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The role of *Drosophila* Piezo in mechanical nociception

Sung Eun Kim¹, Bertrand Coste¹, Abhishek Chadha¹, Boaz Cook^{1,3}, and Ardem Patapoutian^{1,2,3}

¹Department of Cell Biology, The Scripps Research Institute (TSRI), La Jolla, CA 92037, USA

²Genomic Institute of the Novartis Research Foundation (GNF), San Diego, CA 92121, USA

Abstract

Transduction of mechanical stimuli by receptor neurons is essential for senses such as hearing, touch, and pain^{1–4}. Ion channels play a role in neuronal mechanotransduction in invertebrates¹; however, functional conservation of these ion channels in mammalian mechanotransduction is not observed. For example, NOMPC, a TRP ion channel, acts as a mechanotransducer in *Drosophila melanogaster*⁵ and *Caenorhabditis elegans*^{6,7}; however, it has no orthologues in mammals. DEG/ENaC family members are mechanotransducers in *C. elegans*⁸ and potentially in *D. melanogaster*⁹; however, a direct role of its mammalian homologues in sensing mechanical force is not shown. Recently, Piezo1 and Piezo2 were identified as components of mechanically activated (MA) channels in mammals¹⁰. Piezos represent an evolutionary conserved family of transmembrane proteins. It is unknown whether Piezos function in mechanical sensing *in vivo*, and if they do, which mechanosensory modalities they mediate. Here, we study the physiological role of the single Piezo member in *D. melanogaster* (*dpiezo*). *dpiezo* expression in human cells induces mechanically activated currents, similar to its mammalian counterparts [Coste et al., accompanying paper¹¹]. Behavioral responses to noxious mechanical stimuli were severely reduced in *dpiezo* knockout larvae, while responses to another noxious stimulus or touch were not affected. Knocking down *dpiezo* in sensory neurons that mediate nociception and express the DEG/ENaC ion channel *pickpocket* (*ppk*) was sufficient to impair responses to noxious mechanical stimuli. Furthermore, expression of *dpiezo* in these same neurons rescued the phenotype of the constitutive *dpiezo* knockout larvae. Accordingly, electrophysiological recordings from *ppk*-positive neurons revealed a *dpiezo* dependent, mechanically-activated current. Finally, we found that *dpiezo* and *ppk* function in parallel pathways in *ppk*-positive cells, and that mechanical nociception is abolished in the absence of both channels. These data demonstrate physiological relevance of Piezo family in mechanotransduction *in vivo*, supporting a role of Piezo proteins in mechanosensory nociception.

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³To whom correspondence should be addressed, bcook@scripps.edu, apatapou@gnf.org.

AUTHOR CONTRIBUTIONS

S.E.K. conducted most experiments. B.C. (Coste) performed electrophysiology experiments shown in Fig. 3 and Supplementary Fig 5. A.C. performed fly electrophysiology experiments shown in Supplementary Fig. 4. S.E.K., A.P. and B.C. designed experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

D. melanogaster is widely used to study mechanotransduction and genetic studies have identified several ion channels that are essential for mechanosensation^{5,9,12–14}. However, none of these proteins are shown to be activated by mechanical force when expressed in heterologous systems. Since expression of mouse Piezos in a variety of mammalian cells induces large mechanically activated currents¹⁰, we set out to test if the fly counterpart is also sufficient to induce mechanosensitivity. Similar to its mammalian counterparts, the *Drosophila piezo* gene (CG8486) is predicted to consist of a large number of transmembrane domains (39, Supplementary Fig. 1). Albeit fly and mammalian *piezos* do not exhibit extensive sequence conservation (24% identity), expression of *Drosophila piezo* in cultured human cells induced large mechanically activated cationic currents, suggesting a role of *piezo* in mechanotransduction¹¹.

To characterize *dpiezo* expression in flies we used a fusion between the *dpiezo* enhancer/promoter region and GAL4 (dPiezoP-GAL4). Four independent dPiezoP-GAL4 transgenic insertions were examined to avoid insertional effects on GAL4 expression. We used UAS-GFP for labeling cells except for arborized neurons that were optimally visualized using the membrane-targeted UAS-CD8::GFP. We found fluorescent labeling induced by *dpiezo* enhancer/promoter region in all types of sensory neurons and several non-neuronal tissues in both adults and larvae (Supplementary Fig. 2). This diverse pattern of *dpiezo* expression observed in *Drosophila* is in accord with the expression of Piezo1 and Piezo2 in mice¹⁰.

