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ANTI-NOCICEPTIVE EFFECT OF PERIPHERAL SEROTONIN 5-HT$_{2B}$ RECEPTOR ACTIVATION ON NEUROPATHIC PAIN

Running foot: Anti-nociceptive effect of 5-HT$_{2B}$ receptor on neuropathic pain

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Abstract

Neuropathic pain remains difficult to manage. Antidepressants are among the main treatments for neuropathic pain, although the role of serotonin is poorly understood. In a rat model of neuropathy induced by sciatic nerve constriction (CCI), we demonstrated an antinociceptive effect of the 5-HT$_{2B}$ receptor. CCI resulted in a biphasic upregulation of 5-HT$_{2B}$ receptor expression in lumbar dorsal root ganglia, consisting of a transient early increase (23-fold), two days after surgery, before the development of neuropathic pain, followed by a steady, five times increase, levels remaining constant thereafter until the pain disappeared. 5-HT$_{2B}$ receptors were immmunolocalized mostly on primary sensory neurons and infiltrating macrophages. Intrathecal injection of RS127445, a selective 5-HT$_{2B}$ receptor antagonist, enhanced mechanical and cold allodynia. A single application of BW732C86, a 5-HT$_{2B}$ agonist, to the sciatic nerve immediately after ligature completely prevented mechanical allodynia and significantly decreased cold allodynia. This effect was dose-dependent and reversed by a 5-HT$_{2B}$ antagonist. We also observed a marked decrease in macrophage infiltration of the sciatic nerve, neuropathic pain markers and cytokine induction. Our data reveal the relationships between serotonin, the immune system and neuropathic pain, and demonstrate a critical role of 5-HT$_{2B}$ receptors in blood-derived macrophages.

Key words: analgesia; dorsal root ganglion cells; immune response; macrophage; sciatic nerve constriction

INTRODUCTION

Painful peripheral sensory neuropathies developing after peripheral nerve injury involve complex changes to nociceptor function. The sensitization of peripheral nociceptors, and of spinal and supraspinal relay cells leads to a persistent pain syndrome including abnormal pain sensations, such as hyperalgesia (enhanced pain in response to a painful stimulus) and allodynia (pain sensation after an innocuous stimulus) [3,28,36,52,62]. There is increasing evidence to suggest that the immune system plays a critical role in both the development and maintenance of clinical symptoms [5,15].

The principal treatments for neuropathic pain include antidepressants, mixed serotonin (5-HT) and noradrenaline reuptake inhibitors [4,22]. They may exert their analgesic effects at peripheral or central levels. However, despite major alterations to serotoninergic functions in neuropathic pain, specific serotonin (5-HT) reuptake inhibitors (SSRI) are not very effective for the relief of neuropathic pain. Indeed, pain modulation by 5-HT is complex and may involve pro- or antinociceptive effects, depending on the cellular targets and receptor subtypes involved. In the spinal cord, 5-HT acts through bulbospinal descending projections [30,38,54], whereas, in the periphery, it sensitizes nociceptors following both inflammation and nerve injury [53]. By contrast, the role of 5-HT in the immune mechanisms associated with neuropathic pain has never been addressed, despite the abundance of data implicating 5-HT in immune functions [1,9,21,33,44]. Interestingly, one study [61] has already described an antinociceptive effect of dexfenfluramine, a 5-HT releaser that is metabolized in norfenfluramine, a preferential 5-HT$_{2B}$ receptor agonist [23].

We have shown that the 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) is involved in peripheral and spinal sensitization in two rodent models of neuropathy [57,59], whereas the 5-HT$_{2C}$ receptor (5-HT$_{2C}$R) displays antinociceptive properties [41,42,46]. However, very little is known about the role in neuropathic pain of the 5-HT$_{2B}$ receptor (5-HT$_{2B}$R), which has a similar molecular structure, pharmacology and signal transduction pathways [8]. We therefore investigated the role of this receptor at the peripheral and/or spinal levels and investigated its possible relationship to immune cells.

MATERIALS AND METHODS

Animals and ethics statements

Male Sprague-Dawley rats (Janvier, Le Genest St Isle, France) weighing 250 to 350 g were used for experiments. The animals were kept under a regular 12-h day/12-h night cycle in controlled temperature and humidity conditions, with free access to food and water. Experiments were performed according to the European Community Council Directive of 26 May 2010 (2008/0211[COD]) and were in accordance with French "Ministère de l'Agriculture et de la Pêche" rules, authorization number 75-819.

In vivo drug administration

RS127445, a selective 5-HT$_{2B}$R antagonist kindly provided by Roche Bioscience (Indianapolis, IN, USA), was found to have a subnanomolar affinity for 5-HT$_{2B}$R (pKi = 8.22 ± 0.24) and a selectivity for this receptor 1,000 times stronger than that for other receptors and monoamine uptake sites [12,19,20]. A 1 mg/ml stock solution of RS127445 was prepared in DMSO. Injectable solutions were prepared in saline (0.9 % NaCl). Intrathecal injections were performed under volatile anesthesia (see next paragraph). We injected 20 µl (30, 60, 125 ng RS127445) of the antagonist or vehicle directly through the intact skin, between the L5 and L6 vertebrae, with a Hamilton syringe equipped with a 26-gauge needle. We applied 10 µl of solution (125 ng) to the sciatic nerve, as described below. BW723C86 (Sigma, France), a mixed 5-HT$_{2B}$R/5-HT$_{2C}$R agonist [6,17], was prepared at a concentration of 1 mg/ml in distilled water (stock solution). Injectable solutions were prepared in saline. We applied 10 µl of solution to the sciatic nerve (15, 30 or 64 ng BW723C86), as described above. In some cases, a mixture of BW723C86 (64 ng) and RS127445 (125 ng) was applied to the sciatic nerve. Control animals received the same volume of the vehicle via the same route of administration.

Chronic constriction injury of the sciatic nerve

Chronic constriction injury (CCI) of the sciatic nerve was induced as previously described [10]. Under volatile anesthesia with isoflurane (Aerrane, Baxter, Maurepas, France), the left sciatic nerve was exposed at mid-thigh level, by blunt dissection through the biceps femoris muscle. Proximal to the trifurcation, 10 mm of nerve was carefully freed from adhering tissue. Four chromic catgut (4-0, Ethicon, Norderstedt, Germany) ligatures were tied loosely around the nerve at intervals of about 1 mm. The ligatures reduced the diameter of the nerve but did not interrupt the epineurial circulation. In some rats, 10 µl of BW732C86 was applied directly to the sciatic nerve via a small piece of sterile gauze (8 x 4 mm) impregnated in the solution and wrapped around the nerve. The drug was allowed to diffuse for one minute. The gauze was then sewn up around the nerve and the muscle and skin were closed. Sham-operated rats underwent the same surgical procedure without nerve ligature.

Behavioral tests

Behavioral experiments were carried out between 10 a.m. and 4 p.m., in a quiet controlled-temperature room reserved for the tests, to avoid variations linked to the environment. Stress was minimized by allowing the rats to get used to the behavioral testing apparatus and environment over a period of at least five days before the first test. All tests were performed by the same experimenter, blind to the treatment applied.

Mechanical allodynia was assessed with calibrated von Frey filaments (Bioseb, Chaville, France), as previously described [13]. Animals were placed on an elevated grid and confined within a transparent plastic cylinder. They were allowed to acclimate for 30 min before the test. The filament was applied, for 5s, to the mid-plantar area, until the filament began to bend. A positive response was indicated by a sharp withdrawal of the paw. The 50% withdrawal threshold was determined as described by Dixon [18], with the stimulus progressively increased until a positive response was obtained, and then decreased until a negative response was observed. The protocol was repeated until three changes in behavior had been observed. A table of the positive and negative responses was drawn up. The 50% withdrawal threshold was determined as [10 (Xf+kD)]/10,000, where Xf is the value of the last von Frey filament used, k is the Dixon value for the positive/negative pattern, and D is the logarithmic difference between stimuli.

Cold allodynia was evaluated by the acetone test. Rats were placed in the plastic cylinder and a drop (0.1 ml) of acetone was applied to the mid-plantar area of the hind paw. The response of the rat was monitored over a period of one minute. Responses were graded according to a four-point scale [24]: 0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking (> 2) of the paw; 3, repeated flicking of the paw with licking of the paw. Acetone was applied three times to each paw alternately, and the responses were scored. Cumulative scores were then obtained by adding the three scores for each rat. The minimum score was 0 (no response in any of the three trials) and the maximum was 9 (repeated flicking and licking in all three trials).

