Whole-cell patch-clamp recording

DRG neurons were bathed in extracellular solution containing, in mM: 128 NaCl, 5 $MgCl_2$, 1.8 $CaCl_2$, 5.4 KCl, 5.6 glucose, 20 HEPES, pH 7.4. Acidic solutions were buffered by 10 mM MES and 10 mM HEPES. Pipettes contained, in mM: 100 KCl, 30 NaCl, 2 $MgCl_2$, 10 EGTA, 20 HEPES and 1 ATP. All cells exhibited depolarization-activated currents. Methods to assess pH-gated currents in small diameter neurons and membrane properties of isolated mechanoreceptors were as described[30]. Under current-clamp conditions action potentials were evoked with current injection. Neurons with broad action potentials displaying a hump on the falling phase (a marker of nociceptor type neurons) were tested for pH 5-induced currents. The amount of current required to initiate action potentials in large diameter neurons with narrow spikes was also measured. Cells were used from at least four mice per genotype in each experimental series.

Single fibre recording

We used an *in vitro* skin/nerve preparation to record from functionally single primary afferents in micro-dissected teased filaments of the saphenous nerve as described[16,17]. A standard ascending series of displacement stimuli were applied to the receptive field at 30-s intervals. Each displacement was maintained for 10 s, and neurons that maintained a discharge throughout the stimulus were characterized as SA mechanoreceptors. Those neurons that responded only at the beginning and end of the 10-s stimuli were classified as RA mechanoreceptors. Each stimulus–response function started at threshold as the probe was adjusted so that the first 5-μm displacement evoked spikes.

A group of C-fibre nociceptive neurons (axonal conduction velocity $<1.0\ m\ s^{-1}$) was characterized with the same stimulus series ($n = 46$). C-fibres were not divided into C-MH and C-M fibres, as these two groups have essentially identical mechanosensitivity[16]. A separate series of experiments were carried out to characterize the heat and pH sensitivity of nociceptive C-fibres (80 C-fibres tested)[22]. A small metal ring isolated the receptive field and a heated Ringer solution was rapidly applied. The measured intradermal temperature increased to over 49 °C for about 3 s; this stimulus activates all heat-sensitive nociceptors. One min after the heat stimulus, the solution was replaced with oxygenated Ringer solution with pH 5.0 for exactly 2 min. Raw electrophysiological data were collected with a Powerlab 4.0 system (AD Instruments) and spikes discriminated off line with the spike histogram extension of the software.

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Urinary bladder hyporeflexia and reduced pain-related behaviour in $P2X_3$-deficient mice

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Extracellular ATP is implicated in numerous sensory processes ranging from the response to pain to the regulation of motility in visceral organs[1]. The ATP receptor $P2X_3$ is selectively expressed on small diameter sensory neurons[2–4], supporting this hypothesis. Here we show that mice deficient in $P2X_3$ lose the rapidly desensitizing ATP-induced currents in dorsal root ganglion neurons. $P2X_3$ deficiency also causes a reduction in the sustained ATP-induced currents in nodose ganglion neurons. $P2X_3$-null mice have reduced pain-related behaviour in response to injection of ATP and formalin. Significantly, $P2X_3$-null mice exhibit a marked urinary bladder hyporeflexia, characterized by decreased voiding frequency and increased bladder capacity, but normal bladder pressures. Immunohistochemical studies localize $P2X_3$ to nerve fibres innervating the urinary bladder of wild-type mice, and show that loss of $P2X_3$ does not alter sensory neuron innervation density. Thus, $P2X_3$ is critical for peripheral pain responses and afferent pathways controlling urinary bladder volume reflexes. Antagonists to $P2X_3$ may therefore have therapeutic potential in the treatment of disorders of urine storage and voiding such as overactive bladder.

