

## Article

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# Age-related changes in afferent pathways and urothelial function in the male mouse bladder

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## Key points

- The prevalence of bladder conditions such as overactive bladder syndrome and urinary incontinence significantly increases with age, but how bladder function is altered by ageing is unclear.
- Sensory nerves together with the epithelial lining of the bladder known as the urothelium play a key role in mediating bladder function.
- In aged male mice we find a significant increase in natural bladder voiding, augmented afferent nerve firing during bladder filling and a significant increase in urothelial responses to purinergic receptor stimulation.
- This suggests that with ageing there is increased purinergic transmission in the mouse bladder which may lead to increased sensation and result in bladder hypersensitivity.
- These findings help us better understand how the function of the bladder may be affected by advancing age.

**Abstract** The prevalence of lower urinary tract storage disorders such as overactive bladder syndrome and urinary incontinence significantly increase with age. Previous studies have demonstrated age-related changes in detrusor function and urothelial transmitter release but few studies have investigated how the urothelium and sensory pathways are affected. The aim of this study was to investigate the effect of ageing on urothelial-afferent signalling in the mouse bladder. Three-month-old control and 24-month-old aged male mice were used. *In vivo* natural voiding behaviour, sensory nerve activity, urothelial cell function, muscle contractility, transmitter release and gene and protein expression were measured to identify how all three components of the bladder (neural, contractile and urothelial) are affected by ageing. In aged mice, increased voiding frequency and enhanced low threshold afferent nerve activity was observed, suggesting that ageing induces overactivity and hypersensitivity of the bladder. These changes were concurrent with altered ATP and acetylcholine bioavailability, measured as transmitter overflow into the lumen, increased purinergic receptor sensitivity and raised P2X<sub>3</sub> receptor expression in the urothelium. Taken together, these data suggest that ageing results in aberrant urothelial function, increased afferent mechanosensitivity, increased smooth muscle contractility, and changes in gene and protein expression (including of P2X<sub>3</sub>). These data are consistent with the hypothesis that ageing evokes changes in purinergic signalling from the bladder, and further studies are now required to fully validate this idea.

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**Abbreviations** ACh, acetylcholine; DRG, dorsal root ganglia; OAB, overactive bladder syndrome; UI, urinary incontinence

## Introduction

Current demographic forecasts have predicted a worldwide increase in the proportion of people aged 65–80 (United Nations DoEaSA, Population Division, 2013). Understanding how physiological function alters as we age is now vital to ensure the progress of drug discovery and treatment of disease in an ageing society. Previous studies have shown that ageing dramatically affects the function of the urinary bladder. Overactive bladder syndrome (OAB) is a common and distressing disorder of the lower urinary tract which arises due to compromised storage ability of the bladder. Its cardinal symptoms are frequent micturition, urgency (the compelling and urgent sensation to empty the bladder) with or without urinary incontinence (UI, the involuntary leakage of urine from the bladder) and nocturia in the absence of other pathologies. The aetiology of OAB and UI is not fully understood, although studies suggest that the epithelial lining of the bladder, known as the urothelium and suburothelium, and the sensory innervation play a key role in normal function. Strikingly, the prevalence of bladder and continence conditions increases considerably with age (Dubeau, 2006).

Normal bladder function is dependent on the integration of autonomic and somatic mechanisms which coordinate a complex cycle of filling and emptying. This process is instigated and ultimately regulated by the sensory signalling pathways, which detect changes in bladder filling (volume and distension) and convey information to the CNS. The sensory innervation from the bladder is carried in the pelvic and hypogastric afferent nerves, the cell bodies of which, lie in the dorsal root ganglia (DRG). They consist of myelinated A $\delta$  fibres and unmyelinated C fibres which have polymodal sensitivity responding to a host of mechanical and chemical stimuli.

To date, there are relatively few studies that have investigated how bladder function and neural activity are affected by ageing. Morphological studies suggest that there may be an age-dependent reduction in the numbers of unmyelinated afferents innervating the bladder, although the general pattern of innervation appears to be preserved (Nakayama *et al.* 1998; Mohammed & Santer, 2002; Aizawa *et al.* 2010). At the level of the DRG, the sensory neuronal markers calcitonin gene related peptide and substance P are markedly reduced in aged rats (Mohammed & Santer, 2002) but how bladder projecting afferents are specifically affected is unknown. Functional and histological studies have produced contradictory reports, with some investigators finding an age-dependent fall in contractile ability, detrusor muscle thinning and collagen deposition and others seeing no functional change and/or increased muscle mass (Lluel *et al.* 2000; Smith *et al.* 2012). This has made it difficult to fully understand how the function of the bladder changes with age and to specifically assess how

afferent signalling is affected. Clinical studies estimate that one-third of elderly patients with UI have overactivity of the detrusor muscle (spontaneous contractile activity) during bladder filling but diminished contractile activity during emptying (Resnick & Yalla, 1987). Potentially, this 'overactivity' could generate hypersensitivity of bladder afferents during the storage phase, driving the sensory symptoms associated with OAB (urgency and frequency). In one previous study, bladder afferent nerve responses to increasing volume (but not pressure) were shown to decline in the aged rat, but in another study C-fibre mediated afferent discharge was shown to increase in aged rats (Hotta *et al.* 1995; Aizawa *et al.* 2010).

It is now well established that the urothelium, traditionally believed to act as a barrier to prevent noxious agents in the urine from entering the circulation, can actively modulate sensory nerve firing by releasing a host of excitatory and inhibitory signalling molecules in response to mechanical and chemical stimulation. These mediators act on neighbouring urothelial cells via paracrine and autocrine signalling mechanisms and also act directly at the afferent terminal to excite or inhibit afferent activity. The most well-characterised example is the graded release of ATP from the urothelium, which occurs as the bladder fills. This ATP acts at purinergic receptors (P2X<sub>2</sub> and P2X<sub>3</sub>) located on nerve terminals to initiate afferent nerve firing (Burnstock, 2009). One theory suggests that excitatory and inhibitory agents are released from the urothelium in balance and that bladder dysfunction may be caused by a shift in this balance towards either excitatory or inhibitory pathways (Smith *et al.* 2008). Yoshida *et al.* (2001) demonstrated an age-dependent rise in ATP release from the human urothelium and an age-dependent fall in acetylcholine (ACh) release, suggesting that ageing causes a shift in urothelial function. However, to date, there are few studies that have directly investigated the impact of ageing on the urothelium.

The aim of this study was to investigate how natural ageing affects bladder afferent activity, contractility and the function of the urothelium.

## Methods

### Ethical approval and animals

All experiments were performed in accordance with the University of Sheffield's Animal Care Committee, under an approved UK protocol and project licence. Three- to 4-month-old adult (herein referred to as control) and 24-month-old (herein referred to as aged) C57/BL6 male mice from Charles River (Margate, Kent, UK) and Harlan (UK) were used in all experiments. Mice were killed by cervical dislocation following anaesthesia with isoflurane. Maintenance and killing of the animals followed principles of good laboratory practice in compliance with UK laws and regulations.

