Supporting Information

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SI Methods

Animals. The experiments were performed in Wistar male rats, C57BL/6 male mice, PI3Kγ-deficient mice (PI3Kγ$^{-/-}$), and neuronal nitric oxide (NO) synthase-deficient mice. All animals were housed in the animal-care facility of the Faculty of Medicine of Ribeirão Preto-University of Sao Paulo, Brazil. Animals were taken to the testing room at least 1 hour before experiments and were used only one time. Food and water were available ad libitum. Animal care and handling procedures were in accordance with the International Association for the Study of Pain guidelines (1) for those animals used in pain research, and the procedures were approved by the Committee for Ethics in Animal Research of the Faculty of Medicine of Ribeirão Preto-University of Sao Paulo.

Nociceptive Tests. The term hypernociception rather than hyperalgesia or allodynia is used to define the decrease of nociceptive-withdrawal threshold. It was assessed using three different methods: the constant-pressure rat-paw test, the electronic pressure-meter test, and the Hargreaves tests for mice. Quantification of nociception was performed by an observer unaware of the treatment given to the animals (blind). Multiple paw treatments with saline did not alter basal reaction time, which was similar to that observed in noninjected paws.

Constant-Pressure Rat-Paw Test. Mechanical hyperalgesia was tested in rats (180–200 g) as previously described (2). In this method, a constant pressure of 20 mmHg (measured using a sphygmomanometer) is applied (through a syringe piston moved by compressed air) to a 15-mm^2 area on the dorsal surface of the hind paw and discontinued when the rat presents a typical freezing reaction. This reaction is comprised of brief apnea, concomitant with retraction of the head and forepaws, and reduction in the escape movements that animals normally make to free themselves from the position imposed by the experimental situation. Usually, the apnea is associated with successive waves of muscular tremor. For each animal, the latency to the onset of the freezing reaction is measured before administration (zero time) and at different times after administration of the hypernociceptive agent. The intensity of mechanical hyperalgesia is quantified as the reduction in the reaction time, calculated by subtracting the value of the second measurement from the first measurement. Reaction time was 31.9 ± 0.2 s (mean ± SEM; $n = 36$) before injection of the hypernociceptive agent.

Electronic Pressure-Meter Test. Mechanical hypernociception was tested in mice (20–30 g) and rats as previously reported (3, 4). In a quiet room, mice or rats were placed in acrylic cages (12 × 10 × 17 cm) with wire-grid floors 15–30 min before the start of testing. The test consisted of evoking a hind-paw flexion reflex with a hand-held force transducer (electronic aesthesiometer; IITC Life Science) adapted with a 0.5- (mice) or 0.7-mm^2 (rats) polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was automatically recorded, and the final value for the response was obtained by averaging the three measurements. The animals were tested before and after treatments. The results are expressed by the delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the time-interval mean measurements. Withdrawal threshold was 9.1 ± 0.2 g for mice (mean ± SEM; $n = 30$) and 38.8 ± 0.7 for rats (mean ± SEM; $n = 20$) before injection of the hypernociceptive agents.

Thermal Nociceptive Test. The latency of paw withdrawal to radiant heat stimuli was measured using a Plantar Ugo Basile apparatus (Stoelting) as previously described (5). Mice can move freely in this apparatus on an elevated glass surface with plastic boxes above as the top cover. Mice are given a 1-hour acclimation period before testing to become calm and motionless. A calibrated infrared light source of high intensity was applied perpendicular on the plantar surface of each mouse's hind paw. The end point was characterized by the removal of the paw followed by clear flinching movements. Latency to paw withdrawal was automatically recorded. Each hind paw was tested alternately with an interval of 5 min for four trials. Paw-withdrawal latency of four trials from both hind paws of each mouse was averaged and recorded as mean ± SEM.

