Supplementary Information

Toll-like receptor-7 Mediates Pruritus

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Supplementary Methods

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Supplementary Methods

Animals

Adult mice (male, 25-32 g) were used in all behavioral experiments. All transgenic mice including *Tlr7* knockout mice (B6.129S1-*Tlr7*^{tm1Flv}/J), *Trpv1* Knockout mice (B6;129P2-*Gt(ROSA)26Sor*$^{tm1(Trpv1-ECFP)Mde}$/J), and mast cell deficient SASH mice with *Kit* mutation (B6.Cg-KitW-sh/HNihrJaeBsmJ) were purchased from The Jackson Laboratory and bred in Thorn Animal Facility at Harvard Medical School. Since all these mice have C57BL/6 background, we used C57BL/6 mice as controls. *Tlr7* knockout mice were made by replacing a segment of exon 3 with a *lacZ* gene and *loxP*-flanked neomycin resistance cassette. The targeting vector was introduced to 129S1/Sv-p^+ Tyr^+ $Kitl^+$-derived CJ7 embryonic stem (ES) cells. This line was backcrossed ten times to C57BL/6Ncr by the donating laboratory. Homozygotes are viable, fertile, have no obvious developmental problems, but are poor breeders for unknown reasons. *Tlr7* knockout mice were made by Heterozygote x Hemizygote (Female x Male). In addition, adult male CD1 mice (Charles River Laboratories) were used included for some experiments. Because itch responses could be sex- and strain-dependent, there are limitations in this study by focusing on males and C57B/6 strain due to the fact that *Tlr7* knockout mice have C57BL/6 background. All animal experiments were approved by Harvard Medical School Institutional Animal Care and Use Committee and performed in accordance with the guidelines of guidelines of the International Association for the Study of Pain. Animals were housed with 12 h/12 h light/dark cycle. All of the behavioral tests were done by the observers blinded to the treatment or genotype of the animals.

Reagents

We purchased imiquimod, R848 and loxoribine from Invivogen, histamine, compound 48/80, chloroquine, serotonin, capsaicin, and resiniferatoxin (RTX), formalin, and carrageenan from Sigma-Aldrich. We also purchased histamine-trifluoromethyl-toluidine (HTMT) from Tocris, endothelin-1 (ET-1) from ALEXIS Biochemicals, mustard oil (allyl isothiocyanate, AITC) from Fluka, and the PAR2 agonist H-Ser-Leu-Ile-Gly-Arg-Leu-NH2 (SLIGRL-NH2) from Bachem.

Itch behavior test

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Itch behavior was tested blindly. Mice were habituated to the testing environment daily for at least two days before testing. The room temperature and humidity remained stable for all experiments. Animals were shaved at the back of the neck in an area of approximately 15 × 10 mm on the day before the injection of pruritic agents. Animals were put in small plastic chambers (14 × 18 × 12 cm) on an elevated metal mesh floor and allowed 30 min for habituation before examination. Mice were then briefly removed from the chamber and given an intradermal injection (50 μl) of a pruritogen in the nape of the neck under a brief anesthesia with isoflurane. After the injection, the number of scratches in every 5 min for 30 min was counted [5,16]. A scratch was counted when a mouse lifted its hindpaw to scratch the shaved region and returned the paw to the floor or to the mouth. On the basis of previous reports [5,8] and our preliminary studies, the following drug doses were selected: 500 μg for histamine, 100 μg for HTMT, 100 μg for compound 48/80, 25 ng for ET-1, 20 μg for 5-HT, 200 μg for chloroquine, and 100 μg for SLIGRL-NH2.

We also produced the "cheek model" of itch that can distinguish itch and pain responses as previous described [9]. Briefly, mice cheek was shaved after a brief anesthesia with isoflurane at least two days before experiments. On the day of experiment, mice were placed in chambers and allowed to habituate for one hour. Then, mice were removed from the chambers and given an intradermal injection of imiquimod, R848 or loxoribine (20 μg, 15 μl) into the cheek under isoflurane anesthesia. Immediately after the injection, the number of wiping by forearm and scratching by the hindpaw on the cheek were counted simultaneously for 20 min.

To examine the roles of TRPV1-expressing C-fibers in the imiquimod-induced itch, we destroyed the C-fibers by daily treatment with the potent TRPV1 receptor agonist resiniferatoxin (RTX, 30, 70 and 100 μg/kg, subcutaneously) for 3 consecutive days starting 10 days before the imiquimod injection as described previously[18].

Behavioral testing for baseline pain, inflammatory pain, and neuropathic pain

We performed the following tests blindly to examine pain sensitivity in normal, inflammatory pain, and neuropathic pain conditions.

von Frey test: Mice were put in boxes on an elevated metal mesh floor and allowed 30 min for habituation before examination. The plantar surface of each hind paw was stimulated with a

series of von Frey hairs with logarithmically incrementing stiffness (Stoelting), presented perpendicular to the plantar surface. The 50% paw withdrawal threshold was determined using Dixon's up-down method[19].

Randall-Selitto test: Mice were stimulated with ascending pressure applied to the tail (one third from the tip), and the threshold was determined when animal showed a clear sign of discomfort or escape. A cutoff threshold of 250 g was set to avoid tissue damage.

Tail immersion test: The tail (one third from the tip) of a mouse was kept in hot water at 48, 50, or 52°C, and the tail flick latency was recorded. A cutoff time of 10 seconds was set to avoid tissue damage.

Hargreaves test[20]: Animals were put in plastic boxes and the plantar paw surface was exposed to a beam of radiant heat (IITC). The paw withdrawal latency was determined after repeated measures for 3 times. A cutoff time of 20 seconds was set to avoid tissue damage.

Capsaicin test: Capsaicin (5 μg in 20 μl 10% DMSO) was intraplantarly injected in one hindpaw and the time spent on licking and flinching was recorded for a 5-minute period.

Mustard oil test: Mustard oil (10 μg in 20 μl 0.2% ethanol) was injected into a hindpaw and the time spent on licking and flinching was recorded for a 5-minute period.

Formalin test: Diluted formalin (5% formalin in 20 μl saline) was injected into the plantar surface of one hind paw. The time spent in licking and flinching of the injected paw was monitored in the first phase (1-10 min) and second phase (10-45 min)[19].

Carrageenan test: Carrageenan (1%) was injected into the plantar surface of one hind paw[19]. Heat hyperalgesia was tested using radiant heat (Hargreaves).

Neuropathic pain test: Neuropathic pain was produced by ligation of the L5 spinal nerve under isoflurance anesthesia[21,22]. Heat hyperalgesia and mechanical allodynia after nerve injury were tested using radiant heat (Hargreaves) and von Frey hairs.

Primary DRG neurons culture

DRGs from all spinal levels of 4-week old mice were removed aseptically and incubated with collagenase (1.25mg/ml, Roche)/dispase-II (2.4 units/ml, Roche) at 37°C for 90 min, then digested with 0.25% trypsin (Cellgro) for 8 min at 37°C, followed by 0.25% trypsin inhibitor (Sigma). Cells were mechanically dissociated with a flame polished Pasteur pipette in the presence of 0.05% DNAse I (Sigma). DRG cells were plated glass cover slips and grown in

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a neurobasal defined medium (with 2% B27 supplement, Invitrogen) in the presence of 5 μM AraC, at 36.5°C, with 5% carbon dioxide. DRG neurons were grown for 24 hours before use.