### Voiding pattern analysis and urine collection

Aged and control mice were housed singly in cages lined with filter paper (Whatman number 1) for 4 h with free access to food and water (similar protocols as previously described by Uvin *et al.* 2013). Urine spots were detected using an ultraviolet transilluminator, photographed and digitised and spot sizes were measured using Image J software. In separate experiments, naturally voided urine samples were collected and osmolality was determined using an osmometer.

### Afferent nerve recording

Nerve recording was conducted using an *in vitro* model (Daly *et al.* 2007). The whole pelvic region was dissected and placed in a recording chamber (30 ml), continually perfused with gassed (95% O<sub>2</sub>, 5% CO<sub>2</sub>) Krebs-bicarbonate solution (composition in mmol l<sup>-1</sup>: NaCl 118.4, NaHCO<sub>3</sub> 24.9, CaCl<sub>2</sub> 1.9, MgSO<sub>4</sub> 1.2, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 11.7) at 35°C. The urethra and dome were catheterised to enable recording of intravesical pressure and enable evacuation of fluid. Multiunit pelvic and hypogastric nerves were dissected into fine branches, and afferent activity was recorded by a neurolog headstage (NL100; Digitimer Ltd, Welwyn Garden City, UK), amplified, filtered (NL215, band pass 300–4000 Hz) and captured by a computer via a power 1401 interface and Spike 2 software (version 7, Cambridge Electronic Design, Cambridge, UK).

### Bladder contraction

Following removal of the urothelium, whole detrusor muscle was cut into half and strips were mounted in a 10 ml organ bath (filled with gassed Krebs-bicarbonate solution at 37°C) and connected to a UF1 force transducer. Resting tension was adjusted to 1 g for 30 min and the Krebs solution was changed every 10 min. ATP (10 μM, 100 μM and 1 mM) and KCl (25–65 mM) were bath applied with a 20–30 min washout period between concentrations. Bethanechol was bath applied cumulatively (1, 10 and 100 μM). Tension recordings were obtained using a PowerLab data acquisition system and Chart software (ADInstruments, Colorado Springs, CO, USA). Contraction amplitude was normalised to tissue weight.

### Spontaneous contractions

Whole intact bladders were removed and catheterised via the urethra with a dual-lumen cannula, placed in a 500 μl organ bath (filled with gassed Krebs-bicarbonate buffer at 37°C) and distended to 10 mmHg. Preparations were held under isovolumetric conditions and equilibrated for

30 min. Spontaneous contractions were detected as small transient rises in intraluminal pressure, recorded using Spike 2 software and measured using a custom designed script courtesy of Cambridge Electronic Design.

### Measurement of ATP, NO and ACh

Using the isolated whole bladder preparation described above, sequential ramp distensions were performed (with 0.9% NaCl, to 50 mmHg). Intraluminal fluids were collected and the amount of ATP, ACh and NO in the samples was determined using the luciferin-luciferase ATP bioluminescent assay (Sigma-Aldrich, St Louis, MO, USA), Amplex Red ACh assay (Molecular Probes, Carlsbad, CA, USA) and the nitric oxide fluorometric assay (BioVision, Cambridge, UK).

### Calcium imaging of cultured urothelial cells

Bladders from aged and control mice were dissected and pinned with the urothelium upmost and incubated with minimal essential media containing 2.5 mg ml<sup>-1</sup> dispase (2–3 h at 21°C). Cells were collected by gentle scraping and dissociated in 0.025% trypsin EDTA at 37°C (5–15 min) and re-suspended in keratinocyte serum free media and plated on collagen (IV)-coated coverslips. Urothelial cells (20–24 h) were loaded with 2 μM fura-2-acetoxymethyl ester (fura-2AM) for 30 min at 37°C and washed with Hepes buffer (Composition: Hepes 10 mM, NaCl 135 mM, KCl 5 mM, glucose 10 mM, CaCl<sub>2</sub> 2 mM and MgCl<sub>2</sub> 1 mM, pH 7.4). Cells were stimulated with ATP, bethanechol, αβmethylene ATP or βγmethylene ATP for 3 min and changes in intracellular calcium, [Ca<sup>2+</sup>]<sub>i</sub>, were monitored in real time. Ionomycin (5 μM) was applied as a positive control. Results are expressed as relative fluorescence (RF), and [Ca<sup>2+</sup>]<sub>i</sub> is represented as the ratio between the fluorescence signal at 350 nm/380 nm ('n' numbers are presented as  $N = \text{number of mice}$  and  $n = \text{number of cells}$ )

### Quantitative RT-PCR (qRT-PCR)

The whole bladder mucosa was dissected from the detrusor muscle under a microscope and both were stored separately in RNA later. Total RNA was extracted (RNeasy mini Kit, Qiagen, Valencia, CA, USA). cDNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). qRT-PCR reactions were performed in duplicate using TaqMan gene expression master mix (Applied Biosystems) and specific TaqMan primer/probe mix (IDT) for the purinergic P2X (P2X<sub>1-7</sub>) and muscarinic (M1–M5) receptors. Results were expressed as relative expression to the housekeeping gene GAPDH (1/ΔCt), and fold change was calculated using the equation  $2^{-\Delta(\Delta Ct)}$ .



## Western blotting

The whole bladder mucosa was dissected from the detrusor muscle under a microscope and fixed in 4% paraformaldehyde for 24 h at 4°C. Tissues were washed with PBS and stored at 4°C in PBS-azide. Fixed urothelial tissues were lysed using a Qproteome FFPE Tissue kit (Qiagen) and stored at -80°C. Protein levels were quantified using a Bradford assay (BioRad, Hercules, CA, USA). In total, 20 µg of protein was separated on an 8% SDS-PAGE gel and transferred to nitrocellulose membranes (Protran). After blocking, the membranes were incubated with rabbit polyclonal anti-P2X<sub>3</sub> (1:250) or anti-β-actin (1:1000) (Biorbyt, Cambridge, UK) at 4°C overnight. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using the ECL detection system (Pierce, Rockford, IL, USA) and protein expression levels were evaluated by densitometry (Image J), and normalised to β-actin.

## Data analysis

Data are presented as means ± SEM. Statistical analysis was carried out using one- or two-way analysis of variance (ANOVA) and Bonferroni *post hoc* test or Student's *t* test, where appropriate, and significance was confirmed at  $P < 0.05$ .

## Results

### Ageing increases voiding frequency and afferent nerve activity

In control mouse urine samples, osmolality was  $1662 \pm 150.2$  mosmol kg<sup>-1</sup> ( $n = 6$ ). This was not significantly altered in aged urine samples ( $1799 \pm 200.6$  mosmol kg<sup>-1</sup>,  $n = 6$ ), suggesting no change in renal function or fluid intake as a result of ageing. Both control and aged mice tended to urinate in one corner of the cage; voiding pattern analysis found no significant difference in the total area of urine voided, although significantly more urine spots were detected on papers from aged mice than from controls, suggesting an age-related increase in the number of voiding events ( $P < 0.01$ , Student's *t* test,  $n = 6$  and 6, respectively, Fig. 1).