Tissue preparation

For RT-PCR, animals were perfused with 100 ml of saline under 60 mg/kg pentobarbital anesthesia. The L4-L6 lumbar dorsal root ganglia (DRG), sciatic nerve and lumbar spinal cord were rapidly dissected. The ligatures on the sciatic nerve were carefully removed, keeping the endoneurial sheet intact. The DRG and sciatic nerve were frozen on a metal plate cooled with dry ice and stored at -80°C until use. The spinal cord was divided into left and right parts, and into their dorsal and ventral regions by cutting through the central canal. The blocks of tissue were frozen by immersion in liquid nitrogen and stored at -80°C until use. For immunocytochemistry, animals were perfused intracardially with 100 ml of saline, followed by 600 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 (PB) under pentobarbital anesthesia (60 mg/kg). L4-L6 lumbar DRG and sciatic nerves were dissected out and postfixed overnight in the same fixative at 4°C. They were transferred to 20% sucrose in PB for 24-48 h, embedded in Tissue Freezing Medium™ (Jung, Leica, Nanterre, France) and frozen on a metal plate cooled with dry ice. They were stored at -20°C until use.

Preparation of anti-5-HT$_{2B}$R antibodies

The antibodies against 5-HT$_{2B}$R prepared by GEMACBIO (Saint-Jean D’Illac, France) were directed against the C-terminal sequence of the receptor. A 27-amino acid sequence was coupled to keyhole lumpet hemocyanin (KLH) with glutaraldehyde by the N-terminus. Three injections were performed at two-week intervals. The IC_{50} for the immunizing peptide was 1 x 10^{-4} mg/ml.

Radioligand binding assays and immunocytochemistry on transfected cells

COS-7 cells were transfected with 10 µg of rat receptor plasmid, with the Nanofectin kit (PAA Laboratories, France). After 24 h, transfected cells were re- and plated in cell culture dishes for binding or immunocytochemistry assays. Cells were then incubated overnight in serum-free Dulbecco's modified Eagle's medium (DMEM). The next day, cells were harvested by scraping, collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4) for binding assays. Membranes were collected by centrifugation and were frozen at -80°C after removal of the supernatant. Radioligand binding assays were set up in a 96-well plate (1 ml/well capacity), with 5 nM $[^3H]$LSD (PerkinElmer Life and Analytical Sciences, Boston, MA) and various concentrations (10^{-11}-10^{-6} M) of RS127445. The process was terminated by immersing the tubes in ice-cold buffer and passing their contents rapidly through Whatman GF/B filters. Radioactivity was measured by liquid scintillation counting. Binding data were analyzed with the iterative non linear fitting software GraphPad Prism 4.0, to estimate dissociation constants (K_D) and the maximum number of sites (B_{max}).

For immunostaining experiments, coverslips seeded with cells were initially rinsed with Ca^{2+}- and Mg^{2+}-containing PBS, and then fixed in 4% PFA. After three washes with PBS, cells were permeabilized in PBS supplemented with 0.25% Triton X-100 for 5 min, and incubated in blocking solution PBS containing 3% bovine serum albumin (PBS-BSA) for 30 min. Cells were incubated with rabbit anti-5-HT_{2B}R polyclonal antibody (1:5000) for 3 h, and then with an FITC-conjugated anti-rabbit secondary antibody (1:1000, 1 h, Invitrogen, Cergy-Pontoise, France). Cells were then washed three times with PBS and covered with mounting medium and a coverslip. Immunofluorescence images were generated using a Leica DM6000 microscope (Leica, Wetzlar, Germany). Antibodies did not work in western blot.

Immunocytochemistry on dorsal root ganglion and sciatic nerve sections

Frozen PFA-fixed DRG or sciatic nerves were serially cut into 14 µm-thick sections, with alternate sections placed on Super-Frost® Plus slides (Menzel-Glaser, Braunschweig, Germany). Slides were incubated in PBS-BSA supplemented with 0.1% Triton X-100 (PBS-BSA-0.1TX) and then incubated overnight with primary antibodies diluted in PBS-BSA-0.1TX. The following antibodies were used: 1/2000 rabbit anti-rat 5-HT$_{2B}$R (see above), 1/700 goat anti-Iba1 (Abcam, Paris, France), 1/2000 rabbit anti-ATF3 (Santa Cruz, Tebu, Le Perray en Yvelines, France), or 1/5000 mouse anti-GFAP (Sigma, France). Slides were then incubated for 2 h in 1/2000 anti-species IgG antibodies coupled to Alexa 555 or 488 (Invitrogen). They were then washed in PBS and mounted in Vectashield mounting medium supplemented with DAPI (Vector, AbCys, Paris, France). For double labeling, primary and secondary antibodies were incubated simultaneously with the slides. We checked for an absence of cross-reaction by omitting one of the two primary antibodies. To further control the specificity of the 5-HT$_{2B}$R immunolabeling rat anti-Fc (Serotec) was added in the incubation medium. The background due to the anti-rabbit IgG antibodies was controlled by incubating slides in the absence of anti-5-HT$_{2B}$R antibodies. The prior incubation of tissue sections in 1-100 µg/ml of the immunizing peptide for 2 h at room temperature before the addition of 5-HT$_{2B}$R antibodies led to a progressive decrease in immunolabeling. Images were acquired with a Zeiss axioVision imager M1 (Zeiss, Le Pecq, France) equipped with an Axiocam HRc camera (Zeiss).

Quantification of Iba1 immunolabeling in sciatic nerve sections

Quantification was performed by an observer blind to the experimental protocol. Alternate 14 µm-thick sciatic nerve sections were placed on 15 glass slides. Immunocytochemistry was performed as described above, on three slides from each rat, taken from sites at least 70 µm apart. After mounting, the slides were kept overnight in the dark at 4°C to normalize the immunoflurorescence labeling. Quantification was carried out on images acquired with a x 20 objective, with Image J software (NIH, Bethesda, USA) under the same light illumination from the region adjacent to the ligature (0.5 mm-1.5 mm). Four to five sections per slide (3 slides per rat) from each animal (n = 5 for each experimental condition) were scanned for counting. We determined the number of Iba1-positive cells per 1 mm^2, and this result is expressed as the mean ± SEM.

Quantitative real-time PCR

RNA was isolated from DRG, spinal cord or sciatic nerve kept at -80°C, with the NucleoSpin RNA II extraction Kit (Macherey-Nagel, Hoerdt, France). RNA concentration was measured by determining absorbance on a NanoDrop spectrophotometer (Thermo Scientific, Labtech, Palaiseau, France). First-strand cDNA (0.5 µg total RNA per 20 µl reaction) was synthesized with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Courtaboeuf, France). Real-time PCR amplification of each sample was performed in triplicate, on an ABI Prism 7300 (Applied Biosystems), with the ABgene absolute QPCR ROX Mix (ABgene). Assay-on-Demand gene TaqMan PCR probes (Applied Biosystems) were used for target genes: 5-HT_{2B}R (Rn00568450_m1), 5-HT_{2C}R (Rn00562748_m1), GFAP (Rn00566603_m1), IL-6 (Rn00568450_m1), ITGAM (Rn00755092_mL) and ATF3 (Rn00563784_m1). Semi-quantitative studies were carried out with glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn99999916_s1) as a reporter gene. Data are expressed relative to control mRNA levels.

Statistical analysis

Behavioral tests after pharmacological experiments were analyzed by two-way ANOVA for repeated measures, followed by Bonferroni post hoc tests. RT-PCR data and cell quantification data were analyzed by one-way ANOVA followed by the Newman-Keuls post-hoc test, except in figures 1C and 3A, in which a *t*-test was used. Statistical significance was defined as $p < 0.05$. Statistical analysis was performed with Prism 5 GraphPad software (La Jolla, CA, USA).

RESULTS

Intrathecal injection of a 5-HT_{2B}R antagonist enhances CCI-induced mechanical and thermal allodynia

In rats with CCI receiving vehicle, mechanical allodynia reached a plateau 14 days after surgery, with a withdrawal threshold significantly lower than that of sham-operated rats (3.64 ± 0.56 g versus 13.91 ± 1.09 g, $p < 0.001$) (Fig. 1A). The development of cold allodynia followed a similar time-course, with cold scores of 5.80 ± 0.20 A.U. versus 1.10 ± 0.41 A.U. in sham-operated rats ($p < 0.001$) on day 14 (Fig. 1B). The pain behavior of sham-operated rats remained constant throughout the experiment. We assessed the effect of 5-HT_{2B}R antagonism on CCI-induced allodynia, by injecting the antagonist into the rats twice, on days 17 and 18, after the full development of neuropathic pain. Animals were tested 60 min after injection, to allow them time to recover from anesthesia. Intrathecal injection of 125 ng RS127445 decreased the paw withdrawal threshold, from the first injection (0.86 ± 0.47 g versus 3.52 ± 0.21 g in CCI rats). This effect remained significant ($p < 0.001$) two days after the second injection (Fig. 1A). Cold allodynia was also significantly enhanced after the first RS127445 injection, with cold score of 9.00 ± 0.41 A.U., versus 6.00 ± 0.01 A.U. for CCI rats + vehicle ($p < 0.001$). The second RS127445 injection induced an increase in CCI-induced cold score statistically different ($p < 0.01$) than that for CCI rats injected with vehicle (8.50 ± 0.50 A.U. versus 6.4 ± 0.40 A.U.) that lasted 24 h (Fig. 1B). Lower concentrations of RS127445 (30 or 60 ng) were ineffective or less effective (not shown). These results suggest that 5-HT has an anti-nociceptive effect via spinal and/or peripheral 5-HT_{2B}R. We then investigated whether the receptor was expressed in the DRG and/or the spinal cord and whether its expression was regulated by neuropathy.

