Supporting Information

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SI Materials and Methods

Animals. Male Wistar rats (100 g for in vitro tests; 180–200 g for behavioral tests) were housed in temperature-controlled rooms (22–25 °C) with an alternating 12-h light–dark cycle. Water and food were provided ad libitum. All experiments were conducted in accordance with National Institutes of Health Guidelines for the Welfare of Experimental Animals (1) and with the methodology approved by the Ethics Committee of the School of Medicine of Ribeirão Preto (University of São Paulo). Each animal was used only in a single experimental group.

Drugs. The substances used in this study were PGE_2 (Sigma/RBI; stock solutions at 1 μg/μL were prepared in 10% (vol/vol) ethanol, and additional dilutions were made in saline; the final concentration of ethanol was ∼1%); NMDA (*N*-methyl-D-aspartate receptor agonist; Tocris; diluted in saline); the NMDAR antagonist D-AP-5 (kindly provided by J. Garthwaite, Welcome Foundation, London; diluted in saline); the nonselective COX inhibitor indomethacin (1-[P-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid; Indo; Sigma; diluted in Tris·HCl buffer, pH 8.0); AMPA [(RS)-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPA receptor agonist; Tocris; diluted in saline]; and the AMPA receptor antagonist DNQX (Tocris Neuramin; diluted in saline).

ODNs. The AS-ODNs specific for each NMDAR subunit used in this study were purchased from Bioneer and designed based on published rat cDNA sequences available in online databases (PubMed–Medline). From the many possible sequences, the choices followed the criteria established in the literature (2) related to the size and proximity to the initial extremity of the mRNA and the number of guanine and cytosine residues. The AS-ODN sequences were as follows: NR1: TCTTCCAAGAGCCGTC; NR2A: TATCGGAGGCGTGGTCGTG; NR2B: TAGGTGACAGTGTGCGTGG; NR2C: TAAGGCGCCAACTCTCGGT; NR2D: TGAAGGTAGAGCCACCAGG; NR3A: TATCACCTGGCTTCGTGCG; and NR3B: TGTTGCCTCAGATCCGCCT.

As controls, the missense-ODN sequences corresponded to the AS-ODN sequences with the exception of four changed bases. Before administration into the right L5-DRG, each ODN was reconstituted in saline to a concentration of 10 μg/μL. Each ODN was administered via the i.gl. route (4 μg/μL) once daily for four (Fig. S6) or five (Fig. 5 and Fig. S5) consecutive days, depending on the experiment.

Drug Administration. The drugs were administered via the i.t., i.gl., or i.pl. route. Under light anesthesia [2% (vol/vol) isoflurane], the lumbar region of each rat was elevated with a small cylinder placed underneath the abdomen. The i.t. and i.gl. injections were performed in this position.

i.t. administration. A 26-gauge needle was directly inserted into the subarachnoid space between the L4 and L5 vertebrae (3). The correct position of the needle tip was confirmed by a characteristic flinch of the tail. A volume of 10 μL of the test agent was then slowly injected. The entire procedure, from the induction of anesthesia until recovery of consciousness, lasted ∼4 min.

i.gl. administration. A calibrated catheter prepared as described (4, 5) was used. Briefly, the point of skin puncture was defined at 1.5 cm lateral to the vertebral column and ∼0.5 cm caudal to a virtual line passing over the rostral borders of the iliac crests. An initial puncture with a larger needle (25 × 10; 19 gauge) was made to facilitate penetration of the injecting needle through the skin of the rat. In sequence, the injecting needle was inserted through the punctured skin toward the intervertebral space between the L5 and L6 vertebrae until the tip touched their lateral regions. To reach the space between the transverse processes of the L5 and L6 vertebrae, delicate movements of the needle were made until the bone resistance was diminished and an ipsilateral paw flinch reflex was observed as a sign that the needle tip had penetrated the DRG of the fifth lumbar spinal nerve (L5-DRG), located underneath the transverse process of the L5 vertebra. Once the L5-DRG was located, a volume of 5 μL of the solution was injected.

i.pl. administration. A 27-gauge hypodermic needle was introduced in the s.c. tissue near the third digit, with the needle tip reaching the middle of the plantar area (6), and a volume of 50 μL was slowly injected.

