Supplementary Methods

μ-receptor binding. To determine the binding affinities (K_d) and the number of μ-receptors expressed on DRG cells (B_{max}) plasma membranes from DRG neurons were prepared as described (1), and saturation binding was performed using the μ-receptor ligand [^{3}H] [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO). Briefly, cell membranes (200-300 μg) were incubated in assay buffer with increasing doses of DAMGO (0.02-2 nM at 65 Ci/mmol) (Amersham Pharmacia Biotech, Buckinghamshire, England) in the absence or presence of 10 μM naloxone. Membranes were incubated in a final volume of 1 ml for 1 h at 30°C in assay buffer. Filters were soaked in 0.1% (w/v) polyethyleneimine solution for 30 min before use. Bound and free ligands were separated by rapid filtration under vacuum through Whatman GF/B glass fiber filters. Bound radioactivity was determined by liquid scintillation spectrophotometry at 70% counting efficiency for [^{3}H] after overnight extraction of the filters in 3 ml scintillation fluid (EG&G Wallac, Turku, Finland).

[^{35}S] guanosine-5`-O-(γ-thio)-triphosphate (GTPγS) saturation binding at μ-receptors. GTP_S saturation binding experiments were conducted by a modification of the procedure previously described for G-protein activation by cannabinoid receptors (2), _-adrenoceptors (3), orphanin FQ-receptors (4), serotonin H_5-HT_{1B} receptors (5), adenosine A1 receptors (6), and opioid receptors (7-9). Isotopic saturation analysis of DAMGO-stimulated [^{35}S]GTPγS binding was used to determine the apparent affinity of [^{35}S]GTPγS (apparent $K_{d\ Gprotein}$) and the apparent number of G-proteins activated (apparent $B_{max\ Gprotein}$). Various concentrations (0.05-2 nM) of [^{35}S]GTP_S (1250 Ci/mmol; New England Nuclear Corp., Boston, USA) were incubated with 50 μm GDP in assay buffer for 2 h at 30°C. At each concentration of [^{35}S]GTPγS, basal binding was assessed in the presence of GDP and absence of DAMGO, whereas specific binding was determined in the presence of 10μM DAMGO. Basal (unstimulated) [^{35}S]GTPγS binding was subtracted from DAMGO-stimulated binding at each measurement to determine net DAMGO-stimulated [^{35}S]GTPγS binding. The resulting isotherms were best fitted by a one-site nonlinear regression analysis. The relative amplification factor (B_{max} of net DAMGO-stimulated [^{35}S]GTPγS binding / B_{max} of DAMGO binding) was calculated according to (9) and represents the number of G-proteins activated per μ-receptor.

Immunofluorescence. Immunofluorescence imaging of DRG were performed in cultures and sections of native DRG neurons from 7 animals/group as described previously (10). For cultures, rats were killed by

isoflurane anaesthesia and lumbar (L3-5) DRG were removed. Cells were plated in 4-well glass slides (Lab-Tek Chamber Slide System; NalgeNunc International, Naperville, USA) in MEM Earle growth media without horse serum at 37°C for 30 min in an atmosphere of 5% CO_2. For sections of native DRG rats were deeply anesthetized with isoflurane and perfused transcardially with 100 ml 0.1 M PBS (pH 7.4) and 300 ml cold phosphate buffered saline (PBS) containing 4% paraformaldehyde and 0.2% picric acid. DRG were removed and cryoprotected overnight at 4 °C in PBS containing 10% sucrose. The tissues were then embedded in Tissue-Tek compound (OCT, Miles, Inc., Elkhart, USA) and frozen. Consecutive sections (10 µm thick) prepared on cryostat were mounted onto gelatine-coated slides. Immunfluorescence staining for µ-receptors was performed as described previously (10). Briefly, DRG cultures and sections were incubated overnight at 4 °C with rabbit anti-µ-receptor antibody (1:1000 dilution) and then incubated with goat anti-rabbit conjugated with texas red (Vector Laboratories, Burlingame, USA). The sections were viewed under a confocal laser scanning microscope (Zeiss, Jena, Germany) by an experimenter blinded to the treatment regime. Controls included: (1) pre-absorption of diluted antibodies with their respective immunizing peptides and (2) omission of either the primary antisera or the secondary antibodies. These control experiments did not show staining. For β-endorphin (END) detection, the sections were incubated overnight at 4°C with a rabbit polyclonal antibody against END (Peninsula Laboratories, USA, 1:1000 dilution). Staining was performed with a Vectastain avidin-biotin peroxidase complex (ABC; Vectastain Elite Kit, Vector Laboratories, Burlingame, USA), as described previously (10).

