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Purinergic signalling underlies transforming growth factor-β-mediated bladder afferent nerve hyperexcitability

Eric J. Gonzalez[1], Thomas J. Heppner[2], Mark T. Nelson[2,3] and Margaret A. Vizzard[1]

[1] *Department of Neurological Sciences, University of Vermont College of Medicine, Burlington, VT 05405, USA*
[2] *Department of Pharmacology, University of Vermont College of Medicine, Burlington, VT 05405, USA*
[3] *Institute of Cardiovascular Sciences, University of Manchester, Manchester, UK*

Key points

- The sensory components of the urinary bladder are responsible for the transduction of bladder filling and are often impaired with neurological injury or disease.
- Elevated extracellular ATP contributes, in part, to bladder afferent nerve hyperexcitability during urinary bladder inflammation or irritation.
- Transforming growth factor-β1 (TGF-β1) may stimulate ATP release from the urothelium through vesicular exocytosis mechanisms with minimal contribution from pannexin-1 channels to increase bladder afferent nerve discharge.
- Bladder afferent nerve hyperexcitability and urothelial ATP release with CYP-induced cystitis is decreased with TGF-β inhibition.
- These results establish a causal link between an inflammatory mediator, TGF-β, and intrinsic signalling mechanisms of the urothelium that may contribute to the altered sensory processing of bladder filling.

Abstract The afferent limb of the micturition reflex is often compromised following bladder injury, disease and inflammatory conditions. We have previously demonstrated that transforming growth factor-β (TGF-β) signalling contributes to increased voiding frequency and decreased bladder capacity with cystitis. Despite the functional presence of TGF-β in bladder inflammation, the precise mechanisms of TGF-β mediating bladder dysfunction are not yet known. Thus, the present studies investigated the sensory components of the urinary bladder that may underlie the pathophysiology of aberrant TGF-β activation. We utilized bladder–pelvic nerve preparations to characterize bladder afferent nerve discharge and the mechanisms of urothelial ATP release with distention. Our findings indicate that bladder afferent nerve discharge is sensitive to elevated extracellular ATP during pathological conditions of urinary bladder inflammation or irritation. We determined that TGF-β1 may increase bladder afferent nerve excitability by stimulating ATP release from the urothelium via vesicular exocytosis mechanisms with minimal contribution from pannexin-1 channels. Furthermore, blocking aberrant TGF-β signalling in cyclophosphamide-induced cystitis with TβR-1 inhibition decreased afferent nerve hyperexcitability with a concomitant decrease in urothelial ATP release. Taken together, these results establish a role for purinergic signalling mechanisms in TGF-β-mediated bladder afferent nerve activation that may ultimately facilitate increased voiding frequency. The synergy between intrinsic urinary bladder signalling mechanisms and an inflammatory mediator provides novel insight into bladder dysfunction and supports new avenues for therapeutic intervention.

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Corresponding author M. A. Vizzard: University of Vermont College of Medicine, Department of Neurological Sciences, D405A Given Research Building, Burlington, VT 05405, USA. Email: margaret.vizzard@uvm.edu

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Abbreviations BFA, Brefeldin A; CYP, cyclophosphamide; Det, detrusor smooth muscle; ICC, interstitial cells of Cajal; P2X/P2Y, purinergic receptor; Panx1, pannexin 1; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; PSS, physiological saline solution; TGF-β, transforming growth factor-β; TβR, transforming growth factor-β receptor.

Introduction

The transitional epithelial cells that line the bladder lumen are now recognized to act as a passive barrier to urinary metabolites (Min *et al.* 2003) and contribute to the sensory transduction of bladder filling (Birder, 2005). These cells, termed the urothelium, respond to mechanical (Olsen *et al.* 2011) or chemical (Everaerts *et al.* 2010) stimuli by releasing urinary proteins (Deng *et al.* 2001) or neuroactive signalling molecules (Ferguson *et al.* 1997; Birder *et al.* 2002; Hanna-Mitchell *et al.* 2007; Nile & Gillespie, 2012) from their apical and basolateral surfaces (Lewis & Lewis, 2006). The release of these mediators presumably permits the urothelium to communicate with nerve endings (Morrison, 1999; Andersson, 2002) and other cell types such as interstitial cells of Cajal (ICC) (Sui *et al.* 2008; Drumm *et al.* 2014) or smooth muscle cells (Hashitani *et al.* 2004) that are proximal to the mucosa. Given the role that the urothelium has in bladder sensation, any disruption to these signalling mechanisms, such as what may occur with inflammation (de Groat & Yoshimura, 2009), may contribute to the development of lower urinary tract symptoms (Gonzalez *et al.* 2014*b*).

Purinergic signalling has garnered much of the focus in bladder sensory processing ever since the urothelium was discovered to release ATP (Ferguson *et al.* 1997). The mechanisms of ATP release from the urothelium have since been demonstrated to include vesicular exocytosis (Wang *et al.* 2005; Sui *et al.* 2014; McLatchie & Fry, 2015), ion channels (Wang *et al.* 2005; Sui *et al.* 2014; Beckel *et al.* 2015; McLatchie & Fry, 2015) and transporters (Wang *et al.* 2005). Extracellular ATP and other associated nucleotides may then bind to purinergic receptors located on various cell types (e.g. urothelial (Tempest *et al.* 2004; Chopra *et al.* 2008), smooth muscle (Heppner *et al.* 2005) and ICC (Wu *et al.* 2004)) or nerve terminals (Namasivayam *et al.* 1999; Andersson, 2002) to activate autocrine or paracrine pathways and facilitate bladder sensory signalling (Birder & Andersson, 2013). Purinergic signalling in these cell types has been demonstrated to increase sensory neuron excitability (Chen *et al.* 1995) and to modulate smooth muscle tone (Sui *et al.* 2014). Furthermore, aberrant purinergic signalling in the urothelium has been suggested to contribute to bladder dysfunction in injury or disease since ATP release is increased in many functional disorders of the urinary bladder (Sun *et al.* 2001; Ruggieri, 2006; Sun & Chai, 2006; Silva-Ramos *et al.* 2013).

In addition to alterations in the intrinsic properties of the urothelium, functional disorders of the bladder that activate an inflammatory response may also involve an upregulation of proinflammatory mediators (Gonzalez *et al.* 2014*a*). Previous studies from our laboratory have established the roles of cytokines (Malley & Vizzard, 2002) and chemokines (Arms *et al.* 2010, 2013) in the maintenance and development of lower urinary tract symptoms. In particular, we have demonstrated that transforming growth factor-beta (TGF-β) overexpression contributes to the afferent limb of the micturition reflex with cyclophosphamide (CYP)-induced cystitis (Gonzalez *et al.* 2013). In addition to its role(s) in cystitis, increased urinary TGF-β has also been observed in diabetic nephropathy (Chen *et al.* 2003) and other nephropathic conditions (Goumenos *et al.* 2002; De Muro *et al.* 2004) with bladder dysfunction (Golbidi & Laher, 2010). Despite its functional presence in the bladder inflammatory milieu, the precise mechanisms of TGF-β mediating voiding dysfunction are not yet known. Therefore, we hypothesized that TGF-β signalling contributes to afferent nerve excitability through purinergic mechanisms that may ultimately facilitate increased voiding frequency. A comprehensive understanding of the downstream signalling effectors that interact in the afferent limb of the micturition reflex with bladder dysfunction will allow for novel therapeutic approaches to improve the quality of life of these patients.

Methods

Ethical approval

Experimental procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (protocol 08–055) and experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (8th edn).

Animals

Male C57Bl/6 mice (3–6 months old) purchased from Jackson Laboratories (Bar Harbor, ME, USA) were housed with littermates and maintained in standard laboratory conditions with food and water available *ad libitum*.

Induction of CYP-induced cystitis

Male C57Bl/6 J mice ($n = 4$) received a 150 mg kg^{-1} I.P. injection of CYP (Sigma-Aldrich, St. Louis, MO, USA). Mice were harvested 48 h following CYP treatment for bladder–pelvic nerve electrophysiology or ATP release experiments.

