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Creators	Konthepakdee, N, Grundy, L, O'Donell, T, Garcia-Caraballo, S, Brierly, S, Grundy, D and Daly, Donna

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

2 N Konthapakdee ¹, L Grundy ^{2,3,4}, T O'Donnell ^{2,3,4}, S Garcia-Caraballo ^{2,3,4}, S M. Brierley
3 ^{2,3,4}, D Grundy ⁵ and D M Daly ⁶

4

5 Affiliations:

6 ¹ Department of Physiology, Faculty of Science, Prince of Songkla University,
7 Hat Yai, Songkhla 90112, Thailand.

8 ² Visceral Pain Research Group, Centre for Neuroscience, College of Medicine
9 and Public Health, Flinders University, Bedford Park, South Australia, 5042, Australia.

10 ³ Hopwood Centre for Neurobiology, Lifelong Health Theme, South Australian Health
11 and Medical Research Institute (SAHMRI), North Terrace, Adelaide, South Australia
12 5000, Australia.

13 ⁴Centre for Nutrition and Gastrointestinal Diseases, Discipline of Medicine, University of
14 Adelaide, North Terrace, Adelaide, South Australia 5000, Australia.

15 ⁵ Department of Biomedical Science, University of Sheffield, Sheffield, England ⁶
16 University of Central Lancashire, Preston, England.

17

18

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

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

21

22 Co-corresponding authors

23 Professor David Grundy

24 Department of biomedical science

25 University Of Sheffield

26 Western bank

27 Sheffield

28 S10 2TN

29 d.grundy@sheffield.ac.uk.

30

31 Dr Donna Daly

32 School of Pharmacy and Biomedical Science

33 University of Central Lancashire

34 Fylde Road

35 Preston

36 PR1 2HE

37 Ddaly3@uclan.ac.uk

38 **Key Points**

39

40 • Functional disorders (i.e., interstitial cystitis/painful bladder syndrome and irritable
41 bowel syndrome) are associated with hyperexcitability of afferent nerves innervating
42 the urinary tract and the bowel respectively.

43 • Various non-5-HT₃ receptor mRNA transcripts are expressed in mouse urothelium and
44 exert functional responses to 5-HT.

45 • Whilst 5-HT₃ receptors were not detected in mouse urothelium, 5-HT₃ receptors
46 expressed on bladder sensory neurons plays a role in bladder afferent excitability under
47 both normal conditions and in a mouse model of chronic visceral hypersensitivity
48 (CVH).

49 • These data suggest that the role 5-HT₃ receptors play in bladder afferent signaling
50 warrants further study as a potential therapeutic target for functional bladder disorders.

51

52

53 **Abstract**

54 Serotonin (5-HT) is an excitatory mediator, which in the gastrointestinal (GI) tract, plays
55 a physiological role in gut-brain signaling and which is dysregulated in functional GI
56 disorders such as irritable bowel syndrome (IBS). Patients suffering from IBS frequently
57 suffer from urological symptoms characteristic of interstitial cystitis/painful bladder
58 syndrome, which manifests due to cross-sensitization of shared innervation pathways
59 between the bladder and colon. However, a direct modulatory role of 5-HT in bladder
60 afferent signaling and its role in colon-bladder neuronal crosstalk remain elusive. The
61 aim of this study was to investigate the action of 5-HT on bladder afferent signaling in
62 normal mice and mice with chronic visceral hypersensitivity (CVH) following
63 trinitrobenzenesulfonic acid (TNBS) induced colitis. Bladder afferent activity was
64 recorded directly using *ex vivo* afferent nerve recordings. Expression of 14 5-HT receptor
65 subtypes, the serotonin transporter (SERT) and 5-HT producing enzymes were
66 determined in the urothelium using RT-PCR. Retrograde labelling of bladder projecting
67 dorsal root ganglion (DRG) neurons was used to investigate expression of 5-HT₃
68 receptors using single cell RT-PCR, while sensory neuronal and urothelial responses to
69 5-HT were determined by live cell calcium imaging. 5-HT elicited bladder afferent firing
70 predominantly via 5-HT₃ receptors expressed on afferent terminals. CVH animals
71 showed a downregulation of SERT mRNA expression in urothelium, suggesting
72 increased 5-HT bioavailability. Granisetron, a 5-HT₃ antagonist, reversed bladder afferent

73 hypersensitivity in CVH mice. These data suggest 5-HT exerts a direct effect on bladder
74 afferents to enhance signaling. 5-HT₃ antagonists could therefore be a potential
75 therapeutic target to treat functional bladder and bowel disorders.

76

77 **Introduction**

78 Information regarding the state of bladder distension is carried via sensory afferents that
79 project via the pelvic and hypogastric nerves into the dorsal horn of the spinal cord,
80 feeding into autonomic reflex and micturition centers within the brainstem to maintain
81 continence (see Grundy *et al.* 2018a for review). Bladder afferents are found innervating
82 both the detrusor smooth muscle and the urothelium (Spencer *et al.*, 2018), consisting
83 of myelinated A δ fibres and unmyelinated C fibres that exhibit polymodal sensitivity to a
84 host of mechanical and chemical stimuli (Su and Gebhart, 1998; Zagorodnyuk *et al.*,
85 2006; Zagorodnyuk *et al.*, 2007). Hypersensitivity of these bladder afferents to bladder
86 distension may underlie the symptoms of urgency, frequency and nocturia in urological
87 disorders such as overactive bladder syndrome (OAB) and interstitial cystitis/painful
88 bladder syndrome (IC/PBS).

89

90 Serotonin (5-HT) is a key neuromodulator, regulating enteric and viscerosensory function
91 as well as acting in the central nervous system (Berger *et al.*, 2009; Gershon and Tack,
92 2007; Grundy, 2008). Out of the seven members of the 5-HT receptor family (5-HT₁₋₇),
93 six are G-protein coupled receptors (GPCRs) and one, the 5-HT₃ receptor is a
94 ligand-gated ion channel (McCorvy and Roth, 2015). Within the bladder, activation of 5-
95 HT_{1A}, 5-HT₂ and 5-HT₃, but not 5-HT₄ and 5-HT₇ can generate altered bladder contraction
96 in rodent models (Chetty *et al.*, 2007; Kodama and Takimoto, 2000; Mittra *et al.*, 2007).
97 5HT_{3A} and 5-HT_{3B} are expressed in the bladder urothelial layer and detrusor, where it is
98 considered to play a role in neurogenic contraction (Chetty *et al.* 2007), whilst 5-HT
99 receptor subtypes 5-HT_{1D}, 2A, 4 and 5-HT₆ are present in urothelial cells. 5-HT_{1A}, 1B, 1D, 2A,
100 2B, 2C, 3A, 4, and 5-HT₇ have also been reported in urothelium-denuded tissues
101 (MatsumotoMiyai *et al.*, 2016). However, a modulatory role of 5-HT on bladder afferent
102 excitability has yet to be explored.

103

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

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

116

117 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis is a commonly used animal
118 model of IBS, triggering chronic visceral hypersensitivity (CVH) that persists after the
119 initial inflammation has resolved (Castro et al., 2017; de Araujo et al., 2014; Hughes et
120 al., 2009; Osteen et al., 2016). Furthermore, it has been shown that these CVH mice
121 exhibit bladder afferent hypersensitivity and abnormal voiding patterns, characteristic of
122 an 'overactive' phenotype (Grundy et al., 2018b). 5HT plays an essential role in health
123 and the development of colonic afferent hypersensitivity associated with irritable bowel
124 syndrome (IBS) in humans (Gershon and Tack, 2007; Grundy, 2008) and animal models
125 (Keating et al., 2008; Linden et al., 2003; Linden et al., 2005). As such, this raises the
126 possibility that bladder hypersensitivity associated with TNBS-induced colitis may involve
127 a 5-HT₃ receptor dependent mechanism.

