The University Of Sheffield.

Role of 5-Hydroxytryptamine (5-HT) in Urinary Bladder Signalling and Colon-Bladder Cross-Organ Sensitization

By

Nipaporn Konthapakdee

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TABLE OF CONTENTS

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SUMMARY OF THESIS

This thesis investigates the role of 5-HT in bladder sensory (afferent) signalling in healthy animals and in a mouse model of colon-bladder cross-sensitization. We primarily address the effect of 5-HT and involvement of different 5-HT receptor subtypes on afferent nerve activity using *in vitro* extracellular nerve recordings. 5-HT is known to be a key neurotransmitter that regulates many essential roles in the body including the bowel but less is known about 5-HT's role in the urinary bladder. Moreover, research has focused on the role of 5-HT on bladder efferent nerves and muscle contraction, while the role of 5-HT on bladder afferent nerves is still an enigma.

We have investigated 5-HT receptor expression in the urothelium and determined the effect of 5-HT on urothelial signaling and its contribution to bladder afferent activity, examining both mechanosensitive and spontaneous nerve firing. In addition, the role of 5-HT on bladder afferent activity was investigated in a TNBS-induced colonic inflammation model of colon-bladder cross-sensitization. Finally we examined whether the urinary bladder has an endogenous source of 5-HT.

We have made a number of novel findings: (i) various 5-HT receptors transcripts were expressed in mouse urothelium with a notable exception of 5-HT3 receptors. Cultured urothelial cells examined using calcium imaging responsed directly to 5-HT demonstrating that these 5-HT receptors are functional; (ii) 5-HT exerted excitatory effect on spontaneous afferent firing but attenuated mechanosensitive responses to distension, these actions were mainly mediated through 5-HT3 receptors, and were independent from muscle contraction; (iii) the effects of 5-HT on spontaneous and mechanosensitive firing were attenuated in the post-inflammatory state of colonic TNBS-treated mice. There was an accompanying downregulation in SERT mRNA expression in the urothelium; (iv) citalopram, a selective 5-HT reuptake inhibitor, attenuated mechanosensitive afferent discharge which was reversed by the 5-HT3 antagonist, granisetron. mRNA expression of 5-HT producing enzymes, TPH1 and TPH2, and SERT were detected in the urothelium. 5-HT positive cells were expressed in mouse urethra but not in the bladder dome.

We conclude that 5-HT has the potential to modulate bladder afferent signaling by direct actions on the afferent nerves and indirect effects via the urothelium with nitric oxide playing a modulatory influence. The urothelium contains the necessary molecular machinery for endogenous 5-HT production but the extent to which this 5-HT contributes to bladder signaling in normal and diseased states requires further investigation and may represent a novel therapeutic target to treat bladder symptoms.

ABBREVIATIONS

2-Me-5-HT	2-methyl-5-hydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxy-L-tryptophan
5-MT	5-methoxytryptamine
ACh	acetylcholine
CGRP	calcitonin gene related peptide
CNS	central nervous system
DRG	dorsal root ganglia
EC	enterochromaffin cell
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCRs	G protein-coupled receptors
HT	high threshold
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IC	interstitial cystitis
ICC	interstitial cells of Cajal
IL	interleukin
IP_3	inositol triphosphate
L-NAME	L-NG-Nitroarginine methyl ester
LT	low threshold
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
NK	neurokinin
NMDA	N-methyl-D-aspartate
NO	nitric oxide
OAB	overactive bladder syndrome
NPY	neuropeptide Y
OCT	optimal cutting temperature
P2X	purinergic receptor 2X
P2Y	purinergic receptor 2Y
PAG	periaqueductal gray
PCA	principal component analysis
PKA	protein kinase A
PLC	phospholipase C
PYY	peptide Y
ROS	reactive oxygen species
ROCK	Rho kinase
RTX	resiniferatoxin
SERT	serotonin reuptake transporter
SP	substance P
TNBS	2,4,6-trinitrobenzene sulphonic acid
TNF	tumor necrosis factor
TPH	tryptophan hydroxylase
TRP	transient receptor potential
VIP	vasoactive intestinal polypeptide

CHAPTER 1 GENERAL INTRODUCTION

1.1 ANATOMY OF THE URINARY BLADDER AND CELLULAR COMPOSITION OF THE BLADDER WALL

The lower urinary tract is composed of the urinary bladder, the ureters, and the urethra. The urinary bladder is a muscular hollow organ which has a major role to periodically store and empty the urine. The human bladder has maximum capacity of 1000 mL but an average capacity is 300-500 mL.

The bladder morphology consists of three main regions as follows;

- *The dome* primarily consists of the smooth muscle which is unique in both structural and function from the smooth muscle of the trigone and the urethra.
- *The trigone* is a triangular shape tissue that is from the entry points of the ureters and the base, connecting urethra. The trigone has the highest density of afferent nerve innervation.
- *The bladder neck* is the lower part of the bladder that connects the bladder to the urethra.

A schematic diagram of bladder structure is shown in figure 1.1.

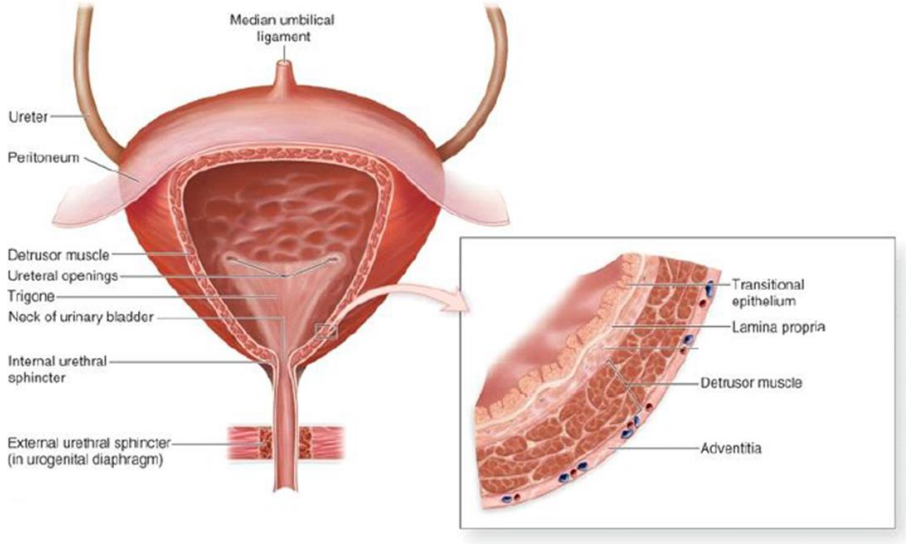


Figure 1.1 Schematic diagram representing the structure of the urinary bladder (Mckinley, 2009).

The bladder wall comprises various kinds of cells organized into complex structures related to their cellular function. The structure of the bladder wall is divided into;

- *Urothelial layer* is the most inner layer consisting of transitional epithelial cells called 'urothelial cells' are the first line barrier of the urinary bladder. In addition, glycosaminoglycan (GAG) covers the apical site of the urothelium, which is beneficial for barrier function of the urothelium (see review by Birder and Andersson 2013).

- *Suburothelial layer* is beneath the urothelial layer and is separated from the urothelium by the basement membrane. The lamina propria has a number of cell types including interstitial cells of Cajal (ICC), blood vessels, fibroblasts, muscularis mucosae and nerve terminals. These play a major role in signal integration from the urothelium and the muscle layer (see review by Andersson and McCloskey 2013).

- *Muscularis layer* consists of three layers of smooth muscle called 'detrusor muscle'. The detrusor smooth muscle contains inner and outer longitudinal muscles with the circular muscles in the middle surrounding by connective tissue i.e., collagen (see review by Andersson and Arner 2004).

- *Adventitia and serosa* layers cover the outer layer of the bladder. This layer mainly contains loose connective tissue (McKinley, 2009).

A schematic diagram of the cellular components of the bladder wall is depicted in figure 1.2

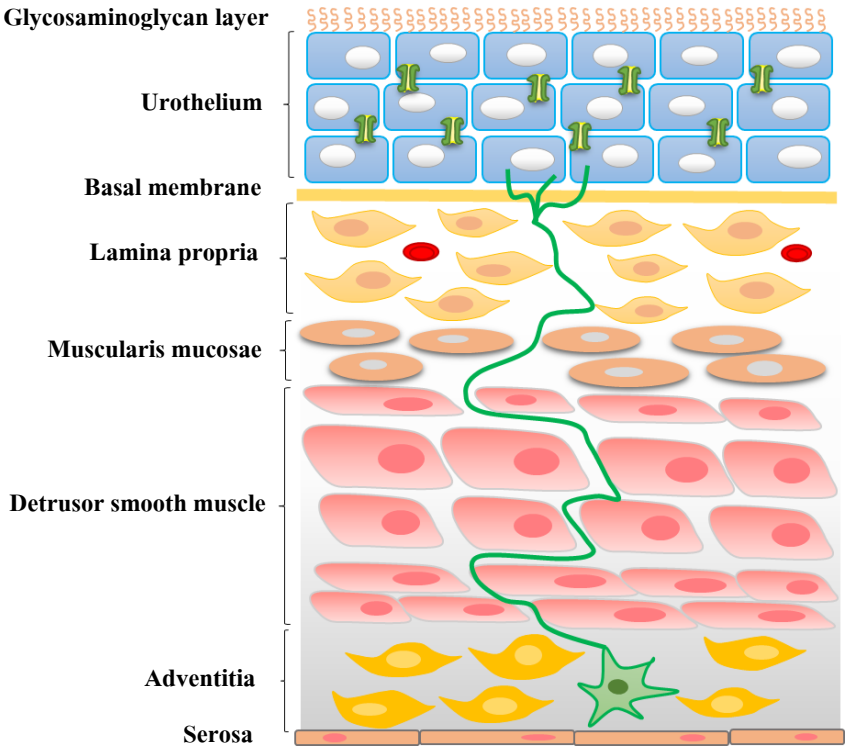


Figure 1.2 Structure of the bladder wall (modified from Merrill *et al.* 2016).

1.2 STRUCTURE AND FUNCTION OF THE BLADDER WALL

1.2.1) UROTHELIUM

Urothelial cells are transitional epithelial cells located between the bladder lumen and the basal membrane, which are found in the renal pelvis, ureters, bladder body, upper part of urethra, and glandular structure of prostate glands (Khanderwal *et al.* 2009). Urothelial cells consist of at least 3 types; (i) umbrella cells (ii) intermediate cells and (iii) basal cells. Umbrella cells are the most superficial layer of cells; they have a hexagonal shape with an average cell diameter between 25-250 µm (Apocada, 2004; Khanderwal *et al.* 2009). They are connected via

superficial proteins; uroplakins, assembled together with tight junction proteins; claudins and occludins. Intermediate cells are found between the umbrella cell and the basal cell layers (Hicks *et al.* 1974; Liang *et al*. 2001). The thickness of the intermediate layers varies between species. In humans, it has been reported to range from 15-50 μm (Jost *et al.* 1989). Some of the intermediate cells and umbrella cells project cytoplasmic processes to the basal cells layer that attach to the basal membrane, which help to maintain flexibility and bladder structure when the bladder is dilated (Battifora, 1964; Jost *et al.* 1989; Apodaca, 2004) (Figure 1.3).

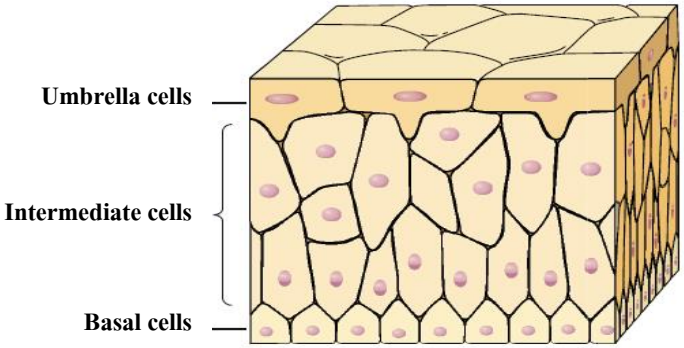


Figure 1.3 Schematic diagram depicting umbrella cells, intermediate cells and basal cells layers in the urothelium (Birder and Anderson 2013).

Barrier function of the urothelium

Multiple layers of urothelial cells strengthen the barrier function to protect the rest of the bladder from urine and luminal pathogens. In addition, there is a glycosaminoglycan (GAG) layer that covers the surface of the urothelium, this is not only beneficial as a physical layer, but also acts as a defense mechanism against bacteria or toxic substances in the urine (Grist and Chakraborty 1994). The GAG layer is composed of several classes of mucopolysaccharides (i.e., heparin sulfate, hyaluronic acid, and chondroitin sulfate) which have hydrorepellent properties. Alterations in the GAG layer exposes the urothelium to toxic agents in the urine and may contribute to bladder inflammation (Bassi *et al.* 2011; Cervigni 2015). In addition, uroplakin, superficial proteins with four transmembrane domains on the urothelial surface, plays an essential role in permeability of the urothelium to control access of proteins, ionic, and non-ionic substances (Acharya *et al*. 2004; Apocada, 2004).

Sensory function of the urothelium

Many reports have purported a sensory role of urothelial cells to detect various stimuli i.e., mechanical, chemical, and noxious stimuli via an array of "sensor molecules" (i.e., channels and receptors) expressed on the cells including, purinergic receptors (P2X and P2Y) (Birder *et al*. 2004; Burnstock 2001), muscarinic and nicotinic receptors (Beckel and Birder 2012; Beckel *et al.* 2006), adrenergic receptors (Birder *et al.* 2003; Kullmann *et al.* 2011), TRP channels (Birder *et al.* 2007; Birder *et al.* 2002; Birder *et al.* 2001), TrkA (Murray *et al.* 2004), and prostaglandin receptors (Wang *et al.* 2008) as summarised in table 1.1.

In response to mechanical and chemical stimulation, urothelial cells also have ability to release mediators i.e., ATP, ACh, substance P, prostaglandin, and NO (Birder *et al.* 1998; Birder and Andersson 2013; Birder *et al.* 2003; Ferguson *et al.* 1997). This chemical message is possibly sent via an autocrine and/or paracrine manners to the nearby cells (e.g. nerve terminals, blood vessel, and ICC) in order to modulate bladder functions (Apodaca 2004; de Groat 2004; Birder and Andersson 2013).

Birder and co-workers have shown that activation of ß-adrenergic receptors mediated NO release from urothelial cells via an increase of cAMP and intracellular Ca^{2+} (Birder *et al.* 2002). In addition, activation of urothelial cells with capsaicin or resiniferatoxin induced an increase in intracellular Ca^{2+} levels and also enhanced release of ATP and NO (Birder *et al*. 2002; Vyklický *et al.* 2008; Yamada *et al.* 2009; deVries and Blumberg 1989), which may further act on nearby cells to modulate bladder function.

Table 1.1. Properties of ionic channels/receptors expressed within the urothelium (Birder and Andersson 2013).

Channel/Receptor	Activator(s)
TRPV1	Heat (>43°C), low pH, anandamide, vanilloids
TRPV2	Noxious heat (>53°C), mechanical
TRPV4	Moderate heat (>24°C), cell swelling, 4α-PDD, 5'6'-EET
TRPM8	Cold (8–28°C), menthol, icilin
TRPA1	Mechanical, cinnamaldehyde, isothiocyanate
P2X (1–7)	ATP
P2Y (P2Y2/P2Y4)	Nucleotides (ATP; UTP; ADP)
P1	Adenosine
Adrenergic alpha (–1, –2)	Endogenous catecholamines (norepinephrine; epinephrine)
Adrenergic beta (–1, –2, –3)	Endogenous catecholamines (noreadrenaline; adrenaline), isoproterenol
Cholinergic muscarinic (M1–M5)	acetylcholine
Cholinergic nicotinic (α3, α5, α7, α3, ß4)	acetylcholine, nicotine, choline, cystisine
Estrogen receptor (alpha; beta)	Estradiol, estrogen derivatives, selective estrogen-receptor modulators (SERM)
Degenerin/ENaC (DEG/ENaC) family (ENaC; ASIC)	Low pH, serum proteases, amiloride, mechanical?
Neurotrophins (trkA; p75; trkB)	NGF, BDNF
Bradykinin (B1; B2)	Bradykinin
Tachykinin (NK1; NK2)	Substance P, neurokinin A
Piezo1[a]	Mechanical

[a] (Coste *et al.* 2011)

1.2.2) INTERSTITIAL CELLS OF CAJAL IN THE URINARY BLADDER

Interstitial cells of Cajal (ICC) in the bladder have been reported in the lower urinary tract in various species i.e., guinea pigs, mouse, rabbits, and human (Davidson and McCloskey 2005; Pezzone *et al*. 2003; Lyons *et al.* 2007; Sergeant *et al.* 2000; Hashitani and Lang 2010; Rasmussen *et al.* 2009). Currently, ICC in the bladder have been found to form a network in 2 main areas; in the lamina propria and in the detrusor muscle layers. ICC in the lamina propria (ICC-LP) and inter-muscle bundle ICC (ICC-IB) are stellate shaped, while intramuscular ICC (ICC-IM) are branched and have an elongated morphology.

ICC are also located in the gastrointestinal tract and have been widely studied. They exert a key role as a pacemaker to generate spontaneous electrical slow wave to the nearby smooth muscle cells and initiate spontaneous muscle contraction. ICC could mediate both excitatory (via cholinergic) and inhibitory (motor) motor input to the smooth muscle (Beckett *et al.* 2002; Burns *et al*. 1996; Ward *et al.* 2000). In addition, ICC act as a mechanosensor and transducer to detect mechanical and chemical stimuli and further modulate smooth muscle contraction (Won *et al.* 2005; Ward *et al.* 2000). Moreover, ICC have been suggested to play a role in vagal afferent signalling (Fox *et al.* 2001).

Unlike the gastrointestinal tract, the physiological roles of bladder ICC are still unclear. However, previous studies using immunohistology clearly demonstrated that bladder ICC expressed marker proteins vimentin, c-Kit (or CD117) similar to the gut ICC (Davidson and McCloskey 2005). Previous investigations by immunofluorescence and electron microscopy showed that ICC-LP and ICC-IM are in proximity to the nerve terminals (Johnston *et al*. 2010; Davidson and McCloskey 2005; Johnston *et al*. 2010). The distance between ICC and nerves in the bladder wall is within 20 nm (Johnston *et al.* 2012), close enough to exert neurotransmitter interaction (Burnstock, 1977; Burnstock, 1986). Moreover, previous studies have reported bladder ICC also respond to ATP and carbachol by forming Ca^{2+} transients, indicating that receptors expressed on the cells mediate functional responses (McCloskey and Gurney 2002; Wu *et al*. 2004). Interestingly, Gray and co-workers (2013) studied bladder mucosal strip stimulated with electrical field stimulation (EFS) and monitored intracellular Ca^{2+} change using calcium imaging in guinea pigs. They found that ICC-LP, ICC-IM and perivascular ICC

responded to EFS by forming Ca^{2+} transients, indicating that bladder ICC are functionally innervated by nerves (Gray *et al.* 2013).

ICC connect to other nearby cells e.g. nerve terminals and smooth muscle cells via gap junctions. These gap junctions provide a route for intercellular communication allowing low molecular weight molecules to diffuse to neighbouring cells. Nemeth *et al*. (2000) demonstrated gap junction proteins Connexin 43 in ICC colocallized with c-Kit in colon (Nemeth *et al.* 2000). In addition, low-resistance gap junction proteins provide electrical connection via changing the intracellular Ca^{2+} status in order to modulate the nearby smooth cell excitability (Drumm, 2014).

Recently, a new type of interstitial cells has been reported in bladder and gut. This group of ICC expressed platelets derived growth receptor alpha (PDGFRα) but not c-Kit (Monaghan *et al.* 2012; Koh *et al.* 2012). PDGFRα+ cells are located in close proximity to motor neurons and express neuronal receptors, suggesting that they may exert neuromodulatory function similar to ICC (Monaghan *et al.* 2012; Koh *et al.* 2012; Lee *et al.* 2014). A schematic diagram depicted ICC in the detrusor muscle is shown in figure 1.4. However, the exact physiological role of these cells is still unclear.

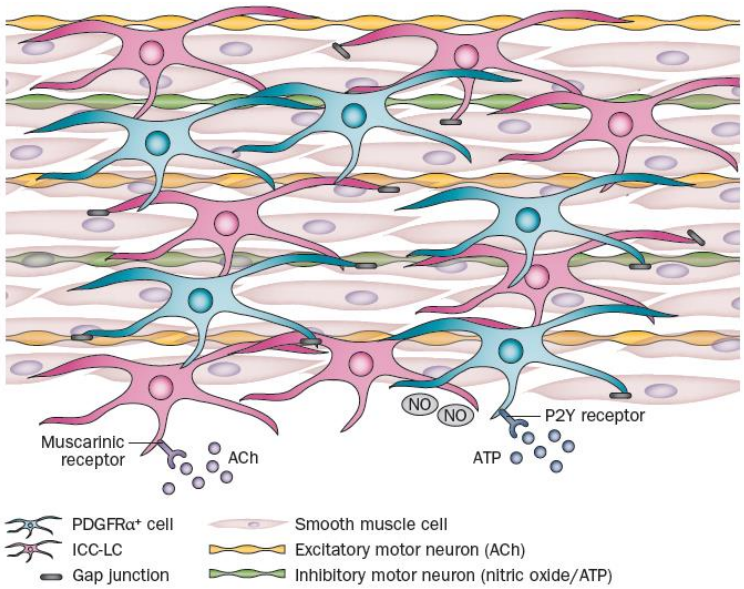


Figure 1.4 A schematic diagram demonstrated interaction of ICC, detrusor smooth muscle cells, and motor neurons in the detrusor smooth muscle layer (Drumm *et al.* 2014).

1.2.3) DETRUSOR SMOOTH MUSCLES

Detrusor smooth muscles of the bladder have typical characteristics with long spindle shaped cells and central nuclei. The muscle cells contain an intermediate filament protein vimentin that is expressed in mesenchymal cells and surrounded by collagens (Drake *et al.* 2006; Smet *et al.* 1997). Detrusor smooth muscle cells express specific receptors in order to respond to particular transmitters. The detrusor muscles are mainly controlled by neurotransmitters /mediators from innervated autonomic nerves and local non-neuronal sources i.e., urothelial cells following mechanical or chemical stimulation. For instance, a previous study using two bladder strips with or without urothelium (denuded) indicated that urothelium produces inhibitory factor(s) (unidentified) to inhibit detrusor contraction-induced by carbachol in pig bladder (Hawthorn *et al.* 2000).

ACh via stimulation of muscarinic receptors (M2 and M3) plays a critical role to generate detrusor contraction. In contrast, detrusor relaxation is mediated through noradrenalin activated ß-adrenergic receptors. A schematic diagram depicting an underlying mechanism of muscarinic and ß-adrenergic receptors induced detrusor muscle contraction and relaxation is shown in figure 1.6.

The mechanism that underlies muscarinic receptor stimulation involves an elevation of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ from the sarcoplasmic reticulum (SR) and Ca^{2+} influx from L-type Ca^{2+} channels. Intracellular $[Ca^{2+}]_i$ is enhanced via Ca^{2+}-induced Ca^{2+} release from the SR. However, detrusor contraction and relaxation is mainly dependent on the phosphorylation state of myosin light chain (MLC), which is potentiated by myosin light chain kinase (MLCK) but reversed by myosin light chain phosphatase (MLCP). Binding of intracellular Ca^{2+} to calmodulin also increases MLCK activity and further enhances phosphorylation of MLC to generate muscle contraction. Another mechanism to enhance muscle contraction is mediated via an inhibition of MLCP activity by the protein kinase; Rho kinase (ROCK) and protein kinase C (PKC) (Figure 1.5).

ß-adrenergic receptor activation promotes detrusor relaxation via two main mechanisms; (i) inactivation of voltage Ca^{2+} channels mediated by hyperpolarization through an activation of K^+

channels (Robertson *et al.* 1993; Xin *et al.* 2014) and (ii) reduction of contractile protein sensitivity or inhibition of Ca^{2+} sensitization that further modulates activity of MLCK and MLCP (Conti and Adelstein 1981; Rembold 1992). Hayashi *et al.* (2016) investigated the mechanism of cAMP-induced muscle relaxation using specific agonists and antagonists of ROCK and PKC. They showed that ROCK but not PKC was inhibited by Ca^{2+} sensitization following carbachol application (Hayashi *et al.* 2016).

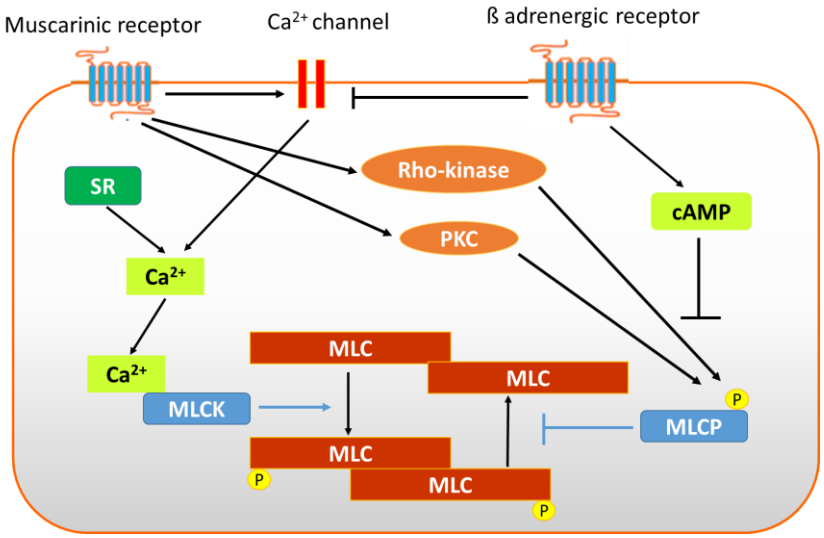


Figure 1.5 A schematic diagram showing underlying cellular mechanisms of muscarinic and ß-adrenergic activation in detrusor smooth muscle (modified from Michel 2015).

1.3 NEURAL INNERVATION OF THE URINARY BLADDER

The micturition reflex of the bladder requires coordinated neuronal control of 2 functional units; a reservoir (the bladder) and an outlet (bladder neck, smooth and striated sphincter muscle of the urethra) (Fowler *et al.* 2008; Morrisson *et al.* 2005). During urine storage, the outlet is tightly closed and the bladder smooth muscle is relaxed to maintain low intravesical pressure. During bladder emptying, the pelvic floor and the striated sphincter muscles are relaxed, the detrusor muscle is contracted and the bladder neck is opened to allow the urine outflow. Co-ordination of these functions is primarily regulated via efferent and afferent pathways (de Groat 1986).

1.3.1) BLADDER EFFERENT PATHWAYS

Parasympathetic efferent signals trigger detrusor contraction. Preganglionic parasympathetic neurons originate from the sacral spinal cord (S2-S4 laminae v-vii) and project axons in the pelvic nerves (Yoshimura and de Groat 1997; de Groat and Yoshimura 2006). Postganglionic parasympathetic nerves contain various neurotransmitters that regulate bladder contraction (Sullivan and Yalla 2002). The major excitatory neurotransmitter to mediate detrusor contraction is acetylcholine (ACh) (Andersson and Arner 2004). ACh regulates detrusor contraction via binding to postganglionic muscarinic receptors. M2 and M3 receptors are expressed in the bladder, however, a study using specific antagonists and knockout animals showed that M3 receptors play a key role to mediate excitatory transmission via increased intracellular Ca^{2+} $[Ca^{2+}]_i$ (Matsui *et al.* 2000, 2002). M2 receptor activation is considered to inhibit adenylate cyclase and may potentiate detrusor contraction via ß-adrenergic inhibition (Andersson and Arner 2004). However, cholinergic activation could be mediated by other signalling pathways (de Groat and Booth 1993), e.g., 5-HT (via 5-HT4 receptors) has been reported to potentiate electrical field stimulation (EFS) induced detrusor contraction in pig bladder (Sellers *et al.* 2000).

In contrast to parasympathetic efferents, sympathetic activation mediates bladder relaxation. Sympathetic efferent neurons originate from thoracolumbar spinal cord (T11-L2), and are carried through hypogastric nerves (de Groat, 1997; de Groat and Yoshimura 2006). The sympathetic post-ganglionic nerves release noradrenaline which activates both α- and β-adrenergic receptors in the bladder body and the urethra. Noradrenaline binds to α-adrenergic receptors in the smooth muscle of the urethra to trigger muscle contraction of the urethral sphincter (Anderson and Arner 2004).

The somatic efferents are conveyed through pudendal nerves which arise from Onuf's nucleus in the ventral horn (T11-L2) of the spinal cord. Pudendal nerves innervate the external urethral sphincter muscle to regulate muscle contraction via binding of ACh to nicotinic receptors (de Groat, 1997; de Groat and Yoshimura 2006). A schematic diagram of bladder efferent innervation is shown in figure 1.6.

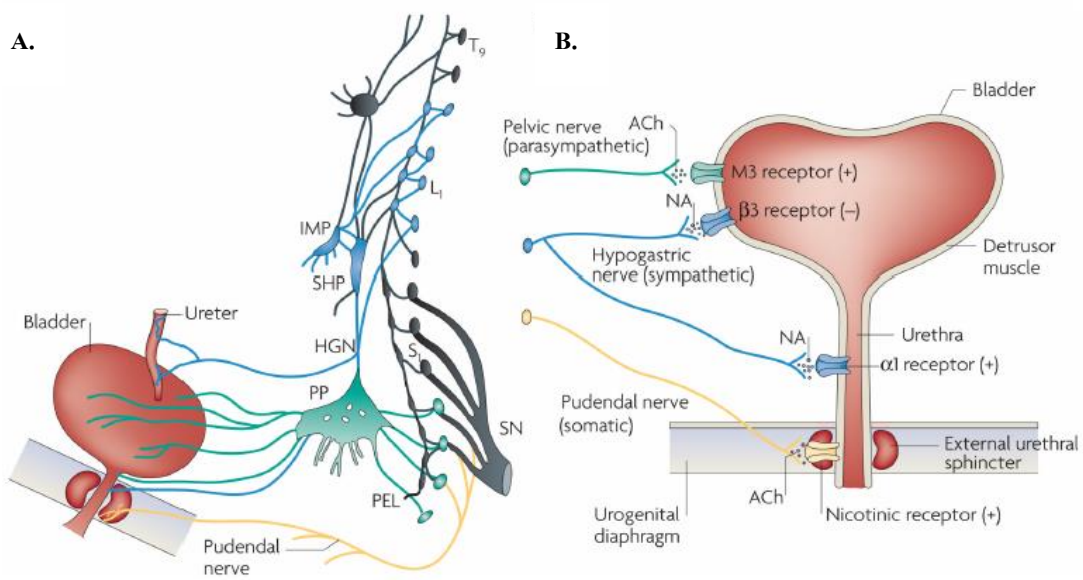


Figure 1.6 Schematic diagram showing efferent nerve innervation of the urinary bladder (Fowler *et al*. 2008). (A) Nerve innervation of parasympathetic (green), sympathetic (blue), and somatic (yellow). **(B)** Neural innervation and neurotransmitter release mechanisms that regulate bladder function. ACh released from parasympathetic nerves binds to muscarinic receptors (M3 receptors) to mediate muscle contraction (green). Sympathetic innervation releases noradrenaline (NA) which binds to ß3 adrenergic receptors in the detrusor smooth muscle to mediate muscle relaxation, while NA activates α1-adrenergic receptor in the urethra to potentiate muscle contraction (blue). Somatic nerves control external urethral sphincter via binding of ACh to nicotinic receptors (yellow). L1=first lumbar root; S1=first sacral root; SHP=superior hypogastric plexus; SN=sciatic nerve; T9=ninth thoracic root; IMP=inferior mesenteric plexus; HGN=hypogastric nerve; PEL=pelvic nerve; PP=pelvic plexus.

1.3.2) BLADDER AFFERENT PATHWAYS

The afferent nerves play an essential role to transmit sensory (mechanical, chemical, and noxious) information from the bladder to the CNS, with the afferent information being carried through pelvic, hypogastric, and pudendal nerves to second-order neurons in the spinal cord. The cell bodies of these fibres are located in the dorsal root ganglia (DRG) and enter the spinal cord at L1, L2, L6 and S1 lumbosacral levels (de Groat 1986; Jänig and Morrison 1986; Yoshimura and de Groat 1997). Pelvic nerves convey both non-painful (bladder fullness) and pain sensation from the detrusor muscle whereas hypogastric nerves provide only minor

innervation to the detrusor muscle. Hypogastric nerves primarily innervate longitudinal and circular smooth muscle layers in the bladder neck and proximal urethra. The sensory input from afferent fibres is projected via the lateral funiculus or the dorsal funiculus to periaqueductal grey matter (de Groat 1997) and then relayed the sensory input to the pontine micturition centre to initiate micturition (Blok *et al.* 1997).

In order to detect various stimuli and responses to chemical messengers, bladder afferent nerves express a wide range of ion channels and receptors i.e., TRPA1, TRPV1, TRPM8, TrkA, TrkB, purinergic, muscarinic, 5-HT, and oestrogen receptors (Bennett *et al.* 2003; Everaerts *et al.* 2008; Vizzard and Boyle 1999; Zhong *et al.* 2003). Moreover, histological studies have shown that afferent nerves contain various kinds of neurotransmitters and mediators including CGRP, substance P, neurofilament protein, neuronal nitric oxide synthase (Gabella and Davis 1998; Smet *et al.* 1997; Uemura *et al.* 1973). Experiments using retrograde transporters have shown that 90% of dorsal root ganglion axons to the bladder at T10-L3 and 60% in L6-S1 spinal level are CGRP positive (Gebella and Davis 1998). Rahnama'i and co-workers (2017) recently reported the differential distribution of bladder afferent nerve characteristic in the suburothelium in mouse bladder using immunohistochemistry. They found that suburothelium afferents in the bladder base had higher CGRP and vesicular ACh transporter (VAChT) than those in the lateral wall, suggesting functional heterogeneity of the bladder afferents (Rahnama'i *et al.* 2017).