Bladder–pelvic nerve electrophysiology

The bladder–pelvic nerve model was developed and described in detail by Namasivayam *et al.* (1998). Briefly, male C57Bl/6 J mice ($n = 3$–4) were killed by an injection of pentobarbital sodium (I.P.) followed by decapitation. The abdominal cavity was opened by a midline incision and the urinary bladder and surrounding tissues (urethra, ureters, major pelvic ganglia, postganglionic nerves and pelvic nerves) were excised and transferred to ice-cold dissection solution. The ureters were isolated, dissected and ligated with nylon suture adjacent to the bladder wall. The pelvic nerves were isolated and the ends were cleaned of connective tissue to facilitate recording. The urinary bladder, urethra, ureters and pelvic nerves were then transferred to a recording chamber on a vibraplane platform and superfused with biological atmosphere gas (20% O_2, 5% CO_2, 75% N_2) in physiological saline solution (PSS) circulating at 37°C. One arm of a triple lumen cannula was inserted through the urethra into the bladder and ligated. The remaining arms were attached to a remote-controlled syringe pump and pressure transducer to monitor intravesical pressure or used to empty the bladder. One of the pelvic nerves was attached to a suction electrode to record distention-evoked multifibre afferent nerve activity. With this recording arrangement, we did not identify the relative contribution(s) of myelinated Aδ fibres and the normally quiescent umyelinated C fibres. Vehicle or drugs were administered at a rate of 30 μl min^{-1} intravesically up to 25 mmHg for the filling phase and then manually emptied for 5–6 cycles each. There was a 10 min rest period between the emptying phase and the start of the next filling phase. For the co-administration studies, bladders were first pretreated with the various inhibitors followed by the inhibitors plus TGF-β1. Administration of drugs began once bladder afferent nerve activity to the vehicle plateaued for two consecutive filling phases (usually after 5–6 filling cycles). Reliable nerve responses to repeated distention could be maintained throughout the course of the experiment (8 h). Bladder afferent nerve activity was collected and amplified with a Neurolog head stage (NL104, Digitimer), filtered (band pass 200–4000 Hz) using a Digitimer NL125/Nl126 filter and digitized with a Power 1401 analog to digital interface (Cambridge Electronic Design, Cambridge, UK). The acquisition rates for nerve activity and bladder pressure were 25,000 Hz and 100 Hz, respectively. Data were analysed offline via Spike 2 software (version 5.11, Cambridge Electronic Design).

Analysis

Action potentials were determined by using twice the root mean square amplitude of the recording signal for detection threshold. Action potential events were quantified from 0 to 25 mmHg and graphed in 5 mmHg increments to determine activity throughout multiple pressures in the filling phase. The mean frequency (impulses s^{-1}) graphed for each condition (vehicle or drug) per animal was the average of two consecutive filling phases. A two-way repeated measures ANOVA followed by Sidak's multiple comparisons test was then performed to compare these frequency group means. Fold change group means were compared with Student's unpaired *t* test.

ATP release

Male C57Bl/6 J mice ($n = 4$–8) were anaesthetized with 2% isoflurane and killed by decapitation. The abdominal cavity was opened by a midline incision and the urinary bladder, urethra and ureters were excised and transferred to ice-cold dissection solution. The ureters were isolated, dissected and ligated with nylon suture adjacent to the bladder wall. The urinary bladder, urethra and ureters were then transferred to a recording chamber with oxygenated (95% O_2–5% CO_2) PSS circulating at 37°C and cannulated through the urethra with a 22 gauge blunt needle. The cannula was attached to a remote-controlled syringe pump and a pressure transducer to monitor intravesical pressure. Vehicle or drugs were administered at a rate of 30 μl min^{-1} intravesically up to 25 mmHg for the filling phase and then manually emptied via a three-way stop cock. There was a 10 min rest between the emptying phase and the start of the next filling phase. After 1–1.5 h of vehicle or drug administration, the instillate was collected from two separate but consecutive emptying cycles and immediately flash frozen until ATP analysis. Upon collection, CYP instillate samples also received adenylyl-imidodiphosphate (AMP-PNP, 200 μM) to limit ATP hydrolysis that may be present from a compromised barrier. ATP quantification was determined with the ATP bioluminescent assay kit (Sigma-Aldrich) following their procedural instructions with the exception of halving the recommended concentration of reagents and samples. ATP measurements were taken with the BioTek H4 Plate Reader (Winooski, VT, USA) within the UVM Advanced Genome Technologies Core.

Analysis

The bioluminescence emitted by the sample was plotted against the calibration curve to determine the final concentration (pmol ml^{-1}) of ATP. Calibration curves were not affected by the drugs used in the current study (data not shown). Statistical analysis was performed on the ATP concentration (pmol ml^{-1}) of the instillate before or after drug administration as Student's paired *t* test for all group means.

Materials

The dissection solution consisted of (mM): 55 NaCl, 6 KCl, 80 MSG, 10 Hepes, 2 $MgCl_2$, 10 glucose,

and adjusted to pH 7.3. The PSS consisted of (mM): 119 NaCl, 4.7 KCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 7 glucose, and adjusted to pH 7.4. Recombinant mouse transforming growth factor-β1 (TGF-β1, R&D Systems, MN, USA) was reconstituted to 50 μg ml^{-1} in 4 mM HCl and 0.1% BSA and stored at -20°C. Bladder tissues respond to a range of TGF-β1 concentrations (Hiti *et al.* 1990; Duan *et al.* 2015) and 10 ng ml^{-1} was selected for the working concentration as it does not induce epithelial–mesenchymal transition (EMT) related genes in the urothelium (Islam *et al.* 2014). SB505124 (R&D Systems) was reconstituted to 100 mM in DMSO (99.5%) and stored at -20°C. SB505124 is a potent inhibitor of TGF-β type I receptor kinase activity with an IC_{50} of 47 ± 5 nM (DaCosta Byfield *et al.* 2004). Before its use, stock solutions were diluted to a working concentration (5 μM) with PSS. Concentrations up to 5 μM maintain inhibition specificity of downstream targets of TβR-1 and other closely related receptors and have been demonstrated to decrease voiding frequency with cystitis (DaCosta Byfield *et al.* 2004; Gonzalez *et al.* 2013). Brefeldin A (BFA, R&D Systems) was reconstituted to 50 mM in DMSO (99.5%) and stored at -20°C. Stock solutions were diluted to a working concentration (10 μM) with PSS to remain consistent with previous literature demonstrating decreased ATP release from the urothelium (Sui *et al.* 2014; McLatchie & Fry, 2015). 10Panx (R&D Systems) was reconstituted to 1 mg ml^{-1} in PSS and stored at -20°C. The IC_{50} of 10Panx for the inhibition of panx1 currents in over-expressed panx1 HEK cells is 52 ± 12 μM (Pelegrin & Surprenant, 2006). We utilized 10Panx at a working concentration of 50 μM because intravesical 10Panx near the IC_{50} concentration was functional in the urinary bladder to decrease voiding frequency (Timoteo *et al.* 2014). Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt (PPADS, R&D Systems) was reconstituted to 100 mM in distilled water and stored protected from light at -20°C to prevent photo-decomposition. A working concentration of 300 μM was selected for PPADS because Vlaskovska *et al.* (2001) reported decreased distention-evoked afferent nerve discharge in control tissues that was not observed at lower concentrations (Yu & de Groat, 2008).

Statistics

All values represent means ± SEM. Outliers were identified using the extreme studentized deviate test on GraphPad Prism (v. 6.07, La Jolla, CA, USA) and excluded from further analysis. Data were then compared with two-way repeated measures ANOVA and two-tailed Student's unpaired or paired *t* test where appropriate. When the *F* test statistic exceeded the critical value at $\alpha = 0.05$, the Sidak's multiple comparisons test was used to compare group means.

Results

TGF-β1 increased bladder afferent nerve discharge and ATP release from the urothelium

The continuous intravesical instillation of recombinant TGF-β1 (10 ng ml^{-1}) had a significant ($P = 0.0447$) treatment effect on mean bladder afferent nerve frequency (impulses s^{-1}) (Fig. 1*A* and *B*). Nerve firing increased (50.5 ± 9.9 to 71.6 ± 13.3 impulses s^{-1}, $n = 4$) by 41.8% from 0 to 25 mmHg with TGF-β1 instillation. More specifically, nerve discharge significantly increased from 5 to10 ($P = 0.0337$), 10 to 15 ($P = 0.0003$), 15 to 20 ($P \leq 0.0001$) and 20 to 25 ($P \leq 0.0001$) mmHg (Fig. 1*B*). Distention-evoked ATP release from the urothelium at 25 mmHg was also significantly ($P = 0.0066$) increased with the intravesical instillation of TGF-β1 (Fig. 1*C*).

The concurrent intravesical instillation of TGF-β1 and SB505124 (5 μM), a TGF-β type I receptor inhibitor, attenuated ($P = 0.4396$) afferent nerve hyper-excitability (76.8 ± 17.7 to 61.8 ± 12.9 impulses s^{-1}, $n = 4$) from 0 to 25 mmHg (Fig. 2*C* and *D*). SB505124 co-administration also attenuated ($P = 0.3245$) TGF-β1-mediated distention-evoked urothelial ATP release at 25 mmHg (Fig. 2*E*). The intravesical administration of SB505124 (5 μM) alone did not have a significant ($P = 0.6506$) treatment effect on mean nerve firing (76.8 ± 17.7 to 63.9 ± 13.6 impulses s^{-1}, $n = 4$) from 0 to 25 mmHg (Fig. 2*A* and *B*). Furthermore, SB505124 did not have a significant ($P = 0.0782$) effect on distention-evoked urothelial ATP release at 25 mmHg (4.8 ± 1.7 to 7.2 ± 2.5 pmol ml^{-1}, $n = 4$).