We created *dpiezo* knockout (KO) flies in which all 31 coding exons were deleted using genomic FLP-FRT recombination¹⁵ (Fig. 1a, see Supplementary Fig. 3 for details). The knockout flies were viable, fertile and did not show uncoordination or a defect in bristle mechanoreceptor potential (Supplementary Fig. 4). We studied whether *dpiezo* KO larvae have mechanical nociception deficits by using a mechanically-induced escape behavior assay^{9,14,16}. Stimulation with von Frey filaments that ranged from 2 to 60 mN demonstrated that *dpiezo* KO larvae have a severe response deficit over a wide range (Fig. 1b). Repeated stimulations of the same larvae resulted in comparable responsiveness in both wild type and *dpiezo* KO, indicating that the stimuli did not induce considerable damage to the sensory system (Fig. 1c, d). A 153 ± 11.0 mN filament elicited responses only to the first of three stimulations in wild type larvae, arguing that this amount of force is damaging (not shown). For further experiments, we chose to stimulate the larvae using a 45 mN filament which has been used in a previous study¹⁴, and elicits a substantial response in both wild type and *dpiezo* mutant larvae. 34 ± 4.4 % of *dpiezo* KO larvae showed a response to 45 mN filament stimulation, compared to over 80 % of wild type or heterozygote larvae (Fig. 1e). As a control for the genetic background, we used larvae that carry the *dpiezo* KO allele on one chromosome and a deficiency in which the entire *dpiezo* genomic region is deleted on the homologous chromosome. The defect in the trans-heterozygous larvae was similar to the KO homozygote phenotype (51 ± 3.9 %, $p = 0.091$). In contrast, *dpiezo* KO larvae were indistinguishable from wild type in an assay for responses to high temperature, a different noxious stimulus that elicits the same escape response¹⁴ (Fig. 1f). Therefore, *dpiezo* KO larvae retain a normal ability to elicit the escape behavior in response to noxious stimuli, while *dpiezo* is specifically required for the mechanical modality of nociception. To evaluate the possible role of *dpiezo* in other modes of larval mechanical sensing, we tested the

sensitivity of *dpiezo* KO to gentle touch which is mediated through ciliated neurons^{17,18}. We observed no defect in the sensitivity of *dpiezo* KO larvae to innocuous gentle touch (Fig. 1g).

A mechanical nociception phenotype was previously observed in *pickpocket* (*ppk*), a DEG/ENaC channel⁹, and *painless* (*pain*), a TRPA ion channel¹⁴. The specificity of *dpiezo* KO to mechanical nociception resembles the phenotype of *ppk* since *pain* is also essential for sensing thermal nociception¹⁴. We therefore tested the role of *dpiezo* in *ppk*-positive cells using *ppk*-GAL4, which labels subclasses of multidendritic (MD) neurons^{19,20}. The MD neurons are non-ciliated receptor cells that tile the body wall of the larvae and respond to a variety of external stimuli such as mechanical forces, temperature and light^{9,14,16,21}. We used a green fluorescent protein driven directly by the regulatory regions of the *ppk* gene (*ppk*-EGFP)²² together with DsRed expression in *dpiezo*-positive cells to probe *dpiezo* and *ppk* co-expression. Indeed we did observe that all *ppk*-positive cells also expressed *dpiezo* (Fig. 2a). Next we used *ppk*-GAL4 to drive the expression of *dpiezo* RNAi to test whether *dpiezo* function is specifically required in *ppk*-expressing cells. The restricted knockdown of *dpiezo* resulted in a mechanical nociceptive phenotype (Fig. 2b) similar to phenotype observed in *dpiezo* KO larvae (Fig. 1e). In a complementary approach, we used *ppk*-GAL4 driven expression of *dpiezo* cDNA in an attempt to rescue the mechanical nociception phenotype of *dpiezo* KO. We used a fusion between dPiezo and GFP to monitor expression levels in *ppk* cells and dPiezo localization within the neurons. GFP-dPiezo fusion protein induces MA currents in human cell lines, similar to untagged dPiezo, confirming functionality (Supplementary Fig. 5a–c). When expressed in the fly, GFP-dPiezo fluorescence was present throughout cell bodies, axons and dendritic arbors of *ppk*-positive neurons (Supplementary Fig. 5d). Importantly, expression of GFP-dPiezo in *ppk*-positive neurons alone was sufficient to rescue the mechanical nociception defect of *dpiezo* KO (Fig. 2c). These data suggests that *dpiezo* functions in *ppk*-positive neurons to mediate mechanical nociception.

To test if the *ppk*-positive neurons respond to mechanical stimuli and if *dpiezo* mediates such responses we performed electrophysiological recordings from isolated cells. Larvae that had GFP labeling in *ppk*-positive neurons were dissociated using enzymatic digestion and mechanical trituration. Plated fluorescent neurons were then tested using patch clamp recordings in the cell-attached configuration and they were stimulated using a negative pressure through the recording pipette¹⁰. Stimulating wild type neurons resulted in a current that was rapidly activated and had a half-maximal activation (P_{50}) of 27.6 ± 7.6 mmHg (Fig. 3). These currents were not observed in the *dpiezo* KO mutant neurons (Fig. 3). Therefore, *ppk*-positive neurons which mediate the avoidance response to noxious stimuli display *dpiezo*-dependent mechanically activated currents.