Afferent recordings remained stable for >4 h and repeated distension elicited reproducible response profiles from both control and aged mice. Spontaneous afferent nerve activity in control mice was significantly lower at  $8.8 \pm 2.3$  impulses s<sup>-1</sup> ( $n = 14$ ) compared to  $26.0 \pm 5.5$  impulses s<sup>-1</sup> in aged mice ( $P < 0.001$ , Student's *t* test,  $n = 7$ , Fig. 2B). As demonstrated previously, ramp distension of the bladder evoked a graded increase in intravesical pressure and afferent nerve discharge (Daly *et al.* 2007). The same distension-response profile was

also observed in preparations from aged mice, although the overall magnitude of firing in response to bladder distension was augmented compared to preparations from controls ( $n = 7$  and  $n = 14$  respectively,  $P < 0.0001$ , two-way ANOVA, Fig. 2C). This increase in afferent activity affected only the low threshold component of the response (0–15 mmHg, Fig. 2D). Bladder compliance as gauged by the pressure-volume relationship was not significantly affected by age (Fig. 2E and F).

### Ageing enhances contractility of the detrusor

Contractile responses to the non-selective muscarinic receptor agonist bethanechol and the non-selective purinergic receptor agonist ATP were significantly increased in muscle strips from aged mice compared to muscle strips from control mice ( $n = 6$  and 6,  $P < 0.001$  Student's *t* test and two way ANOVA with Bonferroni *post hoc* test as appropriate, Fig. 3C and D). Moreover, contractile responses to application of KCl (25 and 65 mM) were also significantly greater from aged mice than from control mice ( $n = 6$ ), in the absence of any change in detrusor weight, suggesting that the overall contractile ability of the detrusor was augmented by ageing ( $P < 0.001$ , Student's *t* test, Fig. 3A and B).

Whole bladder spontaneous contractions were observed in 84% of control preparations and 75% of aged preparations (Fig. 3E,  $n = 6$  and  $n = 8$ ) but there was no significant difference in amplitude or frequency of contractions between the two groups (Fig. 3F).

In muscle samples, qRT-PCR identified relative expression of all five muscarinic receptor genes, with the M2 and M3 subtypes exhibiting the greatest expression, and all seven of the P2X receptor genes, with the P2X<sub>1</sub> subtype exhibiting the greatest expression. No difference in gene expression was detected between aged and control samples ( $n = 6$  and  $n = 9$ , respectively, Fig. 3G and H).

### Altered ATP and ACh overflow with age

Distension of the bladder evoked release of ATP, ACh and NO, which in this study was detected as overflow of these transmitters into the lumen. No significant difference in NO between aged and control bladders was identified ( $n = 8$  and  $n = 7$ , respectively, Fig. 4B), although ATP levels from aged bladder preparations were significantly increased compared to controls ( $n = 8$  and  $n = 7$ , respectively). This was concurrent with a reduction in ACh levels and suggests that ageing may alter transmitter release from the urothelium (Fig. 4C and D).

### Urothelial purinergic receptor signalling is increased

To assess whether purinergic and cholinergic receptor signalling in the urothelium was altered as a result of

ageing, calcium imaging experiments were conducted using isolated urothelial cells from aged and control animals. Preliminary studies found an  $EC_{50}$  for ATP of  $\sim 10 \mu M$  (data not shown). To ensure activation of all purinergic receptors an  $EC_{100}$  concentration ( $100 \mu M$ ) was applied. This application was not deleterious to cells as viability was tested using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (data not shown). The number of urothelial cells responding to purinergic receptor stimulation with ATP was significantly greater from aged mice than from controls ( $N = 4$ ,  $n = 333$  and  $N = 5$ ,  $n = 241$ , respectively). Moreover, the magnitude of the response to ATP was also significantly increased, indicating age-related changes in purinergic receptor signalling. In contrast, urothelial cells from aged mice ( $N = 4$ ,  $n = 226$ ) had similar responses to bethanechol as urothelial cells from controls ( $N = 3$ ,  $n = 190$ ), suggesting that muscarinic receptor signalling in the urothelium was unaffected by ageing (Fig. 5C and D).

In separate experiments, the selective  $P2X_1$  and  $P2X_3$  agonist  $\alpha\beta$ methyleneATP and the selective  $P2X_1$  agonist  $\beta\gamma$ methyleneATP were applied. There was no significant difference in urothelial cell responses to  $\beta\gamma$ methyleneATP

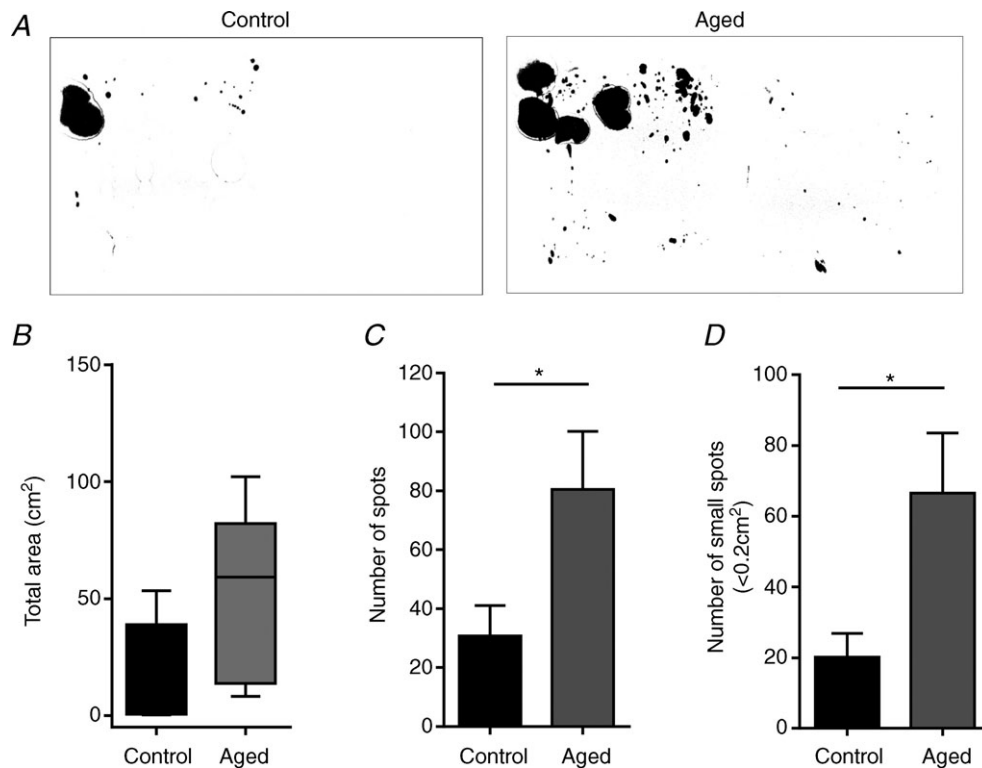
between cells from aged and control mice ( $N = 5$ ,  $n = 239$  and  $N = 4$ ,  $n = 189$ , respectively) although significantly more cells from aged mice responded to  $\alpha\beta$ methyleneATP (Fig. 5E and F) compared to cells from control mice ( $N = 5$ ,  $n = 218$  and  $N = 4$ ,  $n = 175$ , respectively,  $P < 0.001$ , Student's *t* test). The magnitude of the responding cells was also elevated but did not reach significance.