TGF-β1 stimulated ATP release from the urothelium through vesicular exocytosis mechanisms to mediate bladder afferent nerve hyperexcitability

Vesicular ATP release. The continuous intravesical instillation of BFA (10 μM) did not have a significant ($P = 0.8960$) treatment effect on mean bladder afferent nerve firing (68.7 ± 12.6 to 58.7 ± 15.1 impulses s^{-1}, $n = 3$) from 0 to 25 mmHg (Fig. 3*A* and *B*). Similarly, the intravesical co-administration of recombinant TGF-β1 (10 ng ml^{-1}) and BFA (10 μM) did not have a significant ($P = 0.5646$) treatment effect on mean nerve firing (68.7 ± 12.6 to 63.4 ± 21.4 impulses s^{-1}, $n = 3$) from 0 to 25 mmHg (Fig. 3*C* and *D*). The intravesical co-administration of BFA also attenuated ($P = 0.2155$) TGF-β1-mediated distention-evoked urothelial ATP release at 25 mmHg (Fig. 3*E*).

Pannexin-1 channel ATP release. The continuous intravesical instillation of 10Panx (50 μM) did not have a significant (P = 0.1340) treatment effect on mean bladder afferent nerve firing (41.9 ± 1.8 to 42.8 ± 3.9 impulses s^{-1}, n = 4) from 0 to 25 mmHg (Fig. 4*A* and *B*). The intravesical co-administration of recombinant TGF-β1 (10 ng ml^{-1}) and 10Panx (50 μM), however, had a significant (P = 0.0213) treatment effect on mean nerve frequency with nerve firing increased (41.9 ± 1.8 to 50.1 ± 3.4 impulses s^{-1}, n = 4) by 19.4% from 0 to 25 mmHg (Fig. 4*C* and *D*). More specifically, nerve discharge significantly increased from 10 to 15 (P = 0.0014), 15 to 20 ($P \leq$ 0.0001) and 20 to 25 (P = 0.0008) mmHg (Fig. 4*D*). Relative to TGF-β1 alone (1.67-fold, Fig. 1*B*), TGF-β1 and 10Panx co-administration (1.18-fold, Fig. 4*D*) significantly (P = 0.0397) decreased the magnitude of change only from 20 to 25 mmHg. Distention-evoked urothelial ATP release at 25 mmHg was also significantly (P = 0.0465) increased with the intravesical co-administration of TGF-β1 and 10Panx (Fig. 4*E*).

Purinoceptor signalling. The continuous intravesical instillation of PPADS (300 μM) did not have a significant (P = 0.2339) treatment effect on mean bladder afferent nerve firing (75.9 ± 25.1 to 64.0 ± 16.9 impulses s^{-1}, n = 4) from 0 to 25 mmHg (Fig. 5*A* and *B*). The intravesical co-administration of recombinant TGF-β1 (10 ng ml^{-1}) and PPADS (300 μM) attenuated (P = 0.2941) afferent nerve hyperexcitability (75.9 ± 25.1 to 59.2 ± 11.9 impulses s^{-1}, n = 4) from 0 to 25 mmHg (Fig. 5*C* and *D*). The intravesical co-administration of TGF-β1 and PPADS, however, significantly (P = 0.0403) increased distention-evoked urothelial ATP release at 25 mmHg (Fig. 5*E*).

Bladder afferent nerve hyperexcitability and urothelial ATP release with CYP-induced cystitis is decreased with TGF-β inhibition

CYP-induced cystitis had a significant ($P \leq$ 0.0001) treatment effect on mean bladder afferent nerve frequency (impulses s^{-1}) and nerve firing increased (62.2 ± 7.2 to 179.8 ± 20.9 impulses s^{-1}, n = 4–19) by 188.9% from 0 to 25 mmHg relative to control (Fig. 6*A* and *B*). Nerve discharge significantly increased from 5 to 10 ($P \leq$ 0.0001), 10 to 15 ($P \leq$ 0.0001), 15 to 20 ($P \leq$ 0.0001) and 20 to 25 ($P \leq$ 0.0001) mmHg (Fig. 6*B*).

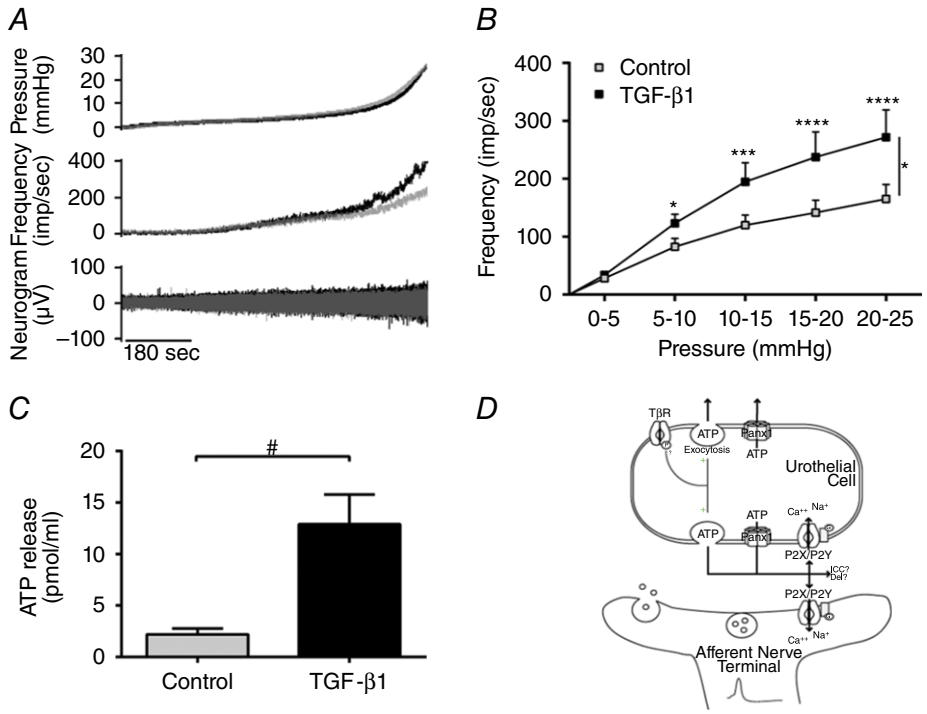

Figure 1. TGF-β1 increased distention-evoked bladder afferent nerve discharge and ATP release
A, representative traces of vehicle (grey) and recombinant TGF-β1 (10 ng ml^{-1}, black) instillation from the same preparation. *B*, TGF-β1 significantly increased mean bladder afferent nerve frequency (impulses s^{-1}) by 41.8% relative to control with significant nerve discharge increase from 5 to 10, 10 to 15, 15 to 20 and 20 to 25 mmHg. *C*, TGF-β1 significantly increased distention-evoked ATP release at 25 mmHg. *D*, proposed mechanisms of purinergic signalling with TβR activation. n = 4–8; values are means ± SEM; $^{*}P \leq$ 0.05, $^{***}P \leq$ 0.001, $^{****}P \leq$ 0.0001 by two-way repeated measures ANOVA followed by Sidak's multiple comparisons test. $^{\#}P \leq$ 0.01 by Student's paired t test.

The continuous intravesical instillation of SB505124 (5 μM) significantly ($P = 0.0028$) decreased mean nerve frequency with CYP-induced cystitis (Fig. 6*C* and *D*). Mean nerve firing decreased (179.8 $\pm$ 20.9 to 103.6 $\pm$ 6.0 impulses s^{-1}, $n = 4$) by 40.6% from 0 to 25 mmHg with a significant decrease in discharge from 5 to 10 ($P = 0.0041$), 10 to 15 ($P = 0.006$) and 15 to 20 ($P = 0.0197$) mmHg following SB505124 instillation (Fig. 6*D*). TβR-1 inhibition with SB505124 also significantly ($P = 0.0190$) decreased distention-evoked urothelial ATP release at 25 mmHg following CYP-induced cystitis (Fig. 6*E*). The vehicle washout of SB505124 increased nerve firing (103.6 $\pm$ 6.0 to 172.8 $\pm$ 21.9 impulses s^{-1}, $n = 4$) and urothelial ATP release (6.3 $\pm$ 2.9 to 17 $\pm$ 3.7 pmol ml^{-1}, $n = 4$) back to baseline.

