Serotonin exerts a direct modulatory role on bladder afferent firing in mice

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Running title: Effect of 5HT on afferent firing from the mouse bladder

Keywords: Afferent, bladder, serotonin, 5HT, visceral hypersensitivity

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Key Points

- Functional disorders (i.e., interstitial cystitis/painful bladder syndrome and irritable bowel syndrome) are associated with hyperexcitability of afferent nerves innervating the urinary tract and the bowel respectively.
- Various non-5-HT_3 receptor mRNA transcripts are expressed in mouse urothelium and exert functional responses to 5-HT.
- Whilst 5-HT_3 receptors were not detected in mouse urothelium, 5-HT_3 receptors expressed on bladder sensory neurons plays a role in bladder afferent excitability under both normal conditions and in a mouse model of chronic visceral hypersensitivity (CVH).
- These data suggest that the role 5-HT_3 receptors play in bladder afferent signaling warrants further study as a potential therapeutic target for functional bladder disorders.

Abstract

Serotonin (5-HT) is an excitatory mediator, which in the gastrointestinal (GI) tract, plays a physiological role in gut-brain signaling and which is dysregulated in functional GI disorders such as irritable bowel syndrome (IBS). Patients suffering from IBS frequently suffer from urological symptoms characteristic of interstitial cystitis/painful bladder syndrome, which manifests due to cross-sensitization of shared innervation pathways between the bladder and colon. However, a direct modulatory role of 5-HT in bladder afferent signaling and its role in colon-bladder neuronal crosstalk remain elusive. The aim of this study was to investigate the action of 5-HT on bladder afferent signaling in normal mice and mice with chronic visceral hypersensitivity (CVH) following trinitrobenzenesulfonic acid (TNBS) induced colitis. Bladder afferent activity was recorded directly using *ex vivo* afferent nerve recordings. Expression of 14 5-HT receptor subtypes, the serotonin transporter (SERT) and 5-HT producing enzymes were determined in the urothelium using RT-PCR. Retrograde labelling of bladder projecting dorsal root ganglion (DRG) neurons was used to investigate expression of 5-HT_3 receptors using single cell RT-PCR, while sensory neuronal and urothelial responses to 5-HT were determined by live cell calcium imaging. 5-HT elicited bladder afferent firing predominantly via 5-HT_3 receptors expressed on afferent terminals. CVH animals showed a downregulation of SERT mRNA expression in urothelium, suggesting increased 5-HT bioavailability. Granisetron, a 5-HT_3 antagonist, reversed bladder afferent

hypersensitivity in CVH mice. These data suggest 5-HT exerts a direct effect on bladder afferents to enhance signaling.5-HT_3 antagonists could therefore be a potential therapeutic target to treat functional bladder and bowel disorders.

Introduction

Information regarding the state of bladder distension is carried via sensory afferents that project via the pelvic and hypogastric nerves into the dorsal horn of the spinal cord, feeding into autonomic reflex and micturition centers within the brainstem to maintain continence (see Grundy *et al.* 2018a for review). Bladder afferents are found innervating both the detrusor smooth muscle and the urothelium (Spencer et al., 2018), consisting of myelinated Aδ fibres and unmyelinated C fibres that exhibit polymodal sensitivity to a host of mechanical and chemical stimuli (Su and Gebhart, 1998; Zagorodnyuk et al., 2006; Zagorodnyuk et al., 2007). Hypersensitivity of these bladder afferents to bladder distension may underlie the symptoms of urgency, frequency and nocturia in urological disorders such as overactive bladder syndrome (OAB) and interstitial cystitis/painful bladder syndrome (IC/PBS).

Serotonin (5-HT) is a key neuromodulator, regulating enteric and viscerosensory function as well as acting in the central nervous system (Berger et al., 2009; Gershon and Tack, 2007; Grundy, 2008). Out of the seven members of the 5-HT receptor family (5-HT_{1-7}), six are G-protein coupled receptors (GPCRs) and one, the 5-HT_3 receptor is a ligandgated ion channel (McCorvy and Roth, 2015). Within the bladder, activation of 5-HT_{1A}, 5-HT_2 and 5-HT_3, but not 5-HT_4 and 5-HT_7 can generate altered bladder contraction in rodent models (Chetty et al., 2007; Kodama and Takimoto, 2000; Mittra et al., 2007). $5HT_{3A}$ and 5-HT_{3B} are expressed in the bladder urothelial layer and detrusor, where it is considered to play a role in neurogenic contraction (Chetty *et al*. 2007), whilst 5-HT receptor subtypes 5-$HT_{1D, 2A, 4}$ and 5-HT_6 are present in urothelial cells. 5-$HT_{1A, 1B, 1D, 2A, 2B, 2C, 3A, 4,}$ and 5-HT_7 have also been reported in urothelium-denuded tissues (MatsumotoMiyai et al., 2016). However, a modulatory role of 5-HT on bladder afferent excitability has yet to be explored.

Significant clinical comorbidity exists between a number of lower urinary tract and colonic disorders, such that the symptoms of bladder dysfunction, including urinary frequency, urgency, and pelvic pain are significantly more common amongst IBS patients than in

control groups (Daly and Chapple, 2013; Grundy and Brierley, 2018). Additionally, onethird of IC patients exhibit concurrent IBS symptoms including abdominal pain, discomfort, constipation and/or diarrhea (Aaron and Buchwald, 2001). The origin of these clinical co-morbidities is embedded within the physiological coordination of pelvic organs to provide efficient and synchronized defecation and micturition responses, and their shared innervation pathways within the DRG and spinal cord (Grundy et al., 2019). As such, sensitization of the afferents innervating one pelvic organ, has the potential to sensitize adjacent organs via cross-organ sensitization (Brumovsky and Gebhart, 2010; Christianson et al., 2007; Grundy et al., 2018b; Malykhina, 2007) .

2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis is a commonly used animal model of IBS, triggering chronic visceral hypersensitivity (CVH) that persists after the initial inflammation has resolved (Castro et al., 2017; de Araujo et al., 2014; Hughes et al., 2009; Osteen et al., 2016). Furthermore, it has been shown that these CVH mice exhibit bladder afferent hypersensitivity and abnormal voiding patterns, characteristic of an 'overactive' phenotype (Grundy et al., 2018b). 5HT plays an essential role in health and the development of colonic afferent hypersensitivity associated with irritable bowel syndrome (IBS) in humans (Gershon and Tack, 2007; Grundy, 2008) and animal models (Keating et al., 2008; Linden et al., 2003; Linden et al., 2005). As such, this raises the possibility that bladder hypersensitivity associated with TNBS-induced colitis may involve a 5-HT_3 receptor dependent mechanism.

Therefore, we performed bladder afferent recordings to investigate the modulatory action of 5-HT on primary afferent signaling in mouse urinary bladder and in a TNBS-induced colitis model of colon-bladder neuronal 'cross talk'. We also used calcium imaging and single cell RT-PCR of retrogradely traced bladder-innervating afferent dosral root ganglion (DRG) neurons to characterise the sensitivity of bladder neurons to 5-HT.

Methods

All experiments were conducted in Sheffield, UK except the induction of TNBS, the retrograde tracing studies and dorsal root ganglion (DRG) experiments (calcium imaging and single cell RT-PCR) which were performed in Adelaide, Australia.

141
142 **Animals**
143 All experiments performed in the UK used C57/BL6 adult male mice (12-16 weeks old,
144 25-30 g) from Charles River (Margate, UK). The animals were acclimatized for 7 days in
145 the laboratory animal husbandry unit under 12-hr light/ 12-hr dark cycle and had free
146 access to water and food. The animals were anaesthetized with isoflurane and humanely
147 sacrificed by cervical dislocation according to UK home office legislation (Scientific
148 procedure Act 1986) and in compliance with Journal of Physiology's ethical guidelines
149 (Grundy, 2015).
150
151 Experiments performed in Australia were approved by and performed in accordance with
152 the Animal Ethics Committees of the South Australian Health and Medical Research
153 Institute (SAHMRI; Application # SAM190, SAM195, and SAM281). 12-16 week male
154 and female mice were acquired from an in-house C57BL/6J breeding program (JAX
155 strain #000664; originally purchased from The Jackson Laboratory; breeding barn MP14;
156 Bar Harbor, ME) within SAHMRI's specific and opportunistic pathogen-free animal care
157 facility. Mice were group housed (5 mice per cage) in specific housing rooms within a
158 temperature-controlled environment of 22°C and a 12-hr light/ 12-hr dark cycle. Mice had
159 free access to food and water.
160
161
162 **2,4,6-trinitrobenzenesulfonic acid (TNBS) treatment**
163 13-week old anesthetized male mice were intracolonically administered with
164 2,4,6trinitrobenzenesulfonic acid (TNBS) 0.01 mL (130 µg mL^{-1} in 30% ethanol) via a
165 polyethylene catheter to induce colonic inflammation. Histological examination of colon
166 and bladder was performed to monitor mucosal architecture and signs of inflammation,
167 i.e., cellular infiltration, crypts abscesses, and goblet cell depletion (data not shown). In
168 this study we investigated the effect of TNBS colitis at 2 time points; acute TNBS (3 days
169 post-treatment) when there is an active inflammatory state (termed acute visceral
170 hyposensitivity, 'AVH') and 28 days post-treatment, when the inflammation has resolved
171 but the bladder afferent hypersensitivity still persist (termed chronic visceral
172 hypersensitivity, 'CVH'). For *ex vivo* experiments mice were humanly sacrificed by
173 cervical dislocation.
174

Extracellular afferent nerve recordings

Bladder afferent nerve activity was determined using an *ex vivo* model previously described (Daly et al., 2007; Grundy et al., 2018a). The whole bladder and surrounding tissues (together with its emanating nerve fibers) was placed in a recording chamber (30 mL). The preparation was continuously perfused at a rate of 5 mL minute^{-1} with oxygenated (95%O_2/5%CO_2) Krebs bicarbonate solution (composition, mM: NaCl 118.4, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KCl 4.7, KH_2PO_4 1.2, glucose 11.7) at constant temperature of 35 °C. A polythene catheter (0.28 mm) was inserted into the urethra to perfuse the bladder with isotonic saline (0.9% NaCl) or pharmacological reagents using a perfusion pump (Genie, Kent, multi-phaser TM model NE-1000) with a rate 100 µL minute^{-1}. The bladder dome was punctured at the apex with a syringe needle (BD microlanceTM, 19G 2") and a dual- lumen catheter was inserted and secured with suture.One arm of the catheter was connected to a pressure transducer (DTXTM plus DT-XX, Becton Dickinson, Singapore) to monitor intravesical pressure and the other arm was connected to the 3 way tap to allow bladder filling (tap closed) or emptying (tap open).Multiunit pelvic and hypogastric nerves were dissected into a fine branches and placed into a suction electrode which was attached to a Neurolog headstage (NL 100, Digitimer, Ltd, UK), amplified with an AC amplifier (NL104) and filtered (NL125, band pass filter. The multi-unit afferent nerve discharge frequency (spikes/sec) was quantified using a spike processor (Digitimer D130). The signal was visualized on a computer running Spike 2 software (Version 7.1, Cambridge Electronic Design, UK).

