

Increased 5-HT₃-mediated signalling in pelvic afferent neurons from mice deficient in P2X₂ and/or P2X₃ receptor subunits

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Abstract Extracellular ATP and 5-hydroxytryptamine (5-HT) are both involved in visceral sensory pathways by interacting with P2X and 5-HT₃ receptors, respectively. We have investigated the changes in P2X and 5-HT₃-mediated signalling in pelvic afferent neurons in mice deficient in P2X₂ and/or P2X₃ subunits by whole-cell recording of L₆-S₂ dorsal root ganglion (DRG) neurons and by multi-unit recording of pelvic afferents of the colorectum. In wildtype DRG neurons, ATP evoked transient, sustained or mixed (biphasic) inward currents. Transient currents were absent in P2X₃^{-/-} neurons, whereas sustained currents were absent in P2X₂^{-/-} DRG neurons. Neither transient nor sustained currents were observed following application of ATP or α,β -methylene ATP (α,β -meATP) in P2X₂/P2X₃^{Db1-/-} DRG neurons. 5-HT was found to induce a fast inward current in 63% of DRG neurons from wildtype mice, which was blocked by tropisetron, a 5-HT₃ receptor antagonist. The percentage of DRG neurons responding to 5-HT was significantly increased in P2X₂^{-/-}, P2X₃^{-/-} and P2X₂/P2X₃^{Db1-/-} mice, and the amplitude of 5-HT response was significantly increased in P2X₂/P2X₃^{Db1-/-} mice. The pelvic afferent response to colorectal distension was attenuated in P2X₂/P2X₃^{Db1-/-} mice, but the response to serosal application of 5-HT was enhanced. Furthermore, tropisetron resulted in a greater reduction in pelvic afferent responses to colorectal distension in the P2X₂/P2X₃^{Db1-/-} preparations. These data suggest that P2X receptors containing the P2X₂ and/or P2X₃ subunits mediate purinergic activation of colorectal afferents and that 5-HT signalling in

pelvic afferent neurons is up-regulated in mice lacking P2X₂ or P2X₃ receptor genes. This effect is more pronounced when both subunits are absent.

Key words pelvic afferents · P2X receptors · 5-HT

Abbreviations

| | |
|-----------------------|--|
| α,β -meATP | α,β -methylene ATP |
| DRG | dorsal root ganglion |
| HBSS | Hank's balanced salt solution |
| 5-HT | 5-hydroxytryptamine |
| DMSO | dimethylsulphoxide |
| PPADS | pyridoxyl 5-phosphate 6-azophenyl-2',4'-disulphonic acid |

Introduction

Extracellular ATP has been implicated in visceral sensory pathways as well as in somatosensory signal transduction. ATP evokes rapid excitation of mesenteric afferent fibres supplying the small intestine in the rat [1], pelvic afferent fibres of the rat colorectum [2] and the mouse urinary bladder [3, 4]. Pelvic afferent sensitivity to distension of the urinary bladder in the mouse and the colorectum in the rat could be attenuated by P2 purinoceptor antagonists. It has been proposed that the underlying mechanism is a purinergic mechanosensory transduction, where mechanical stimulation of tube and sac organs results in the release of ATP from epithelial cells lining these organs, and this ATP, in turn, stimulates afferent terminals in the subepithelial layers [5]. It is generally believed that the actions of ATP on afferent neurons are through interactions with P2X receptors containing the P2X₂ and/or P2X₃ subunits [6, 7]. While homomeric P2X₃ receptors give rise to rapidly desensitising

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responses and are activated by α,β -methylene ATP (α,β -meATP), P2X₂ receptors give a sustained response to ATP but are insensitive to α,β -meATP. Heteromeric P2X_{2/3} receptors give a slowly desensitising response to both agonists. However, pharmacological tools for studying P2X receptor mechanisms are still very limited. The development of P2X₂ and/or P2X₃ null-mutant mice has enabled us to investigate the effects of selective loss of these receptor subunits and, thus, the role of P2X receptors in visceral sensation. P2X₃ knockout studies have revealed an important role of purinergic signalling in sensory control of the urinary bladder [3, 8]. However, while some changes in peristaltic activity were observed *in vitro*, gastrointestinal transit in these animals was little changed [9].

5-Hydroxytryptamine (5-HT) is also known to be an important mediator of visceral afferent activation. 5-HT can rapidly stimulate mesenteric afferent fibres of the small intestine as well as the colonic afferents [10]. Multiple subtypes of serotonin receptors are expressed in sensory neurons. However, it appears that the rapid actions of 5-HT are mediated by 5-HT₃ receptors [11–13], which are part of the nicotinic receptor superfamily of ligand-gated ion channels. 5-HT₃ and P2X receptors are both expressed in dorsal root ganglia (DRG) neurons [14–18]. There is evidence that 5-HT₃ and P2X receptors may interact in a mutual inhibitory manner [19, 20], although the functional significance of this interaction remains unclear.

It has been recognised that deletion of a receptor gene can result in adaptive compensatory changes in related signalling pathways during development [21]. Thus, loss of purinergic afferent signalling may be replaced by up-regulation of other systems. In the current study, we aimed to determine the possible changes in 5-HT₃-mediated signalling in the pelvic afferent neurons from mice with targeted deletion of genes encoding P2X₂ and/or P2X₃ subunits. We performed whole-cell recordings of dispersed DRG cells from the L₆–S₂ segments, which include the primary afferent neurons innervating the colon, and compared the effects of 5-HT in cells from P2X₂ and/or P2X₃ deficient mice and their wildtype controls. We also investigated changes in pelvic afferent sensitivity to colorectal distension and 5-HT in an *in vitro* colorectal preparations using P2X₂/P2X₃^{Db1^{-/-}} mice and their wildtype controls. The results suggest an adaptive increase in 5-HT₃-mediated signalling in pelvic afferent neurons in mice deficient in P2X₂ and/or P2X₃ subunits.