Primary Dorsal Root Ganglia-Neuron Culture. Rats or mice weighing 80–120 g or 20–25 g, respectively, were killed by decapitation under anesthesia. Dorsal root ganglia (DRG) were collected (18–20 ganglia per animal) and transferred to a sterile HBSS (Sigma) containing 10 mM Hepes (HBSS/Hepes). Isolated ganglia were incubated with collagenase II (0.28 U/mL) for 75 min and trypsin (0.25% wt/vol) for 12 min in HBSS/Hepes. Ganglia were washed and resuspended in DMEM (Sigma) containing 10% FBS and penicillin/streptomycin (1000 U/mL). Cells were dissociated by triturating the ganglia with a fire-polished pipette and plated in glass-bottomed Petri dishes (for confocal microscopy; MatTek), six-well plastic plates (for Western blot analysis), or plastic coverslips coated with Matrigel (BD; for patch-clamp analysis). Cell cultures were maintained at 37 °C and 5% CO_2, and experiments were performed within 12–48 hours (6).

Western Blot Analysis. After indicated stimulation, DRG cells were homogenized in a lysis buffer containing a mixture of protease and phosphatase inhibitors (Sigma). The protein concentrations of the lysate were determined using a BCA Protein Assay kit (Pierce), and 30 μg of protein was loaded for each lane. Protein samples were separated on SDS/PAGE gel (10% gradient gel; Bio-Rad) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). The filters were blocked with 5% dry milk and incubated overnight at 4 °C with primary antibody, phosphorylated (p-Ser473) protein kinase B (AKT) AKT (1:500; Cell Signaling Technology) for 1 hour at room temperature (RT) with HRP-conjugated secondary antibody (1:20,000; Jackson ImmunoResearch). The blots were visualized in ECL solution (Amersham Pharmacia Biotech) for 2 min and exposed onto hyperfilms (Amersham Pharmacia Biotech) for 1–30 min. Non-phosphorylated AKT (1:1,000; Cell Signaling Technology) antibody was used as a loading control (7).

DRG Immunohistochemistry. Animals were terminally anesthetized with urethane and perfused through the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4 °C). After the perfusion, DRG were removed and postfixed in the same fixative for 2 hours, which was then replaced overnight with 20% sacarose. All of the DRG were embedded in optimum cutting temperature (OCT) OCT, and DRG sections (14 μm) were cut in a cryostat and processed for immunofluorescence. All of the sections were blocked with 2% BSA in 0.3% Triton X-100 for 1 hour at RT and incubated for 2 hours at 4 °C with a mixture of polyclonal rabbit anti-PI3Kγ

(1:200; SantaCruz), polyclonal goat anti-Transient receptor potential cation channel, subfamily V, member 1 (TRPV1) TRPV1 (1:400; SantaCruz), polyclonal guinea-pig anti-Substance P (SP) SP (1:500; Millipore), and polyclonal mouse anti-Neurofilament 200 (NF200) NF200 (1:100; AbCam) antibodies. Finally, it was incubated with a mixture of AlexaFluo-488 and AlexaFluo-594 conjugated secondary antibodies (Molecular Probes) for 1 hour at RT. For the examination of Isolectin B4 (IB4) IB4-labeled nonpeptidergic nociceptors, IB_4-FITC conjugated antibodies (1:100; Sigma) were incubated together with secondary antibodies. The specificity for antibodies was confirmed by loss of staining in the absence of primary antibodies and single bands in Western blotting. Furthermore, the PI3Kγ immunoreactivity was not detected in DRG from PI3Kγ null mice.

Measurement of NO Production by DRG Neurons with 4,5-Diaminofluorescein Diacetate. NO production by DRG neurons was evaluated as previously described (8) but with modifications. Male Wistar rats weighing 80–120 g were killed by decapitation under anesthesia. Lumbar DRG were collected and transferred to a sterile HBSS (Sigma) containing 10 mM Hepes (HBSS/Hepes). The DRG were incubated in plastic dishes containing HBSS/Hepes in the presence of 10 μM 4,5-diaminofluorescein diacetate (DAF-FM diacetate; Molecular Probe) (9) for 1 hour at 37 °C. After loading with DAF, the DRGs were transferred into DAF-free medium and exposed to the following agonists/inhibitors: medium, morphine (10 μM), morphine (10 μM) plus PI3Kγ inhibitor (AS605240; 100 nM) (10), and morphine (10 μM) plus AKT inhibitor IV (100 nM) (11). The inhibitors were introduced 10 min before morphine. In control experiments, NOS activity was blocked using the nonspecific inhibitor L-NMMA (10 μM). After 1 hour, the DRGs were fixed with 4% paraformaldehyde for 30 min (4 °C) and washed in PBS (pH 7.4) at RT. DRGs were cut in slices and then applied to cover slips in Fluormont diluted in PBS (2:1). Sections were examined using a confocal laser-scanning microscope (Leica SP5) using the 488-nm excitation wavelength. Four to six fields from each DRG were evaluated. Furthermore, we used between three and four DRGs for each treatment. Neurons were considered positive for DAF-fluorescence when their fluorescence was 3-fold greater than background.

