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

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17  
18  
19 **Running title:** Effect of 5HT on afferent firing from the mouse bladder

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

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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-HT<sub>3</sub> receptor mRNA transcripts are expressed in mouse urothelium and  
44 exert functional responses to 5-HT.
- 45 • Whilst 5-HT<sub>3</sub> receptors were not detected in mouse urothelium, 5-HT<sub>3</sub> 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-HT<sub>3</sub> 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-HT<sub>3</sub>  
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-HT<sub>3</sub> 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-HT<sub>3</sub> antagonist, reversed bladder  
73 afferent hypersensitivity in CVH mice. These data suggest 5-HT exerts a direct effect on

74 bladder afferents to enhance signaling. 5-HT<sub>3</sub> 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 $\delta$  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-HT<sub>1-7</sub>),  
93 six are G-protein coupled receptors (GPCRs) and one, the 5-HT<sub>3</sub> receptor is a ligand-  
94 gated ion channel (McCorvy and Roth, 2015). Within the bladder, activation of 5-HT<sub>1A</sub>,  
95 5-HT<sub>2</sub> and 5-HT<sub>3</sub>, but not 5-HT<sub>4</sub> and 5-HT<sub>7</sub> can generate altered bladder contraction in  
96 rodent models (Chetty *et al.*, 2007; Kodama and Takimoto, 2000; Mittra *et al.*, 2007). 5-  
97 HT<sub>3A</sub> and 5-HT<sub>3B</sub> 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<sub>1D</sub>, 2A, 4 and 5-HT<sub>6</sub> are present in urothelial cells. 5-HT<sub>1A</sub>, 1B, 1D, 2A,  
100 2B, 2C, 3A, 4, and 5-HT<sub>7</sub> have also been reported in urothelium-denuded tissues (Matsumoto-  
101 Miyai *et al.*, 2016). However, a modulatory role of 5-HT on bladder afferent excitability  
102 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, one-  
108 third 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 possibility  
126 that bladder hypersensitivity associated with TNBS-induced colitis may involve a 5-HT<sub>3</sub>  
127 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  
159 had 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 2,4,6-  
164 trinitrobenzenesulfonic 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, crypt 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<sup>-1</sup> with  
180 oxygenated (95%O<sub>2</sub>/5%CO<sub>2</sub>) Krebs bicarbonate solution (composition, mM: NaCl 118.4,  
181 NaHCO<sub>3</sub> 24.9, CaCl<sub>2</sub> 1.9, MgSO<sub>4</sub> 1.2, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 11.7) at 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<sup>-1</sup>. The bladder dome was punctured at the apex with a syringe needle (BD  
186 microlance<sup>TM</sup>, 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 (DTX<sup>TM</sup> 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<sup>-1</sup> 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  
202 distensions, pharmacological agents were either perfused into the bladder lumen  
203 (intravesical application) or into the recording chamber (bath application). Following  
204 application of any compound a 'wash out' was conducted using either saline  
205 (intravesical) or Krebs (bath 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  
214 intravesical 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 trypsin-  
227 EDTA was deactivated by adding pre-warm MEM with 10 % Fetal Bovine Serum (FBS)  
228 (Gibco®). The cell suspension was centrifuged at 1500 rpm, 4 °C for 15 minutes. The  
229 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% CO<sub>2</sub>-95% O<sub>2</sub> 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 <sub>1A</sub>	NM_008308	FW: 5' TAAGAACTCCCGCTCCAGT 3'	103	Brain
		RW: 5' AGAAATGCAAGGGGATCTCC 3'		
5-HT <sub>1B</sub>	NM_010482	FW: 5' CCAACACACAATAAATGCTCCT3'	135	DRG
		RW: 5' CCAAGTCAAAGTGCGAGTCT 3'		
5-HT <sub>1D</sub>	NM_008309	FW: 5'TACAAACACCCCTACTAAACGC 3'	310	DRG
		RW: 5'ATGAGTGTTTCAGCGTTGGTT 3'		
5-HT <sub>1F</sub>	NM_008310	FW: 5'GACCAGAGCCCCTTAGCTTC 3'	340	DRG
		RW: 5'TGCAGCTTCCGAGTCACAAT 3'		
5-HT <sub>2A</sub>	NM_172812	FW: 5' CATCTCCCTGGACCGCTAC 3'	150	DRG
		RW: 5' TCATCCTGTAGCCCGAAGAC 3'		
5-HT <sub>2B</sub>	XM_006529146	FW: 5' CCGATTGCCCTCTTGACAAT 3'	120	DRG
		RW: 5' GGCACAGAGATGCATGATGG 3'		
5-HT <sub>2C</sub>	NM_008312	FW: 5' TGAAACTGGTTGCTTAAACTGA 3'	126	DRG
		RW: 5' AGCTGCTACTGGACTTATGGA 3'		
5-HT <sub>3A</sub>	NM_013561	FW: 5' CCACCTTCCAAGCCAACAAG 3'	128	DRG
		RW: 5' CTCCCTTGGTGGTGAAGAG 3'		
5-HT <sub>3B</sub>	NM_020274	FW: 5' TGATTCTTCTGTGGTCCTGC 3'	154	DRG
		RW: 5' GCCTCAGCCCAGTTGTAAAC 3'		
5-HT <sub>4</sub>	NM_008313	FW: 5' ATGTTCTGCCTGGTCCGG 3'	162	DRG
		RW: 5' GCCTCCCAACATTAATGCGA 3'		
5-HT <sub>5A</sub>	NM_008314	FW: 5' AAGACCAACAGCGTCTCCC 3'	124	Brain
		RW: 5' TCCACGTATCCCTTCTGTC 3'		

5-HT <sub>5B</sub>	NM_010483	FW: 5' TCTCCTTCGACGTGTTGTGC 3'	469	Brain
		RW: 5' GAGTCTCCGCTTGTCTGGAA 3'		
5-HT <sub>6</sub>	NM_021358	FW: 5' TGGGCAAAGCTCGAACATCT 3'	386	Brain
		RW: 5' GTCACATACGGCCTGAGCTAT 3'		
5-HT <sub>7</sub>	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'		

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  
268 correspond to the pelvic innervation of the bladder, were isolated and incubated in Hanks  
269 balanced 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  
271 and 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% CO<sub>2</sub>. 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 Cells-  
287 to-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® Thin-  
309 Wall 96-Well Skirted PCR Plates (BIO-RAD, HSP-9665). Prior to running the reactions,  
310 the plates were covered with MicroAmp™ Optical Adhesive Film for 96-Well Plates  
311 (Applied Biosystems, 43111971) and centrifuged briefly to spin down the contents and

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

318

### 319 **Calcium imaging**

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

332

### 333 **Drugs and solutions**

334 5-hydroxytryptamine (5-HT) and 5-methoxytryptamine (5-MT) were obtained from Sigma  
335 Aldrich, UK. 2-Methoxy-5-hydroxytryptamine (2-Me-5-HT) was obtained from  
336 Tocris/Bioscience, UK. Granisetron hydrochloride was obtained from LKT Laboratories,  
337 USA. ML-9 was obtained from Cayman Chemical. Y-27632 was obtained from  
338 Chemdea, USA.