5-HT_{2B}R mRNA levels are strongly upregulated in the dorsal root ganglia of neuropathic rats

In normal rats, the baseline level of 5-HT_{2B}R mRNA was relatively low in the L4-L6 DRG (ΔCT 16.73 ± 0.27 versus GAPDH). In the L4-L6 spinal cord, 5-HT_{2B}R mRNA levels were higher, with a ΔCT of 15 ± 0.08 in the dorsal horn and 14.33 ± 0.07 in the ventral horn. Fourteen days after surgery, when maximal pain behavior was observed, 5-HT_{2B}R mRNA levels were unchanged in the

L4-L6 DRG contralateral to the lesion and bilaterally in the dorsal and ventral spinal cord. By contrast, they had increased significantly in the ipsilateral DRG, as shown by comparisons with sham-operated rats (5.94 ± 0.53 fold, $p < 0.001$) (Fig. 1C), suggesting a critical role of the receptor at this level. The time-course of 5-HT$_{2B}$R mRNA production in the ipsilateral DRG showed early transient overexpression of the receptor in the two days after surgery (28.95 ± 6.25 fold versus DRG from sham-operated rats), well before the observation of pain-related behavior (Fig.1D). Levels of 5-HT$_{2B}$R mRNA then gradually decreased, to levels lower, by a factor of 5.94 ± 0.53, than those of the control on day 14, when neuropathic pain was maximal. CCI-induced 5-HT$_{2B}$R mRNA overproduction persisted until neuropathic pain was observed. The levels of 5-HT$_{2B}$R mRNA levels decreased with decreasing allodynia (enhanced paw withdrawal threshold). For example, on day 35 after surgery 5-HT$_{2B}$R mRNA levels lower than those in sham-operated animals by a factor of 4.57 ± 0.45 still corresponded to a low withdrawal threshold (6.93 ± 1.25 g/15 g for control rats). At day 65, mRNA levels 1.69 ± 0.25 times higher than those in the sham-operated animals corresponded to higher mechanical threshold of 9.09 ± 0.84.

5-HT$_{2B}$R immunoreactivity is localized in neurons and macrophages of the lumbar DRG

We investigated 5-HT$_{2B}$R protein localization in the DRG by immunocytochemical experiments carried out with anti-5-HT$_{2B}$R antibodies directed against the C-terminal sequence of the receptor. We controlled the specificity of the 5-HT$_{2B}$R antibody, by carrying out immunocytochemistry on transfected COS cells. We showed that the anti-5-HT$_{2B}$R antibodies were specific for rat 5-HT$_{2B}$R and did not cross-react with COS cells transfected with the truncated C-terminal tail form of the 5-HT$_{2B}$R, the epitope used to generate the antibodies (Fig. 2A). We confirmed that all the receptors were expressed in COS cells after transient transfection, by carrying out ligand binding assays with a 5-HT2 receptor agonist, [^{3}H] LSD (Fig. 2B). Transiently transfected cells contained 410 ± 18 and 690 ± 22 fmol/mg of total or truncated rat 5-HT$_{2B}$R, respectively. Untransfected COS cells did not display endogenous expression of these receptors. The antibodies did not cross react with rat 5-HT$_{2A}$R and 5-HT$_{2C}$R (not shown). Thus, these results indicate that the anti-5-HT$_{2B}$R antibody used in our study specifically recognizes 5-HT$_{2B}$R.

Immunocytochemical staining for 5-HT$_{2B}$R in the lumbar DRG of naive rats showed an almost complete absence of immunolabeling in the DRG (Fig. 2C). Forty-eight hours after CCI, 5-HT$_{2B}$R immunoreactivity was enhanced in the L4-L6 lumbar DRG ipsilateral to the ligature. Enhanced immunoreactivity was observed in neuronal somata, particularly in small, presumably nociceptive cell bodies (Fig. 2C). Abundant Iba1 labeling was observed in macrophages, which often formed a ring around large cell bodies, whereas Iba1-positive cells were sparse in control DRG. In DRG from CCI rats, 5-HT$_{2B}$R immunolabeling colocalized with Iba1 (Fig. 2C). Omission of the primary antibody led to an absence of immunolabeling and preincubation with the immunizing peptide led to a dose-dependent decrease in labeling (not shown).

5-HT$_{2B}$R expression is upregulated in the sciatic nerve of rats with CCI

As macrophage infiltration also occurs at the lesion site [11,27], we investigated 5-HT$_{2B}$R expression in the sciatic nerve. In control rats, the basal expression of 5-HT$_{2B}$R was weak (ΔCT 16.73 ± 0.27 versus GAPDH) (Fig. 3A). Accordingly, 5-HT$_{2B}$R immunoreactivity did not differ from background, except in resident macrophages (Fig. 3B). Forty-eight hours after CCI, 5-HT$_{2B}$R mRNA levels were 2.06 ± 0.12 times higher ($p < 0.05$) than those of sham-operated rats (Fig. 3A). At the same time point, the number of 5-HT$_{2B}$R-positive macrophages had increased at the lesion site (Fig. 3C). A subpopulation of 5-HT$_{2B}$R cells, probably corresponding to another type of immune cell, was negative for Iba1.

Application of a 5-HT$_{2B}$R agonist to the sciatic nerve entirely prevents CCI-induced mechanical allodynia and decreases cold allodynia

We then investigated the effect of sciatic nerve 5-HT$_{2B}$R activation on pain development. We first showed that the 5-HT$_{2C}$R mRNA was undetectable, in highly sensitive RT-PCR assays, in the sciatic nerves of naïve rats and 48 h after CCI, suggesting that neither resident nor infiltrating macrophages expressed the receptor. We therefore considered a preferential 5-HT_{2B}R agonist, BW723C86, to be selective for 5-HT_{2B}R on the sciatic nerve. A single application of BW723C86 (10 µl, 125 ng) was made to the sciatic nerve just after nerve ligature, and pain behavior was then regularly assessed. After CCI, paw withdrawal thresholds rapidly decreased between days 7 to 14, reaching a plateau, as described above. However, by sharp contrast, CCI rats receiving BW723C86 did not develop mechanical allodynia (Fig. 4A). Their paw withdrawal thresholds were significantly different from those of untreated CCI rats from day 7 to day 17 (i.e. 14.40 ± 0.40 g for CCI + BW723C86 rats versus 4.90 ± 0.25 g for CCI rats on day 10 and 13.35 ± 0.58 g versus 3.28 ± 0.22 on day 17, $p < 0.001$).

After CCI, rats developed cold allodynia between days 7 and 17 (cold score of 3.67 ± 0.54 A.U. on day 7 and 6.33 ± 0.142 A.U. on day 17; sham-operated rats did not respond to acetone). In CCI rats receiving BW723C86, cold allodynia was lower than that measured in CCI rats receiving vehicle (Fig. 4B). Cold scores were decreased at all time tested (i.e. 2.09 ± 0.64 A.U. versus 4.42 ± 0.37 A.U. on day 7, 1.73 ± 0.43 A.U. versus 5.57 ± 0.3 A.U. on day 10, 3.27 ± 0.81 A.U. versus 6.29 ± 0.28 on day 14 and 3.82 ± 0.82 A.U. versus 6.57 ± 0.2 A.U. on day 17, $p < 0.001$). By contrast, local application of the selective 5-HT_{2B}R antagonist RS127445 (125 ng) had no significant effect on mechanical and cold allodynia (Fig. 4A and B). Local application of the vehicle had no effect on CCI-induced cold and mechanical allodynia. The preventive effect of BW723C86 on CCI-induced pain was dose-dependent (Fig. 4C) and antagonized by RS127445 (Fig. 4D).