Materials and methods

Animals

P2X₃^{-/-} mice were generated as described previously in detail [8]. P2X₂^{-/-} mice were generated by the introduction

of a deletion encompassing exons 2–11 and replacement of this sequence with a *LoxP*-flanked neomycin resistance gene into the mouse P2X₂ gene (see [22]). P2X₂/P2X₃^{Db1^{-/-}} mice were generated by the crossing of 129Ola x C57BL/6 P2X₃^{-/-} F2 mice to 129Ola x C57BL/6J P2X₂^{-/-} F2 mice to generate P2X₂/P2X₃ compound heterozygotes, which were bred to generate homozygous P2X₂/P2X₃ double wildtype and double knockout mice [22]. Although many P2X₂/P2X₃^{Db1^{-/-}} mice die before being weaned [23], those that survive go on to become outwardly healthy looking adults. Animals used in this study were 6 to 8 months old.

Whole-cell recording

Mice were killed by inhalation of a rising concentration of CO₂, and death was confirmed by cardiac haemorrhage. DRGs were rapidly dissected out, desheathed and incubated in 4 ml Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (HBSS) with 10 mM HEPES buffer (pH 7.4) (HBSS; Life Technologies) containing 1.5 mg ml⁻¹ collagenase (class II, Worthington Biochemical Corporation, UK) and 6 mg ml⁻¹ bovine serum albumin (Sigma Chemical Co., Poole, UK) at 37 °C for 45 min. The ganglia were then incubated in 4 ml HBSS containing 1 mg ml⁻¹ trypsin (Sigma) at 37 °C for 15 min. The solution was replaced with 1 ml growth medium comprising L-15 medium supplemented with 10% bovine serum, 50 ng ml⁻¹ nerve growth factor, 2 mg ml⁻¹ NaHCO₃, 5.5 mg ml⁻¹ glucose, 200 i.u. ml⁻¹ penicillin and 200 µg ml⁻¹ streptomycin. The ganglia were then dissociated into single neurons by gentle trituration. The neurons were plated onto 35 mm Petri dishes coated with 10 µg ml⁻¹ laminin (Sigma) and maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and were used within 30 h.

Whole-cell voltage-clamp recordings were made from small and medium neurons having a diameter of less than 25 µm which had a membrane capacitance of 39.8 ± 3.2 pF (*n* = 38). Experiments were carried out at room temperature using an Axopatch 200B amplifier (Axon Instruments, Foster City, Calif., USA) with membrane potential held at -60 mV. External solution contained (in millimoles): NaCl 154, KCl 4.7, MgCl₂ 1.2, CaCl₂ 2.5, HEPES 10 and glucose 5.6; the pH was adjusted to 7.4 with NaOH. Recording electrodes (resistance 2–4 MΩ) were filled with internal solution that contained (in millimoles): KCl 120, HEPES 10, tri-potassium citrate 10; EGTA 10. The pH was adjusted to 7.2 with KOH. Current signals were acquired with pClamp software (version 6.1, Axon Instruments) and were plotted using Origin7 (Microcal, Northampton, Mass., USA).

Drugs were applied rapidly through a manifold comprising seven capillaries made of fused silica coated with polyimide, with 250 µm internal diameter (SGE, Milton

Keynes, UK), connected to a single outlet made of the same tubing, which was placed approximately 200 μm from the cell. Solutions were delivered by gravity flow from independent reservoirs. One barrel was used to apply drug-free solution to enable rapid termination of drug applications. Solution exchange measured by changes in open tip current was complete in 200 ms; however, complete exchange of solution around an intact cell was considerably slower (1 s). Nevertheless, solution exchange using this system was fast enough for us to observe rapidly desensitising responses in DRG neurons (see [24]). Agonists were separately applied for 2 s at 4 min intervals, times which were sufficient for responses to be reproducible. Antagonists were pre-applied for 2 min and then co-applied with agonists.

Recording of pelvic afferent activity in an *in vitro* colorectal preparation

The distal colon and rectum were dissected from the pelvis with attached pelvic nerve and placed in a 10 ml bath superfused with Krebs solution (contents in millimoles: NaCl 120; KCl 5.9; NaH_2PO_4 1.2; MgSO_4 1.2; NaHCO_3 15.4; CaCl_2 2.5; glucose 11.5), equilibrated with 95% O_2 5% CO_2 . Both proximal and distal ends of the 30 mm length of bowel were secured to 8.5 F. three-way cannulae, and the lumen was perfused with Krebs solution. Ports on the cannulae were connected to a pressure transducer, large and small drainage tubing, and infusion tubing, which was connected, in turn, to a syringe driver (sp210iw; World Precision Instruments, Sarasota, Fla., USA). Following careful dissection of the pelvic nerve under the microscope into small branches, multi-fibre afferent activity was recorded using a glass suction electrode (tip diameter 50–100 μm) connected to a Neurolog headstage (NL 100; Digitimer Ltd., UK) and an AC amplifier (NL 104). Signals were amplified ($\times 10,000$), filtered (NL 125, band pass 200–4000 Hz) and captured by a computer via a power 1401 analogue-to-digital interface and Spike 2 software (version 4.03, Cambridge Electronic Design, UK). In all cases the tissues were allowed to stabilise in the bath for 60 min before the data were gathered. Branches that failed to respond consistently to repeated distensions were discarded. Control distensions to 40 mmHg with Krebs were repeated at 10 min intervals until nerve responses were stable. The various agonists were applied as a bolus to the serosal surface of the colorectum, and the antagonists were circulated via the pump into the organ bath.