Drug application

After a 30-minute stabilization period the bladder was distended by an intravesical infusion with saline at a rate of 100 µL minute^{-1} to a maximal pressure 50 mmHg (control distensions). This was repeated at 10-minute intervals for 30-60 minutes to establish reproducible afferent responses before starting the protocol. After the control distensions, pharmacological agents were either perfused into the bladder lumen (intravesical application) or into the recording chamber (bath application). Following application of any compound a 'wash out' was conducted using either saline (intravesical) or Krebs (bath application) as appropriate for 30 minutes.

Isovolumetric protocol

In order to evaluate the effect of a pharmacological reagents on spontaneous afferent firing and bladder tone, isovolumetric experiments were conducted. After a 30-60 minute

stabilization period the bladder was filled to an intravesical pressure of 15 mmHg and left to stabilize for 30 minutes to allow the bladder to accommodate to the intravesical volume before bath application of agonists or antagonists. Nerve firing and bladder tone were continuously captured. Bladder contraction was determined as an increase in intravesical pressure.

Isolation of urothelial cells

Urothelial cells were isolated as previously described (Daly et al., 2014). The bladder was dissected longitudinally under a stereo microscope to expose the urothelium to the media (fresh Modified Eagle Media (MEM) (Gibco®) containing 0.7% HEPES and 1% antibiotic-antimycotic (PSF) solution (Gibco®) at 37˚C and transferred to a Sylgard® (Dow Corning) coated dish. The tissue was stretched and pinned. The media was removed and replaced with 2.5 mg/mL dispase in MEM at 37°C. The bladder was incubated in dispase for 2 hours at room temperature. After the dispase had been aspirated, the urothelium was gently scraped with a scalpel under the stereo microscope and the cells were immediately placed in 0.5% trypsin-EDTA (Gibco®). The solution was incubated at 37 °C for 10 minutes, and gently triturated every a few minutes. The trypsinEDTA was deactivated by adding pre-warm MEM with 10 % Fetal Bovine Serum (FBS) (Gibco®). The cell suspension was centrifuged at 1500 rpm, 4 °C for 15 minutes. The solution was gently aspirated and the pellet was resuspended in pre-warmed Keratinocyte-serum free medium (K-SFM) and centrifuged at 1500 rpm, 4 °C for 15 minutes. For calcium imaging experiment, the cells were resuspended in K-SFM 200 µL and plated on collagen IV (Sigma Aldrich Poole, UK) coated coverslips in a 12 wells plate and incubated in 5% CO_2-95% O_2 at 37 °C overnight. For PCR experiments, the cell pellet was washed by adding PBS and centrifuged at 1000 rpm, 4 °C for 5 minutes and stored at -80 °C for RNA isolation process.

Reverse-transcription PCR (RT-PCR)

Urothelial cell pellets were used in this experiment. The total RNA was extracted using an RNeasy mini Kit (Qiagen, Valencia, CA, USA. RNA was transcribed to cDNA by High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Carlsbad, CA, USA).PCR reactions were prepared using Gotaq® Green Master Mix (Promega M7121) according to manufacturer's instructions (Total volume 25 µL; Gotaq Gotaq® Green Master Mix 12.5 µL, forward and reward primers 2.5 µL, cDNA for 50 ng RNA, nuclease-free water was adjusted to volume 25 µL). The samples were run in

triplicate. The list of exon spanning primers used is summarised in Table 1. ß-actin was used as a house keeping gene and either brain, DRG neurons, or duodenum was used as positive control. For negative control reactions, distilled water was used instead of cDNA samples. All the PCR products of detected 5-HT receptors were checked by DNA sequencing and all genes showed a high percentage matching (96-100%) to the original sequences, suggesting that the designed primers were efficient and the detected 5-HT receptor results were valid (data not shown).

Table 1 Summary of primer sequences used in RT-PCR

Receptor subtypes	Accession number	Primer sequences	Product size (bps)	Positive control
5-HT_{1A}	NM_008308	FW: 5' TAAGAACTTCCCGCTCCAGT 3'	103	Brain
		RW: 5' AGAAATGCAAGGGGATCTCC 3'		
5-HT_{1B}	NM_010482	FW: 5' CCAACACACAATAAATGCTCCT3'	135	DRG
		RW: 5' CCAAGTCAAAGTGCGAGTCT 3'		
5-HT_{1D}	NM_008309	FW: 5'TACAAACACCCCTACTAAACGC 3'	310	DRG
		RW: 5'ATGAGTGTTCAGCGTTGGTT 3'		
5-HT_{1F}	NM_008310	FW: 5'GACCAGAGCCCCTTAGCTTC 3'	340	DRG
		RW: 5'TGCAGCTTCCGAGTCACAAT 3'		
5-HT_{2A}	NM_172812	FW: 5' CATCTCCCTGGACCGCTAC 3'	150	DRG
		RW: 5' TCATCCTGTAGCCCGAAGAC 3'		
5-HT_{2B}	XM_006529146	FW: 5' CCGATTGCCCTCTTGACAAT 3'	120	DRG
		RW: 5' GGCACAGAGATGCATGATGG 3'		
5-HT_{2C}	NM_008312	FW: 5' TGAAACTGGTTGCTTAAAACTGA 3'	126	DRG
		RW: 5' AGCTGCTACTGGACTTATGGA 3'		
5-HT_{3A}	NM_013561	FW: 5' CCACCTTCCAAGCCAACAAG 3'	128	DRG
		RW: 5' CTCCCTTGGTGGTGGAAGAG 3'		
5-HT_{3B}	NM_020274	FW: 5' TGATTCTTCTGTGGTCCTGC 3'	154	DRG

		RW: 5' GCCTCAGCCCAGTTGTAAAC 3'		
5-HT_4	NM_008313	FW: 5' ATGTTCTGCCTGGTCCGG 3'	162	DRG
		RW: 5' GCCTCCCAACATTAATGCGA 3'		
5-HT_{5A}	NM_008314	FW: 5' AAGACCAACAGCGTCTCCC 3'	124	Brain
		RW: 5' TCCACGTATCCCCTTCTGTC 3'		
5-HT_{5B}	NM_010483	FW: 5' TCTCCTTCGACGTGTTGTGC 3'	469	Brain
		RW: 5' GAGTCTCCGCTTGTCTGGAA 3'		
5-HT_6	NM_021358	FW: 5' TGGGCAAAGCTCGAACATCT 3'	386	Brain
		RW: 5' GTCACATACGGCCTGAGCTAT 3'		
5-HT_7	NM_008315	FW: 5' AAGTTCTCAGGCTTCCCACG 3'	485	DRG
		RW: 5' CAGTTTTGTAGCACAAACTCGCT 3'		
TPH1	NM_009414	FW: 5' CTAGGAGTTCATGGCAGGTG3'	83	Duodenum
		RW: 5' TTTCGAGTCTTTCACTGCACT 3'		
TPH2	NM_173391	FW: 5' TTCCCAGGGTCGAGTACACA 3'	216	Brain
		RW: 5' GTCTCTTGGGCTCAGGTAGC 3'		
SERT	NM_010484	FW: 5' CATAGCCAATGACAGACAG 3'	352	Duodenum
		RW: 5'CAAAACCAAGAACCAAGAC 3'		

Retrograde tracing of bladder innervating DRG neurons

A small aseptic abdominal incision was made in anesthetised (isoflurane 2-4 % in oxygen) mice. Cholera toxin subunit B conjugated to AlexaFluor® 488 (CTB-488; 0.5 % diluted in 0.1 M phosphate buffered saline (PBS); ThermoFisher Scientific) was injected at four sites into the bladder wall (2µL / injection) using a 5 µl Hamilton syringe attached to a 23-gauge needle. The abdominal incision was sutured closed and analgesic (Buprenorphine; 2.7µg / 30g) and antibiotic (Ampicillin; 50 mg/kg) given subcutaneously as mice regained consciousness. Mice were allowed to recover, housed individually and monitored for four days, in order to visualize CTB-labelled afferent neurons in the DRG.

Isolation of DRG neurons

DRGs from lumbosacral (L5-S1) spinal levels of the mouse spinal cord, which correspond to the pelvic innervation of the bladder, were isolated and incubated in Hanks balanced salt solution (HBSS) (pH 7.4) containing collagenase (4mg/mL), and dispase (4.5mg/mL), at 37°C for 30 minutes. The collagenase/dispase solution was aspirated and replaced with HBSS containing collagenase (4mg/mL) for 10 minutes at 37°C. The collagenase solution was aspirated and replaced with 600µl DMEM (GIBCO) containing 10% FCS (Invitrogen), 2 mM L-glutamine (GIBCO), 100 µM MEM non-essential amino acids (GIBCO) and 100 mg/ml penicillin/streptomycin (Invitrogen). Neurons were dissociated via trituration with a Pasteur pipette and spot plated onto 15 mm coverslips coated with laminin (20 µg/mL) and poly-D-lysine (800 µg/ mL) and maintained in an incubator at 37 °C in 5% CO_2. Calcium imaging recordings were performed on DRG neurons 18-30hrs post isolation.

Single cell RT-PCR

Cells were used 3-8 hrs after plating a cover slips. Under continuous perfusion of sterile and RNA-/DNase-free PBS, retrogradely traced single DRG neurons (N=3/group; HC: total 77 cells, CVH: total 74 cells; 23-27 cells per mouse) were identified using a fluorescent microscope and picked using a micromanipulator into the end of a fine glass capillary. The glass capillary containing the cell was then broken into a sterile Eppendorf tube containing 10 µL of lysis buffer with 1µl DNAse (TaqMan Gene Expression Cellsto-CT Kit; Life Technologies). Samples were treated according to manufacturer's instructions for cDNA synthesis using SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher Scientific) and RT-PCR using TaqMan™ Universal Master Mix (ThermoFisher Scientific). Ready-made TaqMan probes were purchased from LifeTechnologies (Htr3a: Mm00442874_m1). For each coverslip of cells, a bath control was also taken and analysed together with cells. A total of 45 cycles was run and only samples with a complete amplification curve were considered as positive. After lysis and termination of DNAse treatment, samples were immediately frozen on dry ice and stored at -80°C until cDNA synthesis was performed. Tubulin-3 expression (Mm00727586_m1) served as a neuronal marker and positive control and for every coverslip a bath control was taken and analysed together with other samples. GFAP expression (Mm01253033_m1) was measured to exclude contamination with glial cells. PCR products were stored at -20C and resolved on a 3-4% TBE agarose gel (UltraPure

Agarose 1000, cat#16550-100, Invitrogen). All samples were visualized by adding 1µl of Midori Green Direct (NIPPON Genetics) to 20µl of sample and 5µl of samples was loaded onto wells. A 20bp marker (BioRad) was used to check for correct size.