Whole-cell patch clamp recordings in cultured DRG neurons

Whole-cell voltage- and current-clamp recordings were performed at room temperature to measure currents and action potentials (APs), respectively, with Axopatch-200B amplifier (Axon Instruments, Union City, USA). The patch pipettes were pulled from borosilicate capillaries (Chase Scientific Glass Inc., Rockwood, CA, USA). When filled with the pipette solution, the resistance of the pipettes was 4 ~ 5 MΩ. The recording chamber (volume 300 μl) was continuously superfused (2 ~ 3 ml/min). Series resistance was compensated for (> 80%), and leak subtraction was performed. Data were low-pass-filtered at 2 KHz, sampled at 10 KHz. The pClamp8 (Axon Instruments) software was used during experiments and analysis. The pipette solution for voltage-clamp experiments was composed of (in mM): 126 K-gluconate, 10 NaCl, 1 $MgCl_2$, 10 EGTA, 2 NaATP, and 0.1 MgGTP, adjusted to pH 7.4 with KOH, osmolarity 295 - 300 mOsm. Extracellular solution for voltage-clamp experiments contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, adjusted to pH 7.4 with NaOH, osmolarity 300-310 mOsm. Voltage-clamp experiments were performed at a holding potential of -60 mV. The pipette solution for current-clamp experiments was composed of (in mM): 145 K-gluconate, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 5 HEPES, 5 K_2ATP, adjusted to pH 7.3–7.4 with KOH, osmolarity 300 mOsm. Extracellular solution for current-clamp experiments contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH, osmolarity 300-310 mOsm.

Immunohistochemistry

Mice were terminally anesthetized with isoflurane and perfused through the ascending aorta with saline followed by 4% paraformaldehyde. DRGs, and spinal cord segments were removed and postfixed in the same fixative overnight. Tissue sections were cut in a cryostat (14 μm) and processed for immunofluorescence. We also fixed fresh skin tissues in 4% paraformaldehyde overnight and cut free-floating sections (30 μm) in a cryostat. Tissue sections were blocked with 2% goat serum, and incubated over night at 4°C with the following primary antibodies against TLR7 (rabbit, 1:500-2000, Imgenex) TRPV1 (rabbit, 1:1000, Chemicon) or GRP (rabbit,

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1:500, Immnostar). The sections were then incubated for 1 h at room temperature with Cy3- or FITC- conjugated secondary antibodies. For double immunofluorescence, sections were incubated with a mixture of polyclonal and monoclonal primary antibodies followed by a mixture of FITC- and CY3-congugated secondary antibodies. Immunostained slides were examined under a Nikon fluorescence microscope, and images were captured with a high resolution CCD Spot camera (Diagnostic Instruments Inc.) and analyzed with NIH Image software or Adobe PhotoShop.

To enhance the signal of immunostaining, TSA (Tyramide Signal Amplification) kit (Perkin Elmer, MA) was used for some tissue sections. Briefly, after the primary antibody incubation, the sections were incubated with a biotinylated-secondary antibody (1:400, 1 h at room temperature), followed by avidin-streptin incubation (1:100, 1 h at room temperature), and finally by tyramide incubation (1:50, 5 min at room temperature)[19].

Single-cell reverse-transcription polymerase chain reaction (RT-PCR)

Single-cell RT-PCR was performed as previously described[23]. Briefly, a DRG neuron with small size (18-22 μm) was aspirated into a patch pipette with a tip diameter of about 25 μm, gently put into a reaction tube containing reverse transcription reagents, and incubated for 1 hr at 50°C (superscript III, Invitrogen, Carlsbad, CA, USA). The cDNA product was used in separate PCR. The sequences of the primers used are presented in the Table below.

List of DNA primer sequences designed for single-cell RT-PCR.

Target gene (Product length)[a]	Outer primers	Inner primers	Genbank No.
TRPV1 (273 bp, 203 bp)	TGATCATCTTCACCACGGCTG CCTTGCGATGGCTGAAGTACA	AAGGCTTGCCCCCCTATAA CACCAGCATGAACAGTGACTGT	NM_001001445.1
GRP (258 bp, 201 bp)	CCAAGGAGCAAAACAAAACCC GCAAATTGGAGCCCTGAATCT	AGACTGCCTTCTGCAAACGTC AAGCCTAGCTGGAAAAAGCG	NM_175012.2
GAPDH (367 bp, 313 bp)	AGCCTCGTCCCGTAGACAAAA TTTTGGCTCCACCCCTTCA	TGAAGGTCGGTGTGAACGAATT GCTTTCTCCATGGTGGTGAAGA	XM_001473623.1
TLR8 (300 bp, 212 bp)	TATCGGATACCACTGCAGCTG CCCAATCCCTCTCCTCTAAACA	CCACCTCCATGGTTATGTTGG ACTCTCTTCAAGGTGGTAGCGC	NM_133212.2
TLR7 (421 bp, 359 bp)	CAGTGAACTCTGGCCGTTGAGA TGGCGGCATACCCTCAAAA	TTCTCCAACAACCGGCTTGAT TCAGGAGGCAAGGAATTCAGG	NM_133211.3
Mrgpra3 (354 bp, 301 bp)	GGCATCACCTGGTTCCTGTTA CCAGTGAGGCATGTCAAGTCA	CTGGATTGCACCTGGTGTGTT GAGGAGAGTGGACAGTGGTCAA	NM_153067.1

[a] (n, n) indicates product size obtained from outer and inner primers, respectively.

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The first round of PCR was preformed in 50 µl of PCR buffer containing 0.2 mM dNTPs, 0.2 µM "outer" primers, 5 µl RT product and 0.2 µl platinum Taq DNA polymerase (Invitrogen). The protocol included an initial 5-min denaturizing step at 95 °C followed by 40 cycles of 40 s denaturation at 95 °C, 40 s annealing at 55 °C, and 40 s elongation at 72 °C. The reaction was completed with 7 min of final elongation. For the second round of amplification, the reaction buffer (20 µl) contained 0.2 mM dNTPs, 0.2 µM "inner" primers, 5 µl of the first round PCR products and 0.1 µl platinum Taq DNA polymerase. The reaction procedure for these primers was the same as the first round. A negative control was obtained from pipettes that did not harvest any cell contents, but were submerged in the bath solution. The PCR products were displayed on ethidium bromide-stained 1% agarose gels.

RT-PCR

Total RNAs were isolated from DRGs, spleen, skin, spinal cord, and cortex with the RNeasy Mini Kit (QIAGEN) and quantified using A260/A280 absorption. The first-strand cDNA were synthesized with Oligo(dT)$_{12-18}$ primer using SuperScript™ II Reverse Transcriptase (Invitrogen). Mouse PCR primers were designed as follows:

List of DNA primer sequences designed for single-cell RT-PCR.

Target gene (Product length)[a]	primers	Genbank No.
GAPDH (367 bp, 313 bp)	TGAAGGTCGGTGTGAACGAATT GCTTTCTCCATGGTGGTGAAGA	XM_001473623.1
TLR8 (300 bp, 212 bp)	CCACCTCCATGGTTATGTTGG ACTCTCTTCAAGGTGGTAGCGC	NM_133212.2
TLR7 (421 bp, 359 bp)	TTCTCCAACAACCGGCTTGAT TCAGGAGGCAAGGAATTCAGG	NM_133211.3

[a] (n, n) indicates product size obtained from outer and inner primers, respectively.

GAPDH were used as an internal control. cDNA samples were amplified for 40 cycles by Taq DNA Polymerase (Invitrogen), and PCR products were separated on agarose gel. Gel image were captured in Gel Document System (Bio-Rad).

In situ hybridization

Mice were terminally anesthetized with isoflurane and transcardially perfused with PBS and 4% paraformaldehyde. DRGs were collected and post-fixed overnight. DRG tissues were sectioned

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in a cryostat at a thickness of 12 μm and mounted on Superfrost plus slides. The TLR7 riboprobes (0.34 and 0.76kb) was generated by PCR. DNA sequences were transcribed *in vitro* with T7 RNA polymerase (Promega) in the presence of digoxigenin-labeling mix. DRG sections were hybridized with TLR7 riboprobe (1 μg/ml) overnight at 65°C. After washing, sections were blocked with 20% serum for 1 h at room temperature followed by incubation with alkaline phosphatase-conjugated anti-digoxigen antibody (1:2000; Roche Diagnostics) overnight at 4°C. Sections were then incubated with a mixture of nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in alkaline phosphatase buffer for 24-48 h for color development. *In situ* hybridization images were captured with a Nikon microscope under bright-field.

