Supporting Information

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SI Methods

Tissue Fixation. Adult rats, mice, and *Oprd1* exon 1-deleted mice (Jackson Lab) were anesthetized and perfused with 20 mL of warm saline, followed by a warm mixture of 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer (pH 7.4) and then the same but ice-cold fixative (50 mL). The lumbar segments of the spinal cord and DRGs were dissected out and postfixed in the same fixative for 90 min at 4 °C.

Gene Transfection in DRG Neurons. Dissociated DRG neurons were transfected with pCMV-Myc-DOR1 or DOR1-EGFP in pEGFP-N3 by electroporation with Nucleofector II (Amaxa) using Neuron Nucleofector Solution and program O-003 and were then plated on coverslips in DMEM containing 10% (vol/vol) FBS. After 6 h, neurons were cultured in the medium containing 50% (vol/vol) DMEM, 50% (vol/vol) F12, 1% N2, and 100 ng/mL NGF for small neurons or 50 ng/mL NT3 for large neurons for 48–72 h.

Whole-Cell Patch-Clamp Recording. The composition of the pipette solution was 120 mM CsCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM EGTA, 4 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.2, 300 mOsm). Cells were kept in Tyrode's solution containing 135 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM Hepes (pH 7.4, 300 mOsm) until the whole-cell configuration was achieved. The external solution was then exchanged with the Ba^{2+}-containing solution, referred to as Ba-solution, containing 5 mM $BaCl_2$, 150 mM *N*-methyl-D-glucamine, 2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM Hepes (pH 7.4, 300 mOsm,), while holding the cell at −70 mV. The drug stock solutions were diluted in Ba-solution before use. Effects of agonists on barium currents flowing through Ca^{2+} channels were measured on current waveforms activated by 100- or 200-ms command pulses to 0 mV from a holding potential of −70 mV and recorded with a MutiClamp-700B amplifier (Molecular Devices). The data were quantified off-line using custom-made software by measuring the peak current amplitude of the depolarizing test pulses.

Whole-Cell Recording in Spinal Cord Slice. Transverse slices (~600 μm) of L4 and L5 spinal cord segments with an attached dorsal root from adult rats were prepared with a vibrating microslicer and perfused in oxygen-bubbled Krebs' solution (117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose) for blind whole-cell recording (1). Resistance of patch pipettes was typically 4–10 MΩ. The pipette solution contained 135 mM K-gluconate, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM KCl, 5 mM EGTA, 5 mM Hepes, and 5 mM D-glucose. Currents were filtered at 2 kHz and digitized at 5 kHz using the Axopatch 200B (Molecular Devices) and were analyzed by pCLAMP8.5 (Molecular Devices) at a holding potential of −70 mV. Amplitude of mEPSCs was analyzed with an Axograph (Molecular Devices). Dorsal root stimulation, which was sufficient to recruit C-fibers, was delivered with a suction electrode linked to a constant-current stimulator (Electronic Stimulator; Nihon Kohpen). Afferent C-fibers were identified by their estimated evoked EPSC latency at 0.9 ± 0.5 m/s (range: 0.3–1.5 m/s).

Electric Stimulation, Cell Surface Biotinylation, and Immunoblotting. Neurons from L4 and L5 DRGs were cultured in the medium containing an equal volume of DMEM and F12 medium with 1% N2 for 3 d. Cells were stimulated by 10-Hz electric stimulation with platinum wires contacting the culture medium. Each stimulation pulse (0.5 ms, 8–10 V) was sufficient to elicit action potential. Cells were stimulated for 5 min. Surface protein biotinylation was performed after 5-min electrical stimulation by incubating cultured neurons with Sulfo-NHS-LC-Biotin (0.5 mg/mL; Pierce) for 60 min at 4 °C. Cells were then processed for precipitation of biotinylated proteins with ImmunoPure Immobilized Streptavidin (Pierce) and immunoblotting with DOR antibodies (1:3,000; Neuromics) and actin (1:10,000; Santa Cruz). The intensity of DOR bands was normalized to actin.

1. Nakatsuka T, Park JS, Kumamoto E, Tamaki T, Yoshimura M (1999) Plastic changes in sensory inputs to rat substantia gelatinosa neurons following peripheral inflammation. *Pain* 82:39–47.

