerve injury from day 3 after surgery when compared to sham-operated mice (Fig. 7). In contrast, mechanical allodynia and thermal hyperalgesia were significantly attenuated in nerve-injured mice treated with E-57431 throughout the treatment period. Mechanical allodynia was significantly reduced on day 3 in nerve-injured mice receiving subchronically E-57431 (respect to values in vehicle-treated nerve-injured mice) and the antiallodynic efficacy of the treatment increased progressively on days 6 and 10 (Fig. 7A). Regarding thermal hyperalgesia, it was completely blocked on day 3 in nerve-injured mice receiving subchronically E-57431 (values were undistinguishable from values obtained in sham-operated mice) and this level of efficacy was maintained on days 6 and 10 of treatment (Fig. 7B). Neuropathic pain-related

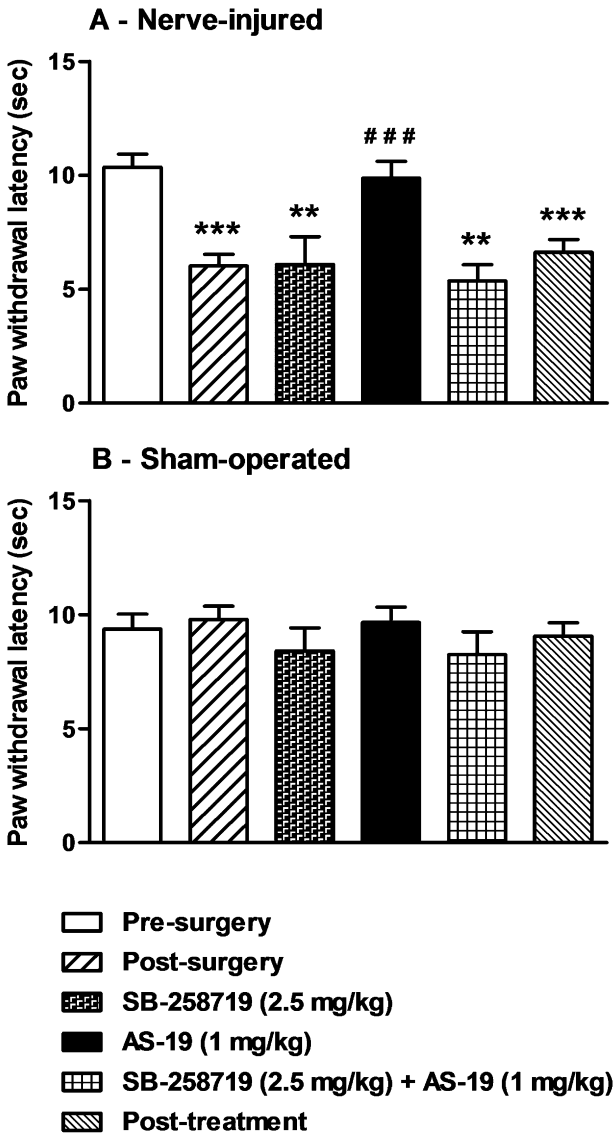

Fig. 6. Reversion of the effects of AS-19 on thermal hyperalgesia. Latency of withdrawal of the ipsilateral hindpaw in response to thermal stimulus (plantar test) in nerve-injured (A) and sham-operated (B) mice before surgery (pre-surgery), on day 10 after surgery following treatment with vehicle (post-surgery), on day 11–13 post-surgery after treatment with SB-258719, AS-19 or their combination, and finally on day 14 post-surgery after treatment with vehicle (post-treatment). Note that AS-19 (1 mg/kg) exerted antihyperalgesic effect in nerve-injured mice whereas SB-258719 (2.5 mg/kg) was devoid of effect. However, when combined, SB-258719 blocked the antihyperalgesic effect of AS-19. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pre-surgery; $^{###}p < 0.001$ vs. post-surgery (ANOVA followed by Newman–Keuls multiple comparison test).