128

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

134

135

136

137 **Methods**

138 All experiments were conducted in Sheffield, UK except the induction of TNBS, the
139 retrograde tracing studies and dorsal root ganglion (DRG) experiments (calcium imaging
140 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 intracolonicly administered with
164 2,4,6trinitrobenzenesulfonic acid (TNBS) 0.01 mL (130 $\mu\text{g mL}^{-1}$ 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

175 **Extracellular afferent nerve recordings**

176 Bladder afferent nerve activity was determined using an *ex vivo* model previously
177 described (Daly et al., 2007; Grundy et al., 2018a). The whole bladder and surrounding
178 tissues (together with its emanating nerve fibers) was placed in a recording chamber (30
179 mL). The preparation was continuously perfused at a rate of 5 mL minute⁻¹ with
180 oxygenated (95%O₂/5%CO₂) Krebs bicarbonate solution (composition, mM: NaCl 118.4,
181 NaHCO₃ 24.9, CaCl₂ 1.9, MgSO₄ 1.2, KCl 4.7, KH₂PO₄ 1.2, glucose 11.7) at constant
182 temperature of 35 °C. A polythene catheter (0.28 mm) was inserted into the urethra to
183 perfuse the bladder with isotonic saline (0.9% NaCl) or pharmacological reagents using
184 a perfusion pump (Genie, Kent, multi-phaser TM model NE-1000) with a rate 100 µL
185 minute⁻¹. The bladder dome was punctured at the apex with a syringe needle (BD
186 microlanceTM, 19G 2") and a dual- lumen catheter was inserted and secured with
187 suture. One arm of the catheter was connected to a pressure transducer (DTXTM plus
188 DT-XX, Becton Dickinson, Singapore) to monitor intravesical pressure and the other arm
189 was connected to the 3 way tap to allow bladder filling (tap closed) or emptying (tap
190 open). Multiunit pelvic and hypogastric nerves were dissected into a fine branches and
191 placed into a suction electrode which was attached to a Neurolog headstage (NL 100,
192 Digitimer, Ltd, UK), amplified with an AC amplifier (NL104) and filtered (NL125, band
193 pass filter. The multi-unit afferent nerve discharge frequency (spikes/sec) was quantified
194 using a spike processor (Digitimer D130). The signal was visualized on a computer
195 running Spike 2 software (Version 7.1, Cambridge Electronic Design, UK).

196

197 **Drug application**

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

206

207 **Isovolumetric protocol**

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

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

215

216 **Isolation of urothelial cells**

217 Urothelial cells were isolated as previously described (Daly et al., 2014). The bladder
218 was dissected longitudinally under a stereo microscope to expose the urothelium to the
219 media (fresh Modified Eagle Media (MEM) (Gibco®) containing 0.7% HEPES and 1%
220 antibiotic-antimycotic (PSF) solution (Gibco®) at 37°C and transferred to a Sylgard®
221 (Dow Corning) coated dish. The tissue was stretched and pinned. The media was
222 removed and replaced with 2.5 mg/mL dispase in MEM at 37°C. The bladder was
223 incubated in dispase for 2 hours at room temperature. After the dispase had been
224 aspirated, the urothelium was gently scraped with a scalpel under the stereo microscope
225 and the cells were immediately placed in 0.5% trypsin-EDTA (Gibco®). The solution was
226 incubated at 37 °C for 10 minutes, and gently triturated every a few minutes. The
227 trypsinEDTA was deactivated by adding pre-warm MEM with 10 % Fetal Bovine Serum
228 (FBS) (Gibco®). The cell suspension was centrifuged at 1500 rpm, 4 °C for 15 minutes.
229 The solution was gently aspirated and the pellet was resuspended in pre-warmed
230 Keratinocyte-serum free medium (K-SFM) and centrifuged at 1500 rpm, 4 °C for 15
231 minutes. For calcium imaging experiment, the cells were resuspended in K-SFM 200 µL
232 and plated on collagen IV (Sigma Aldrich Poole, UK) coated coverslips in a 12 wells plate
233 and incubated in 5% CO₂-95% O₂ at 37 °C overnight. For PCR experiments, the cell
234 pellet was washed by adding PBS and centrifuged at 1000 rpm, 4 °C for 5 minutes and
235 stored at -80 °C for RNA isolation process.

236

237 **Reverse-transcription PCR (RT-PCR)**

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

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

252

253 **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' TAAGAACTCCCGCTCCAGT 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' TGAAACTGGTTGCTTAAACTGA 3'	126	DRG
		RW: 5' AGCTGCTACTGGACTTATGGA 3'		
5-HT _{3A}	NM_013561	FW: 5' CCACCTTCCAAGCCAACAAG 3'	128	DRG
		RW: 5' CTCCTTGGTGGTGGAAAGAG 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'CAAACCAAGAACCAAGAC 3'		

254

255

256 **Retrograde tracing of bladder innervating DRG neurons**

257 A small aseptic abdominal incision was made in anesthetised (isoflurane 2-4 % in

258 oxygen) mice. Cholera toxin subunit B conjugated to AlexaFluor® 488 (CTB-488; 0.5 %

259 diluted in 0.1 M phosphate buffered saline (PBS); ThermoFisher Scientific) was injected

260 at four sites into the bladder wall (2µL / injection) using a 5 µl Hamilton syringe attached

261 to a 23-gauge needle. The abdominal incision was sutured closed and analgesic

262 (Buprenorphine; 2.7µg / 30g) and antibiotic (Ampicillin; 50 mg/kg) given subcutaneously

263 as mice regained consciousness. Mice were allowed to recover, housed individually and

264 monitored for four days, in order to visualize CTB-labelled afferent neurons in the DRG.

265

266 **Isolation of DRG neurons**

267 DRGs from lumbosacral (L5-S1) spinal levels of the mouse spinal cord, which correspond
268 to the pelvic innervation of the bladder, were isolated and incubated in Hanks balanced
269 salt solution (HBSS) (pH 7.4) containing collagenase (4mg/mL), and dispase
270 (4.5mg/mL), at 37°C for 30 minutes. The collagenase/dispase solution was aspirated and
271 replaced with HBSS containing collagenase (4mg/mL) for 10 minutes at 37°C. The
272 collagenase solution was aspirated and replaced with 600µl DMEM (GIBCO) containing
273 10% FCS (Invitrogen), 2 mM L-glutamine (GIBCO), 100 µM MEM non-essential amino
274 acids (GIBCO) and 100 mg/ml penicillin/streptomycin (Invitrogen). Neurons were
275 dissociated via trituration with a Pasteur pipette and spot plated onto 15 mm coverslips
276 coated with laminin (20 µg/mL) and poly-D-lysine (800 µg/mL) and maintained in an
277 incubator at 37 °C in 5% CO₂. Calcium imaging recordings were performed on DRG
278 neurons 18-30hrs post isolation.

279

280 **Single cell RT-PCR**

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

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

303

304 **Quantitative real-time RT-PCR**

305 To determine the quantitative expression of TPH1 2 and SERT in urothelial cell samples
306 from sham and TNBS treated animals, quantitative RT-PCR was used. The quantification
307 of mRNA expression determined using TaqMan Gene Expression Master Mix (Applied
308 Biosystems 4374657). The reaction was prepared on ice and mixed in Hard-Shell®
309 ThinWall 96-Well Skirted PCR Plates (BIO-RAD, HSP-9665). Prior to running the
310 reactions, the plates were covered with MicroAmp™ Optical Adhesive Film for 96-Well
311 Plates

312 (Applied Biosystems, 43111971) and centrifuged briefly to spin down the contents and
313 eliminate any air bubbles from the solutions. The reaction for each sample and gene was
314 run in duplicate. DNase free water was used to replace cDNA as a negative control for
315 each gene and plate. PCR reactions were performed in a BIO-RAD CFX96 Touch™
316 Real-time thermocycler (C1000 Touch™ Thermal Cycler, Bio-Rad Laboratories Ltd.
317 Hercules, USA). Results were expressed as relative expression to the housekeeping
318 gene GAPDH ($1/\Delta Ct$), and fold change was calculated using the equation $2^{-\Delta(\Delta Ct)}$.

319

320 **Calcium imaging**

321 Cultured DRG neurons (18-30 hours) or isolated urothelial cells were loaded with 2 µM
322 fura-2-acetoxymethyl ester (Fura-2) for 15 minutes at 37°C and washed with HEPES
323 buffer (10mM, NaCl 142mM, KCl 2mM, glucose 10mM, CaCl₂ 2mM, MgCl₂ 1mM, HEPES
324 10mM; pH 7.4) for 30 minutes prior to imaging at room temperature (23°C). Fura-2 was
325 excited at 340 and 380 nm. Fluorescence images were obtained every 5s using a 20x
326 objective. Retrogradely traced bladder-innervating DRG neurons were identified by the
327 presence of the CTB-488 tracer. Data were recorded and analysed using MetaFluor
328 software. After an initial baseline reading to ensure cell fluorescence was stable (an
329 indication of healthy cells), DRGs and/or urothelial cells were stimulated with either 5-HT
330 (100 µM), 2-Me-5HT (100 µM) in the absence or presence of granisetron (1 µM), and
331 changes in intracellular calcium [Ca^{2+}]_i were monitored in real-time. Ionomycin or KCl
332 (40 mM) was applied as a positive control for cell viability.