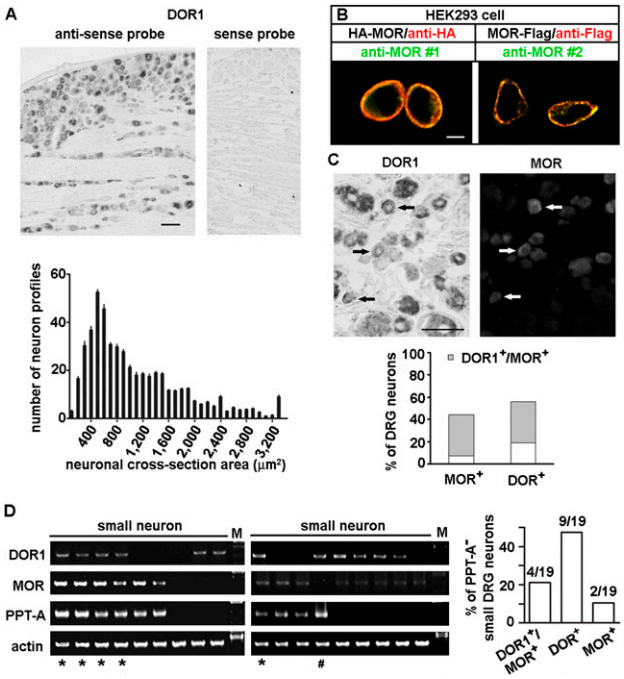

Fig. S1. Coexpression of DORs and MORs in small DRG neurons of rats. (*A*) In situ hybridization shows that DOR1 mRNA is present in both small (<900 μm^2) and large NPs in the rat DRG (500 DOR1$^+$ NPs randomly selected from each DRG, n = 4 DRGs). (Scale bar: 100 μm.) (*B*) Immunostaining shows that two antibodies against MOR$^{384–398}$ (anti-MOR #1, 1:1,000; Neuromics and anti-MOR #2, 1:8,000; Chemicon) recognize MORs in HEK293 cells transfected with the plasmid expressing HA-MOR or MOR-Flag. (Scale bar: 8 μm.) (*C*) In situ hybridization combined with immunostaining shows that DOR1 mRNA is present in MOR-containing small neurons (arrows) in the rat DRG. (Scale bar: 25 μm.) (*D*) Single-cell PCR shows coexpression of DOR1 and PPT-A in small DRG neurons of mice (#). Coexpression of DOR1 and MOR is seen in PPT-A mRNA-containing small neurons (*).

