Extracellular microRNAs activate nociceptive neurons to elicit pain via TLR7 and TRPA1

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I. Supplemental Experimental Procedures

Reagents

We purchased miRNAs from Integrated DNA Technologies. The sequences of miRNAs and control mutant oligoribonucleotide (Mut. oligo) (Lehmann et al., 2012) are shown in Figure 1. The Mut. Oligo has reduced GU content but no changes in the 5′ let-7 seed sequence that is important for post-transcriptional silencing. Fluorescence-conjugated siRNA (Cy3-let-7b) and HC030031 were from Sigma. The inhibitor of let-7b (Lehmann et al., 2012) and kit for measuring miRNAs were from Qiagen.

Animals

Knockout mice lacking *Tlr7*, *Tlr3*, *Trpa1*, *Trpv1*, and *Myd88* and corresponding WT control mice (B6129PF2/J background for *Trpa1*; C57BL/6 background for *Tlr7*, *Tlr3*, *Trpv1*, and *Myd88*) were purchased from Jackson Laboratories and maintained at Duke animal facility. All the knockout mice were viable and showed no developmental defects. Young mice (4-5 weeks) were used for electrophysiological studies in DRG neurons. Adult male mice (8-10 weeks) of knockout mice and corresponding wild-type control mice, as well as CD1 mice were used for behavioral and pharmacological studies. All the animal procedures were approved by the Institutional Animal Care & Use Committee of Duke University.

HEK293 cells and transfection

HEK293 and HEK293XL-mTLR7 cell lines were purchased from InvivoGen. Cells were cultured in high glucose (4.5 g/L) Dulbecco's Modified Eagle's Medium containing 10% (v/v) fetal bovine serum (Gibco). HEK293XL-mTLR7 cells were maintained and subcultured in a growth medium supplemented with 10 μg/ml Blasticidin. Transfection (2 μg cDNA) was performed with Lipofectamine™ 2000 Reagent (Invitrogen) at 80% confluency and the transfected cells were cultured in the same growth medium for 48 h before electrophysiological and biochemical studies. pcDNA3.1-mTRPA1-myc-His plasmid was kindly provided by Ardem Patapoutian from The Scripps Research Institute and the cDNAs of Trpa1, Trpv1, Trpv2, and Trpv4 were kindly provided Dr. Sun Work Hwang from Korea University.

Primary cultures of DRG neurons

DRGs were removed aseptically from mice (4-6 weeks) 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 for 8 min at 37°C, followed by 0.25% trypsin inhibitor. Cells were mechanically dissociated with a flame polished Pasteur pipette in the presence of 0.05% DNAse I (Sigma). DRG cells were plated on glass cover slips and grown in a neurobasal defined medium (with 2% B27 supplement, Invitrogen) with 5 μM AraC and 5% carbon dioxide at 36.5°C. DRG neurons were grown for 24 hours before use.

Whole-cell and inside-out single channel recordings in DRG neurons and HEK293 cells

Whole-cell and inside-out patch clamp recordings were performed at room temperature using an Axopatch-200B amplifier (Axon Instruments). The patch pipettes were pulled from borosilicate capillaries (Chase Scientific Glass Inc.). Pipette resistance was 4-6 MΩ for whole-cell recording or 7-9 MΩ for inside-out recording, and the internal solution contains (in mM): 126 K-gluconate, 10 NaCl, 1 $MgCl_2$, 10 EGTA, 2 NaATP, and 0.1 MgGTP, adjusted to pH 7.3 with KOH and osmolarity 295-300 mOsm. Whole cell recordings were performed in an extracellular solution (in mM): 140 NaCl, 5 EGTA, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, adjusted to pH 7.4 with NaOH and osmolarity 300-310 mOsm.