339

### 340 **Data analysis**

341 Spontaneous afferent firing (also referred to as baseline) was determined by measuring  
342 mean action potential firing rate over 10 seconds either at rest (before a drug application  
343 or before a distension). This is defined as the afferent activity arising from the bladder in  
344 the absence of a stimuli (mechanical or chemical). Mechanosensitive afferent firing was  
345 determined by performing ramp distension of the bladder (as described above) and  
346 measuring the afferent discharge at various intravesical pressure points using a custom-

347 built script from SPIKE 2 version 7 (CED, UK). Data are expressed as means  $\pm$  SEM.  
348 Statistical analysis was analyzed using one- or two-way analysis of variance (ANOVA)  
349 with Dunnett's or Tukey multiple comparison or Student's t test, where appropriate. The  
350 statistical analysis were considered significant at \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  
351 \*\*\*\* $P < 0.0001$ .  
352  
353

## 354 **Results**

### 355 **Intravesical administration of 5-HT agonists excites bladder afferent nerves**

356 In order to determine if bladder afferents functionally express 5-HT receptors, we  
357 investigated the effects of various 5-HT receptor agonists on *ex-vivo* bladder afferent  
358 nerve activity. We found that intravesical infusion of 5-HT (100  $\mu$ M), evoked a dramatic  
359 and sustained increase in baseline afferent nerve firing compared to intravesical saline  
360 (Fig. 1A, 1B). The receptor mechanisms underlying this effect were determined by  
361 comparing the responses to 2-Me-5-HT, a selective 5-HT<sub>3</sub> agonist (100  $\mu$ M), with 5-MT  
362 (100  $\mu$ M) a compound which exhibits selectivity for the GPCR family of 5-HT receptors  
363 including 5-HT<sub>1</sub>, 5-HT<sub>2</sub> and 5-HT<sub>4-7</sub>. The responses to application of 5-HT and 2-Me-5-  
364 HT were comparable. However while the response to 5-MT, was significantly increased  
365 relative to baseline, the magnitude of the firing was reduced compared to that of 5-HT  
366 and 2-Me-5-HT (Fig. 1C, 1D). These data suggest that multiple 5-HT receptors may  
367 influence bladder afferent firing, and that this may occur via both direct and indirect  
368 mechanisms.

369

### 370 **5-HT receptors are expressed on bladder urothelium**

371 Given the established role of the urothelium as a sensory structure, we wanted to  
372 determine if the effects of intravesical 5-HT on bladder afferent firing were secondary to  
373 activation of urothelial cells or a consequence of direct activation of primary afferent  
374 endings innervating the bladder. To this end we investigated serotonergic receptor  
375 expression in the bladder urothelial layer using RT-PCR. The expression profile for the  
376 various 5-HT receptors in the urothelium is shown in Figure 2. Overall, 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>,  
377 5-HT<sub>1D</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, 5-HT<sub>4</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub> receptors were all detected. In contrast,  
378 5-HT<sub>1F</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>3A</sub>, 5-HT<sub>3B</sub> or the 5-HT<sub>5A</sub>, 5-HT<sub>5B</sub> receptors were not detected in the  
379 bladder urothelium. Moreover, expression of the 5-HT producing enzymes, tryptophan  
380 hydroxylase-1, and 2 (TPH-1 and TPH-2), and the sodium-dependent serotonin  
381 transporter (SERT) transcripts were also examined using PCR and all three were shown  
382 to be expressed in the urothelium (Fig. 2). This finding raises the possibility of an  
383 endogenous source of 5-HT in the bladder wall.

384

### 385 **Stimulation of non-5HT<sub>3</sub> receptors on the urothelium can evoke urothelial cell** 386 **activation**

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

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

394

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

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

405

406 The peak afferent response to bath application of 5-HT and 2-Me-5-HT was similar.  
407 Moreover, application of the selective 5-HT<sub>3</sub> receptor antagonist, granisetron (1  $\mu\text{M}$ ),  
408 significantly attenuated afferent responses to 5-HT. To uncouple the direct effect of 5-  
409 HT on the afferent nerves from any indirect nerve response produced by bladder  
410 contraction we used a myosin light chain kinase inhibitor (ML-9, 10  $\mu\text{M}$ ) and/or the rho-  
411 associated kinase inhibitor (Y-27632, 10  $\mu\text{M}$ ) to prevent contraction. Under such  
412 conditions the afferent response to 5-MT was abolished in the absence of contraction,  
413 suggesting that the response to 5-MT was secondary to changes in muscle tone.  
414 Conversely, the afferent response to 2-Me-5-HT persisted despite the absence of  
415 contraction, suggesting that the contractile and nerve responses to 5-HT<sub>3</sub> receptor  
416 stimulation were independent (Fig. 4B and 4C).

417

418 **Chronic visceral hypersensitivity evoked with intra-colonic TNBS caused altered**  
419 **mechanosensitivity and increased spontaneous afferent firing from the bladder,**  
420 **an effect that was reversed by blocking the 5-HT<sub>3</sub> receptor.**

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

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

435

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

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

446

#### 447 **Bladder projecting DRG neurons express 5-HT<sub>3</sub> receptors in both control and CVH 448 mice and display functional response to 5-HT and 2-Me-5-HT activation.**

449 To examine if 5-HT<sub>3</sub> receptors in the afferent terminals play a role in 5-HT-sensitized  
450 bladder afferent firing, we performed retrograde labelling and single cell RT-PCR to  
451 examine 5-HT<sub>3</sub> receptor gene expression in bladder-projecting DRG neurons. The vast  
452 majority of these neurons (91%, 70/77 neurons) expressed 5-HT<sub>3</sub> receptors even in  
453 control animals, which is striking given the absence of any previously documented  
454 endogenous source of 5-HT. Moreover, 95% of bladder-innervating DRG isolated from  
455 mice with CVH expressed 5-HT<sub>3</sub> (70/74 neurons), showing a small increase in the  
456 number of neurons expressing 5-HT<sub>3</sub> occurs during cross-organ sensitization. (Fig. 7A).

457

458 In addition, we performed calcium imaging on bladder innervating (traced) and non-  
459 traced lumbosacral DRG neurons. A representative trace of calcium imaging of  
460 lumbosacral DRG neurons in response to 5-HT (100  $\mu$ M) and 2-Me-5-HT (100  $\mu$ M) is  
461 shown in Figure 7B. 60 out of a total of 69 (87%) bladder projecting DRG neurons (traced  
462 neurons) elicited a calcium signal in response to application of 5-HT, moreover a  
463 significantly greater proportion of the traced DRGs responded to 5HT than the non-traced  
464 DRG neurons. This indicates that expression of 5-HT receptors maybe enriched in  
465 bladder neurons compared to the generalized neuronal population. In addition, a similar  
466 proportion of traced neurons (70%, 36/51) showed an increase in  $Ca^{2+}$  influx after the  
467 application of 2-Me-5-HT (Fig. 7C). The magnitude of the  $Ca^{2+}$  response to 5-HT and 2-  
468 Me-5-HT was similar in traced and non-traced DRG neurons (Fig. 7D). These data are  
469 consistent with a major contribution of 5-HT<sub>3</sub> receptors to 5-HT's ability to sensitize  
470 bladder afferents and enhance firing.