The results for all experiments are presented as means \pm SEMs. Data were compared by chi-squared test, Student's *t*-test, or analysis of variance (ANOVA) as appropriate, and differences were considered statistically significant at $P < 0.05$.

Chemicals

ATP (disodium salt), α, β -meATP (lithium salt), 5-HT, pyridoxyl 5-phosphate 6-azophenyl-2',4'-disulphonic acid (PPADS), suramin, tropisetron and uridine 5'-triphosphate (UTP) were all obtained from Sigma. All chemicals were diluted in Krebs solution to required concentrations before use. All drugs were prepared in deionised water as stock solutions, except for tropisetron, which was dissolved in dimethylsulphoxide (DMSO) to 1 mM, and were diluted to required concentrations before use. The maximum concentration of DMSO in the final solution (1%) did not affect responses.

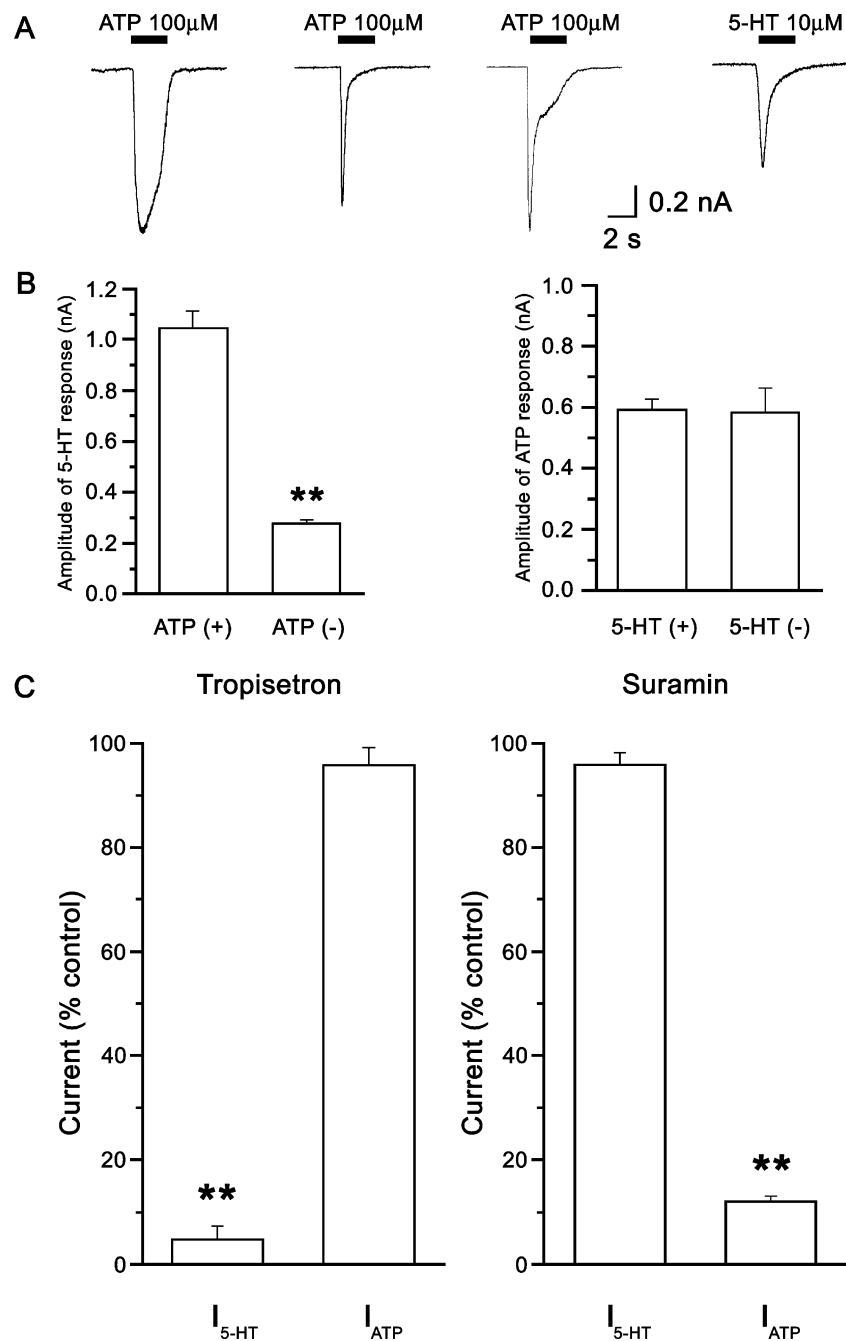
Results

Increased responses to 5-HT in L_6-S_2 DRG neurons

In wildtype mice, 100 μM ATP evoked a slowly desensitising inward current in 24% (13/33) of DRG neurons, with an average amplitude of 1.07 ± 0.12 nA (Figure 1). In 55% (18/33) of neurons, ATP evoked a rapidly desensitising current of 0.28 ± 0.02 nA (Figure 1A). A further 6% (2/33) of neurons gave biphasic responses with both transient and sustained components (Figure 1A). Comparable, transient, sustained or biphasic responses were evoked in these neurons by 100 μM α, β -meATP. Of wildtype DRG neurons, 63% (21/33) responded to 10 μM 5-HT with an inward current, with an amplitude of 0.87 ± 0.06 nA (Figure 1A). However, of the 5-HT-sensitive neurons, $62 \pm 1.1\%$ neurons responded to ATP (100 μM), while, in ATP-sensitive neurons, $88 \pm 0.3\%$ responded to 5-HT (10 μM), which indicated a high degree of overlap between ATP and 5-HT receptor expression. The amplitude of 5-HT evoked currents was 1.04 ± 0.07 nA and 0.27 ± 0.02 nA in ATP-sensitive ($n = 9$) and -insensitive ($n = 7$) neurons, respectively (Figure 1B). There was significant difference between them ($P < 0.01$, Student's *t*-test). In contrast, the amplitude of ATP responses was similar on the 5-HT-sensitive and -insensitive neurons (Figure 1C). As shown in Figure 1C, $I_{5\text{-HT}}$ (10 μM) and I_{ATP} (100 μM) were significantly inhibited by tropisetron (0.3 μM) and suramin (100 μM), respectively. However, tropisetron had no effect on I_{ATP} as suramin did not alter $I_{5\text{-HT}}$.

In $\text{P2X}_3^{-/-}$ mice, application of α, β -meATP (100 μM) did not evoke any inward currents in DRG neurons (22 cells tested) (Figure 2A). No transient currents were evoked by ATP (100 μM) either. However, sustained responses to ATP were observed in 36% (8/22) of neurons, which was not significantly different from the proportion of neurons from wildtype mice (30%), which had a sustained component to the response (Figure 2D). In contrast, $89.3 \pm 3\%$

Figure 1 Response of isolated DRG neurons to ATP and 5-HT. **A** Traces showing the three types of currents evoked by ATP (100 μ M; sustained, transient and biphasic) and current evoked by 5-HT (10 μ M) on DRG neurons in wildtype (WT) mice. **B** Histograms showing the average of the amplitude of 5-HT response in ATP-sensitive [ATP(+)] and -insensitive [ATP(-)] neurons and average amplitude of ATP-evoked current in 5-HT-sensitive [5-HT(+)] and -insensitive [5-HT(-)] neurons. **C** Histograms showing the effect of tropisetron (0.3 μ M) and suramin (100 μ M) on the 5-HT and ATP-evoked responses on DRG neurons from WT mice. ****** P <0.01, Student's *t*-test.