Inside-out patch recordings were performed at room temperature in a bath solution (intracellular side) (in mM): 140 NaCl, 10 EGTA, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, adjusted to pH 7.4 with NaOH and osmolarity 300-310 mOsm. Currents were low-pass filtered at 1 kHz in whole-cell recordings and at 3 kHz in single-channel recordings. Currents were digitized at a sampling rate of 3 kHz in whole-cell recordings and 10 kHz in single channel recordings with Digidata-1440A (Axon Instruments). The pClamp10 (Axon Instruments) software was used during experiments and data analysis. Opening and closing transitions of single channels were detected by using 50% of the threshold criterion. All events were carefully checked before the analysis. Amplitude histograms were obtained using Clampfit software. Single-channel amplitude was calculated from the difference between gauss fitted “closed” and “open” peaks. When superimposed openings were observed, the number of channels in the patch was estimated from the maximal number of superimposed openings. The single-channel open

probability (Po) for the inside-out patches was determined using the following equation: Po=T′/T, where T′ is the total open time for a patch over time T.

Immunocytochemistry

To determine the surface expression of TRPA1, cultured DRG neurons from WT and *Tlr7* knockout mice were stimulated with mustard oil (AITC; 150 μM, 10 min) and then were incubated with anti-TRPA1 antibody (AbE3, 1:50; kindly provided by Dr. Patapoutian A) (Schmidt et al., 2009) for 10 min at 37°C. Cells were then incubated with Cy3-conjugated secondary antibodies (1:400; Jackson ImmunoResearch) for 10 min at room temperature. Finally cells were fixed with 2% paraformaldehyde and examined with a Nikon fluorescence microscope. The percentage of TRPA1-positive neuron was calculated from 200 randomly selected neurons and three independent preparations were included.

293XL-hTLR7 cells were cultured on coverslips, and transfected with pcDNA3.1-mTRPA1-myc-His plasmid, 48 hours later, transfected cells were stimulated 50 μg/ml cy3 labeled let-7b or vehicle for 5 min fixed with 4% paraformaldehyde for 15 min and incubated overnight with a rabbit antibody against TLR7 (1:1000; IMGENEX) mixed with a mouse antibody against myc (1:500; Millipore, Billerica, MA). Then, the cells on coverslips were incubated with secondary antibodies conjugated to FITC and Cy5 (1:100; Jackson ImmunoResearch, West Grove, PA) and examined under a Zeiss LSM 510 inverted confocal microscope (North Chesterfield, VA).

Immunopricipitation and immunobloting

Transfected 293XL-hTLR7 cells were stimulated 50 μg/ml let-7b or vehicle for 5 min and lysed in ice-cold immunoprecipitation buffer (10 mM TrisHCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 10% glycerol). The lysate was immunoprecipitated with 0.5 mg mouse antibody against myc (Millipore, Billerica, MA) and then incubated with protein G-Sepharose beads (Roche, Mannheim, Germany). For immunoblotting, the lysates or beads were incubated in SDS-PAGE loading buffer. The samples were separated on an SDS-PAGE gel, transferred, and probed with antibodies against TLR7 (1:2000; IMGENEX, San Diego, CA), myc (1:1000; Millipore). The immunoreactive bands were then detected with horseradish peroxidase-conjugated secondary antibodies, visualized with enhanced chemiluminescence (Thermo

scientific, Pittsburgh, PA) and quantified with Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MD). Each experiment was repeated at least three times.

Fluorescent In situ Hybridization (FISH) and Immunohistochemistry (IHC)

DRGs were dissected from 4% paraformaldehyde transcardially perfused mice. FISH were carried out according to the manufacturer's guide. Briefly, sections of 10-μm thickness were permeabilized with 70% ethanol and incubated with multiple oligonucleotide probes targeting TRPA1 (Stellaris FISH probes labeled with Quasar® 570 Dye, Bioresearch Technologies) overnight at 37°C. Sections were then washed and incubated overnight with rabbit TLR7 primary antibody (Imgenex) at 4°C following a standard IHC. TLR7 signal was detected by incubation with FITC-conjugated antibody against rabbit (Jackson Laboratories). Images of were acquired with Nikon fluorescence microscopes (Nikon) and FISH and IHC signals superposed by Photoshop (Adobe).