Oligodeoxynucleotides Targeting of PI3Kγ and K_{ATP} Channels. Antisenses oligonucleotides (ODNs) were used to induce a knockdown of PI3Kγ and K_{ATP} channel expression in rat DRG neurons. For PI3Kγ, the ODN antisense was selected from a specific site of the rat p110γ catalytic subunit of PI3Kγ mRNA (accession number NM 001106723). The ODN antisense sequence (5′-AAAAGTTGCAGTCCAGGAGTT-3′) was designed to be complementary to nucleotides 2202–2222 of the coding region of rat PI3Kγ. The mismatch sequence (5′-AAACGTAGCATTCCTCGAGAT-3′) was derived from the respective antisense sequences by scrambling six bases. Rats received an intratecal injection of either antisense one time a day (50 μg/day/10 μL saline) or mismatch (50 μg/day/10 μL saline) for 4 days. For K_{ATP}, ODNs against two different subunits of this channel were used. The ODNs sequences were based on previous studies (12, 13). The sequences were as follow: Kir6.2, antisense (5′-CCTTTCGGGACAGCATGGCT-3′), mismatch (5′-CTTTACGGTACAGTATCGCA-3′), SUR1, antisense (5′-GGC CGA GTG GTT CTC GGT-3′), and mismatch (5′-TGC CTG AGG CGT GGC TGT-3′). Rats receive an intratecal injection of either antisense one time a day (20 μg/day/10 μL saline) or mismatch (20 μg/day/10 μL saline) for 4 days.

On the day after the ODN treatments, the peripheral effect of morphine (6 μg/paw) or NO donor (*S*-nitroso-*N*-acetylpenicillamine (SNAP) SNAP; 200 μg/paw) was evaluated on PGE_2-induced hypernociception followed by the removal of DRG (L4-L-6) of the ipsilateral side of PGE_2 injection. PI3Kγ, Kir6.2, or SUR1 expressions in DRG from antisense and mismatch were evaluated using Western blot analyses. Briefly, DRG cells were homogenized in a lysis buffer containing a mixture of proteinase and phosphatase inhibitors. Proteins were separated by SDS/PAGE 6% and transblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were blocked with 7% dry milk (overnight) and incubated overnight at 4 °C with rabbit polyclonal antibody against PI3Kγ (1:200; SantaCruz), rabbit polyclonal antibody against Kir 6.2 (1:2,000; Sigma), or rabbit polyclonal antibody against SUR1 (1:500; Abcam). After these procedures, the membranes were washed and then incubated for 1 hour at RT with HRP-conjugated secondary antibody (1:20,000; Jackson ImmunoResearch). The blots were visualized in ECL solution (Amersham Pharmacia Biotech) for 2 min and exposed onto hyperfilms (Amersham Pharmacia Biotech) for 2–20 min. β-actin (1:2,000; AbCam) antibodies were used as loading controls.

RT-PCR. mRNA of the p110γ catalytic subunit of PI3Kγ in mice DRG neurons was determined by RT-PCR. Briefly, mice were killed by decapitation under anesthesia, and the DRG (six from each mouse; L4, L5, and L6) were harvested and left in contact with 10 mL of TRIzol reagent (for 10 min). Reverse transcription was performed with 0.5 μg of total RNA. The primers used were as follows: p100γ (sense, 5′-TCA GGC TCG GAG ATT AGG TA-3′; antisense, 5′-GCC CAA TCG GTG GTA GAA CT-3′) and GAPDH (sense, 5′-GCC ATC AAC GAC CCC TTC ATT G-3′; antisense, 5′-TGC CAG TGA GCT TCC CGT TC-3′). The expression of GAPDH mRNA was used as an internal control in all samples. The PCR protocol started with 4 min of incubation at 94 °C followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. The final extension was at 72 °C for 10 min.