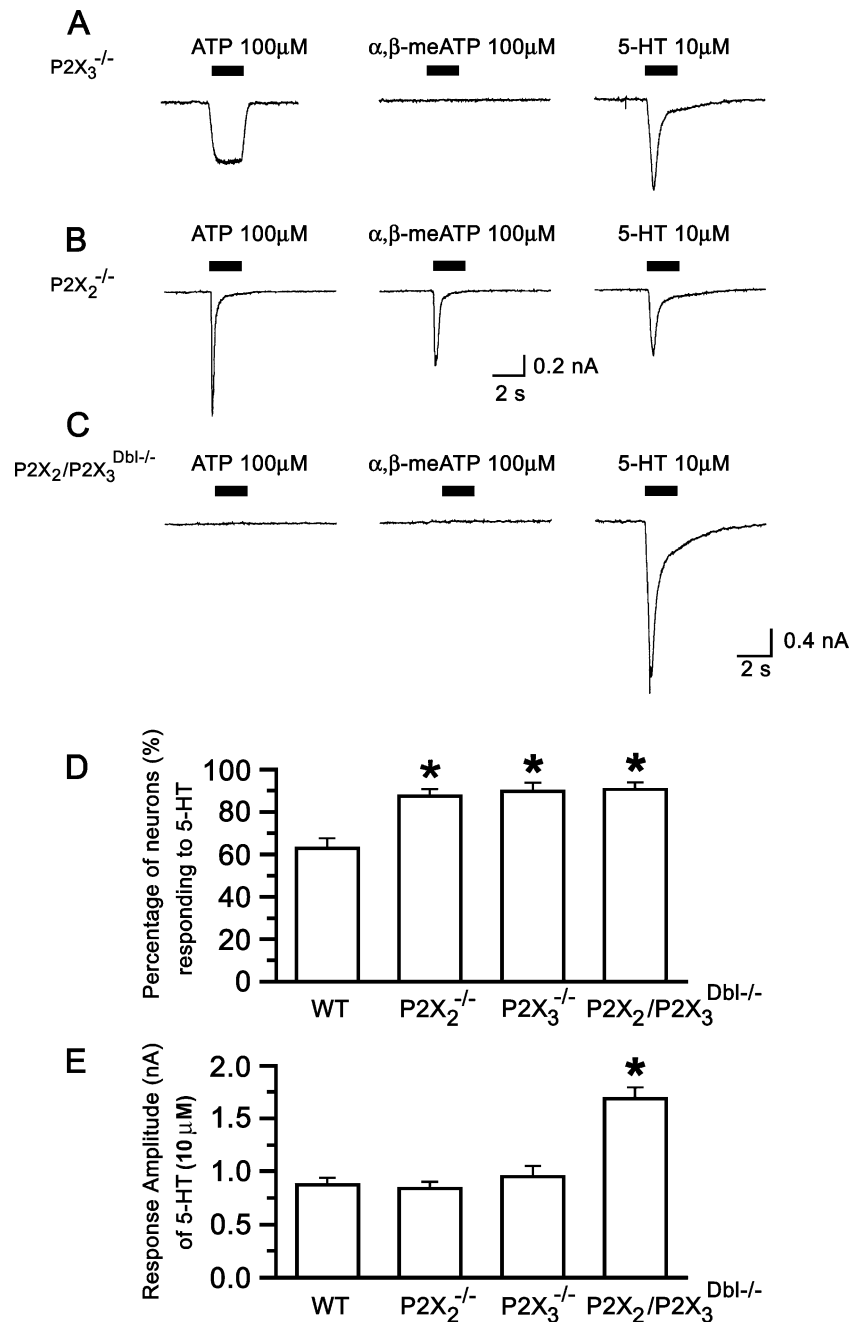


($n = 22$) neurons responded to 5-HT (10 μ M) with the amplitude of 0.94 ± 0.03 nA (Figure 2E). The percentage of neurons sensitive to 5-HT in $P2X_3^{-/-}$ mice was significantly higher than that in wildtype mice ($P < 0.05$) (Figure 2D), although there was no significant difference in the amplitude of 5-HT response in $P2X_3$ wildtype and $P2X_3^{-/-}$ mice ($P > 0.05$) (Figure 2E).

In $P2X_2^{-/-}$ mice, application of either ATP (100 μ M) or α, β -meATP (100 μ M) failed to produce a slowly desensitizing inward current in any of the DRG neurons (31 cells

tested) (Figure 2B). Transient responses to ATP and α, β -meATP were observed in 58% (18/31) of neurons, which was a similar proportion to that in wildtype mice (Figure 2D). In $87.3 \pm 3\%$ ($n = 23$) neurons, 5-HT, 10 μ M, evoked a response with a mean amplitude of 0.83 ± 0.06 nA (Figure 2E). The percentage of neurons responding to 5-HT was significantly higher than that in wildtype mice ($P < 0.05$) (Figure 2D), although the amplitude of 5-HT response was not significantly different in $P2X_2$ wildtype and $P2X_2^{-/-}$ mice ($P > 0.05$) (Figure 2E).

Figure 2 Comparison of responses to ATP and 5-HT in knockout mice. **A** ATP evoked only sustained currents in DRG neurons from $P2X_3^{-/-}$ mice, while the transient response to 5-HT was quite normal. **B** ATP and α,β -meATP, like 5-HT, evoked only transient currents in DRG neurons from $P2X_2^{-/-}$ mice. **C** ATP and α,β -meATP failed to evoke any current in DRG neurons in $P2X_2/P2X_3^{Dbl-/-}$ mice, while 5-HT evoked a significantly larger current. **D** Percentage of neurons responding to 5-HT (10 μ M) from wildtype (WT) and the three knockout strains of mouse. **E** Average amplitude of currents evoked by 5-HT (10 μ M) from WT and the three knockout strains of mouse did not differ between wildtype and knockout neurons. * $P < 0.05$, Student's *t*-test.