The ion-channel subunit $P2X_3$ is one of seven known subunits that form homomeric and heteromeric receptors for ATP[5]. Uniquely, $P2X_3$ is expressed by a subgroup of small sensory neurons of the dorsal root and cranial ganglia[2–4]. ATP and α,β-Me-ATP (a

$P2X_{1,3}$-selective analogue) excite many small calibre afferent neurons from skin, joints and viscera[6]. Studies on ATP release[6,7] and P2 receptor antagonism[8,9] support the involvement of P2X receptors in nociception and the micturition reflex. To investigate the role of $P2X_3$ in sensory processing, we generated $P2X_3$-deficient mice carrying a 1-kb deletion of the $P2X_3$ gene encompassing exon 1 and the initiating codon ATG (Fig. 1a, b).

Normally, $P2X_3$ is selectively expressed by small sensory neurons marked by the lectin IB4 (refs 4, 10). In $P2X_3^{-/-}$ mice, $P2X_3$ immunoreactivity is undetectable in dorsal root ganglion (DRG), spinal cord and peripheral tissues (Fig. 1d, f, h, j). However, staining for IB4 in DRG and spinal cord (Fig. 1e–h), and protein gene product (PGP) 9.5 pan-neuronal staining in the skin (Fig. 1i, j) appears to be unaltered in $P2X_3^{-/-}$ mice. Thus, loss of $P2X_3$ does not appear to affect either peripheral or central innervation patterns. Immunostaining for P2X receptor subunits $P2X_2$, $P2X_5$ and $P2X_6$ was also qualitatively unchanged in DRG, trigeminal and nodose ganglia of $P2X_3^{-/-}$ mice ($P2X_2$ in DRG, Fig. 1c, d; and data not shown), indicating no compensatory changes in other P2X receptor subunits. $P2X_1$, $P2X_4$ and $P2X_7$ receptors were not detected in mouse sensory ganglia.

We analysed dissociated DRG and nodose ganglion neurons using electrophysiology (Fig. 2a–d). In $P2X_3^{+/+}$ DRG, 75% (27/38) and 60% (18/30) of neurons tested responded to ATP and α,β-Me-ATP, respectively. ATP-responsive DRG neurons showed either a rapidly desensitizing inward current (18/27, averaging 0.4 ± 0.08 nA, Fig. 2a), or a slowly desensitizing response (9/27, averaging 0.4 ± 0.22 nA, data not shown). No $P2X_3^{-/-}$ DRG neurons (0/34) responded to either ATP or α,β-Me-ATP with rapidly desensitizing currents (Fig. 2b). The proportion of DRG neurons with slowly desensitizing responses to ATP (4/34,12%) was not significantly different ($P > 0.05$) from that observed in wild-type mice, nor was the proportion of cells responding to GABA (γ-aminobutyric acid) or capsaicin (data not shown). Thus, $P2X_3$ homomers appear to be principally responsible for rapidly desensitizing ATP-activated currents in DRG. However, $P2X_2$ homomers and $P2X_{2/3}$ heteromers may function in a minority of DRG neurons[11–15]. In contrast, in $P2X_3$ wild-type mice, >90% (34/37) of nodose ganglion neurons responded to ATP and α,β-Me-ATP with slowly desensitizing persistent responses (Fig. 2c). In null-mutant mice, the proportion of nodose neurons responding to ATP (43/51, 84%) was similar ($P > 0.1$). However, the mean amplitude of the response (3.2 ± 0.6 nA for 100 μM ATP, $n = 26$) was significantly less ($P < 0.05$) than that observed for $P2X_3^{+/+}$ neurons