Electrophysiology. A whole-cell, patch-clamp technique was employed using an HEKA EPC9 amplifier, ITC 1600 interface, and pulse-pulsefit software (HEKA) (14). DRG neurons (6–12 hours in culture) were placed in a recording chamber where the cells were bathed in modified Tyrode solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 0,5 $MgCl_2$, 10 Hepes, 10 glucose, and 0.1 L-arginine (pH 7.4) adjusted with NaOH. Recording pipettes were pulled from glass tubing (Perfecta) and typically, had a resistance between 1.6–2.0 MΩ when filled with the following solution (in mM): 130 KCl, 5 NaCl, 5 EGTA, 5 Hepes, 0.03 ATP, and 0.1 L-arginine (pH 7.2) adjusted with KOH. After pH correction, the $[K^+]_{internal}$ was 145 mM. The whole-cell configuration was established in modified Tyrode solution. Pipette-voltage offsets were neutralized before the formation of a gigaseal. Series resistance (R_s), and cell capacitance (C_m) were determined. Capacitance compensation and 60% R_s compensation were used. Data were filtered at 2.9 kHz and digitally sampled at 10 kHz. Criteria for cell inclusion in the study were: $R_s < 10$ MΩ and stable recording with 60% R_s compensation throughout the entire experiment. The membrane potential was held at −80 mV, and a voltage step of +50 mV was used to examine the activation of potassium currents. After establishing a stable series of K^+ current recordings, the DRG neurons were perfused with modified Tyrode solution containing morphine alone or morphine in the presence of glibenclamide (a K_{ATP} channel blocker), naloxone, L-NMMA, PI3Kγ inhibitor, or AKT inhibitor. In another set of experiments, neurons were perfused with a NO donor (NOC-18) alone or in the presence of glibenclamide.

Assay for Changes in Membrane Potential. The effect of morphine and PGE_2 on the membrane potential of DRG-culture neurons was evaluated using potentiometric fluorescence dye bis-oxonol [$DiBAC_4(3)$; Molecular Probes]. DRG-cultured neurons were incubated with normal Tyrode solution containing 5 μM $DiBAC_4(3)$ (20 min; 37 °C). $DiBAC_4(3)$ is a bis-barbituric acid oxolol

compound that partitions into the membrane as a function of membrane potential. Hyperpolarization causes extrusion of the dye and decreased fluorescence, whereas depolarization causes enhanced fluorescence. Fluorescence intensity was monitored at 10-s intervals with a confocal microscope (LSM510-Zeiss; Zeiss) using the excitation and emission wavelengths of 470 and 525 nm, respectively. Relative fluorescence intensity was determined with LSM analysis software (Zeiss). $DiBAC_4(3)$ was maintained at 5 μM in all solutions (15). At the end of the experiment, capsaicin (1 μM) was added. Only small-diameter neurons (<30 μm) that depolarized after application of capsaicin were analyzed.

Surgery to Induce Peripheral Neuropathy. Male Wistar rats underwent spinal nerve ligation under isoflurane anesthesia as previously described (16). On the left side, spinal nerves L5 and L6 were isolated and ligated tightly with 5-0 chromed catgut silk sutures distal to the DRG. As a result of this surgery, axons in the L4 spinal nerve were left intact, innervating the plantar hind paw. Some rats (shams) underwent identical surgeries, but the spinal nerves were not ligated. After surgery, animals were housed individually with free access to food and water and allowed to recover for at least 7 days. Left-paw tactile allodynia was confirmed at this time by measuring the hind-paw withdrawal threshold in response to the application of the electronic aesthesiometer as previously described (17).

Drugs. The following drugs were obtained from the sources indicated. Wortmannin, L-NMMA, naloxone chlorhydrate, complete Freund`s Adjuvant (CFA), LY294002, SNAP, dibutyryl cAMP, epinephrine, glibenclamide, morphine chlorhydrate, and PGE_2 were purchased from Sigma Chemical Co. and morphine sulfate was purchased from Prodome Química e Farmaceutica. AS605240 and AKT inhibitor IV were obtained from Calbiochem. N^{ω}-Propyl-L-arginine was obtained from Tocris Bioscience. DETA-NONOate (NOC-18) NOC-18 was obtained from Calbiochem. All reagents used for electrophysiological experiments were purchased from Sigma.