In $P2X_2/P2X_3^{Dbl-/-}$ mice there was no response to ATP (100 μ M) or to α,β -meATP (100 μ M) in 24 neurons tested (Figure 2C). In contrast, $90 \pm 3\%$ ($n = 24$; Figure 2D) neurons responded to 5-HT 10 μ M with the amplitude of 1.68 ± 0.10 nA (Figure 2E). Both the percentage and amplitude of the 5-HT response in $P2X_2/P2X_3^{Dbl-/-}$ mice were significantly higher than those in wildtype mice ($P < 0.05$, $P < 0.05$, Figure 2D,E), although the size of the neurons (as determined from measurement of membrane capacitance) were not significantly different.

The response to 5-HT was activated rapidly and declined in the continued presence of the agonist (Figure 2C).

Pelvic afferent activity from the colorectum in $P2X_2/P2X_3^{Dbl-/-}$ and wildtype mice

To see if changes in receptor signalling at the cell body were mirrored by changes at the nerve terminal, we investigated afferent nerve activity in the colorectum from wildtype and knockout mice. Bolus application of

α, β -meATP 100 μ M in the wildtype preparation resulted in a rapid increase from baseline activity by $174 \pm 10\%$ ($n = 4$), but, in the knockout preparation, the acute response was completely absent. Bolus applications of ATP, 1 mM, to the serosal surface of the colorectum resulted in consistent rises in pelvic nerve activity in the wildtype preparations. The mean increase from baseline activity in the wildtype was $157 \pm 33\%$ (Figure 3A). In $P2X_2/P2X_3^{Dbl-/-}$ preparations, a much slower and smaller increase in activity of $49 \pm 11\%$ ($n = 4$; $P < 0.05$) was observed. In four similar experiments application of the P2Y receptor agonist UTP to the colorectum also increased the nerve activity by $40 \pm 3\%$ in wildtype preparations and, to the greater extent of $76 \pm 6\%$, in the $P2X_2/P2X_3^{Dbl-/-}$ mice ($P < 0.01$; data not shown).

In the wildtype mice 10 μ M 5-HT increased spike frequency in the pelvic nerve by $116 \pm 15\%$. However, in the $P2X_2/P2X_3^{Dbl-/-}$ preparations, the response was $183 \pm 11\%$ over baseline activity ($n = 4$), which was significantly greater than that in the wildtype preparation ($P < 0.05$) (Figure 3B).