### Gene and protein expression in the urothelium is significantly altered by ageing

Western blot analysis found a significantly greater  $P2X_3$  receptor expression in aged bladder mucosa compared to control mucosa ( $n = 4$  and  $n = 4$ ,  $P < 0.01$ , Student's *t* test). However, paradoxically, qRT-PCR experiments showed significantly lower muscarinic and purinergic gene expression in aged samples relative to controls ( $n = 6$  and  $n = 8$ , respectively, Fig. 6).

### Discussion

Given the clear correlation between lower urinary tract disorders such as OAB, UI and advancing age, a number of studies have sought to understand how bladder

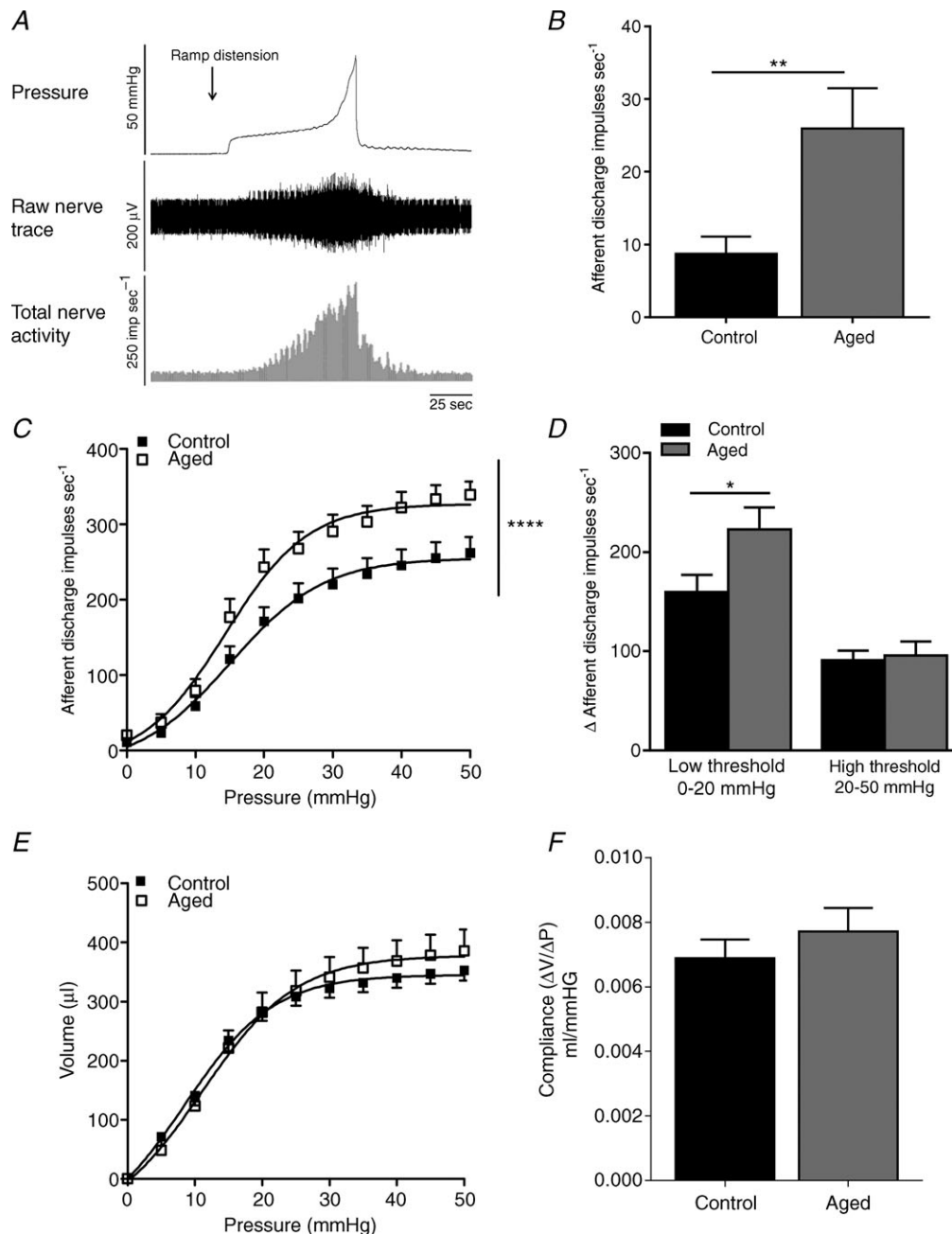


**Figure 1. Urine spot patterns obtained from aged and control mice**

A, representative digitized traces showing the urine spot patterns obtained from a control and an aged mouse over a 4 h period. B, total urine spot area was not significantly different between aged ( $n = 6$ ) and control mice ( $n = 6$ ). C, the number of urine spots detected was greater in aged mice than in controls ( $P < 0.05$ , Student's *t* test). D, the number of small urine spots ( $<0.2 \text{ cm}^2$ ) was significantly greater in aged mice than in controls ( $P < 0.05$ , Student's *t* test).

physiology is affected by ageing. Over the past decade it has become apparent that the sensory innervation of the bladder together with the urothelium plays an integral role in regulating micturition (Birder, 2004, 2009), however it is unclear how these elements are affected by ageing. Afferent