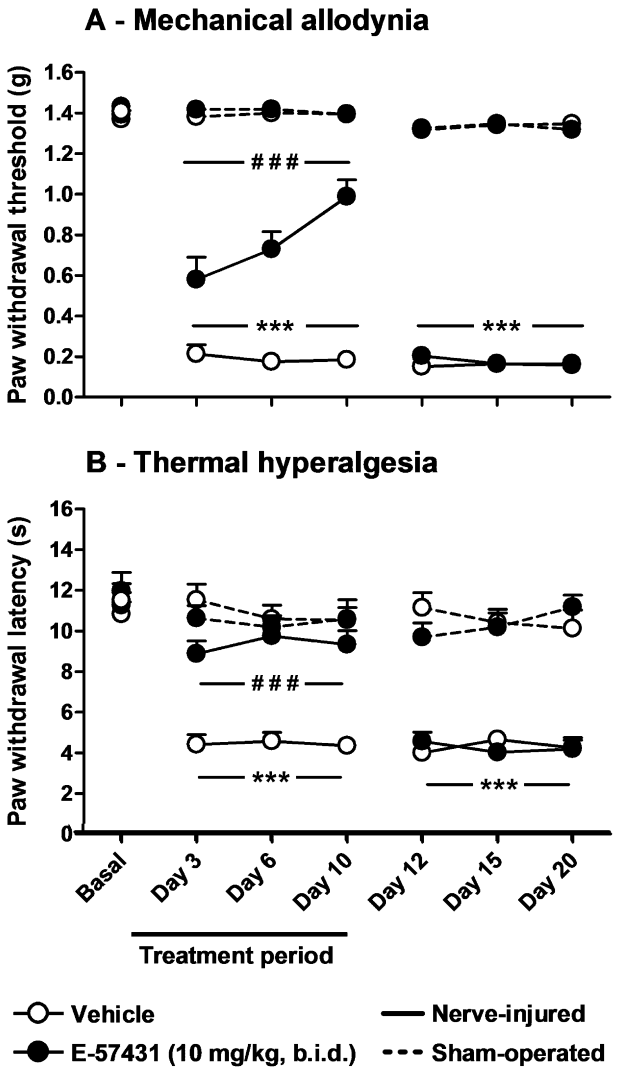

Fig. 7. Effect of repeated administration of E-57431 on the development of neuropathic pain-related behaviors. Mechanical allodynia using manual von Frey filaments (A) and thermal hyperalgesia using the plantar test (B) were assessed in the ipsilateral hindpaw of nerve-injured and sham-operated mice after daily administration of the 5-HT_7 agonist E-57431 (10 mg/kg) or vehicle, twice a day for 11 days. Treatment with E-57431 or vehicle started the day of surgery (day 0) and was maintained up to day 10. Behavioral testing was done before surgery (basal, pre-surgery values), after surgery on days 3, 6 and 10 of treatment (30–45 min after the morning administration), and on days 12, 15 and 20 post-surgery when the treatment was withdrawn (30–45 min after vehicle administration). Note that both mechanical and thermal hypersensitivity were significantly inhibited in nerve-injured mice subchronically treated with E-57431 and that the efficacy of the treatment was maintained (thermal hyperalgesia) or increased (mechanical allodynia) throughout the treatment period. Note also that neuropathic pain-related behaviors reverted back to baseline nerve injury values when the treatment with E-57431 was withdrawn (days 12, 15 and 20). Treatments were devoid of effects in sham-operated mice. $^{***}p < 0.001$ vehicle nerve-injured vs. vehicle sham-operated; $^{###}p < 0.001$ E-57431 nerve-injured vs. vehicle nerve-injured. (ANOVA followed by Bonferroni multiple comparison test).

behaviors reverted back to baseline nerve injury values when the treatment with E-57431 was withdrawn (post-treatment mechanical allodynia and thermal hyperalgesia on days 12, 15 and 20 were undistinguishable from values of nerve-injured mice that received vehicle treatment).

3.6. Effect of AS-19 and E-57431 on motor performance (rotarod test)

Animals treated with different increasing doses of AS-19 and E-57431 were tested in the rotarod test 30, 60, 120 and 180 min post-treatment to rule out possible treatment-related locomotor disturbing effects on the results of the pain experiments.

The latency to fall down from the rotarod was recorded and significant differences between groups were found after the administration of compounds at the highest doses. Both AS-19 and E-57431 induced the maximum effects 30 min after their i.p. administration (data not shown). At 30 min, the dose–response curve revealed significant effects on motor coordination at 40 and 80 mg/kg, but not at 10 and 20 mg/kg for both compounds (Fig. 8). Thus, at the maximum dose used in nociceptive behavioral tests (10 mg/kg), compounds were devoid of motor disturbing effects.