Single-cell RT-PCR

As described in (Liu et al., 2012), a single cell was aspirated into a patch pipette, gently put into a reaction tube containing reverse transcription reagents, and incubated for 1 hr at 50°C (superscript III, Invitrogen). The cDNA product was then used in separate PCR. The sequences of all the primers used for single-cell PCR are described in Table-S2. The 1^{st} and 2^{nd} round PCR was performed using "outer" primers and "inner" primers, respectively. A negative control was obtained from pipettes that did not harvest any cell contents. The PCR products were displayed on ethidium bromide-stained 1.5 % agarose gels.

miRNA quantification by quantitative real-time RT-PCR (qPCR)

Total RNA was isolated from cortex, spinal cord, DRG and skin tissues using Qiazol Lysis Reagent (Qiagen) together with miRNeasy Kit (Qiagen). All RNA samples were immediately used or kept at -80°C until further processing. To convert mature miRNA into cDNA, 6 μl of total RNA solution were reverse transcribed using the miScript II RT Kit (including polyadenylation of miRNAs and reverse transcription using an oligo-dT that binds to an universal RT-sequence, Qiagen). Specific miRNA levels were quantified by qPCR using miScript SYBR Green Kit [Qiagen, including miScript miRNA assay for mmu-let-7b (assay ID

MS00001225) and mmu-let-7a (assay ID MS000032179), together with the universal RT primer], according to the manufacturer's protocol (CFX96 Real-Time system, Bio-Rad). Relative quantities of miRNAs were calculated using the Ct method after normalization to control miRNAs. mmu-SNORD96A (assay ID MS00033733) was used as control for intracellular miRNA, because of its ubiquitous and stable expression in various tissues and cell types (Qiagen). *Caenorhabditis elegans* miRNA-39 (cel-miRNA-39) was included as spiked-in control for extracellular miRNA.

To measure miRNA release, DRG neurons were cultured in 12-well plates at densities of 1500-5000 neurons/well for testing the density-dependent effects of let-7b release and also at the density of 5000 neurons/well for testing the activity-dependent release of let-7a and let-7b. Before the stimulation, DRG cultures were washed three times in Krebs solution. Cells were then incubated with 200 μl of Krebs solution containing formalin (0.01%), ionomycin (10 ng/ml), KCl (50 mM), capsaicin (1 μM), AITC (300 mM), and chloroquine (1 mM) for 30 min at 37°C. Supernatants were harvested and cleared of debris by centrifugation (12000 g for 20 min at 4°C). Isolation of extracellular miRNAs from supernatants was performed using Qiazol Lysis Reagent (Qiagen) together with miRNeasy Serum/Plasma Kit (Qiagen). The concentration of let-7b (pg/ml) was calculated based on a standard curve obtained from synthetic let-7b (Integrated DNA Technologies) and the Ct values were plotted as described previously (Kroh, et al., 2010).

References

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Liu,T., Berta,T., Xu,Z.Z., Park,C.K., Zhang,L., Lu,N., Liu,Q., Liu,Y., Gao,Y.J., Liu,Y.C., Ma,Q., Dong,X., and Ji,R.R. (2012). TLR3 deficiency impairs spinal cord synaptic transmission, central sensitization, and pruritus in mice. J Clin. Invest *122*, 2195-2207.

II. Supplemental Tables

Supplemental Table-1. The GUUGUGU motif contained in miRNAs of Homo sapiens and Mus musculus (related to Figure 1).