These axon containing mediators are distributed in and penetrate the bladder wall into urothelial cells, suburothelial cells, muscularis, and serosal layers. The suburothelial layer has been reported to have the highest density of afferent nerves compared to other layers. The suburotheial plexus is most dense at the bladder neck and trigone regions with the innervation being more diffused in the bladder dome and the lateral wall (Andersson, 2002). Xue and Gebhart identified mechanosensitive afferent fibres according to their receptive fields and their response to mechanical stimuli (probing, stretching and stroking of the urothelium) in mice. They classified pelvic mechanosensitive afferents into 4 classes with different proportions; muscle fibres (63%), muscle/urothelial fibres (14%), serosal fibres (14%), and urothelial fibres (9%) (Xu and Gebhart 2008).

Two major types of bladder afferent fibres have been classified; myelinated Aδ-fibres and unmyelinated C-fibres. In rats, small myelinated Aδ-fibres (2-5 μm diameter) have a conduction velocity greater than 1.3 ms^{-1} and unmyelinated C-fibres (<2 μm diameter) have a conduction velocity less than 1.3 ms^{-1} (Waddell *et al.* 1989). A histological study revealed that the major type of pelvic afferent nerves in rat bladder is unmyelinated C-fibres (~70-80%) (Vera and Madelhaft 1992). Aδ-fibres primarily signal bladder stretching (from tension receptor) during ballder filling, whereas the C-fibres sense noxious stimuli i.e., high pressure bladder distension (Gabella and Davis 1998; de Groat and Yoshimura 2009). This information is related to the anatomical innervation, which Aδ-fibres mainly innervate the muscle layer, while C-fibres are more wide spread in the detrusor and suburothelial layers and come into close proximity to urothelial cells (Wakabayashi *et al.* 1993). Interestingly, a study has shown that some of the C-fibres are volume receptors which respond to slow distension of physiological volumes regardless of intravesical pressure (Morrison, 1999).

The mechanosensitive afferent fibres have also been classified according to the response to mechanical stimulation into low threshold and high threshold fibres, defined from their initial threshold during mechanical stimulation. The low threshold fibres are sensitive to normal bladder filling, while the high threshold fibres are suggested to response only to high pressure stimuli which represent noxious stimuli (Daly *et al.* 2007; Sengupta and Gebhart 1994; Shea *et al*. 2000). In rats, low threshold fibres have been reported to be a major population (~80%). Another class of afferent nerves is silent fibres that are normally insensitive to bladder distension; however, silent fibres can be sensitized by inflammatory mediators and become active in response to mechanical stimulation (Rong *et al*. 2002).

1.3.3) SENSORY NERVES OF THE URINARY BLADDER

Afferent nerves are crucial for bladder sensation in order to control and regulate bladder function. Bladder afferents require different types of receptors to convey various kinds of sensation to the CNS. Zagorodnyuk *et al.* (2009) conducted in *vitro* afferent nerve recordings from guinea pig bladder. The strip tissues were challenged with different types of mechanical stimuli (stretch, von Frey hair stroking and compression) and chemical stimuli (capsaicin, α,β-methylene ATP and hypertonic solution) and the bladder sensory neurons were distinguished into 2 major groups; stretch sensitive and stretch insensitive fibres. Stretch sensitive fibres were subdivided into muscle mechanoreceptors and muscle-mucosal mechanoreceptors. Stretch-insensitive fibres were classified into mucosal mechanoreceptors and chemoreceptors (Zagorodnyuk *et al.* 2009).

1.) Muscle mechanoreceptors

Muscle mechanoreceptors generate afferent firing that is proportional to the intravesical pressure. The stretch sensitive afferents that respond to bladder distension have been suggested to be tension receptors that align 'in-series' with smooth muscle bundles (Iggo 1954; Shea *et al*. 2000). Zagorodnyuk *et al.* (2007) conducted in *vitro* experiment to remove the bladder mucosa and record afferent firings in response to stretching. They reported that mucosal removal did not change afferent firing in response to bladder distension, suggesting that these mechanoreceptors are located in the muscle layer (Zagorodnyuk *et al.* 2007). In addition, some populations of muscle mechanoreceptors are independent of intravesical pressure, exhibiting a plateau in firing or even a decrease at higher intravesical pressures. This group of the receptors has been suggested to be 'volume receptors' which are arranged 'in parallel' to muscle fibres in order to sense volume inside the bladder (Morrison, 1997; Shea *et al.* 2000).

2.) Tension-mucosal receptors

Tension-mucosal receptors are sensitive to stretch, mild stroking with von Frey hair and hypertonic solution (Zagorodnyuk *et al.* 2007), suggesting that the receptive fields may be interspersed between the mucosa and the muscle layers. This type of receptor corresponds to muscle-mucosal receptors in the pelvic afferents supplying the colon, which are unique to the pelvic afferents (Brierley *et al.* 2005).

3.) Mucosal mechanoreceptors

This mechanoreceptor group responds to mucosal stroking with von Frey hair but is insensitive to stretch. The mucosal mechanoreceptors have been identified in splanchnic and pelvic afferents in mouse colon (Brierley *et al.* 2005) and pelvic nerves supplying the mouse bladder (Zagorodnyuk *et al.* 2007). This class of mechanoreceptors shares similar responses characteristic with silent receptors that do not show resting activity but activate responses to chemical stimulation and could generate spontaneous firing following irritation.

4.) Chemoreceptors

This group of the receptors responses to exogenous chemicals and has sensitivity to a variety of autocrine and paracrine mediators. Moss *et al*. (1997) reported a small population of receptors that responded to potassium chloride application in the bladder (Moss *et al.* 1997). Moreover, the chemoreceptor characteristic was further demonstrated in a study by Zagorodnyuk *et al.* 2007 which showed that some population of stretch-insensitive pelvic afferents responded to acid and potassium application to the mucosa (Zagorodnyuk *et al.* 2007).

5.) Silent afferents

These afferent nerves are normally insensitive to high pressure mechanical stimulation or noxious stimuli but become active following irritation or inflammatory response and are therefore termed 'nociceptors'. A study by Habler and colleagues (1990) showed that ~10% of bladder distension unresponsive afferents became activated following bladder irritation with mustard oil (Habler *et al.* 1990). Janig and Koltzenburg (1990) reported that there are ~20-30% of silent afferent nerves in pelvic afferents (Janig and Koltzenburg 1990). However, the mechanism of activation of silent receptors is still unclear.

1.3.4) MECHANISMS OF MECHANOSENSITIVITY

Mechanotransduction is a process of converting mechanical force into electrical signals, which is critical to the function of various tissues and organs including touch, hearing, flow-sensing in kidneys, muscle, bone growth, vascular tone and blood flow (Chalfie, 2009; Hamill and Martinac 2001). The urinary bladder constantly conveys mechanosensory information to the CNS to regulate the micturition reflex. The mechanisms of mechanosensitivity in the bladder are classified into direct and indirect mechanisms.

1.) Direct mechanotransduction

According to an anatomical structure of afferent nerves that penetrate in the bladder wall, this allows the afferents to directly detect mechanical stimulation. A variety of ion channels have been investigated for a role in directly detecting mechanical stimuli in various visceral organs including, transient receptor potential (TRP) families (TRPA1, TRPV1, TRPV4), acid sensing ion channels (ASIC1, ASIC2, ASIC3), Piezo1 and Piezo2 (Gillespie and Walker 2001; Brierley, 2010; Coste *et al.* 2011). In order to sense mechanical force and trigger a response, it requires fast detection and high sensitivity to detect graded stimuli. Therefore, when ion channels are activated, the channels open rapidly and the signals are amplified by entry or efflux of ions promoting graded receptor potentials and action potentials (Gillespie and Walker 2001).

Two models have been proposed for direct mechanosensitivity of these ion channels; (i) bilayer tension and (ii) tethered channels. Bilayer tension model has been explained as mechanical force directly causes tension on the phospholipid cell membrane, a ‘tugging’ of mechanosensitive channels leading to opening of the channels. The mechanism of tethered model is mediated via linking between ion channels to the extracellular matrix and/or intracellular proteins. Changing in tension of the proteins, therefore, opens the transduction channels and allows ion flow to generate mechanotransduction (Gillespie and Walker 2001; Barrit and Rychkov 2005; Christensen and Corey 2007).

2.) Indirect mechanotransduction

Some groups of receptors are chemoreceptors and stretch insensitive which indirectly transfer mechanical signals via release of neurotransmitters/mediators to the nearby cells. A number of studies demonstrated that bladder distension triggers mediator release (i.e., ATP, ACh, and NO) from the urothelium, which could potentially bind to their specific receptors on the urothelial cells and other nearby cells (e.g. nerve terminals and ICC) (Birder and de Groat 2007). The afferent nerves express an array of various sensors to sense these molecules i.e., α-,ß-adrenergic receptors, P2X, P2Y, and muscarinic receptors (Birder *et al.* 1998; Birder *et al.* 2004; Chess-Williams 2002). In addition, ICC which also sit beneath the urothelial layers express both P2X and P2Y receptors (Wu *et al*. 2004). This information suggests that ICC may contribute to indirect mechanotransduction.

Two mechanisms of indirect mechanotransduction have been proposed; (i) adjacent mechanosensitive protein and (ii) ligand release models. The adjacent mechanosensitive protein hypothesis requires mechanical activation of mechanosensitive proteins, leading to release of diffusible second messengers or kinase activation to the ion channels. This further allows channel gating and ion flux (Christensen and Corey 2007). The ligand release model has been used to explain how mechanical force triggers extracellular ligand release which further activates ion channels to promote ionic influx (Christensen and Corey 2007; Brierley, 2010). Figure 1.7 summarizes the direct and indirect hypothetical models.

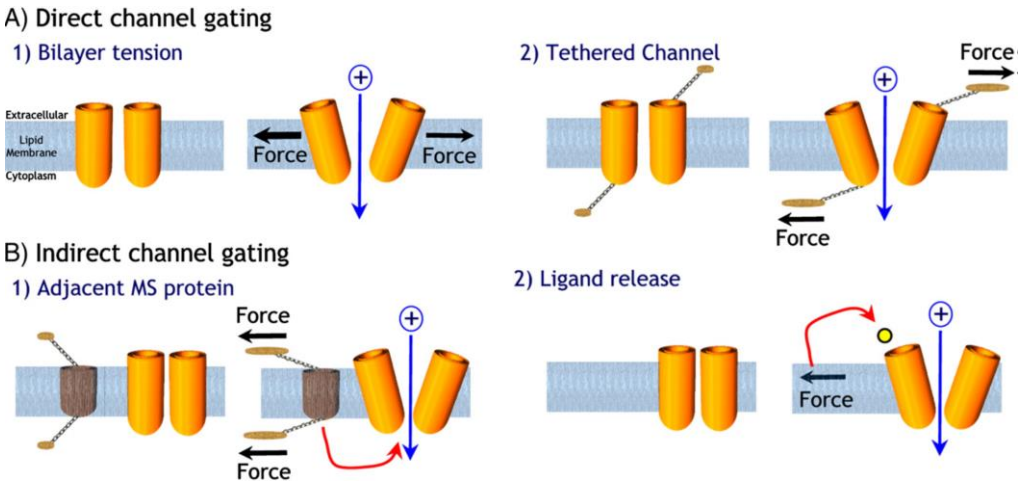


Figure 1.7 Hypothetical model of mechanotransduction channels (Brierley 2010). (A) Direct mechanotransduction mechanisms; 1) bilayer tension and 2) tethered channel, in which a mechanical force directly opens ion channels **(B)** Indirect mechanotransduction mechanisms; 1) adjacent mechanosensitive protein that the nearby proteins stretching promotes opening of the channels and 2) ligand release mechanism requires intermediate mediators to activate and open the channels.

1.4 MICTURITION REFLEX

The micturition reflex is a complicated process that requires co-ordination between the spinal cord and the brainstem (pontine micturition center/Barrington's nucleus) (via the bulbospinal reflex). The neuronal circuits work as an on-off switching mechanism between the urinary bladder and the urethral outlet activity (Mallory *et al.* 1991; Holstege *et al.* 1986; Yoshimura and de Groat 1997). The pontine micturition center also receives many sensory signals from various brain areas including, the periaqueductal gray (PAG), basal ganglia, and hypothalamus (Fowler, 2008). The supraspinal reflex plays a predominant role in voiding, in which bladder afferent signals arrive in the PAG and the signals are then relayed via the thalamus and the insula enabling assessment of sensation. In addition, the higher brain area, prefrontal cortex is involved in making conscious decisions regarding voluntary voiding (Griffiths and Tadic 2008). The micturition reflex comprises the storage phase (bladder filling) and the voiding phase (bladder emptying) as summarized in figure 1.8.

The storage phase

The urinary bladder has an accommodation mechanism that initially allows urine filling without an increase of the intravesical pressure. Bladder accommodation is an intrinsic property of the detrusor (Tang and Ruch 1995) and partial contribution of low-threshold afferent firing during filling which stimulates sympathetic outflow (de Groat and Yoshimura 2006). During urine storage, the parasympathetic system is inhibited. Sympathetic activation leads to release of noradrenaline which acts on ß-adrenergic receptors in the bladder dome and promotes detrusor relaxation. Simultaneously, noradrenaline binds to α-adrenergic receptors in the urethra to potentiate urethral sphincter contraction as a guarding reflex. Increased intravesical pressure during bladder filling further activates sympathetic outflow to allow urine storage (Fowler *et al.* 2008).

The voiding phase

Once the afferent signal reaches the threshold set by the pontine micturition center, the sympathetic and the somatic nervous systems are switched off. Concurrently, the parasympathetic nervous system is activated. Parasympathetic innervation in the bladder body releases acethylcholine (ACh) which further binds to muscarinic receptors (M2 and M3), leading to acute contraction of detrusor smooth muscle (Fowler *et al.* 2008). In addition, the excitatory signal to the bladder urethra is inhibited and parasympathetic neurons release NO to cause urethral sphincter relaxation (Anderson and Persson 1993), allowing urine expulsion through the urethra (Chancellor and Yoshimura 2002).

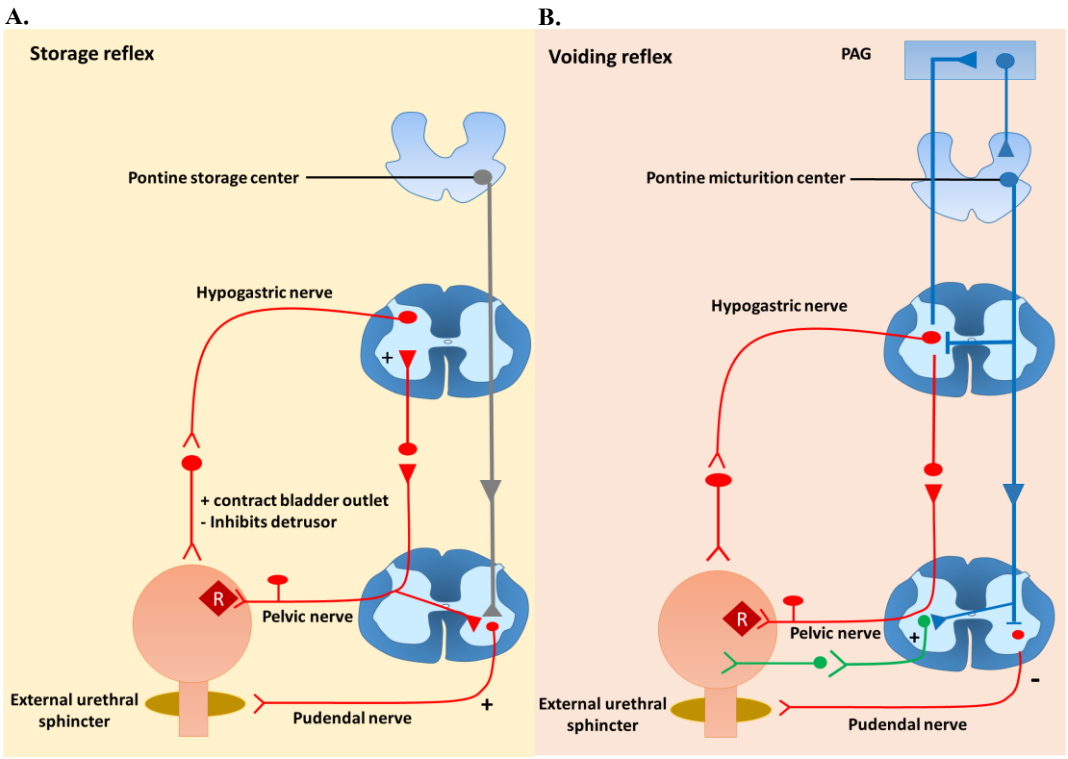


Figure 1.8 Neural circuits to control storage (A) and voiding (B) reflexes (Modified from Fowler *et al.* 2008). (A) Storage reflex: Distention of the bladder wall during urine storage evokes bladder afferent firing. This stimulates sympathetic outflow to contract the bladder outlet (bladder base and urethra) and evokes detrusor relaxation. In addition, the pudendal nerves that innervate the external sphincter are also activated. The pontine storage centre also sends descending signals to increase urethral sphincter activity. All of these mechanisms promote urine storage. **(B)** Voiding reflex: Intense bladder afferent signals via pelvic nerves activate the bulbospinal reflex pathway (blue) and pass through the pontine micturition center. Thereafter, parasympathetic outflow is sent to promote detrusor muscle contraction of the bladder body and relaxation of urethral smooth muscle (green), while the sympathetic and pudendal efferents of urethral outlet are inhibited (red). In addition, ascending pathways from the spinal cord signal to relay neurons in the periaqueductal grey (PAG) before sending to the pontine micturition center.

1.5 5-HT RECEPTORS AND FUNCTION

5-Hydroxytryptamine (5-HT; serotonin) was first identified as a vasoconstrictor substance in the 1940s (Rapport *et al.* 1947). 5-HT is an essential neurotransmitter that is well-documented to regulate many processes in the body. In the brain, 5-HT plays a key role in the regulation of mood, memory, anxiety, sexuality, appetite, and sleep. 5-HT also exerts diverse roles in peripheral organs including cardiovascular, gastrointestinal, pulmonary, genitourinary systems, and nociception (see review by Berger *et al.* 2009).

The major source of 5-HT (95%) is produced in the gastrointestinal tract by enterochromaffin cells (EC cells), but mast cells and myenteric neurons in the gut wall also produce 5-HT (Gershon and Tack 2007), while about 5% is centrally produced from serotonergic neurons originating from raphe nuclei in the brainstem (Berger *et al.* 2009; Bertrand and Bertrand 2010; O'Hara and Sharkey 2007).

Both central and peripheral, 5-HT synthesis requires a rate limiting enzyme, tryptophan hydroxylase (TPH). TPH has 2 isoforms; TPH1 and TPH2. TPH2 is a pre-dominant isoform in neuronal tissues, while TPH1 is broadly expressed and produces 5-HT in non-neuronal tissues (Cote *et al.* 2003; Walther *et al*. 2003). 5-HT biosynthesis requires 2 main steps; (i) hydroxylation of tryptophan into 5-hydroxytryptophan (5-HTP) by TPH and (ii) decarboxylation of 5-hydroxytryptophan into 5-hydroxytrptamine (5-HT) by L-amino acid decarboxylase (L-AADC) (Hakanson *et al*. 1970; Verbeuren 1989). After synthesis, 5-HT is stored in vesicles by the vesicular monoamine transporter 1 (VMAT1) (Rindi *et al.* 2004). Following its release, 5-HT is taken back up into the nearby intestinal epithelial cells and neurones by the serotonin transporter (SERT) (Wade *et al.* 1996).

5-HT which is not stored in the vesicles is degraded by monoamine oxidase (MAO) and aldehyde decarboxylase (ADH). These enzymes convert 5-HT into an inactive metabolite 5-hydroxyindoleacetic acid (5-HIAA) (Egashira and Waddell 1984; Rodriguez *et al.* 2001). A diagram of 5-HT biosynthesis and metabolism in the EC cells is shown in figure 1.9.

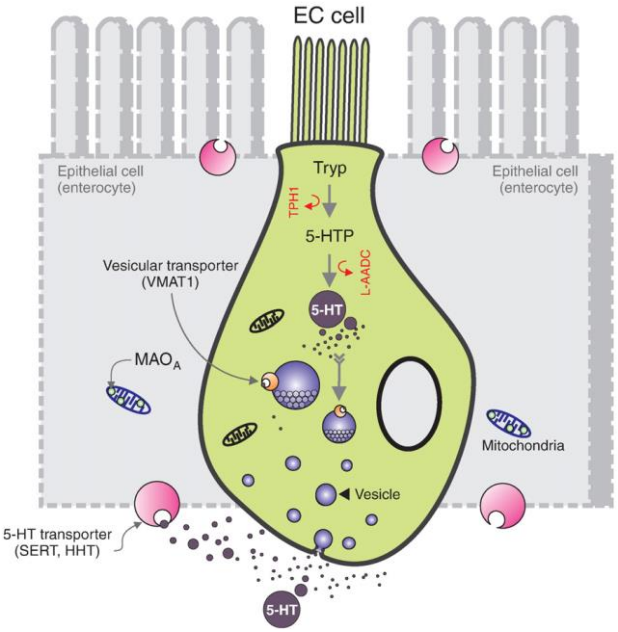


Figure 1.9 Schematic diagram depicting 5-HT production in intestinal enterochromaffin (EC) cells (Bertrand and Bertrand 2010).

1.5.1.) Classification and distribution of 5-HT receptors

5-HT receptors are ubiquitously expressed in the body in both central and peripheral organs. At present 5-HT receptors are mainly classified into 7 subfamilies and at least 14 subtypes have been identified. Most 5-HT receptors are G-protein coupled receptors (GPCR) with the exception of the 5-HT3 receptors which are ligand-gated ion channel (Roth, 2006).

The structure of 5-HT1, 2, 4, 5, 6, and 7 receptors comprises 7 transmembrane domains with an extracellular NH_2 terminal and a cytoplasmic COOH terminal. The third intracellular loop is the active site which interacts with G-proteins (Barnes and Sharp 1999).

5-HT3 receptors are the only group of 5-HT receptor that are ligand gated ion channels. They are member of the Cys loop superfamilies similar to nicotinic acetylcholine, $GABA_A$, and glycine receptors. 5-HT3 receptors comprise four transmembrane domains that form a pore with a pentameric structure. The transmembrane domain two forms a central pore. NH_2 and COOH terminals of 5-HT3 channels are on the extracellular side (Figure 1.10) (Reeves *et al.* 2001; Sine and Engel 2006). The classification, distributions, and the responses of 5-HT receptors are summarized in table1.2.

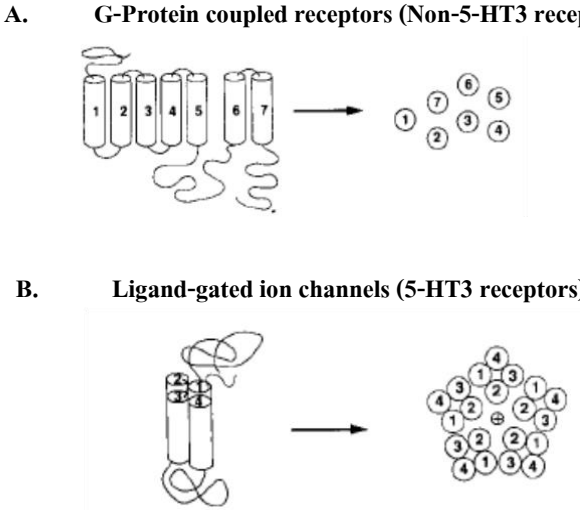


Figure 1.10 Model for subunit structure of 5-HT receptors (Baez *et al.* 1995). **(A)** Subunit model of metabotropic 5-HT receptors (non-5-HT3) showing the seven transmembrane structure of G-protein coupled receptors. **(B)** Subunit model of ligand gated 5-HT3 receptors comprising 4 subunits that form a pentameric structure.

1.) Non-5-HT3 receptors

5-HT1 receptors

5-HT1 receptors are found in both CNS and PNS. In the brain, a dense expression of 5-HT1 receptors have been reported in the dorsal raphe nucleus, cerebral cortex and pyramidal cell layer of hippocampus (Chalmers and Watson 1991; Miquel *et al.* 1991). 5-HT1 receptors are classified into 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E, and 5-HT1F. The main mechanism of 5-HT1 receptors is negatively coupled to adenylyl cyclase via Gi protein (Raymond *et al*. 2006).

Among the receptor subtypes, 5-HT1A receptors are the most extensively studied. It has been suggested that 5HT1 receptors could function as autoreceptors in the raphe nuclei. 5-HT1A activation stimulates neuronal G-protein-gated inwardly rectified (GIRK) K^+ channel (Andrade and Nicoll 1987; Colino and Halliwell 1987; Doupnik *et al.* 1997), leading to neuronal hyperpolarization to prevent over-excitability and inhibit neuronal 5-HT release. In addition, 5-HT1A activation has been reported to stimulate PLC/IP3 and intracellular Ca^{2+} mobilization and coupled to mitogen-activated protein kinase (MAPK) pathway via indirect action of ßγ subunit (Albert and Tiberi 2001).

5-HT2 receptors

5-HT2 receptors are mainly found in the forebrain and motor neurons with the low expression level in the brainstem and spinal cord (Mengod *et al*. 1990). 5-HT2 are classified into 5-HT2A, 5-HT2B, and 5-HT2C. 5-HT2 receptors are coupled to Gq protein to positively regulate phospholipase C (PLC) to increase intracellular Ca^{2+} via activation of the inositol pathway (IP_3) (Hannon and Hoyer 2008).

5-HT4 receptors

5-HT4 receptors are ubiquitously expressed in both central and peripheral organs i.e., brain, heart and gastrointestinal tract (Raymond *et al*. 2006). 5-HT4 receptors activate stimulatory G protein (Gs) and positively coupled to adenylyl cyclase and increase cAMP level, which activate PKA pathway. Activation of 5-HT4 leads to various actions including inhibition of K^+ channel (Fagni *et al.* 1992), facilitation of transmitter release (dopamine, ACh and 5-HT) in the brain (Consolo *et al.* 1994; Bianchi *et al*. 2002; Ge and Barnes 1996), and mediation of colonic relaxation, and activation of L-type Ca^{2+} channels (Ouadid *et al*. 1992).

5-HT5 receptors

5-HT5 receptors are negatively coupled to adenylyl cyclase via Gi proteins, which resemble 5-HT1 receptors, however, 5-HT5 receptors show lower affinity to 5-HT and have sequence homology to other 5-HT receptors of less than 50%. Two subtypes of 5-HT5 are identified; 5-HT5A and 5-HT5B receptors in rats (Matthes *et al.*1993; Erlander *et al.* 1993).

5-HT6 receptors

5-HT6 receptors are primarily expressed in the brain with lower expression in the stomach and adrenal gland (Monsma *et al.* 1993; Ruat *et al*. 1993). The primary cascade of 5-HT6 receptors is linked to stimulatory G-protein which activates adenylyl cyclase and increases cAMP level. (Ruat *et al.* 1993; Grimaldi *et al.* 1998).

5-HT7 receptors

5-HT7 receptors have been found in both in central and peripheral organs. They are highly expressed in the hippocampus, neocortex, and hypothalamus (Lovenberg *et al.* 1993; Thomas and Hagan 2004). 5-HT7 receptors are also widely expressed in the periphery including intestine (Liu *et al.* 2001), stomach (Janssen *et al.* 2004), corneal epithelial cells (Crider *et al.*

2003), adrenal glomerular cells (Lenglet *et al*. 2000), and blood vessels (Heidmann *et al.* 1998). 5-HT7 receptors are positively coupled to adenylyl cyclase and increase cAMP level via Gs stimulation similar to 5-HT4 and 5-HT6 receptors (Shen *et al.* 1993).

2.) Ligand-gated ion channels

5-HT3 receptors

5-HT3 receptors are expressed in both central and peripheral organs including brain, spinal cord, and gastrointestinal tract. 5-HT3 activation evokes rapid depolarization via influx of monovalents (Na^+ and K^+) and lower permeability to divalents (Ca^{2+} and Mg^{2+}) (Peter *et al.* 1988; Lummis, 2012).

5-HT3A and 5-HT3B receptor subunits are the major isoforms that form either homomeric or heteromeric channels. Homomers of 5-HT3A and heteromers of 5-HT3A and 5-HT3B can form functional receptors, whereas homomeric 5-HT3B receptors do not. However, previous studies using patch clamp electrophysiology showed that the composition of 5-HT3 subunits is important in determining channel properties. For example, homomeric 5-HT3A receptors had a lower threshold to reach desensitization compared to hetomeric 5-HT3AB receptors. Additionally, 5-HT3AB receptors had shorter recovery times after desensitization compared to homomeric 5-HT3A receptors (Davies *et al.* 1999; Hapfelmeier *et al.* 2003). However, there are also other factors that could contribute to changing in properties of 5-HT3 desensitization including, extracellular Ca^{2+} level, developmental state of cells, and phosphatases (e.g. calcineurin) (Gunthorpe *et al*. 2000; Lobitz *et al*. 2001; Yakel *et al.* 1993). One explanation why the 5-HT3B subunit can alter the kinetic properties of the 5-HT3 channels is that these channels are more permeable to Na^+ and K^+ compared to $Ca^{2+}$and impermeability to Mg^{2+} and so less likely to cause desensitization (Davies *et al*. 1999). A summary diagram of all signalling pathways of 5-HT receptor subtypes is shown in figure 1.11.

Table 1.2 Summarized 5-HT receptor classification, location and actions (Hoyer *et al.* 1994).

Receptor	Subtypes	Location	Response
5-HT1	5-HT1A	Neuronal, mainly in CNS	Neuronal hyperpolarization
	5-HT1B	CNS and some peripheral nerves	Inhibition of neurotransmitter release
	5-HT1D	Mainly CNS	Inhibition of neurotransmitter release
	5-HT1E	Only CNS	Inhibition of adenylyl cyclase
	5-HT1F	Mainly CNS	Inhibition of adenylyl cyclase
5-HT2	5-HT2A	Vascular smooth muscle, platelets, lung, CNS, gastrointestinal tract	Vasoconstriction, platelet aggregation, bronchoconstriction
	5-HT2B	Mainly peripheral	Rat stomach fundic muscle contraction
	5-HT2C	CNS (high density in choroid plexus)	Increase phosphoinositide turnover
5-HT3		Peripheral and central neurons, gastrointestinal tract	Depolarization
5-HT4		Gastrointestinal tract, CNS, heart, urinary bladder	Activation of ACh release in gut, tachycardia, increased cAMP in CNS neurons
5-HT5	5-HT5A, 5-HT5B	CNS	Not known
5-HT6		CNS	Activation of adenylyl cyclase (HEK 293 cells)
5-HT7		CNS	Activation of adenylyl cyclase (Hela cells and COS cells)

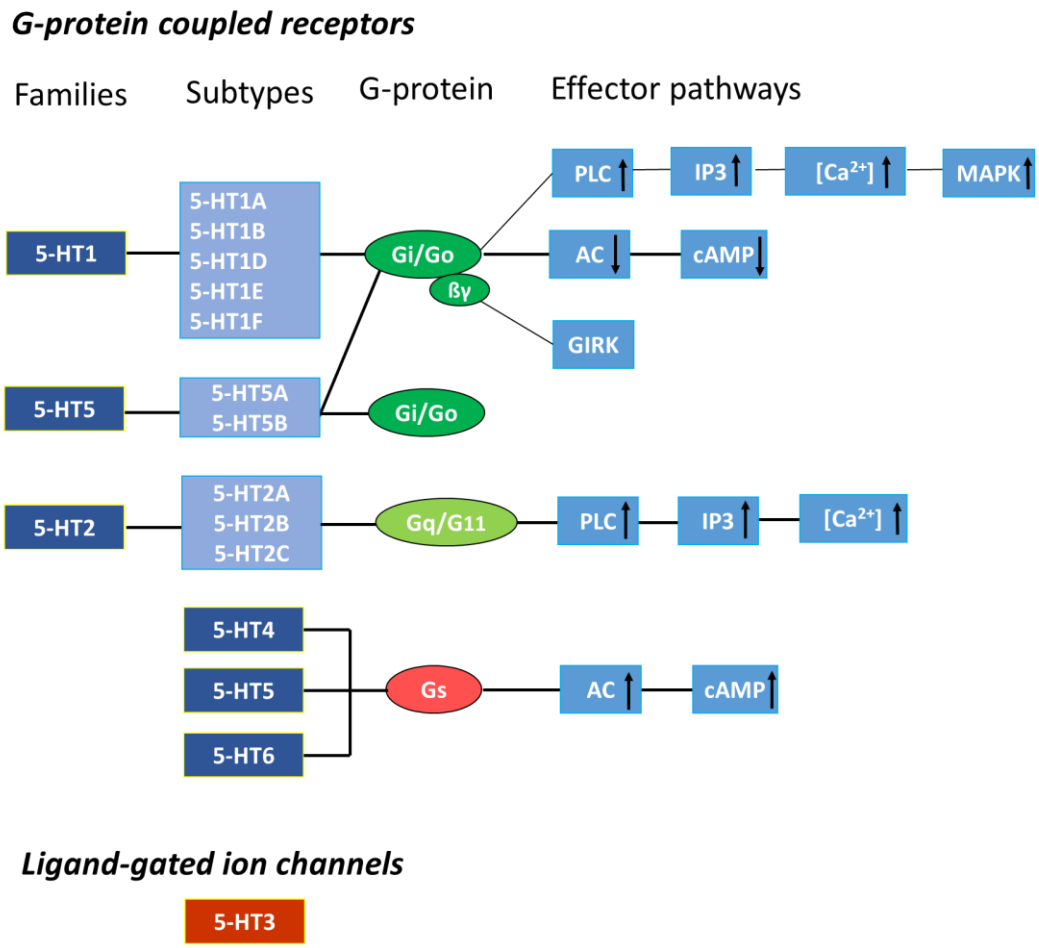


Figure 1.11 Diagram summarizing signal transduction pathways of 5-HT receptor subtypes (Noda *et al.* 2004).

1.6 ROLE OF 5-HT IN THE BOWEL AND THE BLADDER

1.6.1) 5-HT and bowel functions

The bowel is the major source of 5-HT in the body. 5-HT is secreted across the basolateral membrane of EC cells in response to mechanical (intestinal wall distension), chemical (acid or bases), or noxious stimuli (Berger *et al.* 2009; Bertrand and Bertrand 2010; O'Hara and Sharkey 2007). The secreted 5-HT in the bowel has three main functions; (i) bind to 5-HT receptors expressed on either intrinsic or extrinsic nerves, (ii) act as a paracrine mediator to activate nearby enterocytes, (iii) excess 5-HT could enter the circulation through blood vessels in the lamina propria, which may exist in its free form or be taken up into platelets via the serotonin transporter (SERT) (Bertrand and Bertrand 2010). SERT is also expressed on both apical and basolateral sides of the enterocytes (Gill *et al.* 2008).