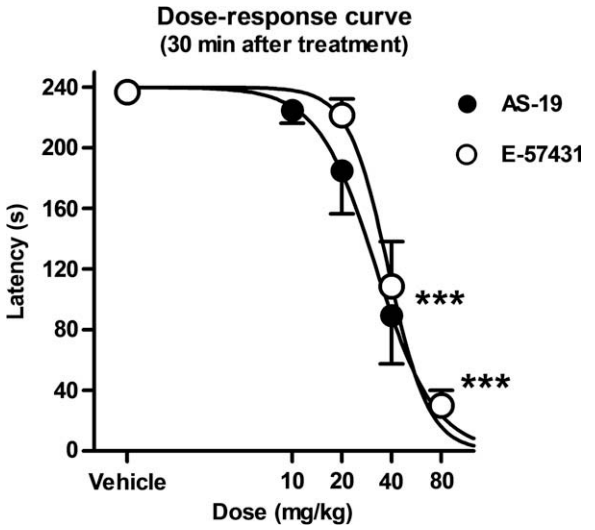

Fig. 8. Effect of AS-19 and E-57431 on the rotarod test. The latency to fall-down from the rotarod was recorded in mice 30 after single administration of AS-19 and E-57431 at different doses. The dose–response curve revealed significant motor disturbing effects at doses higher than 20 mg/kg for both compounds. ED_{50} = 32.45 ± 1.13 mg/kg for AS-19; and 38.35 ± 1.08 mg/kg for E-57431. $^{***}p < 0.001$ vs. vehicle (ANOVA followed by Newman–Keuls multiple comparison test).

3.7. Nerve injury increases 5-HT$_7$ receptor immunoreactivity in the dorsal horn of the spinal cord

We next investigated whether changes in 5-HT$_7$ receptor expression were induced after nerve injury in the spinal cord by immunohistochemistry. Immunolabeling of the 5-HT$_7$ receptor was observed mainly in the two superficial laminae of the dorsal horn. At the light microscope level, immunoreaction was mostly found in the perikarya of some cells (probably neurons based on their distribution) and the neuropile (including probably dendritic processes). Interestingly, when expression levels were quantified on day 11 after surgery, the density of 5-HT$_7$ receptor immunoreactivity was found to be significantly increased in both laminae I–II and III–V of the ipsilateral dorsal horn of L4–L5 segments in nerve-injured compared to sham-operated mice (Fig. 9A–C).

3.8. The 5-HT$_7$ receptor co-localized with GABAergic cells in the dorsal horn of the spinal cord

Double immunofluorescence labeling of 5-HT$_7$ receptor and GABA on spinal cord sections from nerve-injured and sham-operated mice on day 15 after surgery revealed that the 5-HT$_7$ receptor co-localized with GABAergic cells in the dorsal horn of the spinal cord. This co-localization was mainly revealed in cell bodies of GABAergic interneurons in laminae III–V (Fig. 9D–F). In the superficial laminae I and II, immunostaining for 5-HT$_7$ receptor did not clearly co-localized with the GABA neurotransmitter. The number of GABAergic cell bodies that expressed 5-HT$_7$ receptor was similar in the ipsilateral and contralateral horns of both sham-operated and nerve-injured mice.

4. Discussion

In the present work, using the partial sciatic nerve ligation model of neuropathic pain in mice [27], we investigated the effects of 5-HT$_7$ receptor ligands on nerve injury-induced mechanical and thermal (heat) hypersensitivities. A new 5-HT$_7$ receptor agonist, E-57431, with more than 100-fold selectivity over a range of other receptors, was described. The effect of acute and repeated administration of 5-HT$_7$ receptor agonists, the cellular localization of spinal 5-HT$_7$ receptors and changes in 5-HT$_7$ receptor expression in the spinal cord secondary to nerve injury were investigated.