Prevention of CCI-induced neuropathic pain by sciatic nerve 5-HT_{2B}R activation is associated with lower levels of neuropathy marker induction in the DRG and sciatic nerve

We then investigated whether the anti-nociceptive effect applying BW723C86 to the sciatic nerve also involved changes to markers of the activation of glial or neuronal cells in the ipsilateral lumbar DRG and sciatic nerve. Seventeen days after CCI, ITGAM mRNA, a macrophage marker, was upregulated in DRG ipsilateral to the lesion (2.23 ± 0.33 times higher in CCI rats receiving vehicle than in sham-operated rats). These CCI-induced levels of ITGAM mRNA were 34 % lower in DRG from CCI + BW723C86 (x 1.47 ± 0.11 versus 2.23 ± 0.33) (Fig. 5A). A similar decrease (by 37 %) in the CCI-induced upregulation of GFAP mRNA, a satellite cell marker, was observed in DRG from CCI rats receiving BW723C86 (x 3.00 ± 0.25 in CCI + vehicle versus sham-operated rats and 1.89 ± 0.05 in CCI + BW732C86, $p < 0.01$) (Fig. 5B). Levels of activating transcription factor 3 (ATF3) mRNA, a marker of nerve injury [58], were also increased by CCI (x 15.92 ± 3.20 times higher than those in the sham-operated group) and reduced ($p < 0.05$) in DRG from CCI + BW723C86 rats (by a factor of 4.78 ± 2.58) (Fig. 5C). Immunocytochemistry confirmed these data, by showing that BW723C86 decreased the labeling of all markers studied to levels lower than those in CCI rats. In ipsilateral lumbar DRG from rats in the CCI + vehicle group, intense immunolabeling for Iba1was observed around large cell bodies, whereas Iba1 labeling was weak in sham-operated rats. In DRG from CCI + BW723C86 rats, Iba1 immunolabeling was markedly reduced. Nuclear ATF3 labeling, which was absent from sham-operated rats, was induced in CCI + vehicle rats and weaker in CCI + BW723C86 rats than in CCI + vehicle rats. Similar observations were made for GFAP labeling (Fig 5D). We also studied the induction of cytokines classically activated by nerve injury [31,32,40,55]. Seventeen days after surgery, CCI + vehicle-induced IL-6 mRNA upregulation (x 19.40 ± 2.76 versus sham-operated) was 47 % lower in CCI + BW723C86 rats (by a factor of 10.30 ± 3.85 with respect to sham-operated rats) (Fig. 5E). The CCI-induced upregulation of IL-1β mRNA was also decreased by BW723C86 (Fig. 5F).

In the sciatic nerve, CCI-induced ITGAM mRNA upregulation was significantly weaker, by 39.3 % in CCI + BW723C86 rats (factor of 8.06 ± 1.17 versus x 4.89 ± 1.04, $p < 0.05$), 17 days after surgery, (Fig. 6A). Moreover, the increase in the number of Iba1-labeled macrophages 17 days after CCI + vehicle application close to the lesion site (244.35 ± 45.00 cells/mm^2 versus 23.75 ± 3.86 cells/mm^2 in sham-operated rats), was reduced in CCI + BW723C86 rats (81.81 ± 18.02 cells/mm^2, $p < 0.05$) (Fig. 6B). BW723C86 application also markedly decreased, by 47.8%, the increase in IL-6 mRNA levels induced by CCI (x 6.05 ± 0.71 versus x 3.16 ± 0.37 in CCI + BW723C86, $p < 0.01$) (Fig. 6D) and that in IL-1β mRNA levels, by 43.2%, (x 6.88 ± 0.51 versus x 3.91 ± 0.37 in CCI + BW723C86, $p < 0.05$) (Fig. 6E).

DISCUSSION

We provide here, the first demonstration of an anti-nociceptive effect of peripheral 5-HT$_{2B}$R on neuropathic pain. Intrathecal injection of RS127445, a selective 5-HT$_{2B}$R antagonist, markedly enhanced mechanical and cold allodynia, suggesting an effect at the peripheral and/or spinal levels. Indeed, 5-HT$_{2B}$R was expressed in the spinal cord and DRG. In the DRG, basal levels of 5-HT$_{2B}$R mRNA were low, accounting for the lack of detection of this transcript in most studies [34,43,49,63]. Strong CCI-induced upregulation of 5-HT$_{2B}$R mRNA levels in the DRG ipsilateral to the lesion (5-HT$_{2B}$R levels were unaffected in the spinal cord) identified this region as the principal target of the pharmacological effects. Strikingly, 5-HT$_{2B}$ receptor mRNA levels in the dorsal root ganglion displayed biphasic upregulation with a transient early increase, by a factor of up to 23, two days after surgery, well before the development of neuropathic pain, and a five times increase maintained until the pain disappeared. These observations suggest a role for 5-HT$_{2B}$R in both the initiation and maintenance of neuropathic pain. They also suggest that at least two different mechanisms may be involved.

Immunocytochemical data suggested that 5-HT$_{2B}$R was expressed in neurons and macrophages. The antibodies recognized the rat 5-HT$_{2B}$R, as shown by control experiments on transfected cells. However, they did not cross-react with the mouse receptor, precluding checks in 5-HT$_{2B}$R$^{-/-}$ mice, and they could not be used for western blotting. Moreover, the affinity and titer of these antibodies was not compatible with receptor detection in DRG and sciatic nerve from normal rats, in which even 5-HT$_{2B}$R mRNA levels were low. Two days after surgery, neurons were immunolabelled, including the nociceptive C-neurons. Abundant ring-like 5-HT$_{2B}$R-positive macrophages were also observed, particularly around large A-neurons. Observations in the sciatic nerve two days after CCI largely confirmed the 5-HT$_{2B}$R expression in macrophages. Indeed, a doubling of 5-HT$_{2B}$R mRNA levels in the ipsilateral sciatic nerve of CCI rats with respect to the control reflected massive infiltration with 5-HT$_{2B}$R-expressing macrophages. Interestingly, peak levels of 5-HT$_{2B}$R occurred at the time corresponding to reported peak levels of blood-derived macrophage infiltration [64].

Nevertheless, we cannot rule out the possibility that 5-HT$_{2B}$R is also expressed by other cell types in the DRG, including satellite cells, in particular.

As 5-HT$_{2C}$R was absent from the sciatic nerves of control and CCI rats, we used the preferential 5-HT$_{2B}$R agonist BW723C86 to activate 5-HT$_{2B}$R selectively at this level. A single application of a very low dose, 64 ng, of BW723C86 just after CCI was sufficient to prevent the development of mechanical allodynia entirely and to decrease cold allodynia significantly. This effect was particularly remarkable, because few 5-HT$_{2B}$R were detected in the sciatic nerves of control animals: immunoreactivity was observed in scarce resident macrophages and 5-HT$_{2B}$R mRNA levels were low. Activation of the 5-HT$_{2B}$R present in the peripheral branches of sensory neurons is unlikely, because the pharmacological effect was fully antagonized by RS127445, excluding the activation of 5-HT$_{2c}$R, which was expressed 30 times more strongly in the DRG than 5-HT$_{2B}$R (unpublished observations). Interestingly, the 5-HT$_{2B}$R antagonist was ineffective when applied to the sciatic nerve, whereas it significantly increased CCI-induced allodynia when injected intrathecally. Thus, intrathecal, but not local sciatic nerve applications of RS127445, inhibited a tonic effect of 5-HT on 5-HT$_{2B}$R, further supporting the existence of different action mechanisms potentially related to the targeting of neurons or macrophages. An effect on spinal cord cells cannot be ruled out, despite the absence of 5-HT$_{2B}$R regulation by CCI. However, this would appear unlikely, because the opposite effect has been described in the spinal cord, in which C-fiber input is enhanced by the spinal superfusion of BW732C86 after spinal nerve ligation [2]. Alternatively, 5-HT$_{2B}$R may be regulated differently in this model.

The long-term preventive effect of 5-HT$_{2B}$R activation on CCI-induced pain was accompanied by a marked decreased in the increase in ITGAM mRNA levels induced by CCI in the ipsilateral DRG and sciatic nerve. There were also fewer macrophages, suggesting that sciatic nerve 5-HT$_{2B}$R activation may inhibit CCI-induced macrophage infiltration. The precise role of macrophages is not fully understood. On the one hand, they play a key role in the regeneration of damaged nerves through the phagocytosis of cellular debris. On the other, they are involved in pain behavior, via the release of numerous pro-inflammatory mediators [55]. Indeed, the depletion of macrophages or genetic ablation resulting in their absence have been shown to lead to a decrease in hypersensitivity in several neuropathic pain models [7,35,37]. However, several different populations of macrophage have been described in the injured nerve [26], potentially corresponding to different activation states. Macrophage function (inflammation/repair) is probably dependent on environment and time since injury. We found that 5-HT_{2B}R activation initially affected macrophage infiltration, decreasing the number of macrophages in the sciatic nerve of CCI rats treated with BW723C86 by 66% with respect to vehicle. However, the transient overproduction of 5-HT_{2B}R mRNA, between days 1 and 2, suggests that 5-HT_{2B}R expression in macrophages may be regulated. We observed that 5-HT_{2B}R expression in rat peritoneal macrophages was strongly increased by stimulation (unpublished data), suggesting the possibility of a similar phenomenon occurring in sciatic nerve macrophages. Such a phenomenon would cause a switch in the function of macrophages, from injury to repair. We observed marked reduction of CCI-induced IL-6 and IL-1β mRNA increase in the ipsilateral sciatic nerve and DRG after BW723C86. However, a direct relationship between 5-HT_{2B}R activation and the modulation of cytokine production remains to be firmly established. A subpopulation of 5-HT_{2B}R-positive immune-like cells with no labeling for macrophage markers was also observed in the sciatic nerve after CCI, suggesting that other blood-derived cells express this receptor. They may correspond to neutrophils involved in the very early stages following peripheral nerve injury [14,48], with a decrease in infiltration by these cells attenuating mechanical allodynia [25]. Other immune cells express the receptor [1], including T lymphocytes [56]. However, their involvement is unlikely, because T-lymphocyte infiltration occurs three days after the nerve lesion and peaks at day 21 [5] well after the observed peak in 5-HT_{2B}R mRNA levels observed in this study (day 2).