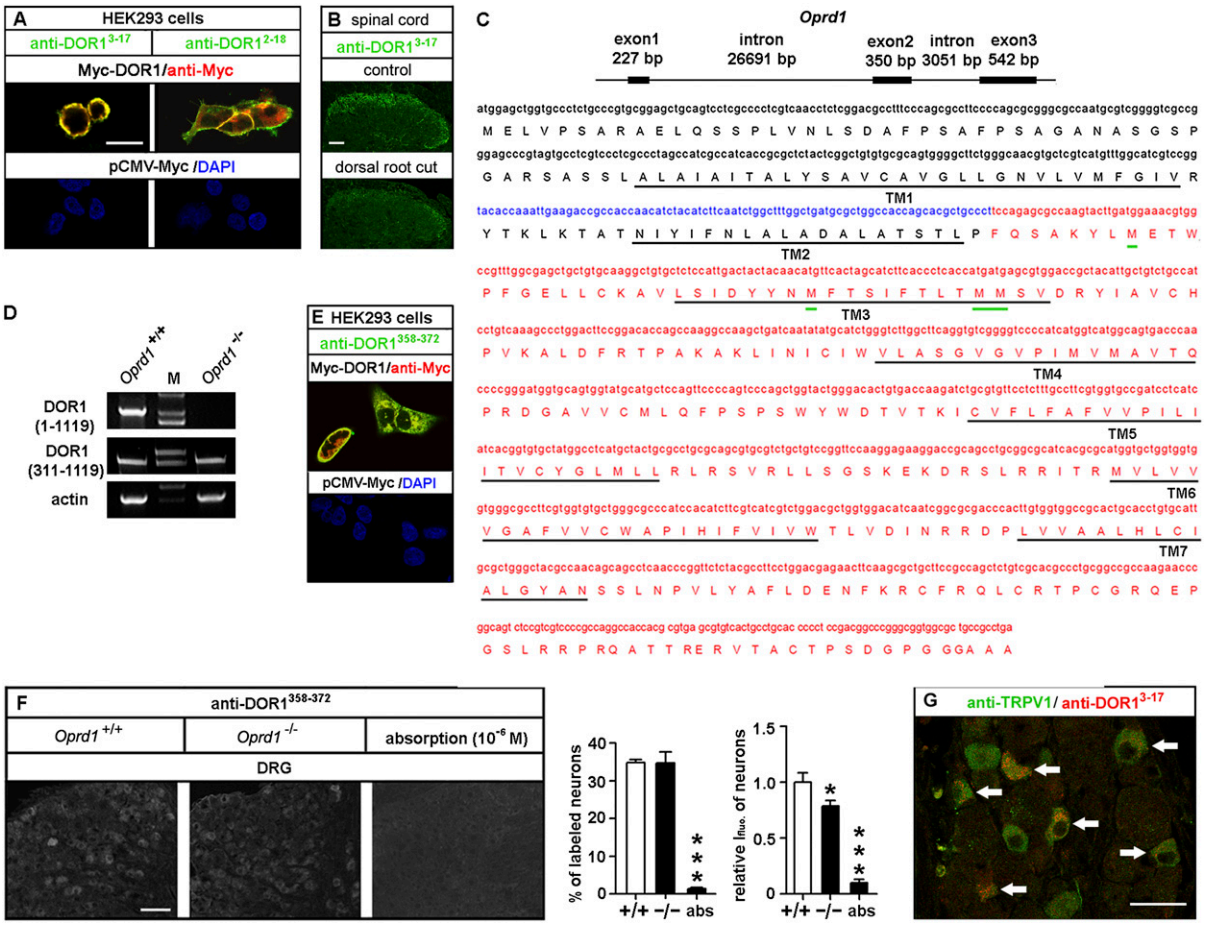

Fig. S2. Analysis on *Oprd1* exon 1-deleted mice. (*A*) Double-immunofluorescence staining shows that antibodies against DOR1$^{3–17}$ (1:500; DiaSorin) or DOR1$^{2–18}$ (1:2,000; Alomone) recognize DORs in HEK293 cells transfected with the plasmid expressing Myc-DOR1 but do not produce any staining in cells transfected with pCMV-Myc vector. Cells are indicated by nuclear DAPI staining. (Scale bar: 20 μm.) (*B*) Immunostaining of DORs in laminae I–II of the mouse spinal cord was markedly reduced after dorsal root transaction. (Scale bar: 80 μm.) (*C*) Scheme of *Oprd1* gene, DOR1 mRNA, and protein. The nucleotide acid sequence of exon 1 is indicated with black letters. The nucleotide acid sequence identified by RT-PCR in exon 1-deleted mice is indicated in red. Green underlining indicates the putative starting codons for translation of truncated protein. TM, transmembrane domain. (*D*) RT-PCR shows the presence of a partial DOR1 mRNA (311–1,119 bp), which contains putative start-codons for translation (indicated by green underlining in *C*), in the spinal cord of *Oprd1* exon 1-deleted mice. Sequencing of the PCR product from the *Oprd1*$^{-/-}$ mice proves the presence of the partial DOR1 mRNA. (*E*) Double-immunostaining shows that in HEK293 cells expressing Myc-DOR1, the Myc-DOR1 is shown by both the antibodies against DOR1$^{358–372}$ (1:500; Lifespan Biosciences) and the antibodies against Myc. (*F*) Immunostaining with antibodies against DOR1$^{358–372}$ (1:10,000) is largely abolished by preabsorption but remains in the *Oprd1* exon 1-deleted mouse with reduced immunostaining intensity. *$P < 0.05$; ***$P < 0.001$. (Scale bar: 80 μm.) (*G*) DORs and vanilloid receptor type 1 (TRPV1) channels are colocalized in many small neurons (arrows) in the mouse DRG. (Scale bar: 40 μm.)