Immunoelectron microscopy. Immunoelectron microscopy of immune cells in hindpaws of animals with CFA inflammation was performed as described previously (11). Briefly, free-floating s.c. paw sections (40 µm) were incubated with antibody against END. The immunostaining was performed in the same way as for light microscopy. The immunoreaction was then visualized by incubation with nickel chloride-enhanced DAB (DAB containing 0.01% H_2O_2 and 0.08% nickel chloride in 0.05 M Tris-buffered saline, pH 7.6) for 3–5 min. The sections were postfixed in 1% tannic acid (in 0.1 M phosphate buffer) and 1% osmium tetroxide solution (in 0.1 M PBS), dehydrated in ethanol, and embedded in Epon. Ultrathin sections were cut on a Reichert Ultracut (Leica, Nussloch, Germany), followed by contrasting with 2% uranyl acetate/lead citrate. Finally, the ultrathin sections were examined under a transmission electron microscope (TEM 10, Zeiss).

Flow cytometry. Cell suspensions from 8 inflamed hindpaws/group were prepared and stained as

described previously (12). Briefly, samples were stained with PE-Cy5-conjugated mouse anti-rat CD45 (4 µg/ml, BD Biosciences, Franklin Lakes, USA) to label all hematopoetic cells. For intracellular stains, cells were fixed with 1% paraformaldehyede and permeabilized with saponin buffer (0.5% saponin, 0.5% BSA, 0.05% NaN_3 in PBS). Permeabilized cells were incubated with PE-conjugated mouse anti-rat RP-1 (recognizing PMN, 12 µg/ml, BD Biosciences, Franklin Lakes, USA), FITC-conjugated mouse anti-rat CD68 (recognizing monocytes/macrophages, 2 µg/ml, Serotec, Raleigh, USA) or mouse monoclonal immunoglobulin $G_{2\_}$ ($IgG_{2\_}$) antibody 3E7 (recognizing the pan-opioid sequence Tyr-Gly-Gly-Phe-Met at the N-terminus of opioid peptides, 10µg/ml, Gramsch Laboratories, Schwabhausen, Germany).

Statistics. Behavioral data are represented as ED_{50} (means ± standard errors) and values were calculated by regression from sigmoidal dose-response curves (Equation: Y = Bottom + (Top – Bottom) / $(1 + 10^{LogED_{50}-X})$, where "Bottom" is the Y value at the bottom plateau and "Top" is the Y value at the top plateau) (GraphPad Prism 4.0, San Diego, USA). All ligand binding and [^{35}S]GTPγS binding data are reported as means ± standard errors of four to six experiments, which were each performed in duplicates. The standard equation for specific radioligand binding to a protein is based on the law of mass action and describes a hyperbolic relationship as follows: $Y = (B_{max} \cdot X) / (X + K_d)$, where B_{max} denotes the maximal density of receptor sites and K_d denotes the radioligand equilibrium dissociation constant. [^{3}H]DAMGO binding experiments and [^{35}S]GTPγS saturation binding experiments were fitted to a one-site binding hyperbola using GraphPad Prism 4.0 (GraphPad, San Diego, USA) to determine K_d and B_{max} values. Amplification factors were defined by DAMGO-activated G-protein B_{max}/µ-receptor B_{max} (see also above). In addition, [^{35}S]GTP_S saturation binding data were displayed using Scatchard plots where the X axis is specific binding (Bound, fmol/mg protein) and the Y axis is specific binding divided by free radioligand concentration (Bound/Free). cAMP accumulation data are reported as means ± standard errors of eight experiments. cAMP after stimulation with FSK alone was set at 100 %. Statistical differences were determined by the unpaired two-tailed Student`s t-test and one way ANOVA on ranks, respectively. All tests were performed using Sigma Stat 2.03 (Jandel, San Rafael, USA) statistical software. $P < 0.05$ was considered significant in all tests.

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