333

334 **Drugs and solutions**

335 5-hydroxytryptamine (5-HT) and 5-methoxytryptamine (5-MT) were obtained from Sigma
336 Aldrich, UK. 2-Methoxy-5-hydroxytryptamine (2-Me-5-HT) was obtained from
337 Tocris/Bioscience, UK. Granisetron hydrochloride was obtained from LKT Laboratories,
338 USA. ML-9 was obtained from Cayman Chemical. Y-27632 was obtained from
339 Chemdea, USA.

340

341 **Data analysis**

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

353

354

355 **Results**

356 **Intravesical administration of 5-HT agonists excites bladder afferent nerves** In
357 order to determine if bladder afferents functionally express 5-HT receptors, we
358 investigated the effects of various 5-HT receptor agonists on *ex-vivo* bladder afferent
359 nerve activity. We found that intravesical infusion of 5-HT (100 μ M), evoked a dramatic
360 and sustained increase in baseline afferent nerve firing compared to intravesical saline
361 (Fig. 1A, 1B). The receptor mechanisms underlying this effect were determined by
362 comparing the responses to 2-Me-5-HT, a selective 5-HT₃ agonist (100 μ M), with 5-MT
363 (100 μ M) a compound which exhibits selectivity for the GPCR family of 5-HT receptors
364 including 5-HT₁, 5-HT₂ and 5-HT₄₋₇. The responses to application of 5-HT and 2-Me-5HT
365 were comparable. However while the response to 5-MT, was significantly increased
366 relative to baseline, the magnitude of the firing was reduced compared to that of 5-HT
367 and 2-Me-5-HT (Fig. 1C, 1D). These data suggest that multiple 5-HT receptors may
368 influence bladder afferent firing, and that this may occur via both direct and indirect
369 mechanisms.

370

371 **5-HT receptors are expressed on bladder urothelium**

372 Given the established role of the urothelium as a sensory structure, we wanted to
373 determine if the effects of intravesical 5-HT on bladder afferent firing were secondary to
374 activation of urothelial cells or a consequence of direct activation of primary afferent
375 endings innervating the bladder. To this end we investigated serotonergic receptor
376 expression in the bladder urothelial layer using RT-PCR. The expression profile for the
377 various 5-HT receptors in the urothelium is shown in Figure 2. Overall, 5-HT_{1A}, 5-HT_{1B},
378 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₄, 5-HT₆, and 5-HT₇ receptors were all detected. In contrast,
379 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
380 bladder urothelium. Moreover, expression of the 5-HT producing enzymes, tryptophan
381 hydroxylase-1, and 2 (TPH-1 and TPH-2), and the sodium-dependent serotonin
382 transporter (SERT) transcripts were also examined using PCR and all three were shown
383 to be expressed in the urothelium (Fig. 2). This finding raises the possibility of an
384 endogenous source of 5-HT in the bladder wall.

385

386 **Stimulation of non-5HT₃ receptors on the urothelium can evoke urothelial cell** 387 **activation**

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

390 with 5-HT triggered an increase in intracellular Ca^{2+} indicative of cellular activation.
391 Consistent with our RT-PCR data identifying an absence of 5-HT₃ receptor expression in
392 the urothelium, both the magnitude of the response to 5-HT and the number of
393 responding cells was unchanged following incubation with the 5-HT₃ selective antagonist
394 granisetron (Fig. 3B, 3C).

395

396 **Activation of 5-HT receptors by bath application of agonist alters afferent nerve** 397 **firing from the bladder and induces contraction of the detrusor**

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

406

407 The peak afferent response to bath application of 5-HT and 2-Me-5-HT was similar.
408 Moreover, application of the selective 5-HT₃ receptor antagonist, granisetron (1 μ M),
409 significantly attenuated afferent responses to 5-HT. To uncouple the direct effect of 5HT
410 on the afferent nerves from any indirect nerve response produced by bladder contraction
411 we used a myosin light chain kinase inhibitor (ML-9, 10 μ M) and/or the rhoassociated
412 kinase inhibitor (Y-27632, 10 μ M) to prevent contraction. Under such conditions the
413 afferent response to 5-MT was abolished in the absence of contraction, suggesting that
414 the response to 5-MT was secondary to changes in muscle tone. Conversely, the afferent
415 response to 2-Me-5-HT persisted despite the absence of contraction, suggesting that the
416 contractile and nerve responses to 5-HT₃ receptor stimulation were independent (Fig. 4B
417 and 4C).

418

419 **Chronic visceral hypersensitivity evoked with intra-colonic TNBS caused altered** 420 **mechanosensitivity and increased spontaneous afferent firing from the bladder,** 421 **an effect that was reversed by blocking the 5-HT₃ receptor.**

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

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

436

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

438 The attenuated responses in the presence of granisetron implies a role for endogenous
439 5-HT in bladder hypersensitivity. We hypothesized that there might be a change in 5-HT
440 bioavailability in the urothelium following chronic visceral hypersensitivity (CVH) induced
441 by intra-colonic TNBS. Quantitative RT-PCR showed that the level of SERT mRNA
442 expression was significantly lower in urothelial cells from bladders in TNBS treated mice
443 compared to sham operated controls (*P<0.05, Fig. 6A). TPH1 and TPH2 mRNA
444 expression was also reduced, but because of the large variability, especially in TPH1
445 levels, this did not reach significance (Fig. 6B, 6C). Nevertheless, these data are
446 consistent with a role for urothelial 5-HT in bladder hypersensitivity.

447

448 **Bladder projecting DRG neurons express 5-HT₃ receptors in both control and CVH** 449 **mice and display functional response to 5-HT and 2-Me-5-HT activation.**

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

458

459 In addition, we performed calcium imaging on bladder innervating (traced) and nontraced
460 lumbosacral DRG neurons. A representative trace of calcium imaging of lumbosacral
461 DRG neurons in response to 5-HT (100 μ M) and 2-Me-5-HT (100 μ M) is shown in Figure
462 7B. 60 out of a total of 69 (87%) bladder projecting DRG neurons (traced neurons) elicited
463 a calcium signal in response to application of 5-HT, moreover a significantly greater
464 proportion of the traced DRGs responded to 5HT than the non-traced DRG neurons. This
465 indicates that expression of 5-HT receptors maybe enriched in bladder neurons
466 compared to the generalized neuronal population. In addition, a similar proportion of
467 traced neurons (70%, 36/51) showed an increase in Ca^{2+} influx after the application of 2-
468 Me-5-HT (Fig. 7C). The magnitude of the Ca^{2+} response to 5-HT and 2Me-5-HT was
469 similar in traced and non-traced DRG neurons (Fig. 7D). These data are consistent with
470 a major contribution of 5-HT₃ receptors to 5-HT's ability to sensitize bladder afferents and
471 enhance firing.

472

473 **Discussion**

474 Over the past decade, a number of studies, including our own, have investigated the
475 chemical and mechanical stimuli that drive afferent transmission from the bladder.
476 Despite this, the mechanisms involved in generating bladder hypersensitivity still remain
477 elusive. There is significant clinical comorbidity between hypersensitivity disorders of the
478 bladder and hypersensitivity disorders of the colon (such as IBS), pointing to a common
479 underlying etiology involving dichotomizing afferent fibres between the bladder and
480 bowel (Grundy and Brierley, 2018; Malykhina, 2007). Serotonin (5-HT) plays integral
481 roles in secretion, motility and visceral sensitivity of the GI tract (Grundy, 2008). However,
482 relatively little is known about how 5HT affects afferent signaling from the bladder in
483 healthy or hypersensitivity states. In this study we investigated the modulatory action of
484 5-HT on primary afferent signaling in the mouse urinary bladder and in a TNBSinduced
485 model of colitis which induces colon-bladder cross-organ sensitization (Grundy et al.,
486 2018b).