Statistical analyses

All the data were expressed as Mean ± S.E.M. Differences between two groups such as mutant vs corresponding wild-type control were compared using student t-test. Differences for more than 3 groups were compared with one way ANOVA. The criterion for statistical significance was $P<0.05$.

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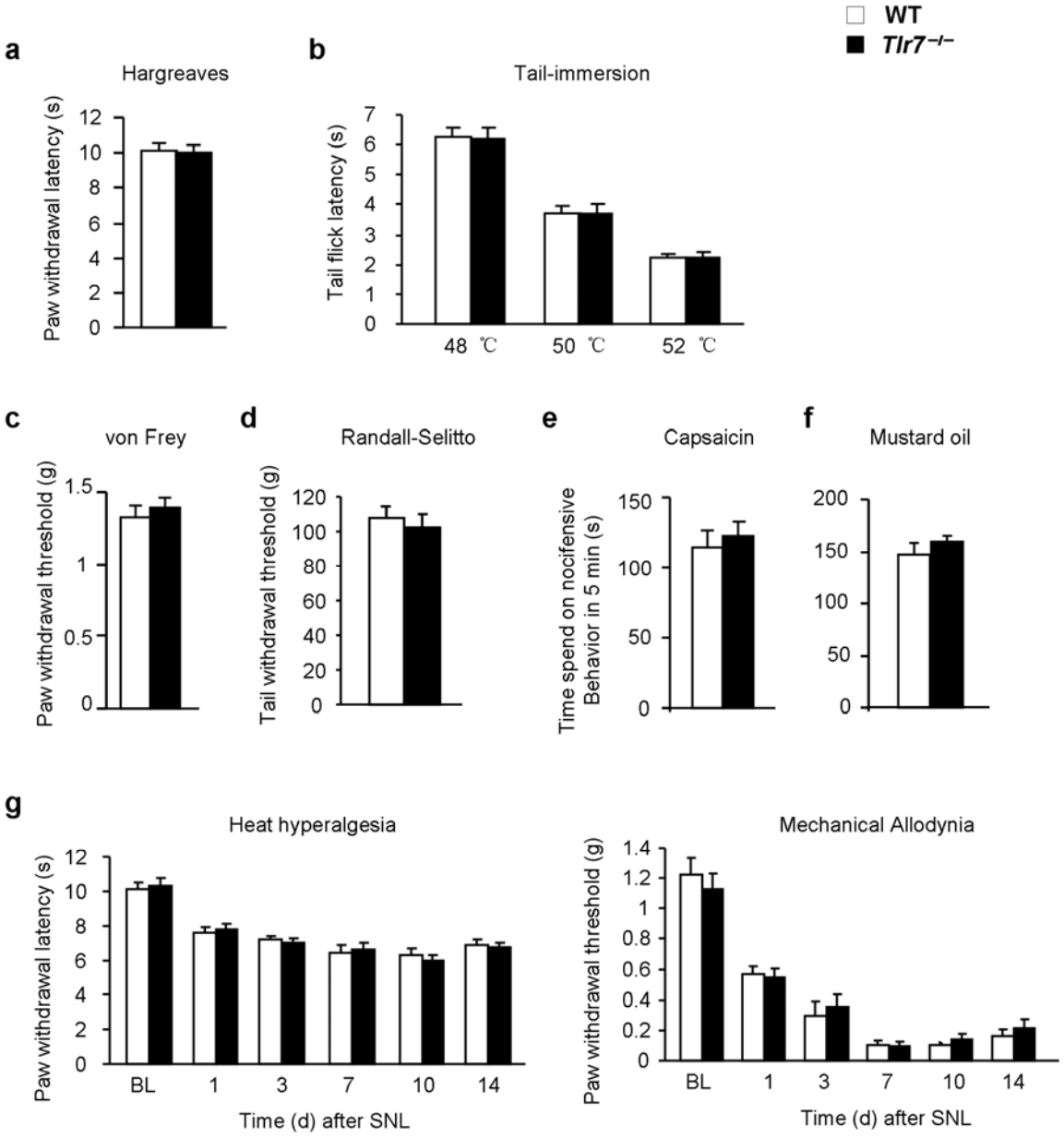
Supplementary Figures



Supplementary Fig 1. Mechanical, thermal, acute inflammatory pain and neuropathic pain in WT and *Tlr7*$^{-/-}$ mice. **(a,b)** Heat pain in responses to radiant heat in Hargreaves test (a) and in hot water tail immersion test (b, n = 8 mice). **(c,d)** Mechanical pain in response to mechanical stimuli by von Frey hairs (c) and noxious mechanical stimuli in the Randall-Selitto test (d, n = 8 mice). **(e,f)** Spontaneous pain (acute inflammatory pain, nocifensive behavior) by intraplantar capsaicin (e, 5 μg) and mustard oil (f, 10 μg; n = 5 mice). **(g)** Heat hyperalgesia and mechanical allodynia induced by spinal nerve ligation (n = 5-6 mice). P >0.05 compared with WT control. All data are means ± s.e.m.

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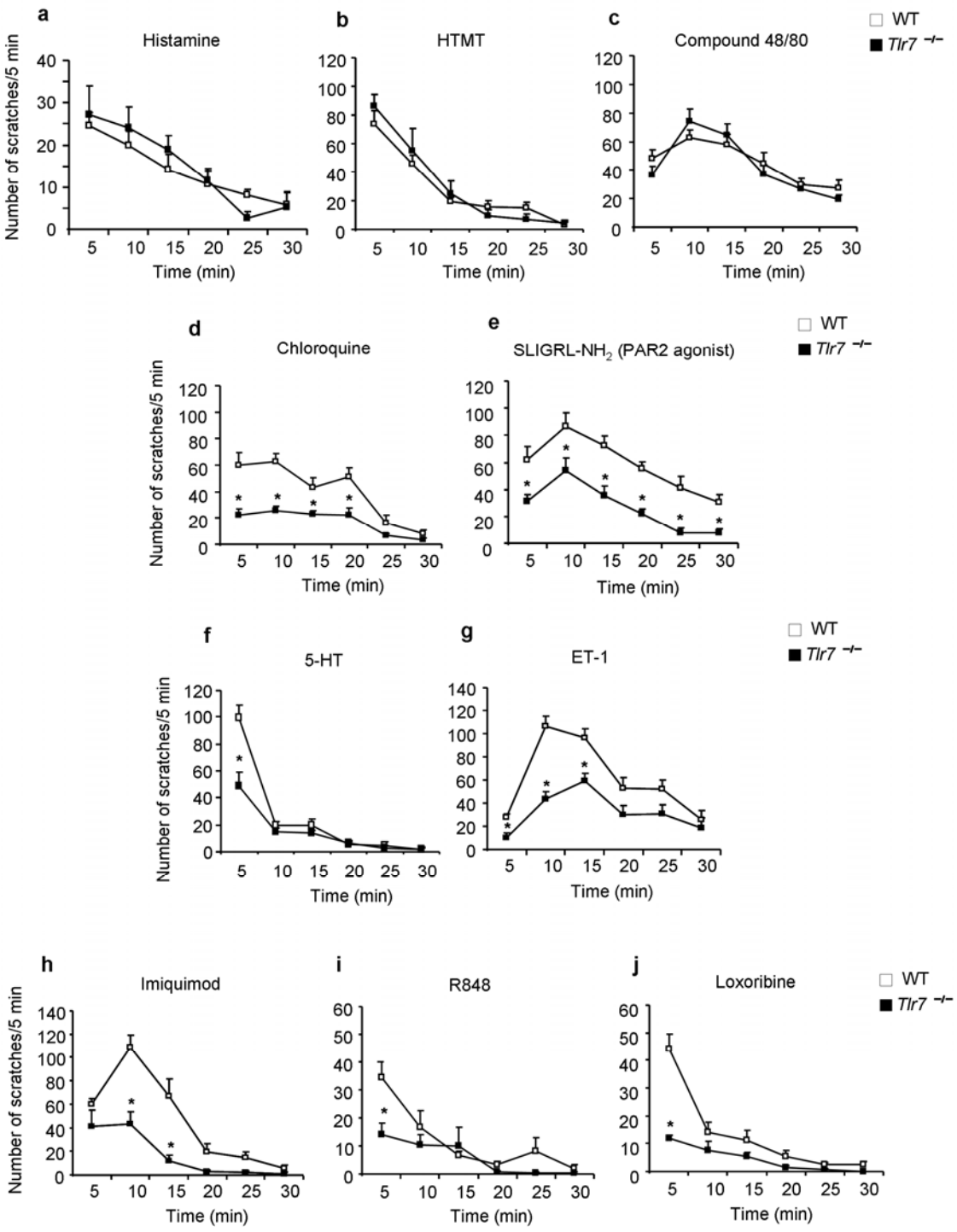