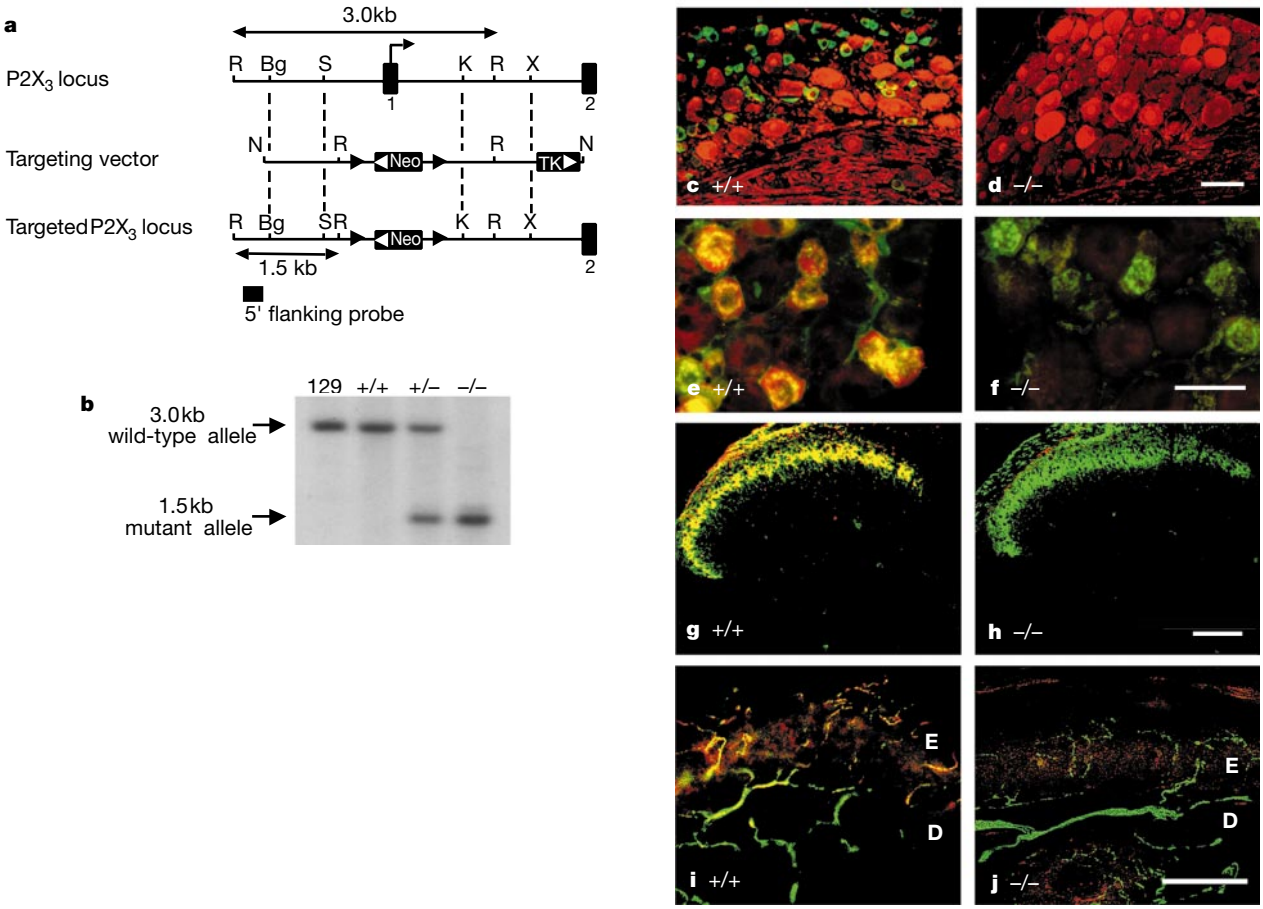

Figure 1 Targeted disruption of the $P2X_3$ gene and immunolocalization studies. **a**, Gene targeting strategy. R, *Eco*R1; Bg, *Bgl* I; S, *Sac* I; K, *Kpn* I; X, *Xho* I; N, *Not* I; Tk, thymidine kinase. **b**, Southern blot of $P2X_3^{+/+}$, $P2X_3^{+/-}$ and $P2X_3^{-/-}$ mice using a 5′-flanking region probe shown in **a**. **c**–**j**, Colocalization of $P2X_3$ with neuronal markers in DRG, spinal cord and skin of $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice. **c**, **d**, Transverse sections (10 μm) of L5 DRG immunostained for $P2X_3$ (green) and $P2X_2$ (red). In $P2X_3^{+/+}$ DRG, $P2X_3$ and $P2X_2$ immunoreactivities are present in small–medium and medium–large cells, respectively. $P2X_2$ staining appears unaltered in $P2X_3^{-/-}$ DRG. **e**–**h**, Transverse sections of L5 DRG (15 μm; **e**, **f**) and lumbar spinal cord (20 μm; **g**, **h**) immunostained for $P2X_3$ (red) and IB4 lectin binding (green). In $P2X_3^{+/+}$ DRG, nearly all $P2X_3$-immunoreactive cells bind IB4 (colocalization, yellow) and IB4 staining appears unaltered in $P2X_3^{-/-}$ DRG. In $P2X_3^{+/+}$ spinal cord, $P2X_3$ and IB4 staining terminals are co-localized (yellow) in inner lamina II of the dorsal horn, with apparently normal distribution of IB4 staining in $P2X_3^{-/-}$ spinal cord. **i**, **j**, Sections (15 μm) of hindpaw plantar skin immunostained for $P2X_3$ (red) and the pan-neuronal marker PGP 9.5 (green). In $P2X_3^{+/+}$ skin, $P2X_3$ and PGP 9.5 immunoreactivities are colocalized (yellow) in some fine epidermal (E) fibres, and to a lesser extent in nerve bundles in the dermis (D). Epidermal innervation is still evident by PGP 9.5 immunoreactivity in $P2X_3^{-/-}$ mice. Scale bars: **c**–**f**, **i**, **j**, 25 μm; **g**, **h**, 75 μm.

(5.2 ± 0.5 nA for 100 μM ATP, $n = 31$) (Fig. 2d). None of the $P2X_3^{-/-}$ nodose neurons tested (0/12) responded to α,β-Me-ATP (Fig. 2d). Together with previous evidence[3,16], these data suggest that nodose ganglion neurons contain significant proportions of homomeric $P2X_2$ and heteromeric $P2X_{2/3}$ channels.