Quantitative real-time RT-PCR

To determine the quantitative expression of TPH1 2 and SERT in urothelial cell samples from sham and TNBS treated animals, quantitative RT-PCR was used. The quantification of mRNA expression determined using TaqMan Gene Expression Master Mix (Applied Biosystems 4374657). The reaction was prepared on ice and mixed in Hard-Shell® ThinWall 96-Well Skirted PCR Plates (BIO-RAD, HSP-9665). Prior to running the reactions, the plates were covered with MicroAmpTM Optical Adhesive Film for 96-Well Plates
(Applied Biosystems, 43111971) and centrifuged briefly to spin down the contents and eliminate any air bubbles from the solutions. The reaction for each sample and gene was run in duplicate. DNase free water was used to replace cDNA as a negative control for each gene and plate. PCR reactions were performed in a BIO-RAD CFX96 TouchTM Real-time thermocycler (C1000 TouchTM Thermal Cycler, Bio-Rad Laboratories Ltd. Hercules, USA). Results were expressed as relative expression to the housekeeping gene GAPDH (1/ΔCt), and fold change was calculated using the equation 2−Δ(ΔCt).

Calcium imaging

Cultured DRG neurons (18-30 hours) or isolated urothelial cells were loaded with 2 µM fura-2-acetoxymethyl ester (Fura-2) for 15 minutes at 37°C and washed with HEPES buffer (10mM, NaCl 142mM, KCl 2mM, glucose 10mM, $CaCl_2$ 2mM, M_gCl_2 1mM, HEPES 10mM; pH 7.4) for 30 minutes prior to imaging at room temperature (23°C). Fura-2 was excited at 340 and 380 nm. Fluorescence images were obtained every 5s using a 20x objective. Retrogradely traced bladder-innervating DRG neurons were identified by the presence of the CTB-488 tracer. Data were recorded and analysed using MetaFluor software. After an initial baseline reading to ensure cell fluorescence was stable (an indication of healthy cells), DRGs and/or urothelial cells were stimulated with either 5-HT (100 µM), 2-Me-5HT (100 µM)in the absence or presence of granisetron (1 µM), and changes in intracellular calcium $[Ca^{2+}]_{i,}$ were monitored in real-time. Ionomycin or KCl (40 mM) was applied as a positive control for cell viability.

Drugs and solutions

5-hydroxytryptamine (5-HT) and 5-methoxytryptamine (5-MT) were obtained from Sigma Aldrich, UK. 2- Methy5- hydroxytrptamine (2- Me- 5- HT) was obtained from Tocris/Bioscience, UK. Granisetron hydrochoride was obtained from LKT Laboratories, USA. ML- 9 was obtained from Cayman Chemical. Y- 27632 was obtained from Chemdea, USA.

Data analysis

Spontaneous afferent firing (also referred to as baseline) was determined by measuring mean action potential firing rate over 10 seconds either at rest (before a drug application or before a distension). This is defined as the afferent activity arising from the bladder in the absence of a stimuli (mechanical or chemical). Mechanosensitive afferent firing was determined by performing ramp distension of the bladder (as described above) and measuring the afferent discharge at various intravesical pressure points using a custombuilt script from SPIKE 2 version 7 (CED, UK). Data are expressed as means ± SEM. Statistical analysis was analyzed using one- or two-way analysis of variance (ANOVA) with Dunnett's or Tukey multiple comparison or Student's t test, where appropriate. The statistical analysis were considered significant at $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.

Results

Intravesical administration of 5-HT agonists excites bladder afferent nerves In order to determine if bladder afferents functionally express 5-HT receptors, we investigated the effects of various 5-HT receptor agonists on *ex-vivo* bladder afferent nerve activity. We found that intravesical infusion of 5-HT (100 µM), evoked a dramatic and sustained increase in baseline afferent nerve firing compared to intravesical saline (Fig. 1*A*, 1*B*). The receptor mechanisms underlying this effect were determined by comparing the responses to 2-Me-5-HT, a selective 5-HT_3 agonist (100 µM), with 5-MT (100 µM) a compound which exhibits selectivity for the GPCR family of 5-HT receptors including 5-HT_1, 5-HT_2 and 5-HT_{4-7}. The responses to application of 5-HT and 2-Me-5HT were comparable. However while the response to 5-MT, was significantly increased relative to baseline, the magnitude of the firing was reduced compared to that of 5-HT and 2-Me-5-HT (Fig. 1*C*, 1*D*). These data suggest that multiple 5-HT receptors may influence bladder afferent firing, and that this may occur via both direct and indirect mechanisms.

5-HT receptors are expressed on bladder urothelium

Given the established role of the urothelium as a sensory structure, we wanted to determine if the effects of intravesical 5-HT on bladder afferent firing were secondary to activation of urothelial cells or a consequence of direct activation of primary afferent endings innervating the bladder. To this end we investigated serotonergic receptor expression in the bladder urothelial layer using RT-PCR. The expression profile for the various 5-HT receptors in the urothelium is shown in Figure 2. Overall, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_4, 5-HT_6, and 5-HT_7 receptors were all detected. In contrast, 5-HT_{1F}, 5-HT_{2C}, 5-HT_{3A}, 5-HT_{3B} or the 5-HT_{5A}, 5-HT_{5B} receptors were not detected in the bladder urothelium. Moreover, expression of the 5-HT producing enzymes, tryptophan hydroxylase-1, and 2 (TPH-1 and TPH-2), and the sodium-dependent serotonin transporter (SERT) transcripts were also examined using PCR and all three were shown to be expressed in the urothelium (Fig. 2). This finding raises the possibility of an endogenous source of 5-HT in the bladder wall.

Stimulation of non-5HT_3 receptors on the urothelium can evoke urothelial cell activation

Calcium imaging of isolated primary mouse urothelial cells (PMUCs) was conducted to investigate the functionality of 5-HT receptors in the urothelium. Incubation of PMUCs

with 5-HT triggered an increase in intracellular Ca^{2+} indicative of cellular activation. Consistent with our RT-PCR data identifying an absence of 5-HT_3 receptor expression in the urothelium, both the magnitude of the response to 5-HT and the number of responding cells was unchanged following incubation with the 5-HT_3 selective antagonist granisetron (Fig. 3*B*, 3*C*).

Activation of 5-HT receptors by bath application of agonist alters afferent nerve firing from the bladder and induces contraction of the detrusor

To elucidate if the 5-HT effects on bladder afferent activity are secondary to induced detrusor contraction, we applied agonists, antagonist and muscle contraction inhibitors under isovolumetric bladder conditions to determine their effect on spontaneous afferent firing and associated intravesical pressure changes. A representative trace of extraluminal 5-HT application on bladder afferent firing and intravesical pressure is illustrated in Fig. 4A. We found that 5-HT (100 µM), 2-Me-5-HT (100 µM) or 5-MT (100 µM) all evoked a marked increase in bladder afferent nerve firing, and a small but robust increase in intravesical pressure indicating detrusor contraction (Fig. 4*B*, 4*C*).

The peak afferent response to bath application of 5-HT and 2-Me-5-HT was similar. Moreover, application of the selective 5-HT_3 receptor antagonist, granisetron (1 µM), significantly attenuated afferent responses to 5-HT. To uncouple the direct effect of 5HT on the afferent nerves from any indirect nerve response produced by bladder contraction we used a myosin light chain kinase inhibitor (ML-9, 10 µM) and/or the rhoassociated kinase inhibitor (Y-27632, 10 µM) to prevent contraction. Under such conditions the afferent response to 5-MT was abolished in the absence of contraction, suggesting that the response to 5-MT was secondary to changes in muscle tone. Conversely, the afferent response to 2-Me-5-HT persisted despite the absence of contraction, suggesting that the contractile and nerve responses to 5-HT_3 receptor stimulation were independent (Fig. 4B and 4C).

Chronic visceral hypersensitivity evoked with intra-colonic TNBS caused altered mechanosensitivity and increased spontaneous afferent firing from the bladder, an effect that was reversed by blocking the 5-HT_3 receptor.

Bladder hypersensitivity has been described in response to colonic inflammation, a phenomenon referred to as cross-organ sensitization. Since 5-HT is implicated in colonic hypersensitivity we investigated the extent to which it may contribute to altered bladder

afferent signalling in a mouse model of TNBS-induced colonic inflammation. We examined 2 time points representing acute inflammation (3 days post-treatment) and a chronic state in which inflammation had resolved (28 days post-treatment). Acute TNBS treatment had no significant effect on spontaneous nerve firing from the bladder and caused a moderate (~25% from sham control) decrease of mechanosensitive afferent firing at 50 mmHg (Fig. 5A). Conversely, in the post-inflammatory CVH state there was a significant increase in spontaneous bladder afferent firing (~160%) and in the afferent response to bladder distension (~27%) when compared to sham controls (*P<0.05, Fig 5). Treatment with granisetron (1 μM) prior to 5-HT application normalized both the spontaneous bladder discharge and the mechanosensitivity observed in the CVH model (Fig. 5A, 5B).

SERT mRNA expression was downregulated in urothelial cells of CVH mice.

The attenuated responses in the presence of granisetron implies a role for endogenous 5-HT in bladder hypersensitivity. We hypothesized that there might be a change in 5-HT bioavailability in the urothelium following chronic visceral hypersensitivity (CVH) induced by intra-colonic TNBS. Quantitative RT-PCR showed that the level of SERT mRNA expression was significantly lower in urothelial cells from bladders in TNBS treated mice compared to sham operated controls (*P<0.05, Fig. 6A). TPH1 and TPH2 mRNA expression was also reduced, but because of the large variability, especially in TPH1 levels, this did not reach significance (Fig. 6*B*, 6*C*). Nevertheless, these data are consistent with a role for urothelial 5-HT in bladder hypersensitivity.

Bladder projecting DRG neurons express 5-HT_3 receptors in both control and CVH mice and display functional response to 5-HT and 2-Me-5-HT activation.

To examine if 5-HT3 receptors in the afferent terminals play a role in 5-HT-senstitized bladder afferent firing, we performed retrograde labelling and single cell RT-PCR to examine 5-HT_3 receptor gene expression in bladder-projecting DRG neurons. The vast majority of these neurons (91%, 70/77 neurons) expressed 5-HT_3 receptors even in control animals, which is striking given the absence of any previously documented endogenous source of 5-HT. Moreover, 95% of bladder-innervating DRG isolated from mice with CVH expressed 5-HT_3 (70/74 neurons), showing a small increase in the number of neurons expressing 5-HT_3 occurs during cross-organ sensitization. (Fig. 7*A*).