471

## 472 **Discussion**

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

486

### 487 **5-HT increases bladder afferent firing and induces contraction of the detrusor**

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

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

497

498 Similar to 5-HT, bath application of the selective 5-HT<sub>1</sub>, 5-HT<sub>2</sub>, 5-HT<sub>4</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub>  
499 agonist, 5 methoxy tryptamine (5-MT) also caused an increase in afferent nerve firing  
500 and evoked a small contraction of the bladder. However, when the contraction was  
501 blocked by preincubation with the muscle blockers (Y-27632 and ML-9), the contraction  
502 was lost and the afferent response to 5MT was abolished suggesting that the afferent  
503 response to stimulation of the non-5HT<sub>3</sub> receptor populations was secondary to changes  
504 in bladder contraction rather than via a direct action at the afferent terminal. A number of  
505 previous studies have demonstrated that 5-HT can induce contraction of bladder muscle  
506 strips *in vitro*, however the receptor that mediates the contraction remains controversial.  
507 Hattori *et al.*, (2017) show that both 5-HT and a selective 5-HT<sub>2</sub> receptor agonist cause  
508 contraction of the bladder, but a lack of efficacy following pre-incubation of selective 5-  
509 HT<sub>2</sub> receptor antagonists suggested that the effect was not mediated by the 5-HT<sub>2</sub>  
510 receptor (Hattori *et al.*, 2017). In another study it was shown that 5-HT potentiates  
511 neurogenic contractions of rat isolated detrusor muscle through both 5-HT<sub>7</sub> and 5-HT<sub>2c</sub>  
512 receptors (Rekik *et al.*, 2011). Moreover, in the guinea pig the contractile response to 5-  
513 HT was suggested to be via the 5-HT<sub>2</sub>, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> receptors (Yoshida *et al.*, 2002).

514

### 515 **5-HT has a direct effect on afferent firing via the 5-HT<sub>3</sub> receptor**

516 Bath application of 2-Me-5-HT caused a robust increase in nerve firing that was not  
517 affected by blocking contractility. 2-Me-5-HT is a potent agonist of both the 5-HT<sub>3</sub> and  
518 the 5-HT<sub>6</sub> receptor (Glennon *et al.*, 2000) suggesting that activation of either 5-HT<sub>3</sub> or 5-  
519 HT<sub>6</sub> located on the urothelium (in the case of 5HT<sub>6</sub>) or at the nerve terminal results in  
520 afferent nerve firing from the bladder. Since we did not use a selective 5HT<sub>6</sub> receptor  
521 antagonist we cannot determine from these data what contribution 5HT<sub>6</sub> made to the 2-  
522 Me-5-HT response. Since the role of 5HT<sub>6</sub> in the lower urinary tract is relatively  
523 understudied this would warrant further investigation. However in this study we focused  
524 on the 5-HT<sub>3</sub> receptor since pre-incubation of the 5-HT<sub>3</sub> selective antagonist granisteron  
525 inhibited the 5-HT-mediated increase in bladder afferent nerve firing by ~65%. Since 5-  
526 HT<sub>3</sub> receptors were not identified in the urothelium via RT-PCR, this suggests that  
527 stimulation of 5-HT<sub>3</sub> receptors can elicit a direct increase in afferent nerve firing by  
528 stimulating receptors on the nerve terminal rather than via an indirect mechanism as a

529 result of muscle contraction or urothelial signaling. Our retrograde tracing studies  
530 revealed that the majority of lumbosacral DRG neurons innervating the mouse urinary  
531 bladder (90%) express the 5-HT<sub>3</sub> receptor. Furthermore, our functional calcium imaging  
532 showed that the majority of these DRGs exhibit functional responses to 5-HT (87%) and  
533 the 5-HT<sub>3</sub> selective agonist, 2-Me-5-HT (70%). **In this study we did not stain the afferent**  
534 **terminals and show expression of 5HT3 in bladder afferent endings, although it is well**  
535 **established that expression in the cell body where the receptor is synthesized is likely to**  
536 **be indicative of expression at the afferent terminal. Therefore** these data suggest that 5-  
537 HT exerts a direct action on 5-HT<sub>3</sub> receptor at the terminal endings of the primary  
538 afferents innervating the bladder. This idea is supported by previous studies which show  
539 that 5-HT<sub>3</sub> receptors are also located in rat DRG neurons (Nicholson et al., 2003) and  
540 that systemic injection of a 5-HT precursor (5-hydroxytryptophan, 5-HTP), triggers  
541 bladder hyper-excitability in a mechanism mediated by spinal 5-HT<sub>3</sub> receptors (Hall et  
542 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-HT<sub>3</sub> receptor.

548

#### 549 **5-HT can alter signaling in the urothelium in a mechanism that is independent of** 550 **the 5-HT<sub>3</sub> 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<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, 5-HT<sub>4</sub>, 5-HT<sub>6</sub>,  
557 and 5-HT<sub>7</sub> 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-HT<sub>3</sub>  
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-HT<sub>3</sub> receptor was not identified in the urothelium suggests that while the 5-HT<sub>3</sub>  
569 receptor may be involved in the direct afferent response to 5HT (i.e. at the 5-HT<sub>3</sub> 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-5HT<sub>3</sub> receptors play in mediating  
572 contractility and urothelial signaling in the bladder.

573

### 574 **5-HT<sub>3</sub> 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 (Antoniou *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 5HT<sub>3</sub> receptor has been linked to intestinal dysfunction in a number of studies. For  
595 example, the selective 5-HT<sub>3</sub> 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, long-  
602 term hypersensitivity of the small intestine was shown to be mediated by changes in 5-  
603 HT<sub>3</sub> 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<sub>3</sub> selective antagonist granisetron, implicating the 5-HT<sub>3</sub> receptor in the  
613 response. The numbers of bladder-projecting DRG neurons expressing 5-HT<sub>3</sub> was also  
614 slightly elevated in the CVH phase.

615

616 Interestingly, mRNA for the 5-HT producing enzymes, TPH1 and TPH2, and the  
617 serotonin transporter, SERT was detected in the normal urothelium and in the urothelium  
618 of mice following TNBS. However, in the urothelium of CVH mice we observed a  
619 significant decrease in SERT expression, which may suggest that following colonic  
620 inflammation there were alterations in the bioavailability of 5-HT. This could potentially  
621 lead to reciprocal changes in 5-HT<sub>3</sub> receptor sensitivity and bladder afferent  
622 hypersensitivity.

