Supporting Information

Kim et al. 10.1073/pnas.1019755108

SI Materials and Methods

Mice. Two-month-old male C57BL/6 mice were purchased from Charles River Laboratories. WBB6F1$^{+/+}$-, WBB6F1-W/W^{v}-, TLR7-, and TRPV1-deficient mice were purchased from Jackson Laboratory. They were crossed with wild-type C57BL/6 (Charles River) to produce heterozygous mice. The resultant heterozygous mice were used as mating pairs. Specifically, the Jackson Laboratory TLR7 mouse was originally established by R. Flavell's laboratory (Yale University, New Haven, CT). Another TLR7-deficient mouse line was originally established by S. Akira's laboratory (Osaka University, Osaka, Japan) and was provided by Maripat Corr's laboratory (University of California, San Diego). According to the Flavell and Corr laboratories, both TLR7 lines were backcrossed 10 generations onto the C57BL/6 background. TLR7 mice were bred and maintained by mating heterozygous female and hemizygous male mice in the University of California, San Diego Animal Facility. Littermates or age-matched mice were generally used as controls. BALB/c and C3H mice were a gift from Kirk Knowlton's laboratory (University of California, San Diego). All experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the recommendation of the International Association for the Study of Pain.

Immunofluorescence. Adult mice were anesthetized with pentobarbital and perfused transcardially with 4% paraformaldehyde and then the dorsal root ganglia (DRG) were collected. The DRGs were cryoprotected in 20% sucrose, frozen in Tissue-Tek O.C.T. (a formulation of water-soluble glycols and resin, which provides a convenient specimen for cryostat sectioning), sectioned with a cryostat at 14-μm thickness, and mounted on Superfrost Plus slides. Guinea pig anti-TRPV1 (1:400; Neuromics) was used for primary antibody. Secondary antibodies were conjugated to Alexa-488 or -568 flurochromes (Molecular Probes). Images were obtained using a confocal microscope system.

Whole-Cell Patch-Clamp Recordings of Mouse DRG Neurons. Acutely dissociated DRG neurons were obtained from C57BL/6 mice (8–10 wk) and attached to coverslips for recording. Before recording, coverslips were incubated in media for at least 1 h. DRG neuron recordings were performed in Hepes buffer of the following composition (in mM): 10 Hepes, 150 NaCl, 10 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5.5 glucose, and 22 sucrose (pH 7.4). Under the upright microscope, whole-cell patch recordings were obtained from acute-isolate DRG neurons in voltage-clamp mode and switched current-clamp configuration for recordings with an Axopatch 700B (Molecular Devices). Pipette resistance ranged from 3 to 6 MΩ. The internal solution consisted of (in mM) 140 K-gluconate, 10 Hepes, 7 NaCl, 4 Mg-ATP, and 0.3 Na_3-GTP (pH 7.4). Firing properties were established for a 1,000-ms duration by applying current injection from −100 to 350 pA with a 50-pA increase. The cells with access resistance less than 20 MΩ that showed less than 20% change during the recordings were included in this study. Records were filtered at 5 kHz and digitized at 2–5 kHz with a Digidata 1322A (Molecular Devices) analog-to-digital board. Data were analyzed and plotted with the pClamp (Molecular Devices), Mini Analysis Program (Synaptosoft), and Origin. All data were collected at room temperature (23–26 °C).

Behavioral Studies. Littermates or age-matched male mice 2–3 mo of age were used in the experiments. Measurement of foot-withdrawal thresholds for the drug administrated to the hind paws was used to assess mechanical allodynia. A 50% withdrawal threshold was measured using a set of von Frey filaments (0.02–4 g; Stoelting), following a modified up–down method (1). Heat hyperalgesia was determined using plantar test instruments (7370; Ugo Basile), following the modified method of Hargreaves et al. (2). A radiant heat source beneath a glass floor was aimed at the plantar surface of the mouse hind paw. Tests were performed five times for each paw, with a 5-min interval between consecutive tests. Mice were tested for their baseline response twice before injection.

The scratching behavioral assay was performed as previously described (3, 4). Imiquimod was purchased from Invivogen; Histamine and chloroquine were from Sigma-Aldrich; SLIGRL-NH_2 was from Bachem. All other compounds mentioned in this study were purchased from Sigma-Aldrich. Briefly, itch-evoking compounds were subcutaneously injected into the rostral back after acclimatization for 1 h, and the resultant bouts of scratching were counted at 5-min intervals over the 30-min observation period. An intraplantar injection of IQ in a volume of 20 μL was made through a 30-gauge needle. Time spent licking the injected hind paw during 20 min at a 10-min interval after injection was measured. All behavioral tests were performed using a blinded protocol.