In addition, we performed calcium imaging on bladder innervating (traced) and nontraced lumbosacral DRG neurons. A representative trace of calcium imaging of lumbosacral DRG neurons in response to 5-HT (100 µM) and 2-Me-5-HT (100 µM) is shown in Figure 7B. 60 out of a total of 69 (87%) bladder projecting DRG neurons (traced neurons) elicited a calcium signal in response to application of 5-HT, moreover a significantly greater proportion of the traced DRGs responded to 5HT than the non-traced DRG neurons. This indicates that expression of 5-HT receptors maybe enriched in bladder neurons compared to the generalized neuronal population. In addition, a similar proportion of traced neurons (70%, 36/51) showed an increase in Ca^{2+} influx after the application of 2-Me-5-HT (Fig. 7C). The magnitude of the Ca^{2+} response to 5-HT and 2Me-5-HT was similar in traced and non-traced DRG neurons (Fig. 7D). These data are consistent with a major contribution of 5-HT_3 receptors to 5-HT's ability to sensitize bladder afferents and enhance firing.

Discussion

Over the past decade, a number of studies, including our own, have investigated the chemical and mechanical stimuli that drive afferent transmission from the bladder. Despite this, the mechanisms involved in generating bladder hypersensitivity still remain elusive. There is significant clinical comorbidity between hypersensitivity disorders of the bladder and hypersensitivity disorders of the colon (such as IBS), pointing to a common underlying etiology involving dichotomizing afferent fibres between the bladder and bowel (Grundy and Brierley, 2018; Malykhina, 2007). Serotonin (5-HT) plays integral roles in secretion, motility and visceral sensitivity of the GI tract (Grundy, 2008). However, relatively little is known about how 5HT affects afferent signaling from the bladder in healthy or hypersensitivity states. In this study we investigated the modulatory action of 5-HT on primary afferent signaling in the mouse urinary bladder and in a TNBSinduced model of colitis which induces colon-bladder cross-organ sensitization (Grundy et al., 2018b).

5-HT increases bladder afferent firing and induces contraction of the detrusor

Stimulation of 5-HT receptors with serotonin caused a large increase in bladder afferent nerve firing, which was concomitant with a small but significant contraction of the bladder. When the contraction was blocked using a combination of muscle blockers the sensory response was significantly attenuated. This suggests that a proportion of the afferent response to 5-HT occurred via the activation of mechanosensory afferent nerves which

were activated in response to bladder contraction. However, since the afferent response to 5-HT was only partially reduced, it is also likely that there was also an additional direct mechanism by which 5-HT stimulated a receptor(s) on the nerve terminal, inducing nerve firing in a mechanism that was independent from changes in the muscle tone.

Similar to 5-HT, bath application of the selective 5-HT_1, 5-HT_2, 5-HT_4, 5-HT_6, and 5-HT_7 agonist, 5 methoxy tryptamine (5-MT) also caused an increase in afferent nerve firing and evoked a small contraction of the bladder. However, when the contraction was blocked by preincubation with the muscle blockers (Y-27632 and ML-9), the contraction was lost and the afferent response to 5MT was abolished suggesting that the afferent response to stimulation of the non-5HT3 receptor populations was secondary to changes in bladder contraction rather than via a direct action at the afferent terminal. A number of previous studies have demonstrated that 5-HT can induce contraction of bladder muscle strips *in vitro*, however the receptor that mediates the contraction remains controversial. Hattori *et al*., (2017) show that both 5-HT and a selective 5-HT_2 receptor agonist cause contraction of the bladder, but a lack of efficacy following pre-incubation of selective 5HT_2 receptor antagonists suggested that the effect was not mediated by the 5-HT_2 receptor (Hattori et al., 2017). In another study it was shown that 5-HT potentiates neurogenic contractions of rat isolated detrusor muscle through both 5-HT_7 and 5-HT_{2c} receptors (Rekik et al., 2011). Moreover, in the guinea pig the contractile response to 5HT was suggested to be via the 5-HT_2, 5-HT_3 and 5-HT_4 receptors (Yoshida et al., 2002).

5-HT has a direct effect on afferent firing via the 5-HT3 receptor

Bath application of 2-Me-5-HT caused a robust increase in nerve firing that was not affected by blocking contractility. 2-Me-5-HT is a potent agonist of both the 5-HT_3 and the 5-HT_6 receptor (Glennon et al., 2000) suggesting that activation of either 5-HT_3 or 5HT_6 located on the urothelium (in the case of 5HT_6) or at the nerve terminal results in afferent nerve firing from the bladder. Since we did not use a selective 5HT_6 receptor antagonist we cannot determine from these data what contribution 5HT_6 made to the 2Me-5-HT response. Since the role of 5HT_6 in the lower urinary tract is relativity understudied this would warrant further investigation. However in this study we focused on the 5-HT_3 receptor since pre-incubation of the 5-HT_3 selective antagonist granisteron inhibited the 5-HT-mediated increase in bladder afferent nerve firing by ~65%. Since 5HT_3 receptors were not identified in the urothelium via RT-PCR, this suggests that stimulation of 5-HT_3 receptors can elicit a direct increase in afferent nerve firing by stimulating receptors on

the nerve terminal rather than via an indirect mechanism as a result of muscle contraction or urothelial signaling. Our retrograde tracing studies revealed that the majority of lumbosacral DRG neurons innervating the mouse urinary bladder (90%) express the 5-HT_3 receptor. Furthermore, our functional calcium imaging showed that the majority of these DRGs exhibit functional responses to 5-HT (87%) and the 5-HT_3 selective agonist, 2-Me-5-HT (70%). In this study we did not stain the afferent terminals and show expression of 5HT3 in bladder afferent endings, although it is well established that expression in the cell body where the receptor is synthesized is likely to be indicative of expression at the afferent terminal. Therefore these data suggest that 5HT exerts a direct action on 5-HT_3 receptor at the terminal endings of the primary afferents innervating the bladder. This idea is supported by previous studies which show that 5-HT_3 receptors are also located in rat DRG neurons (Nicholson et al., 2003) and that systemic injection of a 5-HT precursor (5-hydroxytryptophan, 5-HTP), triggers bladder hyper-excitability in a mechanism mediated by spinal 5-HT_3 receptors (Hall et al., 2015).

A recent study has also shown that bladder-projecting neurons in the caudal raphe nucleus, the area of the brainstem which exhibits supraspinal control of the bladder, also express serotonin (Ahn et al., 2018), suggesting that serotonergic control of the bladder could have both peripheral and central components which involve the 5-HT_3 receptor.

5-HT can alter signaling in the urothelium in a mechanism that is independent of the 5-HT_3 receptor

Over the past decade it has become increasingly clear that the urothelium of the bladder plays an active role in sensation. It is responsive to bladder filling and releases an array of mediators and neurotransmitters which target underlying afferent nerves to modulate firing (urothelial- afferent signaling). Since 5-HT receptor agonists altered sensory signaling from the bladder we wanted to determine what role the urothelium played in the afferent response. mRNA for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_4, 5-HT_6, and 5-HT_7 receptors were identified in the urothelium and our functional calcium imaging experiments demonstrated that ~60% of isolated primary urothelial cells were activated by stimulation with 5-HT. This indicates that there may be functional 5-HT receptors in the urothelium that could play a role in both autocrine urothelial mechanisms and paracrine sensory mechanisms. It is important to note that we did not conduct any western blot analysis or immunocytochemistry to confirm the protein expression of these receptors in the urothelium, nor did we use an array of selective agonist and antagonists

in the calcium imaging experiments, this makes it difficult for us to identify which receptor(s) mediate the calcium responses. However, pretreatment with the 5-HT_3 selective antagonist, granisetron had no effect on the magnitude of the calcium signals or on the numbers of responsive cells. This taken together with the fact that mRNA for the 5-HT_3 receptor was not identified in the urothelium suggests that while the 5-HT_3 receptor may be involved in the direct afferent response to 5HT (i.e. at the 5-HT_3 receptor on the primary afferent terminal) it is not involved in urothelial signaling. Further studies are required to delineate the differntial role that non-$5HT_3$ receptors play in mediating contractility and urothelial signaling in the bladder.

5-HT_3 receptor mechanisms contribute to bladder hypersensitivity in CVH.

Christianson *et al.,* (2007) demonstrated that 21% of all retrograde traced mouse DRGs from the colon also innervated the bladder (dichotomizing neurons) (Christianson et al., 2007). Since co-morbidities between the bladder and bowel are common, the hypothesis of 'cross-organ sensitization' has emerged. Evidence for this phenomenon has come from pre-clinical studies identifying that inflammation or injury to one organ, such as the bowel, can lead to changes in the sensitivity of another closely located organ, such as the bladder, via these convergent neural pathways. A number of models of colon-bladder cross organ sensitization have been developed to study this phenomenon, one such model is the well-established TNBS model of colitis (Antoniou et al., 2016; Grundy et al., 2018b; Malykhina et al., 2006; Qin et al., 2005) . Previous studies using the TNBS induced colitis model suggest that inflammation of the bowel induces hypersensitivity of colonic afferents and changes in spinal neural circuitry that persists after the inflammation as resolved (Brierley and Linden, 2014). This effect is concomitant with hypersensitivity of the bladder and bladder voiding dysfunction indicative of overactive bladder syndrome or painful bladder syndrome, without any obvious morphological changes in bladder structure. Two phases of response have been identified; an acute phase of visceral hyposensitivity (AVH, <15 days post treatment) and a chronic phase of visceral hypersensitivity (CVH, >28 days post treatment) (Grundy et al., 2018b).

