Supplemental Methods.

Neuropathic pain model

The spared nerve injury (SNI) model of neuropathic pain was used. In this partial denervation model two of the three distal branches of the sciatic nerve are ligated and excised. Traditional SNI involves ligating the common peroneal and tibial nerves and sparing the sural nerve. We used a variation in which the tibial nerve was spared (SNI\textsubscript{t}) and the common peroneal and sural nerves were ligated and cut. Baseline responses to mechanical stimuli were determined before surgery. Then mice were deeply anesthetized with isoflurane 1.5–4.0% (Abbott Laboratories, North Chicago, IL), and toe pinch was used to verify that mice were under the surgical plane of anesthesia. Under aseptic conditions, the sciatic nerve was exposed at mid-thigh level through the biceps femoris muscle. The common peroneal and sural nerves were tightly ligated with 6-0 silk and axotomized distal to the ligation. Care was taken to avoid any contact with or stretching of the intact tibial nerve. Muscle and skin were closed in two layers with 4.0 silk and staples, respectively. In sham surgeries, the branches of the sciatic nerve were exposed but not ligated. The animals were returned to their cages after surgery and monitored during recovery. SNI\textsubscript{t} produces early (<24 h) and prolonged (>12 weeks) mechanical hypersensitivity and neuropathic pain predominantly in the area innervated by the tibial nerve. Behavioral experiments were initiated 2 days after surgery and DALDA/morphine analgesia was assessed starting on postoperative day 7.

Immunohistochemistry

To assess the distribution of MOPs in the DRG and spinal cord, we performed immunofluorescence imaging. Mice, aged 6–8 weeks, were deeply anesthetized through isoflurane...
inhalation under saturated vapor pressure at room temperature, and perfused intracardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4, 4°C) followed by fixative (4% formaldehyde and 0.2% [v/v] picric acid in PBS, 4°C). Tissues were cryoprotected in 30% sucrose for at least 24 h before being serially cut into 15-μm sections and placed onto slides. Sections of spinal cord and lumbar DRG were cut on a cryostat and immunostained with guinea pig anti-MOP antibody (Neuromics, Cat #GP10106, RRID:AB_2737108, 1:400). For secondary antibodies, we used Alexa 488-conjugated goat antibody to mouse (Thermo Fisher Scientific, Waltham, MA, Cat #A-10667, RRID: AB_2534057) and Cy3-conjugated goat antibody to guinea pig (Thermo Fisher Scientific, Cat #A-11073, RRID:AB_2534117). All secondary antibodies were diluted 1:100 in blocking solution (PBS + 1% bovine serum albumin [BSA] + 0.1% Triton X-100). Images were taken at 40x with an AXIO Examiner.Z1 confocal microscope (Carl Zeiss AG, Oberkochen, Germany). Tissues from both experimental groups were processed simultaneously during the immunohistochemical process.

Genomic DNA analysis

To detect the presence of the \(Oprm1^{\text{fl/fl}}\) and \(Pirt-Cre^{+/+}\) genes, we performed PCR on genomic DNA obtained from tail samples. \(Oprm1\) primer sequences were 5′-GTTACTGGAGAATCCAGGCCAAGCC-3′ (forward) and 5′-CGCTTGGAATATCTTGTACCTATGACCA-3′ (reverse). \(Pirt-Cre\) primer sequences were 5′-ATCCGTAACCTGGATAGTGAA-3′ (Cre forward), 5′-CAACTTTGTGGTACCCGAAG-3′ (Pirt forward), and 5′-TCCCTGGGACTCATGATGCT-3′ (Pirt reverse).

Western blot analysis
We used western blot analysis to quantify MOP protein expression in lumbar DRG and spinal cord, small intestine, and periaqueductal gray. Samples of each tissue were separated and homogenized for immunoblotting. The tissues were lysed in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.5 mg·ml⁻¹ BSA). After samples were centrifuged at 13,000g (or 12,000 rpm) for 15 min, the protein concentration was determined by using a detergent-compatible protein assay (Pierce™ BCA Protein Assay Kit) with a BSA standard. Samples were separated on a 7.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel by electrophoresis and transferred onto a nitrocellulose membrane (Amersham, Pittsburgh, PA) with a Trans-Blot Transfer Cell system (Bio-Rad, Hercules, CA). Membranes were incubated with the indicated primary antibody overnight at 4°C, and immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham). Antibodies against MOP (1:2000, AB5511, EMP Millipore) and GAPDH (1:50,000, ABS16, EMD Millipore) were used. Western blots were imaged with the ImageQuant LAS 4000 (GE Healthcare Life Sciences) and analyzed with ImageJ 1.46a software. GAPDH staining was used as an internal control for protein loading.

**Preparation of DRG neuronal cultures**

Primary cultures of DRG sensory neurons were obtained from naïve, 3–4-week-old, male C57Bl/6 and Oprm1 cKO mice weighing 12–15 g. Once the mice were anesthetized, the DRGs were carefully removed and immediately placed into fresh, ice-cold DH10 media consisting of DMEM/F-12 supplemented with 10% fetal bovine serum (no DMSO) and 1% penicillin/streptomycin (~1.5 mL per animal), washed with Hanks’ balanced salt solution (HBSS) 1–2×, and then treated with an enzyme solution containing 5 mg·ml⁻¹ dispase and 1 mg·ml⁻¹.
collagenase type I in HBSS without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (Gibco). Cells were shaken slowly for >45 min at 37°C. After trituration (10–15×) and centrifugation, the cells were resuspended in DH10 media containing nerve growth factor (50 U∙ml\textsuperscript{-1}; Upstate Biotechnology) and plated on freshly made poly-D-lysine- (100 µg∙ml\textsuperscript{-1}; Biomedical Technologies) and laminin- (100 µg∙ml\textsuperscript{-1}) coated coverslips. Cultures were then incubated at 37°C in 5% CO\textsubscript{2} until being used in subsequent electrophysiological studies.