Intrathecal Injection. Mice were anesthetized with 1.5% isoflurane before, during, and for 30 min after injection. Intrathecal injection of capsaicin (10 μg; Sigma) or vehicle (10% ethanol and 10% Tween-80 in saline) in a volume of 5 μL was done by direct injection at the level of the pelvic girdle with a Luer-tipped Hamilton syringe to which a 30-gauge needle was attached.

Statistical Analysis. The data were expressed as the mean value with SE. Statistical comparisons were made using Student's t test or two-way ANOVA with Bonferroni posttests. When the p value was smaller than 0.05, the difference was considered to be significant.

RNA Extraction and RT-PCR. Total RNA was isolated from tissues (kidney, spleen, lung, and heart) of WT and TLR7-deficient mice using TRIzol (Invitrogen) according to the manufacturer's instructions and treated with RNase-free DNase I (Ambion) to eliminate genomic DNA contamination. cDNA was generated from the total RNA (2 μg) using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). PCR was performed using Econo Taq DNA polymerase (Lucigen) under the following conditions: 30 cycles at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. The primers used for RT-PCR were 5′-AAGAAAACTGAAAATGGTGTTTTCGA-3′ (TLR7 forward primer), 5′-ACAGGTACACAATTGCATCTTAAATCG-3′ (TLR7 reverse primer), 5′-AGGTCATCCCAGAGCTGAACG-3′ (GAPDH forward primer), and 5′-CACCCTGTTGCTGTAGCCGTAT-3′ (GAPDH reverse primer). The PCR products were resolved by 1% agarose gel electrophoresis and stained by ethidium bromide.

Preparation of Primary Cell Cultures. DRG neurons were prepared from adult mice (~2 mo old) by methods similar to those described previously (3, 5). Briefly, DRG from all spinal levels were collected in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS; Invitrogen) and treated with enzyme solution containing 60 units of papain/cystein (0.33 mg/mL), 5 mg/mL of Dispase, and 4 mg/mL of collagenase (Gibco) for 10 min at 37 °C, respectively. Following trituration and centrifugation, the dispersed cells were

resuspended in DMEM/F12 culture media containing 10% FBS [nerve-growth factor (NGF) or glial cell line-derived neurotropic factor (GDNF) was not included in culture medium] and were plated in 35-mm poly D-lysine precoated glass-bottomed dishes (MatTek) coated with laminin. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and assayed after 16–20 h.

Primary microglia were prepared from P0–P2 (postnatal day 0 to day 2) pups as previously described (6). The brain tissue was mechanically disrupted by triturating with a 30-mL syringe and a 19G ½-inch needle and then seeded into 75-cm^2 flasks. Cells were cultured in DMEM supplemented with 10% FBS, 1 mM Hepes, 2 mM glutamine, and 1× antibiotic/antimycotic (Gibco). Cultures were maintained at 37 °C with 5% CO_2, and the media were changed every 5 d with fresh media containing 5% FBS. After 2 wk, the cells were harvested by gently shaking at 200 × *g* for 3 h on an orbital shaker. Microglia were collected from the flasks and were plated in glial culture media. After 30 min, the dishes were washed with fresh medium to remove unattached astrocytes. The resulting cells were found to be >98% microglia by staining with rabbit anti-Iba-1 antibody (1:2,000; Wako).

Bone marrow-derived macrophage (BMDM) were prepared as previously described (7). BMDM were collected by flushing femurs and tibias of mice (adult C57 female) with BMDM media (DMEM supplemented with 2 mM L-glutamine, 25 mM Hepes, 10% FBS, 10% CMG14-12 cell supernatant, 3.4 μL/L β-mercaptoethanol). Cells were cultured on non-tissue culture treated dishes in BMDM media and differentiated for 5 d. The cells were used on experiment days 6–10 post-isolation.

Splenocytes were prepared as previously described (8). Spleens were aseptically removed from WT and TLR7-deficient mice and then homogenized and strained through 100-μm nylon mesh (BD). Cells collected were centrifuged at 450 × *g* for 5 min, incubated in RBC lysis buffer (Sigma), and washed twice with HBSS (without Ca^{2+}, Mg^{2+}) buffer. Cells were resuspended in DMEM media with 10% FBS and seeded into 96-well plates at a density of 5×10^5 cells/well for proliferation assay or at 1×10^6 cells/well for TNF-α ELISA.

ELISA. Splenocytes from WT and TLR7-deficient mice were stimulated with loxoribine (100 μM), imiquimod (100 μM), or LPS (10 μg/mL) for 24 h, and supernatants were collected. The concentrations of TNF-α secreted in the supernatants were measured using an ELISA kit (BioSource) according to the manufacturer's instruction.