The $5HT_3$ receptor has been linked to intestinal dysfunction in a number of studies. For example, the selective 5-HT_3 antagonists tropisetron and ondansetron significantly reduce signs of inflammatory damage in the bowel in a TNBS-induced colitis model in the rat (Motavallian-Naeini et al., 2012; Motavallian et al., 2013). Bioavailability of 5-HT in the bowel, as measured by expression of the serotonin reuptake transporter (SERT),

599 is reduced in mice (Linden et al., 2005), guinea pigs (Linden et al., 2003) and humans
600 with either inflammatory conditions of the bowel or irritable bowel syndrome (Coates et
601 al., 2004). In addition, in a *Trichinella spiralis* induced model of post-infectious IBS,
602 longterm hypersensitivity of the small intestine was shown to be mediated by changes in
603 $5HT_3$ receptor expression (Keating et al., 2008).
604
605 In the present study, after the induction of colitis with TNBS, the afferent response to
606 bladder distension was actually inhibited by ~20%, suggesting hyposensitivity of the
607 bladder afferent nerves in the acute phase colitis. However, in the post-inflammatory
608 pahse of CVH, the afferent response to bladder distension was significantly increased.
609 This is consistent with previous data (Grundy et al., 2018b), and suggests that there was
610 long-term hypersensitivity of bladder afferents following inflammation of the bowel.
611 Interestingly, the distension-induced hypersensitivity was attenuated via pretreatment
612 with the 5-HT_3 selective antagonist granisetron, implicating the 5-HT_3 receptor in the
613 response. The numbers of bladder-projecting DRG neurons expressing 5-HT_3 was also
614 slightly elevated in the CVH phase.
615
616 Interestingly, mRNA for the 5-HT producing enzymes, TPH1 and TPH2, and the serotonin
617 transporter, SERT was detected in the normal urothelium and in the urothelium of mice
618 following TNBS. However, in the urothelium of CVH mice we observed a significant
619 decrease in SERT expression, which may suggest that following colonic inflammation
620 there were alterations in the bioavailability of 5-HT. This could potentially lead to
621 reciprocal changes in 5-HT_3 receptor sensitivity and bladder afferent hypersensitivity.
622
623 **What is the endogenous source of 5-HT in the bladder?**
624 The majority of 5-HT is the body is produced by enterochromaffin cells in the GI tract
625 (Gershon and Tack, 2007) and in the CNS. Activation of enterochromaffin cells can lead
626 to the release of 5-HT and activation of 5-HT_3 receptors on colonic afferents innervating
627 the colonic mucosa (Bellono et al., 2017). In addition, mast cells also synthesize 5-HT
628 from 5-hydroxytryptophan and express TPH1 in the rat and human gastrointestinal tracts
629 (Dwyer et al., 2016; Weitzman et al., 1985). However, the endogenous source of 5-HT in
630 the bladder has yet to be identified. Since the components needed to produce 5-HT are
631 present in the urothelium (ie TPH1 and TPH2 mRNA), it is possible that urothelial cells
632 produce and release 5-HT which can then exert actions on afferent nerve fibers located
633 in close proximity, or the underlying smooth muscle. Despite the presence of the

molecular architecture responsible for 5-HT synthesis we identified within the urothelium, there have been no previous reports of 5HT release from the bladder in the literature to support this idea. (Fitzgerald *et al.* (2013) showed that there was a significant increase in the number of mast cells in the bladder at 12 days after colonic TNBS administration in rats, raising the possibility that in the post-inflammatory state, 5-HT levels in the bladder rise due to infiltration of circulating mast cells (Fitzgerald et al., 2013). This mechanism could drive altered sensory activity via an interaction with the 5-HT_3 receptor on the primary afferents. Unfortunately, since we did not measure 5-HT levels or mast cell numbers in the bladders from our CVH mice this remains speculative.

Recent studies have demonstrated the presence of 5-HT positive paraneurons in the mouse urethra which have a close association with urethral afferent nerves (Kullmann et al., 2018). In our extracellular recordings the primary afferents we recorded from lie in close proximity to the bladder neck. Early work by Gabella and Davis demonstrated that the majority of afferent innervation to the bladder lies in the lower 1/3 of the bladder body (Gabella and Davis, 1998). Since our electrophysiological recordings consist of multi-unit nerve bundles it is possible that some of the afferents that we recorded from emanated from the bladder neck and urethra. Moreover, it is also possible that the urethral paraneurons could provide an endogenous source of 5-HT, which when released in the urethra either 'diffuses' to reach some of the bladder projecting afferents or via the urethral urothelium alters urothelial- afferent signaling in the bladder body. A recent *in vivo* study lends support to this idea showing that 5-HT released by the urothelial cells located in the urethra can actually activate a urethra-vesical pathway to enhance contraction of the bladder (Coelho et al., 2018). This is a fascinating area for further research and further highlights the complexity of signaling in the lower urinary tract.

Translational Perspectives

These data suggest that 5-HT plays a key role in the modulation of bladder sensory firing and the generation of visceral hypersensitivity. This may have important implications for understanding normal function of the bladder and the changes in bladder function that arise when serotonergic signaling is disrupted, such that occurs in IBS or with depression and anxiety.

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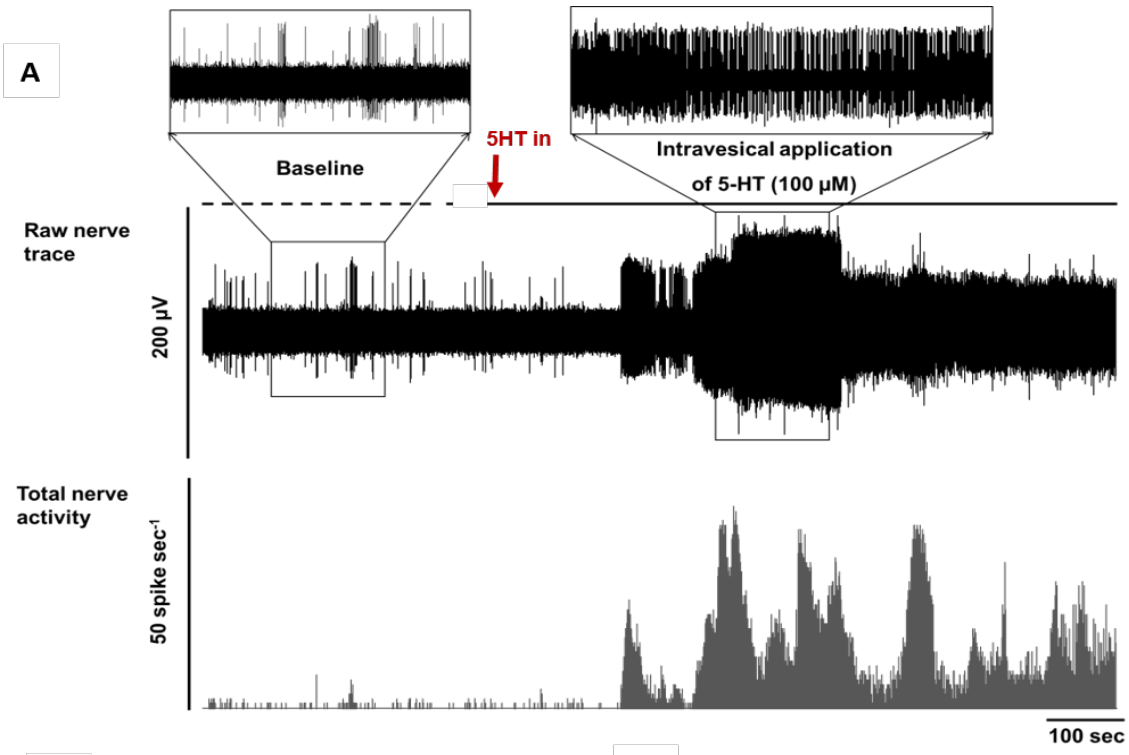
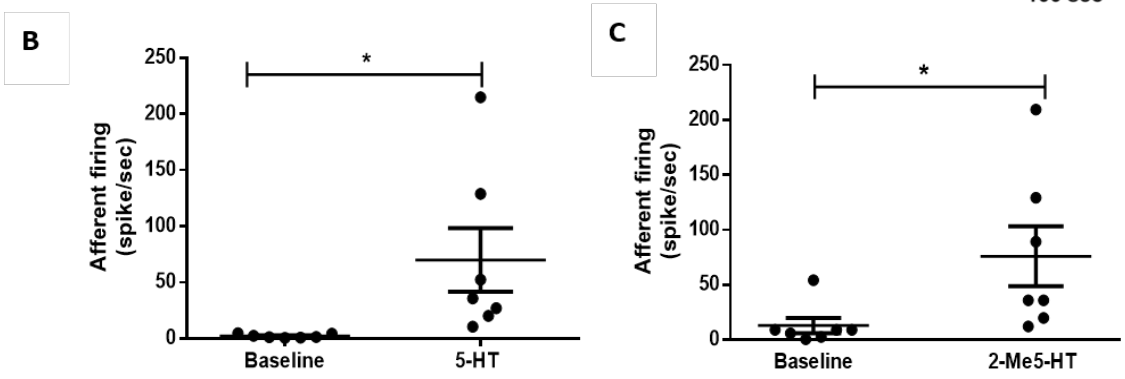
Figures and legends