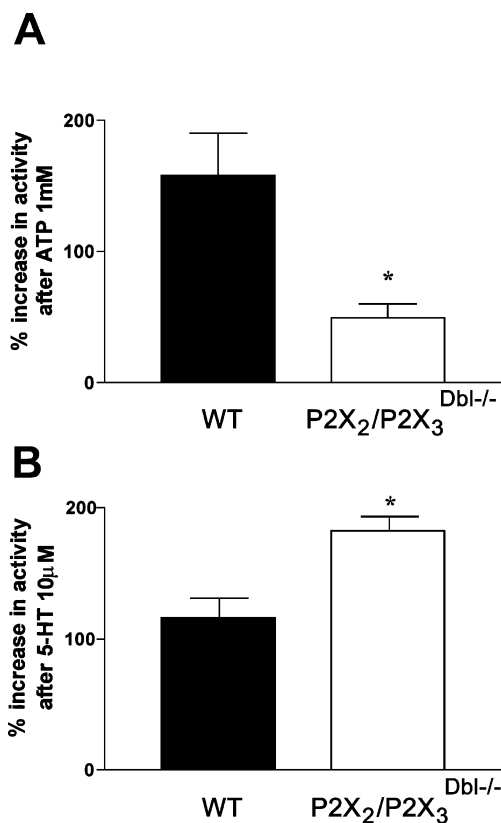


Figure 3 Agonist-evoked responses in pelvic afferents in an in vitro colorectal preparation from wildtype (WT) and $P2X_2/P2X_3^{Dbl-/-}$ mice. **A** Pooled data from four wildtype and four $P2X_2/P2X_3^{Dbl-/-}$ preparations show that the afferent responses to 1 mM ATP was significantly attenuated in $P2X_2/P2X_3^{Dbl-/-}$ preparations. * $P < 0.05$, Student's *t*-test. **B** Pooled data from four wildtype and four $P2X_2/P2X_3^{Dbl-/-}$ preparations indicate an increased response to 5-HT in pelvic afferent fibres from $P2X_2/P2X_3^{Dbl-/-}$ preparations. * $P < 0.05$, Student's *t*-test.

We also compared the responses to colorectal distension in $P2X_{2/3}$ wildtype and knockout mice. Multi-fibre activity in the pelvic nerve was measured at eight different intraluminal pressures from 0 mmHg to 40 mmHg, and these values were plotted as a percentage of the (maximum) activity at 40 mmHg. Figure 4A shows that the relative activity at any given pressure was reduced in the $P2X_2/P2X_3^{Dbl-/-}$ preparations. ANOVA demonstrated that these two plots were significantly different ($P < 0.01$). The effect of the P2 receptor antagonist PPADS, 100 μ M, on the response to distension was tested in the two types of preparation. In the wildtype mice, distension-evoked activity was reduced by PPADS by $32 \pm 2\%$, whereas, in the $P2X_2/P2X_3^{Dbl-/-}$, there was only a $9 \pm 3\%$ reduction ($n = 4$) (Figure 4B). This difference between wildtype and $P2X_2/P2X_3^{Dbl-/-}$ preparations was highly statistically significant ($P < 0.001$). Because application of 5-HT had caused greater excitation of pelvic nerve fibres in the $P2X_2/P2X_3^{Dbl-/-}$ compared to the wildtype, the effect of the 5-HT₃ receptor antagonist tropisetron on the nerve response to distension was evaluated. Figure 4C shows examples of this. Application of tropisetron, 10 μ M, resulted in a $40 \pm 2\%$ reduction in distension-evoked activity in the wildtype preparations, whereas, in the $P2X_2/P2X_3^{Dbl-/-}$, a more pronounced reduction of $53 \pm 2\%$ was observed ($n = 4$; Figure 4C). This difference was statistically significant ($P < 0.01$) (Figure 4D).

Discussion

The pelvic nerves contain afferent fibres innervating pelvic organs such as the urinary bladder, the urethra and the colorectum. Pelvic afferents have their cell bodies in the DRG of the lower lumbar and sacral segments. Previous studies in this laboratory have suggested that extracellular ATP contributes significantly to mechanosensory transduction in the urinary bladder and in the colorectum [2–4]. In the present study we demonstrate that a high proportion of L₆–S₂ DRG neurons responded to 5-HT as well as ATP. Pelvic afferents in mice deficient in the $P2X_2$ and $P2X_3$ subunits had a reduced sensitivity to colorectal distension, whereas the afferent responses to 5-HT were increased in mice deficient in $P2X_2$ and/or $P2X_3$ receptor subunits.

In DRG neurons three types of responses to ATP have been observed: transient, sustained and biphasic (having both transient and sustained components), which suggest the presence of multiple P2X receptor subtypes (see [7]). Although *in situ* hybridisation studies have shown that the mRNAs for $P2X_{1-6}$ are all expressed in sensory neurons [25], the current study is in keeping with the widely held view that P2X receptors containing the $P2X_2$ and/or $P2X_3$ subunits, namely homomeric $P2X_2$, $P2X_3$ and heteromeric