We also observed a significant decrease in CCI-induced GFAP mRNA and protein levels after the application of BW723C86 to the sciatic nerve. Thus, satellite cell activation, which occurs after macrophage invasion [5]; is also clearly reduced by 5-HT_{2B}R activation. As discussed above, the presence of 5-HT_{2B}R in satellite cells cannot be excluded. However, it is interesting to note that 5-HT_{2B}R expression is not regulated in the spinal cord, suggesting a lack of association with the activation of astrocytes and microglia, key players in the neuropathic pain induced by CCI.

Finally, the significantly lower levels of ATF3 mRNA and immunolabeling in the ipsilateral DRG of CCI rats treated with BW723C86 shows that the attenuation of pain behavior is associated with lower neuronal alterations. We showed that sciatic nerve 5-HT$_{2B}$R activation is more effective for preventing CCI-induced mechanical allodynia than cold allodynia. Interestingly, 5-HT$_{2B}$R-positive macrophages are predominantly localized around large cell bodies that are presumably responsible for mechanical transduction. This distribution of macrophages has already been described [60] and a clear relationship was observed between tactile allodynia and macrophage infiltration [16].

In conclusion, our study shows for the first time that peripheral 5-HT$_{2B}$R activation can both prevent and cure CCI-induced neuropathic pain, whereas previous studies have reported opposite findings in other pain models, such as migraine [29,50,51] and visceral pain [45,47]. In these cases, the etiology of pain is quite different from that of neuropathic pain. For example, in migraine, 5-HT$_{2B}$R involvement is linked to vascular activation, and we detected no 5-HT$_{2B}$R expression on sciatic nerve or DRG blood vessels. These observations demonstrate the need for careful comparisons of pain models. Indeed, in neuropathic pain, the involvement of immune, glial and neuronal cells is differs considerably as a function of the etiology of the pain.

We provide the first demonstration of a relationship between 5-HT and the immune system in the genesis of neuropathic pain induced by chronic constriction of the sciatic nerve, by showing that 5-HT$_{2B}$R plays a critical role in blood-derived macrophages. Our data suggest that it may be possible to prevent the neuropathic pain caused by nerve lesions, corresponding to the most severe and frequent peripheral neuropathies, and that selective serotonin reuptake inhibitors, acting at the peripheral level, may be useful for treating peripheral neuropathic pain due to traumatic nerve injury, as frequently observed after surgery.

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FIGURE LEGENDS

Figure 1:

A-B: intrathecal RS127445, a 5-HT_{2B}R antagonist, enhances CCI-induced allodynia.

Two intrathecal injections of RS127445 (125 ng) on days 17 and 18 (arrows), a time corresponding to full pain development, enhanced CCI-induced mechanical (A) and cold (B) allodynia. Paw withdrawal thresholds are expressed in grams (g) and cold scores in arbitrary units (A.U.), as mean ± SEM time after surgery (in days). CCI + vehicle: n = 8, CCI + RS127445: n = 6, sham + vehicle: n = 4. +$p < 0.001$: CCI + vehicle versus sham + vehicle rats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: CCI rats treated with RS127445 versus CCI rats receiving vehicle. Two-way ANOVA for repeated measures was carried out, followed by Bonferroni post-hoc test.

C: Expression profile for 5-HT_{2B}R mRNA in DRG and spinal cord dorsal (DH) and ventral horns (VH) 14 days after CCI. 5-HT_{2B}R mRNA levels are higher in the DRG ipsilateral to the lesion (iDRG) than in the DRG from sham-operated rats. 5-HT_{2B}R mRNA levels are unchanged in the contralateral DRG (cDRG) and in the spinal cord. Data are expressed in arbitrary units, as a relative quantity (R.Q.) with respect to control rats, as the mean ± SEM for four rats/group (*** $p < 0.001$, *t*-test values for comparison of CCI with sham-operated rats). D: Time-course of 5-HT_{2B}R mRNA production in the DRG during neuropathic pain development after CCI. The curve indicates mechanical withdrawal thresholds expressed in grams (g). The basal threshold is 15; the lower threshold corresponds to maximal pain after CCI. After CCI, 5-HT_{2B}R mRNA levels were maximal in the ipsilateral DRG on day 2, well before the development of mechanical allodynia, which appeared between days 8 and 14. High 5-HT_{2B}R mRNA levels are maintained until the pain decreases. n = 4 rats/group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). One-way ANOVA was carried out, followed by Newman-Keuls post-hoc test.

In D: # $p < 0.001$ for mechanical withdrawal threshold in CCI rats compared to baseline values (two way ANOVA for repeated measures, followed by Bonferroni post-hoc test.

Figure 2:

A: 5-HT_{2B}R immunocytochemistry on transfected COS-7 cells: immunopositive cell bodies are observed on COS-7 cells transfected with the rat 5-HT_{2B}R. No labeling is seen after transfection with a C-terminally truncated form of the receptor. Scale bars = 200 µM. B: ^{3}H LSD binding assays on COS-7 cells transfected with the rat 5-HT_{2B}R or with the truncated C-terminal form of the 5-HT_{2B}R and various concentrations of RS127445 are shown. C: 5-HT_{2B}R immunoreactivity in ipsilateral lumbar DRG. Lumbar DRG from sham-operated rats (upper panel) displays an almost complete absence of 5-HT_{2B}R immunoreactivity (green). Forty-eight hours after CCI, 5-HT_{2B}R immunoreactivity is enhanced in neurons, particularly in small cell bodies (white arrows), and is present in numerous macrophages surrounding large cell bodies, labeled with Iba1 in red (open arrows). Scale bars: 50 µm.

Figure 3:

A: Forty-eight hours after CCI, 5-HT_{2B}R mRNA levels are higher in the lesioned sciatic nerve. Data are expressed in arbitrary units, as a relative quantity (R.Q.) with respect to sham-operated rats, as the mean ± SEM (**$p < 0.01$, t-test, n = 4/group). B: In the sciatic nerve from sham-operated rats, 5-HT_{2B}R immunolabelling is almost absent, except in a few resident macrophages labeled for Iba1 (orange). C: 48 h after CCI, 5-HT_{2B}R/Iba1 double-labeling shows a large infiltration of Iba1-positive macrophages also expressing 5-HT_{2B}R close to the ligature (arrow). A subpopulation of 5-HT2BR cells is unlabeled for Iba1 (insert). Scale bars: 60 µm for low magnification, 20 µm for high magnification.

Figure 4:

Sciatic nerve application of BW723C86 (125 ng) totally prevents mechanical allodynia (A), and delays the onset and decreases the severity of the cold allodynia (B) induced by CCI. Application of vehicle or RS127445 has no effect on allodynia. Withdrawal thresholds are expressed in grams (g) and cold scores, in arbitrary units (A.U.) as means ± SEM (sham: n = 9, CCI: n =12, CCI + vehicle: n = 7, CCI + BW723C86: n = 15, CCI + RS127445: n = 12). C: The anti-allodynic effect of BW723C86 is dose-dependent (n = 4 rats/group). D: the effect of BW723C86 is antagonized by RS127445 (n = 5 rats/group). * $p < 0.05$, *** $p < 0.001$: CCI+ BW723C86 versus CCI + vehicle; +++ $p < 0.001$: CCI +

vehicle versus sham-operated. Two-way ANOVA for repeated measures and Bonferroni post-hoc tests were carried out in all cases.