623

#### 624 **What is the endogenous source of 5-HT in the bladder?**

625 The majority of 5-HT in the body is produced by enterochromaffin cells in the GI tract  
626 (Gershon and Tack, 2007) and in the CNS. Activation of enterochromaffin cells can lead  
627 to the release of 5-HT and activation of 5-HT<sub>3</sub> receptors on colonic afferents innervating  
628 the colonic mucosa (Bellono et al., 2017). In addition, mast cells also synthesize 5-HT  
629 from 5-hydroxytryptophan and express TPH1 in the rat and human gastrointestinal tracts  
630 (Dwyer et al., 2016; Weitzman et al., 1985). However, the endogenous source of 5-HT  
631 in the bladder has yet to be identified. Since the components needed to produce 5-HT  
632 are present in the urothelium (ie TPH1 and TPH2 mRNA), it is possible that urothelial  
633 cells produce and release 5-HT which can then exert actions on afferent nerve fibers

634 located in close proximity, or the underlying smooth muscle. Despite the presence of the  
635 molecular architecture responsible for 5-HT synthesis we identified within the urothelium,  
636 there have been no previous reports of 5HT release from the bladder in the literature to  
637 support this idea. Fitzgerald *et al.* (2013) showed that there was a significant increase  
638 in the number of mast cells in the bladder at 12 days after colonic TNBS administration  
639 in rats, raising the possibility that in the post-inflammatory state, 5-HT levels in the  
640 bladder rise due to infiltration of circulating mast cells (Fitzgerald et al., 2013). This  
641 mechanism could drive altered sensory activity via an interaction with the 5-HT<sub>3</sub> receptor  
642 on the primary afferents. Unfortunately, since we did not measure 5-HT levels or mast  
643 cell numbers in the bladders from our CVH mice this remains speculative.