487

488 **5-HT increases bladder afferent firing and induces contraction of the detrusor**

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

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

498
499 Similar to 5-HT, bath application of the selective 5-HT₁, 5-HT₂, 5-HT₄, 5-HT₆, and 5-HT₇
500 agonist, 5 methoxy tryptamine (5-MT) also caused an increase in afferent nerve firing
501 and evoked a small contraction of the bladder. However, when the contraction was
502 blocked by preincubation with the muscle blockers (Y-27632 and ML-9), the contraction
503 was lost and the afferent response to 5MT was abolished suggesting that the afferent
504 response to stimulation of the non-5HT₃ receptor populations was secondary to changes
505 in bladder contraction rather than via a direct action at the afferent terminal. A number of
506 previous studies have demonstrated that 5-HT can induce contraction of bladder muscle
507 strips *in vitro*, however the receptor that mediates the contraction remains controversial.
508 Hattori *et al.*, (2017) show that both 5-HT and a selective 5-HT₂ receptor agonist cause
509 contraction of the bladder, but a lack of efficacy following pre-incubation of selective 5HT₂
510 receptor antagonists suggested that the effect was not mediated by the 5-HT₂ receptor
511 (Hattori et al., 2017). In another study it was shown that 5-HT potentiates neurogenic
512 contractions of rat isolated detrusor muscle through both 5-HT₇ and 5-HT_{2c} receptors
513 (Rekik et al., 2011). Moreover, in the guinea pig the contractile response to 5HT was
514 suggested to be via the 5-HT₂, 5-HT₃ and 5-HT₄ receptors (Yoshida et al., 2002).

515

516 **5-HT has a direct effect on afferent firing via the 5-HT₃ receptor**

517 Bath application of 2-Me-5-HT caused a robust increase in nerve firing that was not
518 affected by blocking contractility. 2-Me-5-HT is a potent agonist of both the 5-HT₃ and the
519 5-HT₆ receptor (Glennon et al., 2000) suggesting that activation of either 5-HT₃ or 5HT₆
520 located on the urothelium (in the case of 5HT₆) or at the nerve terminal results in afferent
521 nerve firing from the bladder. Since we did not use a selective 5HT₆ receptor antagonist
522 we cannot determine from these data what contribution 5HT₆ made to the 2Me-5-HT
523 response. Since the role of 5HT₆ in the lower urinary tract is relatively understudied this
524 would warrant further investigation. However in this study we focused on the 5-HT₃
525 receptor since pre-incubation of the 5-HT₃ selective antagonist granisteron inhibited the
526 5-HT-mediated increase in bladder afferent nerve firing by ~65%. Since 5HT₃ receptors
527 were not identified in the urothelium via RT-PCR, this suggests that stimulation of 5-HT₃
528 receptors can elicit a direct increase in afferent nerve firing by stimulating receptors on

529 the nerve terminal rather than via an indirect mechanism as a result of muscle contraction
530 or urothelial signaling. Our retrograde tracing studies revealed that the majority of
531 lumbosacral DRG neurons innervating the mouse urinary bladder (90%) express the 5-
532 HT₃ receptor. Furthermore, our functional calcium imaging showed that the majority of
533 these DRGs exhibit functional responses to 5-HT (87%) and the 5-HT₃ selective agonist,
534 2-Me-5-HT (70%). In this study we did not stain the afferent terminals and show
535 expression of 5HT₃ in bladder afferent endings, although it is well established that
536 expression in the cell body where the receptor is synthesized is likely to be indicative of
537 expression at the afferent terminal. Therefore these data suggest that 5HT exerts a direct
538 action on 5-HT₃ receptor at the terminal endings of the primary afferents innervating the
539 bladder. This idea is supported by previous studies which show that 5-HT₃ receptors are
540 also located in rat DRG neurons (Nicholson et al., 2003) and that systemic injection of a
541 5-HT precursor (5-hydroxytryptophan, 5-HTP), triggers bladder hyper-excitability in a
542 mechanism mediated by spinal 5-HT₃ receptors (Hall et al., 2015).

543

544 A recent study has also shown that bladder-projecting neurons in the caudal raphe
545 nucleus, the area of the brainstem which exhibits supraspinal control of the bladder, also
546 express serotonin (Ahn et al., 2018), suggesting that serotonergic control of the bladder
547 could have both peripheral and central components which involve the 5-HT₃ receptor.

548

549 **5-HT can alter signaling in the urothelium in a mechanism that is independent of** 550 **the 5-HT₃ receptor**

551 Over the past decade it has become increasingly clear that the urothelium of the bladder
552 plays an active role in sensation. It is responsive to bladder filling and releases an array
553 of mediators and neurotransmitters which target underlying afferent nerves to modulate
554 firing (urothelial- afferent signaling). Since 5-HT receptor agonists altered sensory
555 signaling from the bladder we wanted to determine what role the urothelium played in
556 the afferent response. mRNA for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₄, 5-HT₆,
557 and 5-HT₇ receptors were identified in the urothelium and our functional calcium imaging
558 experiments demonstrated that ~60% of isolated primary urothelial cells were activated
559 by stimulation with 5-HT. This indicates that there may be functional 5-HT receptors in
560 the urothelium that could play a role in both autocrine urothelial mechanisms and
561 paracrine sensory mechanisms. It is important to note that we did not conduct any
562 western blot analysis or immunocytochemistry to confirm the protein expression of these
563 receptors in the urothelium, nor did we use an array of selective agonist and antagonists

564 in the calcium imaging experiments, this makes it difficult for us to identify which
565 receptor(s) mediate the calcium responses. However, pretreatment with the 5-HT₃
566 selective antagonist, granisetron had no effect on the magnitude of the calcium signals
567 or on the numbers of responsive cells. This taken together with the fact that mRNA for
568 the 5-HT₃ receptor was not identified in the urothelium suggests that while the 5-HT₃
569 receptor may be involved in the direct afferent response to 5HT (i.e. at the 5-HT₃ receptor
570 on the primary afferent terminal) it is not involved in urothelial signaling. Further studies
571 are required to delineate the differential role that non-5HT₃ receptors play in mediating
572 contractility and urothelial signaling in the bladder.

573

574 **5-HT₃ receptor mechanisms contribute to bladder hypersensitivity in CVH.**

575 Christianson *et al.*, (2007) demonstrated that 21% of all retrograde traced mouse DRGs
576 from the colon also innervated the bladder (dichotomizing neurons) (Christianson *et al.*,
577 2007). Since co-morbidities between the bladder and bowel are common, the hypothesis
578 of 'cross-organ sensitization' has emerged. Evidence for this phenomenon has come
579 from pre-clinical studies identifying that inflammation or injury to one organ, such as the
580 bowel, can lead to changes in the sensitivity of another closely located organ, such as
581 the bladder, via these convergent neural pathways. A number of models of colon-bladder
582 cross organ sensitization have been developed to study this phenomenon, one such
583 model is the well-established TNBS model of colitis (Antonίου *et al.*, 2016; Grundy *et al.*,
584 2018b; Malykhina *et al.*, 2006; Qin *et al.*, 2005) . Previous studies using the TNBS
585 induced colitis model suggest that inflammation of the bowel induces hypersensitivity of
586 colonic afferents and changes in spinal neural circuitry that persists after the
587 inflammation as resolved (Brierley and Linden, 2014). This effect is concomitant with
588 hypersensitivity of the bladder and bladder voiding dysfunction indicative of overactive
589 bladder syndrome or painful bladder syndrome, without any obvious morphological
590 changes in bladder structure. Two phases of response have been identified; an acute
591 phase of visceral hyposensitivity (AVH, <15 days post treatment) and a chronic phase of
592 visceral hypersensitivity (CVH, >28 days post treatment) (Grundy *et al.*, 2018b).