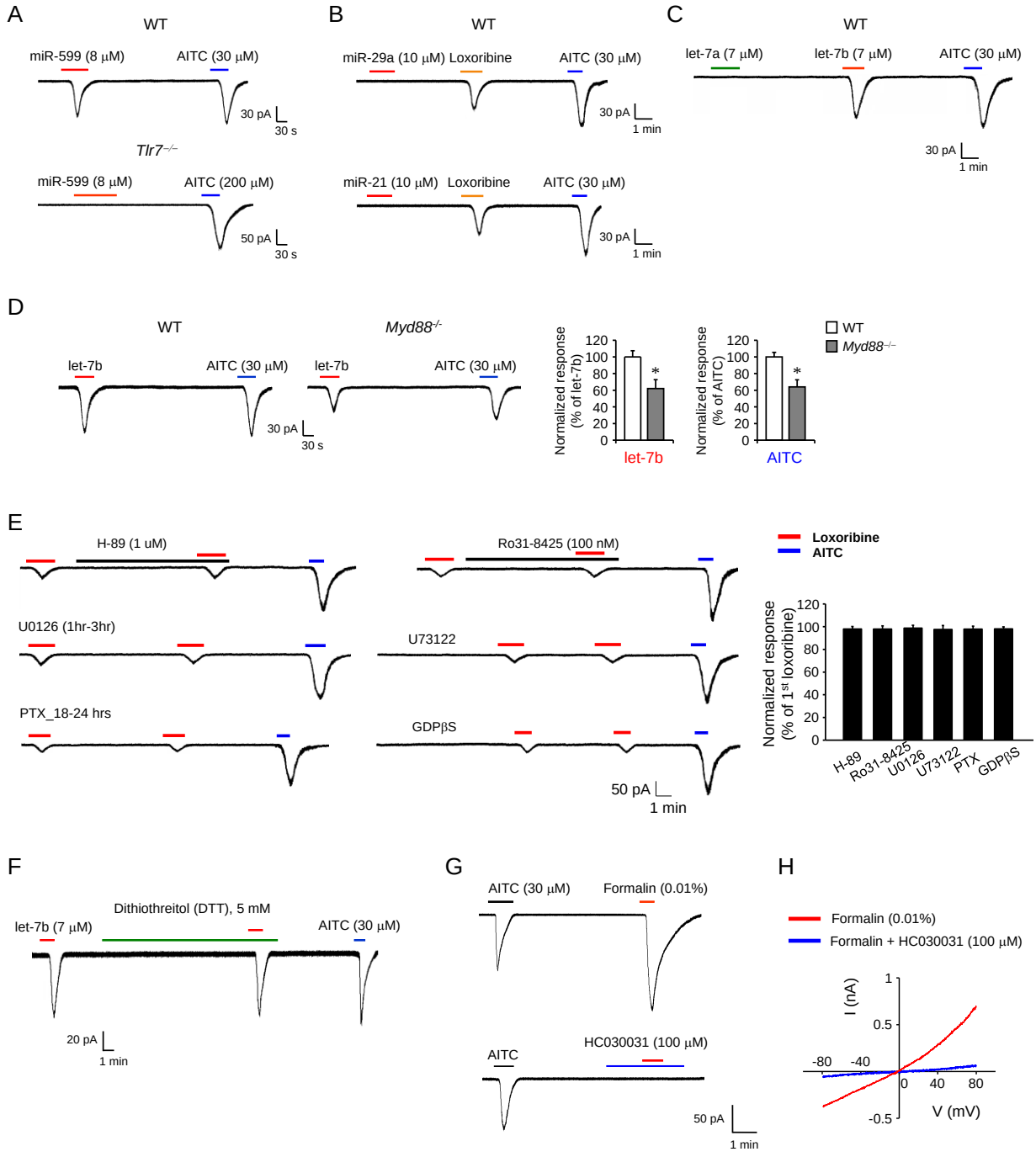
miRNA	Sequence
Homo sapiens	
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
hsa-miR-599	GUUGUGUCAGUUUAUCAAAC
Mus musculus	
mmu-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
mmu-let-7k	UGAGGUAGGAGGUUGUGUG
mmu-miR-669a-5p	AGUUGUGUGUGCAUGUUCAUGUCU
mmu-miR-669c-5p	AUAGUUGUGUGUGGAUGUGUGU
mmu-miR-669f-5p	AGUUGUGUGUGCAUGUGCAUGUGU
mmu-miR-669k-5p	UGUGCAUGUGUGUAUAGUUGUGUGC
mmu-miR-669l-5p	AGUUGUGUGUGCAUGUAUAUGU
mmu-miR-669o-5p	UAGUUGUGUGUGCAUGUUUAUGU
mmu-miR-669p-5p	AGUUGUGUGUGCAUGUUCAUGUCU

Supplemental Table-2. Sequences of outer and inner primers for single-cell PCR (related to Figure 1).

