Supporting Information

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SI Materials and Methods

Animals. Male Wistar rats (180–220 g) were housed in temperature-controlled rooms (22–25 °C), with access to water and food ad libitum. All experiments were conducted in accordance with the International Association for the Study of Pain guidelines (1) and with the approval of the Ethics Committee of the Faculty of Medicine of Ribeirao Preto (University of Sao Paulo). The animals were used only in a single experimental group.

Drugs. The drugs used in this study were dopamine, indomethacin, fluorocitrate, doxycycline, and prostaglandin E_2 (PGE_2) (Sigma), infliximab (Schering-Plough), thalidomide (RBI), and TNFα, IL-1β, and IL-1 receptor antagonist (IL-1ra) (NIBSC). Endotoxin-free rabbit anti-rat CX3CR1 (Torrey Pines Biolabs), anti-rat CX3CL1/fractalkine (neutralizing) (eBioscience) and carrageenin (FMC). A stock solution of PGE_2 (1 μg/μL) was prepared in 10% ethanol, and dilutions were made in 0.9% NaCl (saline); the final concentration of ethanol was 1%. Other drugs were diluted in sterile saline. It is important to mention that, in the present study, the chemokine domain of recombinant rat CX3CL1/fractalkine (amino acids from 22 to 100) was used. This sample was purchased from R&D Systems (catalog no. 568-FR). This active domain of fractalkine has been used by other investigators who evaluate the pronociceptive effects of this chemokine (2).

Drug Administration. ***Intraplantar administration.*** Intraplantar (i.pl.) injections (100 μL) were performed in conscious animals with a 27-gauge hypodermic needle introduced into the s.c. tissue near the third digit, with the needle tip reaching the middle of the plantar area (3).

Intraganglionar administration. The intraganglionar (i.gl.) drug administration was carried out in accordance with ref. 4. Briefly, the rats were anesthetized by the inhalation of 2% isoflurane and placed over a small cylinder to elevate the lumbar region. The point of skin puncture was defined at 1.5 cm laterally to the vertebral column, ∼0.5 cm caudal from a virtual line passing over the rostral borders of the iliac crests. The injecting needle was inserted through the punctured skin, toward the intervertebral space between the fifth and sixth lumbar vertebrae, until the tip touched the lateral region of the vertebrae. To reach the space between the transverse processes of the fifth and sixth vertebrae, smooth movements of the needle were made until the bone resistance was diminished and a paw flinch reflex was observed. The paw flinch reflex was used as a sign that the needle tip penetrated the DRG of the fifth lumbar spinal nerve located underneath the transversal process of the fifth lumbar vertebra. The entire procedure required ∼3 min. The animals regained consciousness ∼1 min after the discontinuation of the anesthesia.

SGC Isolation and Culture. Satellite glial cells (SGCs) were isolated from primary cultures of rat dorsal root ganglion (DRG) (5). DRG cell cultures were prepared from male Wistar rats (80 g) that were killed under urethane anesthesia. The lumbar and thoracic DRG were harvested and transferred to Hank's Buffered Saline Solution containing Hepes (10 mM). The ganglia were incubated in 0.28 U/mL collagenase (type II; Sigma) for 75 min and in 0.25% trypsin (Sigma-Aldrich) for 12 min. After three washes with DMEM containing 10% FCS, the ganglia were dissociated using a fire-polished glass Pasteur pipette. The dissociated cells were plated on Matrigel-coated glass bottom dishes in DMEM containing 10% FCS and penicillin (50 U/mL)/streptomycin (50 mg/mL). The cultures were maintained in a humid 5% CO_2 atmosphere at 37 °C for 24–72 h. After ∼3 d in vitro, the glial cells began to undergo more rapid cell division and approached confluence at ∼5–7 d after dissociation. It was therefore necessary to separate the cells to obtain single cells for stimulation and cytokines and prostanoid evaluation. This condition was achieved by the resuspension of the cells and gentle trituration with a cell scraper; the cells were then centrifuged at 200 × *g* for 4 min. The cells were plated on coated glass bottom dishes in DMEM containing 10% FCS and penicillin (50 U/mL)/streptomycin (50 mg/mL). The cultures were maintained in a humid 5% CO_2 atmosphere at 37 °C for 24–72 h. This procedure virtually eliminated neurons from the cultures because the dishes were not treated with Matrigel, which allows adherence of cell soma to the culture dish. The attachment and growth of the glial cells was unaffected by this procedure. The experiments were performed 6–8 d after the initial dissociation of the ganglia.