593

594 The 5HT₃ receptor has been linked to intestinal dysfunction in a number of studies. For
595 example, the selective 5-HT₃ antagonists tropisetron and ondansetron significantly
596 reduce signs of inflammatory damage in the bowel in a TNBS-induced colitis model in
597 the rat (Motavallian-Naeini *et al.*, 2012; Motavallian *et al.*, 2013). Bioavailability of 5-HT
598 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 5HT₃ 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-HT₃ selective antagonist granisetron, implicating the 5-HT₃ receptor in the
613 response. The numbers of bladder-projecting DRG neurons expressing 5-HT₃ 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-HT₃ 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 in 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-HT₃ 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

634 molecular architecture responsible for 5-HT synthesis we identified within the urothelium,
635 there have been no previous reports of 5HT release from the bladder in the literature to
636 support this idea. (Fitzgerald *et al.* (2013) showed that there was a significant increase
637 in the number of mast cells in the bladder at 12 days after colonic TNBS administration
638 in rats, raising the possibility that in the post-inflammatory state, 5-HT levels in the
639 bladder rise due to infiltration of circulating mast cells (Fitzgerald *et al.*, 2013). This
640 mechanism could drive altered sensory activity via an interaction with the 5-HT₃ receptor
641 on the primary afferents. Unfortunately, since we did not measure 5-HT levels or mast
642 cell numbers in the bladders from our CVH mice this remains speculative.

643

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

659

660 **Translational Perspectives**

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

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Figures and legends

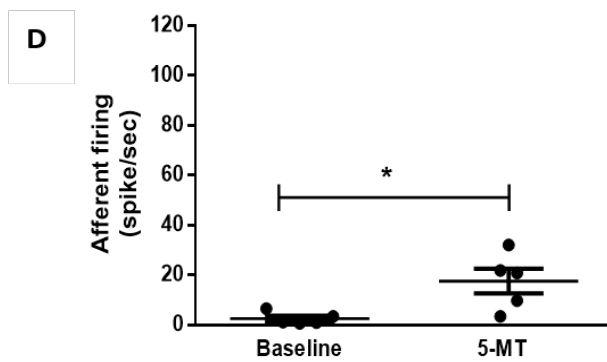
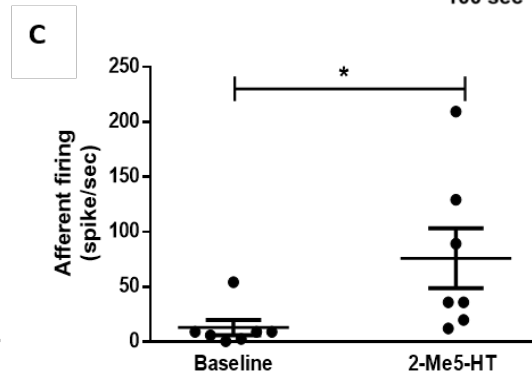
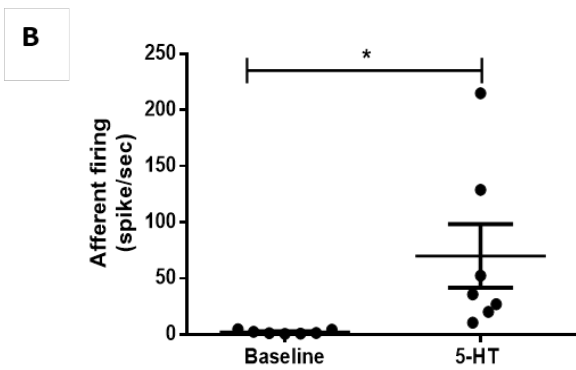
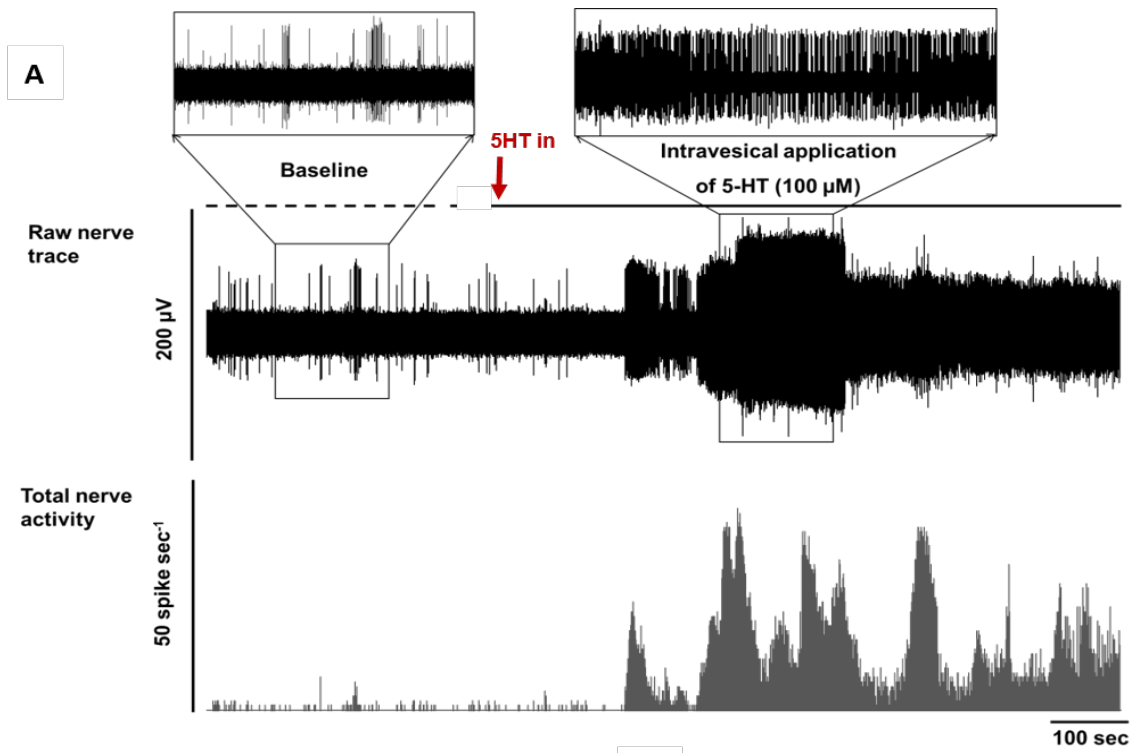


Figure 1 Intravesically applied 5-HT, 2-Me-5HT and 5-MT stimulated bladder afferent firing. (A) Representative trace to illustrate the afferent response to intravesical application of 5-HT (100 μ M). (B-D) The peak afferent response to intravesical application of (B) 5-HT (100 μ M), (C) 2-Me-5HT (100 μ M) and (D) 5-MT (100 μ M). 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).