We next examined sensory deficits in $P2X_3$ wild-type and null-mutant mice. No differences were observed in locomotor activity and rotorod performance (data not shown). Injection of ATP into the hindpaw evoked a nociceptive behavioural response (intermittent hindpaw lifting, licking and biting, as in the rat[17]) in $P2X_3$ wild-type mice that was dose dependent (Fig. 2e). In $P2X_3$ null-mutant mice (Fig. 2e), responses were significantly decreased by 77% and 45% to 100 and 500 nmol of ATP, respectively. Altered pain responses were specific to ATP and not seen with intraplantar injections of 30 μg capsaicin (data not shown). Moreover, the non-selective P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) further reduced the residual hindpaw lifting behaviour of $P2X_3^{-/-}$ mice by about 50% (Fig. 2e). These data suggest that the pain-producing effects of peripheral ATP in humans[18,19] and animals[17,20] are mainly mediated by $P2X_3$ subunits. However, some of this response appears to be derived from other P2 receptors. Responses to noxious thermal and mechanical stimuli were similar in $P2X_3$ wild-type and null-mutant mice (data not shown). In contrast, pain-related behaviour was significantly attenuated in both phases of the formalin test (~50%) in null-mutant mice (Fig. 2f), consistent with previous work[8].

We also investigated the role of $P2X_3$ in urinary bladder sensory function. Bladder afferent activity during filling drives micturition contractions mediated by the central nervous system (CNS)[21,22]. Therefore, we monitored these reflex contractions in $P2X_3$ wild-type and null-mutant mice using two different urodynamic methods. Figure 3a shows representative cystometrograms from conscious mouse cystometry studies in which voiding reflexes were measured in response to a continuous intravesical infusion of saline. $P2X_3$-null mice had significantly decreased micturition frequencies (mean void intervals 9.0 ± 0.8 min versus 5.3 ± 0.3 min in $P2X_3^{+/+}$ mice) (Fig. 3b, left), and significantly increased bladder capacities (mean void volumes 0.41 ± 0.04 ml versus 0.23 ± 0.02 ml in $P2X_3^{+/+}$ mice) (Fig. 3b, middle), but showed no differences in bladder pressures recorded at baseline, micturition threshold (not shown) and micturition peak (Fig. 3b, right). Male and female mice showed similar urodynamic changes. Additionally, cystometric differences are not attributable to influence from the 129Sv background (Fig. 3b).