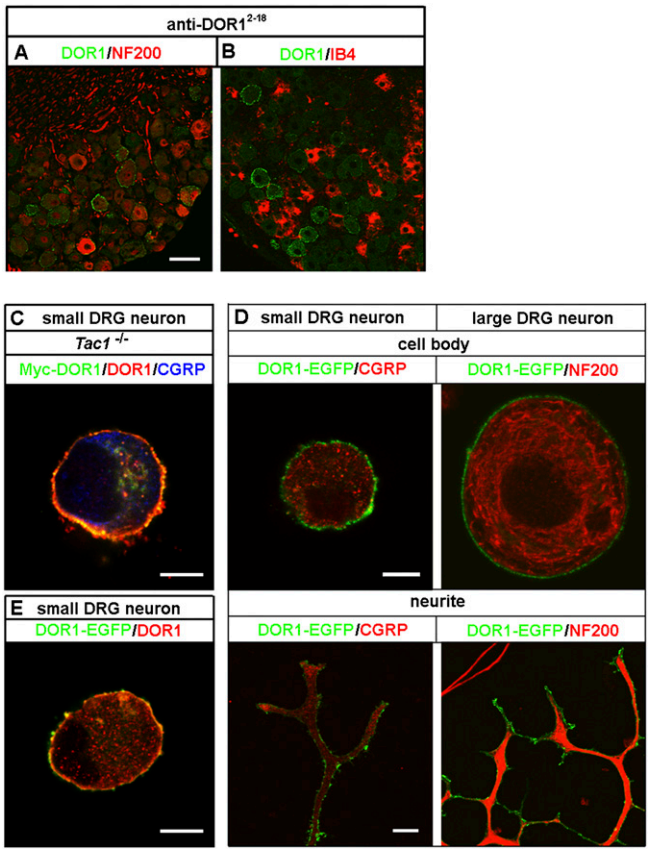

Fig. S3. Cell surface localization of DOR1-EGFP in DRG neurons. DOR^{+} large DRG neurons are also immunostained for neurofilament 200 (NF200) (*A*) but not for IB4 (*B*). (Scale bar: 80 μm.) (*C*) Triple-immunostaining of a small DRG neuron cultured from a *Tac1*$^{-/-}$ mouse and transfected with the plasmid expressing Myc-DOR1 shows that neither Myc-DOR1 nor endogenous DOR is colocalized with CGRP in vesicles, although some DOR-containing vesicular structures are retained in the cytoplasm. (Scale bar: 8 μm.) (*D*) In both CGRP-containing small DRG neurons and NF200-containing large DRG neurons cultured from mice, exogenously expressed DOR1-EGFP is distributed on the cell surface of cell bodies. (Scale bar: 8 μm.) The cell surface localization of DOR1-EGFP is also seen in neurites of both small and large neurons cultured from mice. (Scale bar: 8 μm.) (*E*) In a small DRG neuron of a WT mouse, exogenously expressed DOR1-EGFP is localized on the cell surface but not colocalized with endogenous DORs associated with vesicles in the cytoplasm. (Scale bar: 8 μm.)

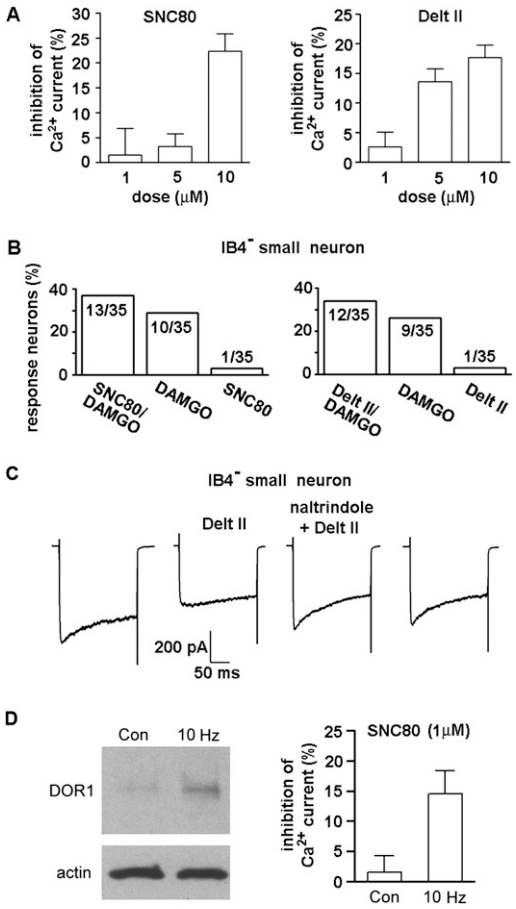

Fig. S4. DOR- and MOR-mediated inhibition in small DRG neurons. (A) Whole-cell patch-clamp recording in IB4$^-$ small neurons freshly dissociated from mouse DRGs. Ca^{2+} currents are apparently inhibited by SNC80 at 10 μM (n = 10) and by Delt II at 5 and 10 μM (n = 9) in a dose-dependent manner. (*B*) In IB4$^-$ small neurons freshly dissociated from rat DRGs, Ca^{2+} currents in a single neuron can be inhibited by both SNC80 (10 μM) and DAMGO (1 μM) or by both Delt II (10 μM) and DAMGO. (*C*) DOR agonist-induced effect in IB4$^-$ small DRG neurons of rats can be blocked by naltrindole (n = 5). (*D*) Cell surface biotinylation and immunoblotting show that the amount of DORs on the cell surface of neurons dissociated from mouse DRGs is markedly increased by electric stimulation (10 Hz, voltage of 8–10 V, duration of 0.5 ms) for 5 min. The inhibitory effect of SNC80 (1 μM) on Ca^{2+} currents is strongly enhanced in IB4$^-$ small DRG neurons 30 min after electric stimulation (10 Hz, voltage of 8–10 V, duration of 0.5 ms) for 1 min (n = 15). Con, control.