Data Analyses and Statistics. All results are presented as means ± SEM. The experiments were repeated at least two times. Two-way ANOVA was used to compare the groups and doses at all times (curves) when the hypernociceptive responses were measured at different times after the stimulus injection. The factors analyzed were treatments, time, and time-by-treatment interaction. When there was a significant time-by-treatment interaction, one-way ANOVA followed by Bonferroni's t test was performed for each time. Alternatively, when the hypernociceptive responses were measured one time after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Bonferroni's t test. Pair-wise comparisons were made with the Student t test. $P < 0.05$ was considered to be significant.

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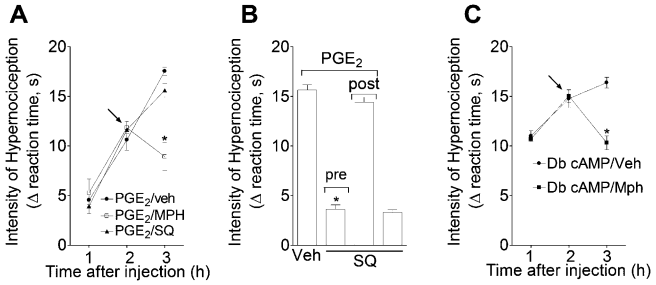

Fig. S1. Inhibition of adenylyl cyclase is not involved in the direct blockade of inflammatory hypernociception by morphine. (*A* and *B*) Mechanical hypernociception in rats was induced by intraplantar injection of PGE_2 (100 ng/paw). (*A*) After 2 hours, morphine (6 μg/paw), SQ22536 (27 μg/paw), or vehicle (veh) were injected in the ipsilateral paws (indicated arrow). Hypernociception was determined 1, 2, and 3 hours after PGE_2 injection ($n = 6$). (*B*) Rats were pretreated (30 min before PGE_2 injection) or posttreated (2 hours after PGE_2 injection) with SQ22536 (27 μg/paw) (18). Hypernociception was determined 3 hours after PGE_2 injection. SQ22536 alone (last bars) did not produce any modification in the nociceptive threshold ($n = 6$). (*C*) Mechanical hypernociception in rats was induced by intraplantar injection of Dibutyryl (Db) cAMP (100 μg/paw). After 2 hours, morphine (6 μg/paw) was injected in the ipsilateral paws (indicated arrow). Hypernociception was determined 1, 2, and 3 hours after Dibutyryl cAMP injection ($n = 6$). *, $P < 0.05$ compared with vehicle treatment.