The main function of SERT is to re-uptake 5-HT into EC cells of the intestinal epithelium and platelets. Storing 5-HT in platelets has beneficial effects to protect and maintain 5-HT levels in the circulation (O'Hara and Sharkey 2007). Free 5-HT is rapidly metabolized in the liver by liver enzymes. One third of 5-HT is degraded by MAO into 5-hydroxyindoleacetic acid (5-HIAA), while two third are processed and turned into 5-HTO-glucurunide. Stored 5-HT in platelets distributes to the other organs via the circulation (Egashira and Waddell 1984; Gershon *et al.* 1989). The schematic diagram of 5-HT production and location of EC cells in the intestinal wall are shown in figure 1.12.

5-HT is well known to regulate many functions in the bowel including, intestinal motility, segmentation, secretion, and vasodilation via activation of both intrinsic (myenteric and submucosal plexuses) afferents and extrinsic (vagal and spinal) afferents. Extrinsic afferent activation of 5-HT regulates gastric emptying, nausea, vomiting, and discomfort (Furness *et al*. 1999; Grundy and Schemann 2005; Grundy 2008). In addition, 5-HT also plays a key role in intestinal inflammation (Keating *et al.* 2008; Wang *et al.* 2007; Mawe *et al.* 2006) and regulation of cell proliferation of neurons and ICC (via activation of 5-HT2B) (Wouters *et al.* 2007). At least 5 subfamilies of 5-HT receptors, 5-HT1, 2, 3, 4 and 7 have been reported and

regulate intestinal functions (Hoyer *et al.* 2002). 5-HT3 and 5-HT4 receptors have been reported to play major roles and have been broadly investigated for their therapeutic potential.

5-HT3 receptors are expressed in epithelial, subepithelial layers, smooth muscle cells, ICC and motor neurons. 5-HT3 receptors are therefore attractive targets to treat many intestinal symptoms including nausea and abdominal discomfort. 5-HT3 antagonists have been widely used to treat diarrhoea in IBD patients (Andersson *et al.* 2008; Rahimi *et al.* 2008). IBS patients treated with alosetron (5-HT3 antagonist) showed increased compliance of the colon (Delvaux *et al.* 1998). In addition, 5-HT3 antagonists have been used to treat nausea from chemotherapy-induced emesis and nausea (Costall *et al.* 1986; Minnie and Sanger 1986). However, there have been reports of adverse effects of 5-HT3 antagonist treatment for constipation (Talley *et al.* 1990). Interestingly, 5-HT3 receptors have been reported to play important roles in afferent hypersensitivity in the bowel, which could be correlated to pathological symptoms in IBS and IBD (Keating *et al.* 2008).

5-HT4 receptors are extensively expressed in the intestinal wall including intrinsic and extrinsic afferent neurons, epithelial cells, globlet cells, and smooth muscle cells (Hoffman *et al.* 2012; Liu *et al.* 2005). 5-HT4 activation increases peristaltic reflexes and gastric emptying. 5-HT4 knockout mice show impair colonic motility (Liu *et al.* 2009). Therefore, 5-HT4 agonist such as cisapride and metoclopramide have been used to treat constipation in IBS patients (Evans *et al.* 2007; Baeyens *et al.* 1984).

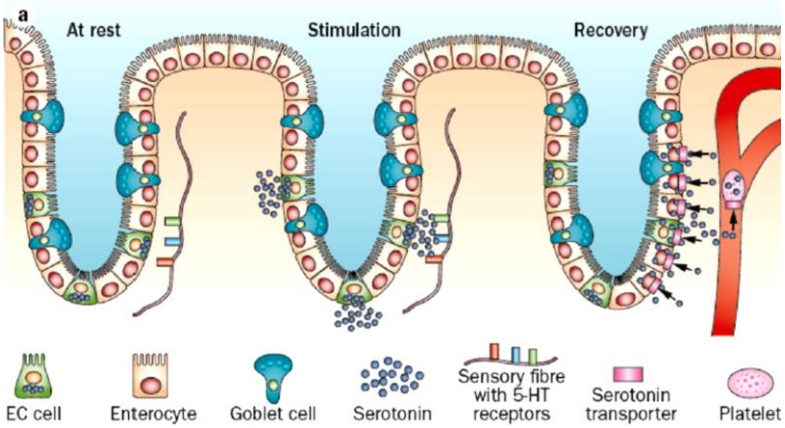


Figure 1.12 Schematic diagram demonsting 5-HT pathways in the intestinal wall (Mawe and Hoffman 2013). 5-HT is released into the lamina propria and directly activates both intrinsic and extrinsic afferent nerves. Some of the 5-HT is transported back to nearby epithelial cells via SERT. 5-HT that enters the blood circulation is taken up and stored in platelets.

1.6.2) 5-HT and urinary bladder functions

Previous studies have investigated the central role of 5-HT in regulating micturition reflexes at both spinal and supraspinal levels (Ishizuka *et al*. 2002; Ramage, 2006). Cell bodies of serotonergic neurons are located in the raphe nuclei in the brainstem and send their projections to many areas of the brain and spinal cord. Lumbosacral parasympathetic, sympathetic and somatic nuclei receive serotonergic innervation from the raphe nuclei. The serotonergic neurons in the raphe nuclei send bulbospinal projection to innervate the superficial of dorsal horn and directly control the sensory information arising from second-order neurons in the dorsal horn which receive the input from primary sensory neurons (Dahlstr and Fuxe 1964; Skagerberg and Bjorklund 1985; de Groat *et al*. 1993).

A study showed that activation of raphe nuclei leads to inhibitory actions on bladder reflex function (McMahon and Spillane 1982; Sugaya *et al.* 1998). Interestingly, a recent study by Chiba and colleagues using a combination of microdialysis in the prefrontal cortex and cystometrography (CMG), showed that 5-HT exerts an inhibitory role on micturition reflexes in the rat. They found a real-time correlation between increased 5-HT levels in prefrontal cortex and increased bladder intercontraction intervals. This effect was reduced by local application of

5-HT1A agonist (8-OH-DPAT) into the prefrontal cortex, which also significantly decreased the intercontraction interval (Chiba *et al.* 2016).

Within the research literature there is clear evidence of species variation which makes interpretation of 5-HT function difficult. Multiple 5-HT receptor subtypes have been found in many regions in the brain and spinal cord area that are involved in controlling micturition. At present, 5-HT1A, 5-HT2, 5-HT3, 5-HT4 and 5-HT7 receptors have been reported for their expression and functional role in controlling bladder activity (Ishizuka *et al.* 2002; Ramage, 2006).

5-HT1A receptor expression has been found in Onuf's nucleus, the sacral parasympathetic nucleus, and raphe nuclei, suggesting roles in controlling micturition reflex (Gobert *et al.* 1995). Even though 5-HT1A receptor activation has inhibitory action on adenylyl cyclase in spinal and supraspinal level, activation of 5-HT1A receptors with the selective 5-HT1A agonist (8-OH-DPAT) results in bladder contraction (Lecci *et al.* 1992) and the inhibition of the receptor using selective receptor antagonist causes decrease in bladder contraction (Testa *et al.* 2000). The potentiation of bladder activity of 5-HT1A receptor has been suggested to have a 'disinhibition' effect. Activation of 5-HT1A autoreceptors reduces 5-HT release from serotonergic neurons, which further decreases an inhibitory action of 5-HT2C receptor activation (Ramage, 2006). In contrast, in cats, 5-HT1A receptors have been reported to play a role only in pathological conditions i.e., bladder irritation with intravesical acetic acid and spinal cord injury (Gu *et al.* 2007).

Lecci and co-workers (1992) suggested different roles for spinal and supraspinal 5-HT1A receptors. They showed that 5-HT depletion by 5,7-dihydroxytrptamine (5,7-DHT) required higher effective dose of intracerebroventricular (i.c.v) administered 8-OH-DPAT, whereas the effective dose of intrathecal (i.t.) 8-OH-DPAT was not changed (Lecci *et al.* 1992). These results suggested that 5-HT1A receptors in the brainstem act as autoreceptors, while 5-HT1A receptors in the sacral spinal cord are heteroreceptors and may have a role in the regulation of the release of other neurotransmitters (Ramage, 2006).

5-HT2 expression has been reported in the Onuf's nucleus, hypothalamus and cortex. The nonspecific 5-HT2 agonist, alpha-methyl-5-hydroxytryptamine maleate (i.c.v), increased bladder contraction and decreased bladder capacity in rats (Ishizuka *et al.* 2002). The literature has conflicting reports as to the role of 5-HT2 receptors which depends on the species used for the study. 5-HT2A receptor activation plays a primary role in activation of EUS activity in rats, whereas 5-HT2C activation inhibits EUS activity in guinea pigs (Thor *et al.* 1990; Danuser and Thor 1996). Overall 5-HT2 receptors have inhibitory role in micturition reflexes but exert stimulatory action on pudendal nerves to increase EUS activity (Ramage, 2006).

Ishizuka *et al*. reported a minor role of supraspinal 5-HT3 receptors using 5-HT3 agonists (2-Me-5-HT) (i.c.v), which correlates with low densitiy of 5-HT3 receptor expression in the brain (Ishizuka *et al.* 2002). Testa and colleagues (2000) have also reported that 5-HT3 receptor antagonist (zalosetron) administration (i.c.v) had no effect on voiding contraction in anesthetized rats (Testa *et al*. 2000). However, the role of spinal 5-HT3 receptors has been reported in cats by Espey and colleagues. Intrathecal administration of the 5-HT3 antagonist (zalosetron) decreased the micturition threshold during bladder filling (Espey *et al.* 1999), suggesting that spinal 5-HT3 receptor may play a role in control bladder function. This information is supported by an investigation by Kidd *et al.* (1993) who showed 5-HT3 receptor expression in the dorsal horn of the spinal cord (Kidd *et al*. 1993).

Using the selective 5-HT4 agonist RS67506 (i.c.v), it was found that supraspinal 5-HT4 receptors enhance the micturition reflex in response to bladder filling (Ishizuka *et al.* 2002). However, this data is in contrast to an investigation in anaesthetized rats by Testa *et al.* (2000). They reported that the 5-HT4 antagonist RS39604 had no effect on the micturition reflex (Testa *et al.* 2000). 5-HT4 receptor expression is high in nigrostriatal and mesolimbic areas, which have been reported to modulate other neurotransmitter release e.g., dopamine and ACh (Barnes and Sharp 1999). The action site and mechanism of 5-HT4 activation is still unclear and it could be direct and/or indirect mechanisms via controlling other neurotransmitters (Ramage, 2006).

Doly and co-workers (2005) reported 5-HT7 receptor expression in the Onuf's nucleus in rats, suggesting its role in micturition (Doly *et al.* 2005). Using the specific 5-HT7 antagonist SB-266970 (i.c.v) administration (10-30 μgkg^{-1}) in rats showed an increase in intravesical pressure

and volume threshold (Read *et al.* 2004). These data suggest that 5-HT7 receptors have a stimulatory action on the micturition reflex (Ramage, 2006).

While many previous studies have purported the central mechanisms of 5-HT in controlling micturition, there is still a lack of information about the modulatory role of 5-HT on bladder afferent activity. Even though, a number of studies have reported 5-HT receptor expression in the bladder urothelium, detrusor muscle, afferent and efferent nerve terminals, most studies have focused on 5-HT efferent function to regulate detrusor contraction. The details of expression profile and their functional roles of 5-HT in the bladder are reviewed in chapter 3 and 4.

1.7 PELVIC CROSS-ORGAN SENSITIZATION

An anatomical and functional relationship between the pelvic organs is important to allow pelvic organs to work harmoniously. A clear example is the alternate function between the distal intestine and the bladder. Bladder contraction is inhibited following rectal or colonic distention (Kock and Pompeius 1963; Floyd *et al.* 1982), which explains the alternative processes between defecation and urination (Vilensky *et al.* 2004).

The concept of neuronal cross-talk or 'viscero-visceral reflexes' refers to change in afferent signals from one stimulated organ affecting afferent activity in another nearby organ. According to previous investigations, cross-sensitization has been proposed to occur by both 'central' and/or 'peripheral' mechanisms (Malykhina, 2007; Brumovsky and Gebhart 2010; Daly *et al.* 2013).

Cross-organ sensitization becomes more prominent after many investigations reported that this mechanism may contribute to an overlapping symptom profile between different pelvic organs e.g., the colon, urinary bladder, uterus, and prostate gland. This mechanism therefore results in difficulty with diagnosis and makes the treatment of patients less precise. Many clinical studies have reported an overlap in pathological symptoms between the distal bowel, bladder, uterus, and prostate gland (Francis *et al.* 1997; Mayer *et al.* 1999; Aaron and Buchwald 2001). For

instance, irritable bowel syndrome (IBS) patients have been reported with bladder symptoms i.e., nocturia, frequency, incomplete voiding, and pain during urination (Whorwell *et al.* 1986). Bladder inflammation reduced uterine contraction (Dmitrieva *et al*. 2001). In addition, uterine inflammation induced extravasation of the bladder and colon (Winnard *et al*. 2006).

Central cross-sensitization is driven by mechanisms in the brain and spinal cord, in which the second order sensory neurons receive convergent sensory information from more than one organ. In the brain, neuronal convergence has been found in thalamus and brain stem. A study has shown that neurons in the Barrington's nucleus of the pontine micturition center respond to both colon and bladder distention (Hubscher and Johnson 2003; Rouzade-Dominguez *et al*. 2003). In the spinal cord, neuronal cross-talk from an inflamed organ could be transferred to uninflamed structures via a direct connection of interneurons that receive afferent signals from both organs (Berkley, 2005; Willis, 1999).

Peripheral cross-sensitization primarily depends on 'axon-reflexes' that are proposed to occur because sensory neurons in the DRG have multiple axons innervating several organs. Therefore, afferent hyperexcitability in one organ could converge with signals from an uninflamed organ resulting in the release of excitatory neurotransmitters which further sensitize afferent nerves of the second organ (Amir and Devor 1992; Brumovsk and Gebhart 2010; Malykhina, 2007). Evidence for peripheral cross-sensitization among the pelvic organs comes from studies showing that local administration of capsaicin in the inflamed colon (Peng *et al.* 2009) or uterus (Peng *et al.* 2008) reduced the effect of urethral reflexes. A schematic diagram summarizing cross-organ sensitization mechanisms is shown in figure 1.13.

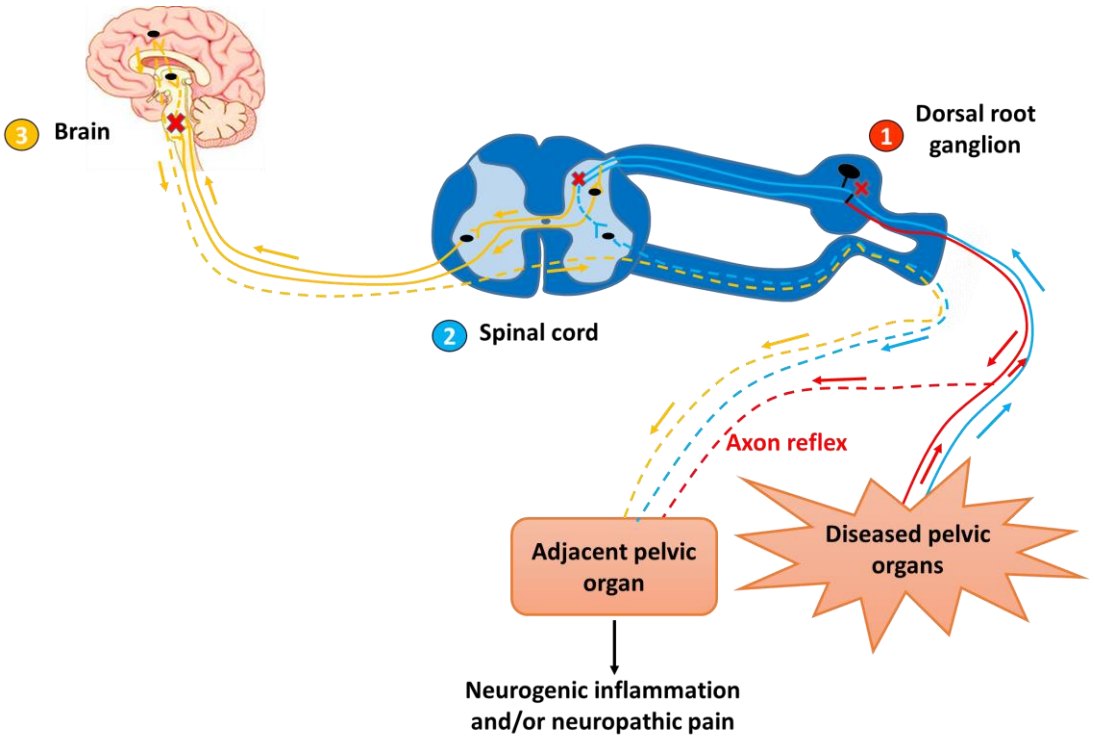


Figure 1.13 Possible mechanisms of neuronal cross-talk between pelvic organs (modified from Malykhina, 2007). The convergence of afferent nerves, possibly occurs either at central or peripheral mechanisms in 3 levels; **(1)** *Dorsal root ganglion (DRG):* DRG neurons that have branching or multiple axons provide a direct neuronal connection between two organs. If the DRG neurons that receive sensory information from an irritated organ have axonal connection with an adjacent organ, changes in the excitability of the excited organ will generate antidromic action potential or "axon-reflexes" via neurotransmitter release to the nearby organ. This mechanism may trigger vasodilation or extravasation, and develop neurogenic inflammation in the adjacent organ (red route). **(2)** *Spinal cord*: The afferent signals from the irritated organ that arrive at the spinal cord converge on the same interneuron with another organ in the dorsal horn, resulting in a convergent action potential to the unirritated organ (blue route). **(3)** *Brain*: Following the convergence of neuronal pathways in the brain, descending signals according to hyperexcitability of the irritated organ is conveyed to the adjacent organ (gold route). Red Cross indicates convergent neurons. Anterograde signal from the brain, spinal cord, and DRG to the organs is shown by dotted lines.

1.8 BLADDER PATHOLOGY

1.8.1) Interstitial cystitis/painful bladder syndrome

Interstitial cystitis/painful bladder syndrome is a chronic bladder condition with bladder pain and related irritant symptoms in the bladder and the pelvic regions lasting more than 6 months. The International Continence Society (ICS) defined interstitial cystitis (IC) or painful bladder syndrome (PBS): "The complaint of suprapubic pain related to bladder filling, accompanied by other symptoms such as increased daytime and nighttime frequency, in the absence of proven urinary infection or other obvious pathology" (Abrams *et al.* 2002). IC/PBS is diagnosed by pain related to urinary bladder and other functional symptoms including, frequency, urgency, and pain during urination without an obvious disease. It has been reported to affect children and adults (Shear and Mayer 2006) with higher incidence in women. IC/PBS does not seem to vary with age or ethnicity (Clemens *et al.* 2007).

The aetiology of IC/PBS is still unclear. At present three main factors have been proposed to contribute to IC/PBS; alteration of urothelial permeability, mast cells activation and C-fiber nerve activation (Sant, 2002).

Alteration of urothelial permeability

Impaired urothelial permeability leads to increased susceptibility to luminal stimuli (Hicks *et al*. 1974). The urothelium is densely coated with glycosaminoglycan (GAG) layers and tight junction proteins which act as a protective barrier to prevent the leakage of potassium ions, bacteria, and noxious substances (Khandelwal *et al.* 2009; Varley *et al.* 2006). The significant role of the GAG layer has been shown by studies where the GAG layer is restored following intravesical application of exogenous GAG (such as chondroitin sulphate). This treatment reverses the permability of the bladder to $^{86}Rb^{+}$ (Hauser *et al.* 2009) and significantly reduces the recruitment of immune cells (Engles *et al.* 2012). A diagnostic test for permeability of urothelium is an intravesicular potassium test (Parson's test) (Bernie *et al.* 2001; Parsons, 2002).

The Parson's test determines changes in bladder permeability by infusion of a diluted potassium solution (40 mEq in 100 mL of water) directly into the bladder. The solution is left in the

bladder for 5 minutes and the patients is asked to rate the degree of provocation of frequency and urgency from zero (no feeling) to five (pronounced feeling) (Parsons, 2002). The positive diagnosis of IC is considered if the patients have a rating higher than two. However, a study has reported sensitivity and specificity of this test are 59% and 69% respectively (Chambers *et al*. 1999) which calls into question its validity in diagnosing IC. Clinical studies have developed a better way by using biomarkers as a tool for the diagnosis. Several biomarkers have been suggested including urinary antiproliferative factor (APF) and urinary glycoprotein (GP-51). However, there have been no studies to confirm if these markers are correlated to cystoscopic and biopsy data (Erickson, 2001; Sant, 2002).

Mast cells activation

Mast cells are a key source of various proinflammatory mediators i.e., prostaglandins, tryptase, leukotriene and histamine (Theoharides *et al*. 2001). Clinical evidence reports that 30-65% of IC/PBS patients have mastocytosis. Previous studies have also reported an increased histamine level in IC patients. Patients with antihistamine treatment (Hydroxyzines) and leukotriene inhibitor showed improvement in IC/PBS symptoms (Bouchelouche *et al.* 2001; Theoharides *et al.* 2001). Animal experiments using TNBS-induced colonic inflammation have shown an increase in mast cell numbers in the bladder, which correlates with an impaired voiding function (decreased voiding interval) (Fitzgerald *et al.* 2013). These studies suggest that IC/PBS may be derived from mast cell activation mechanisms.

C-fiber nerve activation

It has been widely accepted that bladder afferent hypersensitivity correlates with bladder storage symptoms. Activation of C-fiber afferents by inflammatory mediators e.g., substance P and nerve growth factor (NGF) could recruit mast cells and trigger inflammatory mechanisms. Moreover, increased expression of substance P and their receptors in C-fibres has been observed in IC patients (Marchand *et al.* 1998). A clear role for C-fiber afferent activation by neuropeptides contributing to bladder hyperexcitability was shown by Ustinova and co-workers (2007). They showed that depletion of neuropeptides from afferent terminals by systemic administration of capsaicin reduced hyperexcitability of C-fiber afferents in TNBS-induced colonic inflammation. In addition to sensitization of C-fiber afferents, increased mast cells in the bladder was also observed (Ustinova *et al.* 2007). This suggests that the aetiology of IC/PBS

is complex. Bladder dysfunction from alteration of urothelial cells, inflammatory response derived from mast cells and activation of C-fibres may act in a concerted way. Importantly, as described in the previous section with respect to cross-sensitization mechanisms between the pelvic organs, the released mediators from the insulted organ could sensitize neurons in DRG/spinal cord/brain that receive sensory signals from another organ, leading to sensitization of afferent nerve of the other pelvic organs (Sant, 2002). A schematic diagram of hypothesized pathophysiology mechanism of IC/PBS is summarized in figure 1.14.

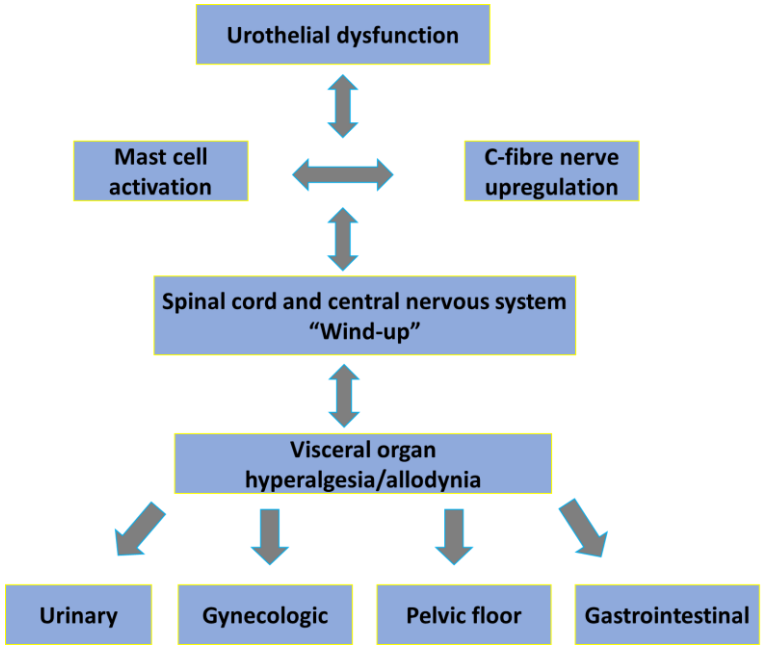


Figure 1.14 Summarized diagram of pathophysiologies of IC/PBS (modified from Sant, 2002).

1.8.2.) Overactive Bladder

Overactive bladder (OAB) is a complex pathophysiological bladder symptom. OAB shares common irritant bladder symptoms to IC/PBS, except that pain is not a feature for this condition. A survey study in 5 countries; Canada, Sweden, Germany, Italy and UK of patient's ages between 18-60 years in both sex showed that average of OAB prevalence is ~12%. It has been reported similar prevalence in both sexes and the incidence tends to increase with age (Irwin *et al.* 2006).

The ICS defined OAB as urgency with or without urinary incontinence (Milsom *et al.* 2001), frequency (voids >8 per day), (Wein and Rover 2002), and nocturia (Abrams *et al*. 2002). All of these pathological symptoms occur without bladder infection or obvious pathology. OAB is associated with urodynamic characteristic of detrusor overactivity (Abrams *et al.* 2002).

The complete aetiology of OAB is not fully understood but it has been suggested to be multifactorial. The aetiology of OAB is classified into two classes:

- *Idiopathic overactive bladder* is referred to non-neurogenic cause that the aetiology is undefined.
- *Neurogenic overactive bladder* is referred to OAB caused by dysregulation or degeneration of neuronal pathways that could occur at multiple levels including, supraspinal, suprasacral and in the level of sacral nerves.

At present, three main theories have been proposed for OAB and detrusor overactivity; urothelium, myogenic, and neurogenic hypotheses.

Modulatory roles of the urothelium on bladder function are increasingly widely studied. Changes in urothelial expression, function, and mediator release may play a role in developing bladder dysregulation. For instance, NGF levels were increased in the bladder and the urine of patients with PBS/IC, neurogenic and idiopathic detrusor overactivity and in animal models exhibiting OAB symptoms (Lui *et al.* 2009; Ochodnicky *et al.* 2011). NGF and its receptors (TrkA) have been found in the urothelium (Ochodnicky *et al.* 2011; Murray *et al.* 2004). Frias *et al.* reported the role of NGF to decrease threshold of TRPV1 signalling in the bladder and TRPV1 is required for NGF mediated bladder symptoms (Frias *et al.* 2012). Moreover, the suburothelial components including ICC and nerve terminals, which lie beneath the urothelium could form an interaction as a functional unit to cause instability of the detrusor muscles. This speculation is supported by an investigation by Ikeda and Kanai (2008) using an optical mapping approach, showing that a mucosal component (urothelium and lamina propria) play a role to regulate spontaneous detrusor contraction in normal and spinal cord transected rats (Ikeda and Kanai 2008).

Myogenic factors are involved in changes in morphology, excitability and coupling of smooth muscle cells that contribute to unstable detrusor contraction. Unstable bladder contractility in

OAB could derive from changes in smooth muscle morphology. Increased connective tissue between muscle fascicles and dense muscle arrangement have been observed in OAB with or without urge incontinence (Morrison *et al.* 2002). Usually, the muscle bundle of normal bladder is not well electrical coupled. Dense muscle arrangement allows a better of electrical coupling, leading to tetanic contraction. This may explain enhanced spontaneous contractile activity in this pathological condition (Steers and Facs 2002).

Impairment of central control and peripheral sensitization also contribute to OAB symptoms. Changes in neuronal types may be a mechanism of developing urgency in OAB. For instance, silent C-fibres that normally do not fire in the normal bladder may become active and provoke micturition in OAB. Rats and cats with spinal cord injury have been shown to have change in spinal micturition reflex (de Groat 1975; Mallory *et al.* 1989). This has been shown by systemic injection of capsaicin which failed to inhibit the micturition reflex in normal cats, while chronic spinal cord injury cats showed a reduction of spontaneous bladder contraction (de Groat *et al.* 1990). Intravesical application of selective C-fiber neurotoxin e.g., capsaicin and resiniferatoin (RTX) alleviated OAB symptoms (Dasgupta *et al*. 2000). In addition, urgency may be derived from altered afferent threshold leading to bladder afferent hypersensitivity and recruitment of silent nerve fibres which become activated (Steers *et al*. 2002). For instance, Vizzard and colleagues have reported that there was an increase in neuronal NOS expression and this contributes to bladder afferent hyperexcitability in chronic cyclophosphamide (CYP) bladder irritation animals which is a model of chronic bladder pain (Vizzard *et al*. 1997).

The first-line treatment of neurogenic OAB are anti-muscarinic drugs e.g. Oxybutynin and Tolterodine, which improve bladder capacity and delay an initial urge to void. These drugs, however, have some unpleasant advert effects e.g. constipation and dry mouth (Kennelly and Devoe 2008). Another option is chemical denervation. Using neurotoxic substances to denervate afferent nerve fibres e.g. capsaicin, RTX, and Botulinum toxin (BTX), could alleviate bladder unstable symptoms by preventing neurogenic inflammation derived from of neuropeptide/mediator release. However, there is ongoing investigation and further investigation still need to be ensure to use for OAB treatment (Fowler, 1999; Chancellor and de Groat 1999; Schurch, 2000).

1.9 GENERAL AIMS AND OBJECTIVES

As mentioned earlier, 5-HT has been suggested to participate in pathology of the bowel especially in sensory functional disorders (i.e., IBS and IBD). The anatomical and functional relationship between the bowel and the bladder raises an interesting question whether 5-HT plays a modulatory role in regulating bladder afferent activity. In addition, there is little information about how 5-HT and its receptor subtypes might contribute to bladder afferent activity. Understanding the role of 5-HT on both spontaneous and mechanosensitive afferent activity will provide additional information about its modulatory role on bladder sensory function which is beneficial to understand mechanisms involved in bladder sensation. Therefore, the overall aim of this thesis is to investigate the modulatory role of 5-HT on bladder afferent firing in normal mice and in a mouse model of colon-bladder cross-sensitization. The specific objectives are as follows;

1.) Examine 5-HT receptor expression in urothelial cells and study whether 5-HT directly activates urothelial cells *in vitro*

2.) Investigate the effect of 5-HT on bladder afferent firing in both spontaneous and mechanosensitive afferents and identify the 5-HT receptor subtype contribution

3.) Study the effect of 5-HT on bladder afferent firing in colon-bladder cross-sensitization using a TNBS-induced colonic inflammation model

4.) Determine whether the urinary bladder could have an endogenous source of 5-HT

CHAPTER 2 MATERIALS AND METHODS

2.1 ANIMALS

This study was performed in adult male mice (12-16 weeks old, 25-30 g) with C57/BL6 mice background purchased from Charles River (Margate, UK). Mice were acclimatized for at least 7 days in the laboratory animal husbandry unit under 12:12-h light-dark cycle and had free access to water and food. The animals were anaesthetized with isoflurane and humanely sacrificed by cervical dislocation according to UK home office legislation regulating Schedule 1 procedures (Scientific procedure Act 1986).

Thereafter, a midline incision was performed to expose the abdominal visceral organs. For afferent nerve recordings, the mouse pelvic region, including the urinary bladder, kidneys, ureter, and urethra, was immediately excised and placed in cold Krebs bicarbonate solution (composition, mM: NaCl 118.4, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7) which was bubled with 95%O_2/5%CO_2. For PCR, immunohistochemistry, and calcium imaging, the bladder was excised and kept in Krebs solution as stated below.

2.2 *In vitro* EXTRACELLULAR NERVE RECORDING

After killing the mice, a midline abdominal incision was performed and mice were bisected at the L1-L2 spinal levels. The tail, hind limbs and gastrointestinal viscera were removed. Both sides of the pelvic bone and the remaining pelvic region consisting of kidneys, ureters, testes, urethra, and the bladder were transported to the recording chamber (Daly *et al*. 2014). The preparation was continuously perfused at a rate of 5 mL minute^{-1} with an oxygenated (95%O_2/5%CO_2) Krebs bicarbonate solution (described in the previous section) at constant temperature at 35 °C.

Under a dissection microscope (Nikon, SMZ645), the tissue surrounding the urinary bladder was removed without damage to the ureters and the bladder. The ureters were ligated using suture (US7/0) to prevent back flow. The pubic symphysis was centrally cut and opened to expose the urethra. A polythene catheter (0.28 mm) was inserted into the urethra to perfuse the bladder with isotonic normal saline (0.9% NaCl) or pharmacological reagents using a perfusion pump (Genie, Kent, multi-phaser TM model NE-1000) with a rate 100 µL minute^{-1}. The bladder dome was punctured at the apex with a syringe needle (BD microlance^{TM}, 19G 2") and a two-lumen catheter inserted. One arm of the catheter was connected to a pressure transducer (DTXTM plus DT-XX, Becton Dickinson, Singapore) to monitor the intravesical pressure and the other was connected to the 3 way tap to close for bladder filling or open for emptying the bladder.

The bladder afferent nerve bundles (a mixture of pelvic and hypogastric nerves) which run from the base of the bladder to the spinal cord were dissected from distal to the base, cut into a fine branch and placed into a suction electrode which was attached to a Neurolog headstage (NL 100, Digitimer, Ltd, UK) and an AC amplifier (NL104) to amplify the signal (x10,000). The multi-unit nerve signal was filtered (NL125, band pass filter), passed through the noise eliminator (Humbug, Quest Scientific), a power 1401 analogue to digital interface, and visualized on a computer running Spike 2 software (Version 7.1, Cambridge Electronic Design, UK). The multi-unit afferent nerve discharge frequency was quantified using a spike processor (Digitimer D130). The action potentials were counted from the pre-set threshold, which was set

roughly at twice the baseline noise level. The schematic diagram and the photograph of the in *vitro* afferent nerve recording set up are shown in figure 2.1 and 2.2.