Acute cystometry carried out under anaesthesia also showed micturition hyporeflexia in $P2X_3$-null mice. Bladder contractions measured in response to distension with a fixed infusion rate of saline (Fig. 3c) resulted in frequent micturition contractions in $P2X_3^{+/+}$ mice, but virtually no contractions in $P2X_3^{-/-}$ mice, up to the cut-off volume. The average number of contractions per cystometrogram was significantly reduced in $P2X_3^{-/-}$ mice (0.6 ± 0.38 compared with 4.9 ± 2.37 in $P2X_3^{+/+}$ mice) (Fig. 3d). Accordingly, the micturition threshold was significantly increased (0.29 ± 0.01 compared with 0.21 ± 0.02 ml in $P2X_3^{+/+}$, $P < 0.05$), and only 23% of $P2X_3^{-/-}$ mice reached the micturition threshold compared with 75% of $P2X_3^{+/+}$ mice. No differences were observed in the intravesical pressure at a volume of 0.3 ml (data not shown).

Finally, we detected $P2X_3$ immunoreactivity on sensory neurons innervating the suburothelial nerve plexus of wild-type mouse

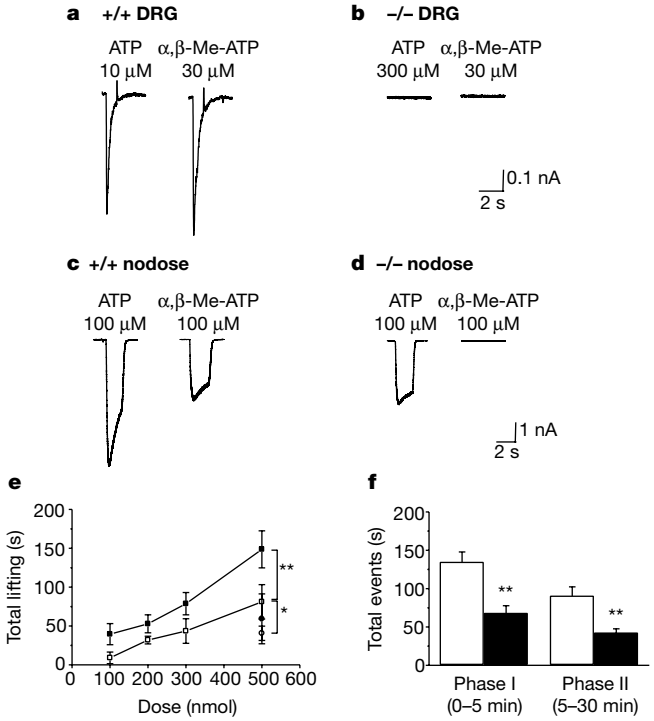

Figure 2 Responses to nucleotide agonists and nociceptive behaviour in $P2X_3$-deficient mice. **a**–**d**, Whole-cell patch-clamp recordings of DRG and nodose ganglion neurons at a holding potential of –60 mV. **a**, $P2X_3^{+/+}$ DRG neurons show rapidly desensitizing inward currents in response to 10 μM ATP and 30 μM α,β-Me-ATP. **b**, $P2X_3^{-/-}$ DRG neurons failed to produce a transient response to either 300 μM ATP or 30 μM α,β-Me-ATP. **c**, $P2X_3^{+/+}$ nodose ganglion neurons show slowly desensitizing inward currents in response to 100 μM ATP and α,β-Me-ATP. **d**, $P2X_3^{-/-}$ nodose ganglion neurons responded to 100 μM ATP, but not to 100 μM α,β-Me-ATP. **e**, ATP-evoked behavioural responses. Hindpaw-lifting responses in 2–3-month-old male $P2X_3^{+/+}$ (filled square) and $P2X_3^{-/-}$ (open square) mice ($n = 10$ to 15) following intraplantar injection of varying doses of ATP in a total volume of 20 μl. Double asterisk, $P < 0.01$ for $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice; analysis of variance. The response to 500 nmol ATP was also measured 20 min following pre-treatment with a 200 mg per kg body weight intraperitoneal injection of PPADS in $P2X_3^{+/+}$ (filled circle) and $P2X_3^{-/-}$ (open circle) mice ($n = 10$ to 15). Asterisk, $P < 0.05$ for $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice; Student's *t*-test (significance shown only for $P2X_3^{-/-}$). **f**, Formalin-induced behavioural responses. Hindpaw-lifting and licking responses in 3–4-month-old male $P2X_3^{+/+}$ (open bars) and $P2X_3^{-/-}$ (filled bars) mice ($n = 10$) following intraplantar injection of 5% formalin in a total volume of 20 μl. Double asterisk, $P < 0.01$ for $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice; Student's *t*-test.

bladder (Fig. 4c), both on small nerve fibres with terminals embedded in the urothelium, as well as on nerve bundles. Bladders from $P2X_3$-null mice showed no $P2X_3$ immunoreactivity (Fig. 4d), but were otherwise histologically normal (Fig. 4a, b), and showed no alterations in sensory innervation patterns as measured by capsaicin receptor (VR-1) immunoreactivity (Fig. 4e, f).

Our findings demonstrate the importance of $P2X_3$ receptors in somatic and visceral sensory function. First, we show that much of the DRG response to ATP is mediated by homomeric $P2X_3$ receptors, while in nodose ganglion neurons homomeric $P2X_2$ and