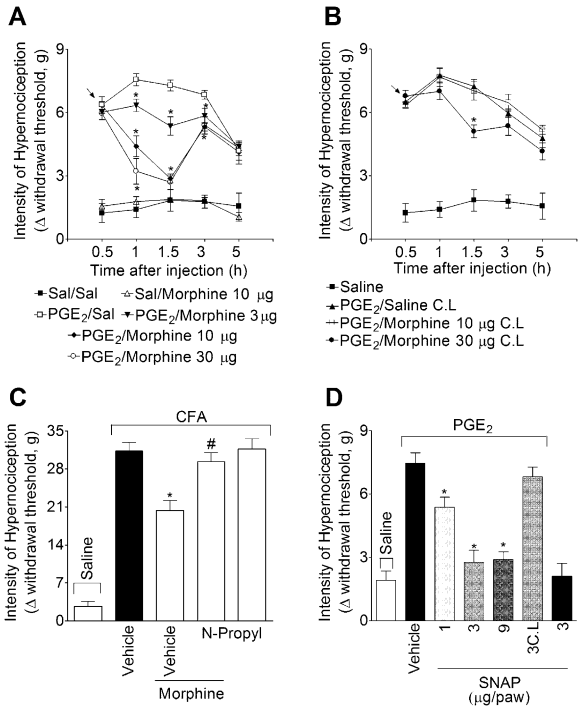

Fig. S2. Peripheral antinociceptive action of morphine (the role of nitric oxide). Mechanical hypernociception was induced in mice by the injection of PGE_2 (30 ng/paw). After 30 min, morphine (indicated arrow) was injected in the (*A*) ipsilateral (1–30 μg/paw; $n = 6$ for each dose) or (*B*) contralateral paws (10–30 μg/paw; $n = 6$ for each dose). *$P < 0.05$ compared with vehicle treatment. (*C*) Mechanical hypernociception in rats was induced by ipsilateral injection of CFA (50 μL/paw). The antinociceptive effect of a local injection of morphine (100 μg/paw 4 hours after CFA injection) on CFA-induced mechanical hypernociception was prevented by treatment with a selective inhibitor of neuronal NO synthase (N-propyl-L-arginine; 30 μg/paw for 20 min before morphine injection; $n = 6$). *$P < 0.05$ compared with vehicle treatment. **$P < 0.05$ compared with morphine. (*D*) Mechanical hypernociception was induced in mice by the injection of PGE_2 (30 ng/paw). After 30 min, SNAP (1–9 μg/paw) was injected in the ipsilateral (1–9 μg/paw; $n = 7$) or contralateral paws (CL; 3 μg/paw; $n = 7$). Hypernociception was evaluated 30 min after SNAP injection. *, $P < 0.05$ compared with vehicle treatment.

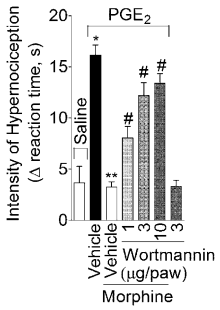

Fig. S3. Effect of wortmannin in the peripheral antinociceptive action of morphine. Mechanical hypernociception in rats was induced by ipsilateral injection of PGE_2 (100 ng/paw). The antinociceptive effect of a local injection of morphine (6 μg/paw 2 hours after PGE_2 injection) on PGE_2-induced hypernociception was prevented by treatment with (30 min before morphine injection) wortmannin (1–10 μg/paw; $n = 10$). *, $P < 0.05$ compared with saline injected group. **$P < 0.05$ compared with vehicle treatment. #, $P < 0.05$ compared with morphine.

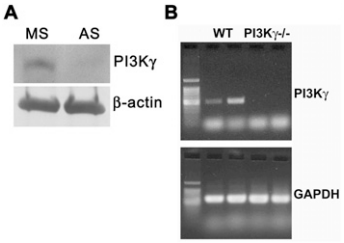

Fig. S4. PI3Kγ expression on DRG of rats and mice. (*A*) Western blot analyses of PI3Kγ expression on DRG neurons from rats after 4 days of treatment with antisense (AS) or mismatch (MS) against p110γ (*B*) mRNA expression of PI3Kγ (p110γ catalytic subunit) in the DRG neurons of wild-type and PI3Kγ null mice. DRG (six from each mouse; L4, L5, and L6 on both sides) were removed from wild-type and $PI3K\gamma^{-/-}$ mice, and the mRNAs were extracted. After the reverse transcriptase reaction, the cDNA was amplified as described in *SI Methods*.

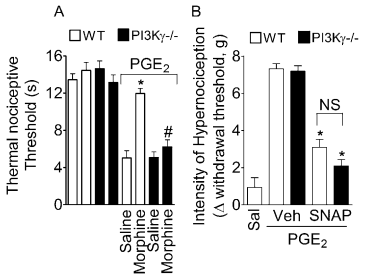

Fig. S5. PI3Kγ mediates morphine inhibition of thermal inflammatory hypernociception, and NO produces peripheral antinociceptive action in PI3Kγ null mice. (*A*) Mechanical hypernociception was induced in mice by the injection of PGE_2 (300 ng/paw). After 15 min, morphine (10 μg/paw) was injected in the ipsilateral paws of wild-type (WT) or $PI3K\gamma^{-/-}$ mice ($n = 6$). Thermal hypernociception was evaluated 1 hour after PGE_2 injection. *$P < 0.05$ compared with vehicle treatment. #, $P < 0.05$ compared with wild-type mice treated with morphine. (*B*) Antinociceptive effect of the NO donor (SNAP; 3μg/paw) in $PI3K\gamma^{-/-}$ mice ($n = 5$) is not different from that of WT mice ($n = 5$; $P = 0.104$). NS, nonsignificant. SNAP was administrated 30 min after PGE_2 injection, and hypernociception was determined 1 hour after PGE_2 injection. *, $P < 0.05$ compared with vehicle treatment.