644

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

660

### 661 **Translational Perspectives**

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

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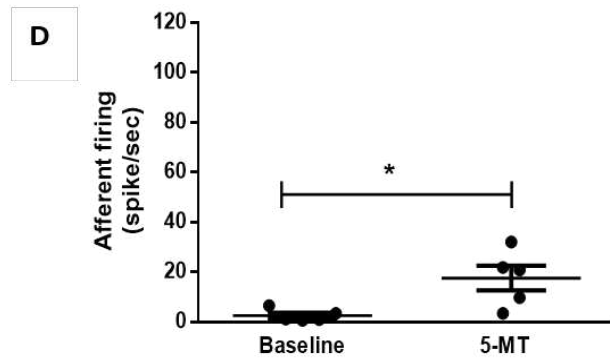
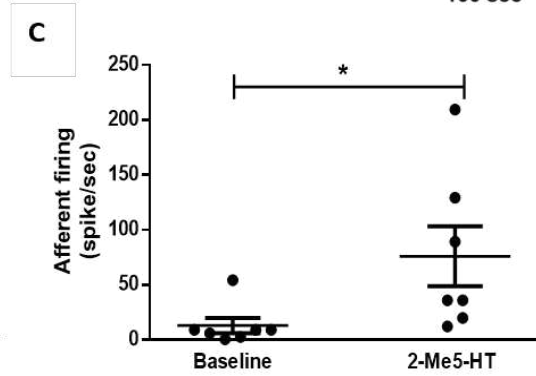
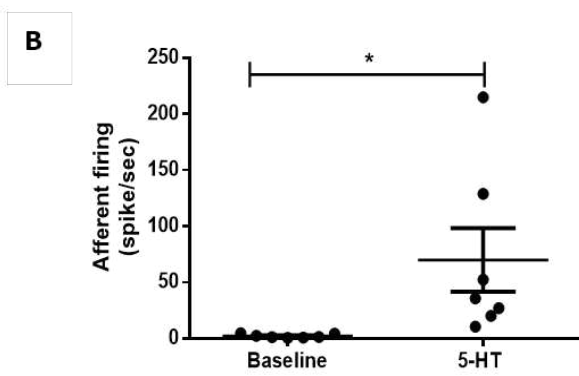
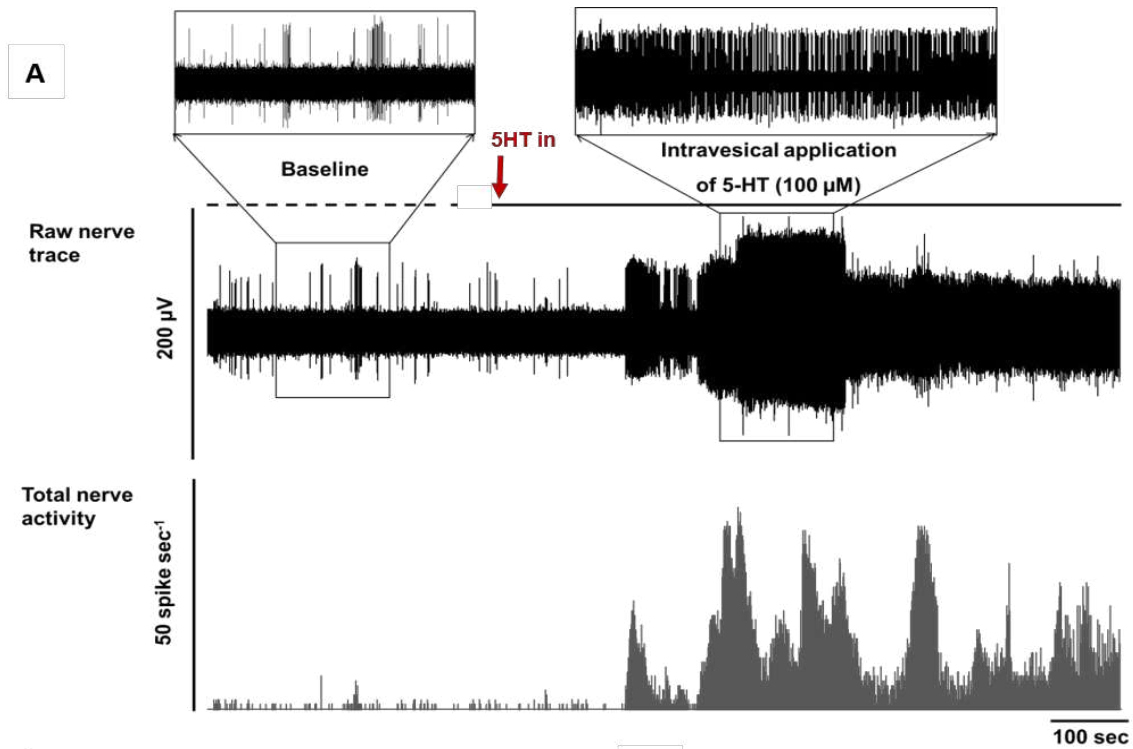
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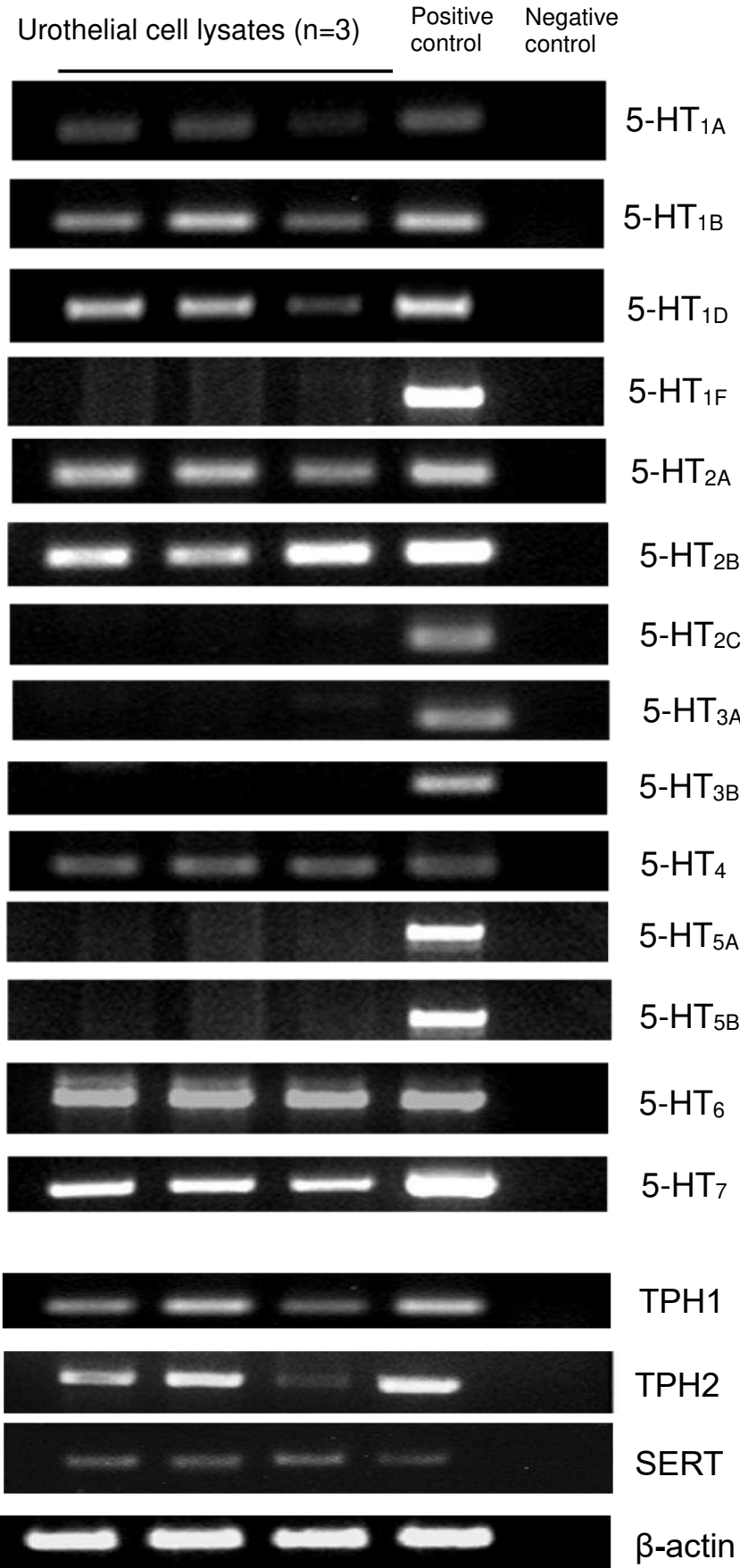
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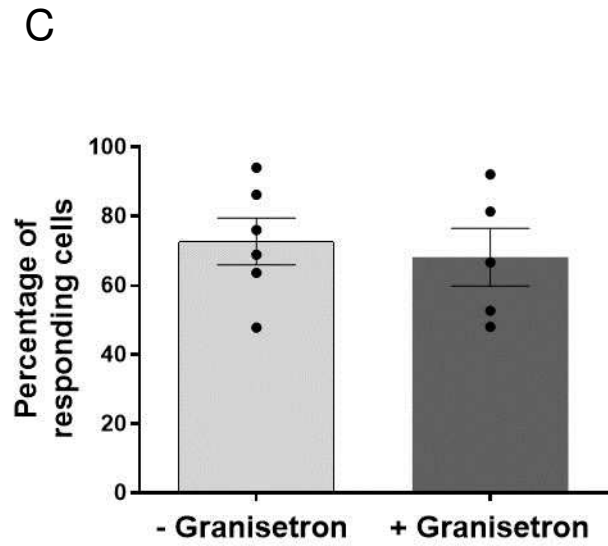
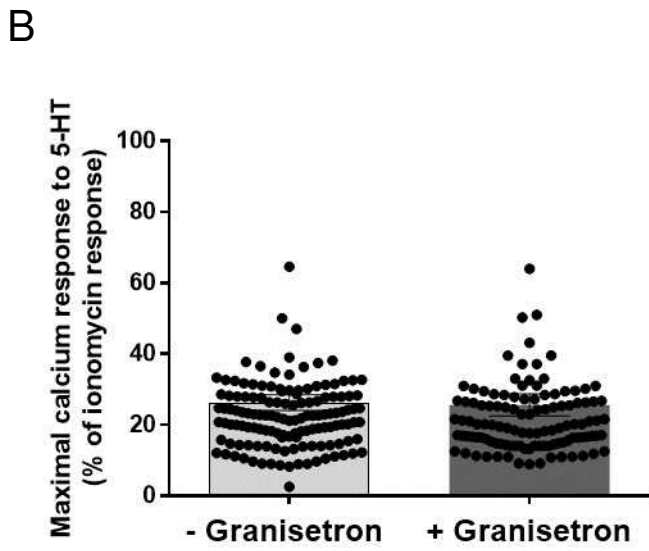
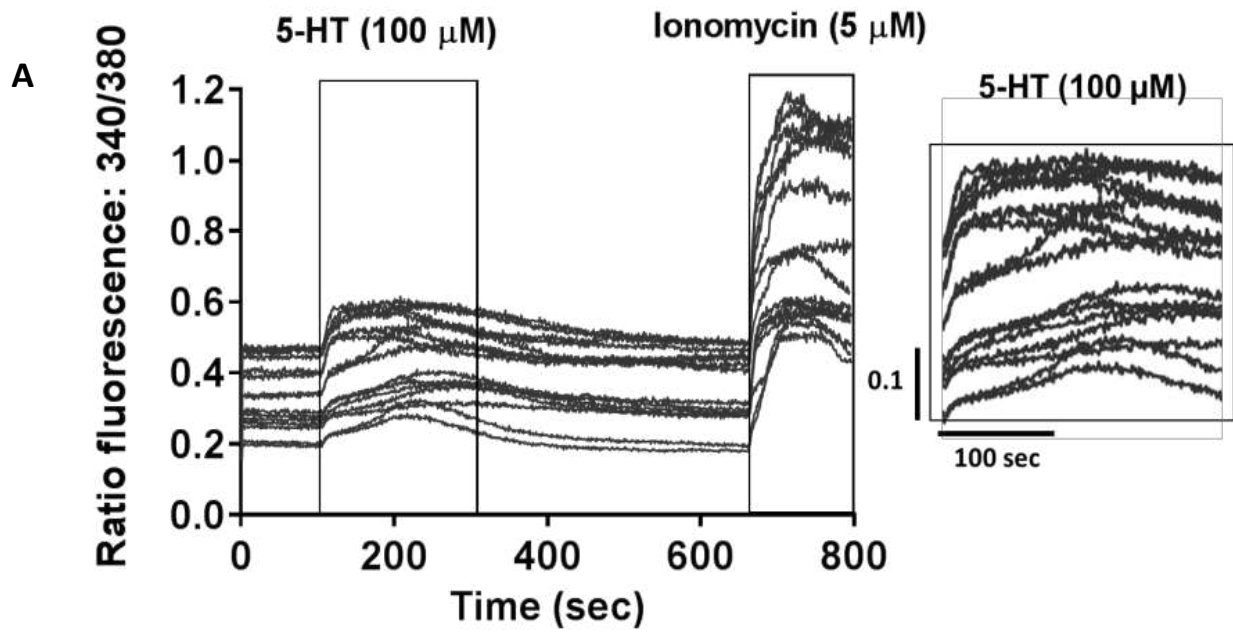
## Figures and legends



**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  $\mu$ M). (B-D) The peak afferent response to intravesical application of (B) 5-HT (100  $\mu$ M), (C) 2-Me-5HT (100  $\mu$ M) and (D) 5-MT (100  $\mu$ 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).

