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PIEZO2 in sensory neurons and urothelial cells coordinates urination

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Abstract

Henry Miller stated that “to relieve a full bladder is one of the great human joys”.

Urination is critically important in health, and ailments of the lower urinary tract (LUT) cause extensive pathological burden. Nevertheless, we take urination for granted, and in-depth mechanistic insight is lacking. We have witnessed advances in understanding the central circuitry in the brain that facilitates urination^{1–3}. Beyond central control, micturition reflexes that govern urination are all initiated by peripheral mechanical stimuli such as bladder stretch and urethral flow⁴. Surprisingly, the mechanotransduction molecules and the cell types that function as the primary stretch and pressure detectors in the urinary tract are mostly unknown. We find that the mechanosensitive ion channel PIEZO2 is expressed in lower urinary tract tissues, where it is required for low-threshold bladder stretch sensing and urethral micturition reflexes. We show that PIEZO2 acts as a sensor in both the bladder urothelium and innervating sensory neurons.

Importantly, both humans and mice lacking functional PIEZO2 have impaired bladder control, and

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Author Contributions

K.L.M. designed and performed all mouse cystometry, behavioral experiments, and tissue histology, analyzed data, and together with A.P. wrote the manuscript. D.S., T.O., C.G.B., and A.T.C. designed and performed the human clinical assessments. Calcium imaging and analysis was performed by N.G., K.L.M., and M.S. Retrograde labeling and fluorescent in situ hybridization (FISH) experiments were performed by K.L.M., A.M.C., and I.D. J.K. and L.T.S. contributed analytical tools for data analysis, technical support and conceptual project design. C.G.B., A.T.C., and A.P. contributed to project design and supervision. All authors discussed results and contributed to manuscript editing.

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humans report deficient bladder-filling sensation. This study pinpoints PIEZO2 as a key mechanosensor in urinary function. These findings enable future work that will unlock how urothelial cells and sensory neurons interact to control urination.

The mechanotransduction channel necessary for urinary reflexes is unknown. Several ion channels are implicated in urinary tract function *in vivo*^{5–7}, but none are shown to be required for micturition reflexes. Moreover, it is not clear which cells are the primary sensors: umbrella cells of the innermost layer in the urothelium have been proposed to be mechanosensory^{8,9}, but the bladder is also innervated by mechanically sensitive afferents from dorsal root ganglia (DRG)^{1,10}. PIEZO2 is the primary mechanosensor that mediates touch, proprioception, and mechanical allodynia in mice^{11–15}. Remarkably, humans with loss-of-function mutations in *PIEZO2* also showed complete deficits in these senses^{13,16}. Furthermore, PIEZO2 mediates interoceptive processes like lung-stretch sensing and baroreception in the mouse^{17,18}, but interoceptive deficits have not been studied in PIEZO2-deficient subjects. Given that urination is driven by mechanical interoceptive reflexes, we investigated whether PIEZO2 is important for urination.

To understand how PIEZO2 contributes to urination in humans, *PIEZO2*-deficient subjects (N=12; ages 5–43) answered questionnaires that are designed to capture pathology and have been validated against healthy controls to screen for voiding and elimination dysfunction¹⁹ (Fig. 1). We also assessed urologic history, previous medical evaluations and non-invasive bladder ultrasounds (Supplementary Table 1). All patients had decreased voiding frequency, reported as low as once or twice daily regardless of hydration status. Notably, the majority of subjects reported they could spend an entire day without feeling the need to void and therefore followed a voiding schedule. Healthy frequency is defined as 5–6 voids per day. Despite the lack of normal sensory feedback, all patients had achieved continence at the time of evaluation except for one 9-year-old. However, many subjects reported sudden urge incontinence where any delay in voiding resulted in urinary accidents. Two individuals reported occasional nocturnal enuresis, and four had stress incontinence caused by laughter, cough and/or postural changes, with one case being severe enough to require treatment. Several patients had a sensation of incomplete voiding and an irregular urinary stream. Three adults described a sensation of pelvic heaviness when their bladder was full, and all independently reported voiding by leaning over or using their hands to apply pressure to their lower abdomen. Overall, these data suggest that PIEZO2 plays an important functional role in human urination.

We next turned to studies in mice to understand where and how PIEZO2 functions in the urinary tract. To test whether *Piezo2* is present in bladder sensory neurons, we used fluorescent in situ hybridization (RNAscope) in bladder and dorsal root ganglion (DRG) tissue taken from three animals after injection of a neuronal tracer into the bladder wall (Fig. 2a, Cholera Toxin B-Alexa 488 (CTB)). Out of 92 bladder-innervating neurons labeled with CTB, 75 expressed *Piezo2* transcript (81.5%). *Piezo2* transcript was also detected in a subset of *Krt20*-positive urothelial cells (Fig. 2b), a marker of umbrella cells that line the bladder lumen and are proposed to contribute to bladder filling detection⁸. Quantification revealed that 74% of umbrella cell nuclei were associated with *Piezo2* transcript, and 12.6% of cells

had high expression (1 SD above mean). *Piezo2*-positive cell density varied across the bladder. These results show that *Piezo2* is expressed in two distinct cell types within the lower urinary tract and could function in detecting relevant mechanical stimuli. We confirmed that urothelial cells express other mechanosensory proteins (Extended Data Fig. 1), but the functional deficits in *PIEZO2*-deficient humans focused our work on this protein.

We next used calcium imaging to determine whether bladder stretch responses in sensory neurons were *Piezo2*-dependent. Whether neurons detect bladder stretch directly or downstream of urothelial cell activation, we expect this stimulus to cause calcium influx. A viral vector carrying Cre recombinase was injected into P0-P3 pups carrying the Cre-dependent calcium indicator GCaMP6f and a conditional *Piezo2* knockout allele, *Piezo2*^{CKO} (*Piezo2*^{f/f}/GCaMP6f^{+/+}, controls were only GCaMP6f^{+/+})¹³. Thus, *Piezo2* was deleted anywhere GCaMP6f was expressed. We observed rapid, robust responses in control S1 DRG neurons in response to manual, high-pressure bladder filling with saline (Fig. 2c), but these responses were dramatically attenuated in *Piezo2*^{CKO} DRG cells (Fig. 2d). Interestingly, cells responding to low-pressure stimuli were completely absent in *Piezo2* knockout DRG (Fig. 2e). Calcium traces for cells in wildtype DRG reveal graded responses to pressure stimuli, with many cells responding to low and high pressures, but *Piezo2*^{CKO} cells were silent at low pressures. *Piezo2*^{CKO} DRG also had fewer bladder-stretch responding cells (Fig. 2f–m) but normal responses to painful pinch (Extended Data Fig. 1). This suggests that PIEZO2 is a key sensor of bladder stretch.