Silencing of *ppk* cells resulted in a complete abolishment of noxious mechanosensation (Supplementary Fig. 6), in accord with a severe defect observed previously¹⁶. In contrast, only a moderate deficit is observed upon eliminating or knocking down *ppk* in the same cells⁹, suggesting that there are multiple pathways for mechanical sensing. We tested mechanical nociception in larvae that are deficient in *dpiezo* and either *pain* or *ppk* to gain insight into cellular pathways that involve mechanotransduction in these cells. Once again,

we used a 45 mN filament, enabling us to monitor both *dpiezo*-dependent and -independent mechanisms (Fig. 1b). The *dpiezo::pain* double mutant had a defect that was comparable to each one of the mutants separately, suggesting that *dpiezo* and *pain* might function in the same pathway (Fig. 4a). Larvae that are heterozygous for both *dpiezo* and *pain* showed a response deficit while each one of them separately was normal (Fig 4b), further demonstrating their role in a common signaling mechanism. Remarkably, combining both *dpiezo* and *ppk* knockdowns resulted in a nearly complete abolishment of responses to noxious mechanical stimuli (Fig. 4c). Importantly, responses to noxious temperatures and touch were normal in larvae with both *dpiezo* and *ppk* knocked-down (Fig. 4d, e). These data suggest that *dpiezo* and *ppk* function in two parallel pathways in *ppk*-positive sensory neurons, and that together they constitute the response to noxious mechanical stimuli. There could be many reasons why the mechanically activated currents we observe are entirely dependent on dPiezo (Fig. 3). This can either be because PPK responds to a different modality of mechanical stimulus or due to the specific experimental settings (e.g. level of applied forces, solutions, applied voltage). Future experiments should resolve this issue.

Using the *Drosophila* model system we have demonstrated that *piezo* is essential for sensing noxious mechanical stimulus *in vivo*. This is the first demonstration that a Piezo family member is essential for mechanotransduction in the whole animal. Indeed, *dpiezo* is to our knowledge the first eukaryotic excitatory channel component shown to be activated by mechanical force in a heterologous expression system and required for sensory mechanotransduction *in vivo*. Piezo2 is expressed in mouse DRG neurons that are involved in sensing nociception, and is required for rapidly-adapting mechanically activated currents in such isolated neurons¹⁰. This study raises the possibility that mammalian Piezo2 is also required for mechanical pain transduction *in vivo*. Furthermore, *Drosophila* genetics can now be utilized to map cellular pathways involved in *piezo*-dependent mechanotransduction in sensory neurons and beyond.

METHOD SUMMARY

Fly stocks

PiggyBacs (PBac{WH}CG8486-f02291, PBac{RB}CG8486-e00109, Exelixis Collection at the Harvard Medical School), *ppk*-GAL4 (Bloomington *Drosophila* Stock Center (BDSC), 32078, 32079), Deficiency (Df(2L)Exel7034/CyO, BDSC, 7807), UAS-*dpiezo*-RNAi (National Institute of Genetics, Japan, 8486R-3), UAS-*ppk*-RNAi (Vienna *Drosophila* RNAi Center, 108683), *ppk*-EGFP5²² (Jan Y.N.), *painless*¹ (BDSC, 27895).

Generating *dpiezo* KO

The *dpiezo* knockout fly was generated by FLP-FRT recombination with two PiggyBac lines as described in Parks et al¹⁵. The recombined KO fly was confirmed by PCR (Supplementary Fig. 2). The genetic background was cleaned using meiotic recombination with w¹¹¹⁸.

Imaging

Fluorescence in adult fly or larva was detected by Nikon C2 Confocal Laser Point Scanning Microscope, Olympus FluoView500 Confocal Microscope or Olympus AX70 microscope.

Cloning

To clone enhancer/promoter of *dpiezo* gene, the genomic region between 1.0 kb upstream of 5'UTR and the start codon of *dpiezo* was amplified by PCR and cloned into pPTGAL vector. GFP-dPiezo construct has three Alanines as a linker between N-terminal GFP and C-terminal dPiezo. The construct was cloned in modified pUAST vector to generate transgenic flies and in modified pIRES2-EGFP vector for electrophysiology recordings.

Behavioral assays and statistics

The mechanical nociception was tested as described previously^{9,14,16} using calibrated von Frey filaments. The thermal nociception was tested as described previously¹⁴ using a 45 °C heated metal probe. All error bars represent mean \pm SEM.

Isolation of ppk-positive neurons

Third instar larvae that had GFP labeling in *ppk*-positive neurons were dissected, digested with collagenase and mechanically triturated. The cells were collected by centrifugation and plated on a poly-D-lysine coated glass coverslip. The fluorescent *ppk*-positive cells were recorded after incubating two hours at room temperature.