Target gene (Product length)[a]	Outer primers	Inner primers	Genbank No.
TRPV1 (273 bp, 203 bp)	TGATCATCTTCACCACGGCTG CCTTGCGATGGCTGAAGTACA	AAGGCTTGCCCCCCTATAA CACCAGCATGAACAGTGACTGT	NM_001001445.1
TRPA1 (371 bp, 303 bp)	GGCTTTTGGCCTCAGCTTTTAT ACACGATGGTGGACCTCTGATC	ATGCCTTCAGCACCCCATT TGCGTAAGTACCAGAGTGGCAG	NM_177781.4
GAPDH (367 bp, 313 bp)	AGCCTCGTCCCGTAGACAAAA TTTTGGCTCCACCCCTTCA	TGAAGGTCGGTGTGAACGAATT GCTTTCTCCATGGTGGTGAAGA	XM_001473623.1
TLR7 (421 bp, 359 bp)	CAGTGAACTCTGGCCGTTGAGA TGGCGGCATACCCTCAAAA	TTCTCCAACAACCGGCTTGAT TCAGGAGGCAAGGAATTCAGG	NM_133211.3

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

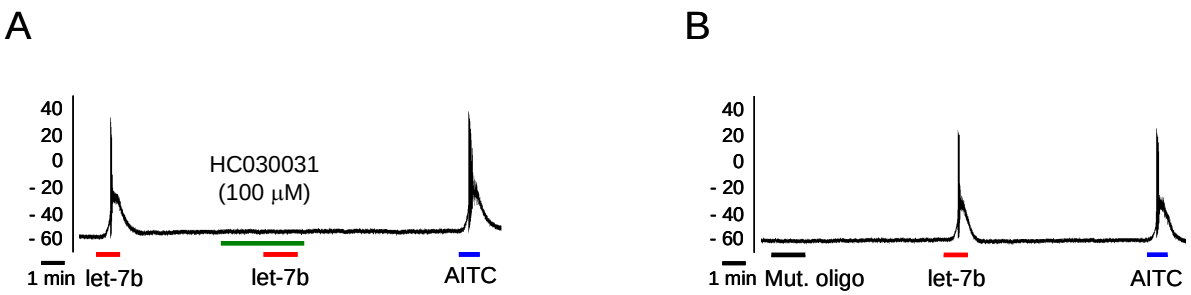
III. Supplemental Figures



Supplemental Figure 1. Inward currents in DRG neurons (related to Figure 1)