Evaluation of Mechanical Nociceptive Threshold. The mechanical nociceptive threshold was measured with an electronic pressure meter. The rats were placed in acrylic cages (12 × 20 × 17 cm in height) with a wire grid floor, 15–30 min before the start of the tests. During this adaptation period, the paws were poked two to three times. Before paw stimulation, the animals should have been quiet, without exploratory movements and not resting over the paws. In these experiments, a pressure meter, which consisted of a hand-held force transducer adapted with a 0.7-mm^2 polypropylene tip (electronic von Frey anesthesiometer; IITC Inc. Life Science Instruments) was used. The investigator was trained to apply the polypropylene tip perpendicularly in between the five distal footpads with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal hind paw. The test consisted of poking the hind paw to provoke a flexion reflex followed by a clear flinch response after the paw withdrawal. The electronic pressure meter automatically recorded the intensity of stimulus when the paw was withdrawn. The upper limit of the calibration range in which the pressure was linearly detectable by the equipment was 80 g. The animals were tested before and after treatments, and the results are expressed as delta reaction force (grams), which was calculated by subtracting the value of the initial measurements from the final ones (3).

RNA Extraction and Real-Time PCR. At 3, 6, and 24 h after the paw administration of carrageenin or saline, the rats were terminally anesthetized, and the ipsilateral DRGs [fifth lumbar vertebra (L5)] were removed. The samples were homogenized in 1 mL of TRIzol (Invitrogen), and total RNA was extracted according to the manufacturer's instructions. The purity of the total RNA was measured with a spectrophotometer, and the wavelength absorption ratios (260/280 nm) were between 1.8 and 2.0 for all preparations. The reverse transcription of total RNA to cDNA was carried out with a reverse transcription reaction (Superscript II, Gibco Life Technologies). Real-time PCR was performed using primers specific for the rat gene GFAP and for the rat housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The reactions were conducted with the ABI Prism 7500 Sequence Detection System using the SYBR-green fluorescence system (Applied Biosystems) (6). The primer pairs for rat *GFAP* and *Gapdh* were as follows: GFAP fwd, 5′-CCT CGG CAC CCT GAG GCA GA-3′; GFAP rev, 5′-AGC CAA GGT GGC TTC ATC CGC-3′; *Gapdh* fwd, 5′-CAG TGC CAG CCT CGT CTC ATA-3′; and *Gapdh* rev, 5′-TGC CGT GGG TAG AGT CAT A-3′.

Western Blot Analysis. After the indicated stimulation, the DRG cells were homogenized in a lysis buffer containing a mixture of protease inhibitors and phosphatase inhibitors (Sigma-Aldrich). The protein concentrations of the lysate were determined using a BCA Protein Assay kit (Pierce), and 10 or 50 μg of GFAP and ionized calcium binding adaptor molecule-1 (IBA-1), respectively, of protein was loaded for each lane. The protein samples were separated on an SDS/PAGE gel and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). The filters were blocked with 7% dry milk, incubated overnight at 4 °C with a primary antibody against GFAP (1:500; Millipore) and IBA-1 (1:300; Wako Chemicals) and then incubated for 1 h at room temperature with an HRP-conjugated secondary antibody (1:3,000; Jackson ImmunoResearch). The blots were visualized in an ECL solution (Millipore) for 2 min and exposed in a ChemiDoc MP Imaging System (Bio-Rad Laboratories) for 1–20 min (7).