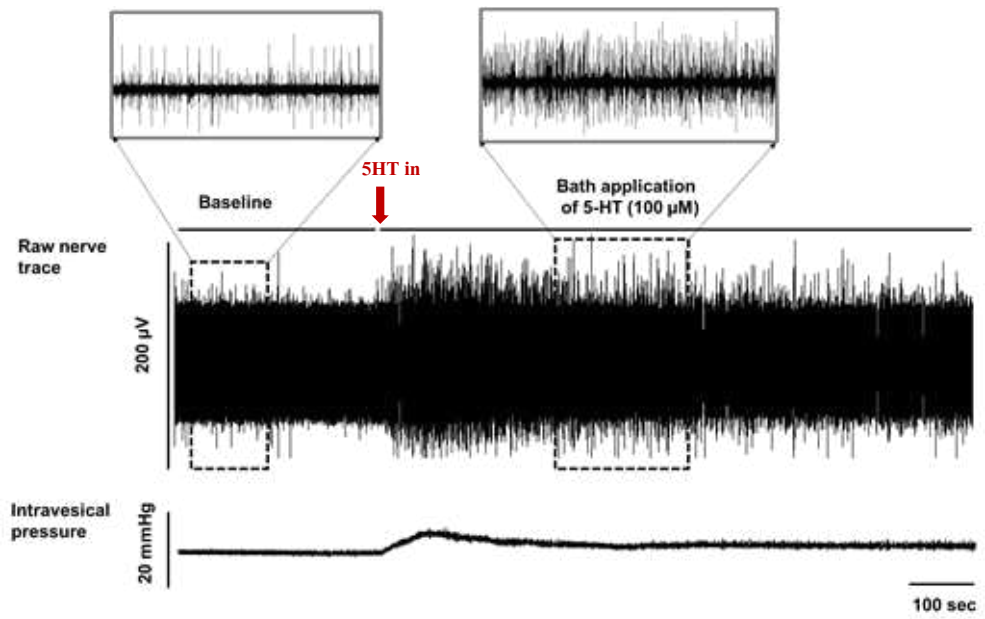


**Figure 2. mRNA expression of 5-HT receptors, TPH1, TPH2, and SERT on the mouse urothelium.** An array of 5-HT receptor transcripts were detected in mouse urothelial cell lysates (5-HT<sub>1A</sub>, 1B, 1D, 2A, 2B, 4, 6, 7). 5-HT<sub>1F</sub>, 2C, 3A, 3B, 5A, and 5B receptors were not detectable. mRNA expression of TPH1, TPH2, and SERT were detected in mouse urothelial cells.  $\beta$ -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).

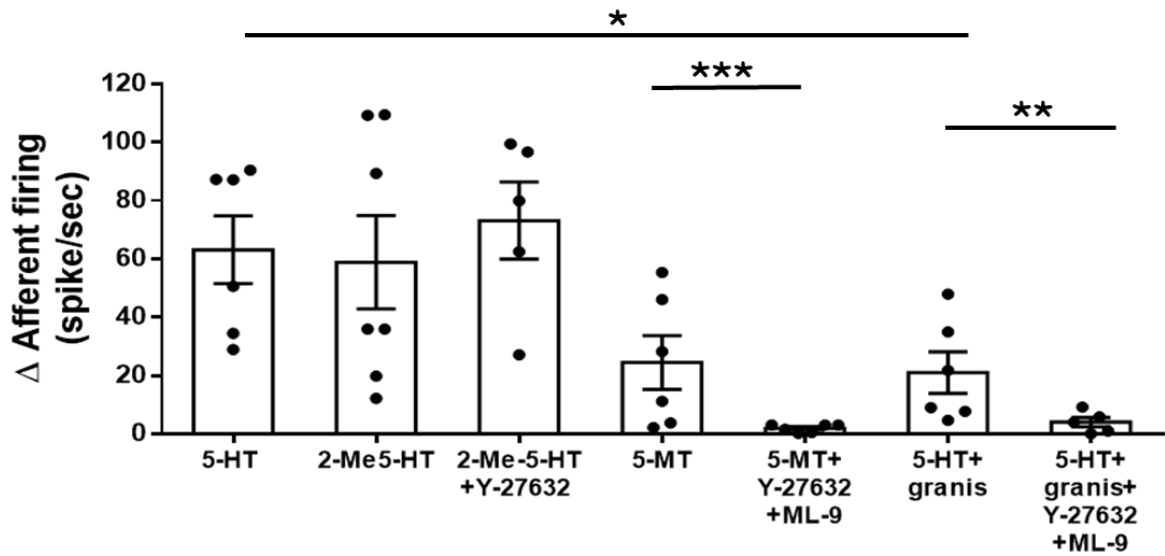


**Figure 3. 5-HT stimulated mouse urothelial cells through a non-5-HT3 receptor mechanism.** (A) A sample trace showing the calcium response of isolated primary mouse urothelial cells to 5-HT (100  $\mu$ M) and ionomycin (5  $\mu$ M) . Each line represents a single urothelial cell. (B) The relative increase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