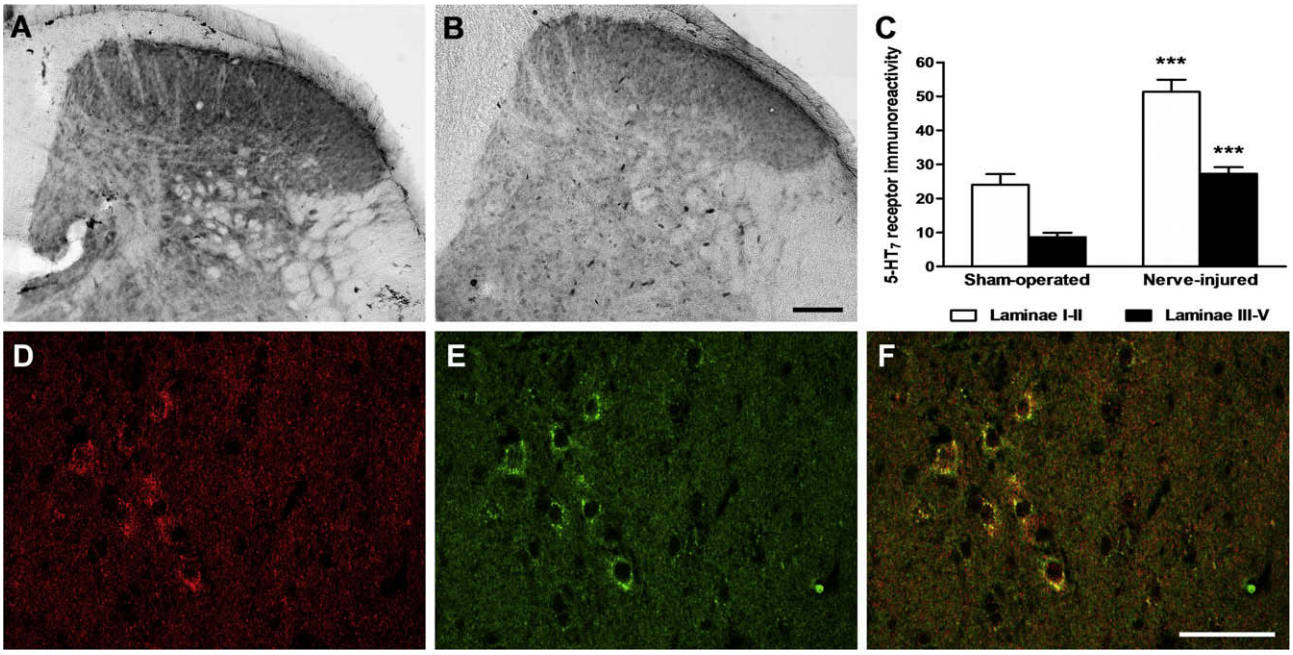

Fig. 9. 5-HT$_7$ receptor immunoreactivity in the dorsal horn of the lumbar spinal cord and co-localization with GABAergic neurones. Immunohistochemical labeling of 5-HT$_7$ receptors in the ipsilateral dorsal horn of the spinal cord 11 days after sciatic nerve injury (A) or sham operation (B). Note that, when quantified, immunoreactivity for 5-HT$_7$ receptors was significantly increased in both laminae I–II and III–V of the ipsilateral dorsal horn at lumbar L4–L5 levels in nerve-injured compared to sham-operated mice (C). Confocal immunofluorescence microscopy showing 5-HT$_7$ receptor (D), GABA (E) and double 5-HT$_7$ receptor/GABA (F) immunostaining in the ipsilateral laminae III–V of the lumbar spinal cord of sciatic nerve-injured mice. $^{***}p < 0.001$ vs. corresponding laminae of sham-operated mice (ANOVA followed by Newman–Keuls multiple comparison test). Scale bar in A and B = 200 μm; in D–F = 50 μm.

Activation of 5-HT_7 receptors by acute systemic administration of the 5-HT_7 receptor agonist AS-19 exerted a clear-cut dose-dependent inhibition of nerve injury-induced mechanical hypersensitivity and thermal hyperalgesia. Co-administration of the selective 5-HT_7 receptor antagonist SB-258719 blocked the effects of the agonist. Similarly, in a previous study describing the effect of systemically administered BBB-penetrant 5-HT_7 receptor ligands following neurogenic sensitization with capsaicin, 5-HT_7 receptor agonists inhibited mechanical hypersensitivity in mice and co-administration of 5-HT_7 receptor antagonists prevented this effect [3]. Here, using the same 5-HT_7 receptor ligands, we show that the overall effect of activating 5-HT_7 receptors is antinociceptive (antiallodynic/antihyperalgesic) in sensitizing conditions involving nerve injury. Interestingly, no tolerance to the effect was evidenced following repeated systemic administration of the selective 5-HT_7 receptor agonist E-57431 twice daily for 11 days. The effectiveness was maintained or even slightly increased throughout the treatment period but neuropathic pain-related behaviors reverted back to baseline nerve injury values when the treatment was withdrawn. This suggests an improvement of "disease symptoms" related to the presence and influence of the drug at the time of the test. Finally, it is important to note that effectiveness of the treatment with 5-HT_7 receptor agonists was not masked by non-specific motor effects, as no motor incoordination was found in the rotarod test at the doses used in both acute and repeated administration experiments.