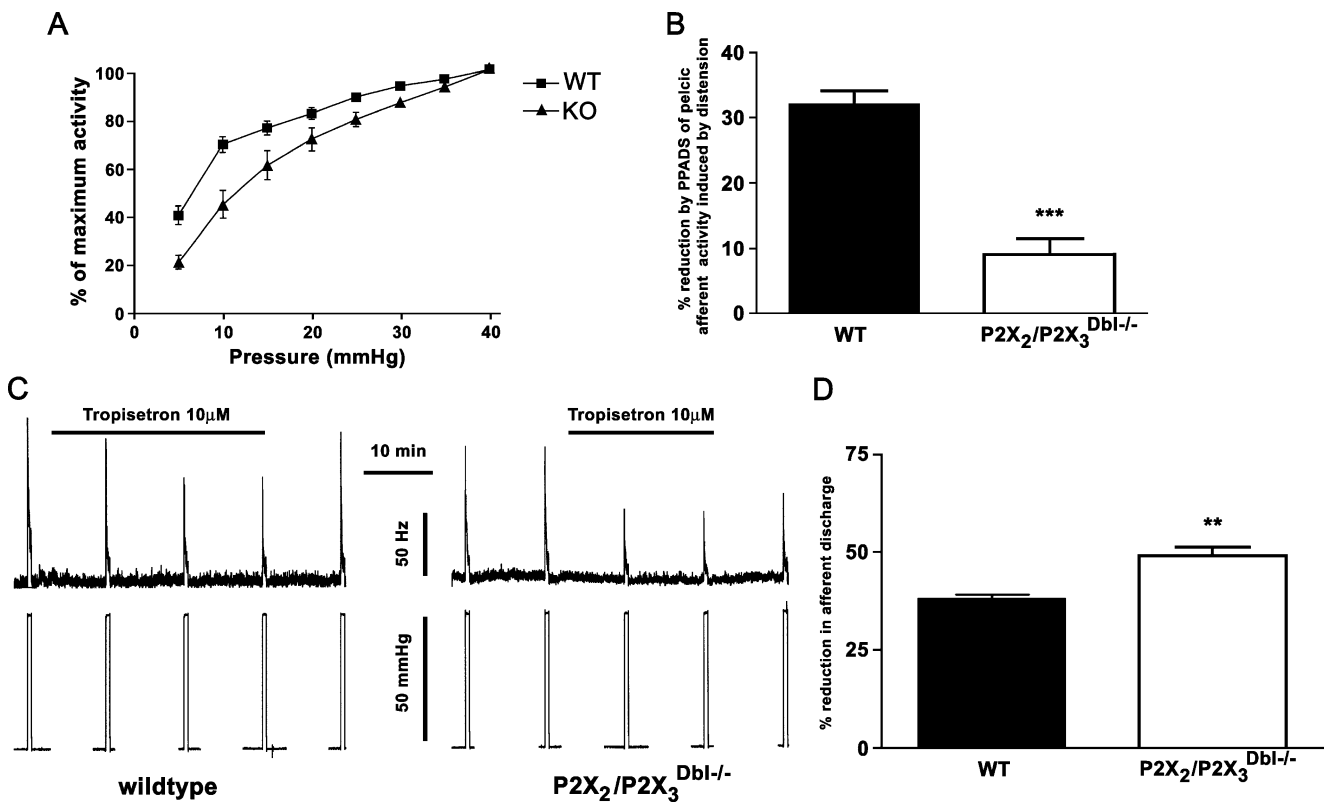


Figure 4 Distension-evoked responses of pelvic afferents from *in vitro* colorectal preparations from wildtype (WT) and P2X₂/P2X₃^{Dbl-/-} mice. (A) A plot of afferent activity (normalised as percentage of the peak activity at 40 mmHg) in relation to intraluminal pressure indicates reduced responsiveness of pelvic afferent to colorectal distension in the P2X₂/P2X₃^{Dbl-/-} preparations. (B) The P2 antagonist PPADS resulted in a greater reduction in the responses of pelvic afferents to colorectal distension in wildtype than in the P2X₂/P2X₃^{Dbl-/-}

^{-/-} mice. ****P* < 0.001, Student's *t*-test. (C) Effects of tropisetron on the responses of pelvic afferents to colorectal distension in preparations from wildtype, where tropisetron produces a reversible decrease in the multi-unit discharge rate, and P2X₂/P2X₃^{Dbl-/-} mice, where tropisetron produces a more dramatic inhibition. (D) Pooled data from four wildtype and four P2X₂/P2X₃^{Dbl-/-} mice confirm a greater reduction in distension-induced afferent discharge in the P2X₂/P2X₃^{Dbl-/-} preparations. ***P* < 0.01, Student's *t*-test

P2X_{2/3} receptors, mediate these responses (see [7]). Thus, in P2X₃^{-/-} mice, no transient response to ATP was detected, whereas, in P2X₂^{-/-} mice, sustained responses to either ATP or α,β -meATP were absent. In P2X₂/P2X₃^{Dbl-/-} mice neither transient nor sustained responses to either ATP or α,β -meATP were detected. Because the rate of desensitisation of the P2X₃ receptor is fast, compared with the speed of agonist application, there will be an underestimate of the amplitude of the P2X₃ receptor-mediated response. However, this error will have been considerably reduced by the use of a high agonist concentration.

We have previously shown in a rat colorectal preparation that ATP and its stable analogue, α,β -meATP, could directly activate pelvic afferents and sensitise their responses to colorectal distension [2]. In contrast, the P2 receptor antagonist PPADS resulted in a reduction in the pelvic afferent responses to colorectal distension, suggesting that ATP contributes to mechanosensory transduction through interactions with P2X receptors. The present study provides further support for this. Firstly, we show that, in

the P2X₂/P2X₃^{Dbl-/-} mice, the responses of pelvic afferent fibres to colorectal distension were reduced compared to those in the wildtype animals. Secondly, the P2 receptor antagonist PPADS resulted in a 32.1% reduction in distension-induced afferent activity in the wildtype but only 9.3% reduction in the knockout preparations, suggesting that P2X receptors containing the P2X₂ and/or P2X₃ subunits are involved in this purinergic mechanosensory transduction.