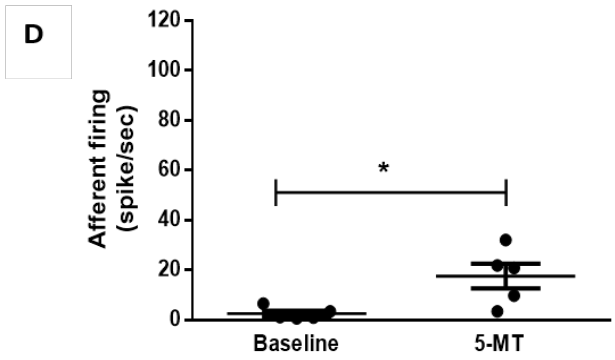


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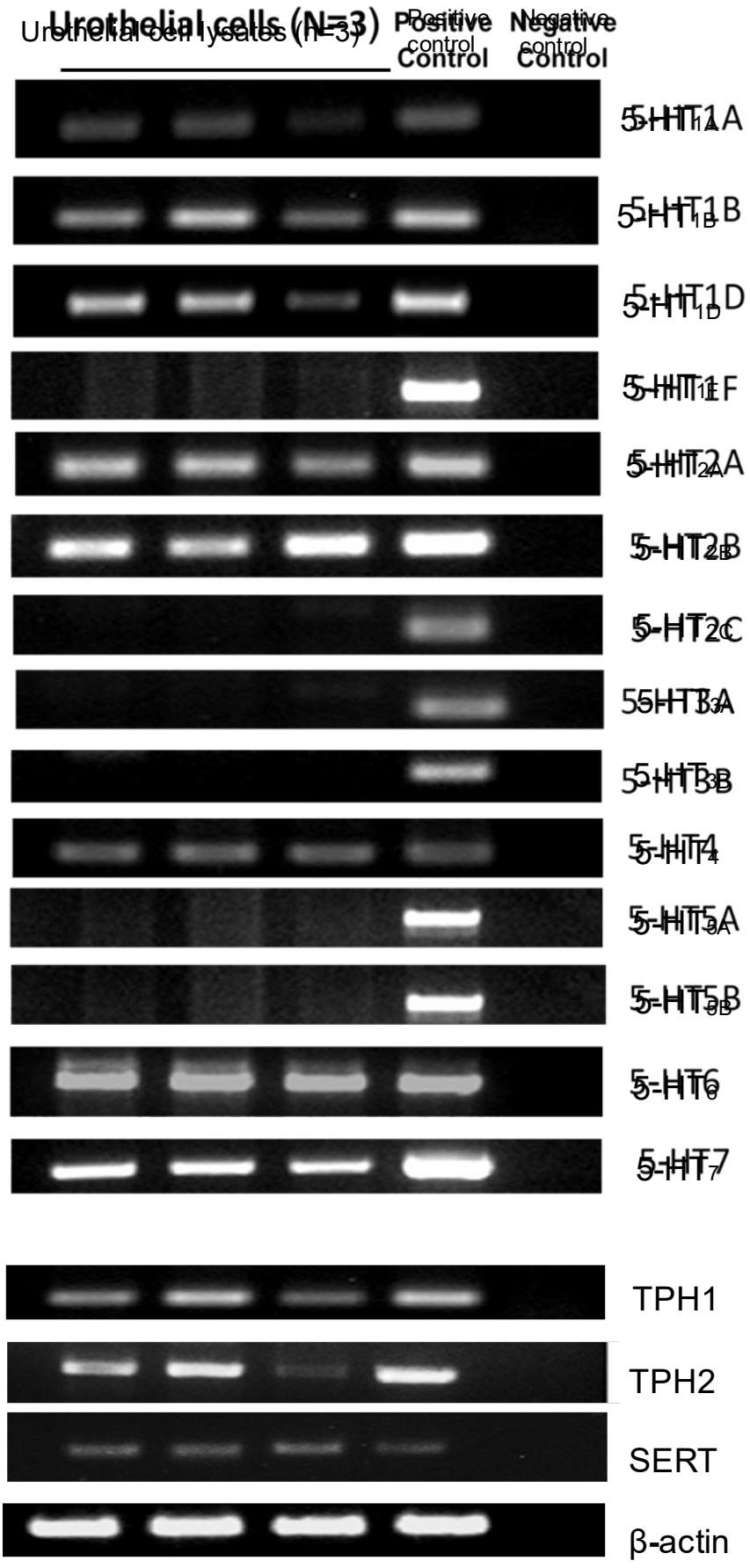
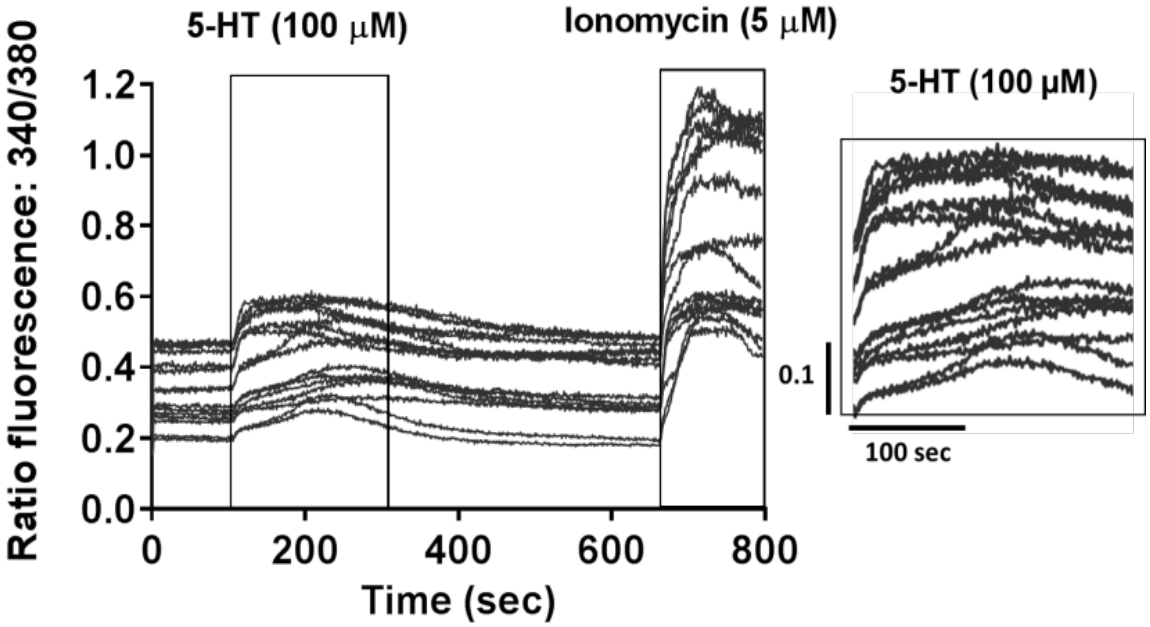
Figure 1 Intravesically applied 5-HT, 2-Me-5HT and 5-MT stimulated bladder afferent firing. (A) Representative trace to illustrate the afferent response to intravesical application of 5-HT (100 μM). (B-D) The peak afferent response to intravesical application of (B) 5-HT (100 μM), (C) 2-Me-5HT (100 μM) and (D) 5-MT (100 μM). Overall, 5-HT, 2-Me-5-HT and 5-MT all evoked increased in afferent firing above baseline (*P<0.05), paired Student's t-test. Each dot represents data from a single preparation (N).


Urothelial cells (N=3)
Positive Control
Negative Control
5-HT1A
5-HT1B
5-HT1D
5-HT1F
5-HT2A
5-HT2B
5-HT2C
5-HT3A
5-HT3B
5-HT4
5-HT5A
5-HT5B
5-HT6
5-HT7
TPH1
TPH2
SERT
β-actin

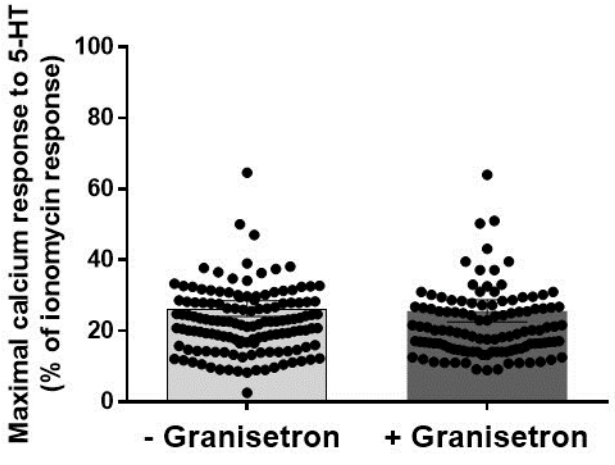
.

Figure 2 mRNA expression of 5-HT receptors, TPH1, TPH2, and SERT on the mouse urothelium. An array of 5-HT receptor transcripts were detected in mouse urothelial cell lysates (5-HT$_{1A, 1B, 1D, 2A, 2B, 4, 6, 7}$). 5-HT$_{1F, 2C, 3A, 3B, 5A,}$ and $_{5B}$ receptors were not detectable. mRNA expression of TPH1, TPH2, and SERT were detected in mouse urothelial cells. ß-actin was used as a house keeping gene. Duodenum was used as a positive control of TPH1 and SERT. Brain was used as a positive control for TPH2 (N=3 mice per tissue).

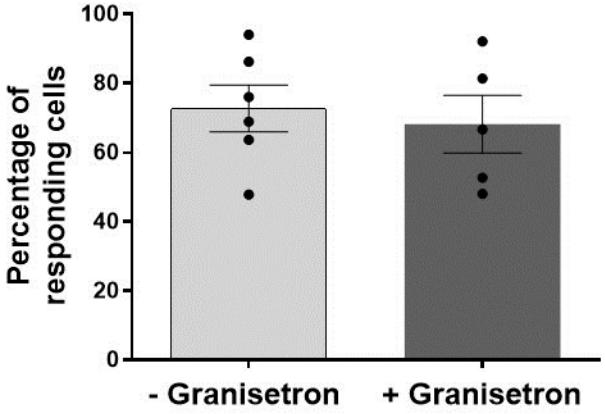
A


5-HT (100 μM)
Ionomycin (5 μM)
5-HT (100 μM)
Ratio fluorescence: 340/380
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
Time (sec)
0.1
100 sec

B

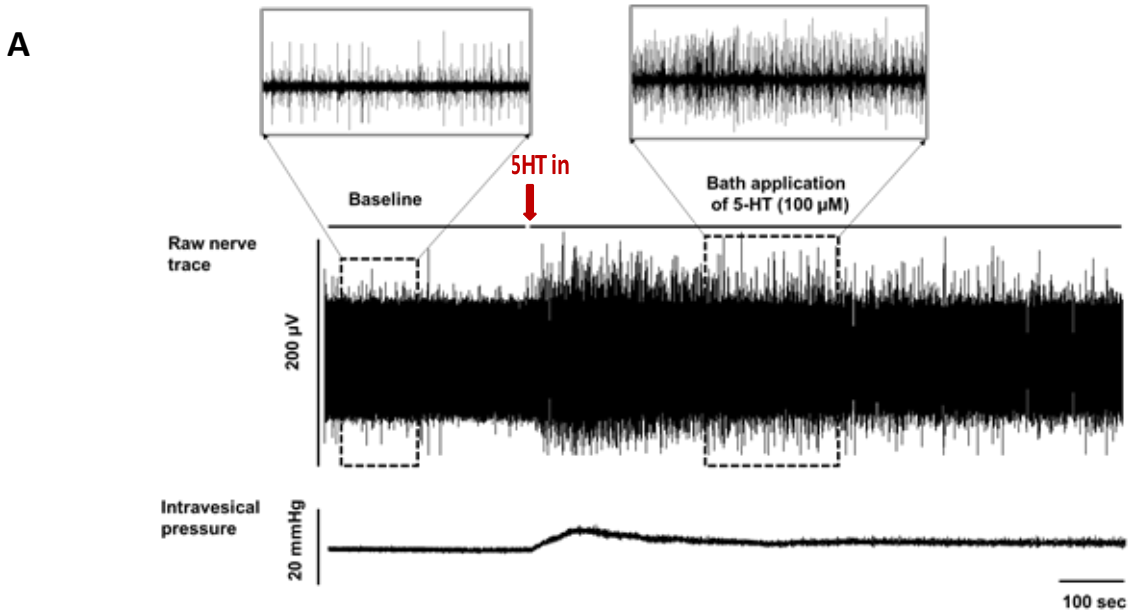

Maximal calcium response to 5-HT
(% of ionomycin response)
100
80
60
40
20
0
- Granisetron
+ Granisetron