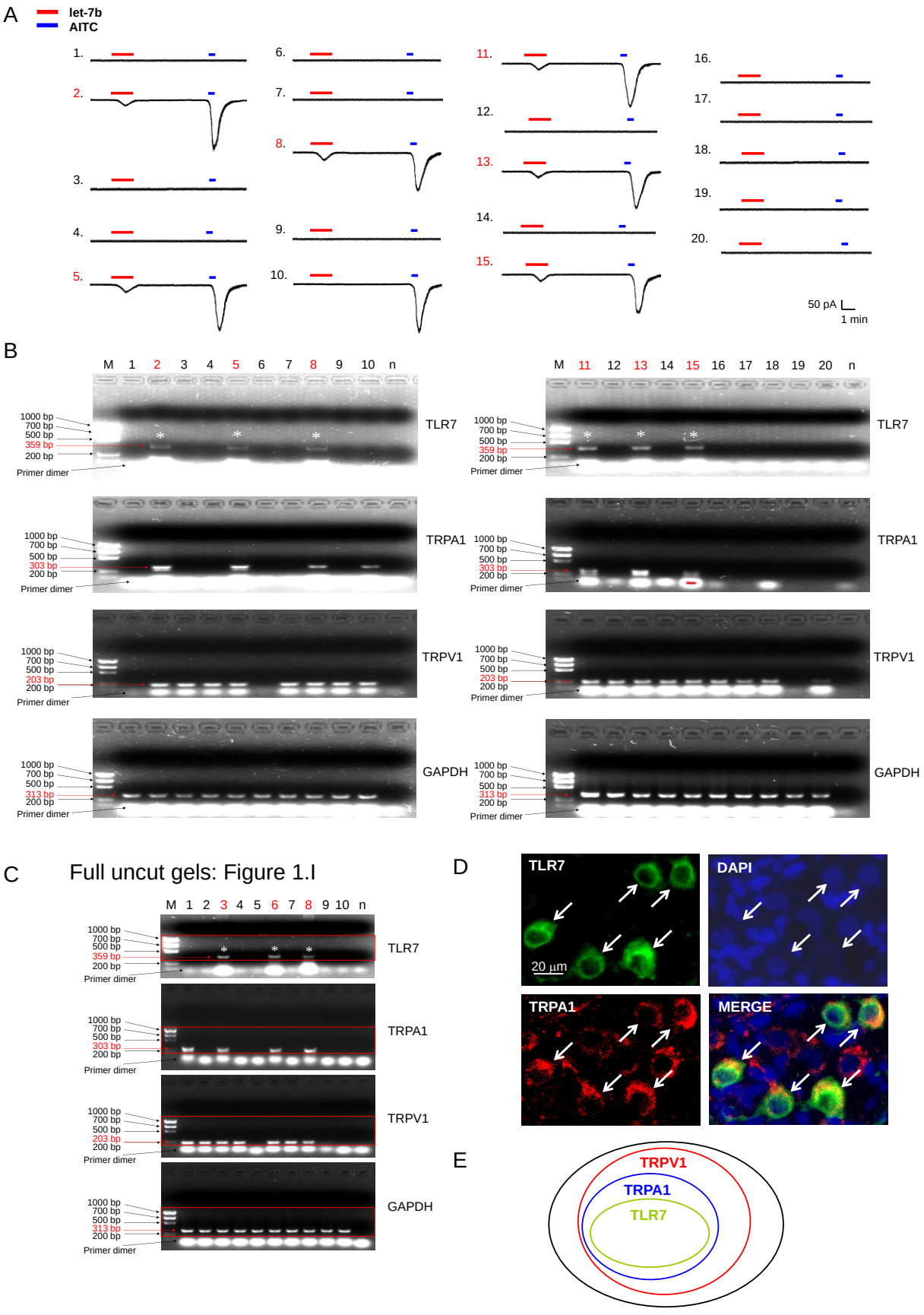
(A) miR-599 but not miR-29a and miR-21 induces inward currents in small-sized DRG neurons via TLR7. miR-599 (8 μM) induces AITC-sensitive inward currents in DRG neurons (7 out of 24) of WT but not *Tlr7*$^{-/-}$ mice (n = 0/25). **(B)** miR-29a (10 μM) and miR-21 (10 μM) do not elicit inward currents in loxoribine- and AITC-sensitive DRG neurons (n = 0/25). **(C)** let-7a (7 μM) fails to induce any inward current in let-7b- and AITC-sensitive DRG neurons (n = 0/10). **(D)** Myd88 is partially required for let-7b-induced inward currents in small-sized DRG neurons. Left, traces of let-7b-induced inward currents. Right, the amplitudes of let-7b (7 μM) and AITC (30 μM)-induced inward currents are slightly reduced in *Myd88*$^{-/-}$ mice (≈30% reduction, $P < 0.05$, *t*-test, compared with WT mice; n = 6 neurons). **(E)** Intracellular signaling pathways are not required for the functional coupling of TLR7 and TRPA1 in DRG neurons. Loxoribine (200 μM)-induced inward currents are not suppressed by inhibitors of PKA (H-89, 1 μM, n = 15), PKC (Ro31-8425, 100 nM, n = 12), ERK (U0126, 10 μM, n = 9), PLC (U73122, 10 μM, n = 8), and G-proteins (pertussis toxin, 0.5 μg/ml, n = 8; GDP βS, 2.5 mM, n = 8). Right, peak amplitude of loxoribine-induced currents in DRG neurons following the treatment of the above-mentioned inhibitors. The data are normalized to the peak amplitude of the first loxoribine response ($P > 0.05$; paired *t*-test versus first loxoribine response). DRG neurons were treated with the inhibitors for 5-6 min before the 2nd loxoribine stimulation. **(F)** Treatment of DRG neurons with dithiothreitol (5 mM, 10 min), a cell-permeable reducing agent, does not alter let-7b (7 μM)-induced inward currents. n = 6 neurons. **(G)** Low concentration of formalin (0.01%) induces TRPA1-mediated inward currents in DRG neurons (n = 8). The formalin-induced inward currents are completely blocked by the TRPA1 antagonist HC030031 (100 μM, n = 7). **(H)** Formalin (0.01%) induced I/V curves with the reversal potential of 0 mV (n = 5). Formalin-induced current is suppressed by HC030031 (100 μM, n = 5). All the data are mean ± SEM.