Supplementary Fig 2. Time course of scratching responses for a period of 30 min in *Tlr7*$^{-/-}$ and WT mice, following intradermal injection of 50 μl of pruritic agents including histamine (**a**, 500 μg), HTMT (**b**, H1 agonist, 100 μg), compound 48/80 (**c**, 100 μg), chloroquine (**d**, 200 μg), SLIGRL-NH2 (**e**, PAR2 agonist); serotonin (**f**, 5-HT; 20 μg), endothelin-1 (**g**, ET-1, 25 ng), imiquimod (**h**, 100 μg), R848(**i**, 100 μg) and loxoribine(**j**, 100 μg). **P*<0.05, versus WT control, *n* = 5~8 mice. All data are means ± s.e.m.

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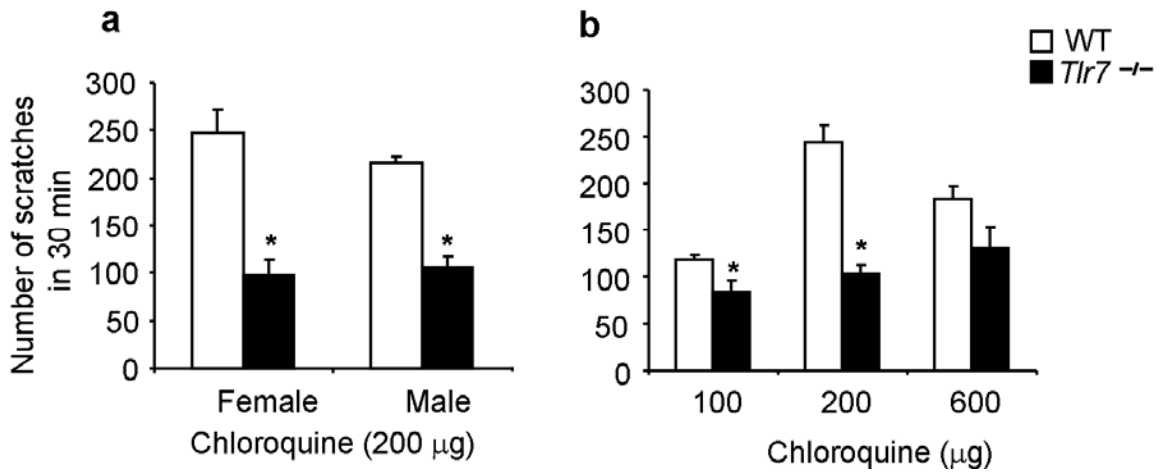

Supplementary Fig 3. Influence of sex and dose on chloroquine (CQ)-induced scratching behavior in WT and *Tlr7*$^{-/-}$ mice. (**a**) Scratching induced by intradermal injection of CQ (200 μg) was reduced in both male and female *Tlr7*$^{-/-}$ mice compared to WT mice. Note that there is no significant sex difference in CQ-induced scratching in WT and KO mice. (**b**) An inverted-U-shaped dose-response curve following injection of CQ in WT mice. Noted that scratching behavior induced by the highest dose of CQ (600 μg) is TLR7-independent. **P*<0.05, *n* = 5~8 mice.

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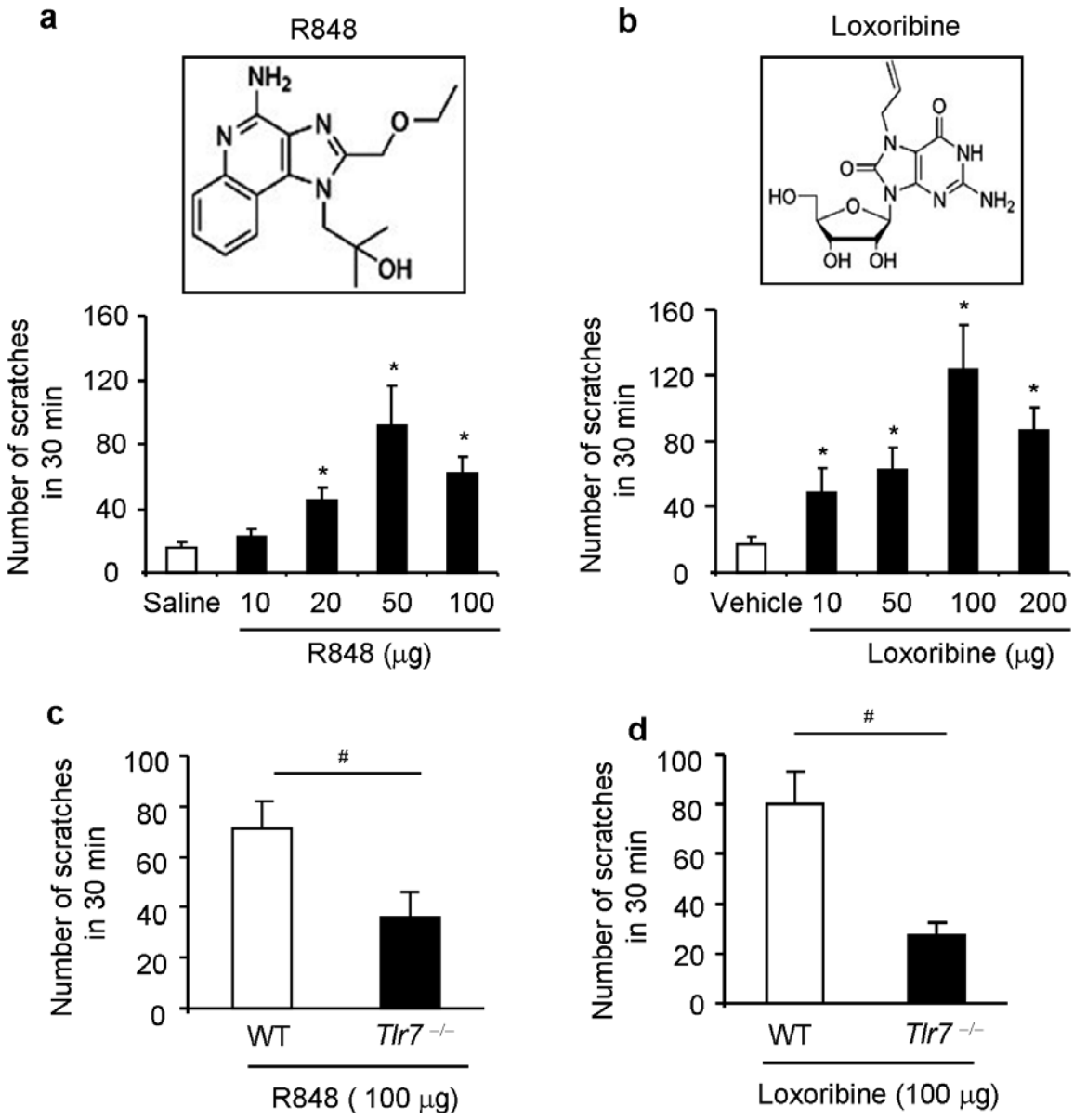