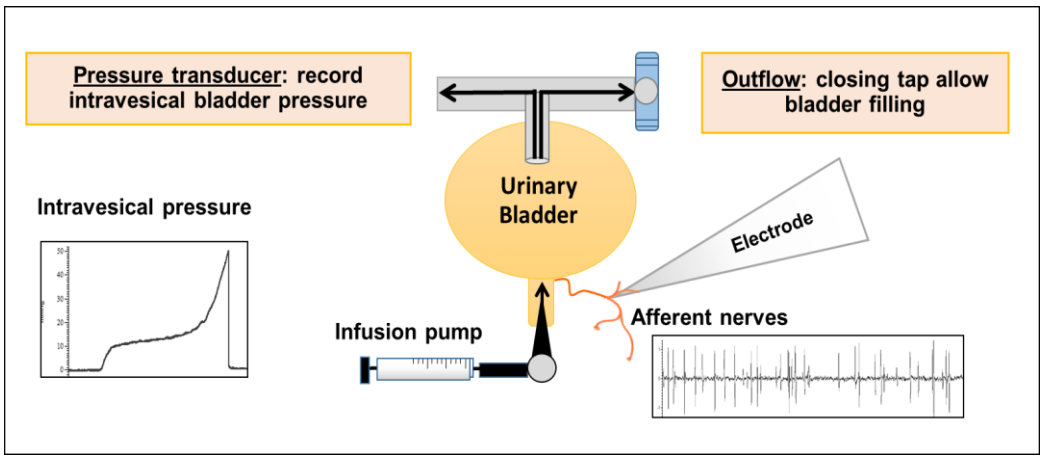


Figure 2.1 *In vitro* afferent nerve recording is shown model to measure afferent nerve activity and intravesical pressure.

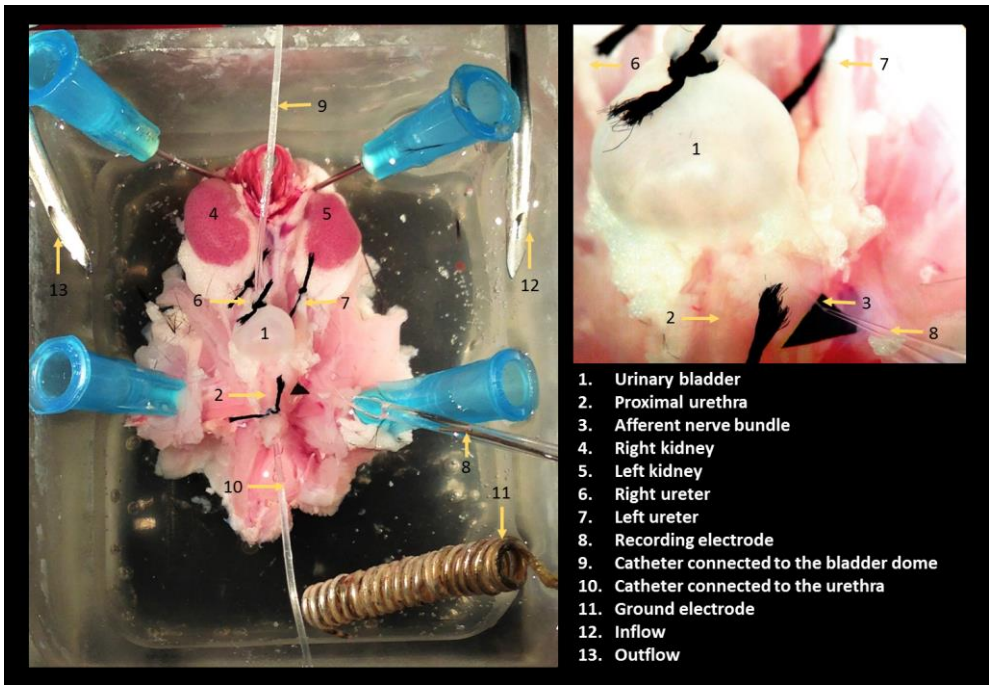


Figure 2.2 Photographs of the *in vitro* afferent nerve recording set up: the bladder afferent nerve bundles and catheters positioned in the bladder dome and urethra are indicated.

2.2.1) Experimental protocols

This part describes the general protocols used in the study. The specific details of the experimental protocols are provided in the methods of each of the result chapters.

1.) Reproducibility of response and control of bladder distension

To evaluate the viability of the afferent nerves, the outflow tap was closed and the urinary bladder was continuously perfused (100 μL minute^{-1}) with normal saline to allow bladder filling until the intravesical pressure reached 50 mmHg when the outflow tap was opened to empty the bladder. Concurrently, the afferent nerve activity was recorded. After the bladder preparation was stabilized for 30 minutes, the bladder was distended by an intravesical infusion with normal saline as described before. The bladder distension was repeated 3 times (10 minutes interval) to establish reproducible bladder afferent responses before starting the experiment (Daly, 2007).

2.) Accommodation of bladder

In order to evaluate the effects of pharmacological reagents on bladder tone, the bladder was filled with normal saline or antagonists to an intravesical pressure of 15 mmHg and the syringe tap was closed. The preparation was stabilized for 30 minutes in order to allow the bladder to accommodate to the change in intravesical volume before application of pharmacological substances.

3.) Applying pharmacological agents

Intravesical application of pharmacological agents

To study the effect of a pharmacological agent on baseline afferent firing, the bladder preparation was continuously perfused via the urethral catheter using a syringe pump with isotonic saline (50 μL minute^{-1}) for 15 minutes to establish a baseline. This was followed by a wash out period with normal saline perfused for 30 minutes.

In order to investigate the effects of pharmacological reagents on mechanosensitive afferent activity, the bladder was perfused with isotonic saline or antagonist at a rate 100 μL minute^{-1} for

30 minutes during which 3 periods of distension were performed (at 10 minutes interval). This was followed by a 30 minute washout during which the bladder distensions were repeated.

Extraluminal application of pharmacological agents

The bladder was distended to an intravesical pressure of 15 mmHg and allowed to stabilize for 30 min. Agonists were dissolved to a pre-determined concentration in Krebs solution which was perfused into the bath (5 mL minute^{-1}) for 20 minutes. Thereafter, Krebs solution was perfused for a further 30 minutes to wash out the drugs. The afferent peak firing and change in intravesical pressure was determined and compared to a control period.

The effects of the pharmacological reagents on mechanosensitive afferent was determined using the distension protocol described above. Following a 30 minute control period with 3 distensions, the bathing medium was switched to Krebs buffer containing drugs dissolved to a pre-determined final concentration for a further 3 distensions before a 30 minute washout period. The afferent response to bladder distension before, during and after drug application was quantified.

2.2.2) Data analysis

Data were analysed either as an absolute level of afferent discharge (spike/sec) or as a percentage change from the control. All data is expressed as mean ± SEM with N representing the number of the animals in each group.

1.) Bladder compliance

Bladder compliance is defined as a relationship between change in bladder volume and change in bladder pressure. In this study the bladder was distended with isotonic saline to an intravesical pressure of 0-50 mmHg and the volume was calculated from time as in equation 1.

$$\text{Equation 1:} \qquad \text{Volume } (\mu L) = \text{Rate } (\mu L \text{ minute}^{-1}) \times \text{Time (minute)}$$

In this study bladder compliance is reflected by the volume infused necessary to generate a particular intravesical pressure. An increase in volume would indicate greater compliance while a decrease in volume would be indicative of increased detrusor tone.

2.) Single unit analysis

The afferent nerve signals recorded in this study were obtained from multi-unit nerve bundles reflecting the firing of a number of distinct single afferent fibres. These single units could be discriminated using the offline spike sorting function of Spike 2 software (version 7.1). Afferent activity was sampled at 25,000 Hz. A spike template was determined from an individual spike with a 2.5 millisecond period and composed of 60 data points. The shape of template spike was used to analyze subsequent spikes and could be matched and colour coded. The same template was used to analyze in all expreriments (Figure 2.3).

After a single unit “wavemark” channel had been obtained, further analysis provided details of firing frequency that could be used in subsequent statistical analysis.

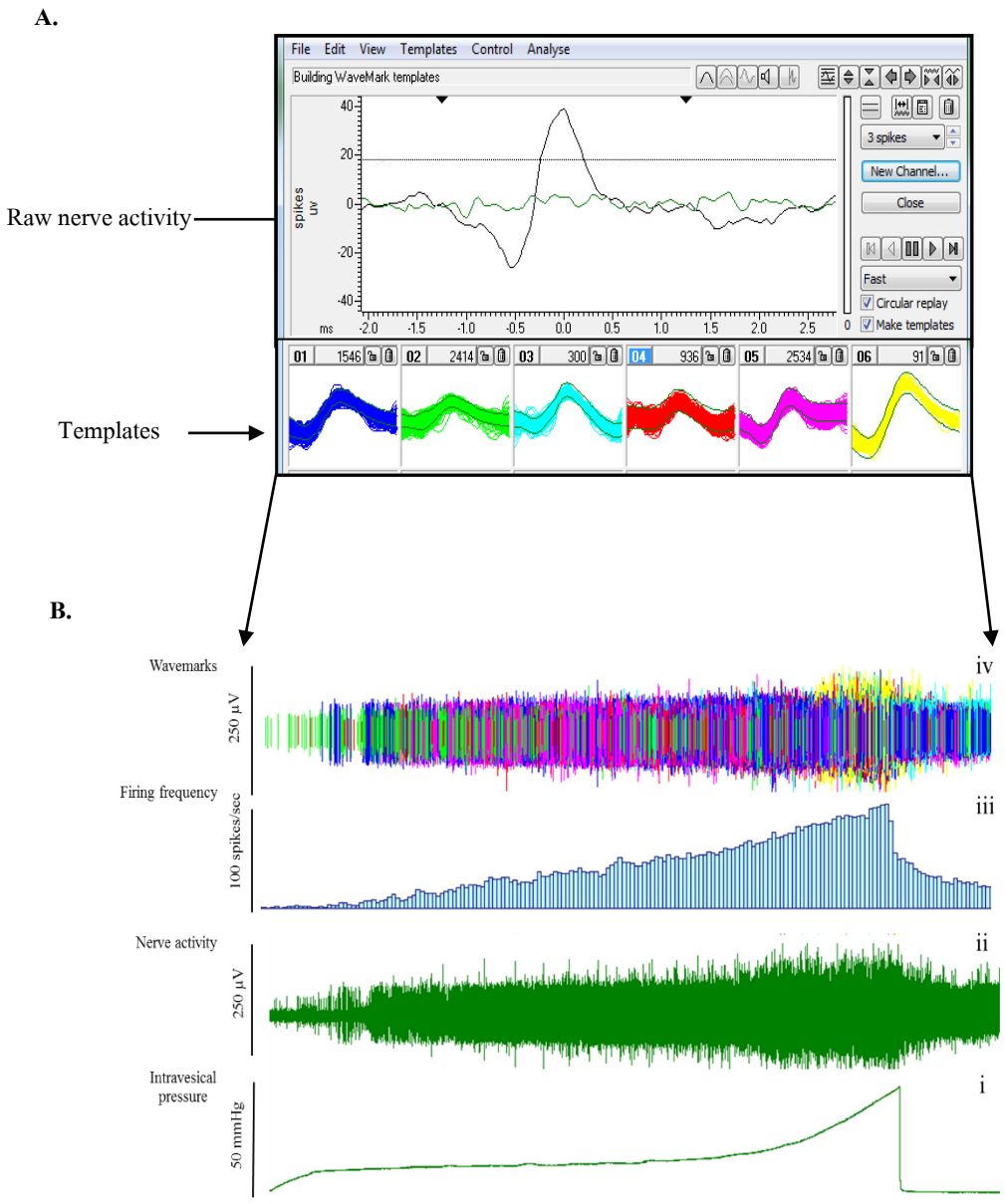


Figure 2.3 Diagramatic representation of single unit analysis. (A) The template generated from the tracing (ii) in B. using off-line function of Spike 2 software. **(B)** An example showing an analyzed bladder afferent firing (ii) in response to pressure rise during bladder distension (i). (iii) is the histogram showing whole nerve firing frequency of spikes passing a pre-set threshold, which is discriminated into the individual single units in (iv).

3.) Principal component analysis (PCA)

Principal component analysis (PCA) was performed after the wavemark analysis in order to ensure the classified single units were sufficiently different to define as a single unit and to demonstrate the variation in spike shapes using offline function from Spike 2 software.

PCA works by identifying the shape of individual single units based on spike shape, amplitude, area, and slope. These analyzed data are normalized and scaled in a 3-dimensional manner and shown as clusters of individual nerve fibres in a different colours. The extent of any overlap between individual clusters could be examined by eye in a 3D display. Overlapping clusters were not classified as distinct fibres.

An example of PCA is shown in figure 2.4. The six clusters of each single unit in figure 2.3 were assigned different colours and coded 01, 02, 03, 04, 05, 06. By examining the stimulus-response to distension it was possible to classify nerve fibres as low threshold fibres (with a threshold <15 mmHg) or high threshold (>15 mmHg) and to reveal firing profile of each single unit in response to drugs.

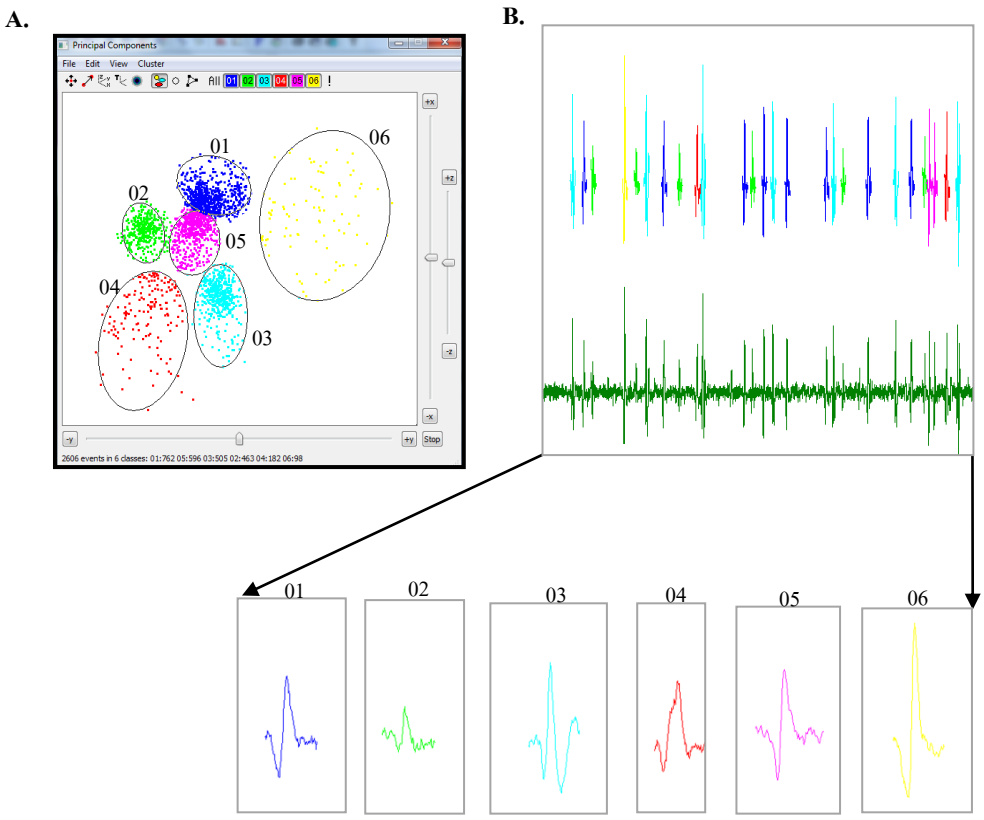


Figure 2.4 Example of principle component analysis. (A) Clusters of 6 individual nerve units representing each template generated by Spike 2 software. **(B)** Wavemark of 6 units and raw spike tracing (green).

2.2.3) Statistical analysis

Various statistical analysis were performed according to experimental design. Student's t test, One-Way ANOVA, and Two-Way ANOVA were considered significant at $P<0.05$. The post hoc analysis was carried out where necessary. All statistical analysis and data plots were performed using Graph Pad Prism (Version 6.00 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com).

2.3 ISOLATION AND CULTURE OF PRIMARY UROTHELIAL CELLS

After cervical dislocation, the visceral organs were removed and the bladder was immediately excised in to fresh Modified Eagle Media (MEM) (Gibco®) containing 0.7% HEPES and 1% Antibiotic-Antimycotic (PSF) solution (Gibco®) at 37°C and transfered to a Sylgard® (Dow Corning) coated dish. The bladder was dissected free of surrounding tissue and cut longitudinally under a stereo microscope to expose the urothelium to the media. The tissue was stretched and pinned. The media was removed and replaced with 2.5 mg/mL Dispase in MEM at 37°C. The bladder was incubated in Dispase for 2 hours at room temperature in the tissue culture hood. After the Dispase had been aspirated, the urothelium was gently scraped with a scalpel under the stereo microscope and the cells were immediately placed in 0.5% trypsin-EDTA (Gibco®). The solution was incubated at 37 °C for 10 minutes, and gently triturated every a few minutes. The trypsin-EDTA was deactivated by adding pre-warm MEM with 10 % Fetal Bovine Serum (FBS) (Gibco®). The cell suspension was centrifuged at 1500 rpm, 4 °C for 15 minutes. The solution was gently aspirated and the pellet was resuspended in pre-warmed Keratinocyte-serum free medium (K-SFM) and centrifuged at 1500 rpm, 4 °C for 15 minutes.

For calcium imaging experiment, the cells were resuspended in K-SFM 200 µL and plated on collagen IV (Sigma Aldrich Poole, UK) coated coverslips in a 12 wells plate and incubated in 5% CO_2-95% O_2 at 37 °C overnight. For PCR, the cell pellet was washed by adding PBS and centrifuged at 1000 rpm, 4 °C for 5 minutes and stored at -80 °C.

2.4 CALCIUM IMAGING

After an overnight incubation of primary urothelial cells at 37 °C, the cells were examined for viability. Cells were incubated with 2 µM Fura 2 acetoxymethyl ester (Fura 2-AM) (Sigma-Aldrich (Poole, UK)) for 30 minutes at 37 °C in the dark. Coverslips with cells were then sealed on a chamber platform (Series 20, Warner Instruments, Hamden, CT, USA). Excitation lights were generated by OptoLED light source (Cairn Research Limited, Kent, UK). Fluorescent intensity of Fura2 was monitored and recorded using a digital camera (C4742-95 Hamamatsu

Corporation, Sewickley, PA, USA), a Hamamatsu camera controller and a computer with SimplePCI software (Version 6.6.0.0, Hamamatsu Corporation).

The cells were continually perfused by gravity with HEPES buffer (composition, mM: HEPES 10, NaCl 135, KCl 15, glucose 10, $CaCl_2$ 2, $MgCl_2$ 1) (1.5 mL $minute^{-1}$) to stabilise and remove any excess Fura 2-AM for 30 minutes before imaging started. The cells was continuously perfused with HEPES for 3 minutes to record the baseline. Thereafter cells were continuously perfused with HEPES containing the pharmacological reagents for 3 minutes and then washed again with HEPES for 15 minutes. At the end of experiment, the calcium ionophore ionomycin (Sigma-Aldrich (Poole, UK)) (5 μM) was applied to the cells as a positive control. Only urothelial cells that responded to ionomycin were included in the analysis. The whole experiment was carried out in the dark and at room temperature.

Data analysis

Intracellular calcium was determined by the ratio of emission fluorescence under 340 nm and 380 nm excitation light. The response of urothelial cells to the drugs was reflected by the change in intracellular calcium calculated as the net difference between baseline 340/380 ratio and the peak ratio during drug application. All data were expressed as mean ± SEM.

2.5 INVESTIGATION OF GENE EXPRESSION

2.5.1) RNA extraction

RNA from mouse urothelial cells and tissues i.e., dorsal root ganglion (DRG) neurons, small intestine and brain was extracted to investigate mRNA expression. RNA purification was performed using a RNeasy Mini Kit (QIAGEN 74104) according to manufacturer's instructions. The tissues up to 20 mg was disrupted and homogenized with TRIzol® reagent in 1.5 mL Pestle and Microtube (VWR Labshop, 47747-366) using a Pestle Motor (VWR Labshop, 47747-370). Isolated RNA was eluted in RNase-free volume 30-50 μL. The RNA concentration and quality were determined by the Thermo Scientific NanoDrop™ 2000c spectrophotometer and the NanoDrop 2000 software version 1.3.1 at absorbance wavelength of 260 and 280 nm. In addition, RNA quality was determined as 260/280 nm ratio in the range of 1.8-2.0. The extracted RNA samples were stored at -80°C.

2.5.2) Reverse transcription

RNA samples was reverse transcribed into cDNA by High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, 4374966) according to manufacturer's instructions. Reverse transcription mix was prepared in a PCR tube (25 µL) kept on ice as shown in table 2.1. The reaction mixture was run using a thermal cycler (TECHNETC-3000X, Stone, UK). A detail of reverse transcription programme is shown in table 2.2. The synthesized cDNA was immediately used for PCR reaction or kept at -20 °C for storage.

Table 2.1 Component of cDNA synthesis reaction mix (for 20 µL per reaction)

Component	Volume/Reaction (µL)
10× RT Buffer	2
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2
MultiScribeTM Reverse Transcriptase	1
RNase Inhibitor	1
Nuclease-free H_2O	3.2
RNA	10
Total	**20**

Table 2.2 Reverse transcription thermal cycle for cDNA synthesis

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

2.5.3) Conventional reverse transcription polymerase chain reaction (RT-PCR)

mRNA expression of interested genes was examined using RT-PCR. PCR reactions were prepared using Gotaq® Green Master Mix (Promega M7121) according to manufacturer's instructions as shown in table 2.3.

Table 2.3 Components of RT-PCR reaction (for 25 µL per reaction)

Component	Volume/Reaction (µL)
GoTaq® Green master mix	12.5
Primers Forward	1.25
Primers Forward	1.25
cDNA	(for 50 ng RNA)
Nuclease-free H_2O	Adjust volume to 25
Total	**25**

All the steps were performed on ice and avoiding DNase contamination. After mixing and spin down, the reaction mixture was incubated in a thermal cycler machine in three steps of the PCR cycle. The thermal changes were continued for 36 cycles The PCR product was either directly studied or kept at -20 °C.

The PCR product (10 µL) and DNA ladder (5 µL) were separated by 1.5% gel electrophoresis under electric field of 100 mA and 120 volts from power supply (MINIS-150VS, Fisher Scientific) for 50 minutes.

The gel was visualized under UV light by a UV transilluminator (Gel Doc™ EZ Imager, BIO-RAD, UK) and the ImageLab software. The gel was photographed to determine the expression of the interested genes. The details of genes and primers are described in subsequent chapters.

2.5.4) Quantitative real-time PCR

The quantification of mRNA expression of genes of interest was determined using TaqMan Gene Expression Master Mix (Applied Biosystems 4374657) according to the manufacturer's instruction as shown in table 2.4. The reaction was prepared on ice and mixed in Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates (BIO-RAD, HSP-9665). Prior to running the reactions, the plates were covered with MicroAmpTM Optical Adhesive Film for 96-Well Plates (Applied Biosystems, 43111971) and centrifuged briefly to spin down the contents and eliminate any air bubbles from the solutions. The reaction for each sample and gene was run in duplicate. DNase free water was used to replace cDNA as blank for each gene and plate. PCR reactions were performed in BIO-RAD CFX96 TouchTM Real-time system (C1000 TouchTM Thermal Cycler, Bio-Rad Laboratories Ltd. Hercules, USA) with the thermal cycling programme as summarized in table 2.5.

Table 2.4 Components of quantitative real-time PCR reaction (for 20 µl per reaction).

Component	Volume/Reaction (µl)
TaqMan Gene Expression Master Mix	10
Probe	1
cDNA	1
Nuclease-free H_2O	8
Total	**20**

Table 2.5 Thermal cycle of real-time PCR used in this study.

Step	UDG incubation	AmpliTaq Gold®, UP Enzyme Activation	PCR	
	Hold	Hold	Cycle (40 Cycles)	
Time	2 minutes	10 minutes	Denature	Anneal/Extend
Temp	50 °C	95 °C	15 seconds	1 minute

Data analysis

Cycle threshold (Ct) refers to the number of the cycle required to reach the point that a specific PCR product is amplified in a linear way, and was determined for each of the genes of interest. Higher Ct value indicated lower levels of mRNA expression. The level of mRNA expression of genes of interest was determined relative to the house keeping gene, ß-actin. The calculation was demonstrated as follows:

Equation 2: ΔCt = Ct tested gene – Ct housekeeping gene

Fold change refers to the relative change of gene expression between different samples and was calculated as follows:

ΔCt sample 1 = Ct tested gene in sample 1 – Ct housekeeping gene in sample 1

ΔCt sample 2 = Ct tested gene in sample 2 – Ct housekeeping gene in sample 2

$\Delta\Delta$Ct = ΔCt sample 1 – ΔCt sample 2

Fold change = $2^{-\Delta\Delta Ct}$

Fold change was used to indicate change of gene expression in CVH animals compared to healthy control animals.

2.6 IMMUNOHISTOCHEMISTRY

2.6.1) Tissue fixation and cryo-embeding

The jejunum and bladder were collected and cleaned in phosphate buffer (0.01M PBS, made from tablet, Sigma P4417), and then the whole tissue was fixed in 4% paraformaldehyde (PFA) at 4 °C overnight. The tissue was cryoprotected through a series of sucrose gradients (10, 15, and 30%), overnight in each solution or until the tissue sunk to the bottom of the vial. Thereafter, the tissue was placed into moulds filled with optimal cutting temperature compound (OCT, Bright Instrument Company, 53581), and bladder and jejunum orientated in a longitudinal and transverse direction respectively. The moulds were kept on dry ice until OCT was fully frozen and stored at -80°C (Figure 2.5).

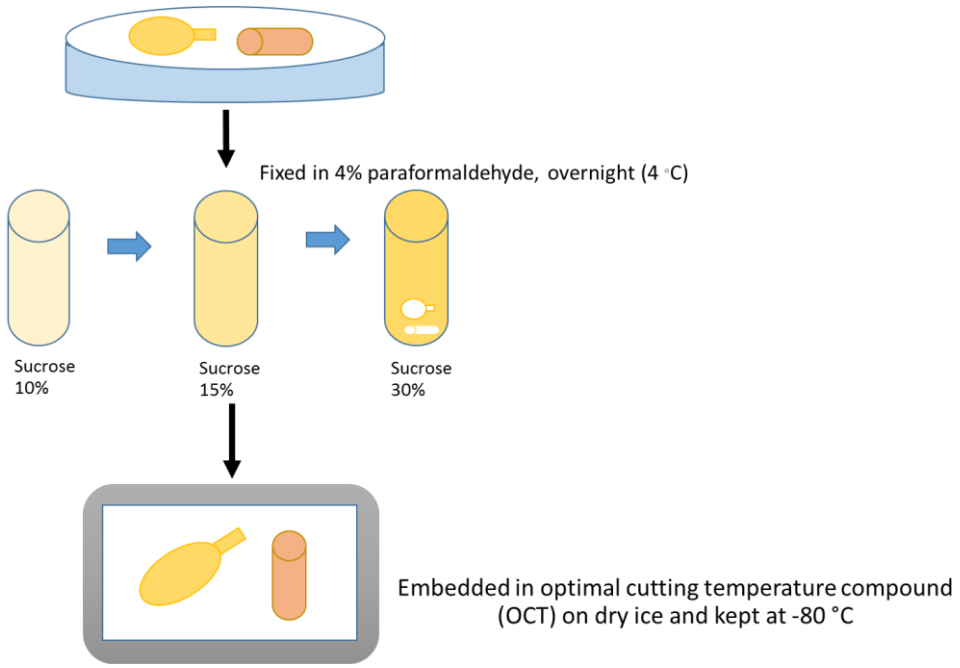


Figure 2.5 Diagram shows bladder and jejunum fixation and cryo-embeding processes.

2.6.2) Cryo-sectioning

The OCT blocks containing tissues were sectioned in a cryostat (Bright Instrument, OTF5000, Huntingdon, UK) at 15 µm. The cryostat was set up at -20°C for specimen temperature and -15°C for the chamber. The whole tissue was sectioned and labelled in order and stored at -20°C.

2.6.3) Immunostaining

The sectioned slides were left at room temperature for 15 minutes before placing in a closed humid box. The slides were washed with PBS for 5 minutes (3 times) to remove OCT. Prior the staining, sections were quenched with 50 mM NH_4Cl (VMR International, A893078) for 20 minutes and permeabilized with 0.1% Triton X for 30 minutes. To block non-specific staining, the slides were incubated with blocking solution for 20 minutes. The blocking solution was made from 5% normal serum of the species in which the secondary antibody was raised. The primary antibody was applied to the slides and covered with parafilm. For each experiment, a negative control was performed, in which the tissue was incubated with blocking solution instead of the primary antibody. The slides was incubated with the antibody at 4 °C overnight. Prior to the application of secondary antibody, slides were rinsed 3 times with PBS to wash any excess primary antibody. Following this, slides were incubated with fluorophores conjugated secondary antibodies for 2 hours at room temperature. Slides were mounted with coverslips using VECTASHIELD Mounting Medium with DAPI (Vector, H1200). The slides were either imaged immediately or kept at 4 °C for later analysis.

2.6.4) Microscopy and image analysis

The stained sections were observed under an Olympus BX51 microscope (Tokyo, Japan). Images were captured using an Olympus ColorView II digital camera. The images were displayed by ImageJ software (1.43u, National Institutes of Health, USA). The light wavelength for excitation and emission of DAPI, FITC, and Fluor 594 are 358/461, 490/525, and 590/617, respectively.

2.7 PHARMACOLOGICAL AGENTS AND SOLUTIONS

The pharmacological reagents used in this study are listed in table 2.6. All the reagents were dissolved in distilled water (dH_20) and Krebs solution where possible or DMSO (Sigma®) to make a stock solution. The regents were either diluted in isotonic normal saline (for intraluminal application) or Krebs solution (for extraluminal application) to get the working concentration. Details of the concentration used are described in the results sections.

Table 2.6 List of pharmacological agents used in this study

	Compounds	Main action	Company	Catalogue number	Solvent to make stock solution
1	5-Hydroxytryptamine (5-HT)	5-HT receptor agonist	Sigma	H9523	dH_2O
2	Granisetrone hydrochoride	5-HT3 receptor antagonist	LKT Laboratories	G6802	dH_2O
3	5-Methoxytryptamine (5-MT)	Full agonist for 5-HT receptor except 5-HT3	Sigma	M6628	dH_2O
4	2-Methy5-hydroxytrptamine (2-Me-5-HT)	5-HT3 receptors agonist	Tocris/Bioscience	0558	dH_2O
5	ML-9	Myosin light chain kinase inhibitor	Cayman Chemical	10010236	DMSO
6	Y-27632	Rho kinase inhibitor	Chemdea	CD0141	dH_2O
7	Citalopram hydrobromide	Selective 5-HT reuptake inhibitor	LKT Laboratories	C3477	dH_2O
8	L-NG-Nitroarginine methyl ester (L-NAME)	Nitric oxide synthase inhibitor	Sigma	N5751	dH_2O

CHAPTER 3

EXPRESSION OF 5-HT RECEPTORS AND THE FUNCTIONAL ROLE OF 5-HT IN MOUSE UROTHELIUM

3.1 INTRODUCTION

A significant role for urothelial cells in modulating bladder function has recently gained support. Several studies have addressed the idea that urothelial cells are not only a passive barrier to protect the urinary bladder from ions and solutes in urine, but also act as sensory epithelium. Urothelial cells express an array of ion channels and receptors on their cell surface which allows the cells to detect mechanical, chemical, and thermal stimuli and transduce these signals by releasing an array of mediators (Apodaca 2004; Birder and Andersson 2013; Merrill *et al.* 2016). The mediators released from the urothelial cells could either exert autocrine action to bind to their receptors expressed on urothelial cells themselves or act in a paracrine manner binding to receptors expressed on nearby cells, including smooth muscle cells, suburothelial nerve plexuses and ICC (Birder, 2011; Birder and Andersson 2013; Merrill *et al*. 2016; Varley *et al.* 2005).

A recent study has reported a direct regulatory role of serotonergic signalling in mouse urothelium. Matsumoto-Miyai and co-workers highlight that 5-HT could modulate bladder distention-induced ATP release from urothelial cells. Using the specific 5-HT1D antagonist, GR-127935 and 5-HT4 antagonist, SB204070, they showed that 5-HT1D receptor inhibits distension-induced ATP release, whereas, 5-HT4 receptor facilitates the ATP released. They also examined mRNA expression of all 5-HT subtypes in mouse urothelial cells. 5-HT1D, 2A, 4, and 6 were detected in the urothelial cells. Moreover, 5-HT1A, 1B, 1D, 2A, 2B, 2C, 3A, 4, and 7 have been reported in urothelial-denuded tissues (Matsumoto-Miyai *et al.* 2016).

In contrast, mRNA expression of 5-HT3A and 3B receptors has been reported in the bladder mucosa (Chetty *et al.* 2007). There is much less information of 5-HT receptor expression in human urothelial cells. Only 5-HT2A receptor has been found in the human urothelium (Ochodnick *et al.* 2012). This reported expression of 5-HT receptors may suggest that 5-HT may exert a sensory role in urothelial cells.

5-HT receptors have also been demonstrated in the detrusor smooth muscle cells and DRG neurons. Unpublished data of Matsumoto-Miyai *et al.* (2015) shows that 5-HT1A, 1B, 1D, 2A, 2B, 2C, 3A, 3B, 4, and 7 are expressed in mouse detrusor smooth muscle cells. Nicholson and colleagues (2003) studied mRNA expression of 5-HT receptors by in situ hybridisation and found that 5-HT1B, 1D, 2A, 2B, 2B, 3A, 3B, and 4 are expressed in rat DRG neurons (Nicholson *et al.* 2003). A summary of 5-HT receptor expression in urothelial cells, detrusor smooth muscles, and DRG neurons is shown in table 3.1

5-HT receptors have also been demonstrated to play a sensory role in other organs. In the gastrointestinal tract, 5-HT1A, 3, and 4 are detected in myenteric and submucosal neurons, ICC, and enteroendocrine cells using immunohistochemistry (Glatzle *et al.* 2002). Mechanical stimulation of mucosa evoke release of 5-HT which further activates 5-HT4 receptors in human sensory CGRP neurons and 5-HT3 and 5-HT4 in guinea pig colon, which are involved in the initiation of the peristaltic reflex (Foxx-Orenstein *et al.* 1996). 5-HT4 receptor activation has been shown to inhibit visceral hypersensitivity in colorectum (Greenwood-Van Meerveld *et al.* 2006; Hoffman *et al.* 2012) and gastric distension-induced visceral pain (Seto *et al.* 2011). In addition, 5-HT4 receptors are also expressed in the epithelial cells of other hollow organs including ovary (Henriksen *et al.* 2012), airway (Murphy *et al.* 2013), and in the mucosa of the esophagus (Yang *et al.* 2012).