Figure 5:

A single sciatic nerve application of BW723C86 decreases “pain markers” 17 days after CCI in the ipsilateral DRG. BW723C86 reduces CCI-induced upregulation in ITGAM (A), GFAP (B) and ATF3 (C) mRNA. D: Immunocytochemistry shows lower levels of Iba1, ATF3 and GFAP labeling in DRG from CCI + BW723C86 than in CCI + vehicle. Scale bars: 40 µm. The overproduction of IL-6 (E) and IL-1β (F) mRNA induced by CCI is decreased by BW723C86 treatment (E). RT-PCR data are expressed in arbitrary units, as relative quantity (R.Q.) with respect to control (saline or sham-operated), as means ± SEM for at least four animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: CCI + vehicle versus sham-operated rats, + $p < 0.05$ and ++ $p < 0.01$: CCI + BW723C86 versus CCI + vehicle. One-way ANOVA followed by Newman-Keuls post-hoc test was applied in all cases.

Figure 6:

A single sciatic nerve application of BW723C86 decreases “pain markers” 17 days after CCI in the sciatic nerve. BW723C86 decreases CCI-induced ITGAM mRNA upregulation (A) and Iba1-labeled cell number (B), as shown in representative micrographs (C). Scale bar = 20 µm. Upregulation of IL-6 (D) and IL-1β (E) mRNA levels by CCI + vehicle versus sham-operated is weaker in CCI + BW723C86 rats. RT-PCR data are expressed as a relative quantity (RQ) with respect to the mean ± SEM for saline, for at least four rats/group. * $p < 0.05$ and ** $p < 0.01$: CCI + vehicle versus sham-operated rats; + $p < 0.05$: CCI + BW723C86 versus CCI + vehicle. Cell counts are expressed as mean cell number/mm^2 ± SEM *** $p < 0.001$: CCI + vehicle versus sham-operated rats; ++ $p < 0.01$: CCI + BW723C86 versus CCI + vehicle. One-way ANOVA followed by Newman-Keuls post-hoc test was applied in all cases.

***Summary**

Preventive and curative effects of peripheral 5-HT_{2B} receptor activation are shown in a rodent model peripheral neuropathy partly involving blood-derived macrophage bearing the 5-HT_{2B} receptor.

Figure 1

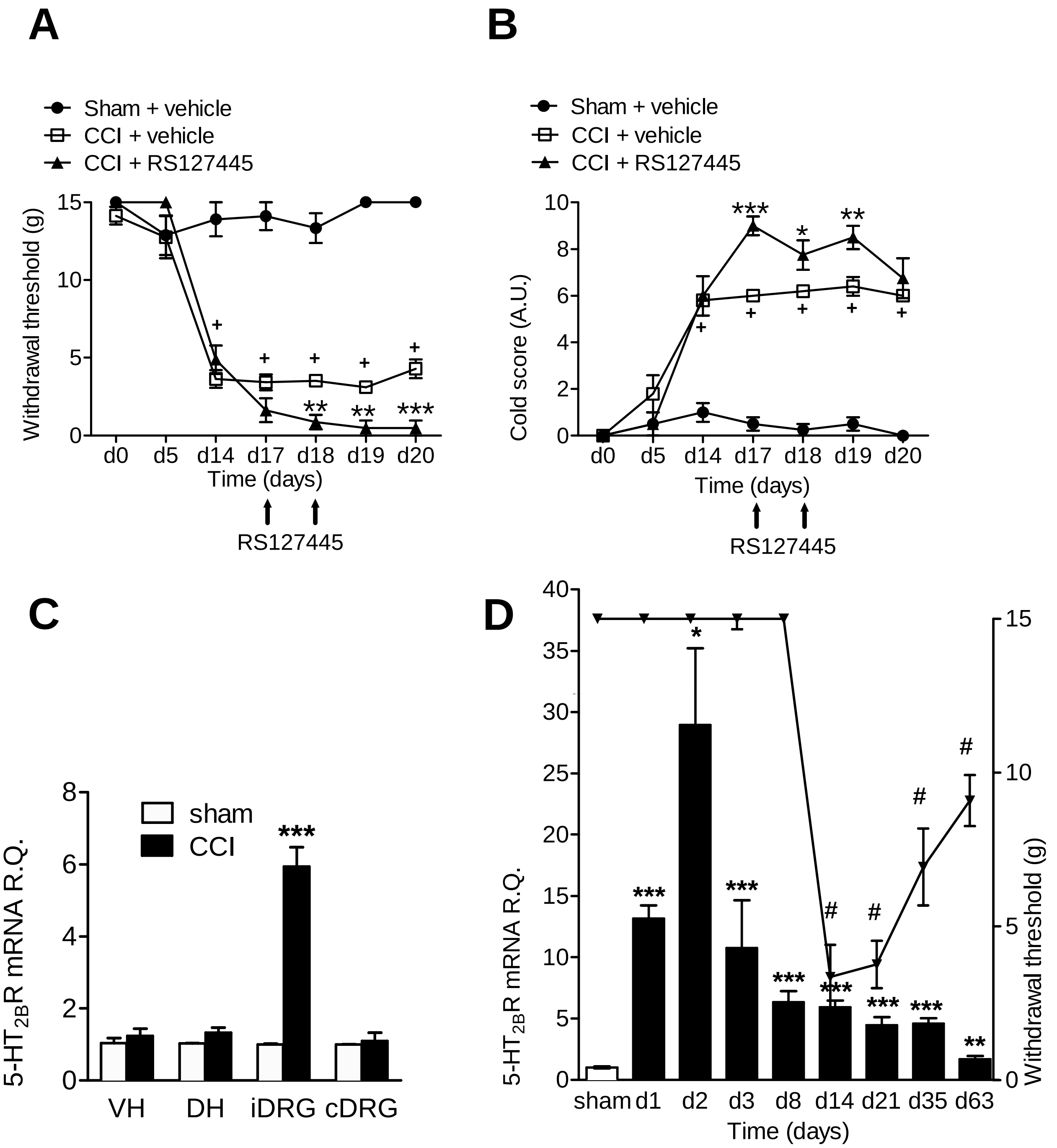

A
Sham + vehicle
CCI + vehicle
CCI + RS127445
Withdrawal threshold (g)
15
10
5
0
+
+
+
+
+
**
**
***
d0
d5
d14
d17
d18
d19
d20
Time (days)
RS127445
B
Sham + vehicle
CCI + vehicle
CCI + RS127445
Cold score (A.U.)
10
8
6
4
2
0
***
*
**
+
+
+
+
+
d0
d5
d14
d17
d18
d19
d20
Time (days)
RS127445
C
sham
CCI
***
5-HT_{2B}R mRNA R.Q.
8
6
4
2
0
VH
DH
iDRG
cDRG
D
40
35
30
25
20
15
10
5
0
*
***
***
***
***
***
***
**
#
#
#
#
#
15
10
5
0
5-HT_{2B}R mRNA R.Q.
Withdrawal threshold (g)
sham
d1
d2
d3
d8
d14
d21
d35
d63
Time (days)