Mechanical Hyperalgesia Evaluation: Electronic von Frey Test. The change in the mechanical threshold was evaluated by the electronic von Frey method, as described by Vivancos et al. (7). In a quiet room, rats were placed in acrylic cages (12 cm × 20 cm × 17 cm) with wire grid floors 15–30 min before the start of testing. During this adaptation period, the paws were tested (probed) two or three times. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer adapted with a 0.7-mm^2 polypropylene tip (electronic von Frey; IITC Life Science). A tilted mirror placed under the grid provided a clear view of the rat's hind paw. The investigator was trained to apply the tip between the paw's five distal footpads with a gradual increase in pressure. The stimulus was automatically discontinued and its intensity recorded when the paw was withdrawn. The maximum force applied was 80 g. The stimulus was repeated (up to six times; usually three) until the animal presented three consistently similar measurements (differences <10%). The endpoint was characterized by removal of the paw in a clear flinch response following paw withdrawal. Animals were tested before and after the treatments. The results are expressed as the Δ paw withdrawal threshold (in grams), which was calculated by subtracting the average of the three measurements after the treatments from the average of the three measurements before the treatments.

Primary DRG Cultures. Dissociated cell cultures were prepared from male Wistar rats (100 g) that were euthanized under anesthesia. Lumbar and thoracic DRGs 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) for 12 min. After three washes with DMEM containing 10% FCS, the ganglionic cells were dissociated by using a fire-polished glass Pasteur pipette. Dissociated cells were plated in glass dishes with Matrigel-coated bottoms in DMEM plus 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 24–36 h. Fig. S4 shows a representative confocal image of a neuron and its closely associated satellite glial cells in cultured DRG responding to administration of NMDA (Fig. S4*B*) or capsaicin (Fig. S4*C*). The $\Delta F/F$ measurements (Fig. S4*E*) demonstrate that the fluorescence changes in satellite glial cells, but not in neurons, immediately after NMDA (250 μM) administration, and in neurons, but not in satellite glial cells, immediately after capsaicin (1 μM) administration. Change in fluorescence

immediately after capsaicin was the criteria used to identify TRPV1⁺ neurons.

In Fig. S7, the confocal images of immunofluorescence assay were performed in a 24-h DRG culture labeled with antibodies for glial fibrillary acidic protein (GFAP) and the NMDAR subunits NR1 and NR3A. The primary DRG cultures were washed in PBS, fixed in paraformaldehyde (4%; 20 min), washed again, and incubated during 30 min in glycine (0.1 M). Cells were permeabilized with Triton X-100 (0.1%) for 5 min and, then, primary antibodies were incubated in PBS containing 3% BSA for 30 min at room temperature. The primary antibodies and the respective dilutions used were anti-GFAP (1:100; Santa Cruz), anti-NR1 (1:100; Abcam), and anti-NR3A (1:100; Millipore). In one plate, primary antibodies were omitted as control for secondary antibody specificity and auto fluorescence. Cultures were washed three times in PBS, and the secondary antibodies were incubated in PBS plus 3% BSA for 1 h at room temperature. Fluorescent secondary antibodies labeled with Alexa 488, Alexa 594, and Alexa 633 against the IgG of primary antibodies were obtained from Invitrogen. Cells were washed again three times in PBS, and images were acquired in a confocal microscope (Leica SP5).

Real-Time PCR.

RNA extraction and cDNA preparation. At the end of experiment shown in Fig. 5, rats were euthanized with an overdose of sodium pentobarbital, and the L5-DRGs were removed. The tissues were homogenized in 1.0 mL of TRIzol reagent for total mRNA extraction, as described by Overbergh et al. (8), and 0.2 mL of chloroform was added to the samples before shaking vigorously for 30 s. The suspension was centrifuged at 13,000 × *g* at 4 °C for 15 min. The aqueous phase was transferred to a fresh tube to which an equal volume of isopropylic alcohol was added. After mixing, the samples were incubated for 15 min at −20 °C and then centrifuged at 13,000 × *g* at 4 °C for 15 min. The RNA precipitate was washed with 0.5 mL of ethanol, and 50 μL of DNase-free water was added. In sequence, 2 μg of the total mRNA were used for reverse transcription, which involved 200 units of the SuperScript II reverse transcriptase (Gibco, Invitrogen). The cDNA obtained was used for specific amplification of the mRNA of interest.