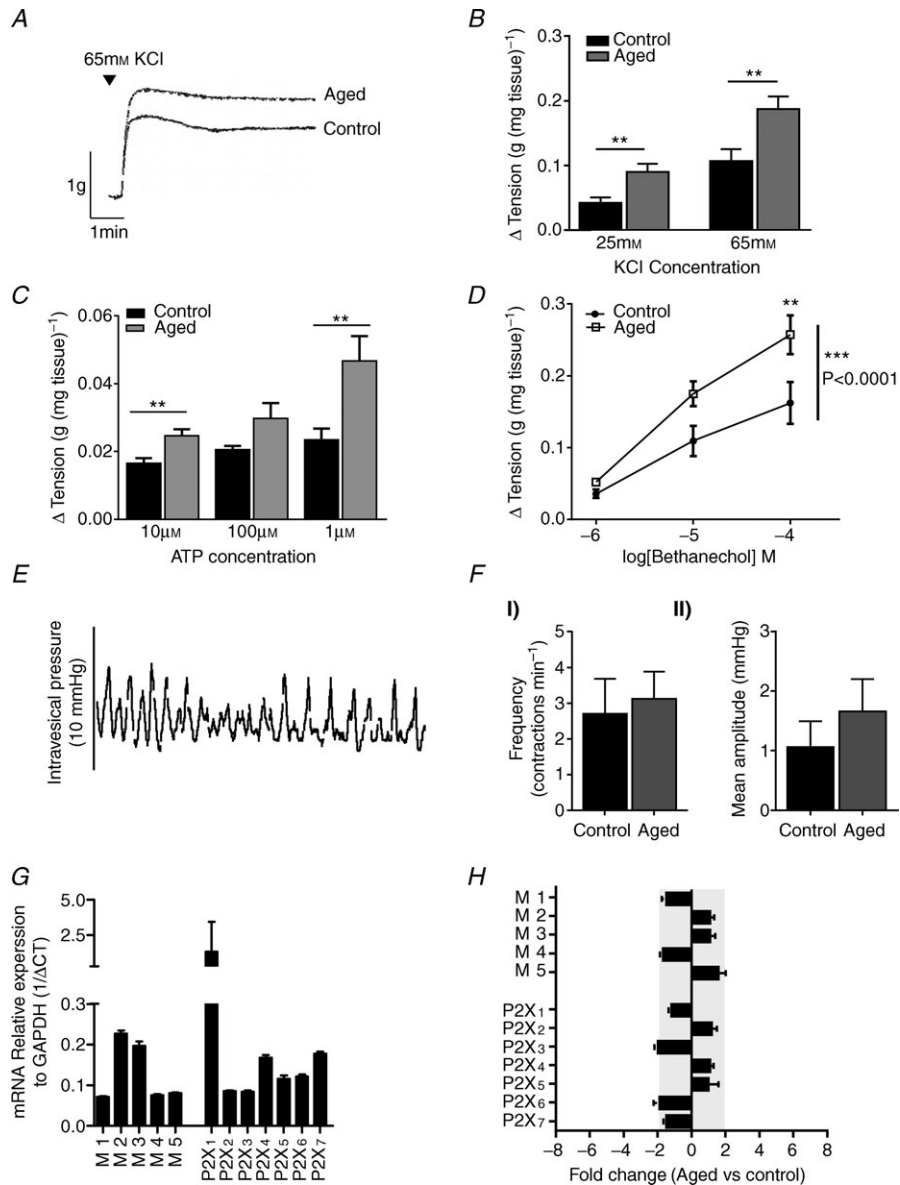
innervation from the bladder is conveyed by the pelvic and hypogastric nerves. Distinct subpopulations of these afferents innervate the muscle, urothelial, suburothelial and serosal layers of the bladder and detect changes in mechanical or chemical environment in the bladder wall



(Zagorodnyuk *et al.* 2007). Changes in the function of any of these components could alter sensory nerve transduction. In this study we sought to examine how ageing affects bladder afferent activity and the function of the detrusor and urothelium.

**Ageing increases bladder mechanosensitivity**

There are relatively few studies that have directly investigated the effect of ageing on bladder sensory pathways. One previous study reported an age-related reduction in the afferent response to bladder filling



**Figure 3. The effect of ageing on detrusor function and gene expression**  
 A, sample trace showing the contractile response to 65 mm KCl in aged and control muscle strips. B, contractile responses to 25 and 65 mm KCl application in denuded muscle strips from aged and control mice. In aged muscle strips contractile response were significantly greater than controls ( $n = 6$ ,  $P < 0.005$ , Student's  $t$  test). C, contractile responses to ATP application in denuded muscle strips from aged and control mice ( $n = 6$ ,  $P < 0.005$ , Student's  $t$  test). D, contractile responses to cumulative application of bethanechol in denuded muscle strips from aged and control mice ( $n = 6$ ,  $P < 0.0001$ , two-way ANOVA and Bonferoni *post hoc* test). E, sample trace showing whole bladder spontaneous contractions. F, frequency and amplitude of spontaneous contractions in bladders from control and aged mice. Spontaneous activity was not affected by ageing ( $n = 6$  and  $n = 8$ ). G, mRNA expression for muscarinic and purinergic receptors in control detrusor samples relative to the housekeeping gene GAPDH ( $n = 9$ ). H, fold change in gene expression between control and aged detrusor samples. No significant difference in gene expression was observed ( $n = 9$  and  $n = 6$ ).



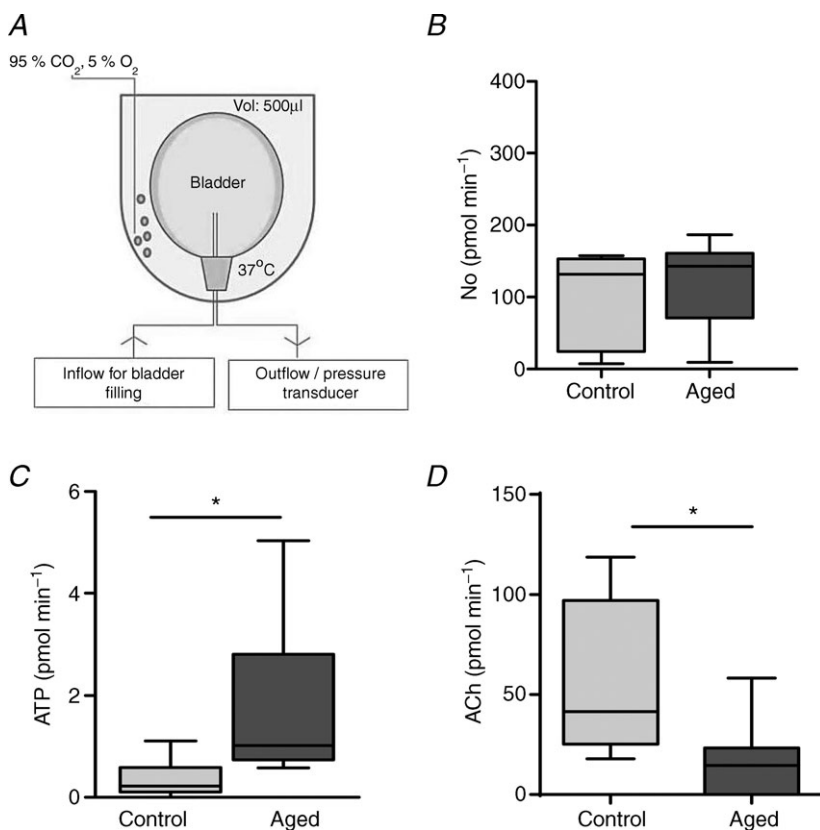
(Hotta *et al.* 1995). However, conversely, a more recent study found increased voiding frequency and higher afferent nerve discharge in aged rats than in younger controls (Aizawa *et al.* 2012). However, both of these previous studies used paradigms in which animals were anaesthetised. As it is not clear what effect this could have on afferent signalling, interpretation of these data is difficult. In this current study we used both voiding pattern analysis (in awake, unanaesthetised freely moving mice), which has been previously shown to provide an accurate assessment of *in vivo* bladder function and correspond to cystometry (Hodges *et al.* 2008), together with direct recordings of pelvic and hypogastric bladder afferents *in vitro*. We identified a significant difference in the voiding patterns of aged and control mice, which indicate that aged mice void smaller volumes of urine more frequently than younger controls. Although fluid intake was not recorded, urine osmolality was unchanged, suggesting that the altered voiding patterns relate to changes in micturition rather than increased urine production or altered renal function. Moreover, this increase in voiding activity *in vivo* was concurrent with a significant increase in spontaneous nerve firing and in low-threshold mechanosensitivity.