Cytokine Measurements. The supernatants of isolated/cultured SGCs were collected 6 h after incubation with fractalkine (1–100 ng/mL) or medium (control) treatment. The supernatants were used to determine the levels of TNF-α and IL-1β by ELISA, as described previously (8). Briefly, microtiter plates were coated overnight at 4 °C with an immunoaffinity-purified polyclonal sheep antibody against TNF-α (2 μg/mL) or IL-1β (2 μg/mL). After the blocking of the plates, recombinant rat TNF-α or IL-1β standards at various dilutions and the samples were added to the plates in duplicate and incubated overnight at 4 °C. Rabbit biotinylated immunoaffinity-purified antibodies against TNF-α (1:500) or IL-1β (1:1,000) were added, followed by incubation at room temperature for 1 h. Then, 50 μL of avidin-HRP (1:5,000 dilution; DAKO) was added to each well. After 30 min, the plates were washed, and the color reagent OPD (200 μg per well; Sigma) was added. After 15 min, the reaction was stopped with 1 M H_2SO_4, and the optical density (O.D.) was read at 490 nm. The results are expressed as picograms (pg) of each cytokine per mg of protein from the lysed cells.

Measurement of PGE_2 in the Culture of Satellite Cells by RIA. The supernatants of isolated, cultured SGCs were collected 6 h after incubation with fractalkine (100 ng/mL) or medium, as described above. The supernatants were centrifuged at 1,500 × *g* for 10 min at 4 °C. The concentration of PGE_2 in these samples was then measured by RIA using a commercially available kit. The results are expressed as picograms of PGE_2 per mg of protein from lysed cells (8).

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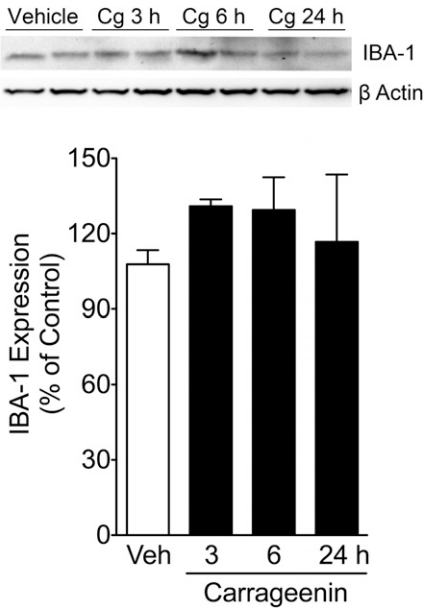

Fig. S1. IBA-1 expression in DRGs (L5) after peripheral inflammation. The rats received an intraplantar injection of carrageenan (Cg; 100 μg per paw) or saline. At the indicated time points, IBA-1 expression was conducted on protein samples isolated from DRGs (L5) 3, 6 and 24 h after carrageenin or saline injection in the rat paw. The Western blot analysis was performed using Image Lab Software, version 3.0. ($n = 6$).

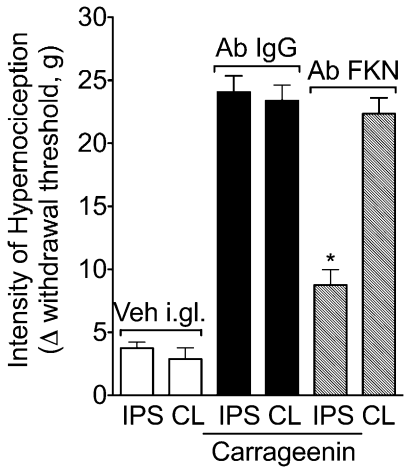

Fig. S2. Effect of the neutralizing antibody against fractalkine (Ab FKN) in inflammatory hypernociception induced by carrageenin. The rats were pretreated (1 h) with Ab FKN (10 μg) or IgG control injected into the DRG (L5) isilateral (IPS) or contralateral (CL), followed by the injection of carrageenin (100 μg per paw) and mechanical hypernociception was evaluated 3 h after carrageenin injection. The data are expressed as the mean ± SEM of five rats per group. "*" indicates statistical significance compared with Ab control-treated group.