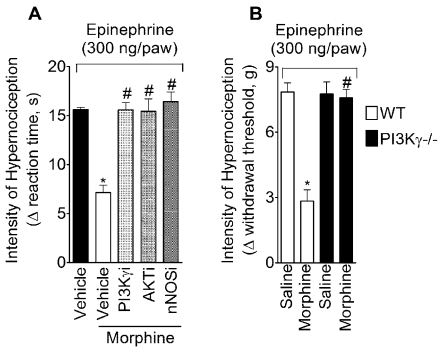

Fig. S6. The PI3Kγ/AKT/NO pathway mediates morphine inhibition of epinephrine-induced inflammatory hypernociception. (*A*) Mechanical hypernociception in rats was induced by ipsilateral injection of epinephrine (300 ng/paw). The antinociceptive effect of a local injection of morphine (6 μg/paw 2 hours after epinephrine injection) on epinephrine-induced mechanical hypernociception was prevented by treatment with selective inhibition of PI3Kγ (AS605240; 30 μg/paw), AKT (AKT inhibitor IV; 10 μg/paw; $n = 10$), or neuronal NO synthase (nNOS inhibitor; 10 μg/paw). All inhibitors were injected 30 min before morphine injection. (*B*) Mechanical hypernociception was induced in mice by the injection of epinephrine (300 ng/paw). After 15 min, morphine (10 μg/paw) was injected in the ipsilateral paws of WT or PI3K$\gamma^{-/-}$ mice ($n = 6$). Mechanical hypernociception was evaluated 1 hour after epinephrine injection. *, $P < 0.05$ compared with vehicle treatment. #, $P < 0.05$ compared with rats or WT mice treated with morphine.

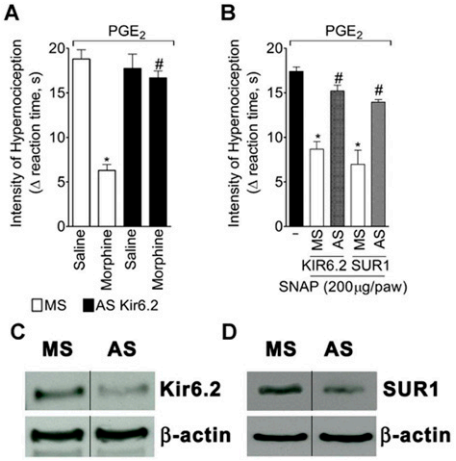

Fig. S7. The K_{ATP} channel is involved in the peripheral antinociceptive effects of morphine and NO. Mechanical hypernociception in rats was induced by ipsilateral injection of PGE_2 (100 ng/paw). The antinociceptive effect of a local injection of morphine (*A*; 6 μg/paw 2 hours after PGE_2 injection) or SNAP (*B*; 200 μg/paw 2 hours after PGE_2 injection) on PGE_2-induced hypernociception was prevented by treatment with ODN antisense (AS) against Kir6.2 or SUR1 ($n = 5$). *, $P < 0.05$ compared with vehicle treatment. #, $P < 0.05$ compared with mismatch (MS) treatment. (*C* and *D*) Western blot analyses exhibited reduction in both Kir6.2 and SUR1 expression in DRG after AS treatment compared with MS treatment.

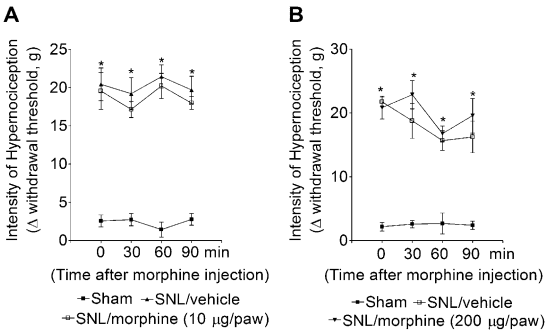

Fig. S8. Effect of morphine on peripheral nerve lesion-induced neuropathic pain. Neuropathic pain was induced by the ligation of L5 and L6 spinal nerves. (*A* and *B*) Mechanical hypernociception was evaluated 7 day after surgery in sham and injured rats using the electronic von Frey test. Rats received an ipsilateral injection of morphine (10 μg/paw or 200 μg/paw; $n = 5$). The effect of morphine was determined 30, 60, and 90 min after injection. *, $P < 0.05$ compared with sham operated rats.