There are a number of components that could contribute to this increased mechanosensitivity. (1) Changes in sensory nerve morphology and function. In

previous studies some moderate changes due to ageing have been described but the general pattern of innervation seems to be conserved with age (Nakayama *et al.* 1998; Mohammed & Santer, 2002; Aizawa *et al.* 2010). One previous study found a 40% reduction in calcitonin gene-related peptide and substance P containing afferents in the DRG of aged rats (Mohammed & Santer, 2002). However, bladder projecting afferents were not identified specifically, and as yet, there are few data concerning how the exact properties of bladder projecting nerves are affected by age. (2) Altered contractility and changes in detrusor tone. Approximately 30% of hypogastric and 80% of pelvic innervation from the bladder arises from afferents whose terminals lie within the muscle layers (Xu & Gebhart, 2008), and thus altered detrusor activity may indirectly influence bladder mechanosensitivity. (3) Changes in urothelial function: the urothelium has a well-established role in modulating neural excitability, and thus changes in urothelial function could indirectly alter afferent excitability.

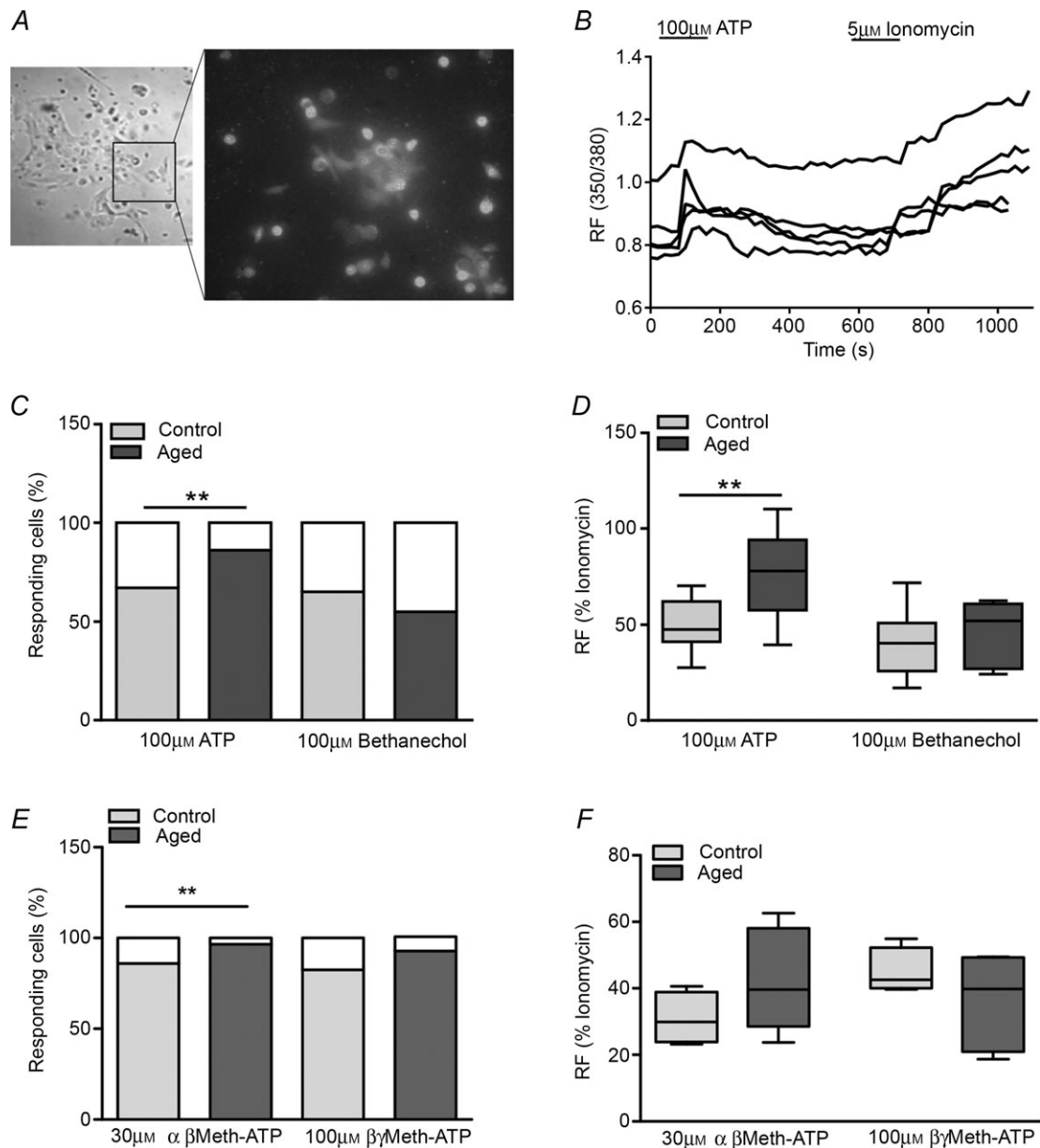
#### Ageing increases contractility of the bladder

Urodynamic studies in humans suggest that ageing is associated with overactivity of the detrusor muscle during bladder filling and storage but diminished detrusor contractility during bladder voiding (Resnick



**Figure 4. The effect of ageing on transmitter releases from the urothelium**

A, schematic diagram of the isolated whole-bladder model used to collect intraluminal samples for analysis of urothelially released NO ATP and ACh. B, NO release from aged ( $n = 8$ ) and control ( $n = 7$ ) mice. Release of NO was not affected by ageing. C, ATP release from aged ( $n = 8$ ) and control ( $n = 7$ ) mice. ATP release was significantly higher in aged bladders than in controls ( $P < 0.04$ , Student's  $t$  test). D, ACh release from aged ( $n = 8$ ) and control ( $n = 7$ ) mice urothelium. In aged bladders, ACh release was significantly lower compared to controls ( $P < 0.02$ , Student's  $t$  test).