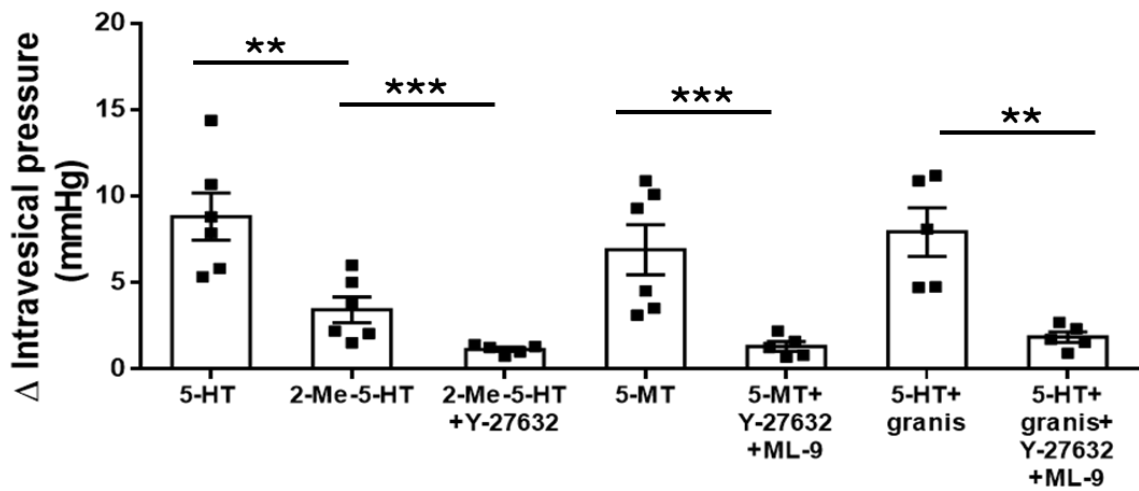
**A**



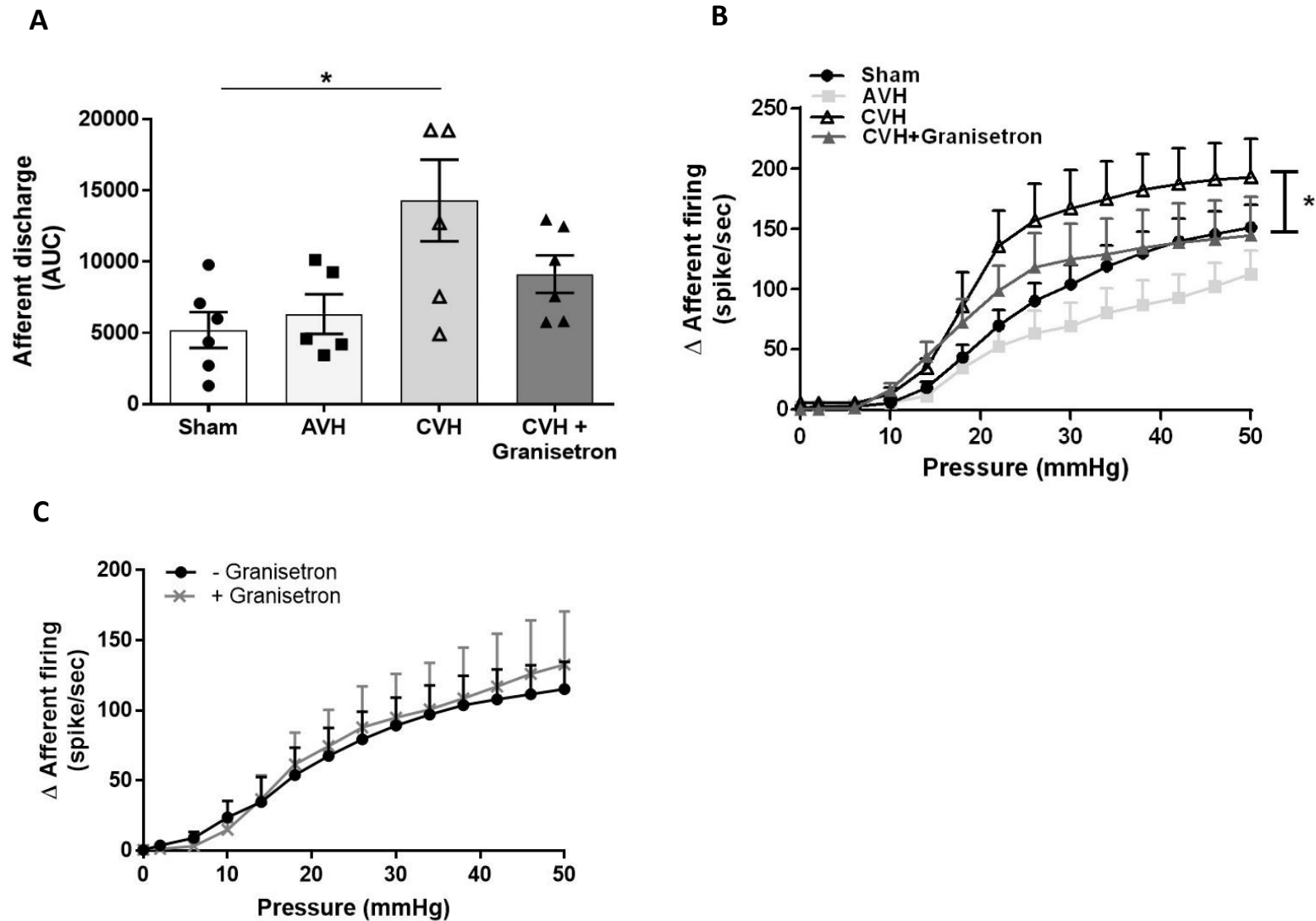
**B**



**C**

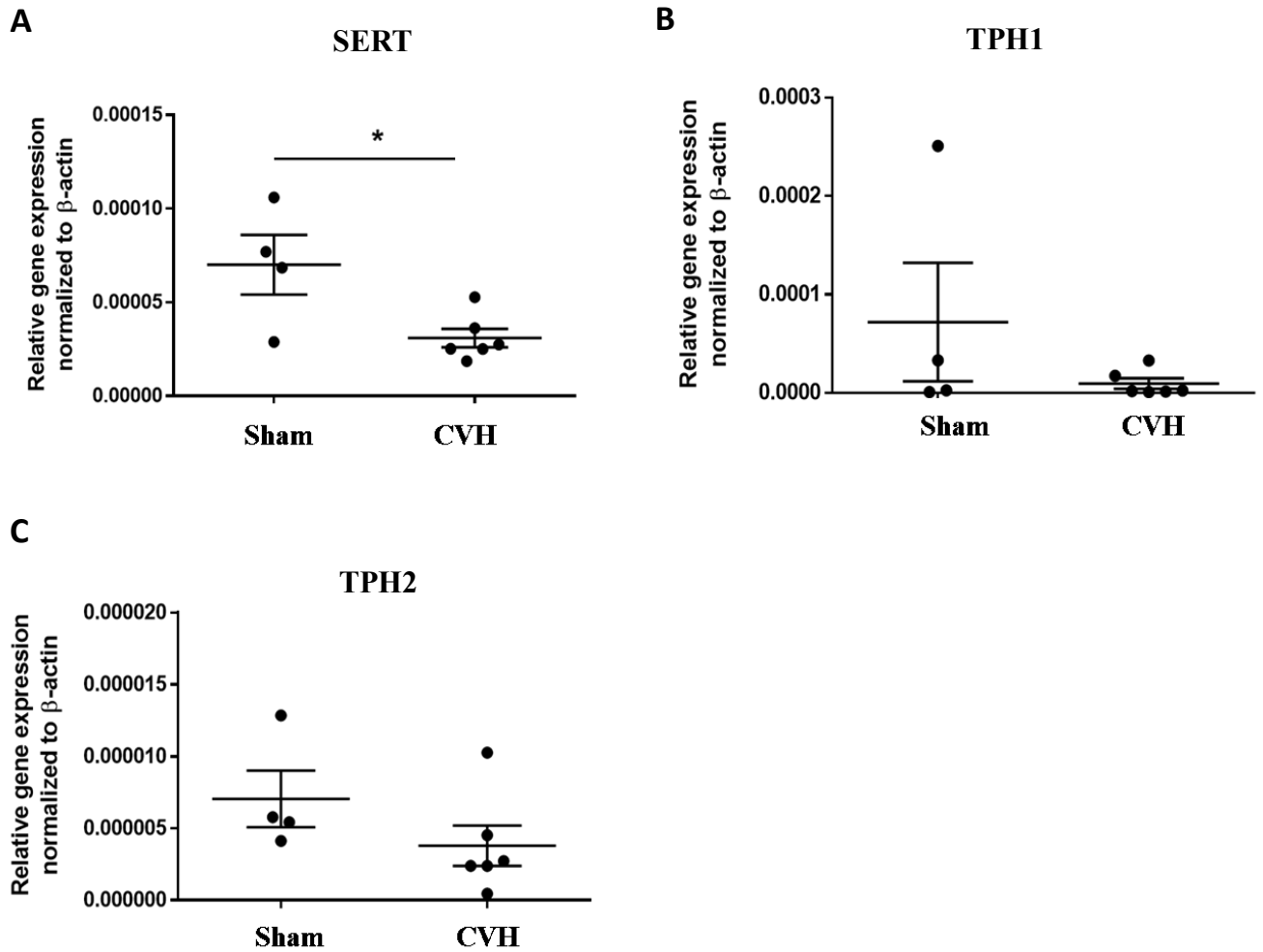


**Figure 4. 5-HT<sub>3</sub> receptors play a role in mediating the action of 5-HT on bladder afferent firing and this effect is independent of detrusor contraction.** (A) A representative trace showing the effect of bath applied 5-HT on afferent nerve firing and detrusor tone. 5HT, 2-Me-5-HT and 5-MT 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-Me-5-HT, 5-MT with and without pre-incubation of the 5-HT<sub>3</sub> 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  $\pm$  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).



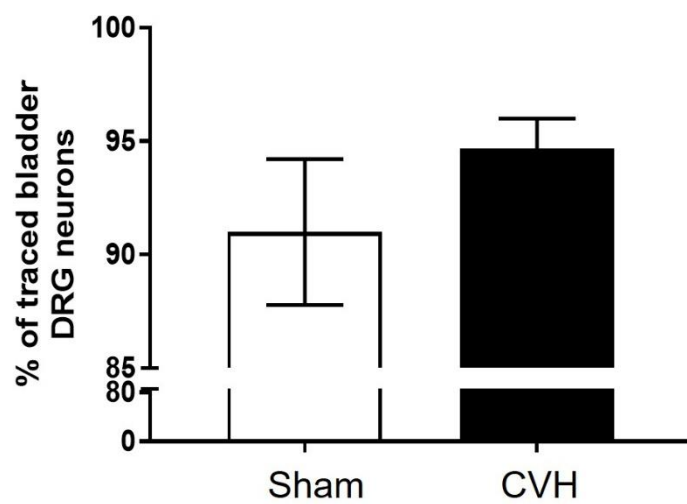
**Figure 5. Mice with chronic visceral hypersensitivity (CVH) evoked by TNBS had increased spontaneous afferent firing and hypersensitivity in response to bladder distension. This effect was partially reversed by application of granisetron.