Cell Proliferation Assay. Splenocytes were seeded into a 96-well plate (5×10^5 cells/well) and stimulated with various concentrations of loxoribine, imiquimod, or Oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanosine motifs (CpG-ODN) for 48 h. After stimulation, 10 μL of cell proliferation detection reagent WST-1 (Daeil Lab) was added to each well and incubated further for 4 h, and absorbance was measured at 450 nm using a microplate reader (Fluostar optima; BMG).

Calcium Assays. The calcium response in macrophage or microglia cells was measured by the FLEXstation system (Molecular Devices). Cells were plated a day before at 150,000 cells/well in 96-well clear-bottom black-walled plates. On the following day cells were incubated for 30 min at room temperature with 180 μL 4 μM Fura-2 AM in HBSS solution containing 25 mM Hepes, 0.5% BSA, and 2.5 mM probenecid (pH 7.5). Cells were washed with HBSS–Hepes–BSA–Probenecid twice and were incubated at 37 °C for 25 min in 75 μL of the buffer. Cells were washed with HBSS–Hepes–BSA–Probenecid and incubated an additional 5 min at 37 °C before the calcium response was assayed. A baseline reading for 40 s was taken before addition of either 25 μL 20 μg/mL IQ or 2.5 μM UDP (control). The response was measured before the 25-μL minimization and maximization calibration buffers were added. Intracellular calcium was calculated by the following equation:

$$[Ca^{2+}]_i = K_d\, Q\, (R - R_{min})/(R_{max} - R),$$

where K_d is 250 nM, R_{min} is the maximum 380-nm value after minimization addition, R_{max} is the minimum 380-nm value after maximization addition, R is the ratio of the reading at 340 nm over 380 nm, and Q is the ratio of R_{min}/R_{max}.

Measurements of $[Ca^{2+}]_i$ at the individual cell level were performed using a confocal laser microscope (FluoView FV1000; Olympus). Fluorescent images were acquired and analyzed with the FluoView software package. Changes in the intracellular Ca^{2+} levels in acutely dissociated DRG neurons of the adult mouse DRG were monitored using the Ca^{2+}-sensitive dye fluo-3.

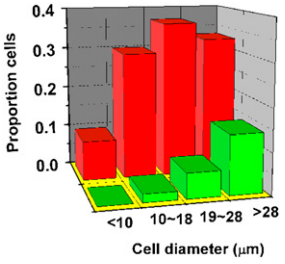

Fig. S1. IQ-induced calcium responses were preferentially observed in medium-to-large-diameter neurons. The cell diameter is the average of the shortest and the longest diameters of the cell. The red bars give the proportion of total neurons at given cell diameters; the green bars indicate proportion of IQ-sensitive neurons. Forty-one percent (57/139) of large-diameter neurons (>28 μm) responded to IQ; 16.5% (26/158) of neurons that have a range of cell diameter from 19 to 28 μm responded to IQ. These two populations (>19 μm) represented 89.2% of IQ-responding neurons. In contrast, only 7.7% (10/130) of small-diameter neurons (<19 μm) were sensitive to IQ, which represented 10.8% of IQ-responding neurons.

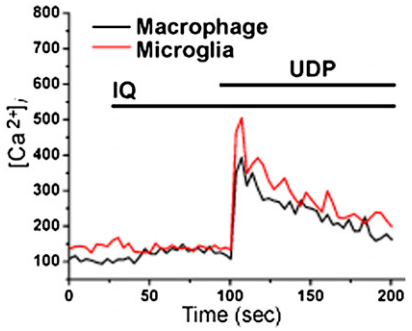

Fig. S2. IQ-induced calcium responses were not detected in macrophage and microglia cells. Macrophage and microglia cells were derived from WT mice. Treatment with IQ (final concentration of 10 μg/mL) did not elicit calcium responses. In contrast, application of UDP (100 μM) gave rise to increases in intracellular calcium in these cells. The calcium response was measured by the FLEXstation system after loading the cells with fura-2.