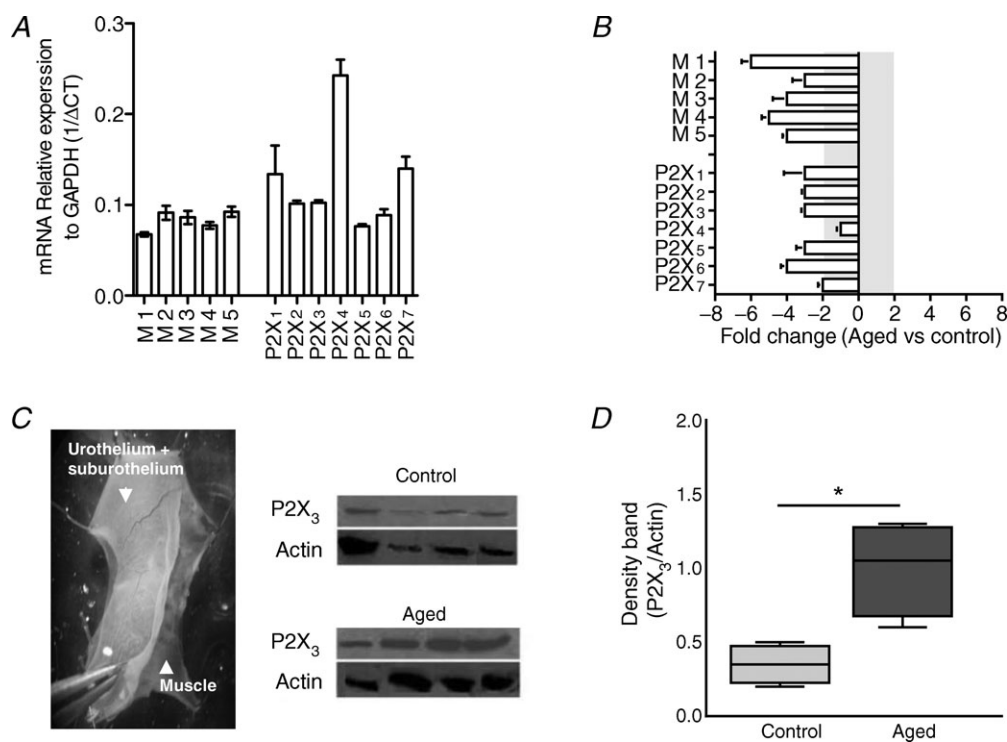
### Figure 5. The effect of ageing on purinergic receptor signalling in the urothelium

**A**, example of primary urothelial cells cultured from aged and control mice and loaded with the calcium-sensitive dye Fura-2AM ( $2 \mu\text{M}$ ). **B**, sample traces showing typical urothelial cell response to ATP ( $100 \mu\text{M}$ ) and the ionophore ionomycin ( $5 \mu\text{M}$ ). **C**, the number of cells responding to application of the purinergic receptor agonist ATP ( $100 \mu\text{M}$ ) and the muscarinic receptor agonist bethanechol ( $100 \mu\text{M}$ ). In aged mice ( $N = 4$ ,  $n = 333$ ), the number of urothelial cells responding to purinergic receptor stimulation with ATP was significantly greater compared to controls ( $N = 5$ ,  $n = 241$ ,  $P < 0.001$ , Student's  $t$  test). However, responses to the muscarinic receptor agonist bethanechol were unchanged. **D**, the magnitude of the response to ATP and ACh. Responses to ATP stimulation were significantly higher in aged urothelial cells compared to controls ( $P < 0.001$  Student's  $t$  test), indicating age-related changes in purinergic receptor signalling. In contrast, urothelial cells from aged mice ( $N = 4$ ,  $n = 226$ ) had similar responses to bethanechol as urothelial cells from controls ( $N = 3$ ,  $n = 190$ ) suggesting that muscarinic receptor signalling in the urothelium is unaffected by aging. **E**, the number of cells responding to application of the P2X<sub>1</sub> and P2X<sub>3</sub> agonist  $\alpha\beta$ methyleneATP ( $30 \mu\text{M}$ ) and the selective P2X<sub>1</sub> agonist  $\beta\gamma$ methyleneATP ( $100 \mu\text{M}$ ). There was no significant difference in urothelial cell responses to  $\beta\gamma$ methyleneATP between cells from aged and control mice ( $N = 5$ ,  $n = 239$  and  $N = 4$ ,  $n = 189$ , respectively) although significantly more cells from aged bladders responded to  $\alpha\beta$ methyleneATP compared to cells from control mice ( $N = 5$ ,  $n = 218$  and  $N = 4$ ,  $n = 175$ , respectively,  $P < 0.001$  Student's  $t$  test). **F**, the magnitude of the response to  $\alpha\beta$ methyleneATP and to  $\beta\gamma$ methyleneATP. Responses to  $\alpha\beta$ methyleneATP were greater in aged urothelial cells compared to controls, although this did not reach significance.  $N$  = number of mice,  $n$  = number of cells. RF, ratio fluorescence.

& Yalla, 1987; Madersbacher *et al.* 1998; Nordling, 2002). Whether this generates hypersensitivity of bladder afferents during the storage phase, driving the sensory symptoms associated with OAB (urgency and frequency), has yet to be established. Moreover, morphological studies seem consistent with age-related changes in the detrusor, showing that with age there is increased collagen deposition but reduced muscle mass and innervation (Lluel *et al.* 2000; Smith *et al.* 2012). In this study we did not examine bladder morphology, but no difference in detrusor weight between control and aged tissue was observed, suggesting that in our hands aged mice appear to have no significant difference in detrusor muscle mass.

There have been some previous muscle bath studies investigating contractility in ageing, but these studies have used a variety of animal models (guinea pig, rat, mouse, pig and human), different age ranges (2–30 months), different sexes and different experimental protocols, which have led to conflicting results. Some studies indicate decreased in contractility with age, and others suggest increased in contractility or no functional alterations whatsoever (Longhurst *et al.* 1992; Munro & Wendt,

1993; Saito *et al.* 1993; Lieu *et al.* 1997; Pagala *et al.* 2001; Yoshida *et al.* 2001; Gomez-Pinilla *et al.* 2011). To examine how muscle function was affected by age in this study, we measured passive bladder compliance *in vitro*, studied contractility using muscle strip experiments and measured whole-bladder spontaneous contractions. Passive bladder compliance and the amplitude and frequency of spontaneous bladder contractions were not significantly different between control and aged mice. Moreover, cholinergic and purinergic gene expression in the detrusor was also unaltered. Interestingly, stimulation of denuded muscle strips with KCl produced greater contractions in aged tissues, suggesting enhanced contractility. This enhanced contractility was also seen in response to muscarinic and purinergic receptor stimulation, suggesting that in our aged mice detrusor contractility was generally increased but that bladder compliance was unaltered. This is in contrast to a recent study by Smith *et al.* (2012) that found increased bladder compliance in aged female mice and impaired responses to bladder filling, but no change in detrusor power or contractile force during voiding (Smith *et al.* 2012). As



**Figure 6. The effect of ageing on gene and protein expression in the mucosa**

A, mRNA expression for muscarinic and purinergic receptors in control mucosal samples relative to the housekeeping gene GAPDH (M1  $n = 4$ , M2–5  $n = 8$ , P2X<sub>1–7</sub>  $n = 8$ ). B, fold change in gene expression between control and aged mucosal samples. A significant reduction in all muscarinic receptor and all P2X receptor genes (except P2X<sub>4</sub>) was seen in aged mucosa relative to controls ( $n = 6$  and  $n = 8$ , respectively). C, mucosal layers were separated from the muscle by blunt dissection under the microscope. Western blot showing the expression of P2X<sub>3</sub> receptors in aged and control mucosal samples. D, relative protein expression compared to  $\beta$ -actin. There was significantly more P2X<sub>3</sub> receptor expression in the mucosa from aged mice ( $n = 4$ ) compared to controls ( $n = 4$ ,  $P < 0.01$ , Student's  $t$  test).

voiding responses were not measured in the present study, it is difficult to ascertain whether the same was true in our aged mice; however, it is important to remember that the *in vivo* situation, where the efferent pathways involved in voiding are intact, may be very different to the *in vitro* where all efferent influences are removed. Moreover the study by Smith *et al.* was performed under anaesthesia, which may inhibit afferent pathways or affect central control centres. In this study we decided to use male mice to avoid complications arising due to hormonal changes, parturition and fertility senescence, these factors could also contribute to the differences seen between this and the previous study by Smith and colleagues.