Table S1. Primers for PCR experiments and probes for in situ hybridization

No.	Gene	Primer name	Primer sequence	Product length, bp
1	*OPRD1* (*NM_013622*)	OPRD1-F	5′-GTGCAAGGCTGTGCTCTCCATTG-3′	770
		OPRD1-R	5′-GTCGGGTAGGTCAGGCGGCAGCGCCACCG-3′	
2	*OPRM1* (*NM_001039652*)	OPRM1-F	5′-ATGGACAGCAGCGCCGGCCC-3′	1,317
		OPRM1-R	5′-TCACCTGCCAAGCTGGCCTTC-3′	
3	*PPT-A* (*NM_009311*)	PPT-A-F	5′-ATGAAAATCCTCGTGG-3′	393
		PPT-A-R	5′-TTATTTACGTCTTCTTTC-3′	
4	*GAPDH* (*NM_008084*)	GAPDH-F	5′-TGCTGAGTATGTCGTGGAG-3′	555
		GAPDH-R	5′-TAGCCCAAGATGCCCTTCAGT-3′	
5	*OPRD1* (*NM_013622*)	OPRD1-F	5′-ATGGAGCTGGTGCCCTCTG-3′	
		OPRD1-F	5′-TCCAGAGCGCCAAGTACTTG-3′	
		OPRD1-R	5′-TCAGGCGGCAGCGCCACCG-3′	
6	*OPRD1* (*NM_013622*)	OPRD1-F	5′-GTGCAAGGCTGTGCTCTCCATTG-3′	770
		OPRD1-R	5′-GTCGGGTAGGTCAGGCGGCAGCGCCACCG-3′	
7	*OPRD1* (*NM012617*)	OPRD1-F	5′-GTGCAAGGCTGTGCTCTCCATTG-3′	770
		OPRD1-R	5′-GCCGGGTAGGTCAGGCGGCAGCGCCACCG-3′	
8	*OPRD1* (*NM_013622*)	OPRD1-F1	5′-TTCTGGGCAACGTGCTCGTC-3′	510
		OPRD1-R1	5′-CATAGCACACCGTGATGATG-3′	
		OPRD1-F2	5′-TGTTTGGCATCGTCCGGTAC-3′	320
		OPRD1-R2	5′-TGAAGCCAAGACCCAGATGC-3′	
9	*OPRM1* (*NM_001039652*)	OPRM1-F1	5′-GTATCTTCACCCTCTGCACC-3′	510
		OPRM1-R1	5′-AGGCAATGCAGAAGTGCCAG-3′	
		OPRM1-F2	5′-AGGCCCTGGATTTCCGTACC-3′	272
		OPRM1-R2	5′-CATGCGGACACTCTTGAGTC-3′	
10	*PPT-A* (*NM_009311*)	PPT-A-F1	5′-ATGAAAATCCTCGTGGCCGT-3′	380
		PPT-A-R1	5′-CTTTCGTAGTTCTGCATCGC-3′	
		PPT-A-F2	5′-CCACTCAACTGTTTGCAGAG-3′	292
		PPT-A-R2	5′-GCTCTTTTGCCCATTAGTCC-3′	
11	β-*Actin* (*X03672*)	β-Actin-F1	5′-GCCAACCGTGAAAAGATGAC-3′	556
		β-Actin-R1	5′-GCACTGTGTTGGCATAGAGG-3′	
		β-Actin-F2	5′-GGCTGTGCTGTCCCTGTATG-3′	391
		β-Actin-R2	5′-GGCTGTGCTGTCCCTGTATG-3′	

Nucleotide sequence of PCR primers is listed with reference to corresponding GenBank accession numbers. Primers 1–4 were used in PCR of three subsets of DRG neurons, primer 5 was used in PCR of *Oprd1* exon 1-deleted mice, primers 6 and 7 were used for probes in situ hybridization, and primers 8–11 were used in single-cell PCR. F: forward; R: reverse. F1 and R1 and F2 and R2 primer pairs were used in the first- and second-round single-cell PCR assays. For each primer pair, forward and reverse primers are located in different exons to avoid the amplification of genomic DNA.