Supplementary Fig 4. Scratching induced by the TLR7 ligands R848 and loxoribine in WT and *Tlr7*$^{-/-}$ mice. (**a, b**) Dose-dependent scratches after intradermal injection of the TLR7 ligands R848 (a) and loxoribine (b). Inset, structure of R848 and loxoribine. Note an inverted-U-shaped dose-response curve following injection of R848 or loxoribine. **P*<0.05, *n* = 5~8 mice. (**c, d**) Scratches induced by intradermal injection of the TLR7 ligands R848 (c, 100 μg) and loxoribine (d, 100 μg) in WT and *Tlr7*$^{-/-}$ mice. Note a marked reduction in R848- and loxoribine-induced scratches in *Tlr7*$^{-/-}$ mice. $^{\#}$*P*<0.05, *n* = 5 mice.

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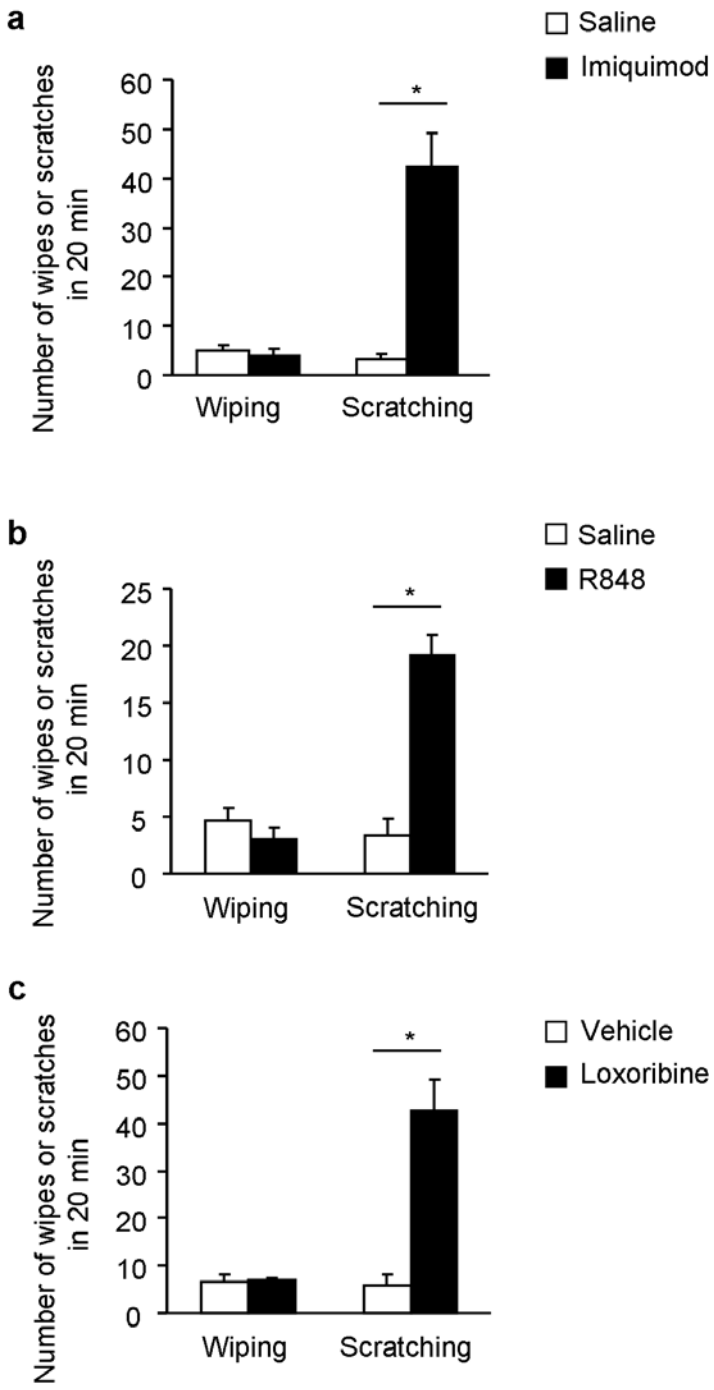

Supplementary Fig 5. Distinct role of TLR7 for mediating pain and itch in the "cheek model". Intradermal injection of TLR7 ligands imiquimod (20 μg, **a**), R848 (20 μg, **b**) and loxoribine (20 μg, **c**) into the cheek induces itch-like scratching by the hind limb but not pain-indicative wiping by forelimbs. $P < 0.05$, $n = 5\sim8$ mice.

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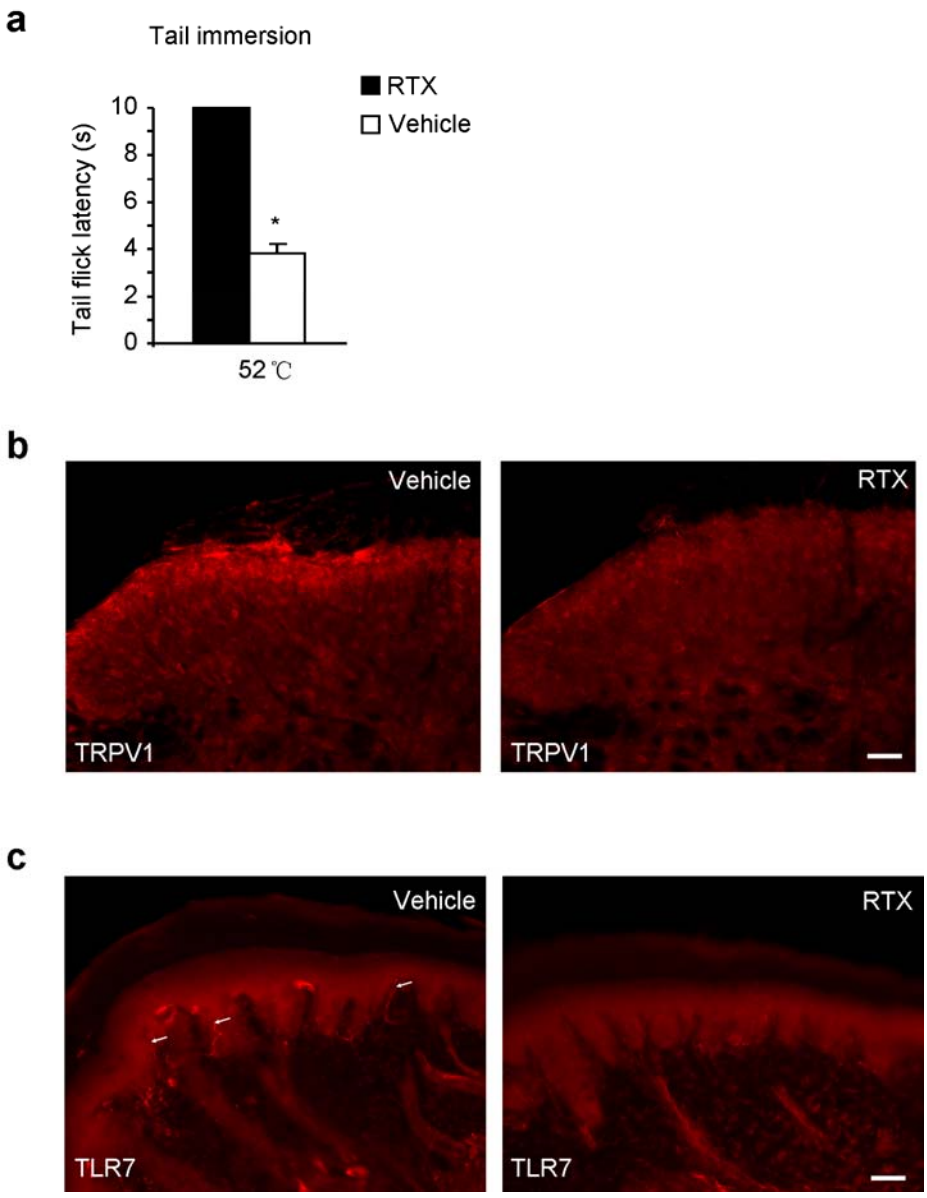