Mechanically-evoked micturition reflexes coordinate the bladder detrusor muscle and the urethral sphincter muscles to mediate efficient urinary control, and are critical for efficient voiding⁴. We hypothesized that reflex responses rely on PIEZO2 function to provide feedback control over bladder pressure and urethra activity. We therefore investigated micturition reflexes in mice lacking PIEZO2 in all caudal tissues. In these *Hoxb8*^{Cre};*Piezo2*^{f/f} animals, Cre recombinase is expressed in bladder-innervating DRG neurons¹⁴ and bladder urothelium (Extended Data Fig. 2a). We used cystometry with urethral EMG to simultaneously monitor bladder pressure and sphincter activity. With continual filling at 30 μ l/min, control animals initiated bladder contractions at regular intervals, which are observed as pressure peaks that correspond to micturition events (Fig. 3a, Extended Data Fig. 2b). *Piezo2* knockout animals displayed irregular micturition timing (Fig. 3b, Extended Data Fig. 2c) and on average, longer intervals between bladder contractions that resulted in urination (Fig. 3c, Extended Data Fig. 2h). Therefore, PIEZO2-deficient animals are less sensitive to bladder filling, as it takes more volume to initiate bladder contractions. Knockout animals also displayed higher bladder pressures five seconds before contraction peaks (Fig. 3d, Extended Data Fig. 2i). In healthy animals, low pressures are maintained during bladder filling because the detrusor muscle relaxes. The higher pressures that were observed before contractions suggest that this relaxation process or bladder compliance is impaired in knockout animals.

We next investigated individual micturition events to determine whether the bladder pressure required for sustaining micturition was abnormal. We observed consistent pressure increases within and between wildtype animals (Fig. 3e, Extended Data Fig. 2d), but bladder pressure traces in knockouts were highly variable (Fig. 3f, Extended Data Fig. 2e). Knockouts had

higher peak pressures (Fig. 3g), and importantly, required significantly more pressure during contractions, suggesting that the detrusor muscle must work harder to accomplish micturition (Fig. 3h, Extended Data Fig 2j). We also assessed whether sensory input via PIEZO2 is important for urethral reflexes, which sustain efficient urination. During bladder contractions in wildtype animals, there was coordinated engagement of the urethra muscle (Fig. 3i). This reliable urethral activity was greatly attenuated in knockouts (Fig. 3j, k, Extended Data Fig 2k). Knockout urethra responses varied from silent or weak coordination to inappropriately timed hyperactivity (Extended Data Fig. 2c). Hyperactivity is a sign of detrusor-sphincter dyssynergia, a condition involving uncoordinated communication between the muscle groups responsible for urination. This indicates that the urethra was not receiving the appropriate sensory input to govern its activity during micturition. Animals lacking PIEZO2 also had more variable, larger void volumes, as longer periods between contractions allows for more bladder filling (Fig. 3l). Together, these data indicate that PIEZO2 sets stretch sensitivity in the lower urinary tract and initiates appropriately timed reflexes that contribute to efficient urination.

Next, we tested if urination behavior is altered in *Piezo2* knockout animals. We placed animals on filter paper for four hours and imaged the resulting urination patterns with UV illumination. We used only female mice to preclude territorial scent-marking behavior. Wildtype mice typically urinated in the corners and edges of the cage in large spots (Fig 3m). Knockout mice had a variety of urination patterns, and some displayed urine leaking (small spots) or large voids towards the cage center (Fig. 3n,o). This phenotype was not attributed to knockouts spending more time in the middle of the cage (Fig. 3p). Thus, knockout mice have abnormal urination behavior, including what appears to be some incontinence.

We next asked if this observed urinary dysfunction in knockout animals led to long-term consequences. Chronic urinary tract dysfunction typically causes tissue remodeling as the bladder wall grows thicker to compensate for inefficient voiding^{20,21}. This remodeling can eventually result in “decompensation”, which is marked by a flaccid, ineffective bladder with sequelae of incomplete voiding, vesicoureteral reflux and increased UTI frequency. Bladder wall thickening was observed by H&E staining in *Piezo2*-deficient animals (Fig. 3q,r,s). The weight of freshly excised bladders also revealed bladder wall remodeling, as knockout bladders were significantly heavier than wildtype littermates (Fig. 3t, Extended Data Fig. 2m). Thus, impaired urinary reflexes in knockout mice lead to detrusor hypertrophy, an indicator of chronic voiding dysfunction.

We next investigated in which cell types PIEZO2 was required. We tested whether lacking PIEZO2 in urothelial cells changed the pressure threshold required to initiate micturition. We used UPKII^{Cre} allele to abrogate functional PIEZO2 in urothelial cells (Extended Data Fig. 3a), which have been proposed to act as stretch sensors and communicate to underlying neurons via ATP^{7,9,22–24}. We found that UPKII^{Cre+;Piezo2^{f/f}} knockout animals displayed similar phenotypes to the Hoxb8^{Cre+;Piezo2^{f/f}} knockout animals, with higher bladder stretch thresholds, greater bladder pressure during micturition and attenuated urethral reflexes (Fig. 4a–h). In combination with expression data from RNAscope (Fig. 2b), these data indicate that PIEZO2 is acting in umbrella cells to help set bladder stretch sensitivity and initiate

appropriate micturition reflexes. These data confirm the proposed role for umbrella cells as mechanosensory cells that participate in initiating micturition⁸.

Interestingly, we again observed these phenotypes in animals that lacked PIEZO2 only in sensory neurons (Fig 4i–p). Deleting PIEZO2 in all sensory neurons is lethal, so we used SNS^{Cre} mice²⁵ to delete PIEZO2 in the Aδ- and c-fiber subsets, which are the primary sensory neuron types described in the bladder^{10,26}. This line does not induce recombination in urothelial cells (Extended Data Fig 3b–e). Sensory neuron knockout animals displayed longer intervals between contractions (Fig. 4i), but interestingly, the pressure before contractions was not different from wildtypes as it was in urothelial and full caudal knockout animals (Fig. 4j). This implies that mechanosensory stimuli activate PIEZO2 in umbrella cells to initiate bladder relaxation during filling (Fig 4b), but neuronal mechanosensing is dispensable for this process. Alternatively, it is possible that bladders become fibrotic and less compliant in UPKII^{Cre} and HoxB8^{Cre} *Piezo2* knockouts, but not SNS^{Cre} *Piezo2* knockouts. Neuronal PIEZO2 knockout animals do require more bladder pressure for micturition and have highly attenuated urethral reflex responses (Fig. 4o,p). These data implicate PIEZO2 in mediating neuronal stretch responses that are critical for downstream urethral reflexes. Interestingly, individual tissue knockout animals did not display the dramatic bladder remodeling that was observed in full caudal knockout animals (Fig 3s,t, Extended Data Fig. 3f,g), suggesting that urothelial or neuronal PIEZO2 alone could still contribute to urinary function. These data indicate that there is a two-part signaling mechanism that utilizes PIEZO2 in umbrella cells and sensory neurons to set bladder sensitivity and promote micturition reflexes. Future work is required to address how these cell types communicate.