Discussion

The present study revealed that purinergic signalling mechanisms underlie TGF-β-mediated bladder afferent

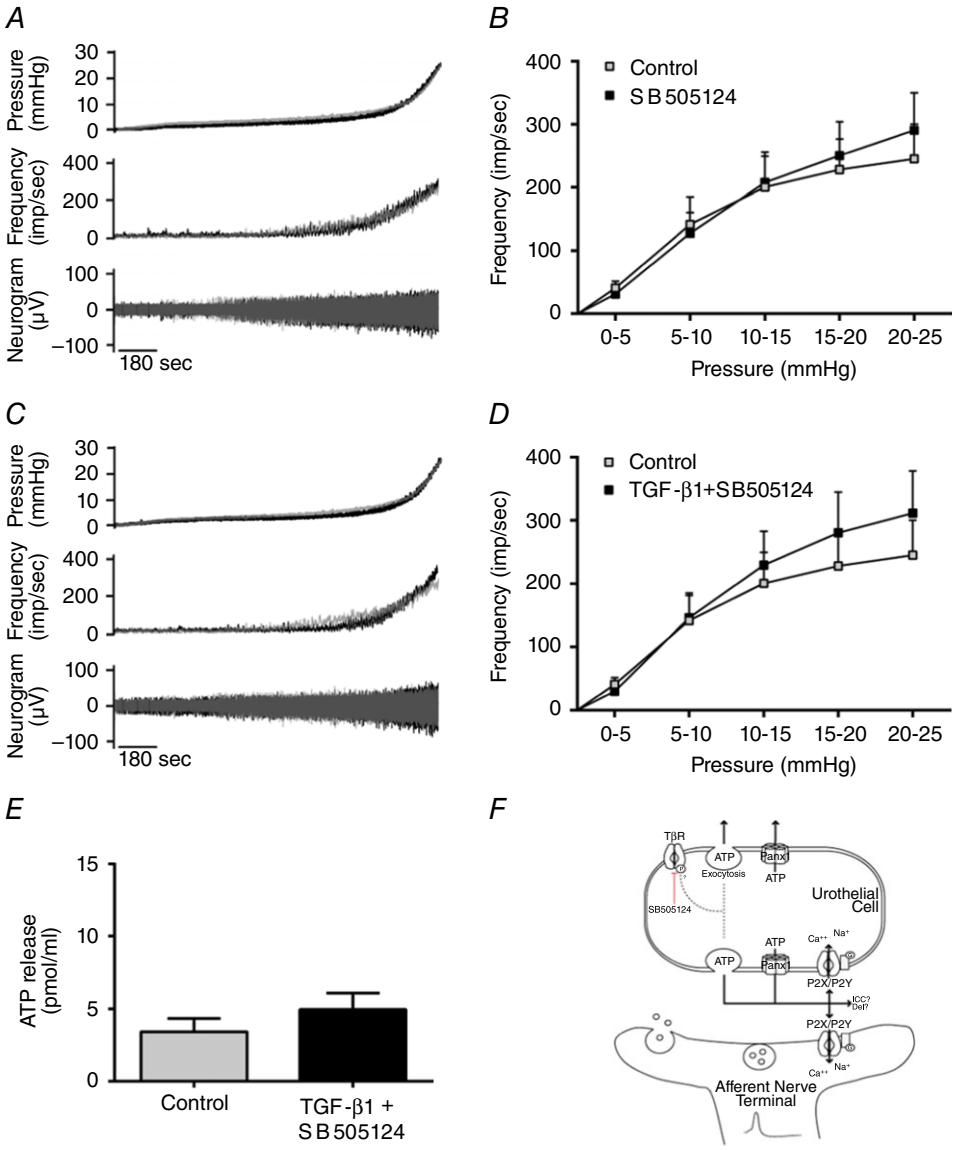

Figure 2. Distention-evoked bladder afferent nerve discharge and ATP release are unchanged with the co-administration of TGF-β1 and SB505124
A, representative traces of vehicle (grey) and SB505124 (5 μM, black) instillation from the same preparation. *B*, SB505124 did not have a significant treatment effect on mean bladder afferent nerve frequency (impulses s^{-1}) relative to control. *C*, representative traces of vehicle (grey) and recombinant TGF-β1 (10 ng ml^{-1}, black) and SB505124 (5 μM, black) co-instillation from the same preparation. Concurrent instillation of TGF-β1 and SB505124 attenuated distention-evoked bladder afferent nerve hyperexcitability (*D*) and ATP release (*E*) mediated by TGF-β1. *F*, proposed mechanisms of purinergic signalling with TβR inhibition. $n = 4$–7; values are means $\pm$ SEM.

nerve excitability in both control and CYP-treated tissues. We determined that TGF-β1 contributed to bladder afferent nerve hyperexcitability through increased ATP release from the urothelium that was consistent with a vesicular exocytosis mechanism. We also demonstrated that blocking aberrant TGF-β signalling in cystitis with TβR-1 inhibition decreased bladder afferent nerve discharge and urothelial ATP release. These studies delineate the sensory components of the urinary bladder that may underlie the interface between the upregulation of inflammatory mediators and bladder dysfunction.

ATP release

The urothelium participates in the sensory processing of bladder filling by releasing neuroactive factors, such as ATP (Wang *et al.* 2005; Birder & Andersson, 2013). The altered release of ATP in many functional disorders

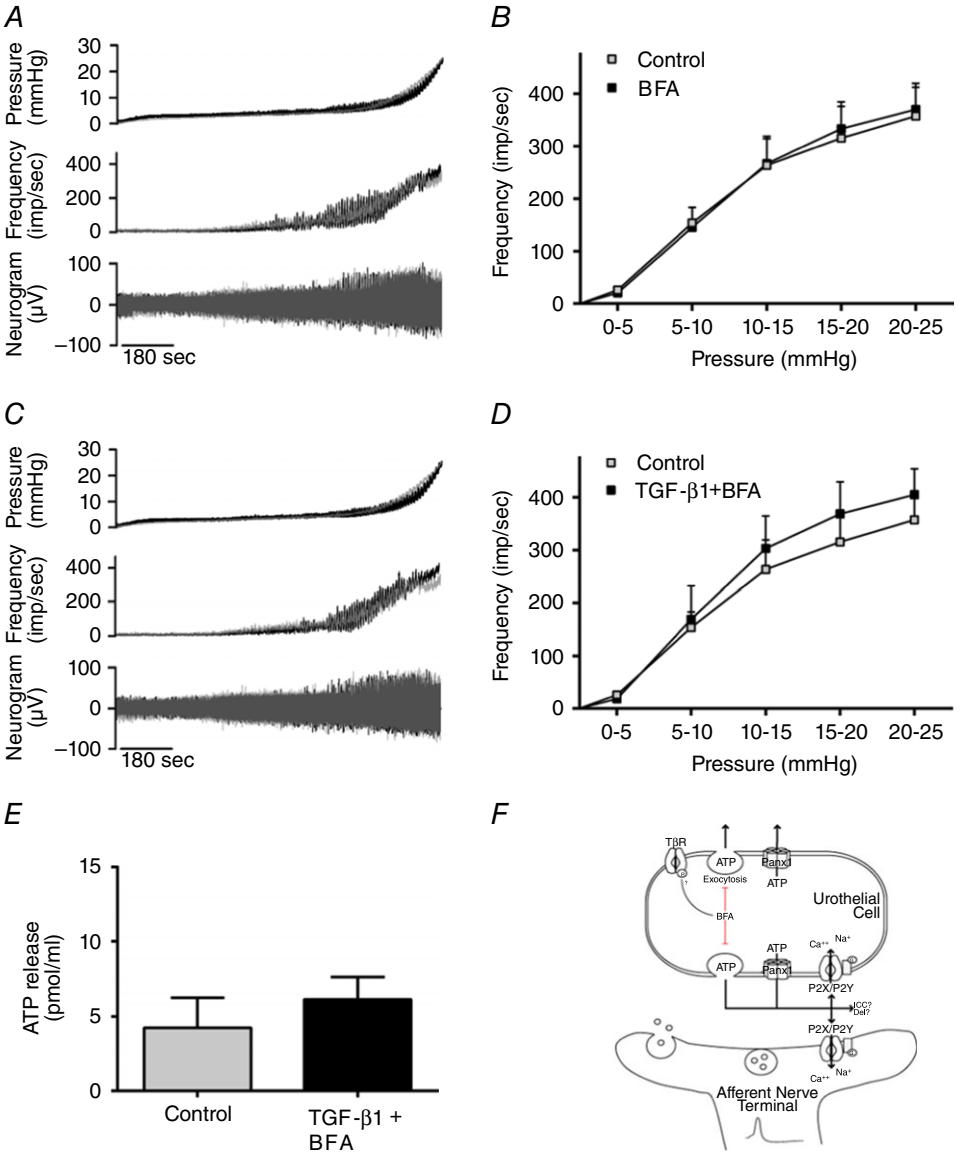

Figure 3. Distention-evoked bladder afferent nerve discharge and ATP release are unchanged with the co-administration of TGF-β1 and BFA
A, representative traces of vehicle (grey) and BFA (10 μM, black) instillation from the same preparation. *B*, BFA did not have a significant treatment effect on mean bladder afferent nerve frequency (impulses s^{-1}) relative to control. *C*, representative traces of vehicle (grey) and recombinant TGF-β1 (10 ng ml^{-1}, black) and BFA (10 μM, black) co-instillation from the same preparation. Concurrent instillation of TGF-β1 and BFA attenuated distention-evoked bladder afferent nerve hyperexcitability (*D*) and ATP release (*E*) mediated by TGF-β1. *F*, Proposed mechanisms of purinergic signalling with TβR activation and BFA. n = 3–8; values are means ± SEM.

of the urinary bladder (Sun *et al.* 2001; Sun & Chai, 2006; Silva-Ramos *et al.* 2013) suggest purinergic signalling may contribute to the pathophysiology of bladder dysfunction (Ruggieri, 2006). We determined that a previously identified inflammatory mediator of cystitis, TGF-β1 (Gonzalez *et al.* 2013), was able to stimulate an increase in distention-evoked release of ATP from the urothelium (Fig. 1*D*). The release of ATP was dependent on ligand/receptor activation because the co-administration of TGF-β1 with a TβR-1 inhibitor, SB505124 (DaCosta Byfield *et al.* 2004), attenuated an elevation in extracellular ATP (Fig. 2*F*). Not surprisingly, TGF-β1 continued to stimulate distention-evoked ATP release from the urothelium when P2 receptors were