Figure 2

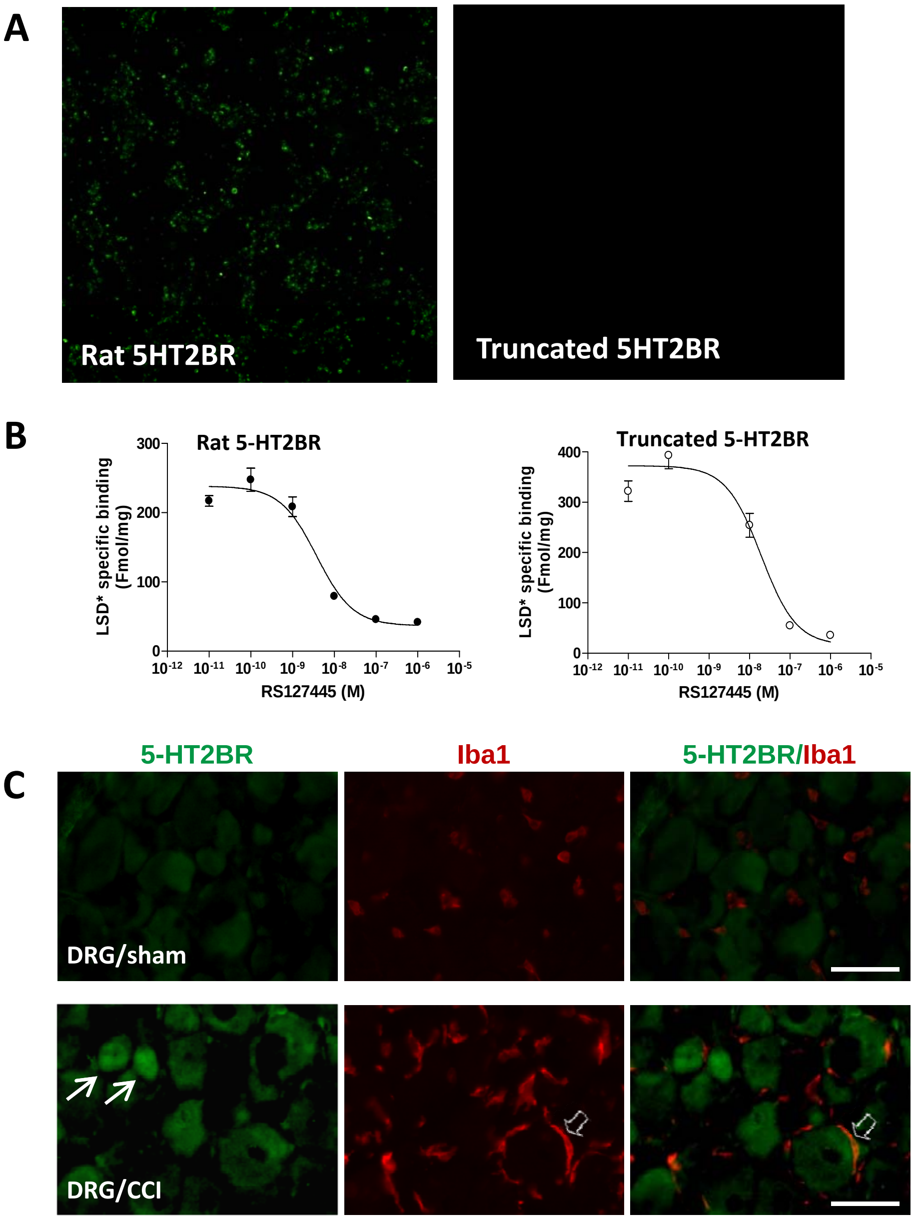

A
Rat 5HT2BR
Truncated 5HT2BR
B
Rat 5-HT2BR
Truncated 5-HT2BR
LSD* specific binding (Fmol/mg)
0
100
200
300
400
10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}
RS127445 (M)
C
5-HT2BR
Iba1
5-HT2BR/Iba1
DRG/sham
DRG/CCI

Figure 3

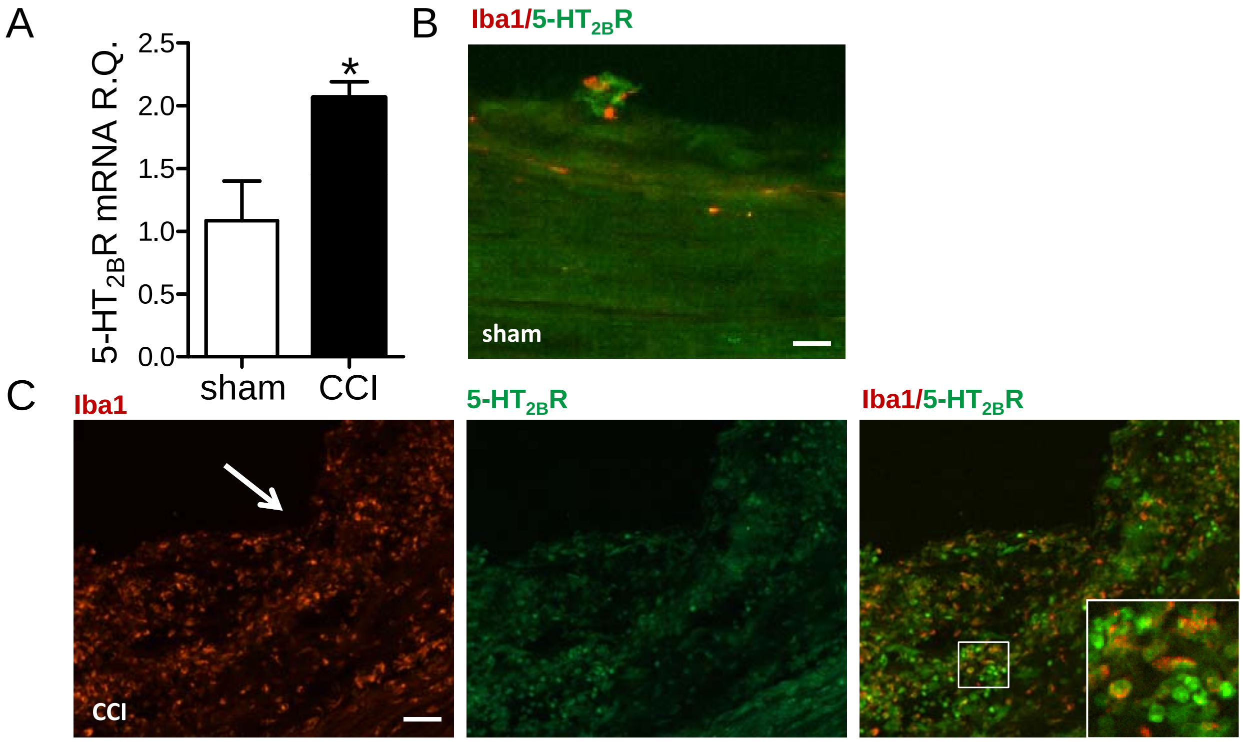

A
5-HT$_{2B}$R mRNA R.Q.
2.5
2.0
1.5
1.0
0.5
0.0
*
sham
CCI
B
Iba1/5-HT$_{2B}$R
sham
C
Iba1
CCI
5-HT$_{2B}$R
Iba1/5-HT$_{2B}$R

Figure 4

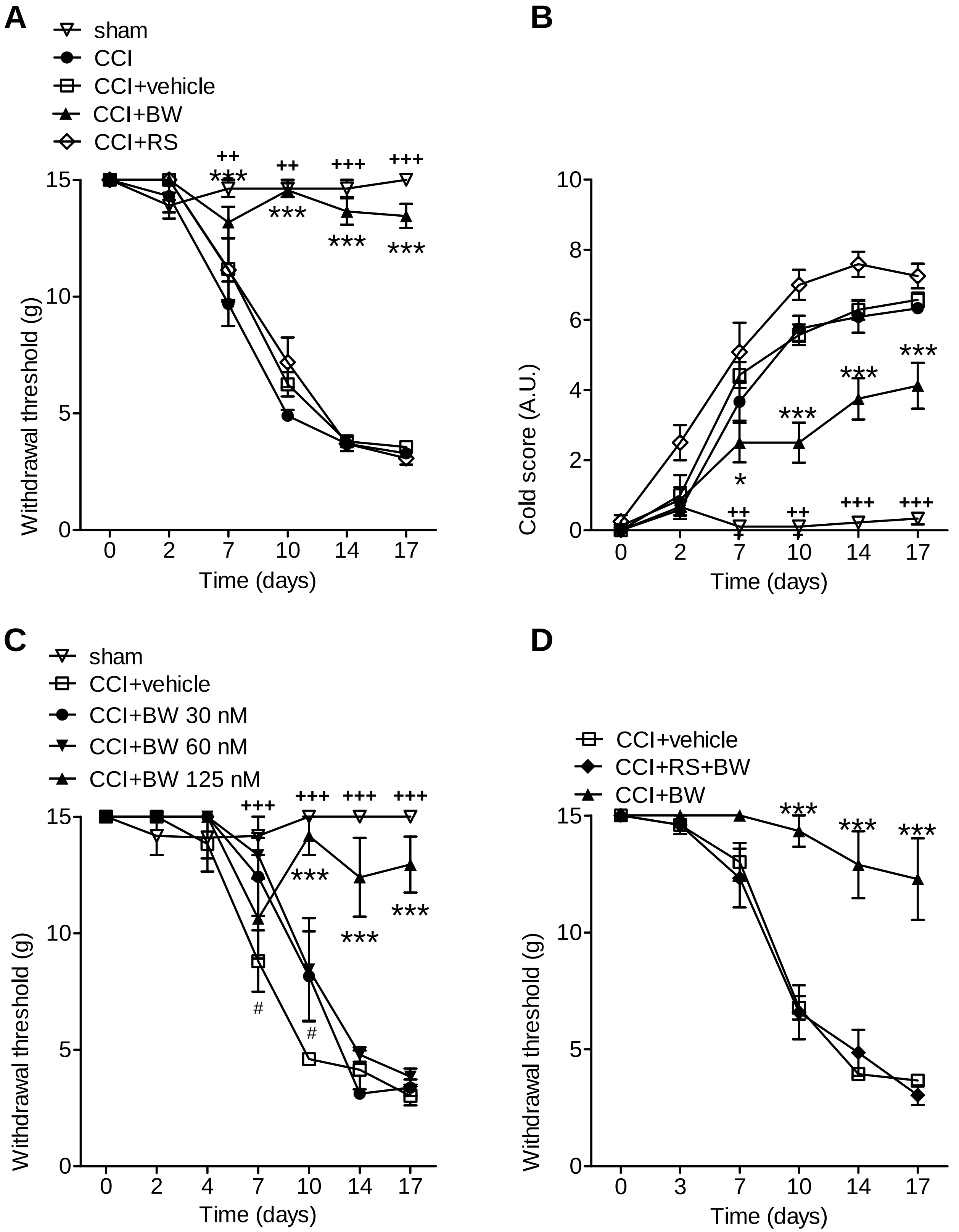

A
sham
CCI
CCI+vehicle
CCI+BW
CCI+RS
++
***
++
***
+++
***
+++
***
Withdrawal threshold (g)
0
5
10
15
0
2
7
10
14
17
Time (days)
B
Cold score (A.U.)
0
2
4
6
8
10
*
***
***
***
++
++
+++
+++
0
2
7
10
14
17
Time (days)
C
sham
CCI+vehicle
CCI+BW 30 nM
CCI+BW 60 nM
CCI+BW 125 nM
+++
+++
+++
+++
***
***
***
#
#
Withdrawal threshold (g)
0
5
10
15
0
2
4
7
10
14
17
Time (days)
D
CCI+vehicle
CCI+RS+BW
CCI+BW
***
***
***
Withdrawal threshold (g)
0
5
10
15
0
3
7
10
14
17
Time (days)