We have used evidence from mice and humans to determine that the mechanotransduction channel PIEZO2 is a critical mediator of urinary tract function. Nonetheless, lacking *Piezo2* in mice does not result in urinary tract paralysis and death, and *PIEZO2*-deficient humans are still able to urinate. This indicates that mechanotransduction proteins beyond PIEZO2 are present in the urothelium and LUT sensory neurons. For example, the mechanotransduction ion channels TMEM63b and PIEZO1 are widely expressed in the urothelium, and PIEZO1 partially mediates urothelial stretch responses *in vitro*²⁷.

Our data puts forth a two-part model of mechanosensory signaling in the urinary tract, which is reminiscent of epithelial cell-neuronal sensory machinery in the skin (Merkel cell-neurite complexes), lung (neuro-epithelial bodies) and intestine (enterochromaffin cells)^{15,17,28,29}. Our data also implicate umbrella cells in mediating bladder relaxation during filling, perhaps by signaling to bladder muscle and/or through stretch-induced cellular changes³⁰. Future studies will address how urothelial cells and sensory neurons collaborate to control urinary function.

Methods

All experiments were performed within the protocols and guidelines approved by the Institutional Animal Care and Use Committees of The Scripps Research Institute in

compliance with regulatory standards established by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Statistics

Unless otherwise noted in the legends, groups were compared using two-tailed Student's *t*-test with Welch's correction, as groups did not have equal variances. For comparisons of groups with an *N* less than 15, we used the non-parametric Mann-Whitney test to assess differences, as we could not assess distributions. These tests were indicated in the figure legends. No statistical test was used to pre-determine sample size. Instead, sample size was determined by animal availability and previous studies in the field, which found these sample sizes sufficient to detect deficits.

Study Design

We established exclusion criteria prior to collecting cystometry data: data from the first 30 minutes of cystometry recording was not used because bladder muscle activity has often not stabilized. Moreover, animals that displayed bladder leaking during recording were excluded from analysis, as leaking indicated a flawed seal and thus inaccurate filling responses. To verify the reproducibility of experimental findings, we restricted the time of day that cystometry recordings were done (Zeitgeber 8–14) and we performed every experiment in a cohort of male and female mice to compare to their wildtype littermates. The order of recordings for different genotypes was randomized. The experimenter was blind to genotype when possible. *HoxB8^{Cre+};Piezo2^{f/f}* knockout mice have obvious motor impairments, so the experimenter was never blind to genotype for these groups.

Mice

Mice were kept in standard housing with 12 h light/dark cycle set with lights on from 6 AM to 6 PM, with room temperature kept around 72 degrees Fahrenheit, with humidity between 30–80 % (not controlled). Adult male and female mice were used as indicated in the text. Age-matched knockout and wildtype littermates were tested at the same age in each cohort, but ages tested ranged from 5–8 months for *HoxB8^{Cre};Piezo^{f/f}*, 6–12 months for *SNS^{Cre}* and 4–6 months for *UPKII^{Cre}*. The *HoxB8^{Cre};Piezo^{f/f}* mouse line has been previously described¹¹. *GCaMP6f^{+/+}* mice (B6;129S-*Gt(ROSA)26Sor^{tm95.1(CAG-GCaMP6f)Hze/J}*, Jackson Laboratory: Ai95, #024105) were bred to *Piezo2^{f/f}* mice as described previously^{13,31}. *Piezo2^{f/f}* mice were mated with *SNS^{Cre}* mice²⁵ or *UPKII^{Cre}* mice (B6(129)-*Tg(Upk2-cre)1Rkl/WghJ*, Jackson Laboratory: #029281) to create sensory-neuron specific and urothelial specific *Piezo2* knockout animals, respectively. Each of these Cre lines was also crossed with Ai9 mice (B6.Cg-*Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J}*, Jackson Laboratory: # 07909) to assess Cre expression. Genotyping was performed using guidelines from Jackson Laboratory.

Retrograde labeling of sensory neurons

Mice were anesthetized with isoflurane. Their lower abdomen was de-haired using Nair (Fisher Scientific: NC0132811), and sterilized with ethanol and iodine, cleaned, and a midline laparotomy was performed to expose the bladder. Three to five injections of 1–2 μ l

of Cholera Toxin B–Alexa 488 (Fisher Scientific: C22841) were made in the bladder wall using a Hamilton syringe. Care was taken to avoid puncturing through to the bladder lumen. The abdominal wall and skin were sutured separately, and mice were given subcutaneous flunixin (0.1 ml / g bw) for post-operative care. We waited three to five days before taking tissue to allow the dye to reach the cell soma.

Fluorescent in situ hybridization

Bladder and DRG tissues were removed immediately from euthanized animals, and flash frozen in liquid nitrogen. The protocol for RNAscope Multiplex Fluorescent Reagent Kit V2 (ACDBio: 323100) was followed according to instructions for fresh frozen tissue. A gentler protease (III) was applied for only 25 min to lessen CTB-fluorophore degradation in DRG tissue and delicate bladder tissue. Probes for *Piezo2* (ACDBio: 400191 or 439971), *Krt20* (ACDBio: 402301), *Piezo1* (ACDBio:) or *Tmem63b* (ACDBio: 431531) were applied to detect transcript. Quantification of was performed in ImageJ using regions of interest to define quantification area and then by measuring mean pixel intensity. Only DRG cells with nuclei visible were quantified to prevent double-counting of the same cells in different sections. Multinucleated umbrella cell borders could not always be defined, so quantification was done per nucleus, with a set ROI size used for all nuclei (roughly 2X the diameter of the nucleus). ROIs were centered on each nucleus and used to measure mean pixel intensity. Control area intensity was measured from tissue background to define an intensity cutoff for negative cells.

DRG calcium imaging

Viral strategy is previously described¹³. Animals were anesthetized with isoflurane and the bladder was exposed (described above). The bladder apex was opened and phlanged PE20 tubing was filled with saline and inserted as a catheter (Stoelting: 51154) into the bladder, and tied off using silk suture (Fisher Scientific: NC9140103). A syringe and lines filled with saline that were connected to the catheter was used to fill the bladder and test for leaking. The abdomen was sewn shut with the catheter extending out. The animal was flipped to prone position, and the vertebral column was exposed. A microdrill was used to open a window in the bone above the first sacral DRG. Epifluorescence imaging was performed using an upright microscope (FVMPE-RS, Olympus) equipped with a 4X, 0.28–numerical aperture air objective. Illumination was provided with a 130-W halogen light source (U-HGLGPS, Olympus), using a standard green excitation/emission filter cube. Images were acquired using an ORCA-Flash 4.0 CMOS camera (Hamamatsu, Japan) at a frame rate of 5 Hz using MetaMorph (Molecular Devices). Analysis was previously described¹³.