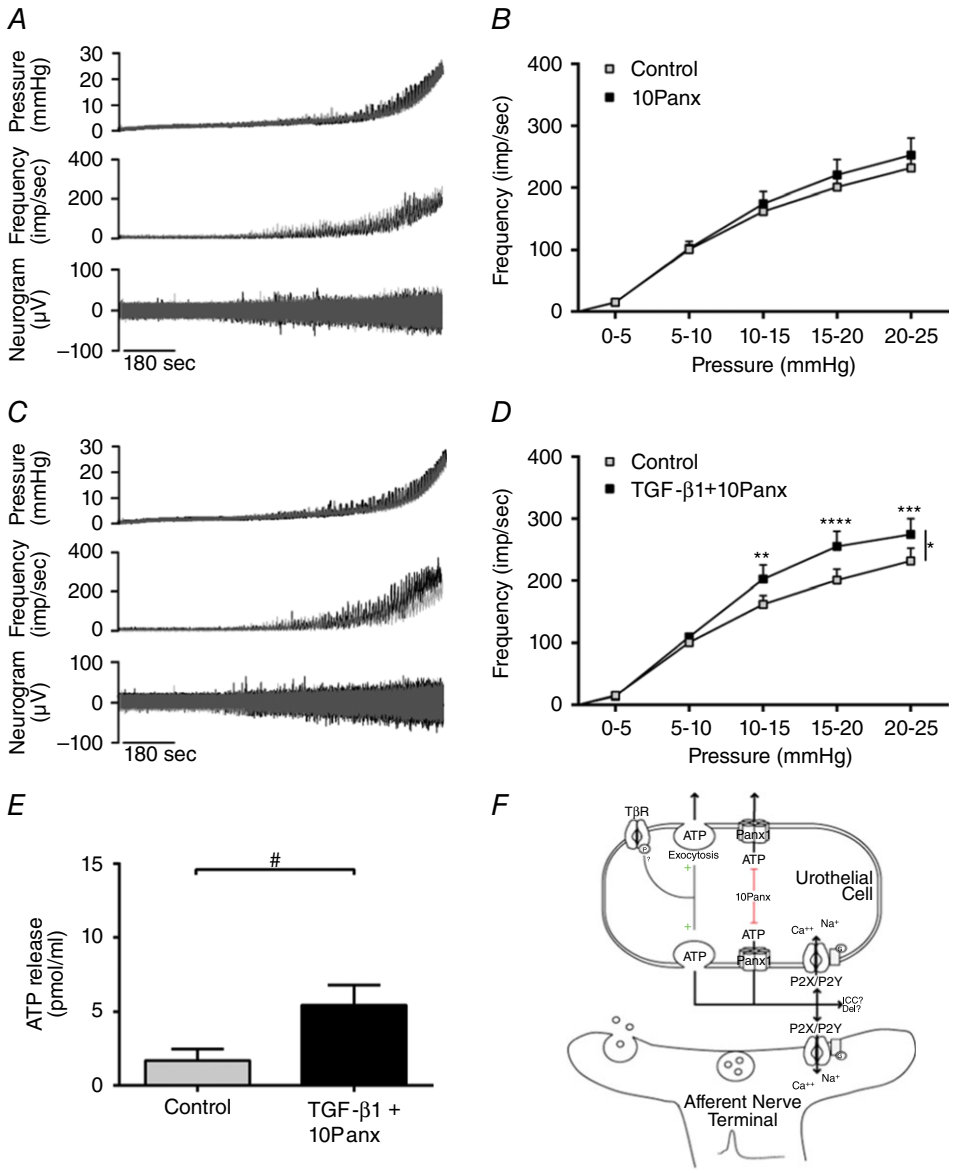

Figure 4. TGF-β1 and 10Panx co-administration increased distention-evoked bladder afferent nerve discharge and ATP release
A, representative traces of vehicle (grey) and 10Panx (50 μM, black) instillation from the same preparation. *B*, 10Panx did not have a significant treatment effect on mean bladder afferent nerve frequency (impulses s^{-1}) relative to control. *C*, representative traces of vehicle (grey) and recombinant TGF-β1 (10 ng ml^{-1}, black) and 10Panx (50 μM, black) co-instillation from the same preparation. *D*, concurrent instillation of TGF-β1 and 10Panx significantly increased mean bladder afferent nerve frequency (impulses s^{-1}) by 19.4% relative to control with significant nerve discharge increase from 10 to 15, 15 to 20 and 20 to 25 mmHg. *E*, TGF-β1 significantly increased distention-evoked ATP release at 25 mmHg when co-administered with 10Panx. *F*, proposed mechanisms of purinergic signalling with TβR activation and 10Panx. $n = 4$–7; values are means ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ by two-way repeated measures ANOVA followed by Sidak's multiple comparisons test. $^{\#}P \leq 0.05$ by Student's paired *t* test.

inhibited downstream of the release pathways with the co-administration of PPADS (Fig. 5*F*). Aberrant TGF-β activation that was previously observed with CYP-induced cystitis (Gonzalez *et al.* 2013) also contributed to altered ATP release. In our studies with cystitis, the inhibition of aberrant TGF-β signalling significantly reduced urothelial ATP release that recovered immediately with vehicle washout (Fig. 6*F*). These results suggest that TGF-β signalling may directly contribute to the altered sensory processing of bladder filling by increasing ATP release from the urothelium.

Urothelial cells release ATP through various mechanisms that include vesicular exocytosis (Wang *et al.* 2005; Sui *et al.* 2014; McLatchie & Fry, 2015), connexin/pannexin channels (Wang *et al.* 2005; Sui *et al.* 2014; Beckel *et al.* 2015; McLatchie & Fry, 2015) and

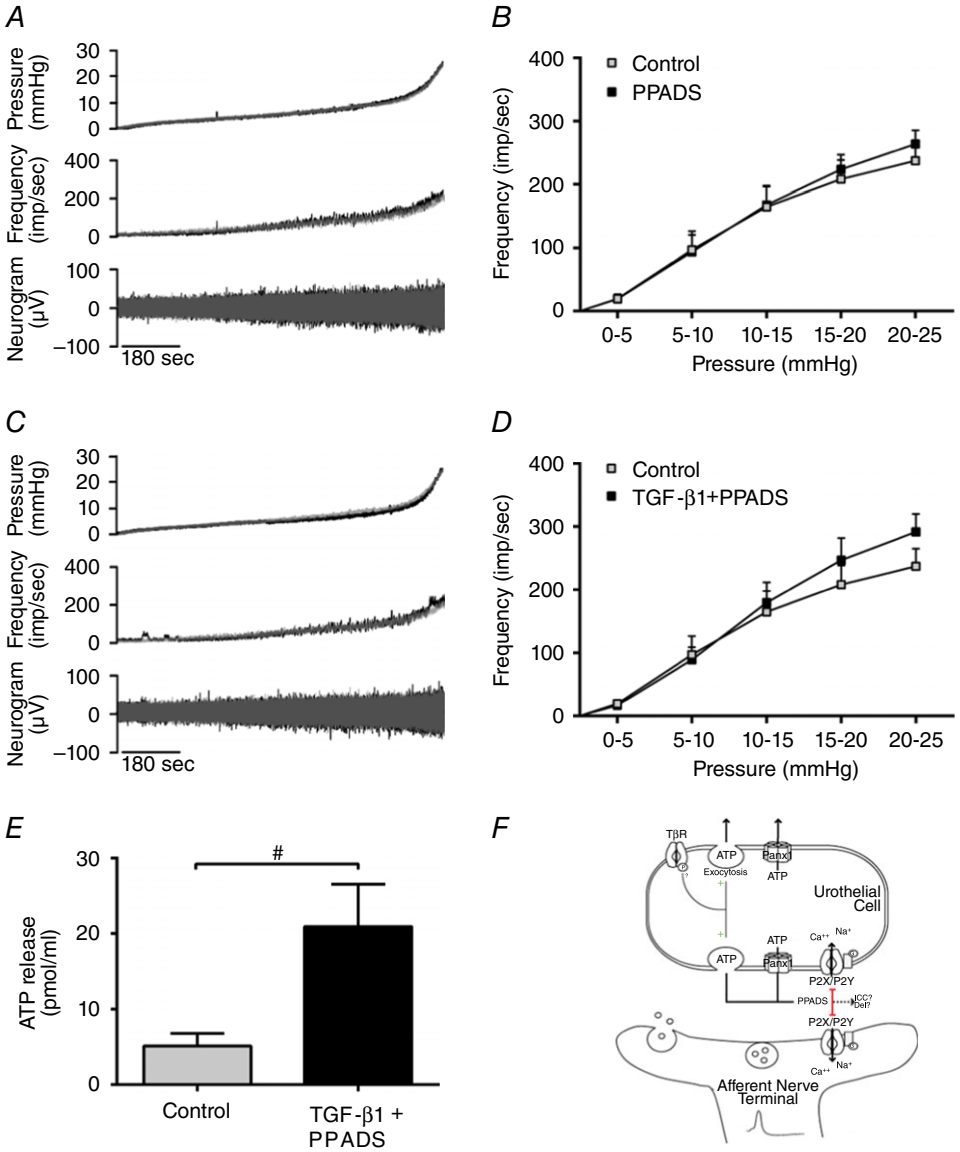

Figure 5. Distention-evoked bladder afferent nerve discharge is unchanged but ATP release is increased with the co-administration of TGF-β1 and PPADS
A, representative traces of vehicle (grey) and PPADS (300 μM, black) instillation from the same preparation. *B*, PPADS did not have a significant treatment effect on mean bladder afferent nerve frequency (impulses s^{-1}) relative to control. *C*, representative traces of vehicle (grey) and recombinant TGF-β1 (10 ng ml^{-1}, black) and PPADS (300 μM, black) co-instillation from the same preparation. *D*, concurrent instillation of TGF-β1 and PPADS attenuated distention-evoked bladder afferent nerve hyperexcitability mediated by TGF-β1. *E*, TGF-β1 significantly increased distention-evoked ATP release at 25 mmHg when co-administered with PPADS. *F*, proposed mechanisms of purinergic signalling with TβR activation and PPADS. $n = 4$–5; values are means ± SEM. $^{\#}P \leq 0.05$ by Student's paired *t* test.