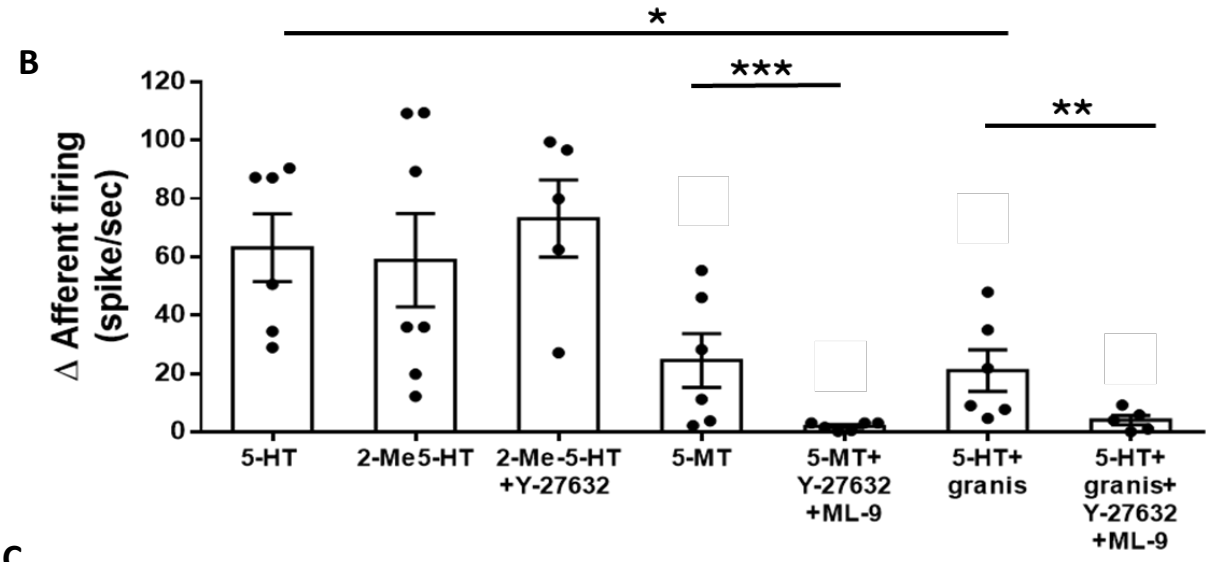
C

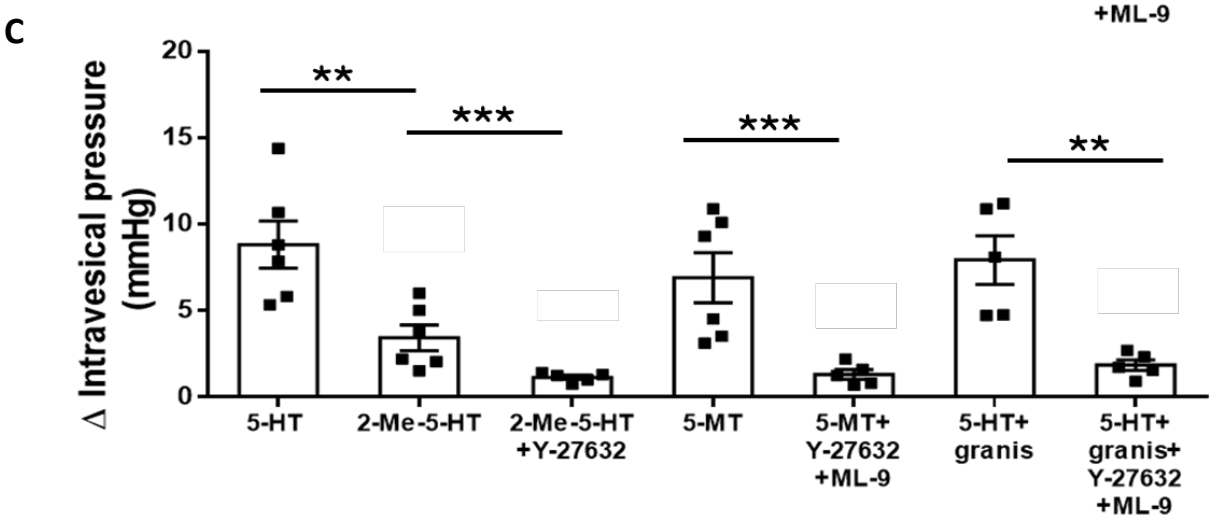

Percentage of
responding cells
100
80
60
40
20
0
- Granisetron
+ Granisetron

.

Figure 3 5- HT stimulated mouse urothelial cells through a non- 5- HT3 receptor mechanism. (**A**) A sample trace showing the calcium response of isolated primary mouse urothelial cells to 5-HT (100 μM) and ionomycin (5 μM) . Each line represents a single urothelial cell. **B**) The relative increase of intracellular Ca^{2+} (Ratio 340/ 380) in response to 5- HT with and without preincubation with granisetron (1 μM), showing that granisetron did not affect 5HT repsonses. (**C**) The percentage of cells responding to 5- HT after preincubation of granisetron, paired Student's t-test, (5-HT, N=6 mice, n=137 cells vs. Granisetron with 5-HT, N=5 mice, n=115 cells), unpaired Student's t-test. N refers to number of animals and n indicates number of cells.


A
5HT in
Baseline
Bath application
of 5-HT (100 µM)
Raw nerve
trace
200 µV
Intravesical
pressure
20 mmHg
100 sec

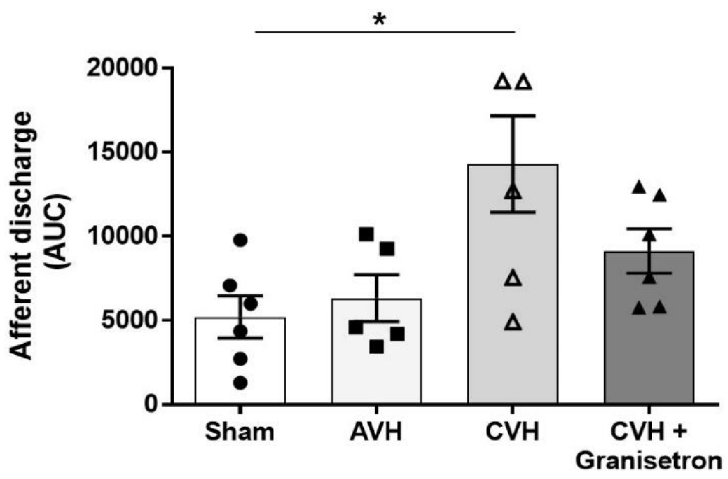

B
Δ Afferent firing
(spike/sec)
120
100
80
60
40
20
0
*
***
**
5-HT
2-Me5-HT
2-Me-5-HT
+Y-27632
5-MT
5-MT+
Y-27632
+ML-9
5-HT+
granis
5-HT+
granis+
Y-27632
+ML-9


C
Δ Intravesical pressure
(mmHg)
20
15
10
5
0
**
***
***
**
5-HT
2-Me-5-HT
2-Me-5-HT
+Y-27632
5-MT
5-MT+
Y-27632
+ML-9
5-HT+
granis
5-HT+
granis+
Y-27632
+ML-9

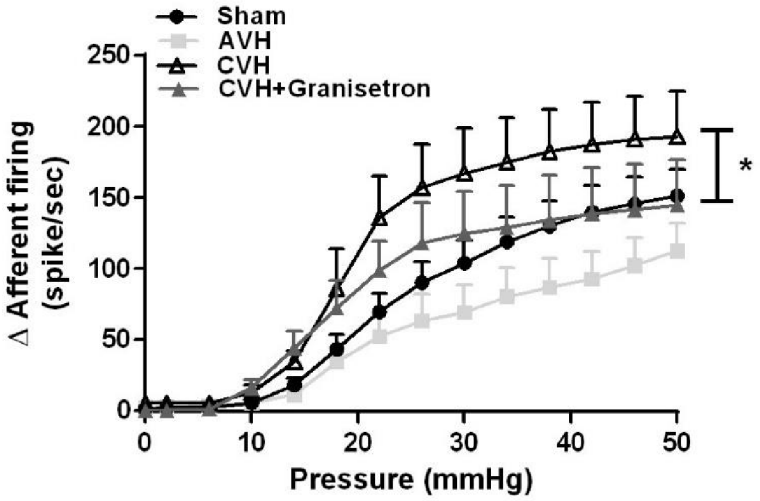
.

Figure 4 5-HT$_3$ receptors play a role in mediating the action of 5-HT on bladder afferent firing and this effect is independent of detrusor contraction. (A) A representative trace showing the effect of bath applied 5-HT on afferent nerve firing and detrusor tone. 5HT, 2-Me-5-HT and 5-MT all evoked an increase in nerve firing which was concomitant with a small but significant bladder contraction. (B) The afferent response to bath application of 5-HT, 2-Me5-HT, 5-MT with and without pre-incubation of the 5-HT$_3$ receptor antagonist granisetron and/or smooth muscle blockers (Y-27632 and ML-9) (C) The effect of the same pharmacological agents on detrusor contractility. Data are represented as mean +/- SEM, each symbol represents a single N number, *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA with Dunnett's multiple comparison (N=5-7).

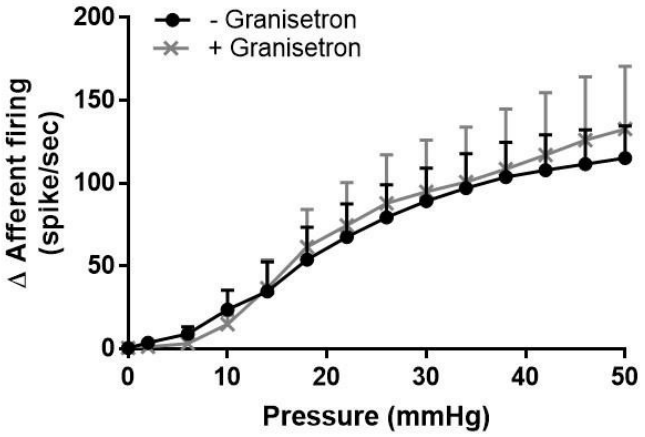
A


*
Afferent discharge (AUC)
20000
15000
10000
5000
0
Sham
AVH
CVH
CVH + Granisetron

B


Sham
AVH
CVH
CVH+Granisetron
Δ Afferent firing (spike/sec)
250
200
150
100
50
0
*
0
10
20
30
40
50
Pressure (mmHg)

C

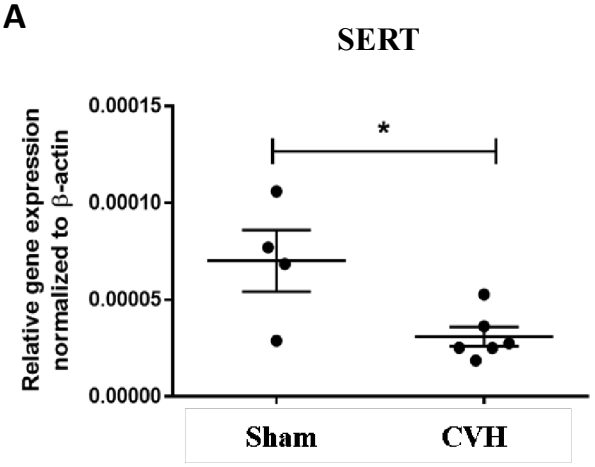

- Granisetron
+ Granisetron
Δ Afferent firing (spike/sec)
200
150
100
50
0
0
10
20
30
40
50
Pressure (mmHg)

.