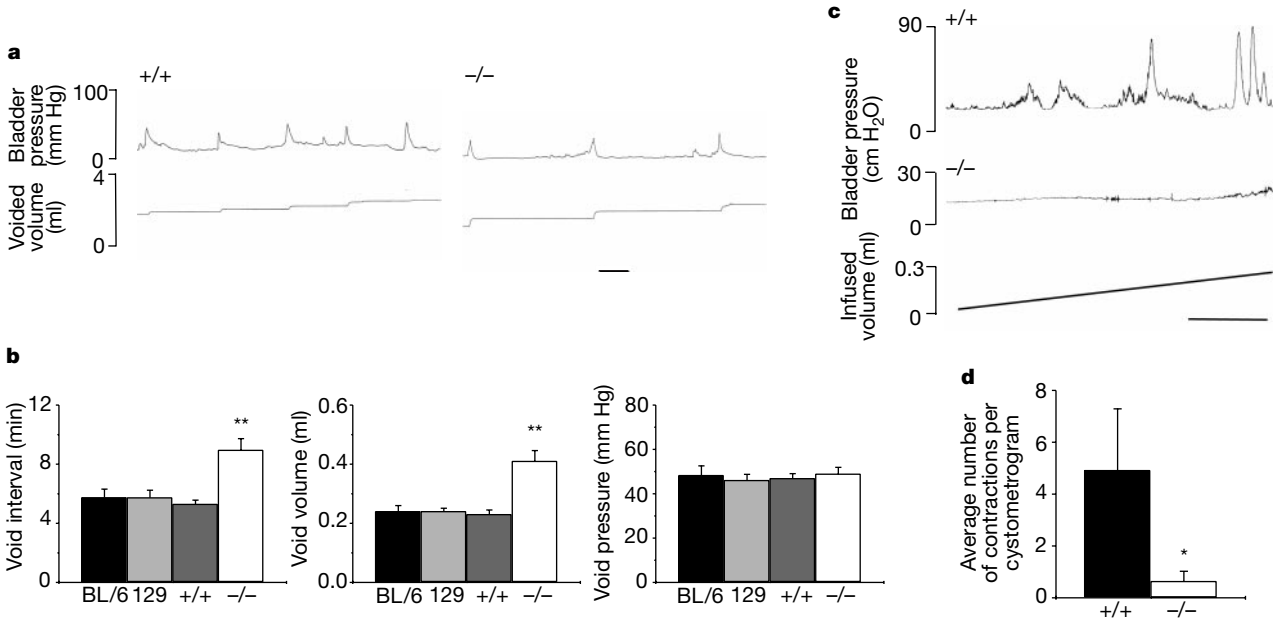

Figure 3 Bladder cystometry in $P2X_3$-deficient mice. **a**, Representative cystometrograms from conscious 5–6-month-old $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice. Traces illustrate bladder pressure recorded in response to a constant intravesical infusion of saline (50 μl min^{-1}), and accumulated void volumes recorded from each micturition (scale bar, 2 min). **b**, Void intervals, void volumes and void pressures were quantified for C57BL/6 (n = 7), 129Sv (n = 6), $P2X_3^{+/+}$ (n = 4 males and 4 females) and $P2X_3^{-/-}$ (n = 4 males and 4 females) mice. $P2X_3^{-/-}$ mice had significantly decreased micturition frequencies (increased void interval) and significantly increased bladder capacities (increased void volume), but no differences in bladder pressures. Double asterisk, $P < 0.01$ for $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice; analysis of variance. **c**, Representative acute cystometrograms recorded from anaesthetized and transurethrally catheterized 5–6-month-old $P2X_3^{+/+}$ and $P2X_3^{-/-}$ mice. Each cystometrogram consisted of intravesical infusion of saline (20 μl min^{-1} for 15 min) (scale bar, 2 min). Contractions