Electrophysiology

HEK cells were studied in the whole cell configuration using a polished glass probe for stimulation¹⁰ and *ppk*-positive neurons were stimulated using a negative pressure in the cell attached configuration¹⁰.

METHODS

Fly stocks

We used the following stocks: PiggyBacs (PBac{WH}CG8486-f02291, PBac{RB}CG8486-e00109, Exelixis Collection at the Harvard Medical School), *ppk*-GAL4 (Bloomington Drosophila Stock Center (BDSC), 32078, 32079), Deficiency (Df(2L)Exel7034/CyO, BDSC, 7807), UAS-*dpiezo*-RNAi (National Institute of Genetics, Japan, 8486R-3), UAS-*ppk*-RNAi (Vienna Drosophila RNAi Center, 108683), *ppk*-EGFP5²² (Jan Y.N.), *painless*¹ (BDSC, 27895) and UAS-DsRed-NLS (Posakony J.W.). The following stocks were from BDSC: UAS-GFP, UAS-CD8GFP, CyO-GFP, w¹¹¹⁸ and Canton-S.

Engineering *dpiezo* knockout

The *dpiezo* knockout fly was generated as described in Parks et al¹⁵. Two PiggyBac lines which carry FRT sequence were selected for FLP-FRT recombination. PBac{WH}CG8486-f02291 is inserted in the first intron and PBac{RB}CG8486-e00109 in the 3' UTR of *dpiezo* gene. After FLP-FRT recombination, 20kb of *dpiezo* gene, including all 31 coding exons was removed and replaced with 7kb of PiggyBac insertion which contains FRT sequence and white gene. The recombined KO fly was confirmed by PCR reactions (Supplementary Fig. 2). The genetic background was cleaned using meiotic recombination with w¹¹¹⁸.

Molecular biology

***dpiezo* promoter cloning**—To clone enhancer/promoter of *dpiezo* gene, the genomic region between 1.0 kb upstream the beginning of the 5'UTR and the start of the *dpiezo* coding region was amplified by PCR using forward primer, 5'-atctggcgccgctatctatttttaactagtggaagtct-3' and reverse primer, 5'-ttactgggtaccatggatgcctccggcgccgttctcctccag-3'. The amplified sequence was cloned into pPTGAL vector (Drosophila Genomic Resource Center, 1225) using NotI and KpnI sites and the sequence was verified.

GFP fusion dPiezo (GFP-dPiezo) cloning—For rescue experiments, *dpiezo* cDNA was amplified from the plasmid reported in Coste et. al. using forward primer 5'-tattagcgccgagctctcagctatgcgtgcatggtg-3' and reverse primer 5'-taattcgggttattgcggttctgtggtgcagttgctccgg-3' and cloned into a modified pUAST vector using NotI and RsrII. NotI restriction enzyme site was used as a linker by providing three Alanine residues between EGFP and dPiezo. The order of sequences in pUAST vector is as following: UAS-kozak-EGFP-3x(Ala)-dPiezo. To generate transgenic flies, DNA was injected into the isogenized w¹¹¹⁸ embryos with 2–3. For electrophysiology experiment, EGFP-dPiezo was cloned into mammalian expression vector with CMV promoter.

Behavior assays

The mechanical nociception was tested as described previously^{9,14,16} using calibrated von Frey filaments. The thermal nociception was tested as described previously¹⁴ using calibrated heated metal probe. For both nociception assays, the number of larvae which showed at least one 360 ° rotation was counted for each trial. The gentle touch assay was made as described previously¹⁷. Each stimulated larva was scored according to Kernan et al.; 0 = no response, 1 = hesitates, 2 = turns or withdraws anterior, 3 = single reverse contractile wave and 4 = multiple waves. For all behavior assays each third instar larva was stimulated only once. All data were generated from at least three trials.

Calibration of von Frey stimulator—The von Frey filaments for larvae behavior experiments were modified from Touch-Test sensory Evaluator (North Coast Medical) or from monofilament fishing lines. Each monofilament was cut to a length of 18 mm, glued into a pipette tip so that 9 mm of it protruded and mounted on a hand manipulator with a 90° angle. Each von Frey filaments was calibrated as described previously⁹. The force of each von Frey stimulator was determined by measuring the weight upon filament bending and converting the value into the force: force (mN) = mass (gram) × gravity acceleration constant, g (9.8). Each stimulator was calibrated 15 times and its mean value was used in figures. The calibrated forces (mean ± SEM) of each stimulator are the following (in mN): 1.78 ± 0.15, 2.59 ± 0.15, 5.04 ± 0.19, 11.2 ± 0.66 and 59.9 ± 1.79.