Cystometry and electromyography

Male and female mice were anesthetized by isoflurane (5% induction, 1–2% maintenance, Kent Scientific SomnoSuite) and the bladder was catheterized and connected to saline lines (described above). Tungsten electrodes were inserted directly into the urethral muscle (A-M systems: 795500). Saline lines were connected to a pressure sensor (Biopac: RX104A-MRI) which connected via pressure transducer (TSD104A) to an MP160 Biopac system amplifier (DA100C). EMG electrodes were connected to a differential amplifier (EMG100C: gain = 1000, sample rate = 10kHz, low pass filter = 5kHz, 60Hz notch filter and 100Hz high pass

filter). Bladder was continuously filled at 20 $\mu\text{l}/\text{min}$ using a syringe pump until regular urination cycles began. Data was not collected until the mouse had been stably cycling (30–40 min after beginning of recording), at which point filling rate was increased to 30 $\mu\text{l}/\text{min}$. Data was logged with Acqknowledge software (version 4.4.2) and processed in MATLAB (version 2018b).

Behavioral assays

Female mice were placed in normal home cages that were bottomless, set on filter paper (Fisher Scientific: 05-714-4) and left in a darkened room for 4 hours. Mice did not have access to water to prevent the water leaks from disturbing urine marks. Paper was imaged using a widefield camera (Logitech C930e) while illuminated by UV light. Images were thresholded and converted to B&W binary in ImageJ (version 2.0.0-rc-49/1.51d), and total number of black pixels was counted. A region of interest corresponding to the middle 50% of the image area was used to count the number of black pixels in the middle of the cage.

Bladder Histology

Bladders used for histology were the same bladders used for the bladder weight measurements. Wildtype and knockout littermate male bladders were collected, opened and blotted on kimwipes before weighing. After recording their freshly excised weight, they were fixed in 10% Neutral Buffered Formalin for 24 hours, and then stored in 70% ethanol. Bladders were paraffin embedded, and central cross-sections were used for Hematoxylin & Eosin staining. Processing and staining was performed by Scripps Histology Core services. Muscle layer thickness was measured in ImageJ at 7–11 different places along the muscle wall per animal perpendicular to the muscle surface, and an average thickness value is shown per mouse.

Clinical assessment

Twelve patients with *PIEZO2* loss-of-function mutations from 11 families (N=4 males and 8 females, ranging in age from 4 to 43) were evaluated at the National Institutes of Health (NIH) under research protocol approved by the Institutional Review Boards of National Institute of Neurological Disorders and Stroke (NINDS, protocol 12-N-0095) between April of 2015 and May of 2020. Written informed consent and/or assent (for minor patients) was obtained from each participant in the study. All of the patients had biparentally inherited bi-allelic homozygous or compound heterozygous nonsense variants that are expected to result in a ‘null’ status for protein expression. Patients with *PIEZO2* loss-of-function either found us or were referred to our group through our network of international collaborators. Genotype information can be found in Extended Data Table 1, along with past treatments and diagnoses. One patient, P10, carried a nonsense and a deleterious splice site variant in compound heterozygosity. Also as stated above, all subjects presented with a profound congenital ubiquitous lack of proprioception, vibration, and specific loss of touch discrimination on glabrous skin. Detailed history, clinical evaluation and testing were conducted including an in-depth review of urinary function, urologic history, review of previous evaluations and non-invasive bladder ultrasound. Patients were recruited from all over the world and their age ranged between 5 to 43 years (see table). 4 adult patients (3 females and 1 male) provided their own history. None of the subjects were taking any

medications that could affect urinary function at the time of the questionnaire. Parents assisted with information gathering from their children.

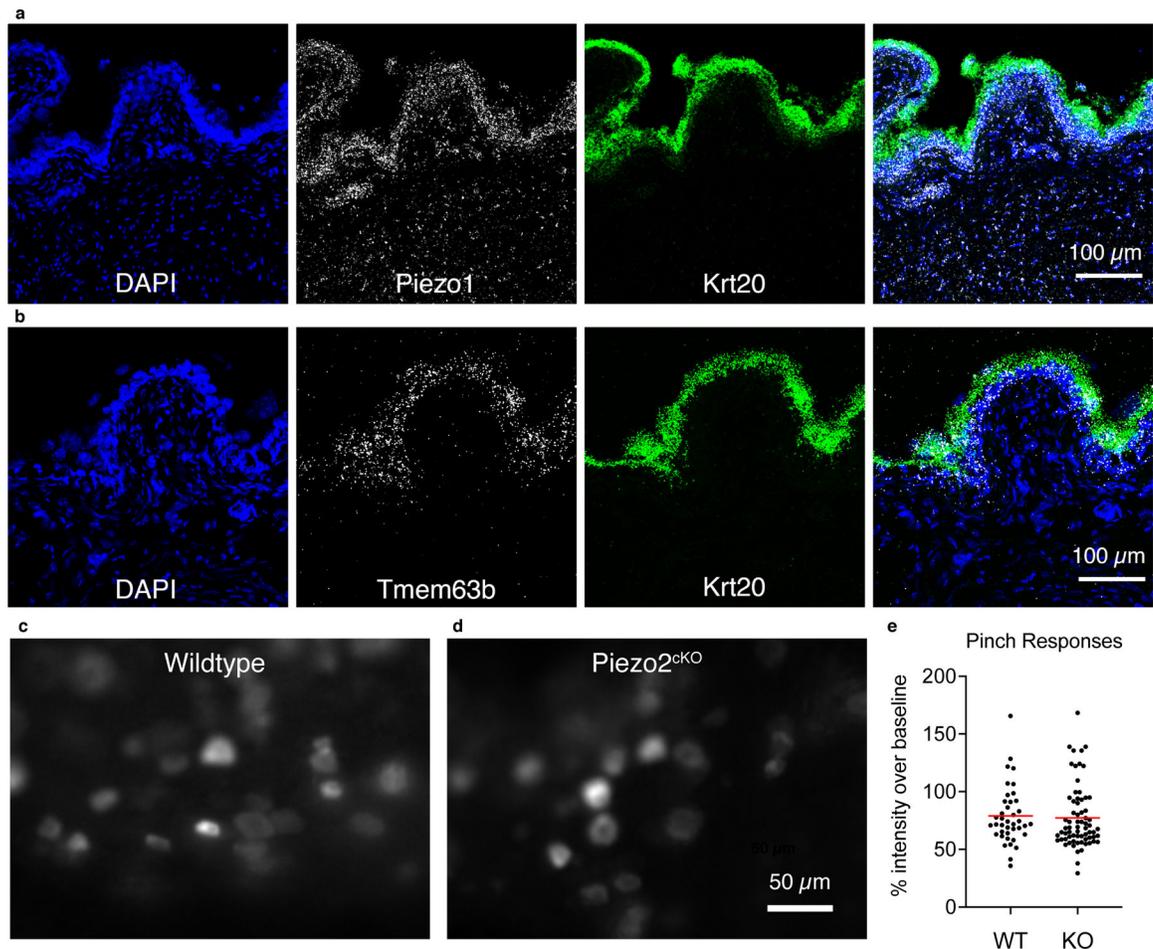
Data Availability

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Code Availability Statement

Code for calcium imaging analysis is previously published¹³. Matlab (version 2018b) code used for cystometry analysis is available at: <https://github.com/PatapoutianLab/cystometry>.