** (A). Afferent firing from bladders taken from sham mice, mice 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 5HT<sub>3</sub> 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)

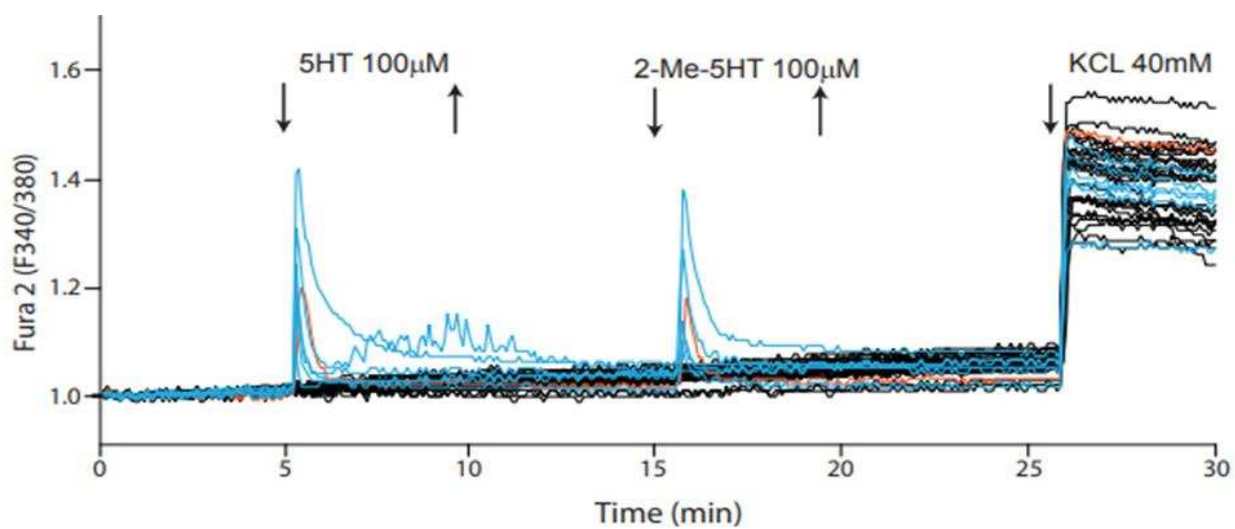


**Figure 6 SERT mRNA expression in the urothelium was significantly attenuated in mice with CVH.** (A) SERT mRNA expression was significantly reduced in urothelial cell cultures from CVH animals compared to control animals (\* $P < 0.05$ , unpaired Student's t-test healthy control;  $N = 4$ , CVH;  $N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



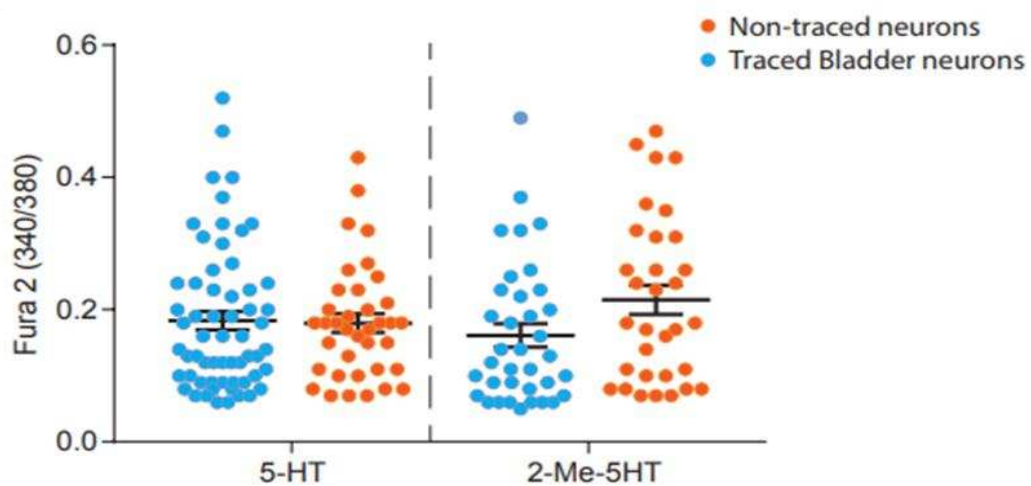
B



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-HT<sub>3</sub> 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 non-traced (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

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### **Competing interests**

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