Supplementary Fig 6. Effects of resininferatoxin (RTX) treatment on thermal sensitivity **(a)**, TRPV1 staining in the spinal cord dorsal horn **(b)** and TLR7 staining in the skin **(c)**. After pretreatment with RTX, mice are insensitive to heat stimulus (52 °C hot water). *P <0.05, n = 5 mice (a). RTX pretreatment also ablated TRPV1+ fibers in the spinal cord dorsal horn (b) and TLR7+ fibers in the skin (c). RTX was given subcutaneously for 3 consecutive days with accumulating doses (30, 70 and 100 μg/kg,), and animals were tested and sacrificed 10 days later. Arrows in c indicate labeled nerve fibers. Scales, 50 μm.

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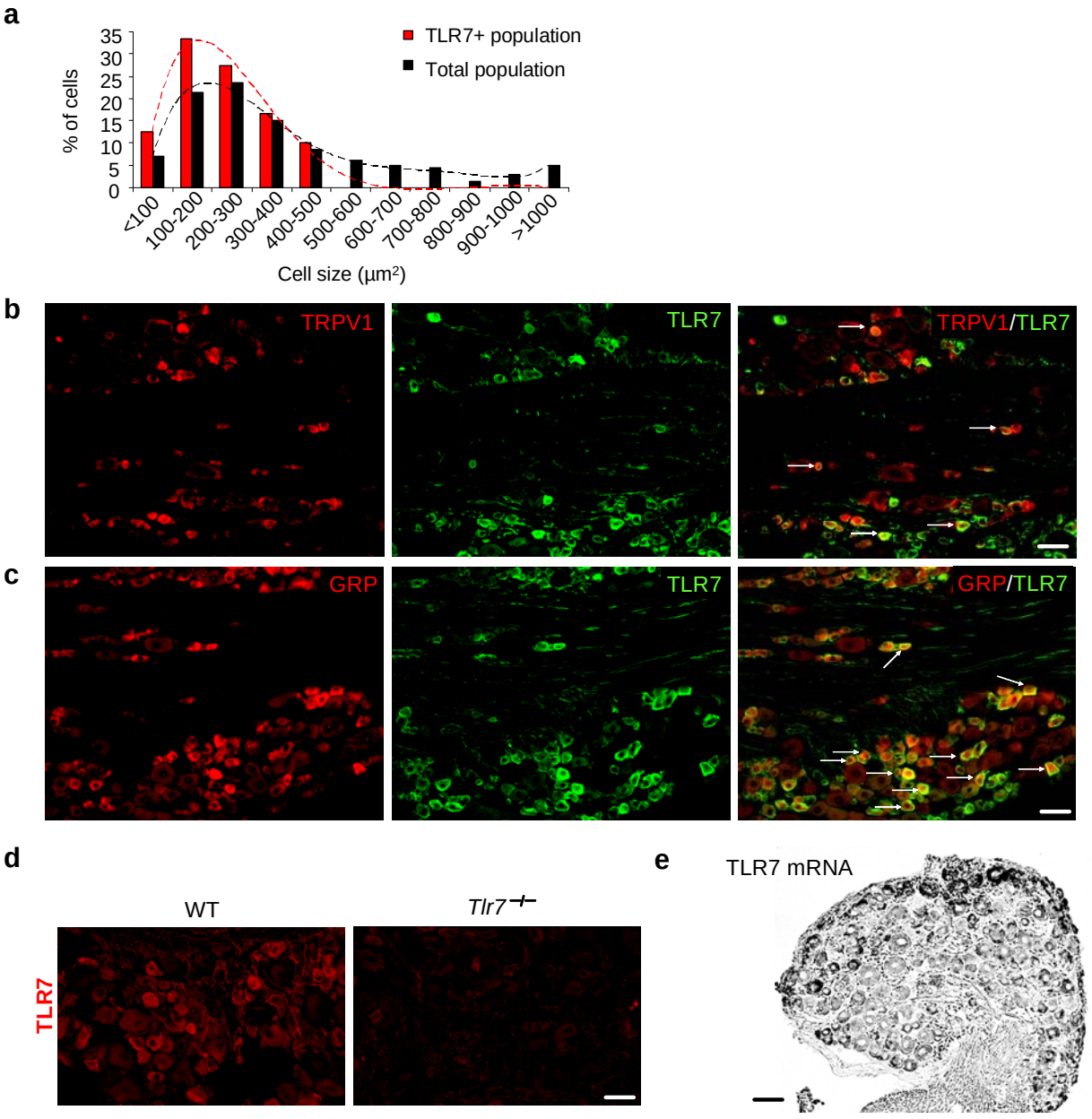

Supplementary Fig 7. Characterization of TLR7 expression in DRG sections. **(a)** Size frequency distribution of TLR7 in DRG neurons. Note that TLR7+ neurons are primarily distributed in the range of small sizes (100-400 μm^2). **(b, c)** Double staining showing co-localization of TLR7 and TRPV1 in dissociated DRG neurons from a culture (b) and in tissue sections of DRG (c). Arrows indicate double-labeled neurons. Note a high degree of co-localization of TLR7 and GRP. **(d)** TLR7 immunostaining in DRG sections of WT and *Tlr7* knockout mice. **(e)** *In situ* hybridization showing TLR7 mRNA expression in DRG neurons. Scales, 50 μm.