Real-time PCR reactions. The quantitative expression of the NMDAR subunit mRNAs was evaluated by using the SYBRGreen system, in an Applied Biosystems device (ABI5700). For the reactions, 12.5 μL of SYBRGreen Master Mix reagent (Applied Biosystems; which contains SYBRGreen 1 fluorophore, the AmpliTaq Gold polymerase enzyme, DNTPs with dUTP, ROX fluorophore—used as passive reference for normalization of the fluorescence levels—and the other buffer elements) was mixed with 5 μL of the previously prepared cDNA, 3.5 μL of diethylpyrocarbonate-treated MilliQ water, and 4 μL of the solution containing each primer (0.1 μg/μL) (from a stock solution, in which each primer was concentrated at 5 μg/μL, and aliquots were prepared to adjust the ideal concentration of the primers to be used in the reactions, for a volume of 4 μL). The primers used were based in refs. 9–12 and are as follows: NMDAR1: Grin1 (UniSTS:465388); sense: ATAGTGACAATCCACCAAGAGCC, antisense: GTAGCTCGCCCATCATTCCGTT; NMDAR2, subunit NR2A: Grin2a (UniSTS:143261); sense: TTATTGGGAGATGTCCCTCG, antisense: CACGTCTATTGCTGCAGGAA; subunit NR2B: Grin2b (UniSTS:143262); sense: ATCAGTGCTTGCTTCACGG, antisense: GGGTTGGACTGGTTCCCTAT; subunit NR2C: Grin2c (UniSTS:143263); sense: CTTTTTGTGCTTGGCCTAGG, antisense: CCTTATCGTGCCTTTGTTCCT; subunit NR2D: Grin2d (UniSTS:531451); sense: CGATGGCGTCTGGAATGG, antisense: AGATGAAAACTGTGACGGCG; NMDAR3, subunit NR3A: Grin3a (UniSTS:621704); sense: CCGCGGGATGCCCTACTGTTC, antisense: CCAGTTGTTCATGGTCAGGAT; subunit NR3B: Grin3b (UniSTS:527371); sense: TGGTGCTTTCCA GTTACACG, antisense: TTTTGTCCCCAACCATGAC. Thermal cycling conditions were 2 min at 50 °C followed by 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. An additional 20-min final cycle was performed, at increasing temperature from 60 °C to 95 °C, to establish the curve of dissociation of the products of the reaction, used to analyze the specificity of the amplification. The system (thermocycler ABI5700 associated with the ABI Prism Software; Applied Biosystems) amplified, detected, and quantified the samples by analyzing the fluorescence level produced by the incorporation of fluorogenic nucleases (SYBRGreen 1) to the products of amplification during the course of the reaction. The results were analyzed based in the cycle threshold values, which allowed the quantitative analysis of the expression of the evaluated factor. All samples were submitted to detection of the mRNA for β-actin, a housekeeping gene, used as positive control for the amplification reactions and normalization of the levels of expression of the target gene. In addition, a negative sample (water) was submitted to reactions with each pair of the primers. The results express the mean values ± SEM of the intensity of the mRNA expression for the target gene, normalized by the expression of β-actin, obtained from the L5-DRGs of 15 rats per experiment [five animals treated with AS-ODN, five with missense, and five naïve (untreated) animals].

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Fig. S1. Mechanical hyperalgesia induced by i.gl. NMDA is not affected by the treatment with indomethacin. The COX inhibitor indomethacin was administered via the i.t. (100 μg per 10 μL; gray bar) or i.p. (5 mg/kg; black bar) route 30 min before NMDA injection (240 ng; i.gl.). Mechanical hyperalgesia was evaluated at the third hour after NMDA injection by the electronic von Frey test. Results represent the means ± SEM of five paws per group. One-way ANOVA followed by Bonferroni posttest showed no significant difference between the groups (NS; $P = 0.9547$).

Fig. S2. Time course of the mechanical hyperalgesia induced by i.pl. injection of PGE_2 (100 ng) in the presence (■) or not (●) of D-AP-5 (9 μg) administered by the i.t. (*Left*) or i.gl. (*Right*) route, concomitantly with PGE_2. Results represent the means ± SEM of five paws per group. $*P < 0.001$ compared with groups □ and ○; $^{\#}P < 0.001$ compared with groups ■ (ANOVA followed by Bonferroni posttest).