Fluorescence imaging

For identifying tissues or cells expressing fluorescence by *dpiezo* promoter, both adult flies and third instar larvae, carrying dPiezoP-GAL4 and UAS-GFP or UAS-CD8::GFP were dissected or whole-mounted. For double fluorescent labeling in md neurons, second instar larvae carrying ppk-EGFP, dPiezoP-GAL4 and UAS-DsRed were whole-mounted. For

imaging *ppk*-cells expressing GFP-dPiezo, third instar larvae carrying *ppk*-GAL4 and UAS-GFP-dPiezo were whole-mounted. Fluorescence images were obtained either by Nikon C2 Confocal Laser Point Scanning Microscope, Olympus FluoView500 Confocal Microscope or Olympus AX70 microscope.

Isolation of larvae *ppk*-positive neurons

In both wild type and *dpiezo* KO larvae, *ppk*-positive neurons were fluorescently labeled by *ppk*-EGFP which is a direct fusion of *ppk* genomic regulatory regions with EGFP. Third instar larvae were dissected in M3 media containing 10% heat inactivated FBS. Each larva was cut twice and its internal organs were removed. The cleaned body wall was treated with 5 mg/ml Collagenase type IV at 25 °C for 1 hour in serum free M3 media and washed with serum containing M3 media. The enzyme treated body wall was triturated with fire-polished Pasteur pipettes in M3 media with 2mM EGTA and 10% FBS. The cuticle and debris were removed by centrifugation at 40 g and the small size cells, including neurons were collected by centrifugation at 360 g for 10min. The cell pellet was resuspended with serum containing M3 media and plated into a poly-D-lysine coated coverslip in a small droplet. After two hours of incubation at room temperature, the coverslips were transferred to the electrophysiology rig for recording.

Electrophysiology

For whole-cell recordings in HEK293T cells, patch pipettes had resistance of 2–3 M Ω when filled with an internal solution consisting of (in mM) 133 CsCl, 10 HEPES, 5 EGTA, 1 CaCl₂, 1 MgCl₂, 4 MgATP, and 0.4 Na₂GTP (pH adjusted to 7.3 with CsOH). The extracellular solution consisted of (in mM) 130 NaCl, 3 KCl, 1 MgCl₂, 10 HEPES, 2.5 CaCl₂, 10 glucose (pH adjusted to 7.3 with NaOH). Mechanical stimulation was achieved using a fire-polished glass pipette (tip diameter 3–4 μ m). The probe had a velocity of 1 μ m/ms during the ramp segment of the command for forward motion and the stimulus was applied for 150 ms.

For cell-attached recordings in *ppk*-positive dissociated neurons, patch pipette had resistance of 3–3.5 M Ω when filled with a solution consisting of (in mM) 130 NaCl, 5 KCl, 10 HEPES, 1 CaCl₂, 1 MgCl₂, 10 TEA-Cl (pH 7.3 with NaOH). External solution used to zero the membrane potential consisted of (in mM) 140 KCl, 10 HEPES, 1 MgCl₂, 10 glucose (pH 7.3 with KOH). Membrane patches were stimulated with brief negative pressure pulses through the recording electrode using a Clampex controlled pressure clamp HSPC-1 device (ALA-scientific). Stretch-activated channels were recorded at a holding potential of –80 mV with pressure steps from 0 to –60 mm Hg (–10 mm Hg increments). Current-pressure relationships were fitted with a Boltzmann equation of the form: $I(P) = [1 + \exp(-(P - P50)/s)]^{-1}$, where I is the peak of stretch-activated current at a given pressure, P is the applied patch pressure (in mm Hg), $P50$ is the pressure value that evoked a current value which is 50% of I_{max} , and s reflects the current sensitivity to pressure.