Table 3.1 5-HT receptors expression profile in bladder afferent pathway (modified from Matsumoto-Miyai *et al.* 2015)

5-HT subtypes	Urothelium (mouse)[a]	Detrusor muscle (mouse)[b]	DRG neurons (rat)[e]	Urothelium (human)[c]	Whole bladder (rat)[d]
5-HT1A	-	+	n.d.	-	-
5-HT-1B	-	++	√	n.d.	n.d.
5-HT1D	++	++	√	n.d.	n.d.
5-HT2A	+	++	√	+	++
5-HT2B	-	+	√	n.d.	++
5-HT2C	-	++	n.d.	n.d.	++
5-HT3A	+*/-	++	√	n.d.	-
5-HT3B	+	+	√	n.d.	n.d.
5-HT4	++	+	√	n.d.	++
5-HT5A	-	-	n.d.	n.d.	-
5-HT6	+	-	n.d.	n.d.	-
5-HT7	-	+	n.d.	n.d.	++

+ moderate expressions, ++ strong expression, - negative results, both positive and negative results, n.d. not determined

[a] Matsumoto-Miyai *et al*. 2016

[b] Unpublished data of Matsumoto-Miyai *et al.* 2015

[c] Ochodnick *et al*. 2012

[d] Sakai *et al.* 2013

[e] Nicholson *et al.* 2003

* Chetty *et al.* 2007

Taken together, the above evidence suggests the important of 5-HT receptors in bladder sensory function. However, especially in the urothelial cells, the expression profile of the receptors are still contradictory. It is also interesting to investigate further whether 5-HT receptors expressed in the urothelial cells could directly respond to 5-HT. Therefore, this chapter aimed to examine mRNA expression profile of 5-HT receptor subtypes in urothelial cells using RT-PCR and examined the direct effect of 5-HT on mouse urothelial cells by calcium imaging.

3.2 EXPERIMENTAL PROTOCOL AND ANALYSIS

mRNA expression of 5-HT receptors in mouse urothelial cells

As described in section 2.5, RNA was extracted from mouse urothelial cells and mRNA expression of all subtypes of all 5-HT receptors was determined by RT-PCR (N=3). The list of primers are summarised in table 3.2. ß-actin was used as a housekeeping gene and loading control. cDNA sample from mouse DRG was used as a positive control for 5-HT1B, 1D, 1F, 2A, 2B, 2C, 3A, 3B, 4, and 7. cDNA sample from mouse brain was used as a positive control for 5-HT1A, 5A, 5B, and 6. For negative control reactions distilled water was used instead of cDNA samples. The mRNA expression was defined to be positive if it was consistent for all three urothelial samples.

DNA sequencing of 5-HT receptors subtypes expressed in mouse urothelial cells

To confirm nucleotide sequences of 5-HT receptors expressed in mouse urothelial cells, PCR product of 5-HT 1A, 1B, 1D, 2A, 2B, 4, 6, and 7 was amplified and extracted from the gels. The extracted DNA sample of each gene was sent to a sequencing service (Faculty of Medicine, the University of Sheffield) to determine the nucleotide sequences. The obtained sequences for each gene were aligned with the expected PCR product and the homology was determined by Chromas Lite 2.1.1 and Clone manager 9.0 software. The percentage match of the PCR product sequences to the genes of 5-HT receptors subtypes was determined to confirm the reliability of the PCR results.

Effect of 5-HT and granisetron on mouse urothelial cells

The direct effect of 5-HT on urothelial cells was investigated using calcium imaging, which represents changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). Detailed calcium imaging protocols and an explanation of the methodology are described in chapter 2 (section 2.4). The methods of urothelial preparation are described in section 2.3. The experimental protocol was summarised in figure 3.1. Following a control period of HEPES buffer perfusion for 3 minutes, 5-HT (100 μM) was constantly perfused into the chamber for 3 minutes. Thereafter the urothelial cells were washed with HEPES buffer for 15 minutes. Finally, ionomycin (5 μM) was applied into the chamber as a positive control to check cell viability.

In a subset of experiments cells were pre-incubated with granisetron (1 μM), a selective 5-HT3 antagonist before 5-HT application. In order to determine the level of urothelial cells response to 5-HT, we compared 5-HT response to ATP. ATP diluted in HEPES (10 μM) was perfused on to cells for 90 seconds. After a wash out period of 15 minutes, the cells were perfused with ionomycin (5 μM) (Figure 3.1).

Fura-2 is excited at 340 nm and 380 nm of light, and emitted at 510 nm. Intracellular Ca^{2+} change is indicated by the fluorescent intensity ratio at wavelength 340 and 380 nm. The background intensity of each cell was subtracted from the response to obtain a mean change in fluorescence intensity (ΔRF). The maximal response during 5-HT and ATP application was calculated as percentages comparing to maximal response to ionomycin and fractional change from baseline ratio of 340/380, which was calculated from area under the curve in equation 3 using Graph Pad Prism software. Data were expressed as mean ± SEM.

Equation 3: Fractional change = (peak response value – baseline value)/baseline value

Table 3.2 Summary of primer sequences used in RT-PCR

Receptor subtypes	Accession number	Primer sequences	Product size (bps)	Positive control
5-HT1A	NM_008308	FW 5' TAAGAACTTCCCGCTCCAGT 3'	103	Brain
		RW 5' AGAAATGCAAGGGGATCTCC 3'		
5-HT1B	NM_010482	FW 5' CCAACACACAATAAATGCTCCT3'	135	DRG
		RW 5' CCAAGTCAAAGTGCGAGTCT 3'		
5-HT1D	NM_008309	Fw 5'TACAAACACCCCTACTAAACGC 3'	310	DRG
		Rw 5'ATGAGTGTTCAGCGTTGGTT 3'		
5-HT1F	NM_008310	Fw 5'GACCAGAGCCCCTTAGCTTC 3'	340	DRG
		Rw 5'TGCAGCTTCCGAGTCACAAT 3'		
5-HT2A	NM_172812	FW 5' CATCTCCCTGGACCGCTAC 3'	150	DRG
		RW 5' TCATCCTGTAGCCCGAAGAC 3'		
5-HT2B	XM_006529146	FW 5' CCGATTGCCCTCTTGACAAT 3'	120	DRG
		RW 5' GGCACAGAGATGCATGATGG 3'		
5-HT2C	NM_008312	FW 5' TGAAACTGGTTGCTTAAAACTGA 3'	126	DRG
		RW 5' AGCTGCTACTGGACTTATGGA 3'		
5-HT3A	NM_013561	FW 5' CCACCTTCCAAGCCAACAAG 3'	128	DRG
		RW 5' CTCCCTTGGTGGTGGAAGAG 3'		
5-HT3B	NM_020274	FW 5' TGATTCTTCTGTGGTCCTGC 3'	154	DRG
		RW 5' GCCTCAGCCCAGTTGTAAAC 3'		
5-HT4	NM_008313	FW 5' ATGTTCTGCCTGGTCCGG 3'	162	DRG
		RW 5' GCCTCCCAACATTAATGCGA 3'		
5-HT5A	NM_008314	FW 5' AAGACCAACAGCGTCTCCC 3'	124	brain
		RW 5' TCCACGTATCCCCTTCTGTC 3'		
5-HT5B	NM_010483	Fw 5'TCTCCTTCGACGTGTTGTGC 3'	469	brain
		Rw 5'GAGTCTCCGCTTGTCTGGAA 3'		
5-HT6	NM_021358	Fw 5'TGGGCAAAGCTCGAACATCT 3'	386	brain
		Rw 5'GTCACATACGGCCTGAGCTAT 3'		
5-HT7	NM_008315	Fw 5'AAGTTCTCAGGCTTCCCACG 3'	485	DRG
		Rw 5'CAGTTTTGTAGCACAAACTCGCT 3'		

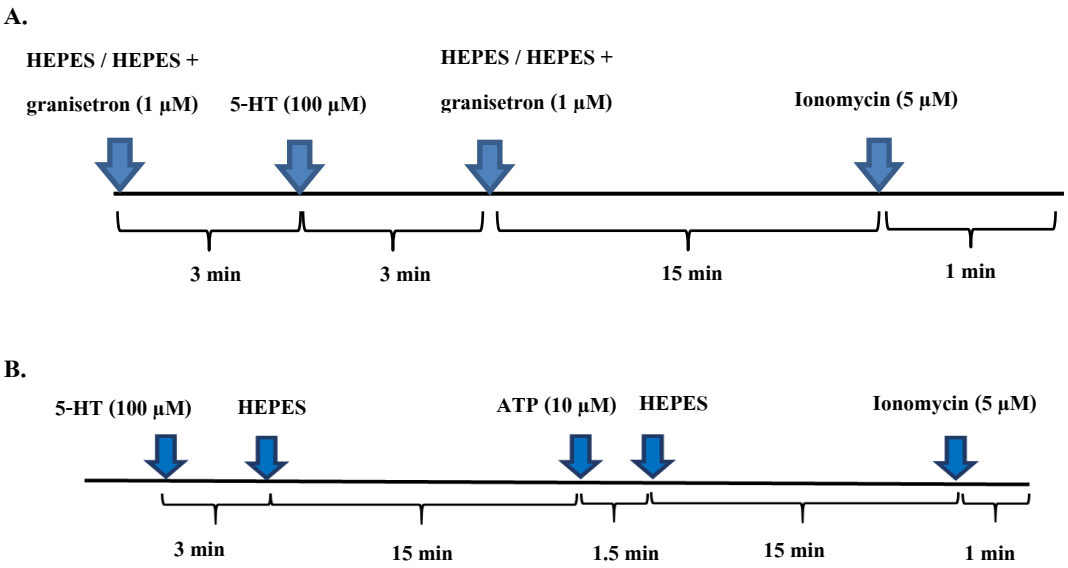


Figure 3.1 Protocols of calcium imaging experiment used in this study. (A) Protocol used to investigate the effect of 5-HT on intracellular Ca^{2+} change in mouse urothelial cells. **(B)** Protocol used to investigate the effect of 5-HT and ATP on intracellular Ca^{2+} change in mouse urothelial cells.

3.3 RESULTS: mRNA EXPRESSION OF 5-HT RECEPTORS IN MOUSE UROTHELIUM

The bands of PCR products from RT-PCR are shown in figure 3.2. mRNA expression 5-HT1A, 1B, 1D, 2A, 2B, 4, 6, and 7 was detected in mouse urothelial cells (N=3) but not 5-HT 1F, 2C, 3A, 3B, 5A, and 5B.

DNA sequencing of 5-HT receptor subtypes expressed in mouse urothelial cells

The percentage match of the PCR product sequences to the genes of 5-HT receptors subtypes was between 96-100% confirming the validity of the primers. mRNA expression of each receptor subtype in mouse urothelial cells (Table 3.3).

Figure 3.2 mRNA expression of 5-HT receptor subtypes in mouse urothelial cells. 5-HT1A, 1B, 1D, 2A, 2B, 4, 6, 7 but not 5-HT 1F, 2C, 3A, 3B, 5A 5B were found in mouse urothelial cells. Urothelial cells for three mice were collected and mRNA expression was determined. ß-actin was used as a housekeeping gene and either brain or DRG neurons was used as positive control. Negative control was similar PCR reaction to others but there was no cDNA in the reactions (N=3).

Table 3.3 Percent match between PCR product sequences of 5-HT receptors subtypes and the sequences from the database

5-HT receptors subtypes	Percent matches
5-HT1A	100
5-HT1B	98
5-HT1D	99
5-HT2A	96
5-HT2B	96
5-HT4	100
5-HT6	98
5-HT7	100

3.4 RESULTS: EFFECTS OF 5-HT ON MOUSE UROTHELIAL CELLS

Since many 5-HT receptor subtypes were found in mouse urothelial cells at gene expression level, it was of interest to investigate whether the expressed 5-HT receptors directly exert their functional response to 5-HT. The response in urothelial cells was compared between 5-HT (100 μM), 5-HT with pre-incubation of granisetron (1 μM), and ATP (10 μM).

A representative image of cultured urothelial cells and sample trace of calcium imaging in response to 5-HT are shown in figure 3.3. 5-HT triggered an increase of intracellular Ca^{2+} (Ratio 340/380). In addition, there was no difference in relative fluorescent ratio between 5-HT and 5-HT with pre-incubation of granisetron (5-HT, 57.11 ± 2.68% vs. granisetron+5-HT, 63.20 ± 1.17%) (Figure 3.4A), which is consistent with analysis of determining fractional change from baseline of ratio 340/380 (5-HT, 0.22 ± 0.01 vs. granisetron+5-HT, 0.26 ± 0.02) (Figure 3.4B). Percentages of responding cells to 5-HT and granisetron pre-incubation was also not different (5-HT, 65.14 ± 10.71% vs. granisetron+5-HT, 70.31 ± 10.51%) (Figure 3.4C). These results indicate that 5-HT could directly bind to non-5-HT3 receptors expressed in the urothelium and trigger an increase in intracellular Ca^{2+} level.

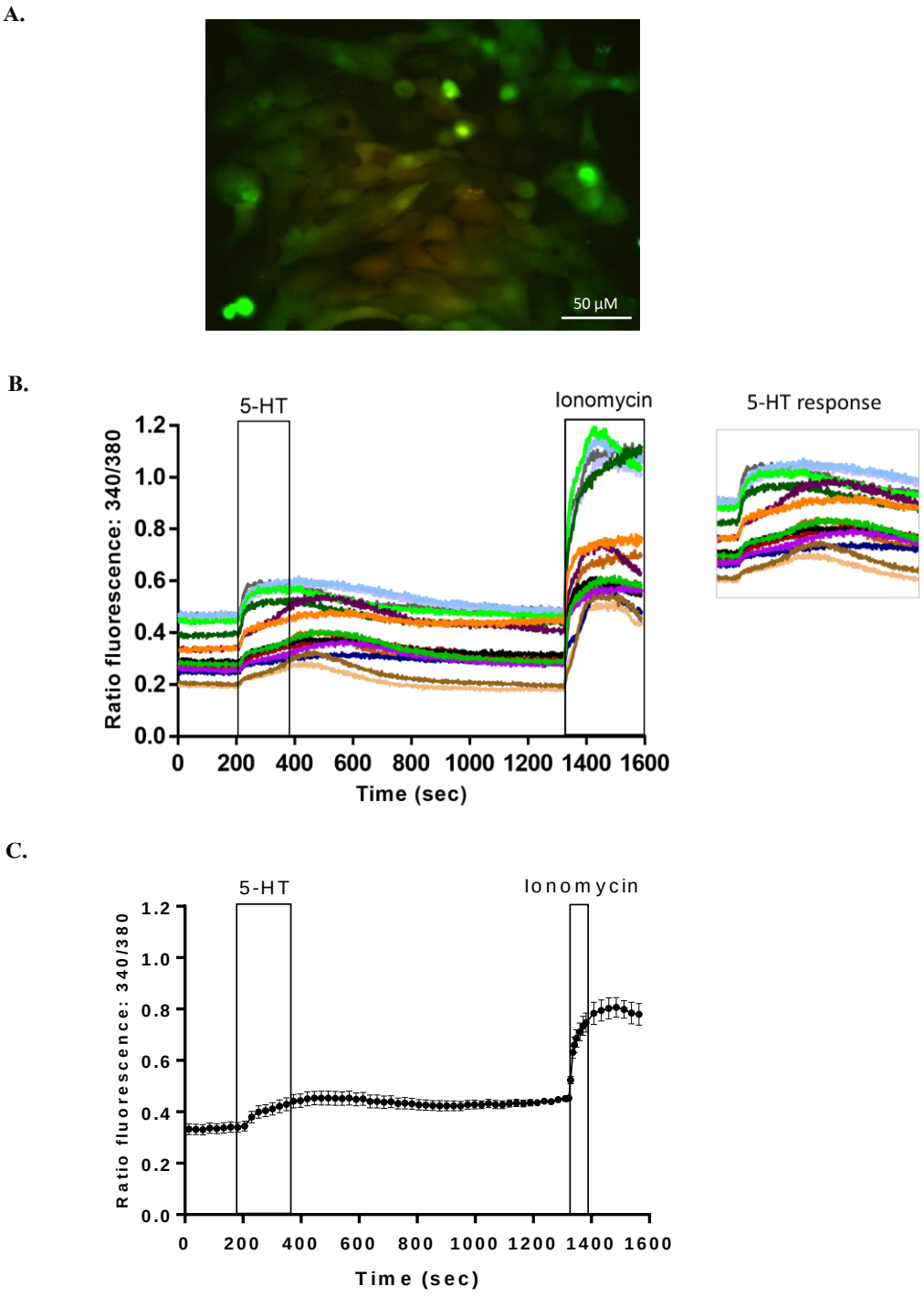


Figure 3.3 Calcium imaging of mouse urothelial cells. (A) a representative image showed urothelial cells labelled by Fura-2. Green and orange colours are software-generated colours to discriminate difference of intracellular Ca^{2+} concentration **(B)** Sample traces showing typical urothelial cell response to 5-HT (100 µM) and ionomycin (5 µM) in calcium imaging experiment. **(C)** Mean ± SEM change in intracellular Ca^{2+} levels of urothelial cells response to 5-HT (100 µM) from a single experiment (n=15).

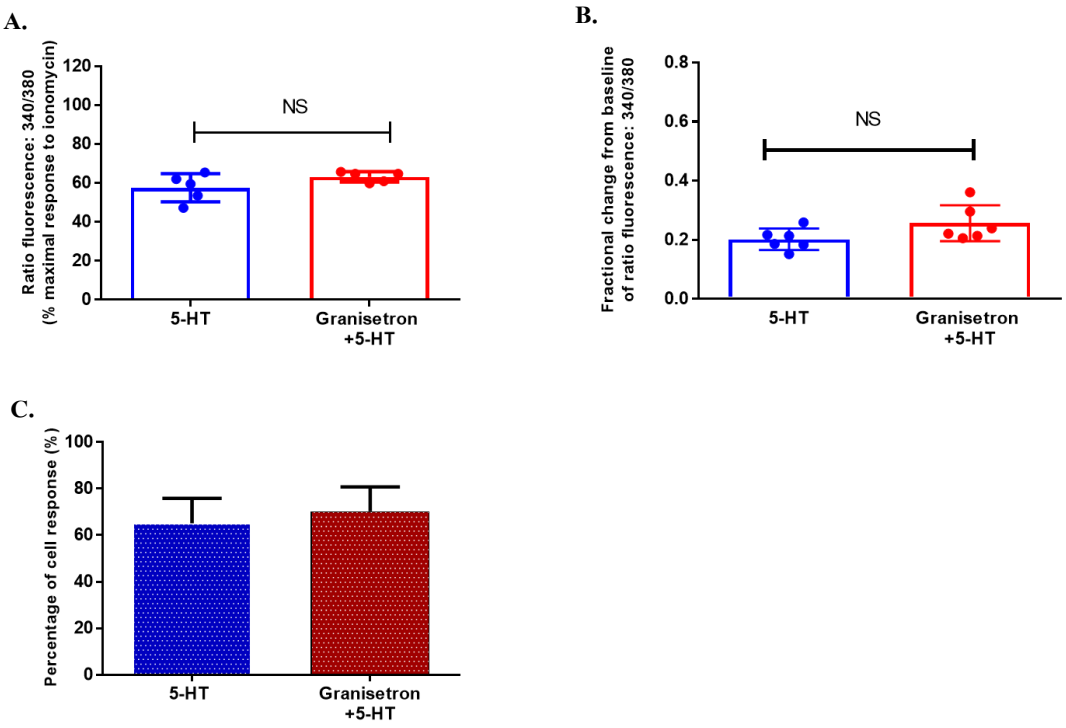


Figure 3.4 5-HT stimulated mouse urothelial cells through non-5-HT3 receptors. (A) Relative increase of intracellular Ca^{2+} (Ratio 340/380) in response to 5-HT and granisetron pre-incubation **(B)** Fractional change from baseline of ratio 340/380 in response to 5-HT and granisetron pre-incubation **(C)** Percentages of responding cells to 5-HT after pre-incubation of granisetron, paired Student's t-test, (5-HT, N=6, n=423 vs. granisetron+5-HT, N=5, n=163), unpaired Student's t-test. N refers to the number of animal and n indicates number of cells.

In addition, pattern and percentages of cell responses to 5-HT and ATP was determined. A representative trace is shown in figure 3.5. 5-HT and 5-HT with granisetron pre-incubation triggered an increase in relative fluorescence ratio less than ATP (5-HT, 57.11 ± 2.68%, granisetron+5-HT, 63.20 ± 1.17% vs. ATP, 89.69± 3.69%) (Figure 3.6A), which is consistent with analysis of determining fractional change from baseline of ratio 340/380 (5-HT, 0.22 ± 0.01 vs. granisetron+5-HT, 0.26 ± 0.02, ATP 0.54 ± 0.02) (Figure 3.6B). Interestingly, time to peak response of 5-HT was significantly longer than ATP (5-HT, 225 ± 21.19 sec, ATP, 92.29 ± 16.07 sec) (Figure 3.6C). The total percentage of cells responding to ATP was 96.5%. 44.8% of cells responded to both 5-HT and ATP. 3.5% of the cells responded to only 5-HT (Figure 3.6D).

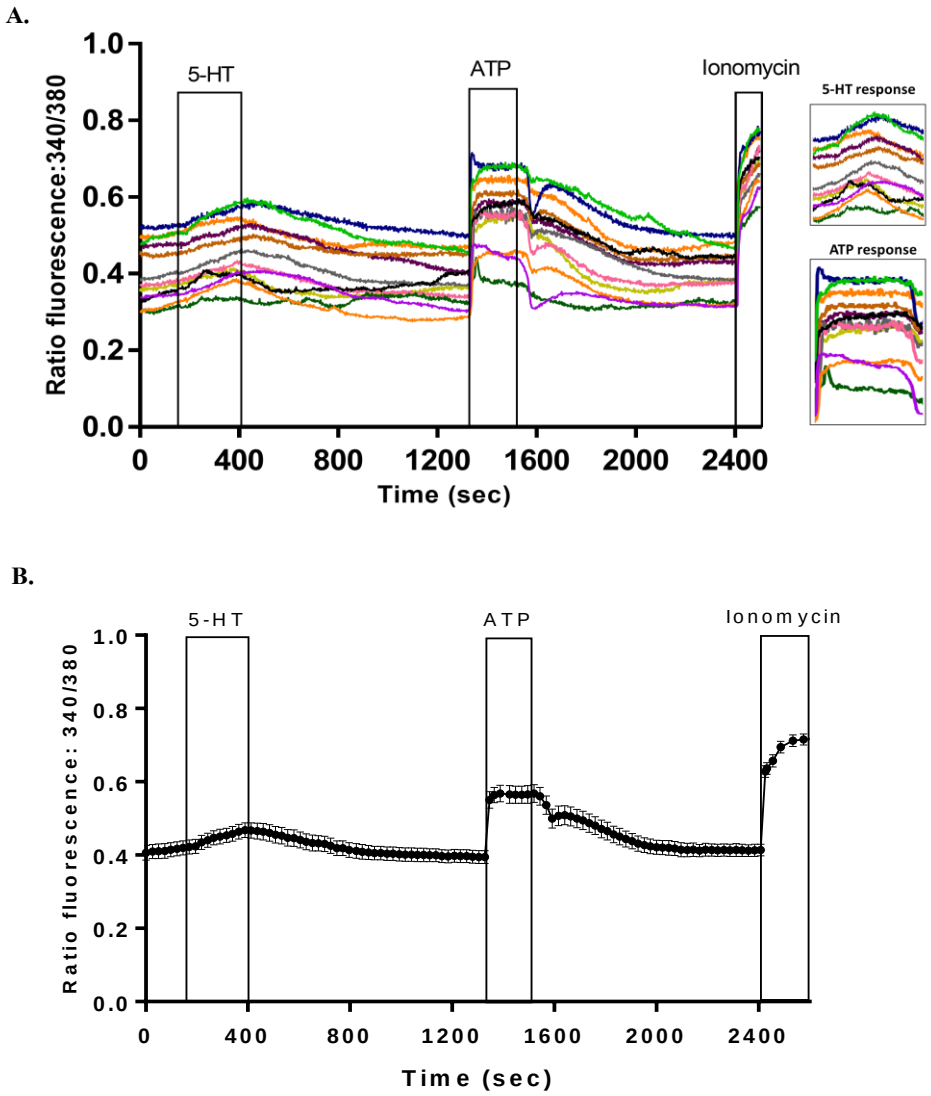


Figure 3.5 Urothelial cell response to 5-HT was lesser than ATP. (A) Sample traces showing typical urothelial cell response to 5-HT (100 µM), ATP (10 µM) and ionomycin (5 µM) **(B)** Mean ± SEM change in intracellular Ca^{2+} levels of urothelial cells response to 5-HT, ATP (10 µM) and ionomycin (5 µM from a single experiment (n=12).

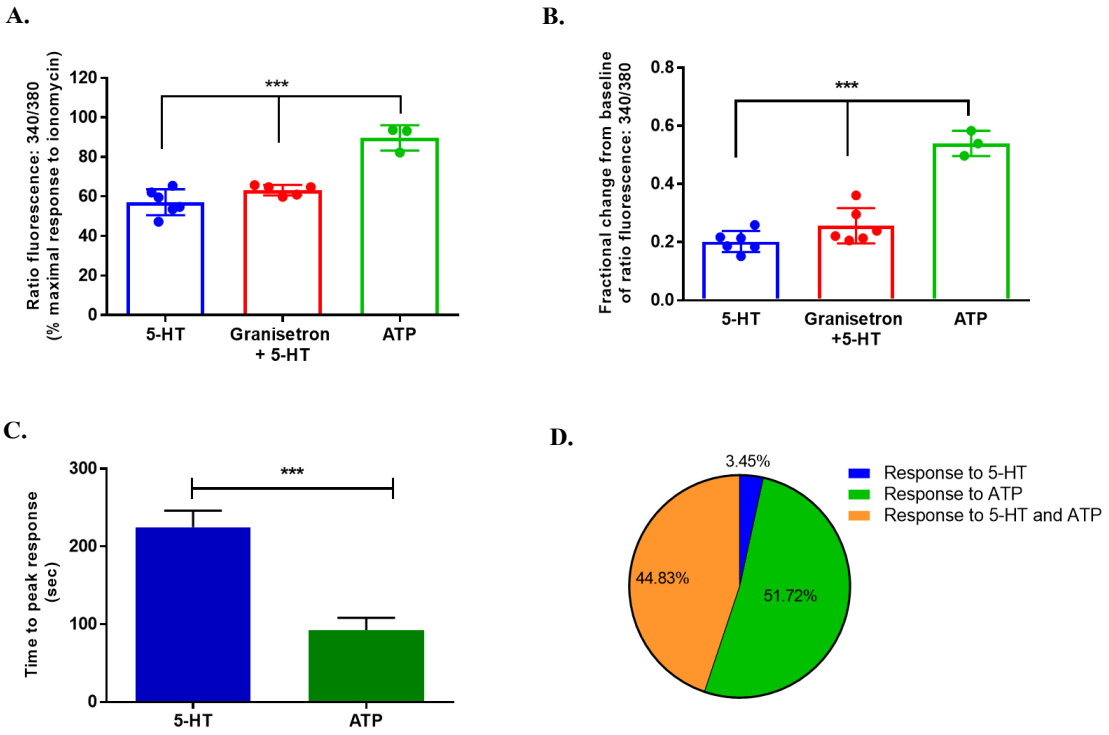


Figure 3.6 Urothelial cell Ca^{2+} responses to 5-HT were reduced relative to ATP. (A) Relative increase in intracellular Ca^{2+} (Ratio 340/380) in response to 5-HT, 5-HT plus granisetron, and ATP **(B)** Fractional change from baseline of ratio 340/380 in response to 5-HT, 5-HT+granisetron and ATP (One-way ANOVA with Tukey multiple comparison, ***P<0.001, N=6, 5, 3 respectively) **(C)** Time to peak response to 5-HT and ATP of the responding cells (***P<0.001, paired Student's t-test) **(D)** Proportion of urotheial cells response to 5-HT, ATP, and both 5-HT and ATP (N=3, n=88). N refers to the number of animal and n indicates number of cells.

3.5 DISCUSSION

The main findings in this chapter are

1.) mRNA of 5-HT 1A, 1B, 1D, 2A, 2B, 4, 6, and 7 but not 5-HT 1F, 2C, 3A, 3B, 5A, and 5B were expressed in mouse urothelial cells.
2.) 5-HT directly activated mouse urothelial cells by a mechanism that is not blocked by granisetron and therefore it is unlikely to be 5-HT3 mediated.

5-HT3 receptors are not expressed on mouse urothelial cells.

In this study we found that 5-HT 1A, 1B, 1D, 2A, 2B, 4, 6, and 7 mRNA, which are all G-protein coupled receptors, were detected in mouse urothelial cells. This correlated to the study of Matsumoto-Miyai and co-workers (2016), which reported mRNA expression profile in urothelium and urothelium-denuded bladder in mice using RT-PCR. The summarised table comparing 5-HT receptor expression in this study and Matsumoto-Miyai *et al.* 2016 is shown in table 3.4.

In the urothelium, 5-HT1D and 5-HT4 receptors are strongly expressed, whereas 5-HT2A and 5-HT6 show weak expression levels. A variety of 5-HT receptor subtypes (5-HT 1A, 1B, 1D, 2A, 2B, 2C, 3A, 4, and 7) were also detected in urothelium-denuded bladder (Matsumoto-Miyai *et al.* 2016). We could also detect 5-HT1D and 5-HT4 subtypes in the urothelial cells, however, we found broader types of the receptors in urothelial cells.

The partial contradiction in PCR result compared to the study by Matsumoto-Miyai and colleagues could be derived from using different primers. Different sets of primers and PCR conditions possibly contribute to a different efficiency to detect and amplify genes. The method used to isolate urothelial cells could also be a reason that we did not detect 5-HT3 receptors in urothelial cells as reported in the study by Chetty and colleagues. The present study determined the expression profile only in the urothelial cells, while in Chetty's study mucosa tissues include lamina propria which could contain other cells expressing 5-HT3 receptors. Another possibility that we could not detect 5-HT3 receptors is the level of 5-HT3 receptors expression in urothelial

cells is very low relative to the other subtypes. However, all the PCR products of detected 5-HT receptors were checked by DNA sequencing and all genes showed a high percentage matching (96-100%) to the original sequences, suggesting that the designed primers are efficient and the detected 5-HT receptor results were valid.

Table 3.4 Summary table of 5-HT receptor subtypes mRNA expression in mouse urothelial cells in this study compared to Matsumoto-Miyai *et al.* 2016.

5-HT subtypes	Urothelium (Mitamamoto *et al.* 2016)	Urothelium (This study)
5-HT1A	-	+
5-HT-1B	-	+
5-HT1D	++	+
5-HT2A	+	+
5-HT2B	-	+
5-HT2C	-	-
5-HT3A	+/-	-
5-HT3B	+	-
5-HT4	++	+
5-HT5A	-	-
5-HT5B	?	-
5-HT6	-	+
5-HT7	-	+

One potential concern of this study is that the collected samples might contain non-urothelial cells. The contamination of non-urothelial cells was previously detected using gene markers of certain cell types such as vimentin for myofibroblasts (Drake *et al.* 2006), c-Kit for ICC (Davidson and McCloskey 2005), and desmin for smooth muscle cells (Council and Hameed 2009). However, the technique used to collect the urothelial cells in this study is a standard method which is also used in other studies (Zeitz *et al.* 2002; Daly *et al.* 2014; Nocchi *et al.* 2014; Matsumoto-Miyai *et al.* 2016). As described in chapter 2 (section 2.3), the urothelial cells

collection was performed gently with concern during cell scraping steps to avoid a contamination of suburothelial cells and smooth muscle cells.

A key finding in this investigation is that there was no ligand-gated 5-HT3 receptors expressed in mouse urothelial cells. Mitsomoto-Miyai and co-workers also reported an absence of 5-HT3 receptors expressed in the urothelial cells, but present in the rest of the bladder (Matsumoto-Miyai *et al.* 2016), which could be the detrusor smooth muscle (Chetty *et al*. 2007). In addition to the detrusor muscle, 5-HT3 receptors have been reported in mucosal layers in mouse bladder (Chetty *et al.* 2007). Apart from the urothelial layers, the suburothelial layers which contains several types of neuronal and non-neuronal cells i.e., fibroblast-like cells, ICC, blood vessels, and the nerve endings (Birder 2011). Kidd and co-workers reported 5-HT3 receptor expression in afferent terminals within spinal dorsal laminae of rats (Kidd *et al.* 1993). Moreover, 5-HT3 receptor expression has been reported in ICC in intestine where it plays a role in enhancing pacemaker activity (Liu *et al.* 2011). It is possible that the other candidate cells that expressed 5-HT3 receptors in suburothelial layers could be efferent and/or afferent nerve terminals and ICC. ICC lining in the lamina propria and the detrusor muscle layers (Davidson and McCloskey 2005) integrate sensory information from the urothelial cells to the detrusor smooth muscle and also modulate spontaneous activity of the detrusor muscles (McCloskey, 2010).

5-HT directly stimulated non-5-HT3 receptors in mouse urothelial cells.

