Supporting Information

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SI Materials and Methods

Chemicals. Capsaicin, tetraethylammonium (TEA) and alpha beta-methylene ATP lithium salt were purchased from Sigma-Aldrich. T16Ainh-A01 (A01) was purchased from Calbiochem. Allyl isothiocyanate (AITC) was purchased from Wako.

Immunohistochemistry. C57BL/6NCr mice were perfused with 20 mL of ice-cold PBS and 4% (vol/vol) paraformaldehyde containing 0.5% methanol (Wako). The DRGs (L1–L6) from one mouse were fixed at 4 °C for 16 h and were immersed twice in 30% (wt/vol) sucrose in PBS at 4 °C for 24 h, embedded in Surgipath FSC22 (Leica Microsystems), and sectioned at a thickness of 10 μm. The sections were incubated with a rabbit anti-TRPV1 antibody (1:2,000) (a generous gift from M. Kido, Kyushu University, Fukuoka, Japan) and a goat anti-ANO1 antibody (1:50; sc-69343; Santa Cruz Biotechnology) at 4 °C for 16 h after blocking. The sections were reacted with Alexa 488 and Alexa 594 (Molecular Probes) at room temperature for 1 h. Blocking, staining, and washing buffers used the PBS solution containing 1% BSA (Sigma-Aldrich) and 0.25% Triton X-100 (Sigma-Aldrich). After staining, the samples were covered with Fluoromount (Diagnostic Biosystems). The anti-TRPV1 antibody from M. Kido was made as follows: A peptide encoding the predicted carboxyl terminus of TRPV1 (EDAEVFKDSMVPGEK) was coupled to keyhole limpet hemocyanin via an N-terminal cysteine and used to immunize rabbits. The affinity and specificity of the anti-TRPV1 antibody were confirmed by both DRG immunohistochemistry and Western blotting analysis (Fig. S4).

Isolation of DRG Neurons. Mice were anesthetized with isoflurane, and the DRG was separated from L4–L6 after perfusion with 10 mL ice-cold artificial cerebrospinal fluid (aCSF: 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 10 mM glucose, 24 mM $NaHCO_3$, equilibrated with 95% O_2 and 5% CO_2 for 1 h on ice). The tissues were incubated with 725 μg collagenase type IX (lots no. SLBG3258V and SLBG3259V; Sigma-Aldrich) in 250 μL Earle's balanced salt solution (Sigma-Aldrich) at 37 °C for 25 min. Next, the DRG neurons were separated mechanically by 10–20 cycles of pipetting using a small-diameter Pasteur pipette and were filtered through a 40-μm cell strainer (BD Falcon). The neurons were centrifuged three times at 300 × *g* for 5 min (4 °*C*). Then supernatants were discarded, and fresh PBS or aCSF was added to wash out collagenase. Finally, the isolated neurons were placed on 12-mm-diameter coverslips (Matsunami) with 40 μL aCSF. They were maintained at room temperature in a 95% O_2 and 5% CO_2 humidified chamber and were used for experiments within 4 h of isolation.

Immunoprecipitation and Western Blotting. Samples were prepared from HEK293T cells expressing both TRPV1 and ANO1, cells expressing TRPV1 alone, cells expressing ANO1 alone, or pcDNA3.1-transfected cells. HEK293T cells were collected by scraping in lysis buffer containing 150 mM NaCl, 20 mM Hepes, and 1 mM EDTA, with 1% IGEPAL CA-630 (Sigma-Aldrich) after 30-min incubation on ice. The samples were centrifuged at 161,000 × *g* at 4 °C for 30 min. The resulting supernatants were incubated with an anti-ANO1 antibody (1:4; ab53213; Abcam) for 16 h. Protein G-coated magnetic beads (GE Healthcare) were added, and the solutions were incubated for 6 h. The proteins were denatured with SDS buffer containing 50 mM Tris·HCL, 2% (wt/vol) SDS, 100 mM DTT, 10% (vol/vol) glycerol, and 0.01% bromophenol blue at room temperature for 30 min and at 95 °C for 10 min and were used in SDS/PAGE. After protein transfer to the Immobilon-P transfer membrane (Millipore), the proteins were reacted with an anti-TRPV1 antibody (1:100; sc-12498; Santa Cruz Biotechnology) in PBS buffer containing 1% BSA and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20; Nacalai Tesque). The BSA concentrations were 3% (wt/vol) and 0% in the blocking and washing solutions, respectively. After the second immunoreaction with an HRP-linked antibody, the membrane was washed once in PBS with Tween 20 and three times in PBS without Tween 20. After washing, the HRP reactions were run with the ECL Prime Western Blotting Detection Reagent (GE Healthcare), and the luminescence was detected with an image analyzer (LAS-3000 mini; Fujifilm).