All experiments were done at room temperature. Currents were sampled at 50 or 20 kHz and filtered at 5 or 2 kHz. Voltages were not corrected for a liquid junction potential. Leak currents before mechanical stimulations were subtracted off-line from the current traces.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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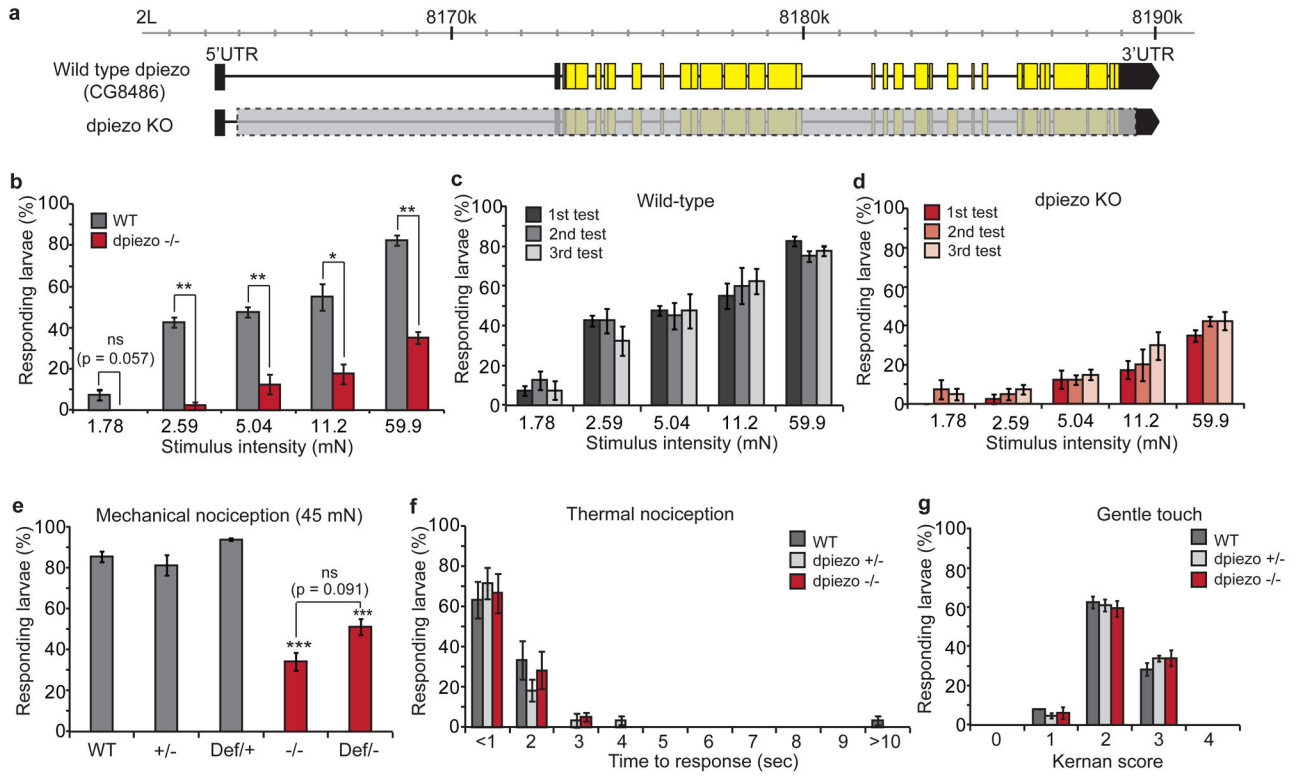


Figure 1. Mechanical nociception defect in *dpiezo* knockout larvae

(a) Genomic map showing wild type *dpiezo* gene (upper) and engineered *dpiezo* KO (lower). Yellow and black boxes represent coding and non-coding exons, respectively. Deleted genomic segment in *dpiezo* KO is marked with a gray box. (b) Mechanical nociception assay using a range of stimulus forces in wild type and *dpiezo* KO larvae. n = 40 from four independent experiments. * p < 0.05, ** p < 0.01 from two tail paired Student t-test. (c, d) Mechanical nociception assay using repeated stimuli of the same larvae. n = 40. (e) Mechanical nociception assay using a 45 mN von Frey filament with wild type (+/+), heterozygous KO (+/-), heterozygous deficiency (Def/+), homozygous KO (-/-) and trans-heterozygous KO (Def/-). n > 85. *** p < 0.001. (f) Thermal nociception assay using heated probe (45 °C). n = 60. (g) Gentle touch assay¹⁷. n > 150.