This is the first study to demonstrate that 5-HT directly triggered an increase in intracellular Ca^{2+} level in the urothelial cells. Granisetron had no effect on urothelial responses to 5-HT. This result supports our RT-PCR data, which suggesting that there are no 5-HT3 receptors expressed in mouse urothelial cells. 5-HT receptor candidates which might play a role in triggers intracellular Ca^{2+} could be 5-HT1A, 1B, 1D, 2A, 2B, 4, 6, and 7. However, some of these receptors exert different transduction pathways. Only 5-HT 2A and 2B are reported to directly augment intracellular Ca^{2+} via coupling to Gq/11 protein and increase IP_3 level, whereas 5-HT4, 6, and 7 are reported to couple with Gs protein and further increase cAMP level. 5-HT1A, 1B, and 1D are reported to couple to Gi/o, which contributes to decrease in cAMP level (

Matsumoto-Miyai *et al.* 2015). This suggests that more than one signal transduction pathways may be involved in 5-HT mediated an increase in intracellular Ca^{2+} in the urothelial cells.

It was clearly showed in the sample tracing that a different pattern of the response has been observed and the majority of 5-HT responded cells (~97%, 37 out of 38 cells), also responded to ATP. Therefore the possibility that 5-HT signalling may also couple to other pathways cannot be excluded. A recent study has established that 5-HT4 receptor play a role in facilitating distension-induced ATP release from mouse urothelium (Matsumoto-Miyai *et al.* 2016). The 5-HT stimulated increase in intracellular Ca^{2+} observed in the present study could possibly be due to ATP release which further acts on P2X receptors expressed on the urothelial cells.

However, our investigation clearly showed that the pattern and time to peak response between 5-HT and ATP was totally different. 5-HT took longer to get the maximum intracellular Ca^{2+} response, whereas the urothelial responded to ATP was more rapid and showed sharp peak response. This different phenotypes of the response is correlated to the characteristic of non-5-HT3 receptors which couple through G-protein pathway and require time to activate second messenger pathway in order to release intracellular Ca^{2+}. In contrast, ATP could directly activated ionotropic P2X receptors and rapidly trigger Ca^{2+}influx (North, 2002).

The heterogeneity of the urothelial cells could contribute to heterogeneity of the pattern of cell response. The mix of different populations of urothelial cell subtypes (umbrella cells, intermediate cells, and basal cells) (Apodaca, 2004; Lewis, 2000) may have different 5-HT receptor distribution. Therefore, this might be a reason that some urothelial cells failed to respond to 5-HT or showed only small increases in intracellular Ca^{2+}.

Taken together, these data demonstrated that various 5-HT receptors are expressed on the mouse urothelium and could exert a functional response to mediate intracellular Ca^{2+} increase. The striking finding is relevant to the next chapter is the absence of 5-HT3 receptor expression and the failure of granisetron to attenuate the response to 5-HT in mouse urothelial cells. The direct modulatory role of 5-HT in urothelial cells could play roles in sensory functions of mouse urinary bladder.

CHAPTER 4

EFFECT OF 5-HT ON BASELINE AND MECHANOSENSITIVE BLADDER AFFERENT FIRING

4.1 INTRODUCTION

Activation of afferent nerves is the first step to send sensory information along the afferent pathway in order to control bladder function. Hyperexcitability of bladder afferents is related to bladder symptoms i.e. pain, urgency, and frequency (Yoshimura *et al.* 2003). As described in chapter 3, various subtypes of 5-HT receptors are expressed in urothelial cells, suggesting 5-HT could play roles in sensory function of the urinary bladder. This chapter addresses the functional role of 5-HT on bladder afferent firing.

All 14 subtypes of 5-HT receptors have been reported to exert regulatory function on the bladder as summarized in table 4.1. 5-HT mediates a different role on bladder function at urothelium, efferent nerve terminals, detrusor muscle, and possibly at the afferent terminals. A number of studies have addressed the peripheral actions of 5-HT on bladder functions as depicted in figure 4.1. Matsumoto-Miyai and co-workers (2016) have addressed regulatory role of 5-HT on ATP release from mouse urothelium during urine storage phase. 5-HT has an inhibitory role in distension-induced ATP release mainly via 5-HT1D receptor, whereas 5-HT4 facilitates the release of ATP at earlier stage (Matsumoto-Miyai *et al.* 2016).

Table 4.1 Transduction pathways and bladder effect of 5-HT receptor subtypes (adapted from Matsumoto-Miyai *et al.* 2015)

5-HT subtypes	Transduction pathway	Smooth muscle (Postjunctional)	Autonomic excitatory nerve terminals (Pre-junctional)	Urothelium	Central pathway
5-HT1A	Gi/o cAMP ↓	Contraction	↓ Cholinergic transmission		↓ Micturition reflex and external urethral sphincter (EUS) (cat); ↑ Micturition reflex and tonic EUS activity (spinal)/bursting EUS activity (supraspinal) (rat)
5-HT-1B	Gi/o cAMP ↓				↓ Rhythmic bladder activity
5-HT1D	Gi/o cAMP ↓			↓ ATP release	
5-HT2A	Gq/11 IP_3 ↑, $[Ca^{2+}]i$ ↑	Contraction	↑ Neurogenic contraction; ↑ Purinergic transmission		↑ Micturition reflex and EUS activity
5-HT2B	Gq/11 IP_3 ↑, $[Ca^{2+}]i$ ↑	Contraction			
5-HT2C	Gq/11 IP_3 ↑, $[Ca^{2+}]i$ ↑	Contraction	↑ Neurogenic contraction		↓ Micturition reflex
5-HT3A	Ligand-gated cation channel	Contraction	↑ Neurogenic contraction; ↑ Cholinergic transmission		
5-HT3B	Ligand-gated cation channel				
5-HT4	Gs cAMP ↑		↑ Neurogenic contraction; ↑ Cholinergic transmission (human)/ purinergic (guinea pig) transmission	↑ ATP release	
5-HT5A	Gi/o cAMP ↓				
5-HT6	Gs cAMP ↑				
5-HT7	Gs cAMP ↑	Relaxation (Pig bladder neck)	↑ Neurogenic contraction; ↑ Cholinergic transmission		↑ Micturition reflex (supraspinal)

Many studies have displayed the roles of 5-HT receptors in detrusor smooth muscle. The 5-HT1A antagonist (WAY-100635) could not prevent 5-HT-induced bladder strip contraction in female rats (Sakai *et al*. 2013). In contrast, the 5-HT1A receptor agonist, 8-hydroxy-2-(di-n-propylamino)tetralin, 8-OH-DPAT-induced isolated detrusor contraction and this effect was reversed by an 5-HT1A antagonist (Mittra *et al.* 2007) in bladder outlet obstruction (BOO) rats. 5-HT2 subtypes have been reported to induce muscle contraction in rats (Kodama and Takimoto 2000) and dog (Cohen 1990). In BOO rat model, 5-HT2A and 5-HT2B antagonists reversed 5-HT-induced contraction (Sakai *et al*. 2013a). A 5-HT3 receptor agonist inhibited detrusor smooth muscle contraction after pre-incubation of the nerve blocker lignocaine, whereas 5-HT3 receptor activation elicited potentiation of bladder contraction following electrical field stimulation in mouse bladder strips, indicating that excitatory 5-HT3 receptors are located in efferent nerve terminals, whereas inhibitory 5-HT3 receptors are located in detrusor smooth muscle (Chetty *et al.* 2007). Both 5-HT4 and 5-HT7 receptor antagonists, GR113808 and SB269970 respectively, failed to prevent 5-HT-induced detrusor contraction in rats (Sakai *et al.* 2013). An opposite action of 5-HT7 receptor has been reported in the electrical field stimulation of the detrusor muscle which the 5-HT7 receptor antagonist, SB258741 antagonized 5-HT-evoked bladder strip contraction in rats (Palea *et al.* 2004). These opposing results could possibly be due to the use of different 5-HT7 antagonists and have variation in the experimental technique used to stimulate the muscle strips. However, 5-HT7 receptors have been established to play a role in smooth muscle relaxation in the pig bladder neck (Recio *et al.* 2009).

In addition to postjuctional sites on detrusor muscle, many 5-HT receptor subtypes are reported to regulate the bladder at prejuctional sites on efferent fibron. 5-HT1A receptors inhibited ACh-release by EFS in isolated human detrusor strips (Agostino *et al.* 2006). 5-HT2A activation in guinea pigs and 5-HT2C activation in rats facilitated EFS-induced neurogenic contractions (Messori *et al.* 1995; Rekik *et al.* 2011). 5-HT2A receptors play a role in purinergic transmission of 5-HT-induced detrusor muscle contraction in guinea pigs (Messori *et al.* 1995). 5-HT3 receptor agonists (2-Me-5-HT and 1-PBG) dose-dependently potentiated neurogenic contraction induced by EFS in mice (Chetty *et al.* 2007). In isolated guinea pig detrusor muscles, 5-HT3 receptors are reportedly involved in ACh release in 5-HT potentiated detrusor contraction by electrical stimulation (Messori *et al.* 1995). 5-HT4 and 5-HT7 receptors are also reported to facilitate neurogenic contraction. However, 5-HT4 mechanisms are different

between. In guinea pigs, 5-HT4 receptor activation leads to a potentiation of purinergic transmission (Messori *et al.* 1995), whereas in humans it is reported to facilitate ACh-release from efferent terminals (Candura *et al.* 1996). 5-HT7 receptors also facilitate ACh-release in human urinary bladder (Agostino *et al.* 2006).

On afferent nerves, Chen and co-workers examined 5-HT subtypes mRNA expression in rat embryonic sensory neurone cultures. Various 5-HT subtypes including 5-HT1B, 1D, 1F, 2A , 2C, 3, 4, 5A, and 5B receptors expressed in embryonic DRG neurons (Chen *et al.* 1998). 5-HT3A and 3B are expressed in afferent terminals in dorsal laminae of the spinal cord (Schmitt *et al*. 2006) and in L4-L5 DRG (Chen *et al.* 1998). In addition, 5-HT3 receptors have been highlighted to play a key role in developing afferent hypersensitivity in post-inflammatory jejujnum mouse model (Keating *et al.* 2008).

Zagorodnyuk *et al.* (2009) conducted functional afferent recording in the bladder. They classified types of mechanoreceptors using von Frey hair stimulation and reported that muscle-mucosal mechanoreceptors are the major group (~75%) that responded to direct application of 5-HT (100 μM) in guinea pigs (Zagorodnyuk *et al*. 2009). In addition, the binding sites of 5-HT1A, 1B, and 3 in the dorsal horn of the spinal cord were decreased in dorsal rhizotomy (Laporte *et al.* 1995). This raises the interesting possibility that these 5-HT receptors are transported to the distal afferents in the bladder wall and play a role in bladder sensory pathways.

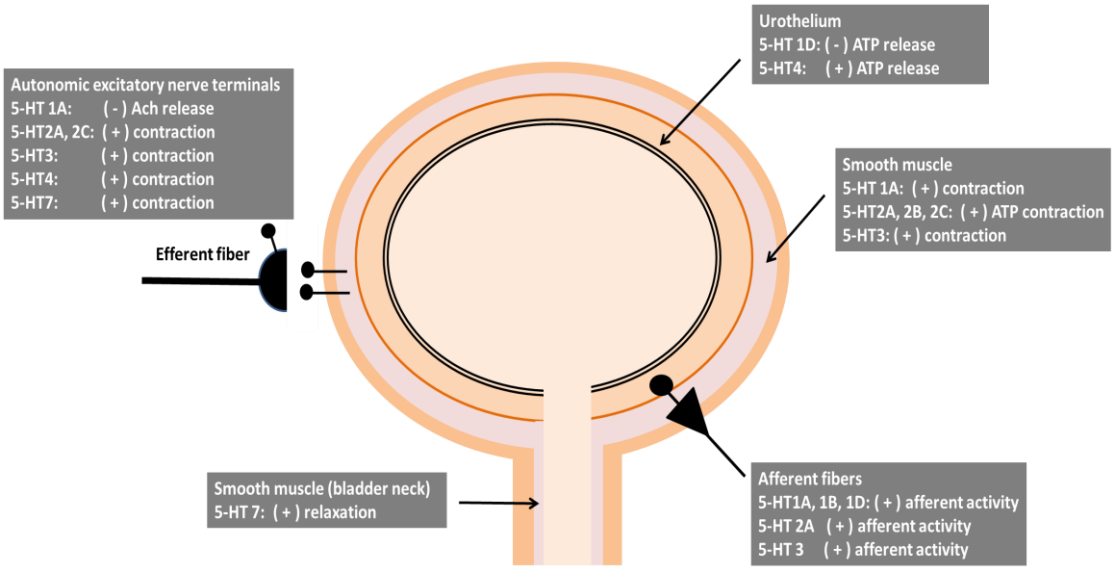


Figure 4.1 Schematic diagram summarising peripheral 5-HT receptors expression and function in the urinary bladder (modified from Matsumoto-Miyai *et al.* 2015).

In response to chemical and mechanical stimulation, urothelial cells release a number of mediators including ATP (Kullmann *et al.* 2008), ACh, and nitric oxide (NO) (Birder 1998). A previous study showed that the NO donor, S-nitroso-N-acetyl-penicillamine (SNAP) and the NO substrate L-arginine, inhibited baseline and mechanosensitive firing in cyclophosphamide (CYP) rats and these effects were reversed by the nitric oxide synthase (NOS) inhibitor, L-N^G-Nitroarginine methyl ester (L-NAME, 20 mM) (Yu and De Groat 2013). NOS has been reported to be expressed in efferent nerves (Vizzard *et al.* 1994), detrusor muscle (Anderson and Persson 1994), afferent nerves (Vizzard 1997), and urothelium (Birder *et al.* 1998; Seo *et al.* 2014). It would be interesting to investigate further if NO could be produced either from urothelium, smooth muscle, or afferent nerves and affect afferent firing.

It has been hypothesized that 5-HT affects bladder afferent discharge either in a direct action on the afferent nerve terminals or secondary to 5-HT induced bladder contraction. 5-HT3 receptors may play a role in bladder afferent firing and NO may be produced and affect bladder afferent firing.

Objectives

This study aimed to investigate the effects of 5-HT on bladder afferent firing and identify the contribution of the 5-HT receptor subtypes that contribute to 5-HT action using an *in vitro* extracellular recording.

4.2 EXPERIMENTAL PROTOCOL AND ANALYSIS

Extracellular nerve recordings were performed to investigate the effect of 5-HT and related pharmacological substances on bladder afferent nerve activity. The detail of the set-up is outlined in chapter 2. After a control ramp distension period, different experimental protocols were used, which are described in detail in each protocol figure.

The effect of drugs on baseline afferent firing was determined by measuring peak firing during the drug application. Mechanosensitive afferent firing was determined by performing bladder-distension evoked afferent firing and the afferent discharge at various intravesical pressure points was determined. In addition spontaneous afferent firing which is the afferent firing occurring between the ramp distensions was determined from the area under the curve (AUC) which was normalized and expressed as a percentage change relative to the control period.

All afferent recordings were subtracted from baseline firing, 3 minutes before drug application. This baseline subtraction was employed to eliminate variability between the bladder preparations. Data are presented as mean ± SEM. Statistical analysis includes Student's t-test, One-way ANOVA, and Two-way ANOVA which were applied in different experiments as described in each result figure.

4.3 RESULTS: EFFECT OF 5-HT, 2-ME-5-HT, GRANISETRON AND 5-MT ON BASELINE AFFERENT FIRING

4.3.1) EFFECT OF 5-HT ON BASELINE AFFERENT FIRING

Firstly, bladder afferent firing in response to 5-HT (10 µM and 100 µM) was determined. The protocol is shown in figure 4.2. After a control period of three ramp distensions, the bladder was constantly perfused with isotonic saline (50 µL minute^{-1}) for 15 minutes to allow the accommodation of the perfusion. Thereafter, 10 µM and 100 µM 5-HT was constantly perfused into the bladder for 15 minutes, respectively. For a wash out period, the bladder was perfused with isotonic saline for 15 minutes at the end of the experiment.

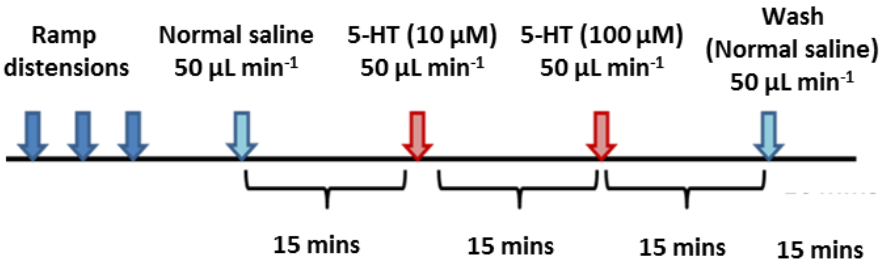


Figure 4.2 Protocol used to study the effect of intravesical 5-HT (10 and 100 µM) application on bladder afferent firing.

5-HT 10 µM did not give a significant increase of baseline affrent firing (baseline, 10.83 ± 5.04 spike/sec, 5-HT 10 µM, 31.58 ± 13.47 spike/sec). 5-HT 100 µM significantly increased the afferent discharge compared to control (5-HT 100 µM, 47.38 ± 16.71 spike/sec, *P<0.05, N=5) (Figure 4.3). Therefore 5-HT 100 µM was selected for further experiments.

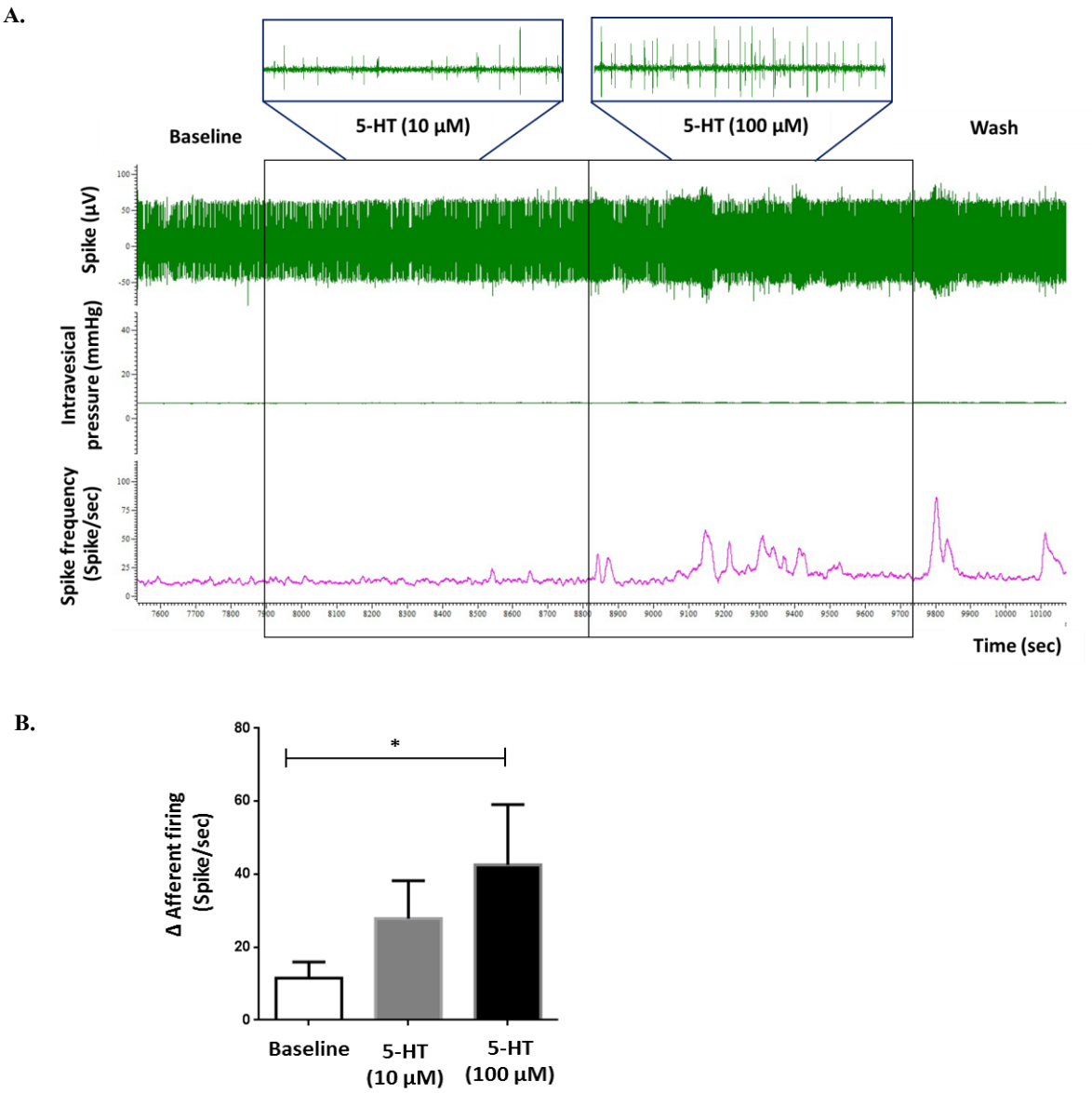


Figure 4.3 5-HT dose-dependently enhanced bladder afferent nerve firing. (A) A representative trace of intravesical 5-HT (10 and 100 µM) application on bladder afferent firing. **(B)** Peak afferent firing in response to 5-HT 10 and 100 µM application. The afferent peak firing in response to 5-HT 100 µM was significantly increased compared to the baseline firing, *P<0.05 N=5, One-way ANOVA with Dunnett's multiple comparison.

In order to investigate the effect of 5-HT on baseline afferent firing, the drug was applied either into the bladder (intravesical) or bath (extraluminal) application. The protocols are summarised in figure 4.4.

A representative trace of intravesical and bath application of 5-HT is shown in figure 4.5. Intravesical 5-HT application activated baseline afferent discharges (baseline, 2.13 ± 0.63 spike/sec vs. 5-HT, 69.93 ± 28.36 spike/sec), paired Student's t-test, *P<0.05 (N=7) (Figure 4.6A). Constant perfusion of 5-HT into the organ bath stimulated baseline firing and triggered an increase in intravesical pressure which represents bladder contraction (baseline, 24.03 ± 9.16 spike/sec vs. 5-HT, 111.30 ± 26.09 spike/sec, *P<0.05) (Figure 4.6B). The time response profile for 5-HT is shown in figure 4.6C where time to peak firing response to 5-HT was shorter in bath application (253.30 ± 39.11 sec) compared to intravesical application (865.7 ± 78.83 sec) (Figure 4.6D).

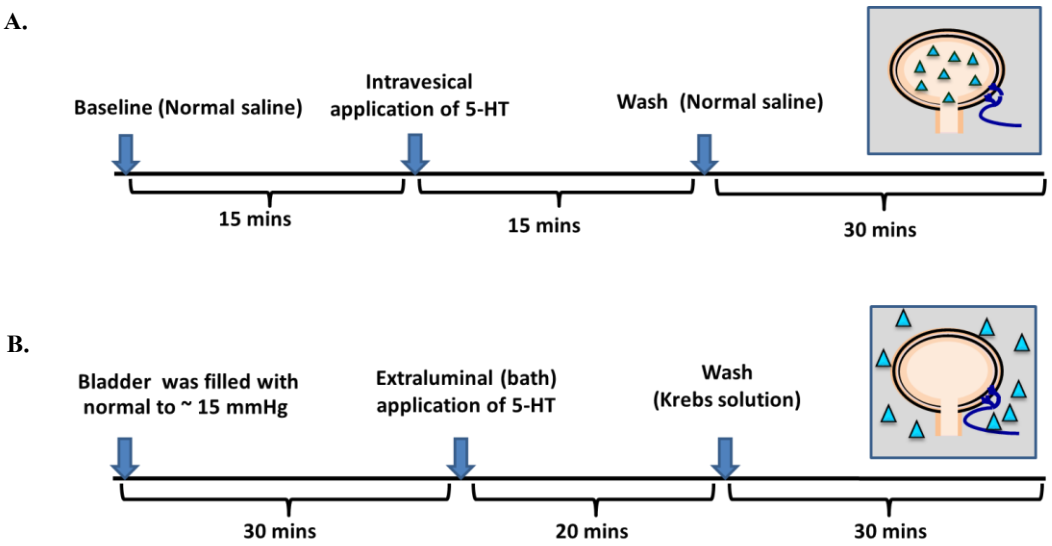


Figure 4.4 Protocol for intravesical and extraluminal (bath) application of 5-HT (Blue arrows = 5-HT in the insert cartoon).

(A) Intravesical 5-HT application protocol: The bladder was constantly perfused with normal saline (50 µL minute^{-1}) for 15 minutes for a baseline period. Thereafter, 5-HT (100 µM) diluted in normal saline was constantly perfused into the bladder for 15 minutes followed by wash out by normal saline for 30 minutes.

(B) Extraluminal (bath) 5-HT application protocol: The bladder was perfused with normal saline until intravesical pressure reached ~15 mmHg for 30 minutes. Thereafter, 5-HT (100 µM) diluted in Krebs solution was continuously perfused into a recording chamber for 20 minutes. The preparation was washed out by Krebs solution for 30 minutes.

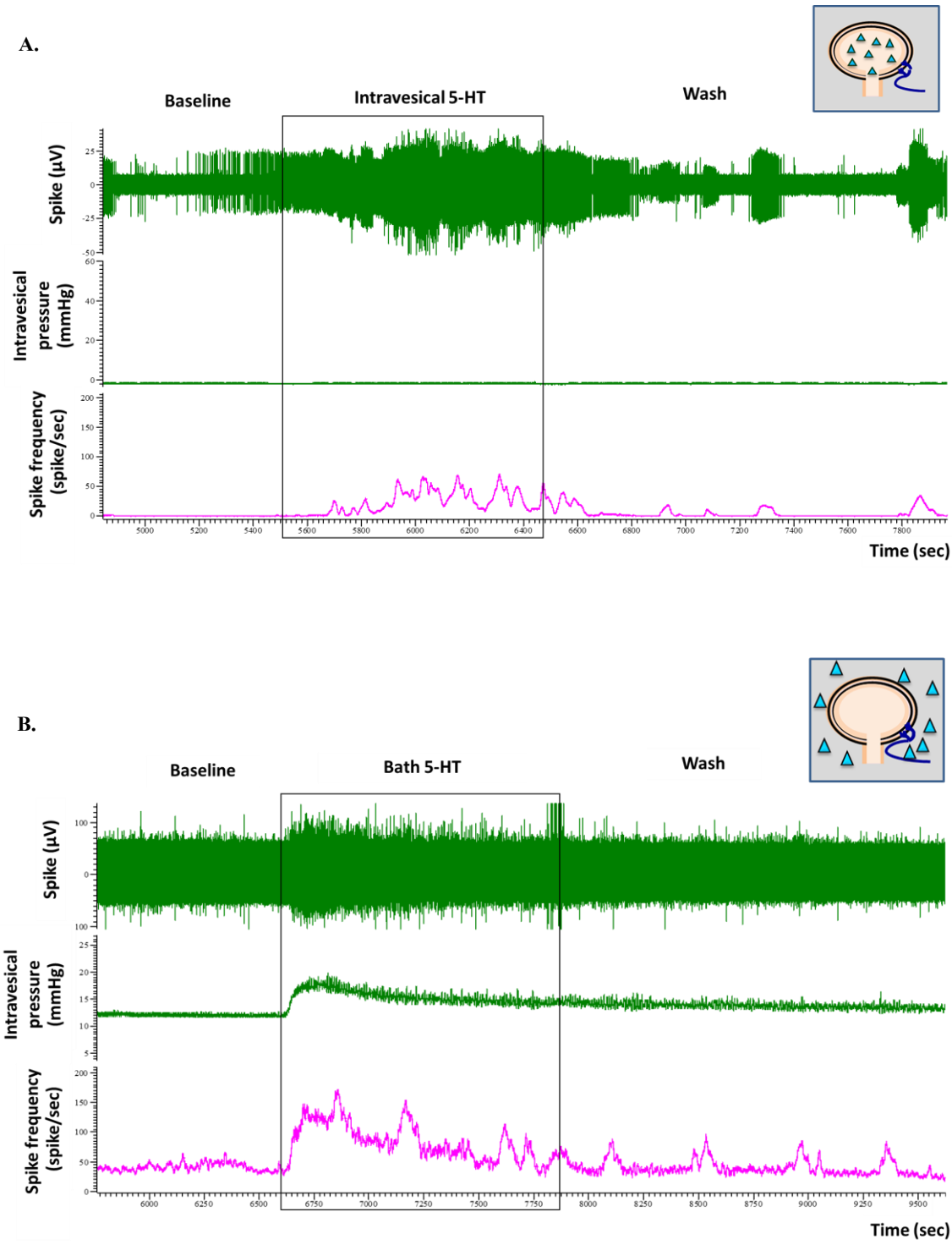


Figure 4.5 Representative traces to illustrate baseline afferent responses to intravesical (A) and bath (B) 5-HT (100 µM) application. (Blue arrows = 5-HT in the insert cartoon)

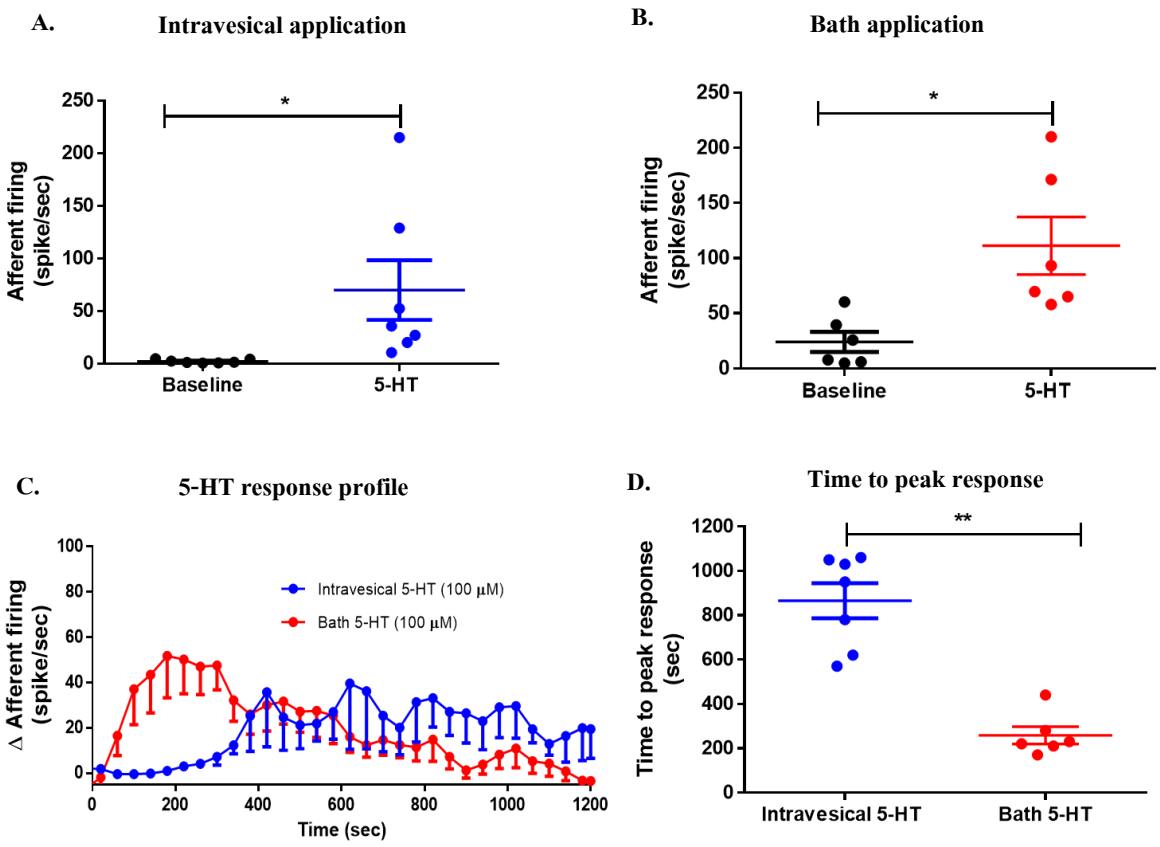


Figure 4.6 5-HT stimulated baseline afferent firing. (A) Peak afferent firing in response to intravesical 5-HT application (100 µM), paired Student's t-test, *P<0.05 (N=7). **(B)** Peak afferent firing in response to 5-HT bath application (100 µM), paired Student's t-test, *P<0.05 (N=6). **(C)** Time response profile of afferent response to intravesical and bath 5-HT application. **(D)** Time to peak firing response to an intravesical application was longer than bath application, **P<0.001 unpaired Student's t-test.

4.3.2) RESULTS: EFFECT OF 2-ME-5-HT AND GRANISETRON ON BASELINE AFFERENT FIRING

In order to examine the contribution of 5-HT3 receptors to the 5-HT-induced activation of baseline afferent firing, a selective 5-HT3 receptor agonist (2-Me-5-HT, 100 μM) and the 5-HT3 receptor antagonist (granisetron, 1 μM), were applied either into the bladder or the recording bath. The protocol used to investigate and representative traces of baseline afferent firing in response to intravesical and bath application of 2-Me-5-HT are shown in figure 4.7 and 4.8 respectively.

Granisetron was applied either into the bladder or into the organ bath for 30 minutes before 5-HT application. The protocol and representative traces of baseline afferent firing in response to pre-incubation with granisetron before intravesical and bath application of 5-HT are shown in figure 4.9 and 4.10 respectively.

Intravesical and bath application of 2-Me-5-HT stimulated baseline afferent discharge (baseline, 12.95 ± 7.01 spike/sec vs. intravesical 2-Me-5-HT, 76.04 ± 27.28 spike/sec) (baseline, 20.14 ± 7.79 vs. bath 2-Me-5-HT, 80.87 ± 19.61 spike/sec) (Figure 4.11A, 4.11B *P<0.05). Interestingly, granisetron pre-incubation reversed 5-HT-induced stimulation of bladder afferent firing both intravesical (5-HT, 67.8 ± 27.73 vs. graniestron+5-HT, 15.48 ± 4.85 spike/sec) and bath application (5-HT, 87.27 ± 16.93 vs. granisetron+5-HT, 46.97 ± 20.48 spike/sec) (Figure 4.11C, 4.11D). Granisetron on its own had no effect on baseline afferent firing (normal saline, 12.33 ± 4.64 spike/sec vs. granisetron, 7.55 ± 3.51 spike/sec) (Figure 4.11E).