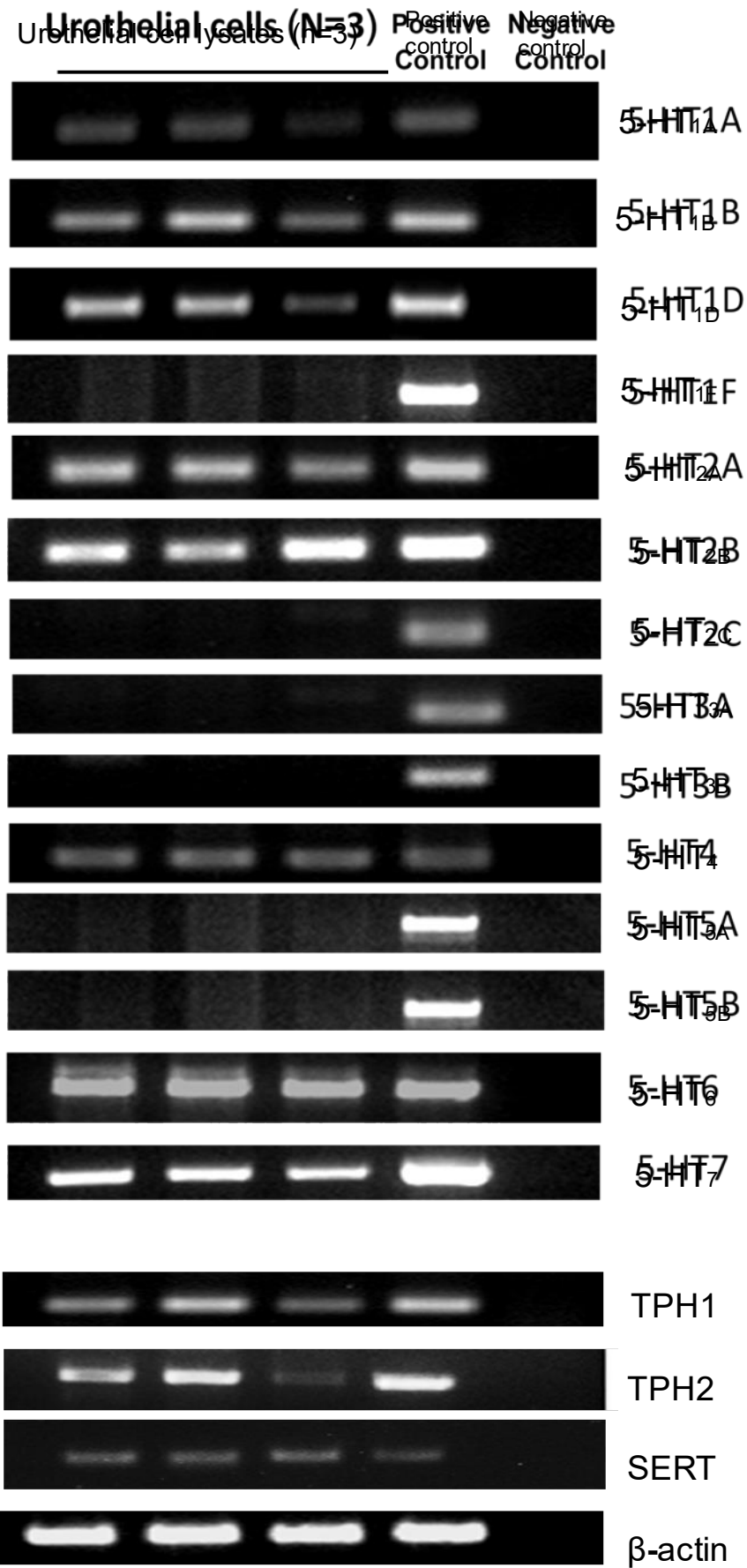
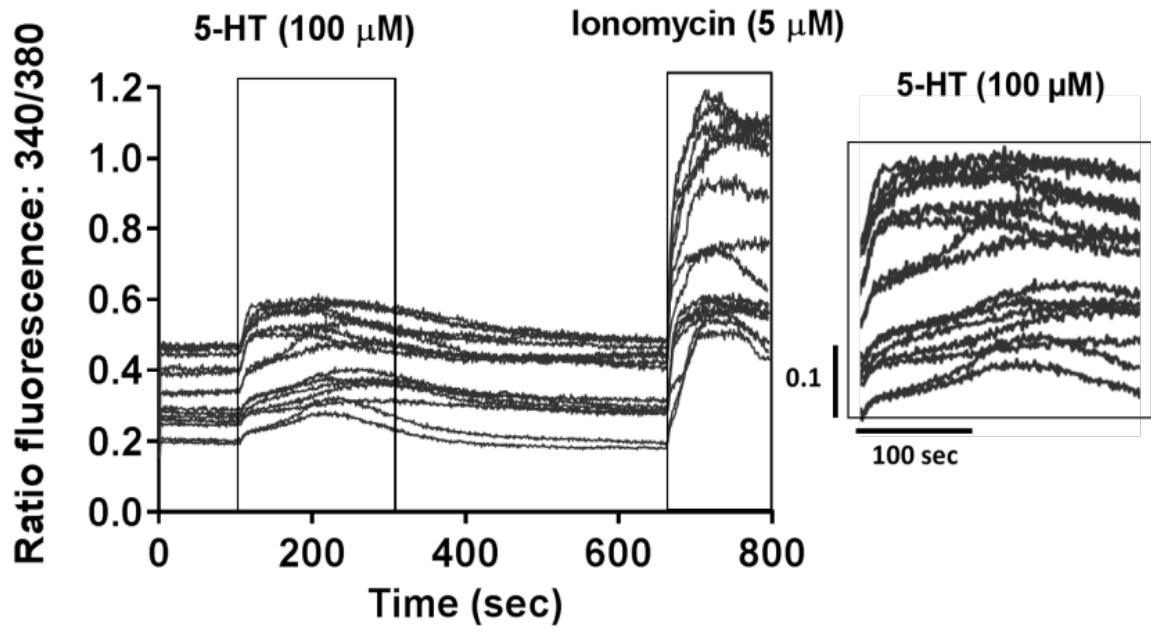
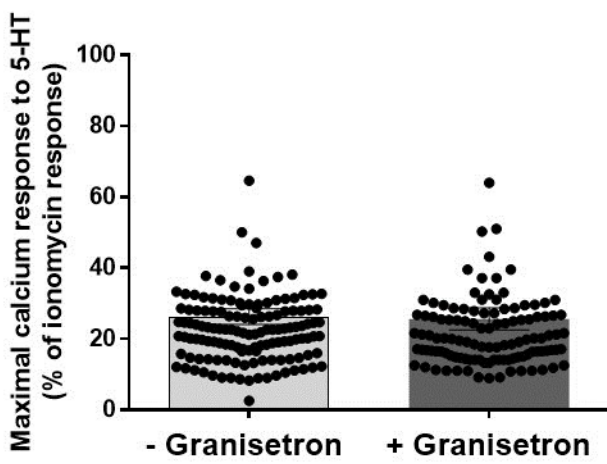


Figure 2 mRNA expression of 5-HT receptors, TPH1, TPH2, and SERT on the mouse urothelium. An array of 5-HT receptor transcripts were detected in mouse urothelial cell lysates (5-HT_{1A}, 1B, 1D, 2A, 2B, 4, 6, 7). 5-HT_{1F}, 2C, 3A, 3B, 5A, and 5B receptors were not detectable. mRNA expression of TPH1, TPH2, and SERT were detected in mouse urothelial cells. β -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



B



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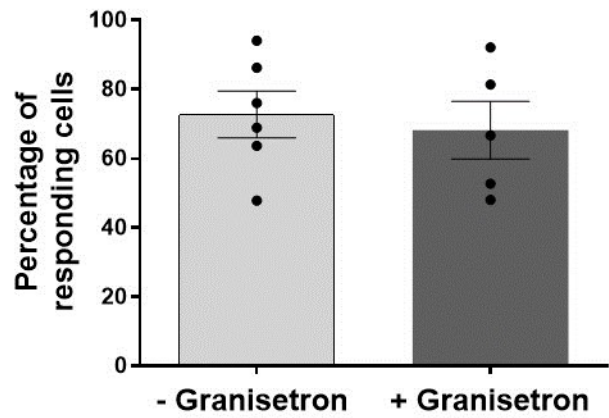


Figure 3 5- HT stimulated mouse urothelial cells through a non- 5- HT3 receptor mechanism. (A) A sample trace showing the calcium response of isolated primary mouse urothelial cells to 5-HT (100 μ M) and ionomycin (5 μ M) . Each line represents a single urothelial cell. **(B)** The relative increase of intracellular Ca^{2+} (Ratio 340/ 380) in response to 5- HT with and without preincubation with granisetron (1 μ M), showing that granisetron did not affect 5HT responses. **(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.