nucleoside transporters (Wang *et al.* 2005). Although each mechanism may play a part in release, our studies focused on the pharmacological manipulation of vesicular exocytosis and pannexin-1 channels due to the disputed role of transporters in uroepithelial cells (Knight *et al.* 2002) and because TGF-β1 downregulates connexin hemichannels in the bladder (Neuhaus *et al.* 2009), suggesting an alternative mechanism for increased ATP release. We intravesically instilled the general secretory inhibitor, BFA, for vesicular release inhibition. BFA has previously been shown to decrease mechanical- and stretch-evoked ATP release from the urothelium (Wang *et al.* 2005; Sui *et al.* 2014; McLatchie & Fry, 2015). We also intravesically instilled the inhibitory peptide, 10Panx, to block pannexin-1 channels. These channels have recently been implicated in distention-evoked ATP

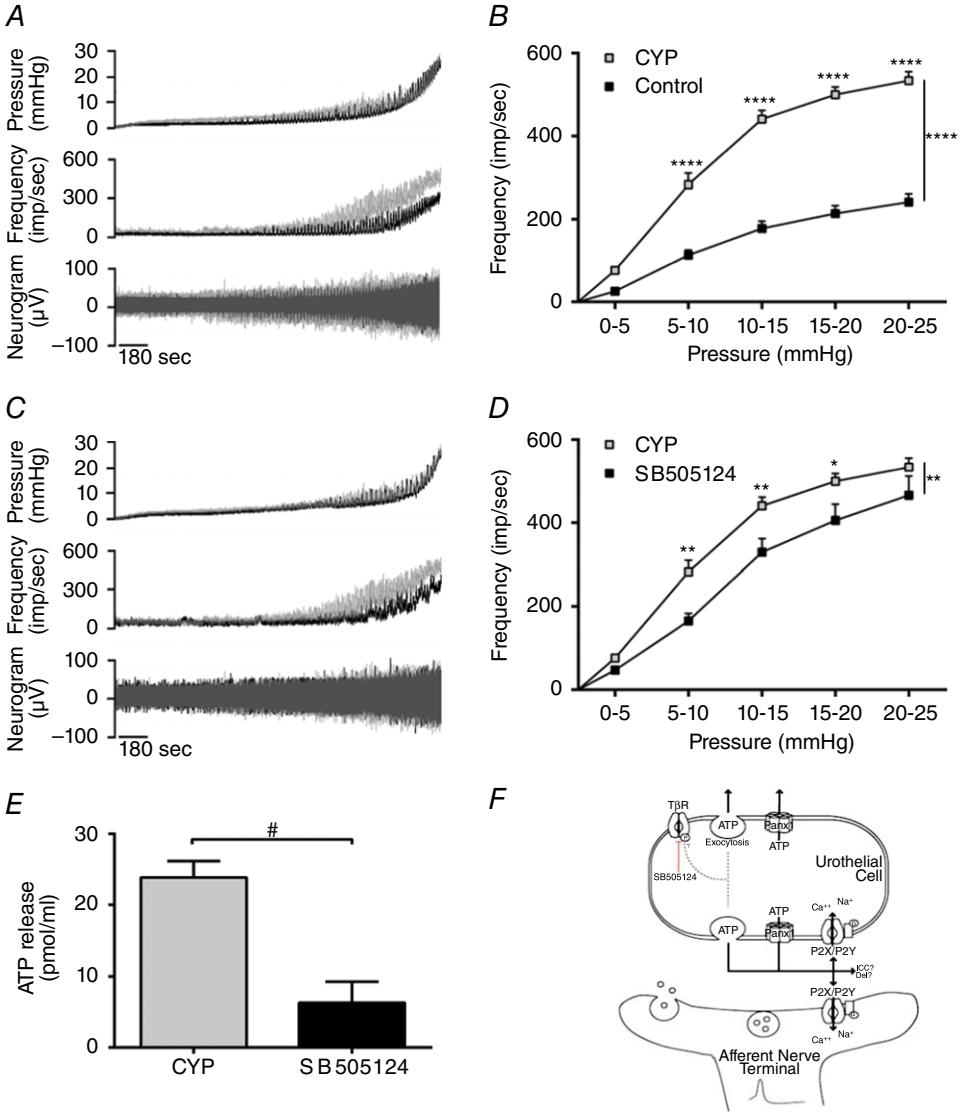

Figure 6. SB505124 decreased distention-evoked bladder afferent nerve hyperexcitability and ATP release with CYP-induced cystitis
A, representative traces of CYP-induced cystitis (grey) and control (black) instillation. *B*, CYP-induced cystitis significantly increased mean bladder afferent nerve frequency (impulses s^{-1}) by 188.9% relative to control with significant nerve discharge increase from 5 to 10, 10 to 15, 15 to 20 and 20 to 25 mmHg. *C*, representative traces of CYP-induced cystitis (grey) and SB505124 (5 μM, black) instillation from the same CYP preparation. *D*, SB505124 significantly decreased mean bladder afferent nerve frequency (impulses s^{-1}) by 40.6% relative to CYP with significant nerve discharge decrease from 5 to 10, 10 to 15 and 15 to 20 mmHg. *E*, SB505124 significantly decreased distention-evoked ATP release with CYP-induced cystitis. *F*, proposed mechanisms of purinergic signalling with TβR inhibition and CYP-induced cystitis. $n = 4$–19; values are means ± SEM; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$ by two-way repeated measures ANOVA followed by Sidak's multiple comparisons test. $^{\#}P \leq 0.05$ by Student's paired *t* test.

release from the urothelium with pharmacological or genetic manipulation (Negoro *et al.* 2014; Timoteo *et al.* 2014; Beckel *et al.* 2015).

Our studies with BFA or 10Panx co-administration with TGF-β1 support the hypothesis that urothelial ATP release stimulated by TGF-β1 may occur through vesicular exocytosis (Figs 3*F* and 4*F*). While these results are consistent with a vesicular secretory mechanism, we are unable to definitively exclude off-target effects of BFA that may also attenuate ATP release, such as the inhibition of the transport of cell surface proteins like hemichannels or TβR-1 (Wang *et al.* 2005). Further studies with more selective inhibitors are needed to separate the effects of vesicle release and hemichannel inhibition. It is, however, likely that the effects that we observed are from vesicular secretory release because TGF-β1 downregulates connexin hemichannels and pannexin-1 channels had a minimal role in TGF-β1-stimulated ATP release. Future studies in our lab will also aim to resolve the intracellular signalling cascade that may promote ATP release through these mechanisms.

Bladder afferent nerve excitability

Purinergic signalling is recognized to directly influence the afferent limb of the micturition reflex and bladder function. The intravesical instillation of ATP enhanced spinal bladder neuron excitability (Munoz *et al.* 2011) and increased bladder activity (Pandita & Andersson, 2002). Non-voiding bladder contractions and voiding frequency, on the other hand, were decreased when ATP release was attenuated with the inhibition of vesicular (Smith *et al.* 2005) or pannexin-1 channel (Timoteo *et al.* 2014; Beckel *et al.* 2015) release mechanisms, respectively. Our studies determined that the decrease in distention-evoked ATP release from the urothelium through vesicular exocytosis or pannexin-1 channels did not significantly contribute to mechanosensitive afferent nerve discharge. This suggests that the decrease in bladder function previously observed occurred through an as-yet-unknown mechanism that is independent of afferent pelvic nerve excitability.

Extracellular ATP may contribute to bladder sensory processing through the activation of purinergic receptors on various cell types within the bladder wall including afferent nerve terminals. The activation of P2 receptors with intravesical α,β-methylene ATP or ATP increased bladder afferent nerve discharge (Yu & de Groat, 2008) that was mediated mainly through capsaicin-insensitive C fibres (Aizawa *et al.* 2011). Following intravesical P2 receptor inhibition, bladder activity decreased (Beckel *et al.* 2015) and afferent nerve discharge decreased (Namasivayam *et al.* 1999; Vlaskovska *et al.* 2001) or remained the same (Yu & de Groat, 2008). Our current study determined that intravesical PPADS did not have a significant effect on afferent nerve discharge at a peak distention pressure of 25 mmHg. Taken together, these discrepant studies indicate that purinergic receptors may only be activated on C fibres during pathological conditions of elevated extracellular ATP to contribute to the sensation of bladder filling.