For immunoprecipitation, tissues were collected after perfusion of ice-cold aCSF and put in TNE lysis buffer [10 mM Tris·HCl, 150 mM NaCl, 1 mM EDTA, complete EDTA-free protease inhibitor mixture (Roche Applied Science), 1 mM Na_3VO_4, and 1% IGEPAL CA-630 (Sigma-Aldrich)]. The samples were homogenized and centrifuged at 161,000 × *g* for 5 min at 4 °C. The supernatants were transferred to a new tube and centrifuged at 161,000 × *g* for 60 min at 4 °C. Immunoprecipitation and Western blotting procedures were similar to the methods used for HEK293T cells; however, we used the Santa Cruz's anti-ANO1 antibody for immunoprecipitation (1:100; sc-69343) and the anti-TRPV1 antibody (from M. Kido) for Western blotting (1:4,000).

Preparation of Spinal Cord Slices. Spinal cord slices were prepared as previously described (1). Mice were anesthetized with urethane (1.2–1.5 g/kg, i.p.) followed by a thoracolumbar laminectomy. Segments of the lumbar spinal cord were placed in ice-cold Krebs solution containing 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MglCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose, equilibrated with 95% O_2 and 5% CO_2. The pia-arachnoid membrane was removed after cutting of all of the ventral and dorsal roots except for the L4 or L5 dorsal root on one side, as reported previously (2). The spinal cord was mounted on a vibratome (Linier Slicer Pro-7; Dosaka) and a 500-μm-thick transverse slice was cut. The slice was placed on a nylon mesh in the recording chamber with a volume of 0.5 mL and was submerged completely and perfused with Krebs solution saturated with 95% O_2 and 5% CO_2 at 36 ± 1 °C at a flow rate of 10–15 mL/min.

Statistical Analyses. Statistical analyses were performed with Origin Pro-8 (OriginLab). Paired or unpaired *t*-tests and Holm–Bonferroni analyses were performed for comparisons. Generation of action potentials was analyzed with the two-sample Kolmogorov–Smirnov test. Behavior tests were analyzed with the Mann–Whitney *u* test. Values of $P < 0.05$ were accepted as statistically significant.

1. Akimoto N, et al. (2013) CCL-1 in the spinal cord contributes to neuropathic pain induced by nerve injury. *Cell Death Dis* 4:e679.

2. Uta D, et al. (2010) TRPA1-expressing primary afferents synapse with a morphologically identified subclass of substantia gelatinosa neurons in the adult rat spinal cord. *Eur J Neurosci* 31(11):1960–1973.