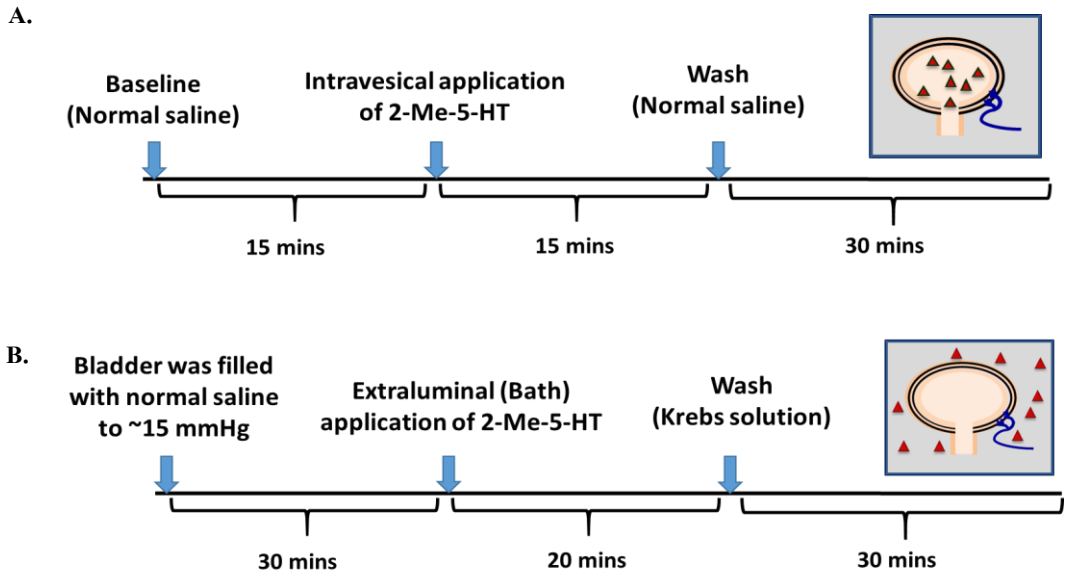


Figure 4.7 Protocol for intravesical and extraluminal (bath) application of 2-Me-5-HT (Red arrows = 2-Me-5-HT in the insert cartoon).

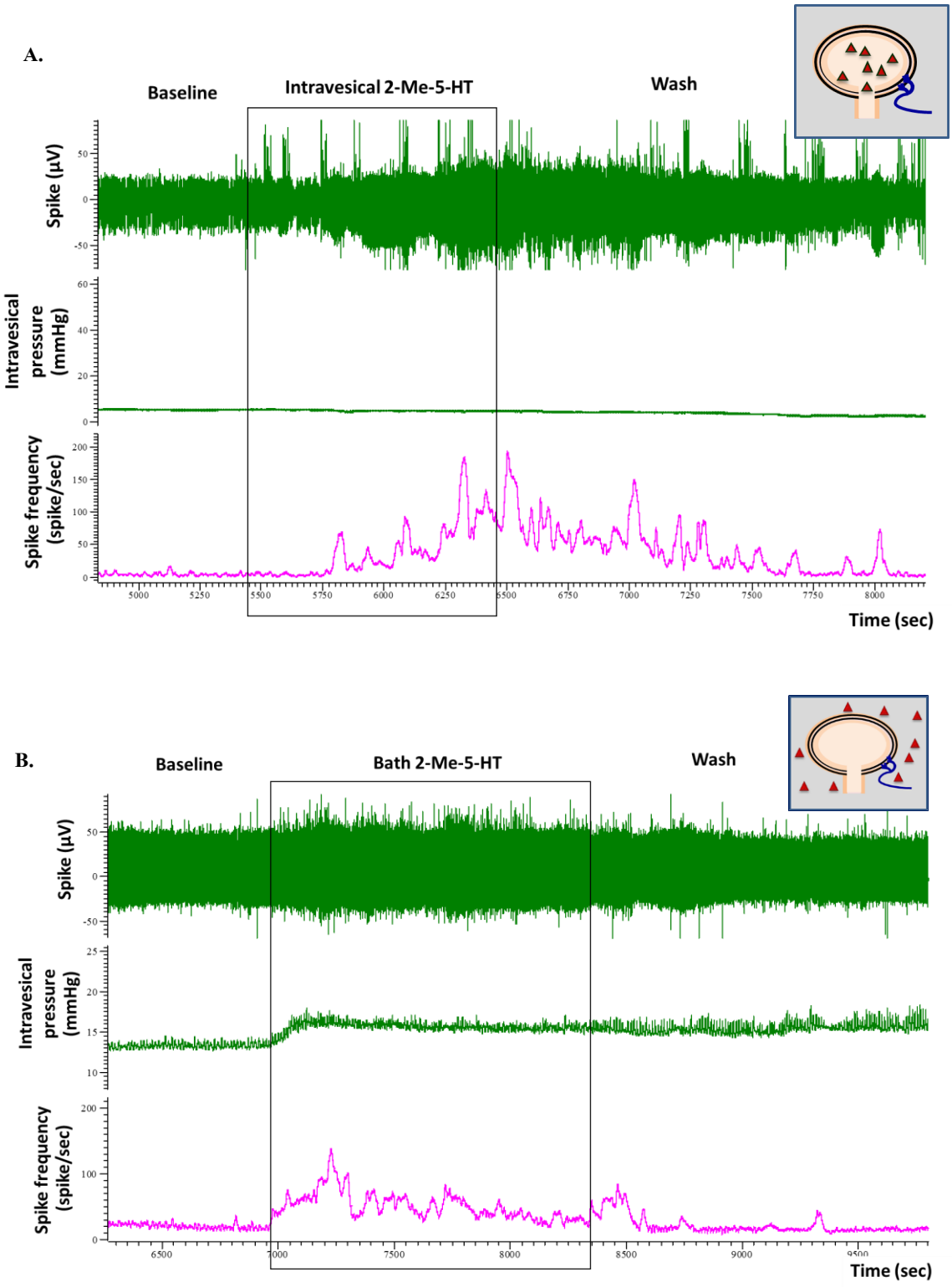


Figure 4.8 Representative traces to illustrate baseline afferent response to intravesical (A) and bath (B) 2-Me-5-HT (100 µM) application. (Red arrows = 2-Me-5-HT in the insert cartoon).

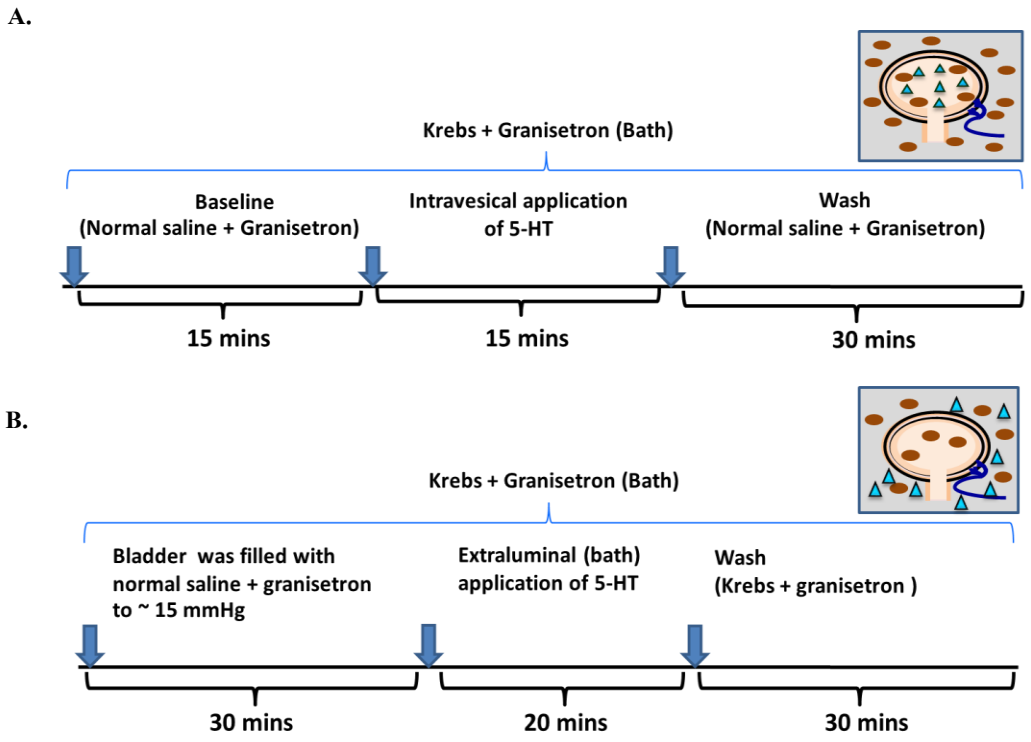


Figure 4.9 Protocol of granisetron pre-incubation before 5-HT application into the urinary bladder in extracellular afferent nerve recording (Brown circles = granisetron, Blue arrows = 5-HT in the insert cartoon). (**A**) Intravesical 5-HT application protocol: The bladder was constantly perfused with normal saline with granisetron (1 µM) (50 µL minute^{-1}) for 15 minutes for a baseline period. Thereafter, 5-HT (100 µM) diluted in normal saline was constantly perfused into the bladder for 15 minutes and the bladder was washed out by normal saline with granisetron for 30 minutes.

(B) Extraluminal (bath) 5-HT application protocol: The bladder was perfused with normal saline with granisetron (1 µM) until intravesical pressure reached ~15 mmHg for 30 minutes. Thereafter, 5-HT (100 µM) diluted in Krebs solution was continuously perfused into a recording chamber for 20 minutes. The preparation was washed out by Krebs with granisetron for 30 minutes.

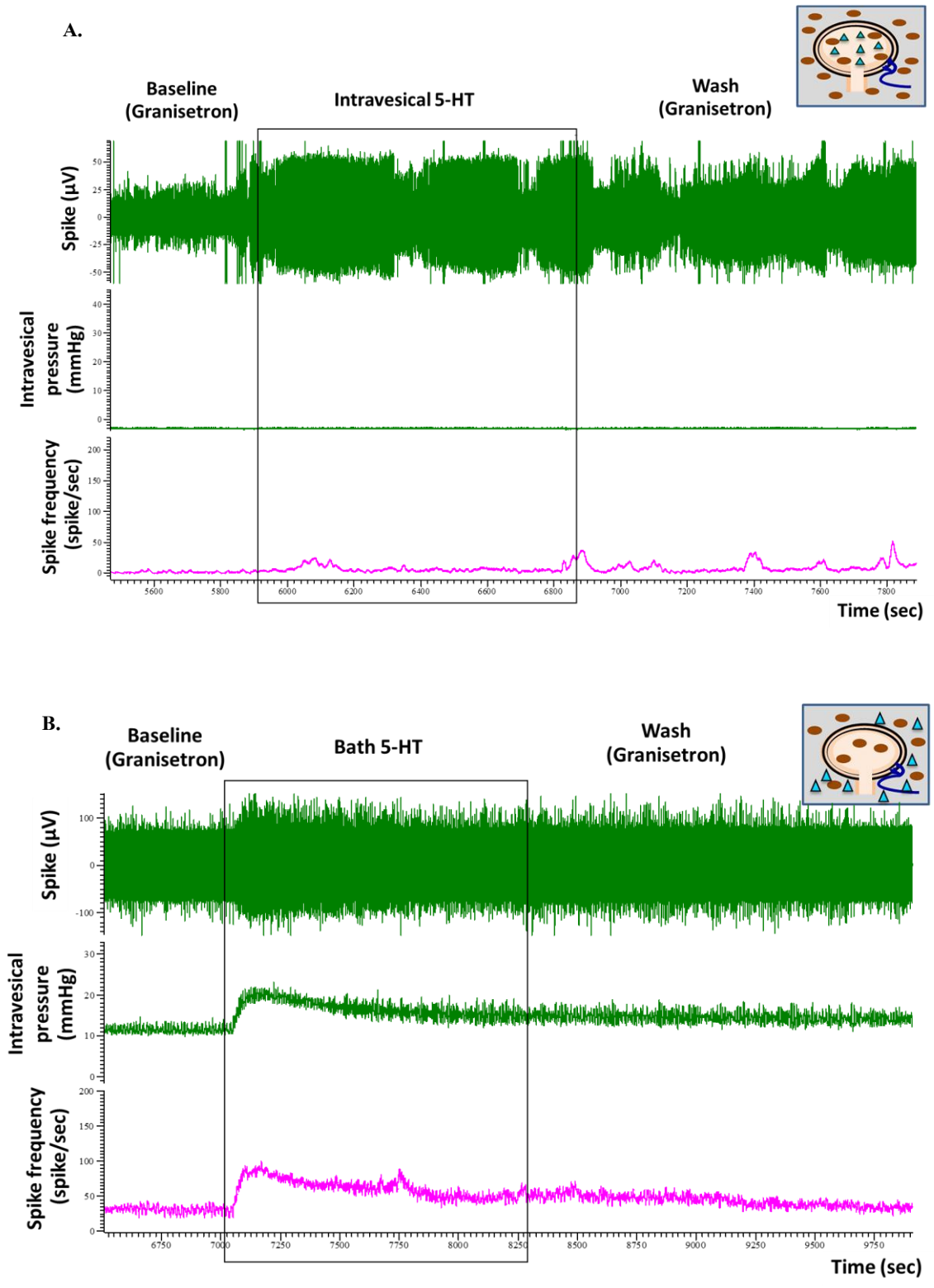


Figure 4.10 Representative traces to illustrate baseline afferent response to intravesical (A) and bath (B) 5-HT (100 µM) application after pre-incubation of granisetron. (Brown circles = granisetron, Blue arrows = 5-HT in the insert cartoon)

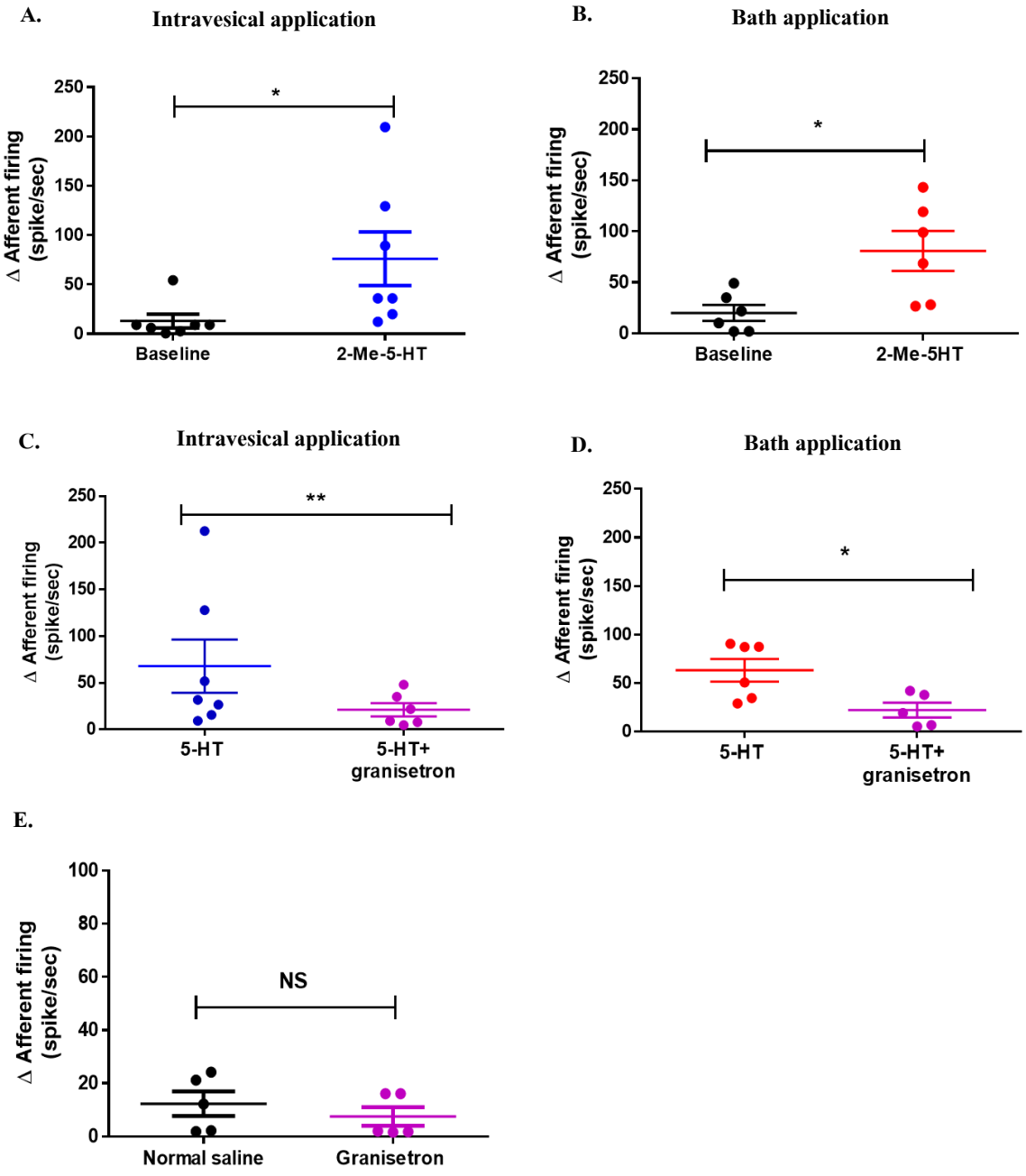


Figure 4.11 2-Me-5-HT stimulated baseline afferent firing and granisetron attenuated 5-HT induced increase in baseline afferent firing. (A) Peak afferent firing in response to intravesical 2-Me-5-HT application (100 μM), paired Student's t-test, *P<0.05 (N=7). **(B)** Peak afferent firing in response to 2-Me-5-HT bath application (100 μM), paired Student's t-test, *P<0.05 (N=6). **(C)** Peak afferent firing in response to intravesical 5-HT application after granisetron pre-incubation paired Student's t-test, *P<0.05 (N=6). **(D)** Peak afferent firing in response to bath 5-HT application after granisetron pre-incubation (N=5). **(E)** Mean afferent firing compared between intravesical normal saline and granisetron application**.**

4.3.3) RESULTS: EFFECT OF 5-MT ON BASELINE AFFERENT FIRING

In order to investigate the role of other 5-HT subtypes, 5-methoxytryptamine (5-MT, 100 µM), a full agonist for 5-HT receptors except 5-HT3, was applied either into the bladder or a recording chamber in order to examine the contribution of 5-HT1, 2, 4-7 receptors on baseline afferent firing. The protocol used to study is shown in figure 4.12.

The representative trace of baseline afferent firing in response to 5-MT are shown in figure 4.13. Intravesical (baseline, 2.55 ± 1.12 spike/sec vs. 5-MT, 17.60 ± 4.99 spike/sec, *P<0.05) and bath application of 5-MT (100 µM) significantly evoked baseline afferent firing (baseline, 25.39 ± 7.93 spike/sec vs. 5-MT, 46.22 ± 10.96 spike/sec, *P<0.05) as shown in figure 4.14.

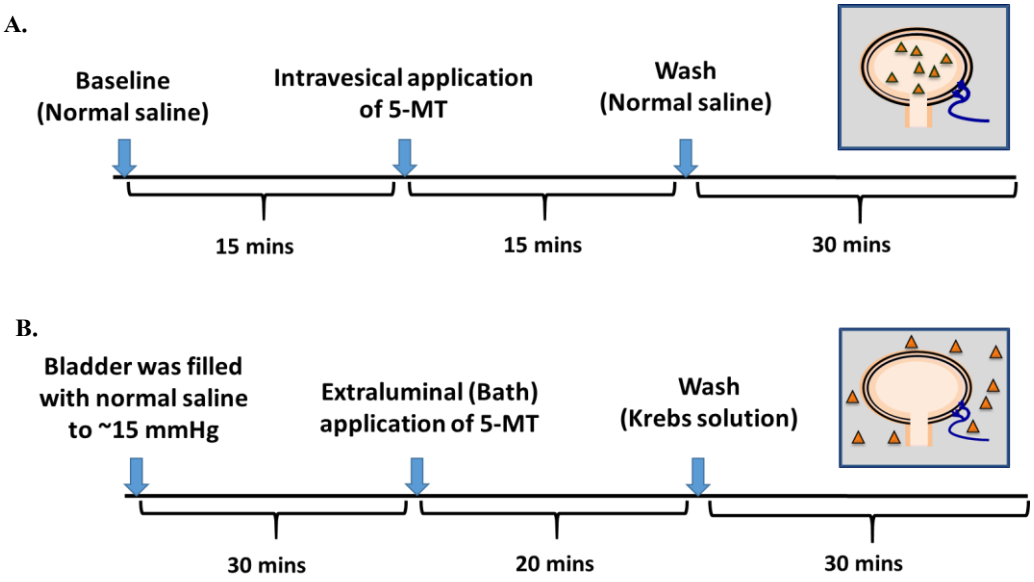


Figure 4.12 Protocol for intravesical and extraluminal (bath) application of 5-MT (Orange arrows = 5-MT in the insert cartoon).

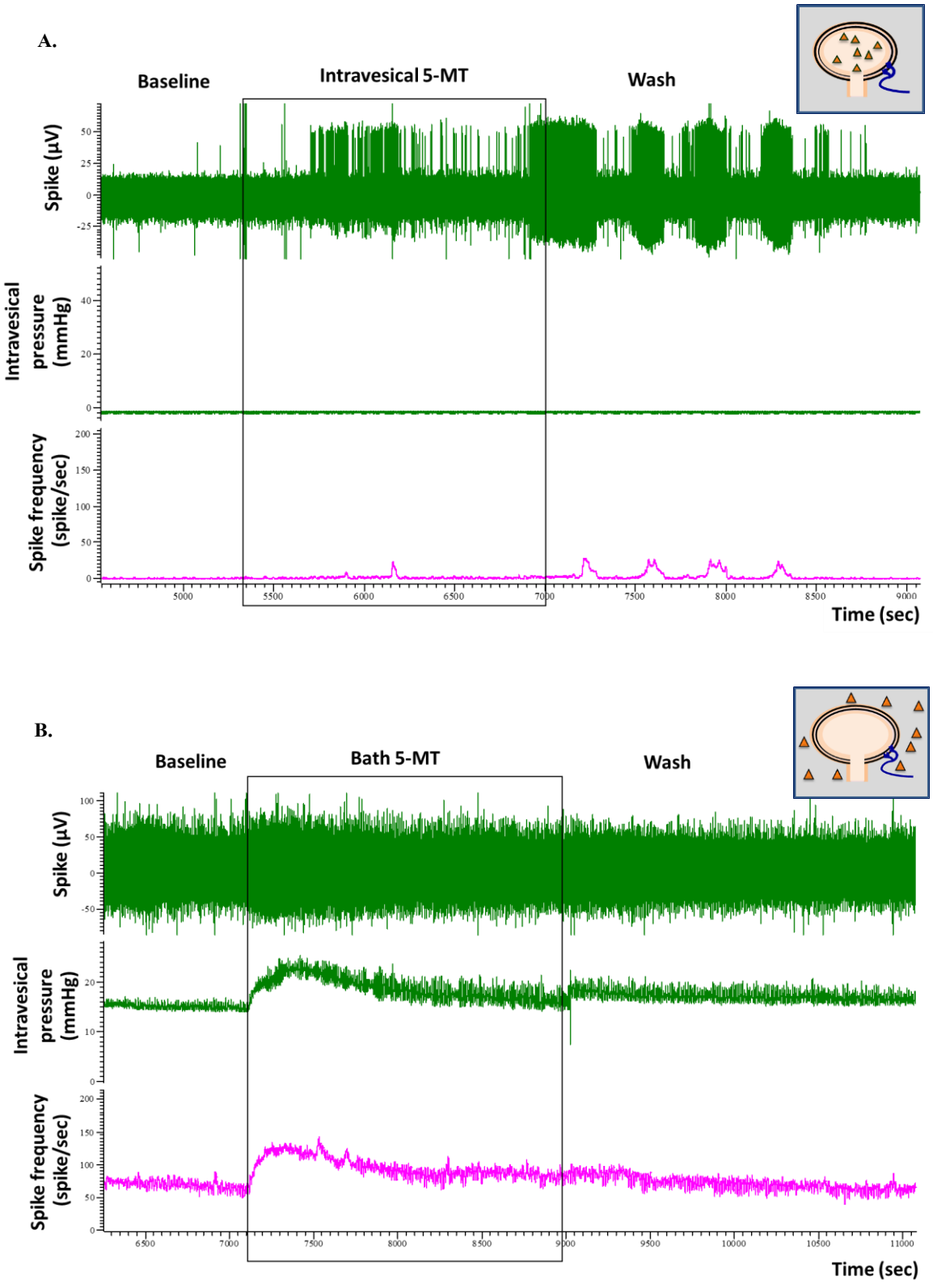


Figure 4.13 Representative traces to illustrate baseline afferent response to intravesical (A) and bath (B) 5-MT (100 µM) application. (Orange arrows = 5-MT in the insert cartoon).

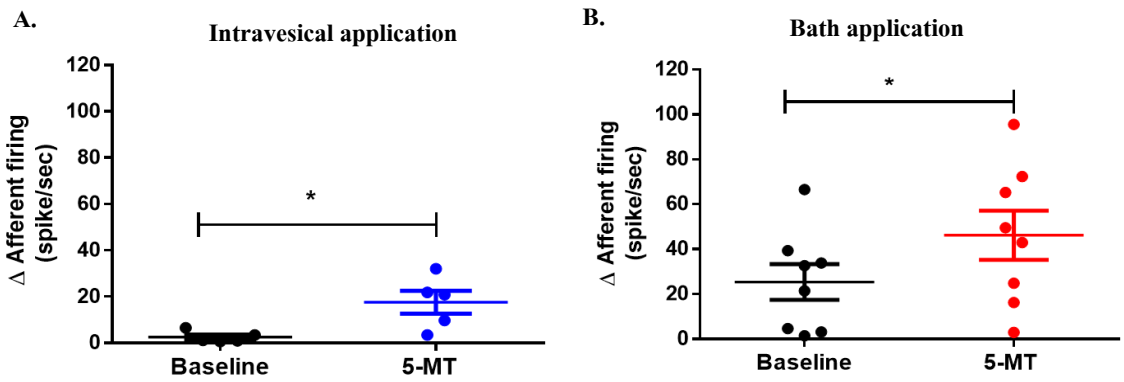


Figure 4.14 5-MT stimulated baseline afferent firing. (A) Peak afferent firing in response to intravesical 5-MT application (100 µM), *P<0.05, paired Student's t-test (N=5). **(B)** Peak afferent firing in response to 5-MT bath application (100 µM), *P<0.05, paired Student's t-test (N=8).

4.3.4) RESULTS: EFFECT OF 5-HT WITH GRANISETRON, 2-ME-5-HT AND 5-MT ON BLADDER CONTRACTION

To determine whether the effect of 5-HT with granisetron, 2-Me-5-HT, and 5-MT which induced an increase in baseline afferent discharge could be secondary to an induction of muscle contraction, Y-27632 (a Rho kinase inhibitor, 10 µM) was applied to the bladder to inhibit contractile components prior to 2-Me-5-HT applicatiton to inhibit detrusor muscle contraction. The protocol and representative trace are shown in figure 4.15.

Similarly, to study whether the effect of 5-HT with granisetron pre-incubation and 5-MT on baseline afferent activation was due to potentiation of muscle contraction, Y-27632 and ML-9 (myosin light chain kinase (MLCK) inhibitor, 10 µM) were pre-incubated on the bladder prior to 5-HT or 5-MT application. The protocols and representative traces are shown in figure 4.16 and 4.17 respectively.

Intravesical pressure was significantly decreased in Y-27632 application prior to 2-Me-5-HT (2-Me-5-HT, 3.07 ± 0.57 mmHg vs. 2-Me-5-HT with Y-27632 pre-incubation, 1.62 ± 0.13 mmHg, *P<0.05) (Figure 4.18A), indicating that Y-27632 could prevent detrusor contraction. However, there was still an excitation of afferent firing (2-Me-5-HT, 87.43 ± 23.78 spike/sec vs. 2-Me-5-HT with Y-27632 pre-incubation, 73.16 ± 13.26 spike/sec) (Figure 4.18B), suggesting that 5-HT activated 5-HT3 receptors directly on the afferent nerve terminals and this was not a secondary effect from induction of bladder contraction.

As expected, Y-27632 and ML-9 application with granisetron prior to 5-HT application significantly decreased the 5-HT-induced increase in bladder contraction (5-HT, 8.83 ± 1.37 mmHg vs. 5-HT with granisetron, Y-27632 and ML-9 pre-incubation, 1.85 ± 0.85 mmHg) (Figure 4.18C). Baseline afferent discharges in response to 5-HT was also significantly attenuated after pre-incubation with granisetron and muscle contraction blockers (5-HT, 63.16 ± 11.62 spike/sec vs. 5-HT with granisetron, Y-27632 and ML-9 pre-incubation, 4.26 ± 1.54 spike/sec) (Figure 4.18D).

Pre-incubation with Y-27632 and ML-9 also inhibited bladder contraction from 5-MT application (5-MT, 5.57 ± 1.12 mmHg vs. 5-MT with Y-27632 and 5-MT pre-incubation, 0.52 ± 0.30 mmHg) (Figure 4.18E). In contrast to 2-Me-5-HT, 5-MT did not activate baseline afferent firing after incubation of muscle contraction blockers (5-MT, 48.04 ± 13.47 spike/sec vs. 5-MT with Y-27632 and ML-9 pre-incubation, 12.36 ± 4.187 spike/sec) (Figure 4.18F). This indicates that other 5-HT receptors (5-HT1, 2, 4-7) mediated 5-HT-induced baseline firing activation through potentiation of bladder contraction.

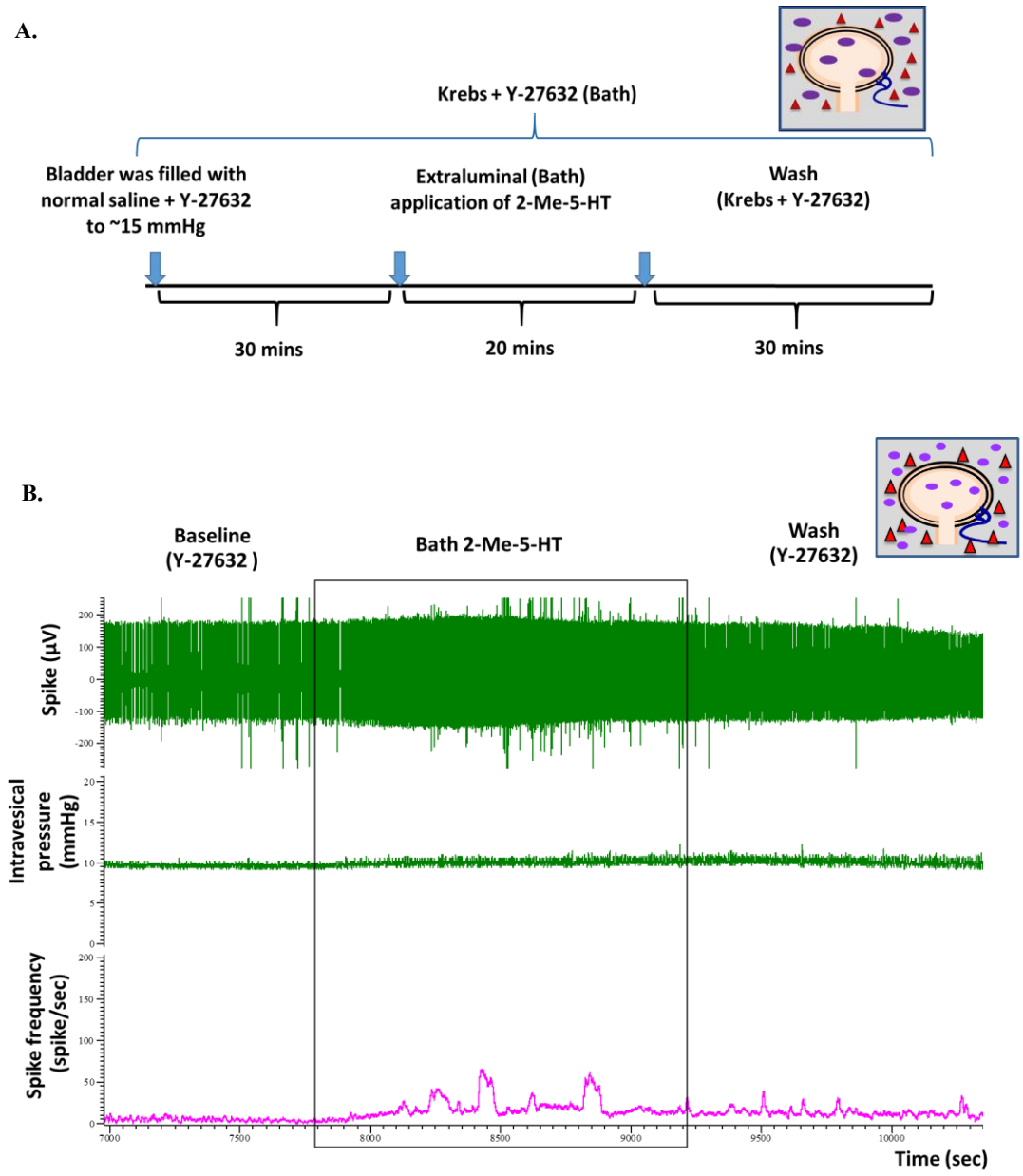


Figure 4.15 (A) Protocol used to investigate the effect of Rho kinase inhibitor (Y-27632) prior to 2-Me-5-HT bath application. (B) Representative trace to illustrate baseline afferent responses to bath application of 2-Me-5-HT in the presence of Y-27632 (Red arrows = 2-Me-5-HT, Purple circles = Y-27632 in the insert cartoon).