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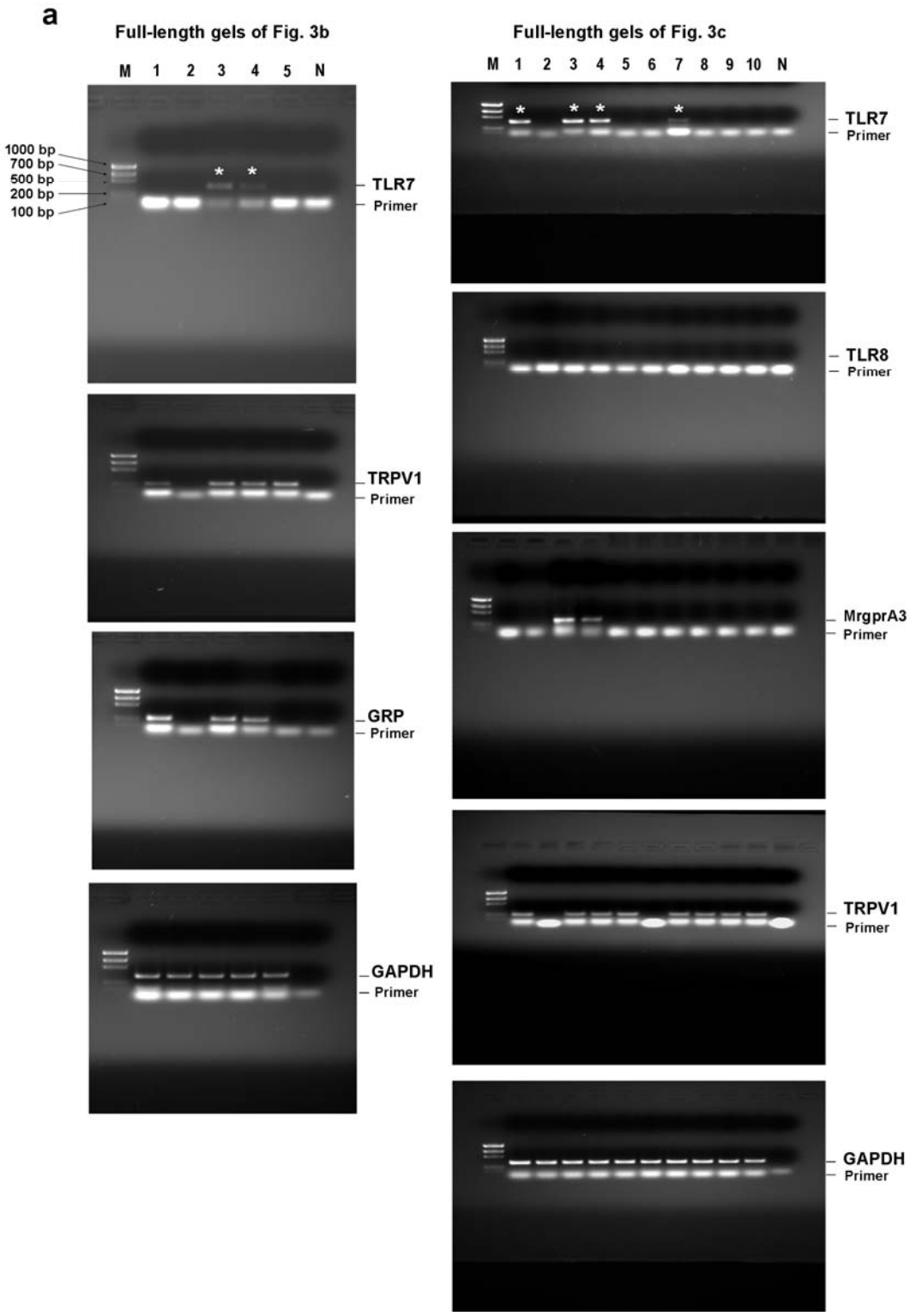

Supplementary Fig 8a. Full length gels of Fig. 3b and Fig. 3c. Single cell RT-PCR analysis in small-size DRG neurons shows colocalization of TLR7 with TRPV1, GRP, and MrgprA3. M, molecular weights; N, negative control with pipette only. * indicates TLR7+ neurons. Numbers on the top of the gels indicate individual neurons.

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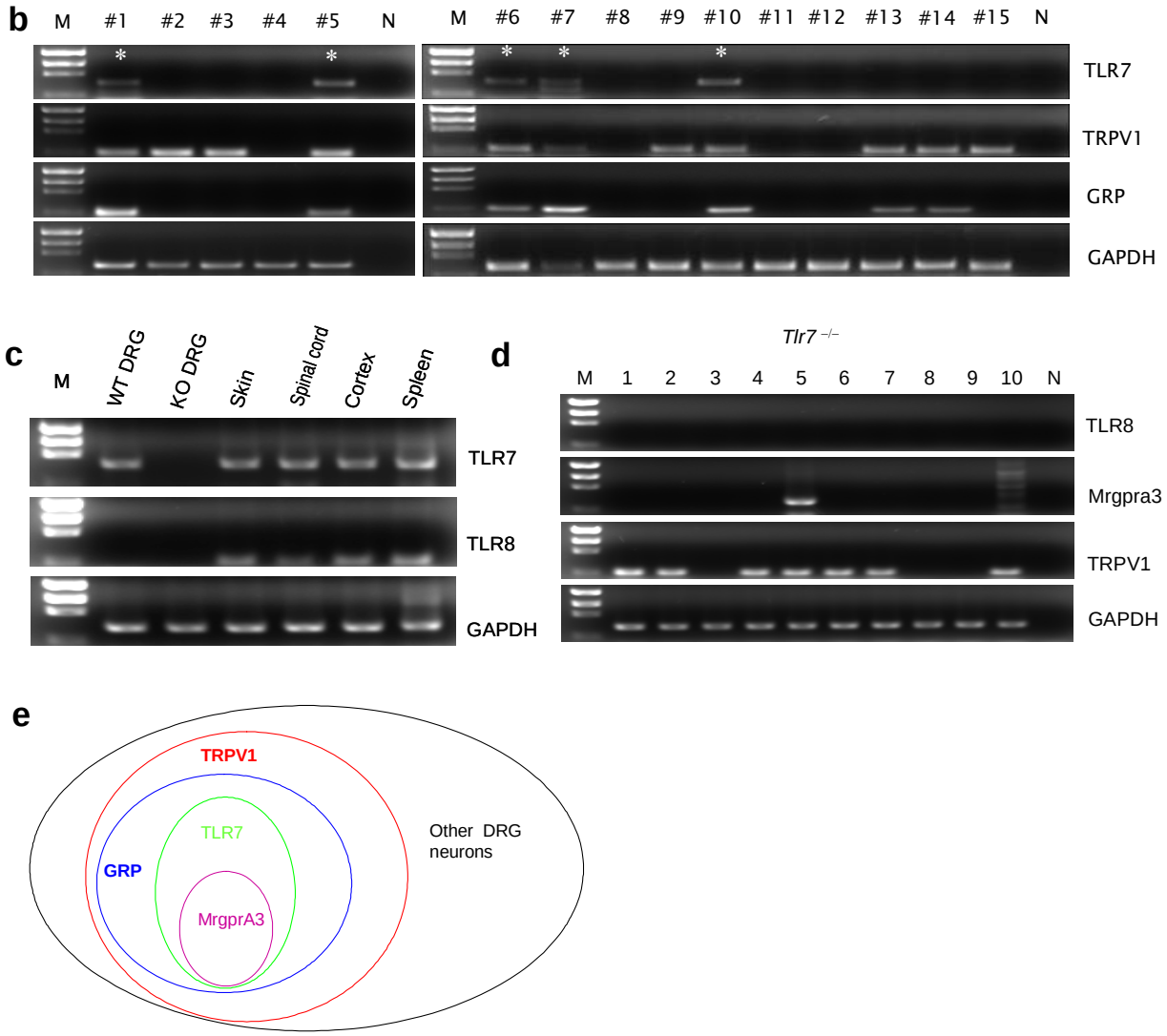

Supplementary Fig 8b-e. Characterization of TLR7 expression in dissociated DRG neurons. **(b)** Single cell RT-PCR analysis in 15 small-size DRG neurons showing co-localization of TLR7 mRNA with TPRV1 and GRP mRNA. M, molecular weights; N, negative control with pipette only. * indicates TLR7+ neurons. Note that all TLR7-expressing neurons express GRP and TRPV1 and all GRP-expressing neurons express TRPV1. **(c)** Expression of TLR7 and TLR8 in different tissues of adult WT mice. Noted that TLR8 is not expressed in mouse DRG and TLR7 is absent in DRG of *Tlr7* KO mice. **(d)** Single cell RT-PCR in 10 small-sized DRG neurons of *Tlr7* KO mice. Numbers on the top of the gels indicate individual neurons. **(e)** The Venn diagram showing the relationship of TLR7+, GRP+, MrgprA3+, and TRPV1+ populations in the DRG.