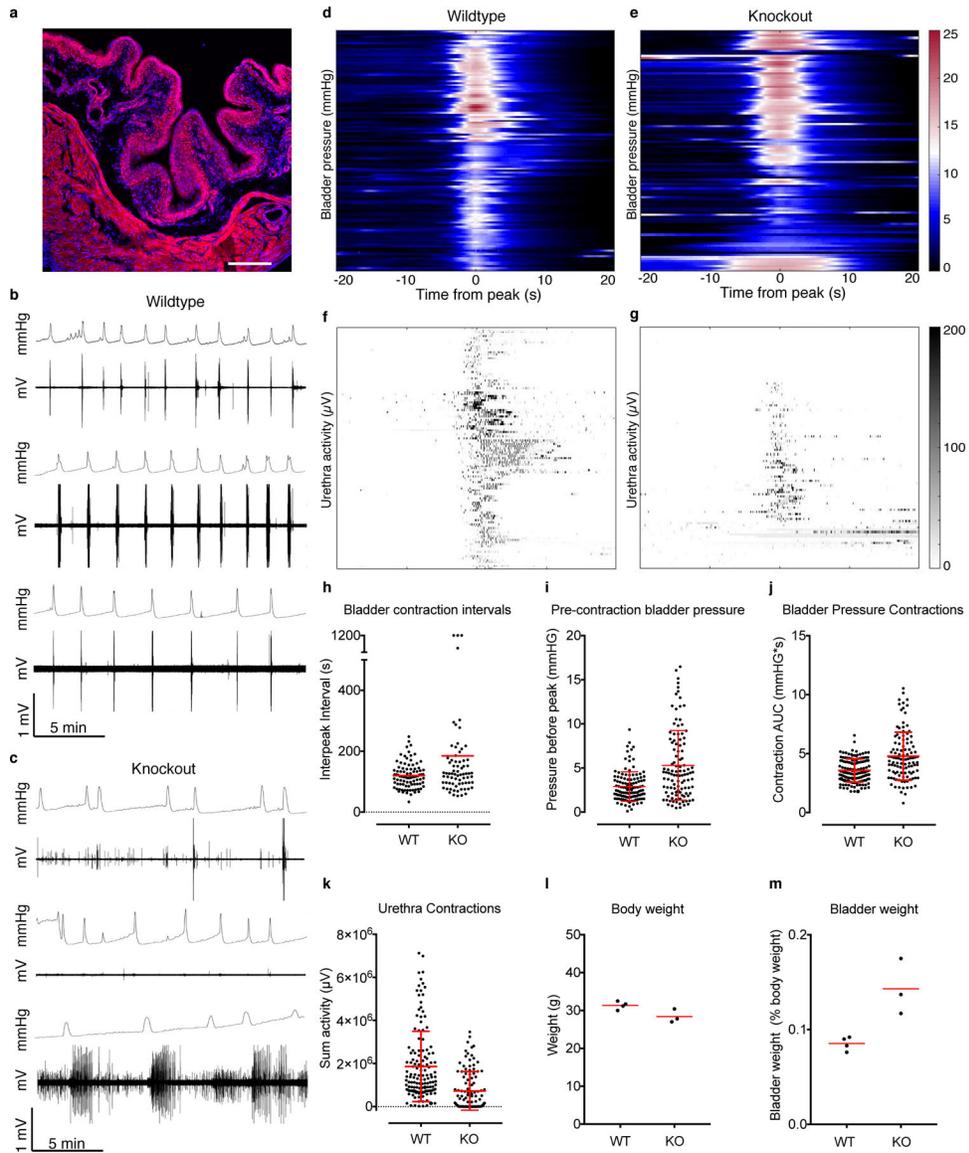
Extended Data



Extended Data Figure 1.

a, FISH in bladder tissue with probes against Krt20 (green) and Piezo1 (white). DAPI in blue. **b**, FISH in bladder tissue with probes against Krt20 (green) and Tmem63b (white). DAPI in blue. **c**, z-projection of the standard deviation of responses from genital pinch in WT and **d**, *Piezo2*^{CKO} DRG. **e**, quantification of peak responses during pinch shown as

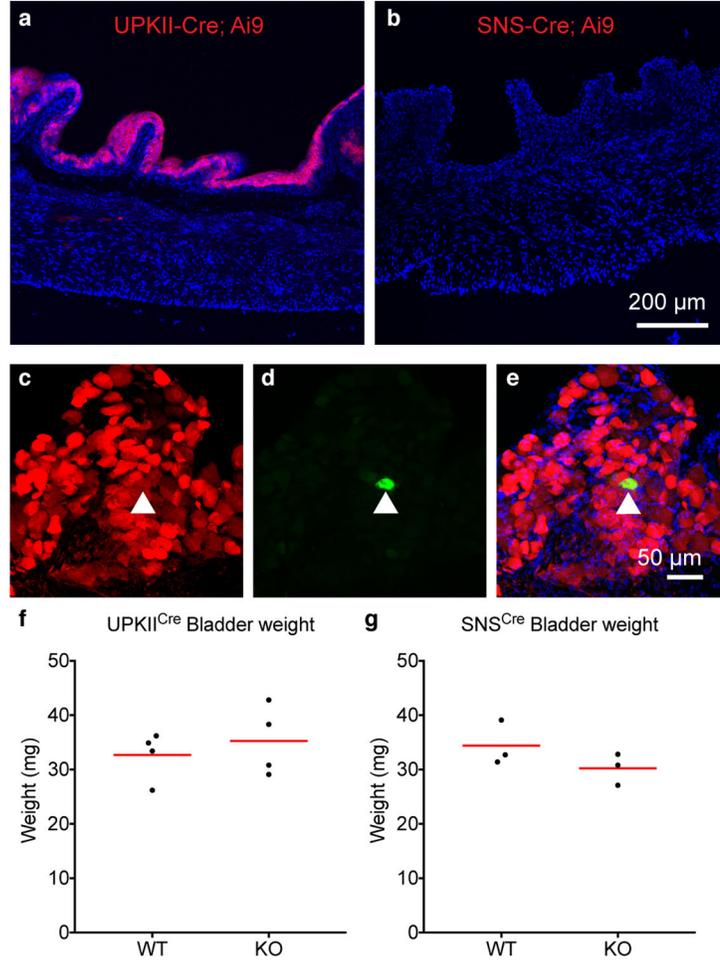
percent of baseline (each data point is one cell). N=3 DRGs, 40 cells for WT, 4 DRGs and 69 cells for *Piezo2^{Cre}* DRGs.