The aforementioned contributions of purinergic signalling to bladder sensory transduction and function suggests that an insult, like inflammation, that alters ATP release may also affect nerve excitability. Given the functional overexpression of TGF-β in CYP-induced cystitis (Tyagi *et al.* 2009; Zhang & Qiao, 2012; Gonzalez *et al.* 2013) and its ability to stimulate ATP release in our current studies, we sought to determine if TGF-β1 also contributes to bladder afferent nerve hyperexcitability. The intravesical instillation of TGF-β1 increased nerve excitability by 41.8% and its significant effects on nerve discharge were observed nearly throughout the entirety of the filling phase (Fig. 1*D*). Nerve hyperexcitability was contingent on receptor activation and subsequent release of ATP because the intravesical co-administration of TGF-β1 and a TβR-1 inhibitor, SB505124, attenuated ATP release and bladder afferent nerve discharge (Fig. 2*F*).

In addition to the necessity of TGF-β receptor activation, we also demonstrated that TGF-β1 may stimulate ATP release through vesicular exocytosis to increase afferent nerve firing. Decreasing the release of ATP from the urothelium with BFA co-administration attenuated TGF-β1-mediated afferent nerve hyperexcitability (Fig. 3*F*). The co-administration of TGF-β1 and 10Panx, however, significantly increased distention-evoked ATP release and afferent nerve discharge, suggesting minimal contribution from pannexin-1 channels in this response (Fig. 4*F*). The elevated extracellular ATP stimulated by TGF-β1 is likely to be activating nerve terminals within the bladder wall to increase excitability. We demonstrated that the intravesical co-administration of PPADS attenuated TGF-β1-mediated bladder afferent nerve hyperexcitability, suggesting a role for these purinergic receptors in suburothelial afferent nerve firing (Fig. 5*F*). Intravesical PPADS may also be working at the level of the urothelium to inhibit further basolateral release of ATP or other mediators that enhance excitability (Fig. 5*F*) (Sun & Chai, 2006). Future studies are still needed to determine the basolateral release of ATP in this model because the concentrations of ATP that we quantified in the lumen likely under-represents basolateral release onto sensory fibres due to volume differences in the interstitium, tissue-associated ectonucleotidases/exonucleotidases (Wang *et al.* 2005) and cell-type-specific ATP release (Cheng *et al.* 2011; McLatchie & Fry, 2015). Currently, the direct activation of purinergic receptors within the wall of an intact urinary bladder remains difficult to investigate without disrupting normal bladder physiology.

TGF-β is increased in the urine (Tyagi *et al.* 2009), urothelium (Tyagi *et al.* 2009; Gonzalez *et al.* 2013) and detrusor smooth muscle (Zhang & Qiao, 2012; Gonzalez *et al.* 2013) following CYP-induced cystitis. CYP-induced cystitis is characterized by voiding dysfunction and TGF-β is considered to contribute, in part, to the development of these lower urinary tract symptoms because TβR-1 inhibition decreased voiding frequency and increased bladder capacity (Gonzalez *et al.* 2013). We confirmed that bladder afferent nerve hyperexcitability is increased in CYP-induced cystitis (Yu & de Groat, 2008) and demonstrated a functional role for aberrant TGF-β signalling in this response (Fig. 6*F*). Our current studies determined that TβR-1 inhibition may improve voiding dysfunction by decreasing ATP release from the urothelium and decreasing nerve firing by 40.6%. These effects were dependent on aberrant TGF-β activation because vehicle washout increased ATP release and recovered nerve hyperexcitability to baseline. The decreased release of ATP with TβR-1 inhibition may normalize purinergic signalling and work comparably to intravesical P2X inhibition that has been previously reported to decrease afferent nerve excitability in CYP-induced cystitis (Yu & de Groat, 2008). It should also be noted that while TβR-1 inhibition significantly decreased distention-evoked ATP release and afferent nerve excitability, nerve frequency did not reach control activity, suggesting that other neuroactive mediators may be influencing mechanosensitive nerve discharge with cystitis.

Conclusion

In summary, our studies highlight the role of purinergic signalling in TGF-β-mediated bladder afferent nerve hyperexcitability. The apparent synergy between a component of the bladder inflammatory milieu and intrinsic signalling mechanisms discussed here will contribute to the understanding of the pathophysiology of bladder injury or disease. The distinct interactions of multiple signal transducers underscore the challenges for single target therapies and support the development of combinatory therapeutics for bladder dysfunction.

References

Aizawa N, Igawa Y, Andersson KE, Iijima K, Nishizawa O & Wyndaele JJ (2011). Effects of intravesical instillation of ATP on rat bladder primary afferent activity and its relationship with capsaicin-sensitivity. *Neurourol Urodyn* **30**, 163–168.

Andersson KE (2002). Bladder activation: afferent mechanisms. *Urology* **59**, 43–50.

Arms L, Girard BM, Malley SE & Vizzard MA (2013). Expression and function of CCL2/CCR2 in rat micturition reflexes and somatic sensitivity with urinary bladder inflammation. *Am J Physiol Renal Physiol* **305**, F111–F122.

Arms L, Girard BM & Vizzard MA (2010). Expression and function of CXCL12/CXCR4 in rat urinary bladder with cyclophosphamide-induced cystitis. *Am J Physiol Renal Physiol* **298**, F589–F600.

Beckel JM, Daugherty SL, Tyagi P, Wolf-Johnston AS, Birder LA, Mitchell CH & de Groat WC (2015). Pannexin 1 channels mediate the release of ATP into the lumen of the rat urinary bladder. *J Physiol* **593**, 1857–1871.

Birder LA (2005). More than just a barrier: urothelium as a drug target for urinary bladder pain. *Am J Physiol Renal Physiol* **289**, F489–F495.

Birder LA & Andersson KE (2013). Urothelial Signaling. *Physiol Rev* **93**, 653–680.

Birder LA, Nealen ML, Kiss S, de Groat WC, Caterina MJ, Wang E, Apodaca G & Kanai AJ (2002). Beta-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells. *J Neurosci* **22**, 8063–8070.

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G & Wood JN (1995). A P2X purinoceptor expressed by a subset of sensory neurons. *Nature* **377**, 428–431.

Chen S, Jim B & Ziyadeh FN (2003). Diabetic nephropathy and transforming growth factor-beta: transforming our view of glomerulosclerosis and fibrosis build-up. *Semin Nephrol* **23**, 532–543.

Cheng Y, Mansfield KJ, Sandow SL, Sadananda P, Burcher E & Moore KH (2011). Porcine bladder urothelial, myofibroblast, and detrusor muscle cells: characterization and ATP release. *Front Pharmacol* **2**, 27. DOI: 10.3389/fphar.2011.00027.

Chopra B, Gever J, Barrick SR, Hanna-Mitchell AT, Beckel JM, Ford AP & Birder LA (2008). Expression and function of rat urothelial P2Y receptors. *Am J Physiol Renal Physiol* **294**, F821–F829.

DaCosta Byfield S, Major C, Laping NJ & Roberts AB (2004). SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* **65**, 744–752.

de Groat WC & Yoshimura N (2009). Afferent nerve regulation of bladder function in health and disease. *Handb Exp Pharmacol*, 91–138.

De Muro P, Faedda R, Fresu P, Masala A, Cigni A, Concas G, Mela MG, Satta A, Carcassi A, Sanna GM & Cherchi GM (2004). Urinary transforming growth factor-beta 1 in various types of nephropathy. *Pharmacol Res* **49**, 293–298.

Deng FM, Ding M, Lavker RM & Sun TT (2001). Urothelial function reconsidered: a role in urinary protein secretion. *Proc Natl Acad Sci USA* **98**, 154–159.

Drumm BT, Koh SD, Andersson KE & Ward SM (2014). Calcium signalling in Cajal-like interstitial cells of the lower urinary tract. *Nat Rev Urol* **11**, 555–564.

Duan LJ, Qi J, Kong XJ, Huang T, Qian XQ, Xu D, Liang JH & Kang J (2015). MiR-133 modulates TGF-beta1-induced bladder smooth muscle cell hypertrophic and fibrotic response: implication for a role of microRNA in bladder wall remodeling caused by bladder outlet obstruction. *Cell Signal* **27**, 215–227.

Everaerts W, Vriens J, Owsianik G, Appendino G, Voets T, De Ridder D & Nilius B (2010). Functional characterization of transient receptor potential channels in mouse urothelial cells. *Am J Physiol Renal Physiol* **298**, F692–F701.

Ferguson DR, Kennedy I & Burton TJ (1997). ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes – a possible sensory mechanism? *J Physiol* **505**, 503–511.

Golbidi S & Laher I (2010). Bladder dysfunction in diabetes mellitus. *Front Pharmacol* **1**, 136. DOI: 10.3389/fphar.2010.00136.