Fig. S2



Supplementary Figure 2. **Action potentials in DRG neurons (related to Figure 1).**

(A) let-7b-induced action potentials are blocked by HC030031 (100 μM). **(B)** Action potentials induced by let-7b and AITC but not by Mut. oligo in DRG neurons. n = 5-10 neurons.


A
let-7b
AITC
1.
2.
3.
4.
5.
6.
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8.
9.
10.
11.
12.
13.
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15.
16.
17.
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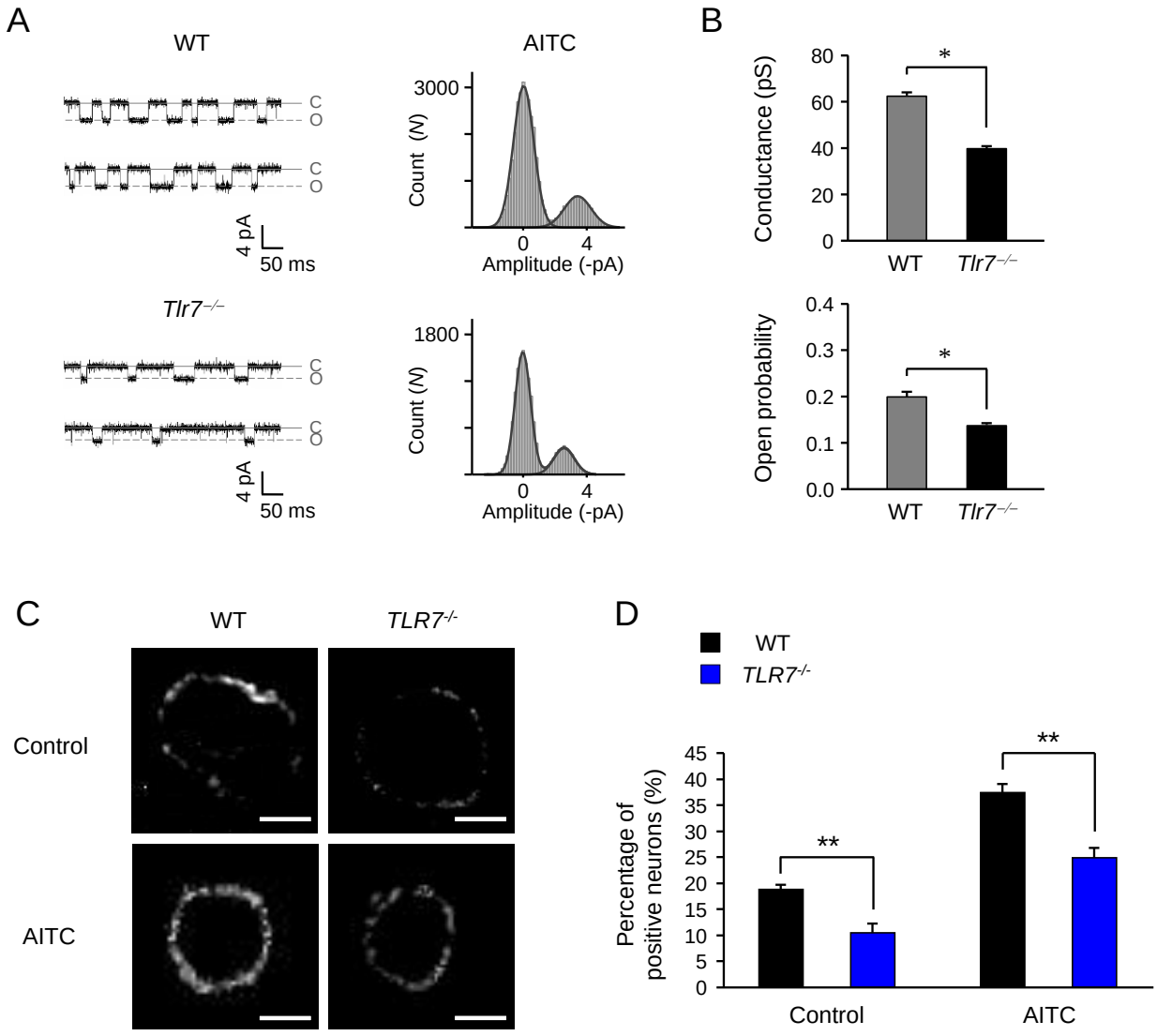
19.
20.
50 pA
1 min
B
M 1 2 3 4 5 6 7 8 9 10 n
M 11 12 13 14 15 16 17 18 19 20 n
1000 bp
700 bp
500 bp
359 bp
200 bp
Primer dimer
TLR7
303 bp
TRPA1
203 bp
TRPV1
313 bp
GAPDH
C
Full uncut gels: Figure 1.I
D
TLR7
DAPI
20 µm
TRPA1
MERGE
E
TRPV1
TRPA1
TLR7