greater than 10 cm H_20 were taken as micturition contractions. **d**, Quantification of the average number of contractions per cystometrogram for $P2X_3^{+/+}$ (n = 8) and $P2X_3^{-/-}$ (n = 11) mice confirms the altered micturition reflex in $P2X_3^{-/-}$ mice. Asterisk, $P < 0.05$; Student's t-test.

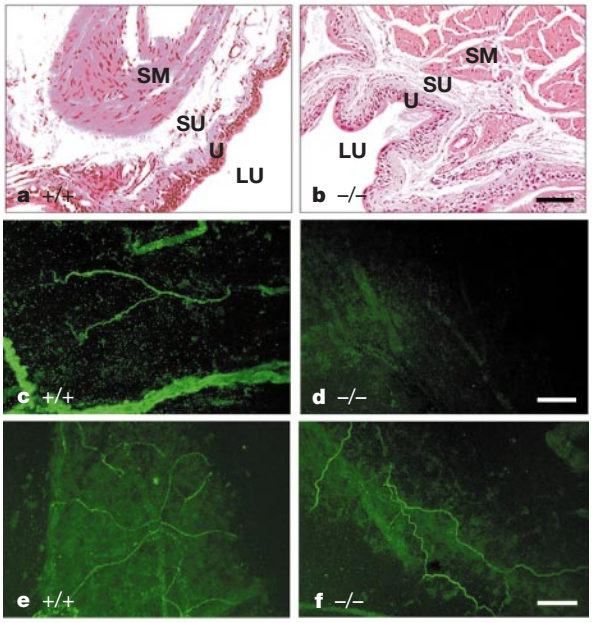

Figure 4 Immunolocalization in the mouse urinary bladder. **a**, **b**, Haemotoxylin and eosin stained transverse sections (10 μm) showing different layers of the urinary bladder (near trigone): LU, lumen; U, urothelium; SU, suburothelium; SM, smooth muscle. Loss of $P2X_3$ does not cause degenerative or hyperplastic changes in $P2X_3^{-/-}$ bladder. **c**, **d**, Confocal images of whole mount bladder exposing the suburothelial sensory nerve plexus. In $P2X_3^{+/+}$ bladder $P2X_3$ immunoreactivity is detected on small nerve fibres with terminals embedded in the urothelium, and on a large nerve bundle (**c**). In $P2X_3^{-/-}$ bladder, $P2X_3$ immunoreactivity is absent (**d**), but sensory innervation to the urinary bladder appears to be intact as evidenced by staining for the sensory neuron-specific capsaicin (VR-1) receptor (**e**, **f**). Scale bars, 50 μm.