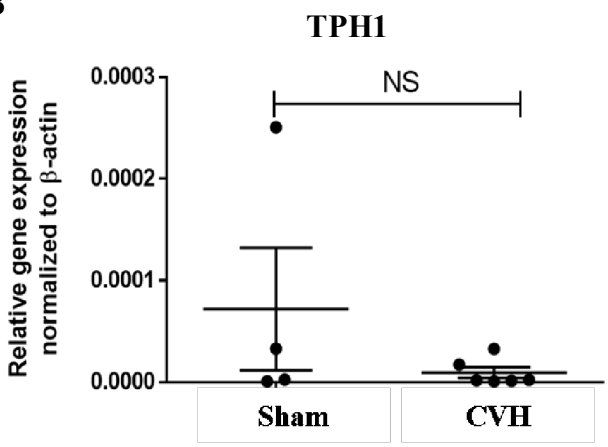
Figure 5. Mice with chronic visceral hypersensitivity (CVH) evoked by TNBS had increased spontaneous afferent firing and hypersensitivity in response to bladder distension. This effect was partially reversed by application of granisetron. (A). Afferent firing from bladders taken from sham mice, mice with acute visceral hypersensitivity (AVH) and mice with chronic visceral hypersensitivity (CVH), showing that firing rate was higher with chronic visceral hypersensitivity and partially rescued by application of the 5HT3 receptor

antagonists granisetron. (B). The afferent response to bladder filling in bladders taken from sham mice, mice with acute visceral hypersensitivity (AVH) and mice with chronic visceral hypersensitivity (CVH) showing that firing rate was higher with chronic visceral hypersensitivity and partially rescued by application of the 5HT3 receptor antagonists granisetron. *P<0.05, and One-way ANOVA with Dunnett' s multiple comparison and Two- way ANOVA respectively (healthy control and CVH, N=6; acute TNBS, N=5; CVH, N=6; CVH+granisetron, N=6). (C). The afferent response to bladder filling in bladders taken from normal control mice before and after application of granisetron. These data show that in bladders taken from healthy control animals granisteron has no effect on the afferent response to bladder distension (N=5)

A


SERT
Relative gene expression normalized to β-actin
0.00015
0.00010
0.00005
0.00000
*
Sham
CVH

B


TPH1
Relative gene expression normalized to β-actin
0.0003
0.0002
0.0001
0.0000
NS
Sham
CVH

C

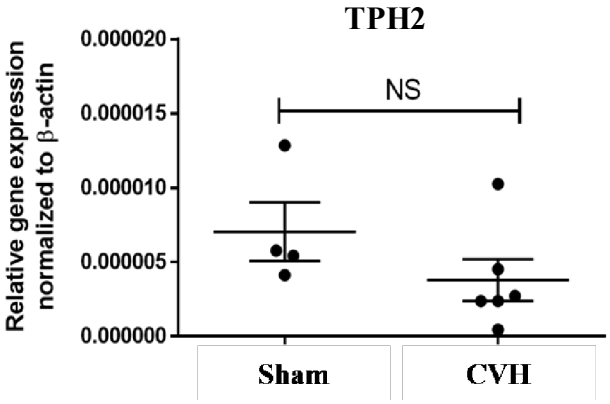
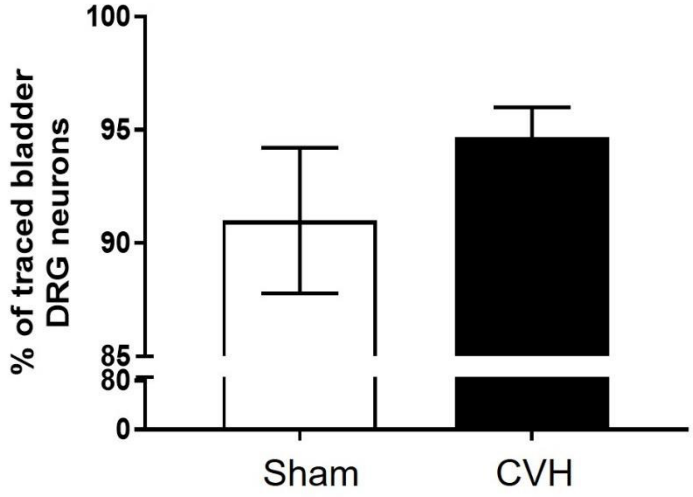
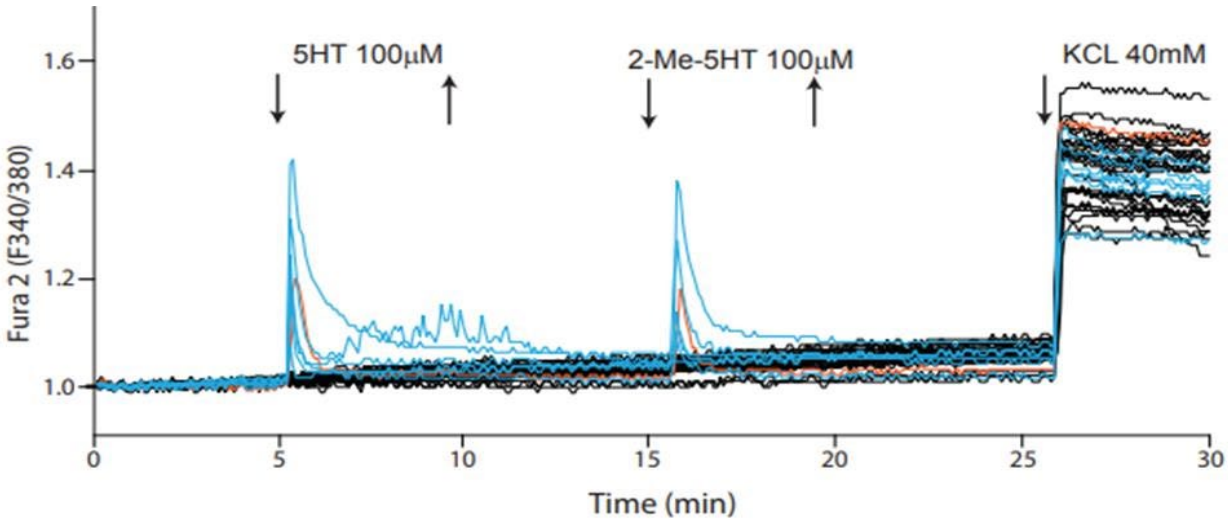

TPH2
Relative gene expression normalized to β-actin
0.000020
0.000015
0.000010
0.000005
0.000000
NS
Sham
CVH

Figure 6 SERT mRNA expression in the urothelium was significantly attenuated in mice with CVH. (A) SERT mRNA expression was significantly reduced in urothelial cell cultures from CVH animals compared to control animals (*P<0.05, unpaired Student's t-test healthy control; N=4, CVH; N=6). (B) TPH1 and (C) TPH2 mRNA expression was not significantly altered. (NS, unpaired Student's t-test healthy control; N=4, CVH; N=6).

A



B



C

	Traced Bladder Neurons	Non-traced Neurons
5-HT	60/69 (87.0%)	37/457 (8.1%)
2-Me-5HT	36/51 (70.6%)	32/349 (9.2%)

D

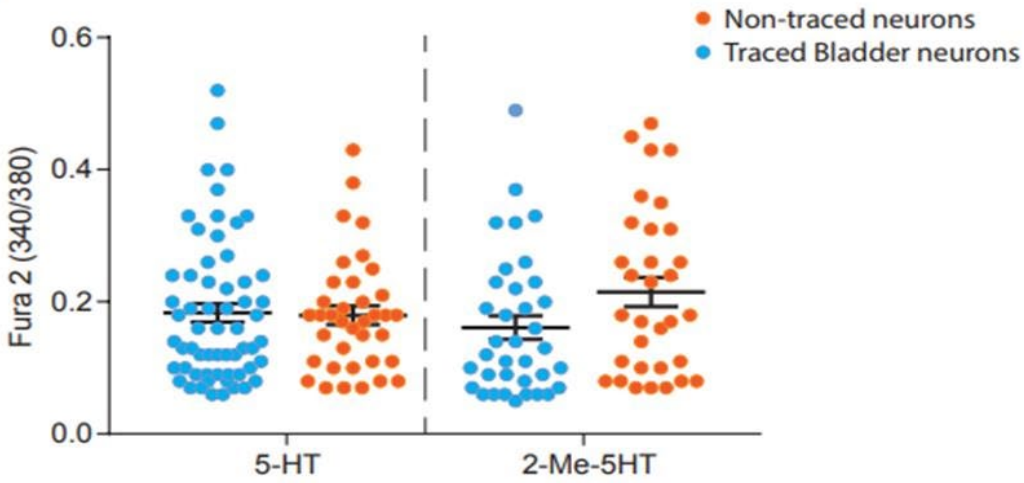


Figure 7. 5-HT and 2-Me-5HT induce calcium transients in bladderinnervating DRG neurons. (A) A high percentage of bladder projecting DRG neurons in both sham (91%) and mice with CVH (95%) express 5-HT$_3$ receptors (sham, N=3, n=77; CVH, N=3, n=74; N refers to number of animals, n indicates number of DRG neurons). (B) Calcium responses elicited by 5-HT (100 µM) and 2-Me-5HT (100 µM) from isolated bladder-innervating (traced), and non-traced LS DRG neurons. Each line represents an individual neuron. Bladder traced neurons are represented in blue, non-traced neurons are represented in black, and responding non-traced neurons are represented in orange. (C) Table

showing number of bladder-innervating and non-traced LS DRG neurons responding to 5-HT (100 μM) (N=4 mice) and 2-Me-5HT (100 μM) (N=4 mice). The percentage of bladder-innervating neurons responding to either to 5-HT and 2-Me-5HT is greater than responding neurons from the general DRG population. (D) Maximum change in fluorescent ratio (F340/380) of individual neurons in response to 5-HT and 2-Me-5HT in bladder-innervating (blue dots) and nontraced (orange dots) LS DRG. The magnitude of the calcium response elicited by 5-HT (100 μM) is equivalent to that elicited by 2-Me-5HT (100μM) for both bladder-innervating DRGs ($p \geq 0.05$, One-way ANOVA, Tukey post-hoc test) and in the non-traced DRGs ($p \geq 0.05$, One-way ANOVA, Tukey post-hoc test).

Author contributions

All experiments were performed in Professor Grundy's laboratory at the University of Sheffield, United Kingdom except for TNBS-induced experiments which were conducted in the Visceral Pain Reserach Group Laboratory at the South Australian Health and Medical Research Institute (SAHMRI), Adelaide, Australia. The following contributions were:

- Conducting experiments: NK, TO, SGC
- Design of experiments, analysis, interpretation of data, - DD, DG, NK and LG
- Production and editing of manuscript- DD, DG, NK, SMB and LG
- Funding and research support- DG and SB

Funding

This research was supported by a full PhD scholarship provided to N.K. from the Development and Promotion of Sciences and Technology Talents Project (DPST), Thailand. An ICS research fellowship was awarded to N.K. to conduct experiments at the SAHMRI, Adelaide, Australia. S.M.B is a National Health and Medical Research Council of Australia (NHMRC) R.D Wright Biomedical Research Fellow (APP1126378). Work in Adelaide was funded in part by NHMRC Australia Project Grant #1140297 to S.M.B.

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Competing interests

We can confirm that none of the authors has any conflicts of interest.