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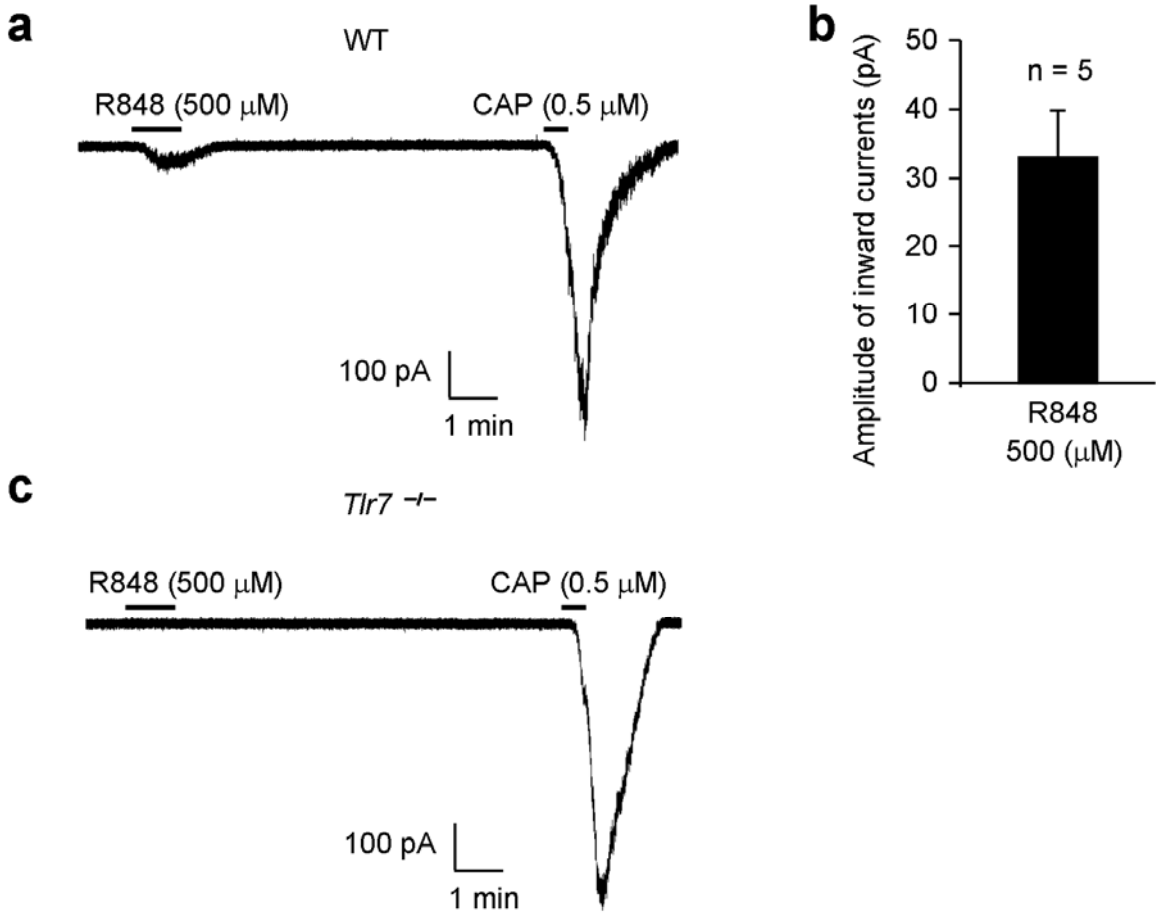

Supplementary Fig 9. R848 induces inward currents in DRG neurons of WT but not *Tlr7*$^{-/-}$ mice. **(a)** Inward currents evoked by R848 (500 μM) and capsaicin (0.5 μM) in small-sized DRG neurons of WT mice. Five out of eleven neurons respond to R848. **(b)** Amplitude of inward currents evoked by R848 (500 μM). n=5. **(c)** R848 fails to induce inward currents in all 10 neurons from *Tlr7*$^{-/-}$ mice.

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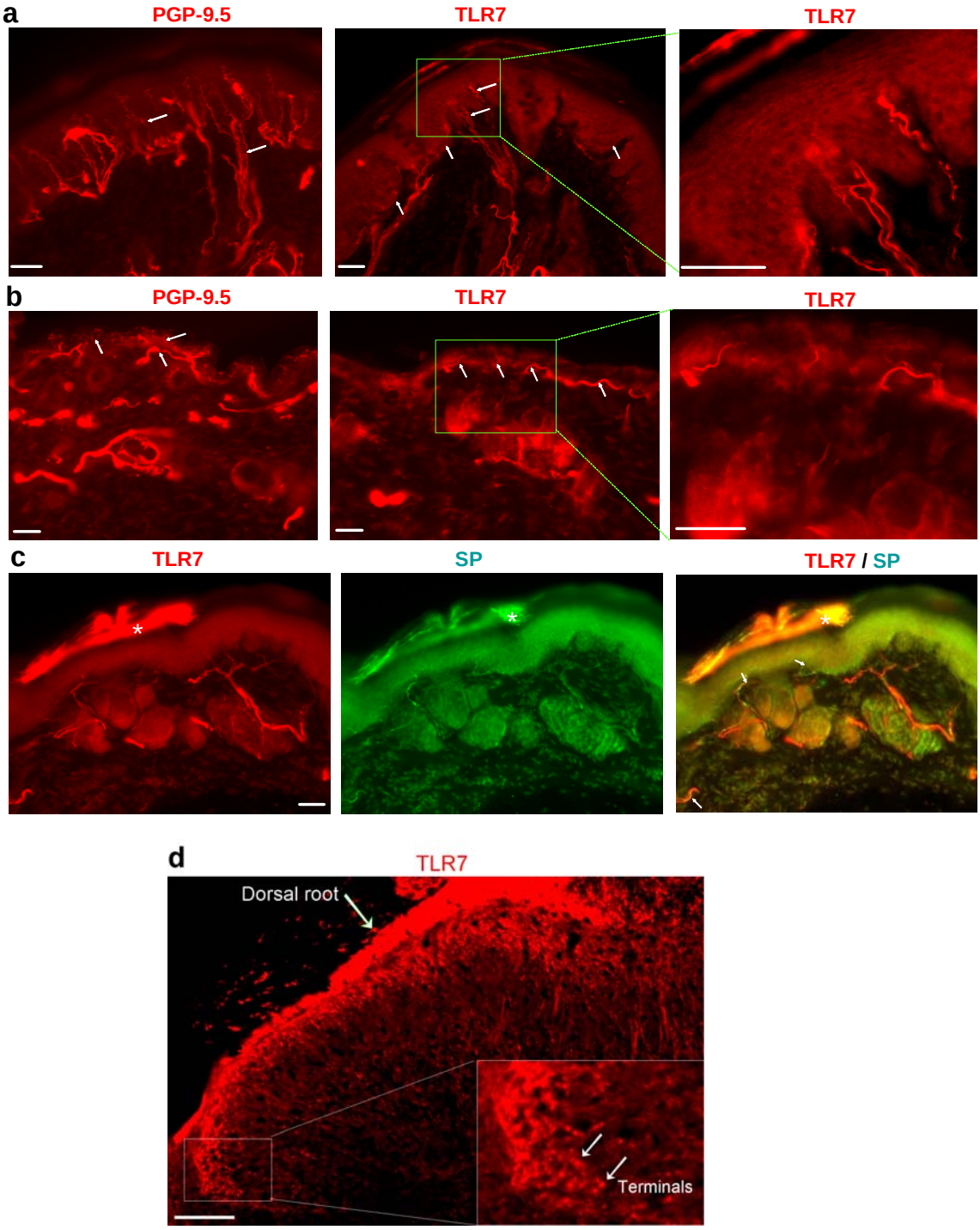

Supplementary Fig. 10. Immunohistochemistry showing TLR7 expression in the skin and spinal cord. **(a, b)** Expression of PGP-9.5 and TLR7 in nerve branches and terminals in the hindpaw footpad skin (a) and hairy back skin (b). **(c)** Colocalization of TLR7 with substance P (SP) in hindpaw skin. **(d)** TLR7 expression in spinal cord dorsal horn. Arrows in (a-c) indicate labeled nerve fibers. Small arrows in (d) show TLR7 staining in axonal terminals in the superficial dorsal horn. Scales, 50 μm.

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