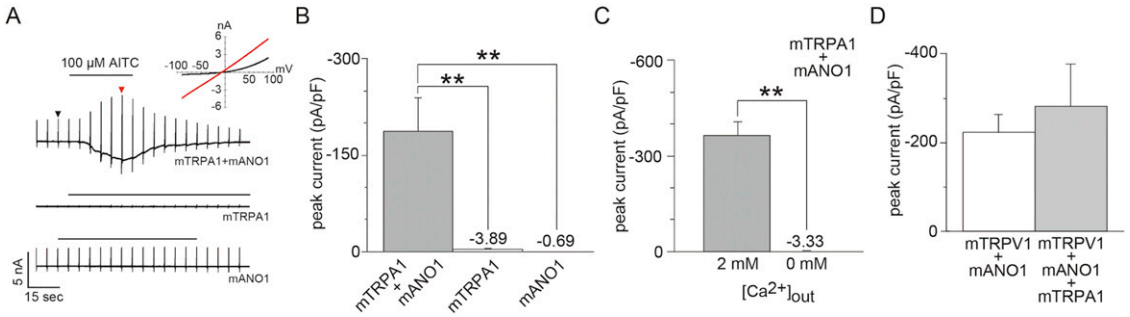

Fig. S1. TRPA1–ANO1 interaction. (*A*) Representative traces of whole-cell currents activated by AITC in HEK293T cells expressing mTRPA1 and mANO1 (*Top*), cells expressing mTRPA1 alone (*Middle*), and cells expressing mANO1 alone (*Bottom*). (*B*) Comparison of the peak currents activated by AITC in cells expressing mTRPA1 and mANO1, cells expressing mTRPA1 alone, and cells expressing mANO1 alone in the presence of extracellular calcium ($n = 5$ or 6 cells). (*C*) Comparison of the peak currents activated by AITC with ($n = 5$ cells) and without extracellular calcium ($n = 7$ cells). (*D*) Comparison of the peak currents activated by capsaicin in HEK293T cells expressing mTRPV1 and mANO1 ($n = 5$ cells) and cells expressing mTRPV1, mANO1, and mTRPA1 ($n = 4$ cells). Data are shown as mean ± SEM; $^{**}P < 0.01$.

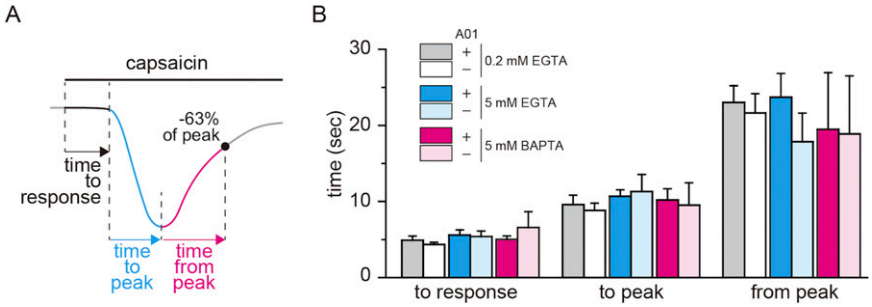

Fig. S2. Kinetic analysis of the capsaicin-activated currents in DRG neurons with or without A01. (*A*) Definition of the measured parameters: time to response, to peak response, and from peak to 1–1/*e* (~0.63). (*B*) Comparison of the capsaicin-activated currents in the pipette solution containing 0.2 or 5 mM intracellular EGTA or 5 mM BAPTA without ($n = 24$, 13, and 10 cells, respectively) and with ($n = 20$, 11, and 9 cells, respectively) A01. Data are shown as mean ± SEM.

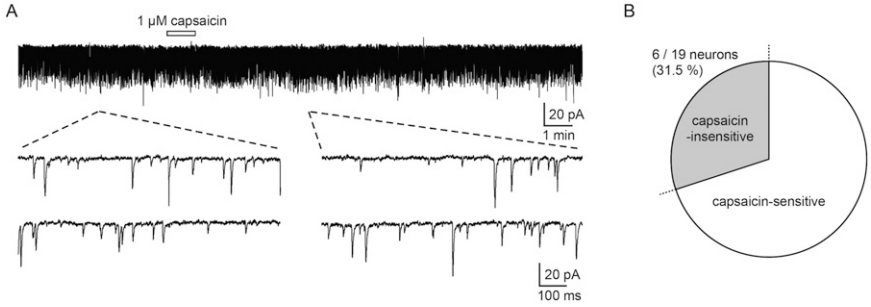

Fig. S3. Capsaicin-insensitive SG neurons in spinal cord. (*A*) A representative trace of sEPSCs in capsaicin-insensitive neurons. (*B*) Proportion of the capsaicin-sensitive and -insensitive neurons in all recorded neurons.