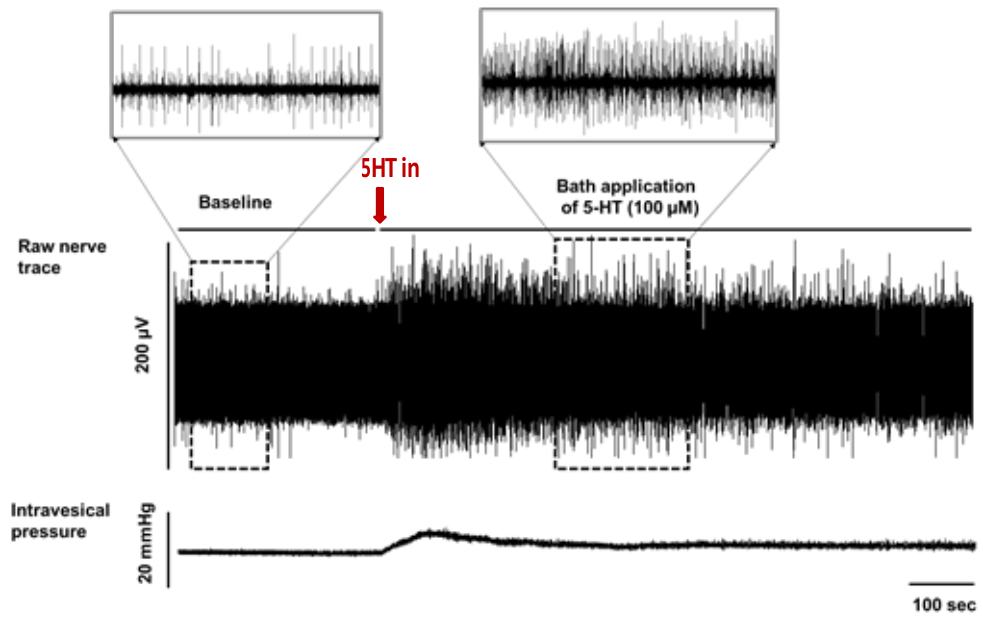
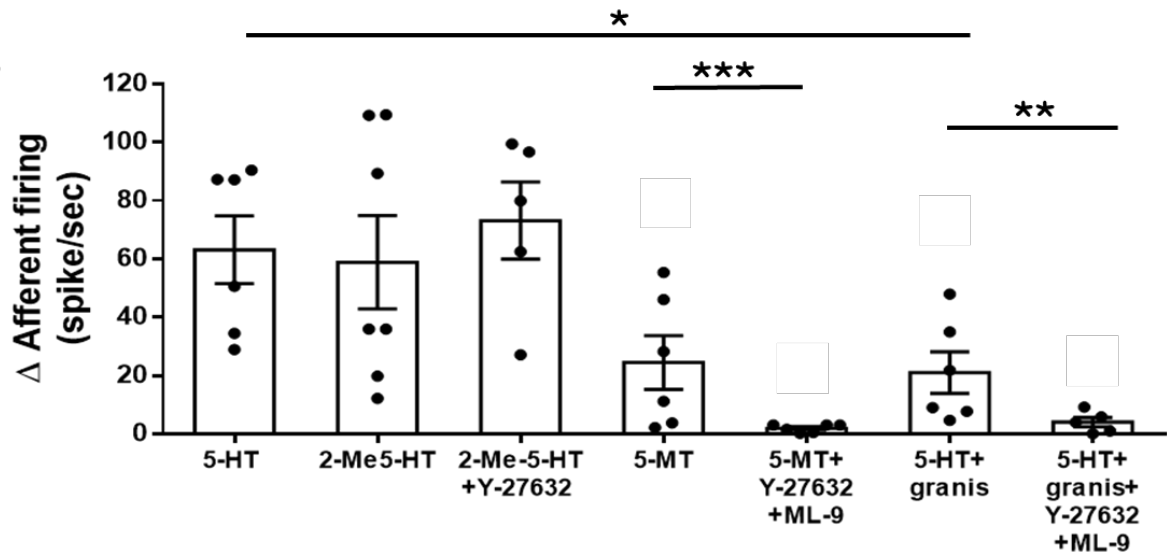
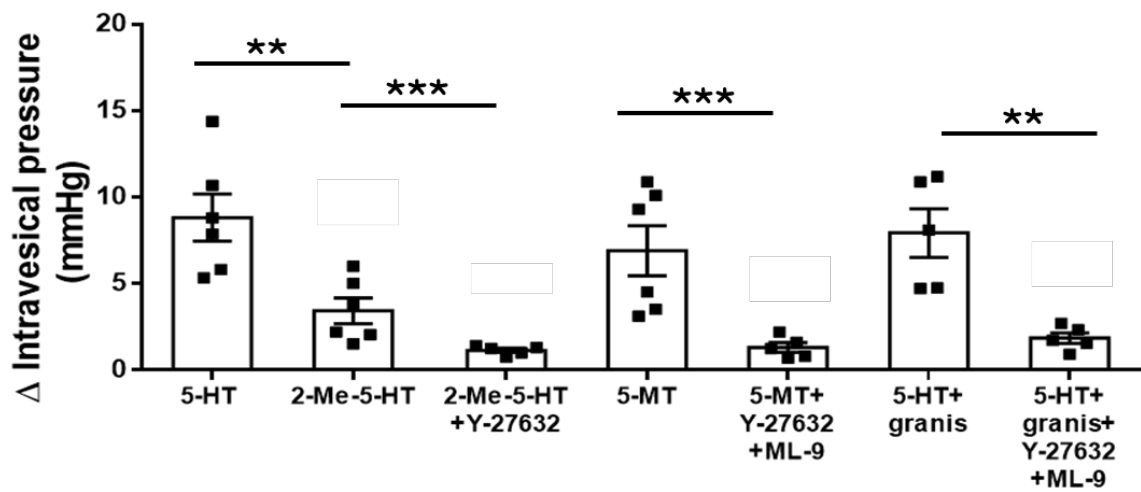
A**B****C**

Figure 4 5-HT₃ 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-HT₃ 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).

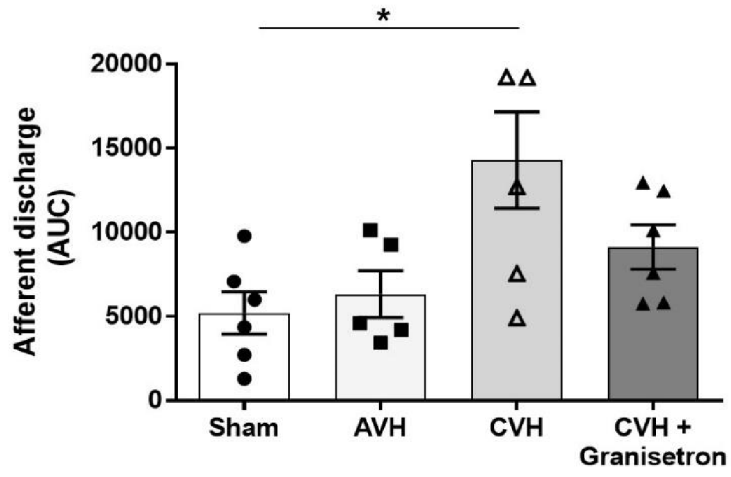
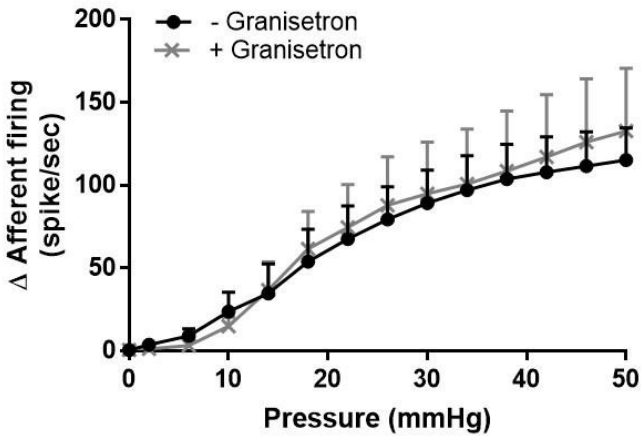
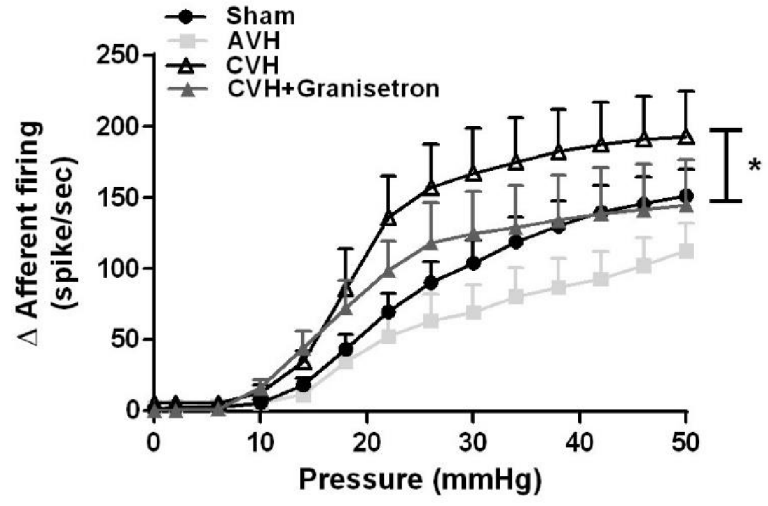
A**B****C**

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)