Extended Data Figure 2.

a, *HoxB8^{Cre};Ai9* bladder tissue, fixed, frozen and mounted to show tdTomato (red) throughout the tissue, labeled with DAPI (blue). Scale is 100 μm . Expression was evaluated in two mice. **b**, Example pressure and urethra activity traces from three wildtype males and **c**, three *HoxB8^{Cre};Piezo2^{f/f}* knockout male littermates. **d**, heatmap of individual bladder contraction events in wildtype and **e**, knockout male mice, with corresponding urethra activity below in **f** and **g** respectively. **h**, Bladder contraction intervals for males. **i**, bladder pressures five seconds before peak contraction for males. Note: 1200 s was the length of one recording. These dots represent recording periods where the animal had no successful urination events. **j**, Total bladder pressure for males and **k**, sum of urethra activity during

bladder contractions. $N = 6$ males per group. $P < 0.0001$ for graphs in **h**, **i**, **j** and **k**, two-sided Student's t test with Welch's correction. **l**, body weights from a subset of mice whose bladder weights are shown in Figure 2t, and **m**, bladder weights from animals in **l**, shown as a percentage of body weight. Red horizontal lines indicate means, vertical red bars indicate \pm standard deviation (shown where possible).



Extended Data Figure 3.

a, UPKII^{Cre}; Ai9 bladder tissue fixed, frozen and mounted to show tdTomato (red) throughout the urothelium, labeled with DAPI (blue). Expression was evaluated in two mice. **b**, SNS^{Cre}; Ai9 bladder tissue fixed, frozen and mounted to show tdTomato (red) is not present. Expression was evaluated in two mice. Thin cryosections made neuronal endings difficult to visualize. Scale: 200 μ m, applies to **a** and **b**. **c**, SNS^{Cre}; Ai9 DRG tissue showing tdTomato (red) in the majority of neurons, and **d**, a cell backlabeled with CTB-Alexa 488 injected into bladder. **e**, merge of **c** and **d**, DAPI in blue. 9/9 backlabeled bladder cells analyzed from two mice were tdTomato positive. **f**, Quantification of freshly excised bladder weights from four UPKII^{Cre}; Piezo2^{f/f} knockout and wildtype littermates. Age-matched littermates were 10–11 months old, which could account for greater variability. **g**, Bladder weights from age-matched SNS^{Cre}; Piezo2^{f/f} knockout mice and wildtype littermates, 7–8 months old. Red lines indicate mean values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Patient	1	2	3	5	7	9	10	12
Age	23	13	16	18	10	9	5	36
Sex	F	F	M	M	F	F	F	F
In a normal day I go to the washroom to pee (# times):	*	3—4	1—2	1—2	1—2	3—4	3—4	5—6 ^
I pee in my underwear during the day:				1 day/wk			1 day/wk	
When I pee in my underwear, they are:				damp			damp	
I feel that I have to rush to the washroom to pee:								
I hold my pee by crossing my legs or sitting down:								
It hurts when I pee:								
I wet my bed at night:								
I wake up to pee at night:	3-4 nights /month							
When I pee, it stops and starts:								
I have to push or wait for my pee to start:								

Figure 1. Human *PIEZO2*-deficient subjects have urinary dysfunction.

Patient numbers correspond to those in Supplementary Table 1. Grey indicates a neutral or non-pathological answer. Urinary frequency information is scored differently than other questions, and is color coded by the pathological score assigned to the answer in the questionnaire. Asterisk indicates an unanswered question. Unless otherwise noted, answers follow the scale: grey: never (no pathology), blue: less than half of the time (pathology score of 1), yellow: half of the time (pathology score of 2), orange: more than half of the time (pathology score of 3), red: every day or every night if nighttime is indicated in question (pathology score of 4). *Question not answered. ^Subject answered 2X per day during clinical interview.

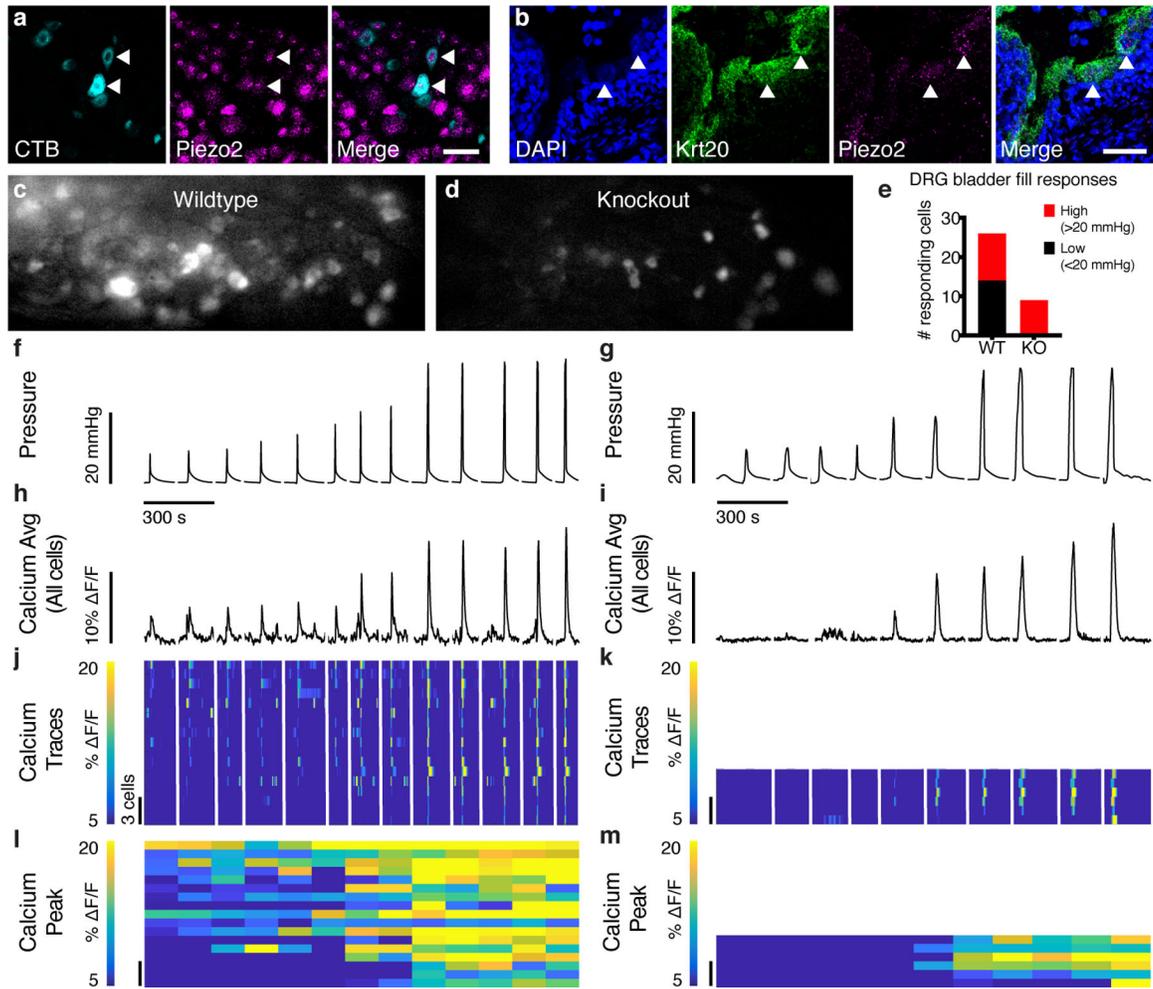


Figure 2. The lower urinary tract expresses *Piezo2*, and sensory neurons require *PIEZO2* for detecting low-pressure bladder filling.