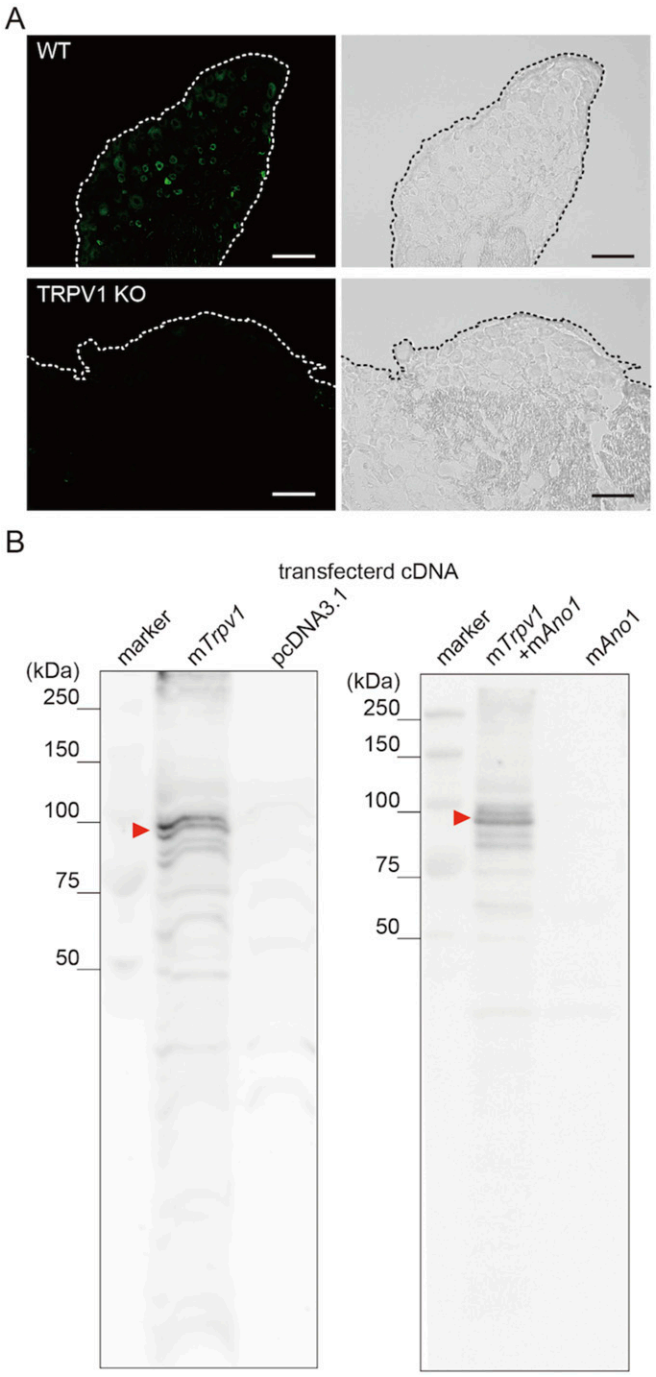

Fig. S4. Basic characterization of the anti-TRPV1 antibody. (*A*, *Left*) Immunofluorescent images with an anti-TRPV1 antibody (from M. Kido; green) in 10-μm slices of DRG (L4–L6) from wild-type and TRPV1 knock-out (TRPV1KO) mice. (*Right*) Bright-field images. (Scale bars, 50 μm.) (*B*, *Left*) SDS/PAGE with the anti-TRPV1 antibody (from M. Kido) in HEK293T cells transfected with m*Trpv1* cDNA or pcDNA3.1. (*Right*) Cells transfected with m*Trpv1* and m*Ano1* cDNAs or with m*Ano1* cDNA alone. Red arrowheads indicate TRPV1 bands (molecular mass ∼95 kDa).