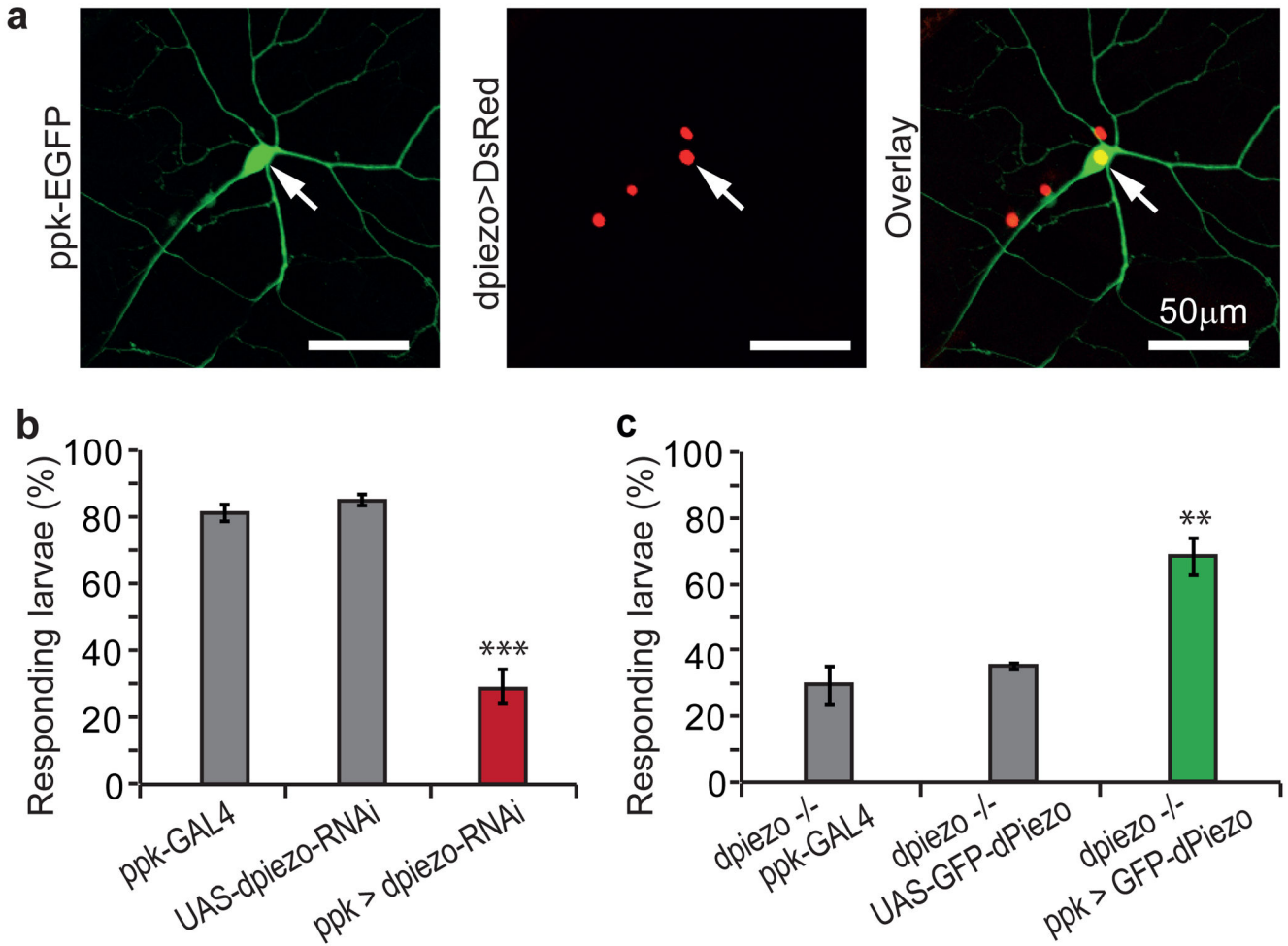


Figure 2. *dpiezo* functions in *ppk*-positive type II sensory neurons

(a) Double fluorescence labeling using *ppk*-EGFP (green) and dPiezoP-GAL4 that drives the expression of the nucleus targeted UAS-DsRed-NLS (red). A representative high-magnification image shows one *ppk*-positive neuron (arrow). All three *ppk*-positive cells in each hemisegment expressed *dpiezo* in all segments. (b) Mechanical nociception assay with *dpiezo* knockdown larvae in *ppk*-expressing cells by *ppk*-GAL4 and UAS-*dpiezo*-RNAi. $n > 85$, *** $p < 0.001$. (c) Mechanical nociception assay in rescued *dpiezo* KO. GFP-dPiezo was expressed in *ppk*-cells using *ppk*-GAL4 and UAS-GFP-dPiezo. $n > 60$. ** $p < 0.01$.