a, DRG neurons were retrogradely labeled using CTB-488. (cyan, left) fluorescent in situ hybridization (FISH) of DRGs with probes targeting *Piezo2* (magenta, middle). Arrowheads point to *Piezo2*⁺ bladder neurons. Scale: 50 μ m. Tracing experiment was repeated using three mice, N=22–36 cells analyzed per animal. **b**, FISH of bladder, probes against *Krt20* (green) and *Piezo2* (magenta). DAPI in blue. Arrowheads point to *Piezo2*⁺ umbrella cell examples. Scale: 50 μ m. RNAscope was performed on three bladders, with two technical replications. Analysis was performed on 80–117 nuclei per bladder. **c**, Image z-stack from GCaMP6f^{+/+} control mouse S1 DRG during bladder fill. **d**, Example image from *Piezo2*^{CKO} mouse S1 DRG during bladder fill. **e**, Count of cells responding to low- (black) and high-pressure (red) stimuli in WT (N=3 animals) and KO (N=4 animals) DRG. **f**, Example pressure trace from a wildtype DRG and **g**, knockout DRG. Stimuli were interleaved during recording, but are shown sorted low to high, hence the discontinuous line. Data below these graphs is sorted together with the respective pressure peaks. **h**, **i**, Average percent change in calcium fluorescence for all responding cells during the pressure peaks shown above in **f** and **g**, respectively. **j**, Calcium traces for individual wildtype cells that responded to pressure stimuli in **f** (N=17). Scale for cell number applies to **j-m**. **k**, Calcium traces from all

knockout cells responding to pressure stimuli shown in **g** (N=6). Each cell's responses are shown on the same horizontal line. Cells are sorted by cumulative response to the four lowest-pressure stimuli. **l, m**, Maximum calcium response for the cells shown above, 1 s after pressure peak.

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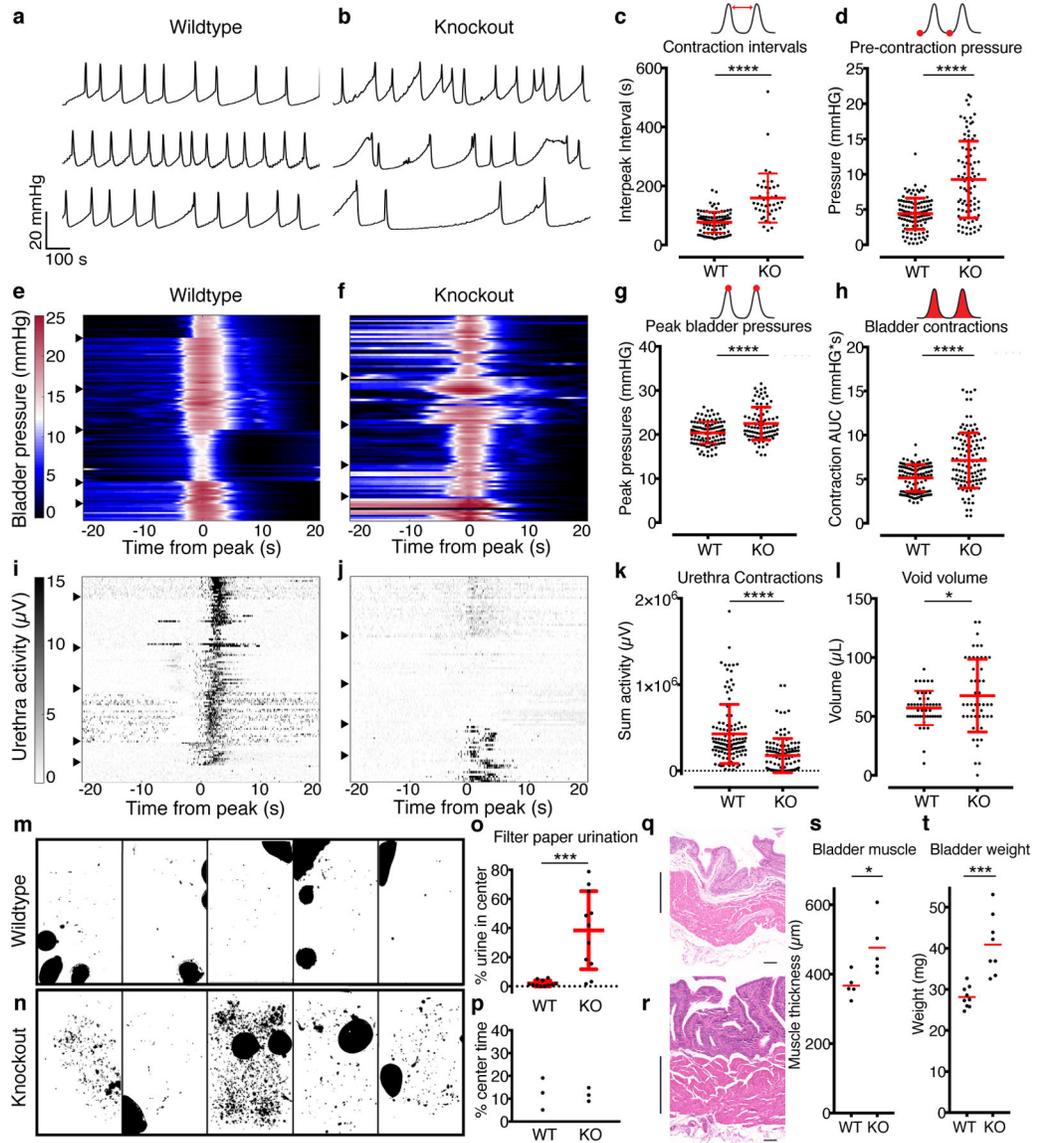


Figure 3. PIEZO2 is required for efficient micturition reflexes.

a, Example pressure traces from three female WT mice and **b**, three female *HoxB8^{Cre};Piezo2^{f/f}* KO mice during continuous bladder filling. Scale in **a** also applies to **b**. **c**, Bladder contraction intervals ($P < 0.0001$). **d**, bladder pressure five seconds before contraction peaks ($P < 0.0001$). **e**, Heatmaps showing bladder contractions for six WT female mice and **f**, five *HoxB8^{Cre};Piezo2^{f/f}* female KO mice. Color scale in **e** also applies to **f**. Each row represents bladder pressure during a single micturition event, with peaks aligned at 0. Arrows mark where data from one animal ends and another begins. **g**, Peak bladder pressures ($P < 0.0001$) and **h**, area under the curve for bladder contractions ($P < 0.0001$). **i**, Heatmap showing urethra activity, with each row from corresponding to bladder contraction events in **e**. **j**, Urethra activity from the *HoxB8^{Cre};Piezo2^{f/f}* KO bladder contraction events shown in **f**. Scale in **i** also applies to **j**. **k**, Urethra activity during micturition ($P < 0.0001$). **l**, Void volume measurements ($P = 0.03$). P values from Student's t-tests with Welch's

correction. N=6 WT and N=5 KO female mice for **c** through **l**. N=10–29 bladder contractions analyzed per mouse. **m**, Urination patterns of five WT mice and **n**, five HoxB8^{Cre};Piezo2^{f/f} KO mice. **o**, Quantification of urine in the middle 50% of the cage (P=0.0001). N=11 female mice per group. **p**, Mice spend the same amount of time in cage center. **q**, H&E staining from WT and **r**, KO bladder sections, from littermates, 6–7 months, scale 100 µm. Muscle layer is marked by vertical lines. Scale for q and r is 200 µm. **s**, Bladder muscle wall thickness (N=5, P=0.016) and **t**, total bladder weights (N=9 and 8, P=0.0002). For **o**, **s** and **t**, Mann-Whitney test, all others are two-sided Student's t-tests with Welch's correction. Red lines indicate mean \pm standard deviation.