Gonzalez EJ, Arms L & Vizzard MA (2014*a*). The role(s) of cytokines/chemokines in urinary bladder inflammation and dysfunction. *Biomed Res Int* **2014**, 120525.

Gonzalez EJ, Girard BM & Vizzard MA (2013). Expression and function of transforming growth factor-beta isoforms and cognate receptors in the rat urinary bladder following cyclophosphamide-induced cystitis. *Am J Physiol Renal Physiol* **305**, F1265–F1276.

Gonzalez EJ, Merrill L & Vizzard MA (2014*b*). Bladder sensory physiology: neuroactive compounds and receptors, sensory transducers, and target-derived growth factors as targets to improve function. *Am J Physiol Regul Integr Comp Physiol* **306**, R869–R878.

Goumenos DS, Tsakas S, El Nahas AM, Alexandri S, Oldroyd S, Kalliakmani P & Vlachojannis JG (2002). Transforming growth factor-beta$_1$ in the kidney and urine of patients with glomerular disease and proteinuria. *Nephrol Dial Transplant* **17**, 2145–2152.

Hanna-Mitchell AT, Beckel JM, Barbadora S, Kanai AJ, de Groat WC & Birder LA (2007). Non-neuronal acetylcholine and urinary bladder urothelium. *Life Sci* **80**, 2298–2302.

Hashitani H, Brading AF & Suzuki H (2004). Correlation between spontaneous electrical, calcium and mechanical activity in detrusor smooth muscle of the guinea-pig bladder. *Br J Pharmacol* **141**, 183–193.

Heppner TJ, Bonev AD & Nelson MT (2005). Elementary purinergic Ca^{2+} transients evoked by nerve stimulation in rat urinary bladder smooth muscle. *J Physiol* **564**, 201–212.

Hiti AL, Rideout WM, 3rd, Laug WE & Jones PA (1990). Plasminogen activator regulation by transforming growth factor-beta in normal and neoplastic human urothelium. *Cancer Commun* **2**, 123–128.

Islam SS, Mokhtari RB, El Hout Y, Azadi MA, Alauddin M, Yeger H & Farhat WA (2014). TGF-β1 induces EMT reprogramming of porcine bladder urothelial cells into collagen producing fibroblasts-like cells in a Smad2/Smad3-dependent manner. *J Cell Commun Signal* **8**, 39–58.

Knight GE, Bodin P, De Groat WC & Burnstock G (2002). ATP is released from guinea pig ureter epithelium on distension. *Am J Physiol Renal Physiol* **282**, F281–F288.

Lewis SA & Lewis JR (2006). Kinetics of urothelial ATP release. *Am J Physiol Renal Physiol* **291**, F332–F340.

McLatchie LM & Fry CH (2015). ATP release from freshly isolated guinea-pig bladder urothelial cells: a quantification and study of the mechanisms involved. *BJU Int* **115**, 987–993.

Malley SE & Vizzard MA (2002). Changes in urinary bladder cytokine mRNA and protein after cyclophosphamide-induced cystitis. *Physiol Genomics* **9**, 5–13.

Min G, Zhou G, Schapira M, Sun TT & Kong XP (2003). Structural basis of urothelial permeability barrier function as revealed by Cryo-EM studies of the 16 nm uroplakin particle. *J Cell Sci* **116**, 4087–4094.

Morrison J (1999). The activation of bladder wall afferent nerves. *Exp Physiol* **84**, 131–136.

Munoz A, Somogyi GT, Boone TB & Smith CP (2011). Lumbosacral sensory neuronal activity is enhanced by activation of urothelial purinergic receptors. *Brain Res Bull* **86**, 380–384.

Namasivayam S, Eardley I & Morrison JF (1998). A novel *in vitro* bladder pelvic nerve afferent model in the rat. *Br J Urol* **82**, 902–905.

Namasivayam S, Eardley I & Morrison JF (1999). Purinergic sensory neurotransmission in the urinary bladder: an *in vitro* study in the rat. *BJU Int* **84**, 854–860.

Negoro H, Urban-Maldonado M, Liou LS, Spray DC, Thi MM & Suadicani SO (2014). Pannexin 1 channels play essential roles in urothelial mechanotransduction and intercellular signaling. *PLoS One* Zhang & Qiao, 2012; Gonzalez *et al.* 2013, e106269.

Neuhaus J, Heinrich M, Schwalenberg T & Stolzenburg JU (2009). TGF-beta1 inhibits Cx43 expression and formation of functional syncytia in cultured smooth muscle cells from human detrusor. *Eur Urol* **55**, 491–497.

Nile CJ & Gillespie JI (2012). Interactions between cholinergic and prostaglandin signaling elements in the urothelium: role for muscarinic type 2 receptors. *Urology* **79**, 240.e217–223.

Olsen SM, Stover JD & Nagatomi J (2011). Examining the role of mechanosensitive ion channels in pressure mechanotransduction in rat bladder urothelial cells. *Ann Biomed Eng* **39**, 688–697.

Pandita RK & Andersson KE (2002). Intravesical adenosine triphosphate stimulates the micturition reflex in awake, freely moving rats. *J Urol* **168**, 1230–1234.

Pelegrin P & Surprenant A (2006). Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. *EMBO J* **25**, 5071–5082.

Ruggieri MR Sr (2006). Mechanisms of disease: role of purinergic signaling in the pathophysiology of bladder dysfunction. *Nat Clin Pract Urol* **3**, 206–215.

Silva-Ramos M, Silva I, Oliveira O, Ferreira S, Reis MJ, Oliveira JC & Correia-de-Sa P (2013). Urinary ATP may be a dynamic biomarker of detrusor overactivity in women with overactive bladder syndrome. *PLoS One* **8**, e64696.

Smith CP, Vemulakonda VM, Kiss S, Boone TB & Somogyi GT (2005). Enhanced ATP release from rat bladder urothelium during chronic bladder inflammation: effect of botulinum toxin A. *Neurochem Int* **47**, 291–297.

Sui G, Fry CH, Montgomery B, Roberts M, Wu R & Wu C (2014). Purinergic and muscarinic modulation of ATP release from the urothelium and its paracrine actions. *Am J Physiol Renal Physiol* **306**, F286–F298.

Sui GP, Wu C, Roosen A, Ikeda Y, Kanai AJ & Fry CH (2008). Modulation of bladder myofibroblast activity: implications for bladder function. *Am J Physiol Renal Physiol* **295**, F688–F697.

Sun Y & Chai TC (2006). Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis. *Am J Physiol Cell Physiol* **290**, C27–C34.

Sun Y, Keay S, De Deyne PG & Chai TC (2001). Augmented stretch activated adenosine triphosphate release from bladder uroepithelial cells in patients with interstitial cystitis. *J Urol* **166**, 1951–1956.

Tempest HV, Dixon AK, Turner WH, Elneil S, Sellers LA & Ferguson DR (2004). P2X and P2X receptor expression in human bladder urothelium and changes in interstitial cystitis. *BJU Int* **93**, 1344–1348.

Timoteo MA, Carneiro I, Silva I, Noronha-Matos JB, Ferreirinha F, Silva-Ramos M & Correia-de-Sa P (2014). ATP released via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial P2Y6 receptors. *Biochem Pharmacol* **87**, 371–379.

Tyagi P, Tyagi V, Yoshimura N, Witteemer E, Barclay D, Loughran PA, Zamora R & Vodovotz Y (2009). Gender-based reciprocal expression of transforming growth factor-beta1 and the inducible nitric oxide synthase in a rat model of cyclophosphamide-induced cystitis. *J Inflamm (Lond)* **6**, 23.

Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne DA, Ford AP & Burnstock G (2001). P2X3 knock-out mice reveal a major sensory role for urothelially released ATP. *J Neurosci* **21**, 5670–5677.

Wang EC, Lee JM, Ruiz WG, Balestreire EM, von Bodungen M, Barrick S, Cockayne DA, Birder LA & Apodaca G (2005). ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. *J Clin Invest* **115**, 2412–2422.

Wu C, Sui GP & Fry CH (2004). Purinergic regulation of guinea-pig suburothelial myofibroblasts. *J Physiol* **559**, 231–243.

Yu Y & de Groat WC (2008). Sensitization of pelvic afferent nerves in the in vitro rat urinary bladder-pelvic nerve preparation by purinergic agonists and cyclophosphamide pretreatment. *Am J Physiol Renal Physiol* **294**, F1146–F1156.

Zhang QL & Qiao LY (2012). Regulation of IGF-1 but not TGF-beta1 by NGF in the smooth muscle of the inflamed urinary bladder. *Regul Pept* **177**, 73–78.

Additional information

Competing interests

The authors declare no competing interests.

Author contributions

Experiments were performed in the laboratories of M.T.N. and M.A.V., E.J.G., T.J.H., and M.A.V. contributed to the conception or design of the work. E.J.G., T.J.H., M.T.N., and M.A.V. contributed to the acquisition, analysis, or interpretation of data for the work. E.J.G., T.J.H., M.T.N., and M.A.V. contributed to drafting the work or revising it critically for important intellectual content. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All those who qualify for authorship are listed.

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