**Whole-cell patch-clamp electrophysiology of cultured DRG neurons**

Whole-cell patch-clamp techniques were utilized to examine the electrophysiologic and pharmacologic properties of high-voltage-activated (HVA) calcium currents in DRG neurons from adult naïve mice. After a 48–72-h incubation period, cultured DRG neuron preparations were placed into a submersion-type recording chamber (RC-22; Warner Instruments, Hamden CT, USA), secured to an inverted microscope, and visualized with a bright-field imaging system (Eclipse TE2000-U; Nikon). Patch-clamp electrodes were constructed from single-filament borosilicate glass (1.5 mm outer diameter and 0.84 mm inner diameter; World Precision Instruments) with a microelectrode puller (P-1000; Sutter Instruments, Novato, CA). Electrode tip impedance ranged from 2 to 4 MΩ and formed seal resistances > 1 GΩ when filled with an internal recording solution composed of (in mM) 140 tetraethylammonium chloride, 10 EGTA, 1 MgCl\textsubscript{2}, 10 HEPES, 0.5 GTP, and 3 ATP (pH = 7.4 by 1 M N-methyl-D-glucamine [NMDG]; ~300 mOsm, adjusted with sucrose, measured by a Wescor Vapro 5600, ELITech Group). Cultured neuron preparations were maintained under constant gravity-driven perfusion of an oxygenated external solution consisting of (in mM) 130 NMDG chloride, 5 BaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, and 10 glucose...
(pH = 7.4 by HCl; ~310-315 mOsm adjusted with sucrose), delivered at a rate of 1–2 mL·min\(^{-1}\) at room temperature. Tetraethylammonium chloride and NMDG chloride were added to each solution to block voltage-dependent K\(^+\) conductance and Na\(^+\) conductance, respectively. Ba\(^{2+}\) was added to the external solution to function primarily as a preferential charge carrier through the HVA channels, but also as a background K\(^+\) conductance blocker. We did not correct for the junction potential between the internal and external solutions.

For patch-clamp recordings of HVA Ca\(^{2+}\) (HVA-\(I_{\text{Ca}}\)) currents in small diameter (<20 µm) DRG neurons, we compensated and maintained series resistance, if necessary, at <20 MΩ approximately 1–2 min after the whole-cell configuration was established. The voltage protocol used to evoke HVA-\(I_{\text{Ca}}\) was modified from that of prior publications.\(^{2,3}\) Briefly, neurons were held at -80 mV, and a 40 mV square wave voltage pulse was applied via the patch electrode (evoked to -40 mV) for 20 ms to activate low-voltage-activated (LVA) Ca\(^{2+}\) channels. The holding voltage was then set at -60 mV for 20 ms followed by a 50 mV voltage application delivered via the electrode (evoked to -10 mV) for 20 ms to evoke HVA Ca\(^{2+}\) channels. After we recorded baseline HVA-\(I_{\text{Ca}}\) for 1 min to assess the stability of each evoked current, we applied either DALDA or morphine (1 µM) to the neurons using a six-channel perfusion valve control system (VC-6; Warner Instruments) for a period of 2 min (time of full bath exchange) followed by a 5-min washout with the external solution. This HVA stimulation protocol was run every 10 s for a total of 8 min. Acquired recordings of HVA-\(I_{\text{Ca}}\) were filtered at 4 kHz with a -3 dB, 4-pole, lowpass Bessel filter, sampled at a rate of 20 kHz, and stored on a personal computer (Dell) using pClamp 9.2 and a digitizer (Digidata 1322A, Molecular Devices). Offline, currents were digitally filtered by using a
lowpass Gaussian filter with a -3 dB cutoff set to 2 kHz (Clampfit software; pClamp 9.2, Molecular Devices).
**Supplemental Figure 1. Mouse genotypes.** Representative images of ethidium bromide-stained agarose gels show PCR genotyping of *Pirt-Cre* (top) and *Oprm1* (bottom) from tail snip tissue samples. Higher band in *Pirt-Cre* panel indicates *Pirt-Cre* allele, and lower band indicates wild-type (WT) allele (i.e., Pirt gene without Cre [Pirt-Cre^+/+]). Single band in *Oprm1* panel indicates null gene (i.e., floxed allele [Oprm1^+/−]).
Supplemental Figure 2. Behavioral and motor activity of male and female wild-type and mu-opioid receptor conditional knockout (Oprm1<sup>+/−</sup> Pirt-cre<sup>+</sup>) mice. (A) Paw withdrawal latency in response to radiant heat stimulus. (B) Response latency to hot plate. (C) Paw withdrawal frequency in response to high-force (0.4 g) von Frey monofilaments. (D) Latency to fall during rotarod test. N = 5 per group. Data are shown as means ± SEM, and comparisons were made with Welch’s t-test.
Supplemental Figure 3. Time course of nerve injury. (A) Time course of paw withdrawal frequency in Oprml conditional knockout (Pirt-cre+/− Oprml+/flo) mice after spared nerve injury (SNI) or sham surgery. #p<0.05 (ipsilateral) and *p<0.05 (contralateral) compared to sham (same time point). (B) Time course of latency to fall during rotarod test. *p<0.05 versus respective baseline, #p<0.05 versus wild-type. N = 10, with 5 males and 5 females. Data are shown as means ± SEM. Comparison were made by repeated measures two-way ANOVA with Bonferroni’s post hoc test.
References