Fig. S3. AMPA receptors involved in the hyperalgesia induced by i.pl. PGE_2 are not located in the DRG. *A* shows the hyperalgesic effect of i.t. or i.gl. administration of AMPA (40 ng) 3 h after injection. $*P < 0.05$ compared with vehicles or i.gl. AMPA groups. *B* shows the inhibitory effect of the AMPA receptor antagonist DNQX (10 nmol) on AMPA-induced hyperalgesia when both were injected at the same site—i.e., by the i.t. route. $*P < 0.05$ compared with the controls and AMPA+DNQX (i.t.). (*C*) However, when DNQX was injected in the DRG, no difference was observed in the hyperalgesia compared with the AMPA plus vehicle group (NS). (*D*) Administration of DNQX by the i.t., but not by the i.gl., route attenuated the hyperalgesia induced by i.pl. PGE_2 3 h after injection. DNQX (10 nmol) was administered via the i.t. or i.gl. route 1 h before the evaluation of the hind paw withdrawal threshold by the electronic von Frey test. Results represent the means ± SEM of five paws per group (one-way ANOVA followed by Bonferroni posttest).

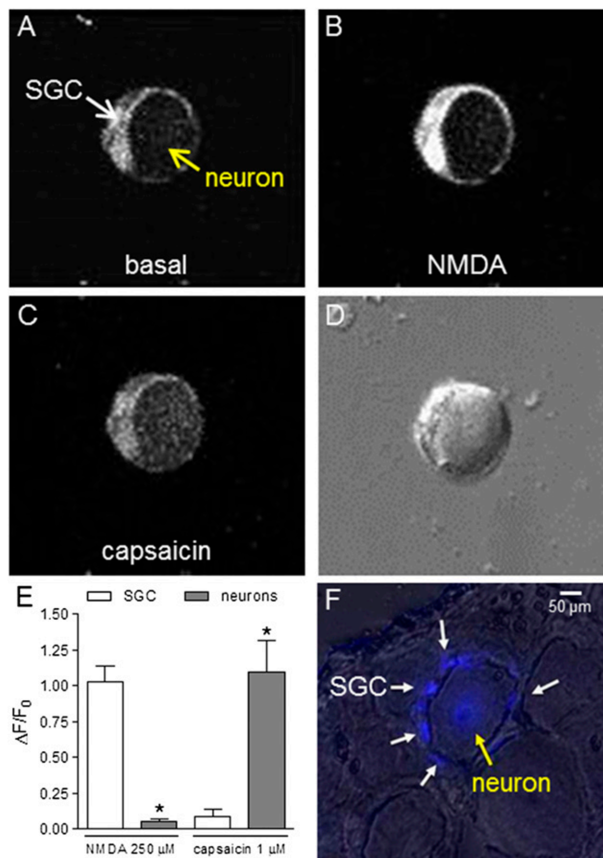

Fig. S4. Calcium transients induced by NMDA and capsaicin in satellite glial cells (SGC) and neurons. *A–D* show images of primary DRG cells cultured for 24 h, in which satellite cells are found attached to the primary sensory neuron soma. (*A–C*) Optical slice of the cell obtained using confocal microscopy. (*D*) Image of the entire cell in phase contrast, as if the cell was observed from above, obtained by using the same microscope, but not in the confocal setting—i.e., it is a bright-field image not obtained through laser scanning of a fluorescent-labeled cell. (*A*) Basal fluorescence of DRG cells. (*B*) Fluorescence changes immediately after NMDA (250 μM) administration. (*C*) Fluorescence observed immediately after capsaicin (1 μM) administration. (*D*) The same neuron observed in bright field with phase-contrast microscopy. Experiments were performed in the presence of glycine (10 μM) in Mg^{2+}-free buffer. (*E*) Maximum fluorescence increase induced by NMDA (250 μM) or capsaicin (1 μM) administration. Results represent the means ± SEM of six to eight cells. *$P < 0.001$ compared with the effect in SGC (ANOVA followed by Bonferroni posttest). (*F*) SGC surrounding the cell body of a L5-DRG neuron. The image, obtained by fluorescence microscopy, clearly shows the satellite cells attached to the primary sensory neuron soma, both labeled by DAPI, a marker for the cell nucleus (50 μm; 40×).