Figure 5

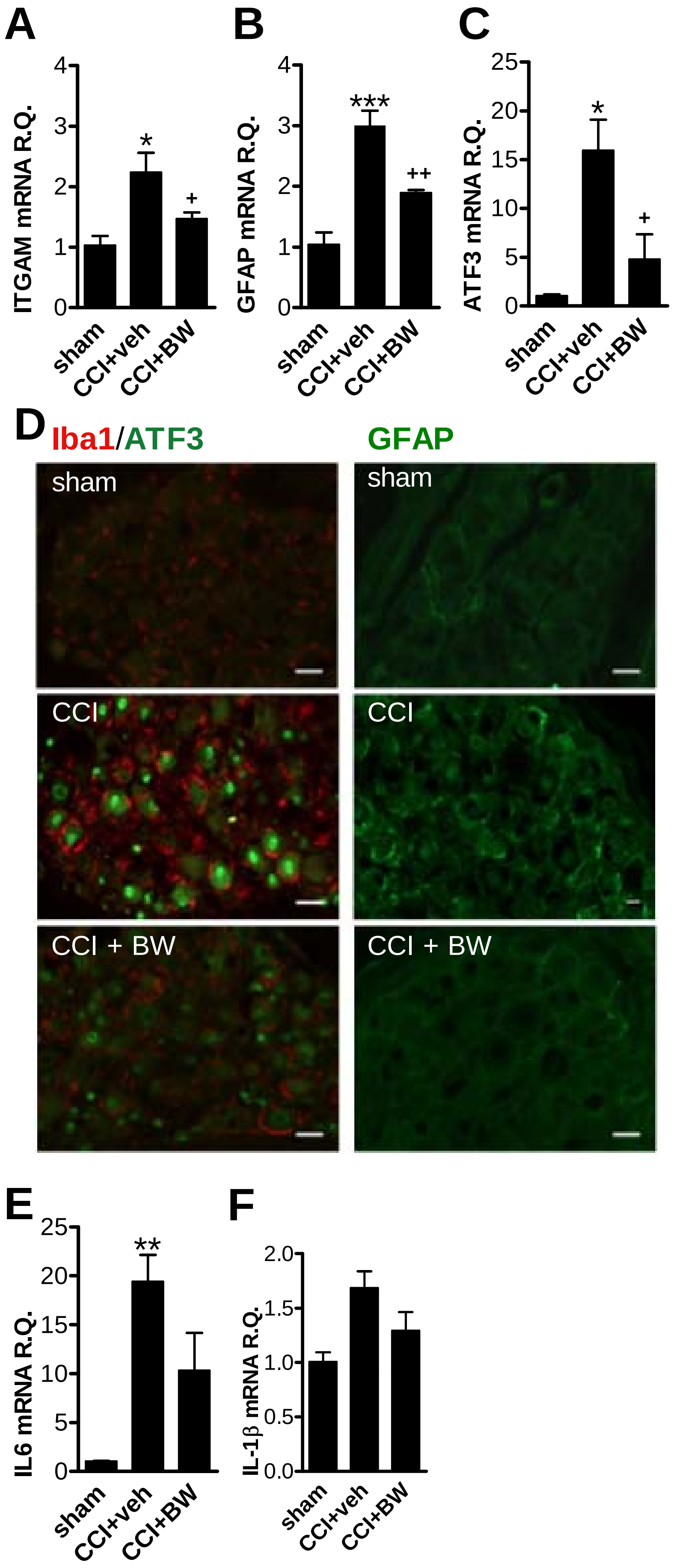
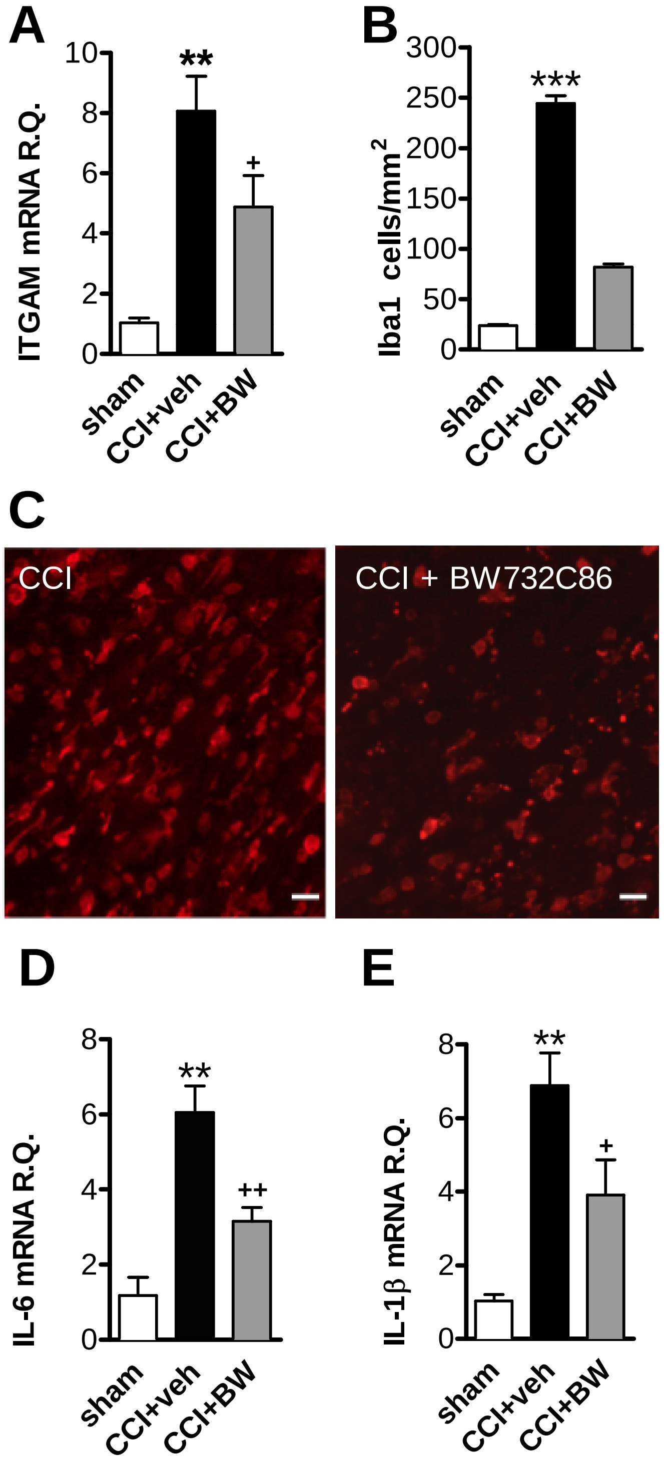

A
ITGAM mRNA R.Q.
0
1
2
3
4
*
+
sham
CCI+veh
CCI+BW
B
GFAP mRNA R.Q.
0
1
2
3
4
***
++
sham
CCI+veh
CCI+BW
C
ATF3 mRNA R.Q.
0
5
10
15
20
25
*
+
sham
CCI+veh
CCI+BW
D
Iba1/ATF3
GFAP
sham
CCI
CCI + BW
sham
CCI
CCI + BW
E
IL6 mRNA R.Q.
0
5
10
15
20
25
**
sham
CCI+veh
CCI+BW
F
IL-1β mRNA R.Q.
0.0
0.5
1.0
1.5
2.0
sham
CCI+veh
CCI+BW

Figure 6


A
ITGAM mRNA R.Q.
0
2
4
6
8
10
**
+
sham
CCI+veh
CCI+BW
B
Iba1 cells/mm^2
0
50
100
150
200
250
300
***
sham
CCI+veh
CCI+BW
C
CCI
CCI + BW732C86
D
IL-6 mRNA R.Q.
0
2
4
6
8
**
++
sham
CCI+veh
CCI+BW
E
IL-1β mRNA R.Q.
0
2
4
6
8
**
+
sham
CCI+veh
CCI+BW