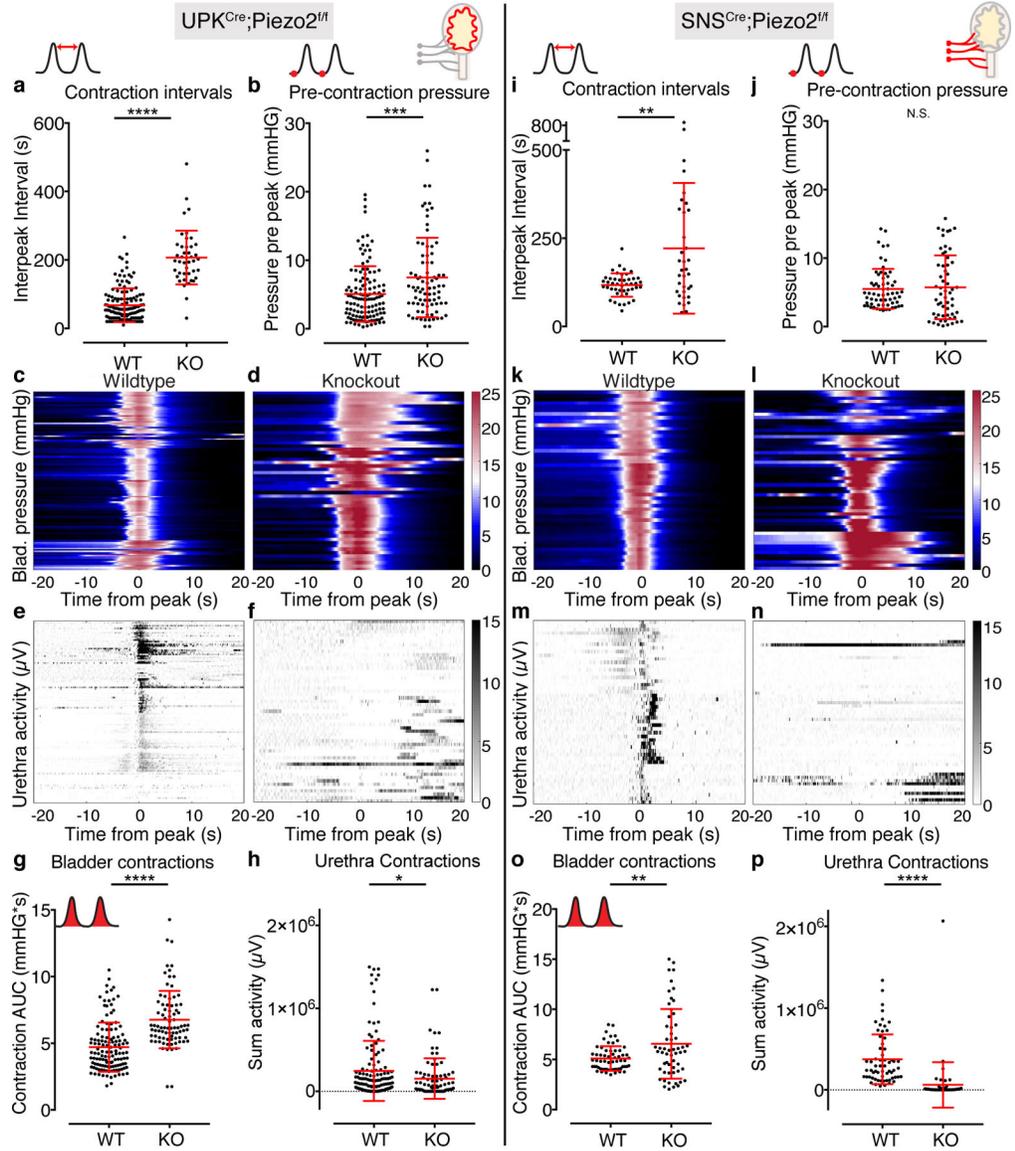


Figure 4. PIEZO2 functions in both bladder urothelium and sensory neurons.

a-h, Cystometry data from UPKII^{Cre};Piezo2^{f/f} animals, N=5 WT and 4 KO female mice, N=18–49 bladder contractions analyzed per mouse. and **i-p**, SNS^{Cre};Piezo2^{f/f} animals, N=3 WT and 3 KO female mice, 11–24 contractions per mouse. Cartoons in the top right depict the lower urinary tract, with *Piezo2* KO tissue in red. **a**, Intervals between bladder contraction voids ($P < 0.0001$) and **b**, bladder pressures five seconds before peak contraction ($P = 0.001$) in UPKII^{Cre};Piezo2^{f/f} knockout mice and wildtype littermates. **c**, Bladder pressure events during continuous filling cystometry in wildtype and **d**, UPKII^{Cre};Piezo2^{f/f} knockout mice. Color scale in **d** also applies to **c**. **e**, Urethra activity recorded during the bladder contraction events shown in **c**. **f**, Urothelial Piezo2 KO urethra activity during bladder contraction events shown in **d**. **g**, Bladder pressure during micturition events ($P < 0.0001$) and **h**, urethral reflex responses during micturition ($P = 0.03$). **i**, Intervals between bladder contractions in SNS^{Cre};Piezo2^{f/f} and wildtype littermates ($P = 0.002$). **j**, Bladder pressures

five seconds before peak contraction. **k**, Bladder pressure events during continuous filling cystometry in wildtype and **l**, SNS^{Cre};Piezo2^{f/f} knockout mice. Color scale in **l** also apply to **k**. **m**, Urethra activity recorded during the bladder contraction events shown in **k**. **n**, Urethra activity during bladder contraction events shown in **l**. **o**, Bladder pressure during micturition events (P=0.004) and **p**, urethral reflex responses during micturition (P<0.0001). Red horizontal lines indicate means, vertical red bars indicate +/- standard deviation. All P values are from two-sided Student's *t* test with Welch's correction. * is P 0.05, ** is P 0.01, *** is P 0.001, **** is P 0.0001.

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