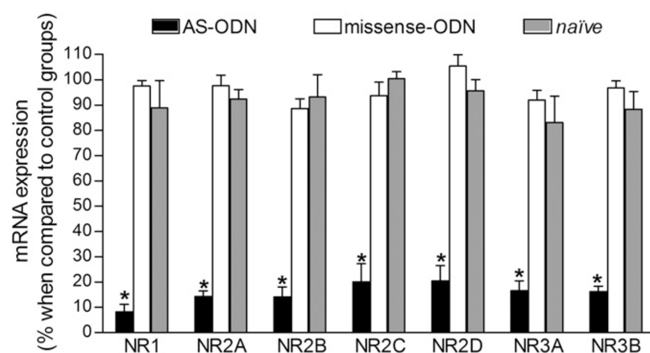

Fig. S5. Expression of mRNA for NMDAR subunits in the L5-DRGs after treatment with the AS-ODN or missense for five consecutive days. After the behavioral tests (Fig. 5), to confirm the knockdown of the NMDAR subunits, the L5-DRGs were harvested, and the real-time PCR analysis of the expression of the respective mRNAs was performed. Results are expressed as the mean ± SEM of five animals per group and represent the comparison (percent) of the expression of the NMDAR subunits in untreated rats (naïve; gray bars) or rats treated with the AS-ODN (black bars) or missense-ODN (white bars), normalized by the expression of β-actin. *$P < 0.001$ compared with the missense and naïve groups (one-way ANOVA followed by Bonferroni posttest).

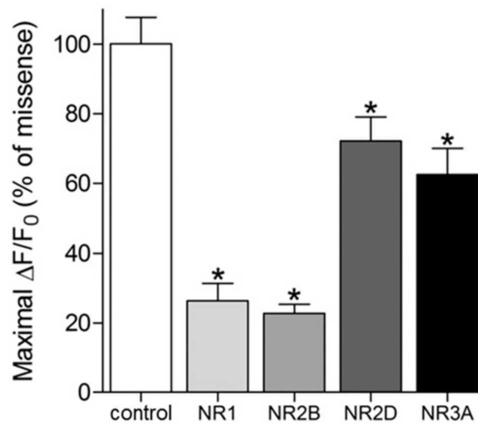

Fig. S6. NMDA-induced calcium transients in satellite cells of primary DRG cultures treated with AS-ODN selective for different NMDAR subunits. AS-ODN against the NMDAR subunit NR1, NR2B, NR2D, or NR3A (20 μg per 5 μL) was administered into the L5-DRG of rats for four consecutive days. After the last injection on the fourth day, the L5-DRGs were harvested, and the cells were immediately isolated for preparation of the cultures. Results show the maximum increase in fluorescence after NMDA administration (250 μM) in the presence of glycine (10 μM) in Mg^{2+}-free buffer for each AS-ODN treatment and represent the means ± SEM of 16–37 cells in three different experiments. *$P < 0.05$ compared with the effect of NMDA on the control cells (one-way ANOVA followed by Bonferroni posttest).

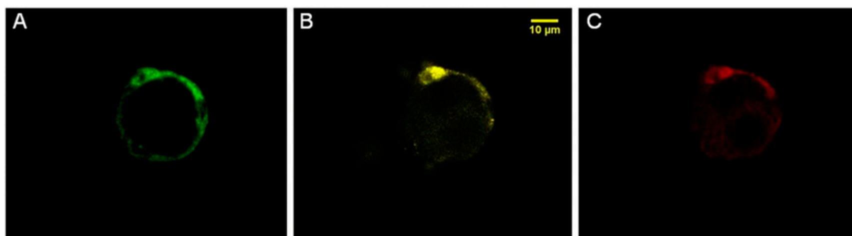

Fig. S7. NMDAR subunits NR3A and NR1 expressed in satellite cells surrounding a neuronal soma. Confocal images of immunofluorescence assay performed in a 24-h DRG culture labeled for NR3A (*A*), NR1 (*B*), and GFAP (*C*). Although the NR1 subunit has been also observed in some of the cell bodies (not shown), the NR3A subunit was found only in the satellite cells.