Desensitization and down-regulation following agonist exposure are common among G protein-coupled receptors, but there is some discrepancy at this regard for 5-HT_7 receptors. Down-regulation has been described in some studies (e.g., in the hypothalamus after fluoxetine treatment for 21 days) [44] but data indicating that 5-HT_7 receptors are not readily down-regulated by long-term exposure to agonists is also available [21]. Actually, significantly increased 5-HT_7 receptor mRNA expression has been reported in raphe nuclei, hippocampus and prefrontal cortex after treatment with the 5-HT_7 receptor agonist AS-19 [35]. In the present study, activity of the 5-HT_7 receptor agonist E-57431 in nerve-injured mice was maintained throughout subchronic (11 days) treatment. Therefore, if desensitization and/or down-regulation phenomena occur, they do not have noticeable consequences in the particular conditions of our study (e.g., they could require longer exposure to the agonist to become apparent) or are compensated by nerve injury-induced receptor up-regulation (see discussion later).

Available data in the literature suggest a pronociceptive role of 5-HT_7 receptors when activation occurs at the periphery [29,39]. This inference is based on the use of non-selective agonists (5-HT, 5-CT, 8-OH-DPAT) locally administered (intraplantarly or intra-articularly) in the context of tissue injury and inflammation. Peripheral tissue injury causes the release of 5-HT from platelets and mast cells, and the endogenous indolamine acts in combination with other inflammatory mediators to excite and sensitize afferent nerve fibers [45]. Activation of 5-HT_{2A} and 5-HT_3 receptor subtypes present on C-fibers was already shown to underlie such a peripheral pronociceptive effect of 5-HT [32,45]. However, whether or not peripheral 5-HT_7 receptors contribute to 5-HT-evoked pain in inflammatory and neuropathic pain conditions needs to be confirmed.

In contrast, an antinociceptive role for central 5-HT_7 receptors has been suggested at both spinal [9,10] and supraspinal [8,16] levels based on the effects of local (intrathecal, intracerebroventricular or intrathalamic) administration of non-selective ligands in nociceptive (tail-flick, paw-flick or tailshock) or inflammatory (intrarticular, gout-like) pain models. Regarding such an antinociceptive role, it is important to note that agonists acting at 5-HT_7 receptors cannot directly inhibit primary afferents, second-order nociceptive dorsal horn neurons or third order supraspinal neurons because stimulation of the 5-HT_7 receptor has excitatory effects [5]. Therefore, an indirect action through the activation of 5-HT_7 receptors localized on inhibitory enkephalinergic or GABAergic interneurons, to evoke the release of enkephalins or GABA, is presumably required to inhibit nociceptive transmission. In this way, immunohistochemical studies revealed that the 5-HT_7 receptor is located postsynaptically on local interneurons within the superficial laminae of the dorsal horn [11,29]. Our observations at the confocal microscope level showing the co-localization of 5-HT_7 receptors and GABA in neurons of the dorsal horn of the spinal cord provide further support to this hypothesis. In addition, it has been recently reported that spinal GABAergic interneurons are involved in 5-HT_7 receptor-mediated antinociception [2,48]. This is based on the finding that intrathecal pre-treatment with the $GABA_A$ receptor antagonist bicuculline, but not with the $GABA_B$ receptor antagonist phaclofen or the opioid receptor antagonist naloxone, prevented the antihyperalgesic effects exerted by 5-HT_7 receptor agonists in rats with constriction injury to the sciatic nerve [2,48]. Accordingly, the activation of spinal inhibitory GABAergic interneurons could underlie or at least contribute to the analgesic effects of 5-HT_7 receptor agonists.