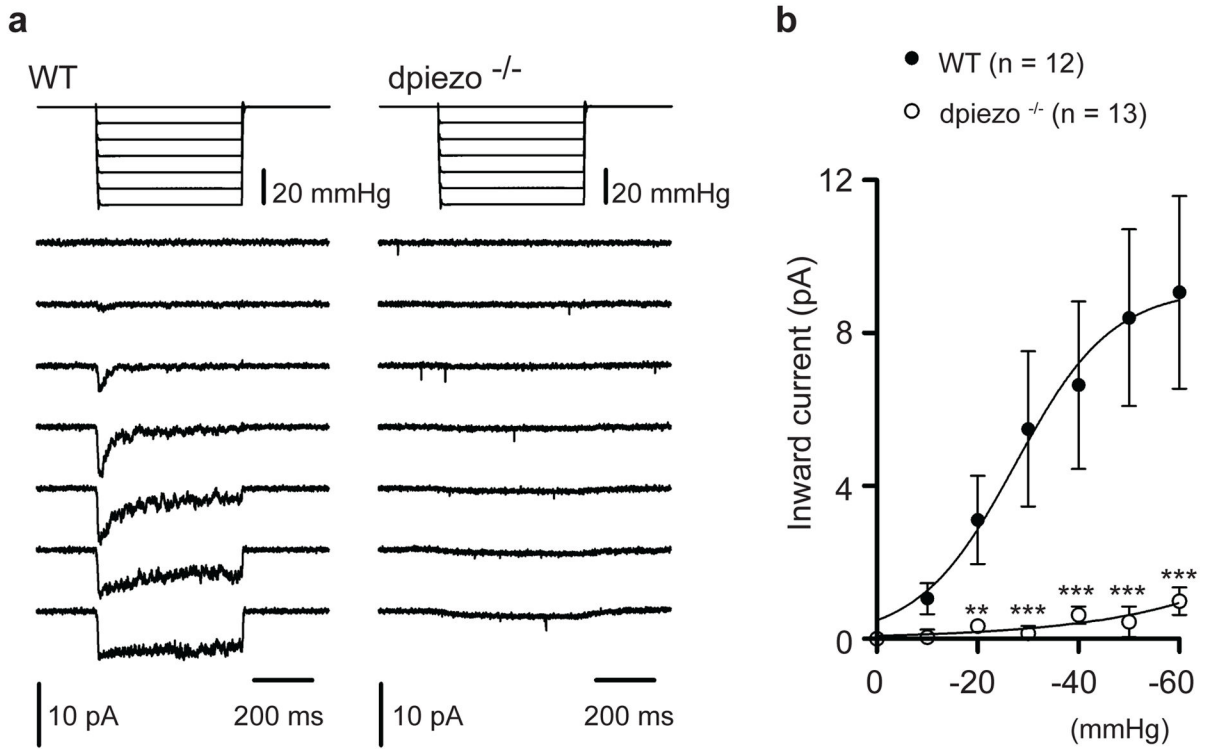


Figure 3. *dpiezo* mediates mechanically-activated currents in *ppk*-positive neurons

(a) Representative currents elicited by negative pipette pressure (0 to -60 mmHg, 10 mmHg) in cell-attached configuration at -80mV in wild type (left) and *dpiezo*^{-/-} (right). (b) Average peak current-to pressure relationship of stretch activated currents in wild type (n = 12 cells) and *dpiezo*^{-/-} (n = 13 cells). Data points are mean ± SEM. fitted with a Boltzmann equation. ** P < 0.01, *** P < 0.001, Mann-Whitney test.

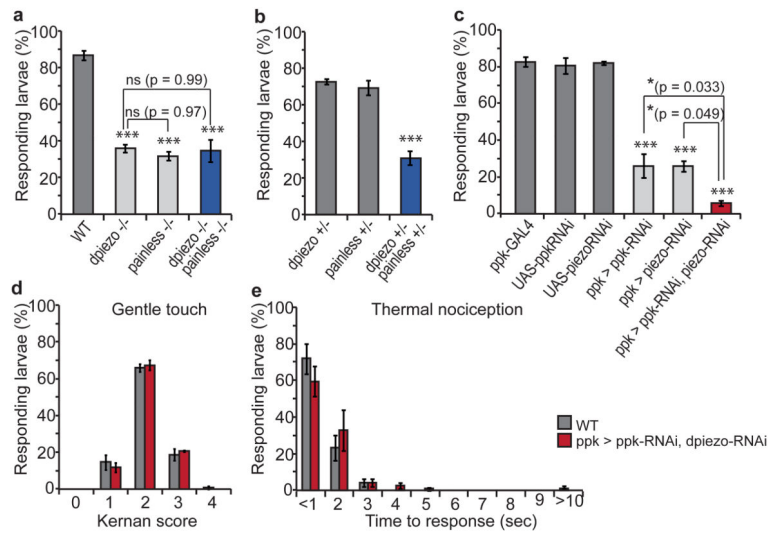


Figure 4. *dpiezo* and *ppk* function in parallel pathways

(a) Mechanical nociception assay using a 45 mN von Frey filament with double null mutant of *dpiezo* and *piezoless*. Single knockout strains were used as controls and the wild type strain is *w*¹¹¹⁸. *n* > 60. (b) Mechanical nociception assay on heterozygous larvae for *dpiezo* and/or *piezoless*. *n* (heterozygote *dpiezo* KO) = 74 from three trials, *n* (heterozygote *piezoless*¹) = 169 from five trials, *n* (trans-heterozygote) = 166 from five trials. (c) Mechanical nociception assay with PPK and Piezo knockdown. *ppk* and/or *piezo* RNAi were driven by *ppk*-GAL4. *n* > 90. * *p* < 0.05, *** *p* < 0.001. *n* (wild type, *w*¹¹¹⁸) = 66, *n* (UAS-ShiDN/+) = 75 and *n* (*ppk*-GAL4/+; UAS-ShiDN/+) = 73 with three trials. (d) Gentle touch sensitivity assay with *ppk* and *dpiezo* knockdown. Wild type is *w*¹¹¹⁸. *n* > 90. (e) Thermal nociception assay using 45 °C probe with *ppk* and *dpiezo* knockdown. *n* > 75.