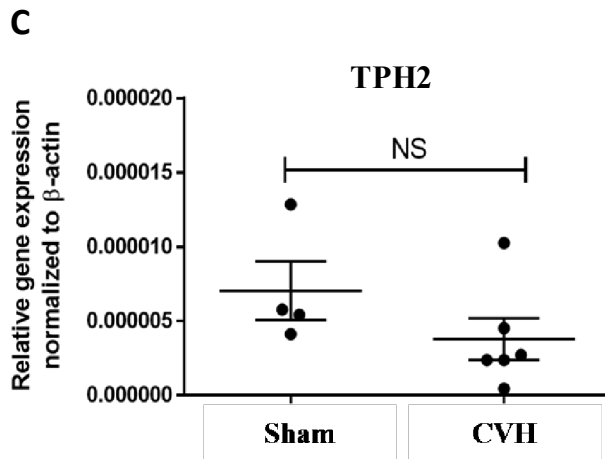
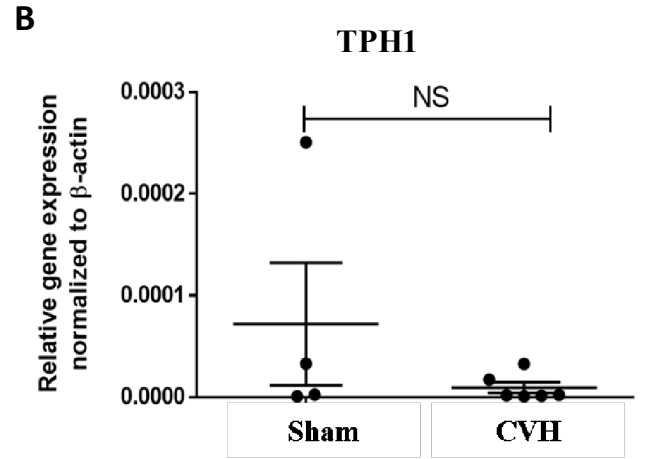
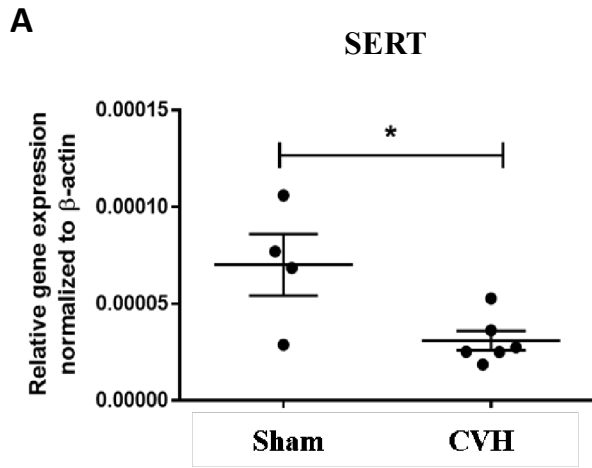
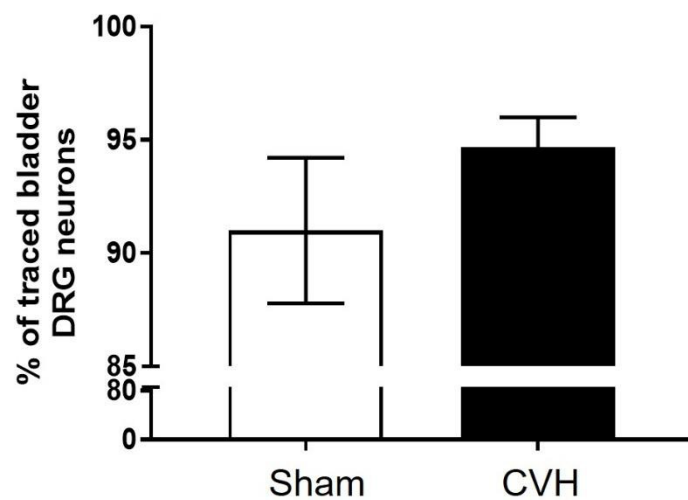
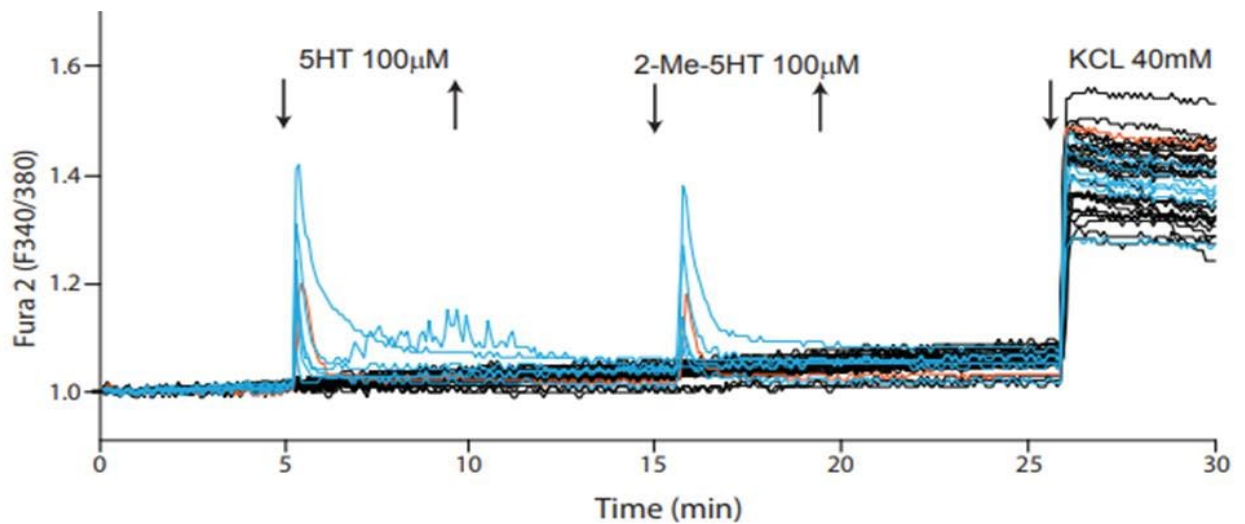


Figure 6 SERT mRNA expression in the urothelium was significantly attenuated in mice with CVH. (A) SERT mRNA expression was significantly reduced in urothelial cell cultures from CVH animals compared to control animals (* $P < 0.05$, unpaired Student's t-test healthy control; $N = 4$, CVH; $N = 6$).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

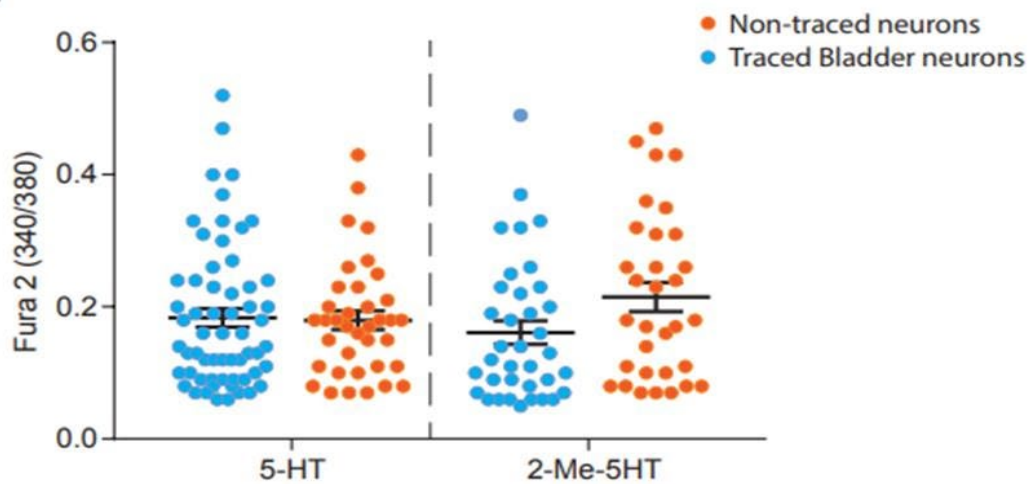


Figure 7. 5-HT and 2-Me-5HT induce calcium transients in bladder-innervating DRG neurons. (A) A high percentage of bladder projecting DRG neurons in both sham (91%) and mice with CVH (95%) express 5-HT₃ receptors (sham, N=3, n=77; CVH, N=3, n=74; N refers to number of animals, n indicates number of DRG 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 μ M) (N=4 mice) and 2-Me-5HT (100 μ M) (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 μ M) is equivalent to that elicited by 2-Me-5HT (100 μ M) 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

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Competing interests

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