heteromeric $P2X_{2/3}$ receptors appear most important. Second, our formalin test data are consistent with a role for ATP activation of $P2X_3$ in mediating some nociceptive responses to tissue damage. Finally, we show that $P2X_3$ is critical in regulating micturition reflex excitability. One explanation for these data is that ATP, released in response to stretch during distension and filling of the urinary bladder, excites primary afferent voiding circuitry through direct interaction with $P2X_3$ receptors. ATP is released from rabbit urothelium in response to stretch[7], and $P2X_3$ is clearly present on nerve fibres innervating urinary bladder[23] (Fig. 4). Electrophysiological evidence also indicates that α,β-Me-ATP directly activates and desensitizes mechanosensitive pelvic afferents arising from rat urinary bladder[9]. Thus, loss of $P2X_3$ might impair sensory neuron activity during bladder filling, raising the volume threshold for activation of the micturition reflex. As loss of compliance and lowered volume thresholds are a component of many bladder storage disorders (for example, overactive bladder)[24], selective modulation of $P2X_3$ may provide new therapies. The potential for similar $P2X_3$ roles in mechanosensation in other hollow organs (for example, GI tract and lung)[25] needs to be explored. □

Methods

Physiological studies

F_2 and F_3 mice were used for *in vitro* and *in vivo* studies, respectively. All experiments were performed blind. Dissociation of neurons and whole-cell patch-clamp recording was carried out as described previously[26]. Agonists were applied rapidly by microperfusion from a 4-barrel manifold controlled by computer-driven solenoid valves. Exchange of solution around the cell was complete in less than 100 ms. Time between applications was 2 min (nodose) and 3.5 min (DRG), allowing sufficient time to achieve reproducible responses. The minimum detectable response was 20 pA. Traces were acquired using FETCHEX (pCLAMP V.6.04 software, Axon Instruments), and plotted using ORIGIN V.4.1 (Microcal). Pain-related responses to injection of ATP into the hindpaw were measured essentially as described for rat[17]. The hindpaw lifting time was measured for a total of 4 min following injection of ATP. Thermal sensitivity was assessed using a radiant heat stimulus and tail immersion in a 52 °C water bath. Mechanical sensitivity was assessed using a set of calibrated von Frey filaments. For the formalin test, the hindpaw lifting and licking time was measured for a total of 30 min. Conscious mouse cystometry was performed essentially as described for rat[27]. Recovery following catheter implantation was for 7 days, and intravesical saline infusion was at a rate of 50 $\mu l\ min^{-1}$. For transurethral cystometry, bladder reflexes were assessed in urethane-anesthetized mice essentially as described for rat[28]. Each cystometrogram consisted of intravesical distension to a total volume of 0.3 ml, at a rate of 20 $\mu l\ min^{-1}$. Contractions greater than 10 cm of H_20 were taken as micturition contractions.

Generation of $P2X_3$ receptor-deficient mice and immunohistochemistry methods are described in Supplementary Information.

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Warm-coding deficits and aberrant inflammatory pain in mice lacking $P2X_3$ receptors

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ATP activates damage-sensing neurons (nociceptors) and can evoke a sensation of pain[1]. The ATP receptor $P2X_3$ is selectively expressed by nociceptors[2,3] and is one of seven ATP-gated, cation-selective ion channels[4–6]. Here we demonstrate that ablation of the $P2X_3$ gene results in the loss of rapidly desensitizing ATP-gated cation currents in dorsal root ganglion neurons, and that the responses of nodose ganglion neurons to ATP show altered kinetics and pharmacology resulting from the loss of expression of $P2X_{2/3}$ heteromultimers. Null mutants have normal sensorimotor function. Behavioural responses to noxious mechanical and thermal stimuli are also normal, although formalin-induced