Together, these data suggest that aged mice exhibit some moderate changes in agonist-induced muscle contraction; however, spontaneous contractions and bladder tone were not significantly changed with age, suggesting that altered afferent activity during bladder filling may also be driven either by changes in nerve function or by alterations in the urothelium.

### Ageing alters purinergic signalling in the urothelium

It is now well established that the urothelium contributes to afferent pathways as it is able to sense and monitor mechanical and chemical changes in the bladder wall and modulate afferent firing via the release of a host of excitatory and inhibitory neurotransmitters. It has also been shown that in disease states such as OAB urothelial transmitter release is altered (Chuang, 2009). Yoshida *et al.* (2004) showed that increasing age was associated with a decrease in ACh release and an increase in ATP release from the human bladder. In this study, we saw the same trend in the aged mouse. However, we measured the concentration of these neurotransmitters in the lumen of the intact bladder, and thus it is not clear exactly which cells release these agents, whether it is release or breakdown of the molecules that is changed, or the exact concentration of these mediators in the urothelium/suburothelium and at the nerve terminal. Nevertheless, it is tempting to speculate that this increase in luminal ATP concentration corresponds to changes in transmitter release from the urothelium. Previous studies have clearly shown that ATP released from the urothelium acts at afferent terminals to alter nerve activity, although the mode of action of urothelially released ACh is controversial. It could play a role in detrusor contraction; however, the plexus of blood vessels which lie in the suburothelium are likely to act as a diffusion barrier preventing it from reaching the muscle layers, which would suggest that, instead, urothelially released ACh acts at the urothelium (via autocrine or paracrine mechanism) or on the afferent terminals to alter urothelial-afferent signalling.

In calcium imaging experiments ageing had no effect on urothelial responses to the muscarinic agonist

bethanechol, but clear differences were seen in purinergic receptor-mediated calcium signals. Both the maximal calcium signal and the number of urothelial cells responding to ATP were increased in urothelial cells from aged mice. This altered purinergic signalling is likely to be mediated via the P2X<sub>3</sub> receptor as studies with the selective P2X<sub>1</sub> and P2X<sub>3</sub> agonist produced greater signals in cells from aged mice but the selective P2X<sub>1</sub> agonist did not. These findings may explain the hyperactivity seen in the electrophysiology and voiding pattern studies. Increased urothelial cell excitability could enhance afferent activity either via a direct communication with the afferent terminal or via increased release of excitatory neurotransmitters such as ATP, which then acts downstream at the afferent terminal to enhance mechanosensitivity and/or sensory excitability.

Western blot analysis identified higher P2X<sub>3</sub> receptor expression in aged mucosa compared to controls. Paradoxically, this increased receptor expression was concurrent with the increased ATP release and may suggest that the feedback mechanisms normally in place to induce receptor down-regulation are aberrant. Moreover, qRT-PCR found a general reduction in purinergic and cholinergic receptor gene expression despite an increased protein expression, suggesting that in ageing, mechanisms involved in P2X<sub>3</sub> receptor turnover in the urothelium could be altered. It is also important to bear in mind that within the mucosal layers there are also afferent and efferent nerve terminals that would express the P2X<sub>3</sub> receptor protein contributing to the overall mucosal expression; however, as the cell bodies of these nerves are located elsewhere, gene expression would not be affected. Thus, the increased P2X<sub>3</sub> receptor expression in aged samples could also indicate changes in purinergic receptor expression on nerve terminals in addition to or instead of the other cell types within the mucosa. Unfortunately, it is not possible to identify where P2X<sub>3</sub> receptor expression is increased from this study.

In this study we have focused mainly on urothelial signalling but it is important to note that downstream signalling events at the level of the afferent terminal (including receptor expression), neuronal excitability and central processing in the CNS could also be disrupted in ageing. Further characterisation of these pathways may provide greater insight into how bladder physiology alters with age.

### Conclusion

It is clear that the incidence of bladder conditions such as OAB increases in line with advancing age for both males and females (Milsom *et al.* 2001; Irwin *et al.* 2006), although the aetiopathogenesis of this is unclear. Our data are consistent with the hypothesis that purinergic signalling in the bladder is altered with



age. In this study we show that ageing causes enhanced bladder activity and peripheral sensory transmission in the mouse. This is associated with altered purinergic signalling in the urothelium via the P2X<sub>3</sub> receptor, increased P2X<sub>3</sub> protein expression and increased overflow of ATP. However, more studies are still required to fully validate this hypothesis. Understanding how the urothelium and afferent mechanisms are affected by ageing may yield a better understanding of normal and aberrant bladder function and could potentially reveal novel targets for the treatment/prevention of bladder conditions in humans.

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## Additional information

### Competing interests

C.C. is an advisor/consultant for Astellas, Pfizer, Allergan, Recordati, Lilly, ONO and Xention.

### Author contributions

All experiments were performed in Professor Grundy's laboratory at the University of Sheffield except for the muscle

bath experiments which were performed in Dr McKay's laboratory at Hallam University. D.M.D., L.N. and D.G. were responsible for the conception and design of experiments. D.M.D., L.N. and M.L. were responsible for the collection, analysis and interpretation of data and all authors were involved in drafting the article and revising it critically for important intellectual content. All authors approved the final version of the manuscript and all persons designated as authors qualify for authorship.

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## Translational perspectives

OAB and in particular urinary incontinence pose no risk to life expectancy, but they severely impair patient quality of life and dramatically increase in incidence with age. In particular, they are major contributing factors to debilitation and medical complications in the institutionalisation of the elderly. OAB is characterised by the cardinal symptom of urgency, which is a compelling desire to void and is the consequence of a combination of bladder sensory dysfunction and loss of central neurological control of the pontine micturition centre. In 40–60% of women and 60–90% of men there is associated overactivity of the bladder. As OAB is a symptom syndrome it is not possible to model the condition in animals, although in this study we show that naturally aged mice exhibit hypersensitivity and hyperactivity similar to that observed in humans. We believe that these mice provide a unique tool to study altered bladder physiology resulting from ageing as well as insight into both peripheral and central neurosensorimotor mechanisms underlying bladder overactivity. It has been known for some time that the urothelium/suburothelium together with the sensory nerves play a key role in normal bladder function. However, our data also suggest that changes in urothelial function and purinergic signalling may underlie age-associated bladder-related symptoms such as OAB. Understanding how these changes alter bladder function and identifying the exact mechanisms involved may provide new targets for selective and effective drug therapies.