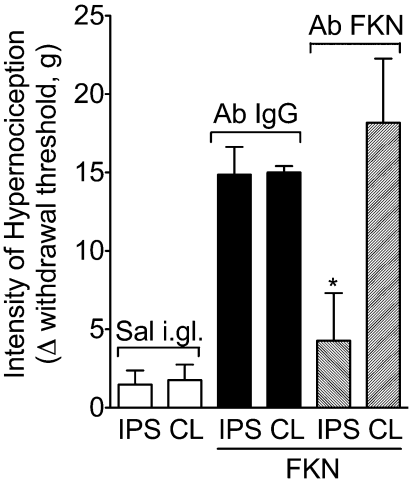

Fig. S3. Effect of the neutralizing antibody against fractalkine (Ab FKN) in hypernociception induced by ig.l. fractalkine. The rats were pretreated (1 h) with Ab FKN (10 μg) or Ab IgG control injected into the DRG (L5) ipsilateral or contralateral, followed by the injection of FKN (50 ng per i.gl.), and mechanical hypernociception was evaluated 1 h after FKN injection. The data are expressed as the mean ± SEM of five rats per group. "*" indicates statistical significance compared with Ab control-treated group.

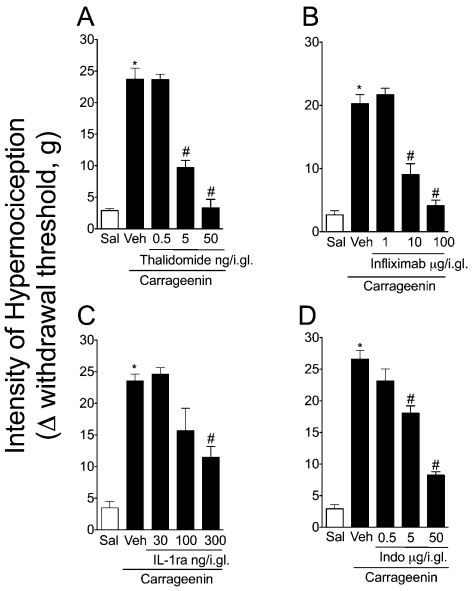

Fig. S4. Role of TNF-α, IL-1β, and prostanoids released into the DRG in the genesis of inflammatory pain. The rats were pretreated with (*A*) thalidomide (0.5–50 ng per DRG), (*B*) infliximab (1–100 μg per DRG), (*C*) IL-1ra (30–300 ng per DRG), or (*D*) indomethacin (0.5–50 μg per DRG) injected into the DRG. After 1 h, carrageenin (100 μg per i.pl.) was injected into the ipsilateral paw, and mechanical hypernociception was evaluated 3 h after carrageenin injection. The data are expressed as the mean ± SEM of 10 per experiment. "*" indicates statistical significance compared with the saline-injected (paw) group; "#" indicates statistical significance compared with the vehicle-treated group. $P < 0.05$, one-way ANOVA, followed by the Bonferroni correction.

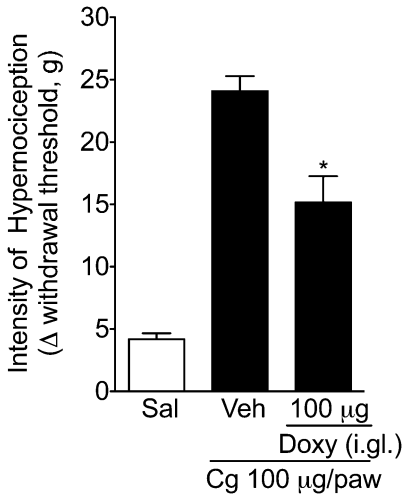

Fig. S5. Effect of the doxycycline (Doxy) in inflammatory hypernociception. The rats were pretreated i.gl. with doxycycline (100 μg per DRG). After 1 h, carrageenan (Cg) (100 μg per i.pl.) or saline (Sal) was injected into the ipsilateral paw, and mechanical hypernociception was evaluated 3 h after carrageenin injection. Data are expressed as the mean ± SEM of five rats per experimental group. "*" indicates statistical significance compared with the vehicle-injected (paw) group.