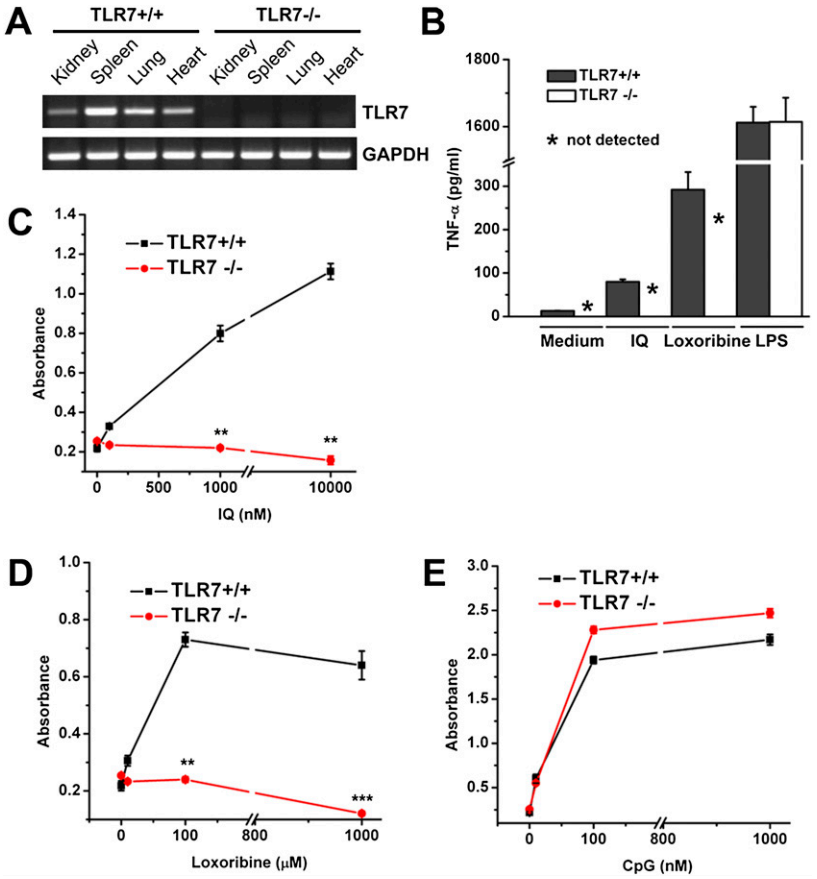

Fig. S3. IQ- or loxoribine-induced TNF-α production and cell proliferation were abrogated in splenocytes derived from TLR7-deficient mice. (*A*) TLR7 mRNA expression in various tissues of WT and TLR7-deficient mice was measured by RT-PCR. (*B*) Splenocytes of WT and TLR7-deficient mice were stimulated with medium alone, IQ (100 μM), loxoribine (100 μM), or LPS (10 μg/mL) for 24 h, and then TNF-α secreted into the medium was measured by ELISA. IQ and loxoribine induced TNF-α production in WT but not in TLR7-deficient splenocytes. (*C–E*) WT and TLR7-deficient splenocytes were stimulated with IQ, loxoribine, or CpG ODN (TLR9 agonist) for 48 h, and then cell proliferation was assessed (Student's *t* test: $^{**}P < 0.01$, $^{***}P < 0.001$). IQ- and loxoribine-activated splenocyte proliferation is completely abolished in TLR7-deficient cells, whereas a TLR 9 agonist (CpG) induced proliferation is not impaired. All data are mean ± SEM of triplicate samples of one representative experiment.

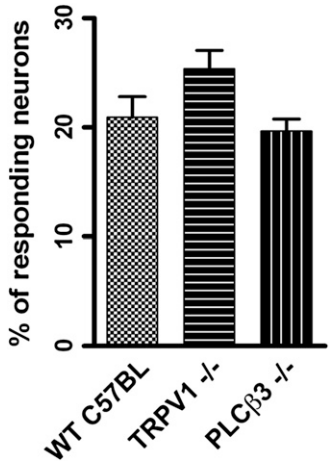

Fig. S4. The calcium responses to IQ were not impaired in either PLCβ3- or TRPV1-deficient DRG neurons. Calcium imaging experiments showed that the percentage of mutant DRG neurons responding to IQ (20 μg/mL) was similar to that of WT neurons (n = 4 or 5/genotype).

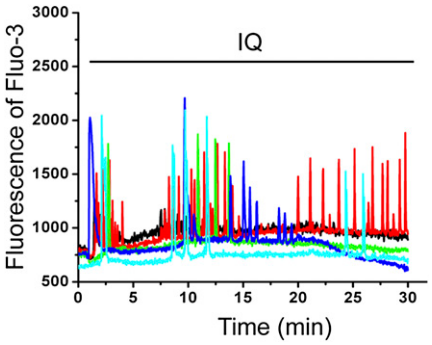

Fig. S5. IQ-induced calcium oscillations frequently lasted over 30-min observation periods in DRG neurons. Each trace indicates representative individual neurons that show calcium oscillation. The oscillations did not occur with regularity, but rather intermittently without significant alteration of amplitude. The frequency of oscillation was significant within 5 min after IQ treatment and reached a maximum between 7–12 min and declined gradually thereafter.

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