A dose-dependent promotion of mechanical hypersensitivity, but not heat hyperalgesia, was observed after treatment with the 5-HT_7 receptor antagonist SB-258719. Differential endogenous 5-HT tone and/or modulation by 5-HT_7 receptors of sensory/nociceptive pathways depending on the nature (mechanical vs. thermal) and intensity (allodynic/subthreshold vs. hyperalgesic/suprathreshold) of the stimulus could explain the different effects on the response to mechanical and thermal stimuli exerted by the 5-HT_7 receptor antagonist. Interestingly, treatment with SB-258719 not only promoted mechanical hypersensitivity in nerve-injured mice but it also induced mechanical hypersensitivity in sham-operated mice. We found a comparable result in the capsaicin model: two different 5-HT_7 receptor antagonists, SB-258719 and SB-269970, promoted mechanical hypersensitivity when administered to mice subplantarly injected with a low subactive dose of capsaicin [3]. This suggests that endogenous activation of the 5-HT_7 receptor occurs, thereby allowing antagonists to exert a counteracting effect.

Descending 5-HTergic pathways projecting into the spinal cord can either suppress (descending inhibition) or potentiate (descending facilitation) nociceptive messages depending on the 5-HT receptor involved and its localization [9,30,33,47]. Regarding the descending inhibitory control, blockage of receptors involved in such a tonic brake control by 5-HT would suppress the inhibitory tone thus promoting hypersensitivity of nociceptive pathways. It is thus plausible on the basis on the present study showing that 5-HT_7 receptor agonists inhibit and a 5-HT_7 receptor antagonist promotes mechanical hypersensitivity, that 5-HT_7 receptors could participate, in concert with other 5-HT receptors [19,42], in the endogenous 5-HTergic inhibitory control of pain. In this way, the RVM is an important source of descending modulation of pain at the level of the spinal cord, and the antinociceptive effect of morphine microinjected into the RVM is known to involve the activation of spinal 5-HT_7 receptors [9,10].

Increased expression of 5-HT_7 receptors was found in the ipsilateral dorsal horn of the spinal cord of nerve-injured compared to sham-operated mice. In particular, we found a significant increase of 5-HT_7 immunoreactivity in laminae I–II and III–V of the dorsal horn in the ipsilateral side of the spinal cord eleven days after nerve injury. Increased 5-HT_7 receptor expression induced by nerve injury in the dorsal horn could represent a physiological,

compensatory, protective spinal mechanism relevant to the control of nociception in neuropathic pain conditions.

5-HT has been reported to exert algesic or analgesic effects depending on its site of action and the receptor subtype it acts on [12,19,30,33,47,50]. Based on the results reported here in the partial sciatic nerve ligation model and those recently reported in the capsaicin model in mice [3] as well as after constriction injury to the sciatic nerve in rats [2,48], it is clear that systemically administered 5-HT_7 receptor agonists crossing the BBB and acting in the CNS [3,14,34] exert an analgesic (antiallodynic/antihyperalgesic) effect in sensitizing neurogenic/neuropathic conditions. We hypothesize that, if a balance exists between pro- and antinociceptive actions depending on the localization of the 5-HT_7 receptor, the antinociceptive effect at some CNS sites may counteract the pronociceptive effect at the periphery or at other CNS sites. Activation of inhibitory GABAergic interneurons in the spinal cord, and possibly in other CNS locations, seems to be the most likely mechanism of action accounting for antinociception. The up-regulation of 5-HT_7 receptors in the dorsal horn of the spinal cord after sciatic nerve injury suggests a "pain"-triggered regulation of receptor expression that may be relevant for the effectiveness of 5-HT_7 receptor agonists.

Taken together, the results of the present study support the involvement of the 5-HT_7 receptor subtype in the control of pain and point to a new potential use of 5-HT_7 receptor agonists for the treatment of neuropathic pain. Nevertheless, this study is limited to a specific type of the experimental neuropathic pain. Further studies in different experimental pain conditions, using recently developed ligands with high affinity and selectivity for the 5-HT_7 receptor and focusing on the site and mechanism of action underlying 5-HT_7 receptor-mediated analgesia would be particularly useful.

Summary

The results of the present study support the involvement of the 5-HT_7 receptor subtype in the control of pain.

Conflicts of interests

The authors state that there were no conflicts of interests in respect to the work reported in the paper.

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