In the *in vitro* colorectal preparation from P2X₂/P2X₃^{Dbl-/-} mice, ATP still evoked an increase in pelvic afferent discharge. Although these responses might involve other P2X receptor subtypes, the slow kinetics of the response and the lack of effect of α,β -meATP would be in keeping with the involvement of metabotropic receptors, possibly adenosine receptors (i.e., P1) or P2Y receptors. ATP can easily be broken down to ADP, AMP and adenosine under the activity of ecto-nucleotidases, which are widely distributed in different tissues [26]. ADP is a potent agonist of certain P2Y receptors, whereas adenosine is an endoge-

nous agonist of A1 receptors, which are present on afferent terminals. The observation that UTP evoked a greater increase in pelvic afferent discharge in P2X₂/P2X₃^{Db1-/-} mice suggests that P2Y₂, P2Y₄ or P2Y₁₁ receptor-mediated signalling might be up-regulated in these mice.

5-HT is recognised as an important mediator of afferent excitation in the gut [27, 28]. The primary source of 5-HT in the body is a population of enterochromaffin cells in the intestinal mucosa. In patients with irritable bowel syndrome (IBS) there was an increase in the population of enterochromaffin cells in the colon [29]. 5-HT activates vagal afferent endings in the upper gastrointestinal tract via 5-HT₃ receptors [11, 12, 30]. More recently, Hicks et al. [10] demonstrated excitation of colonic afferent fibres in the hypogastric nerve by 5-HT, and the effects were partly mediated by 5-HT₃ receptors. However, so far, there has been no direct demonstration of the effects of 5-HT on pelvic afferents of the colorectum. In this study we first showed that a high proportion of L₆-S₂ DRG neurons responded to 5-HT with a fast inward current and that bath application of 5-HT resulted in rapid excitation of the pelvic afferents of the colorectum. The responses to 5-HT in the DRG neurons were blocked by tropisetron, suggesting that 5-HT₃ receptors mediated this response. This is consistent with previous *in situ* hybridisation and immunohistochemical data, which demonstrated the presence of 5-HT₃ along with other 5-HT receptor subtypes in DRG neurons [17, 31]. Furthermore, we showed that tropisetron attenuated the responses of the pelvic afferents to colorectal distension. *In vitro* release of 5-HT from the colon has been shown before [32, 33]. The present results strongly suggest that 5-HT released during distension contributes to mechanosensory transduction in the colorectum.

5-HT can modulate primary afferent input to the spinal cord via the activation of multiple 5-HT receptor subtypes [34]. The rapid effects of 5-HT in mechanosensory transduction are mediated by 5HT₃ receptors and are antagonised by tropisetron. Whether 5-HT has additional modulatory effects in mechanosensory transduction, mediated through other 5HT receptor subtypes, remains to be determined.

There appears to be a clear correlation between the P2X and 5-HT₃-mediated signalling in pelvic afferent neurons. Firstly, there was a high degree of overlap in the neurons that respond to ATP or 5-HT, and the I_{5-HT} was significantly greater in ATP-sensitive neurons than in ATP-insensitive neurons, indicating that more than half of the pelvic afferent neurons express both P2X and 5-HT₃ receptors. Secondly, a greater proportion of L₆-S₂ DRG cells responded to 5-HT in mice deficient in P2X₂ and/or P2X₃ subunits, and the amplitude of 5-HT-induced currents was significantly higher in P2X₂/P2X₃^{Db1-/-} than in the wildtype mice. The

relationship between receptor activation and action potential discharge in a multi-unit recording is likely to be complex. Our results from dissociated DRG neurons suggest that there is not only an increase in the density of 5HT₃ receptor expression but also an increase in the number of neurons expressing these receptors in P2X₂/P2X₃^{Db1-/-} knockout mice. If these changes occur *in vivo* at the level of the afferent nerve terminals, then a greater number of afferent fibres will be activated by distension-evoked 5-HT release. Consequently, there will be a greater antagonism of the response by a high concentration of tropisetron. Thirdly, 5-HT evoked a greater increase in pelvic afferent discharge in the P2X₂/P2X₃^{Db1-/-} colorectal preparation than in the wildtype preparations, and tropisetron resulted in a greater reduction in distension-induced afferent discharge in the knockouts than in the wildtype preparations. Previous evidence indicates that P2X receptors might interact with nicotinic acetylcholine and 5-HT₃ receptors [35]. In the submucosal neurons of guinea pig ileum, simultaneous activation of P2X and 5-HT₃ receptors resulted in mutual inhibition of ATP and 5-HT-induced currents [19, 20]. Thus, one possibility for the increased 5-HT signalling is by the removal of ATP-mediated inhibitory modulation of the 5-HT receptors. The other possibility is that, over the course of development of the P2X₂/P2X₃^{Db1-/-} mice, there is an adaptive increase in the expression of 5-HT₃ receptors.

In summary, our results suggest that P2X receptors and 5-HT₃ receptors are functionally associated, such that the removal of P2X receptors results in adaptive increase in 5-HT₃-mediated signalling in the pelvic afferent neurons.

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