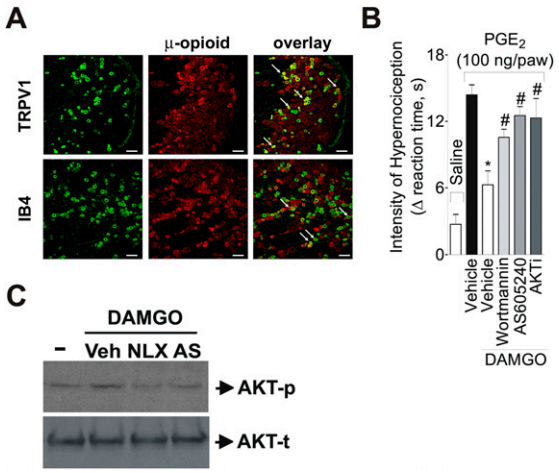

Fig. S9. A selective μ-opioid receptor agonist produces peripheral antinociception through activation of PI3Kγ/AKT in rats. (*A*) Confocal images of anti–μ-opioid receptor immunoreactivity in subpopulations of DRG neurons from rats labeled using binding to IB4 or antibodies to TRPV1. Arrows indicate double-labeled neurons. (Scale bars, 50 μm.) (*B*) Mechanical hypernociception in rats was induced by ipsilateral injection of PGE_2 (100 ng/paw). The antinociception of [D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin (DAMGO) DAMGO (1 μg/paw) on PGE_2-induced hypernociception was prevented by treatment of rat paw with a nonselective inhibitor of PI3K, (wortmannin; 3 μg/paw; $n = 6$), a selective inhibitor of PI3Kγ (AS605240; 30 μg/paw; $n = 6$), or a selective inhibitor of AKT (AKTi; 10 μg/paw; $n = 6$). *, $P < 0.05$ compared with vehicle treatment. #, $P < 0.05$ compared with DAMGO treatment. (*C*) Western blot analyses of phosphorylated-AKT 5 min after DAMGO (1 μM) incubation. Naloxone (NLX; 1 μM) or AS605240 (AS; 100 nM) preincubation (10 min) prevented AKT phosphorylation induced by DAMGO.

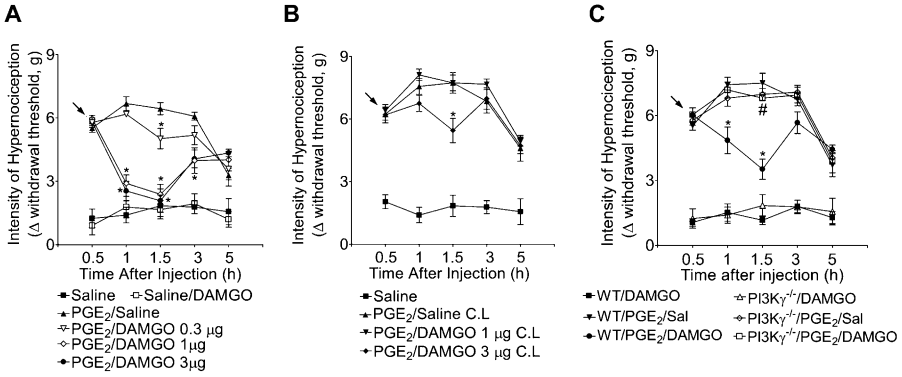

Fig. S10. Peripheral activation of μ-opioid receptors produce antinociception in mice (the role of PI3Kγ). (*A*) Mechanical hypernociception was induced by the injection of PGE_2 into mice (30 ng/paw). After 30 min, DAMGO (0.1–3 μg/paw; indicated arrow) was injected in the (*A*) ipsilateral or (*B*) contralateral paws. *$P < 0.05$ compared with vehicle treatment. (*C*) Peripheral effect of DAMGO (1 μg/paw) was also tested in PI3K$\gamma^{-/-}$ mice ($n = 6$). *, $P < 0.05$ compared with vehicle treatment. #, $P < 0.05$ compared with WT treated with DAMGO.