Supplemental Figure 3. let-7b induces inward currents in small-diameter DRG neurons that co-express TLR7 and TRPA1 (related to Figure 1).

(A and B) Inward currents (A) and single-cell RT-PCR (B) in 20 small-sized DRG neurons. Note that let-7b induces inward currents exclusively in TLR7-expresing neurons. Six neurons (#2, 5, 8, 11, 13, and 15, highlighted in red) respond to let-7b (7 μM) by displaying inward currents, and these neurons also express *Tlr7*, *Trpa1*, and *Trpv1* mRNAs. M, molecular weights; n, negative control. Asterisks indicate TLR7-positive neurons. **(C)** Full uncut gels of Figure 1I. The molecular weights for specific bands are highlighted in red. **(D)** Double staining of TLR7 immunofluorescence (green) and TRPA1 in situ hybridization (red) in DRG sections shows co-colocalization of TLR7 and TRPA1. Arrows indicate double-labeled neurons with TLR7 and TRPA1. The nuclei were stained with DAPI. **(E)** Venn diagram shows the relationship of TLR7+, TRPA1+, and TRPV1+ populations in DRG. Note that all TLR7+ cells express TRPA1 and TRPV1 and all TRPA1+ cells express TRPV1.

Fig. S4



Supplementary Figure 4. TRPA1 function and expression is impaired in DRG neurons of *Tlr7*$^{-/-}$ mice (related to Figure 2).

(A and B) Inside-out patch recordings (-60 mV) in membrane excised from small-sized DRG neurons showing reduced TRPA1 single channel activity in *Tlr7*$^{-/-}$ mice. (A) Left, representative AITC (200 μM)-activated single channel activities from wild-type and *Tlr7*$^{-/-}$ mice. Right, all-point histograms of single-channel opening events (5 min recordings) at -60 mV. The averages of single channel currents are -3.75 ± 0.1 pA and -2.4 ± 0.07 pA in wild-type and *Tlr7*$^{-/-}$ mice, respectively. c:

closed state; o: open state. (B) AITC-induced single channel conductance and open probability are reduced in *Tlr7*$^{-/-}$ mice. *$P < 0.05$, *t*-test. n = 7-8 neurons. **(C and D)** TRPA1 surface expression in DRG neurons, either before or after AITC-stimulation, is reduced in *Tlr7*$^{-/-}$ mice. (C) Immunocytochemistry showing TRPA1 expression in DRG neurons in WT and *Tlr7*$^{-/-}$ mice, before or after AITC stimulation. DRG neurons were stimulated with AITC (150 μM) for 10 min, and fresh (non-fixed) DRG neurons were incubated with TRPA1 antibody for 10 min, followed by Cy3-conjugated 2nd antibody. AITC increases TRPA1 surface expression, and both basal and AITC-induced TRPA1 surface expression are reduced in the KO mice (scale bar = 10 μm). (D) Percentage of TRPA1-positive DRG neurons in WT and *Tlr7*$^{-/-}$ mice, before or after AITC stimulation. *$P < 0.05$, *t*-test. n=4 cultures from separate experiments. All the data are mean ± SEM.