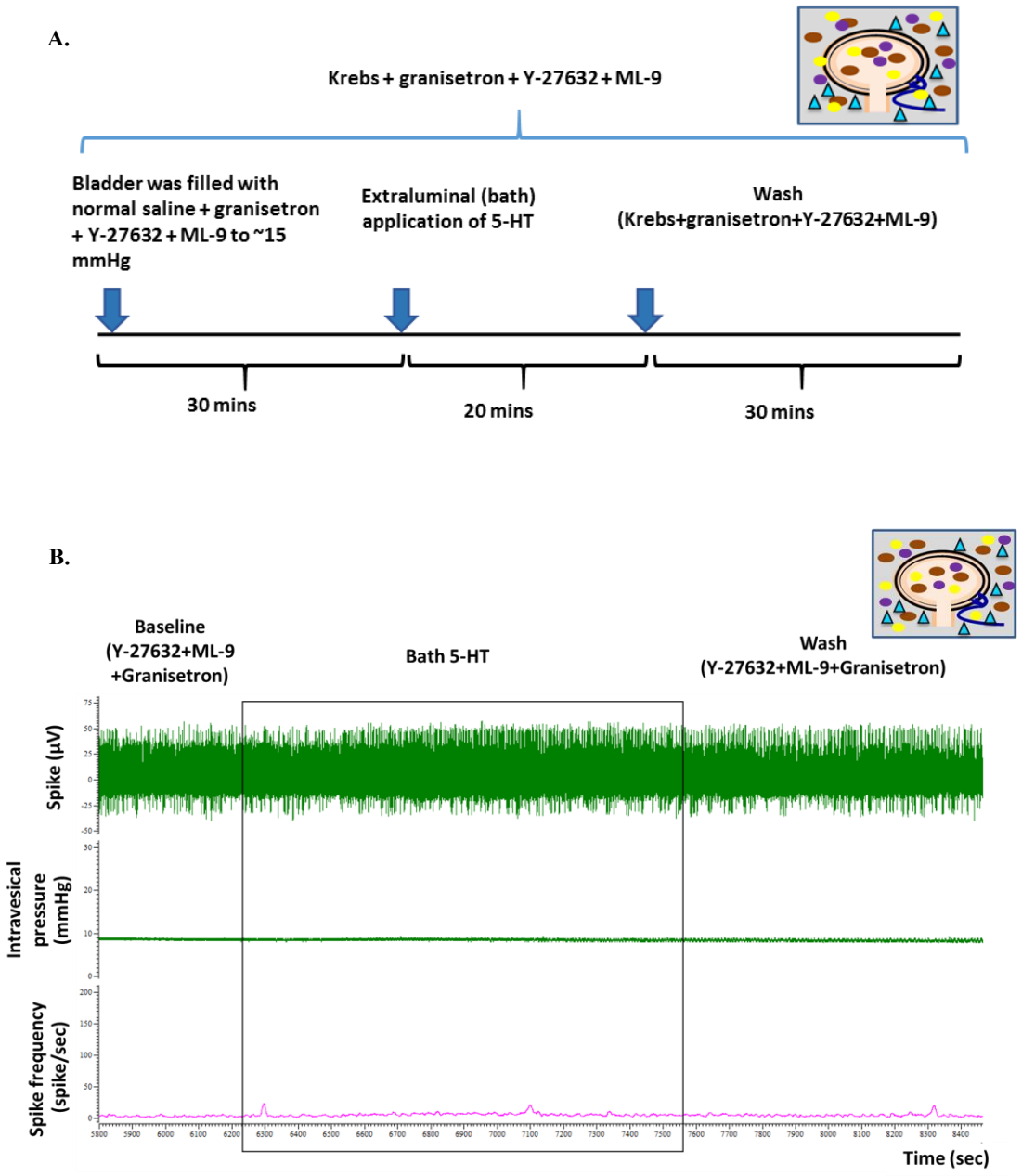


Figure 4.16 (A) Protocol used to investigate the effect of the Rho kinase inhibitor (Y-27623), MLCK inhibitor (ML-9), and granisetron prior to 5-HT bath application. (B) Representative trace to illustrate baseline afferent responses to bath application of 5-HT in the presence of Y-27632, ML-9, and granisetron (Blue arrows = 5-HT, Purple and Yellow circles = Y-27632 and ML-9 respectively, Orange circles = granisetron in the insert cartoon).

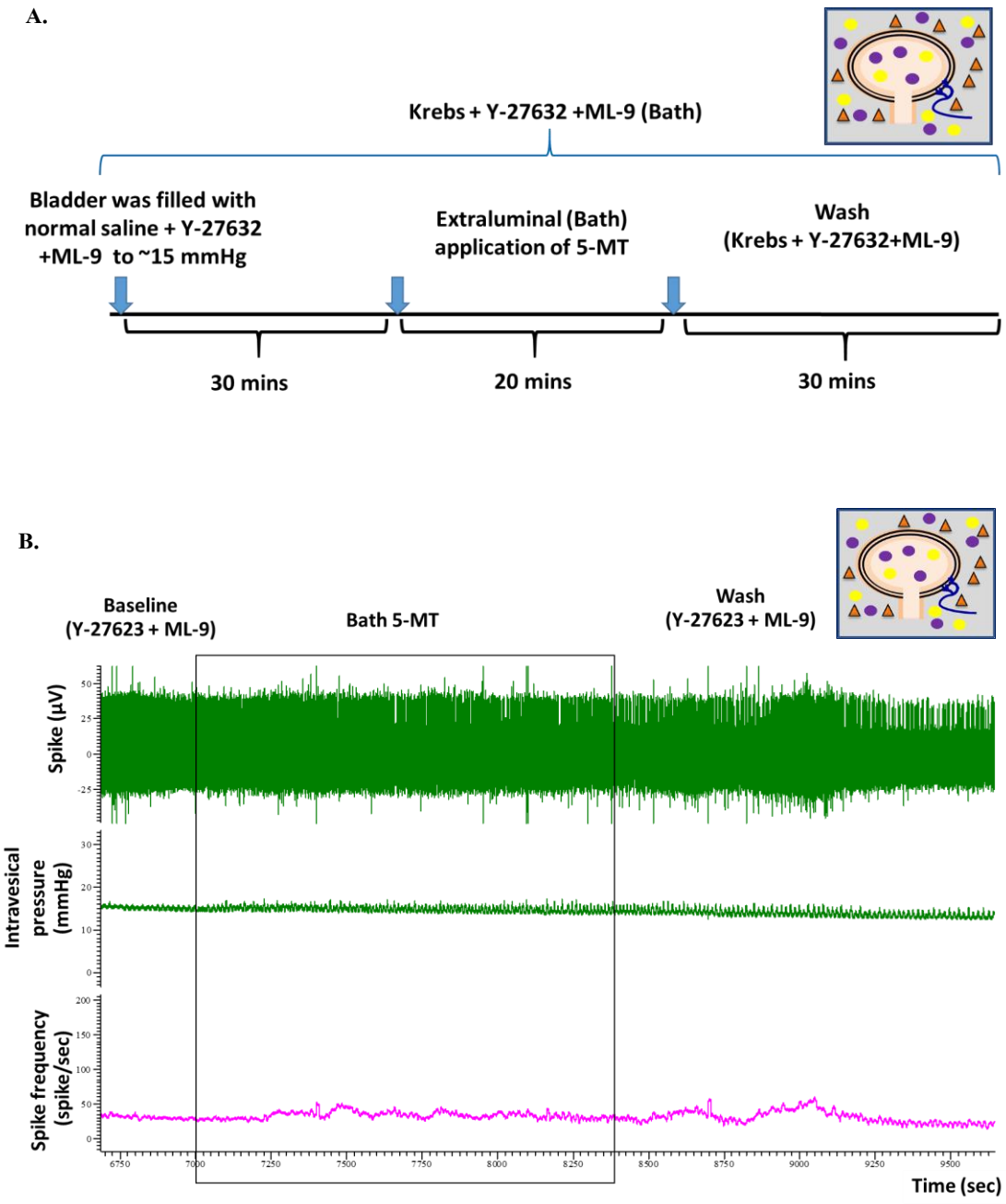


Figure 4.17 (A) Protocol used to investigate effect of Rho kinase inhibitor (Y-27632) and MLCK inhibitor (ML-9) prior to 5-MT bath application. (B) Representative trace to illustrate baseline afferent responses to bath application of 5-MT in the presence of Y-27632 and ML-9 (Orange arrows = 5-MT, Purple and Yellow circles = Y-27632 and ML-9 respectively in the insert cartoon).

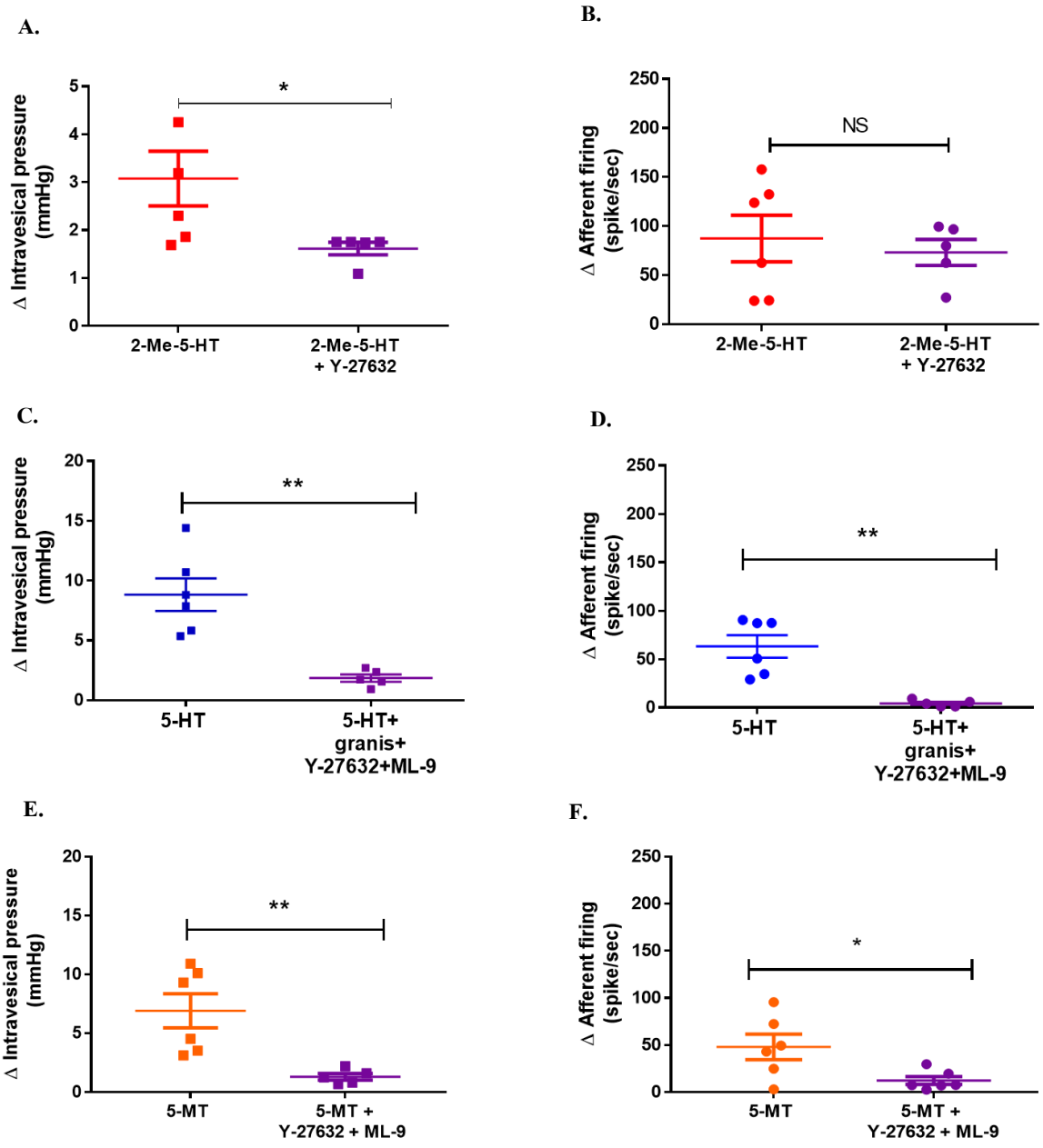


Figure 4.18 Y-27632 and ML-9 pre-incubation decreased the effect of 5-HT with granisetron and 5-MT-induced stimulation of baseline afferent discharges but failed to reduce 2-Me-5-HT activation. Comparison of intravesical pressure change and peak firing of baseline afferent firing in response to 2-Me-5-HT and 2-Me-5-HT with Y-27632 pre-incubation (N=5) **(A-B)**, 5-HT and 5-HT+granisetron with Y-27632+ML-9 (N=6 and 5 respectively) **(C-D)**, 5-MT and 5-MT with Y-27632+ML-9 **(E,F)** (N=6). *P<0.05, **P<0.01, unpaired Student's t-test.

Comparison of peak afferent discharges following bath application of various pharmacological substances is showed in figure 4.19A. There was no significant difference in peak firing in response to bath application of 5-HT and 2-Me-5-HT. In addition, Y-27632 application prior to 2-Me-5-HT did not attenuate baseline afferent firing. The afferent discharges in response to 5-MT and 5-HT was decreased after pre-incubation with granisetron compared to 5-HT alone (*P<0.05 and **P<0.01, respectively) and Y-27632 and ML-9 further attenuated these effects (***P<0.001).

To determine the effects of various pharmacological reagents on bladder contraction, change in intravesical pressure in response to the substances was compared to 5-HT alone. As shown in figure 4.19B, there was no significant difference in the change in intravesical pressure in response to 5-HT, 5-MT, and 5-HT after granisetron. The level of 2-Me-5-HT triggered bladder contraction was significantly lower than 5-HT (*P<0.05). Pre-incubation of muscle contractile component blockers prior to 2-Me-5-HT, 5-MT, and granisetron with 5-HT attenuated intravesical pressure change. The effect of all pharmacological reagents on bladder contraction and baseline afferent discharges is summarised in table 4.2.

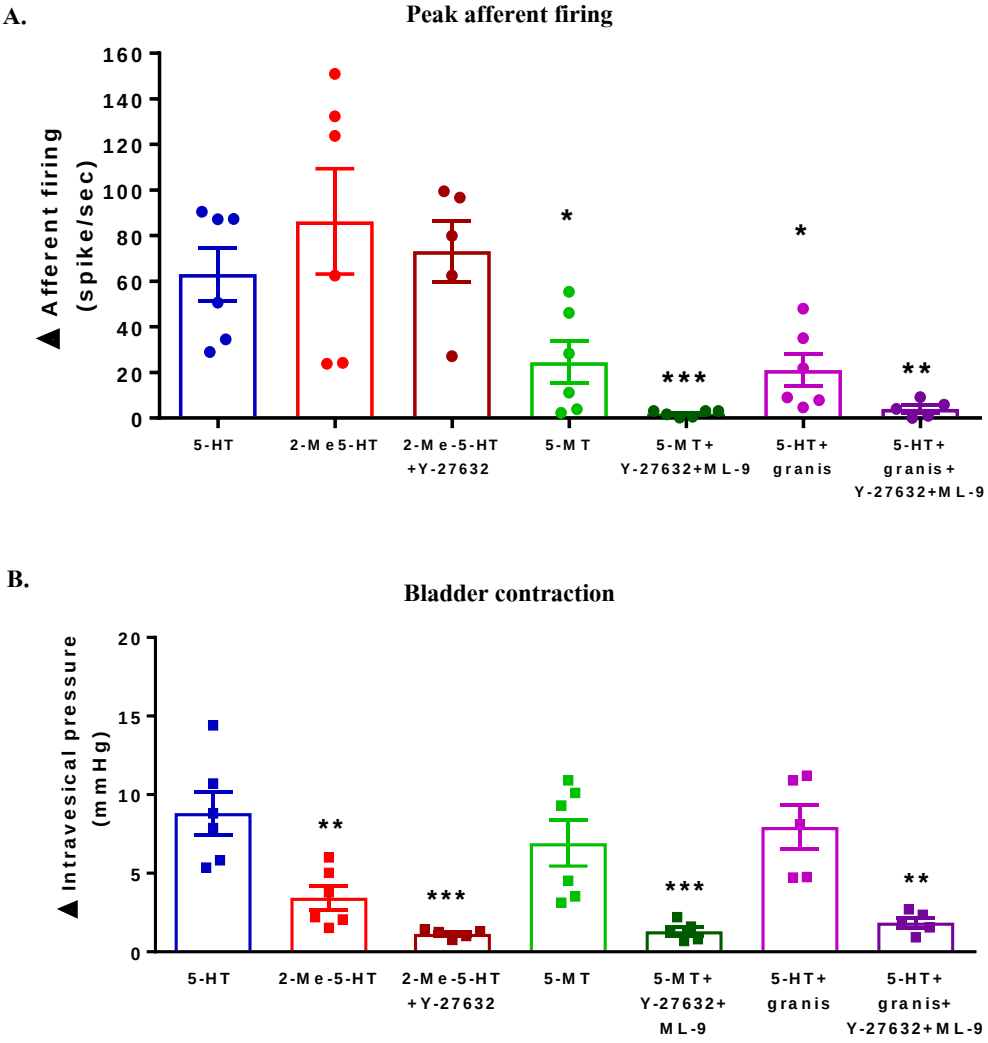


Figure 4.19 Comparison of baseline afferent firing (A) and bladder contraction (B) in response to bath application of 5-HT, 2-Me-5-HT, 5-MT, and pre-incubation with granisetron and muscle blockers (Y-27632 and ML-9). *P<0.05, **P<0.01, ***P<0.001, One-way ANOVA with Dunnett's multiple comparison.

Table 4.2 Summarised effects of 5-HT and related pharmacological reagents on baseline afferent firing and bladder contraction

	5-HT	2-Me-5-HT	2-Me-5-HT + Y-27632	5-MT	5-MT + Y-27632+ ML-9	5-HT + granis	5-HT + granis +Y-27632 +ML-9
Afferent discharge	+++	+++	+++	++	+	++	+
Contraction	+++	++	+	+++	+	+++	++

+++ strong, ++ moderate, +basal level

4.4 RESULTS: EFFECT OF 5-HT, 2-ME-5-HT, GRANISETRON AND 5-MT ON BLADDER MECHANOSENSITIVITY

The effect of 5-HT and all pharmacological substances on mechanosensitive afferent discharges were also investigated. The bladder ramp distensions were performed in the presence of agonists. The protocols used in this study are summarised in figure 4.20A. After a control period of ramp distension, the substance of interest was continuously applied to the organ bath 2 minutes prior the next distension. After 3 consecutive distensions, the bladder was washed with Krebs solution for 30 minutes. Mechanosensitive afferent firing was determined at a range of an intravesical pressures at 2, 15, and 25 minutes after the drug application. In a subset of experiments the bladder was pre-incubated with granisetron, both intravesically and via bath perfusion prior to 5-HT application (Figure 4.20B).

Representative traces showing mechanosensitive firing in response to 5-HT, 2-Me-5-HT, granisetron, and 5-MT are displayed in figure 4.21 and 4.22, respectively. 5-HT attenuated mechanosensitive afferent firing at 15 and 25 minutes after application (*P<0.05) (Figure 4.23A). This effect was reversed by granisetron pre-incubation (Figure 4.23C). 2-Me-5-HT had a similar effect to 5-HT showing an inhibitory effect on mechanosensitive afferent firing at 15 and 25 minutes after application (**P<0.001, ***P<0.001, respectively) (Figure 23B). In contrast, 5-MT did not change the afferent response to bladder distention at any time points (Figure 4.23D).

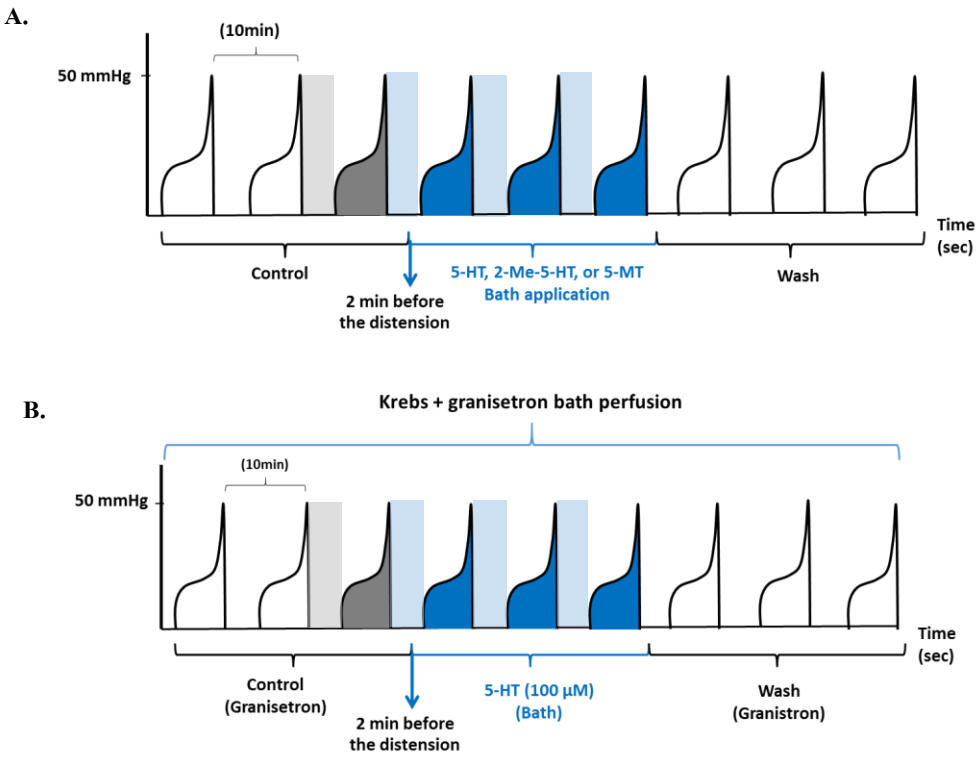


Figure 4.20 Protocol used to investigate the effect of agonists and antagonists on bladder mechanosensitive and spontaneous afferent firing.

(A) Protocol used to investigate effect of 5-HT, 2-Me-5-HT, and 5-MT (100 µM) on mechanosensitive afferent firing, **(B)** Protocol used to investigate effect of granisetron (1 µM) on 5-HT-induced change in mechanosensitive afferent firing. Grey and blue areas indicate where the data of control and response to agonist application were analysed at 2, 15, and 25 minutes respectively.

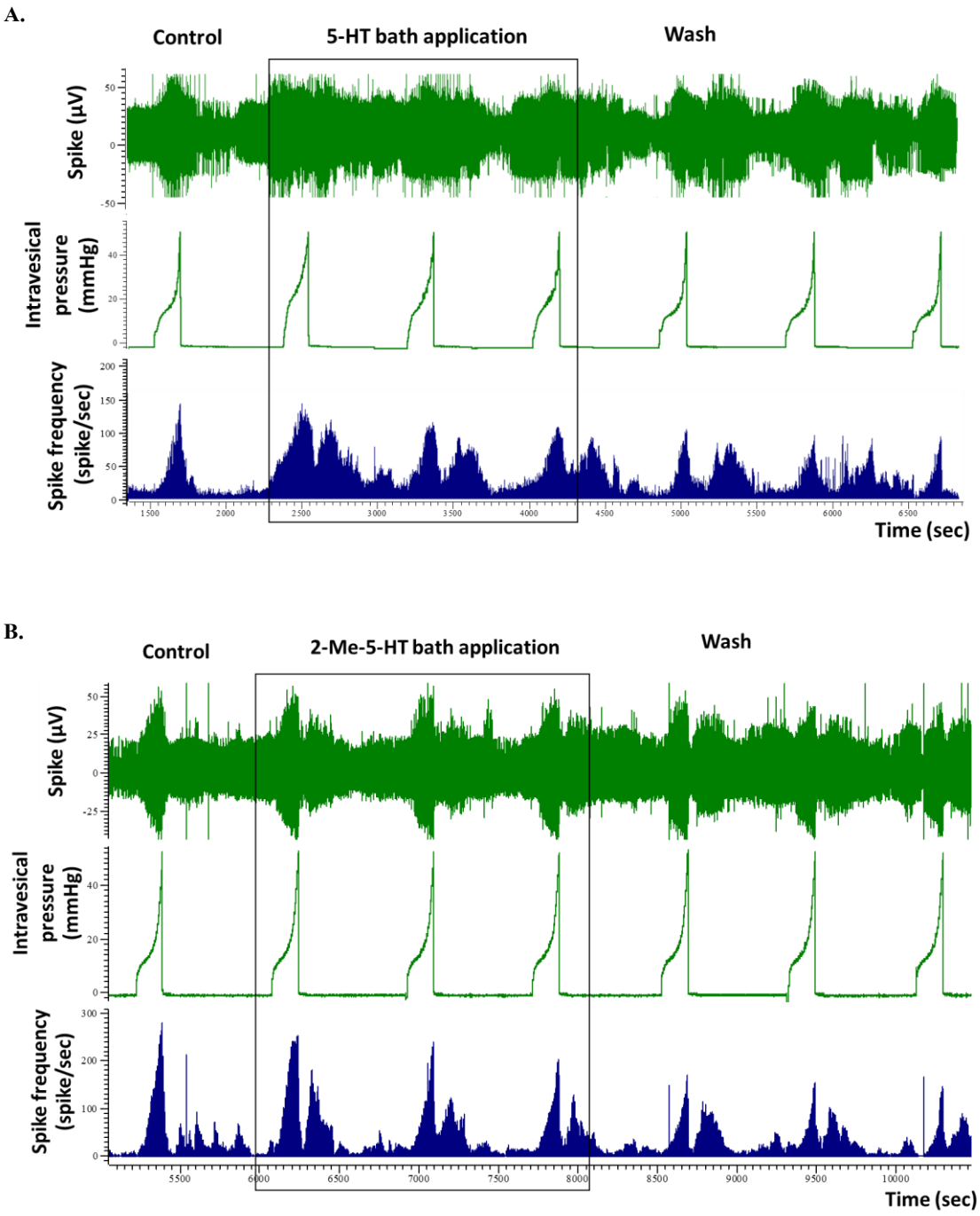


Figure 4.21 Representative traces to illustrate mechanosensitive and spontaneous afferent responses to bladder ramp distension during 5-HT (A) and 2-Me-5-HT (B) bath application.

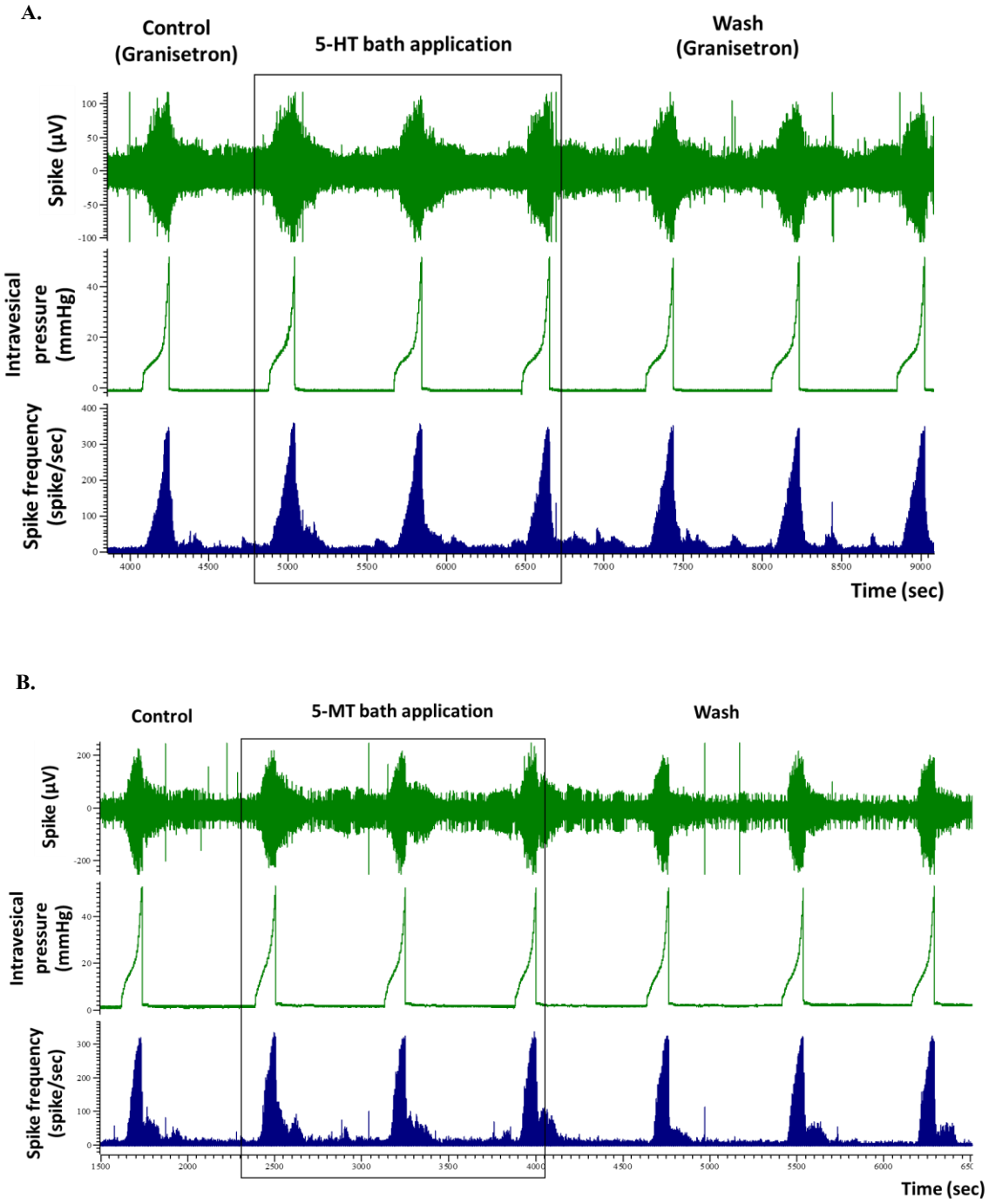


Figure 4.22 Representative traces to illustrate mechanosensitive and spontaneous afferent responses to bladder ramp distention during granisetron+5-HT (A) and 5-MT bath application (B).

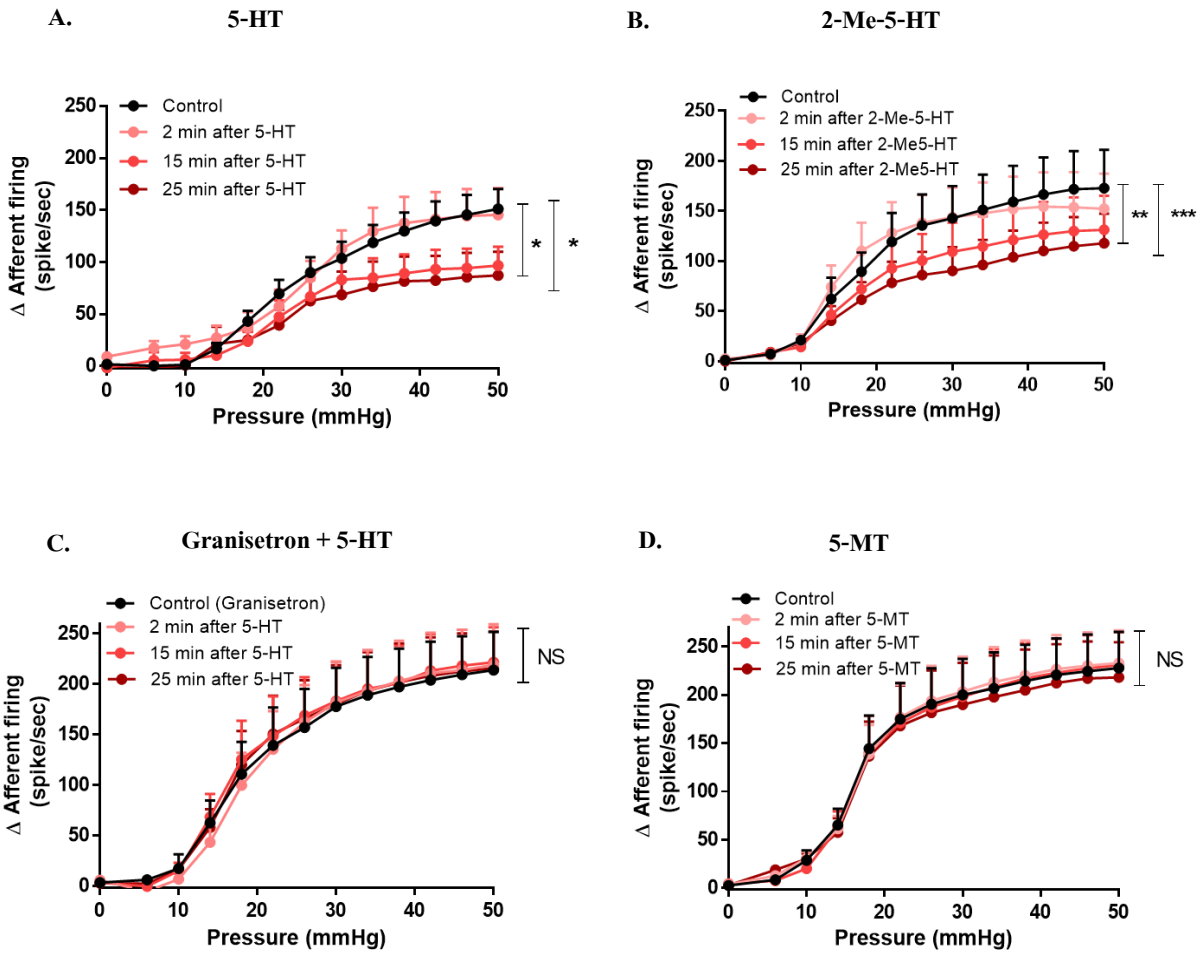


Figure 4.23 5-HT attenuated mechanosensitive afferent response to ramp distensions mediated via 5-HT3 receptors. (A-D) Comparison of bladder afferent responses to ramp distension at 2, 15, and 25 minutes after bath application of 5-HT, 2-Me-5-HT, granisetron+5-HT, and 5-MT, respectively *P<0.05, **P<0.01. ***P<0.001 vs. control, Two-way ANOVA (N=5-6).

Even though 5-HT and 2-Me-5-HT attenuated afferent responses to bladder ramp distensions, the spontaneous firing between the ramp distensions was augmented. 5-HT activated spontaneous burst firing at 2 and 15 minutes after drug application (*P<0.05, ***P<0.001) (Figure 4.24A). 2-Me-5-HT evoked spontaneous firing at 2 minutes (*P<0.05) (Figure 4.24B). Granisetron decreased the 5-HT effect on spontaneous activation (Figure 4.24C). 5-MT had no effect on spontaneous afferent firing (Figure 4.24D). In addition, application of 5-HT, 2-Me-5-HT, 5-HT with granisetron pre-incubation, and 5-MT did not change bladder compliance (Figure 4.25).

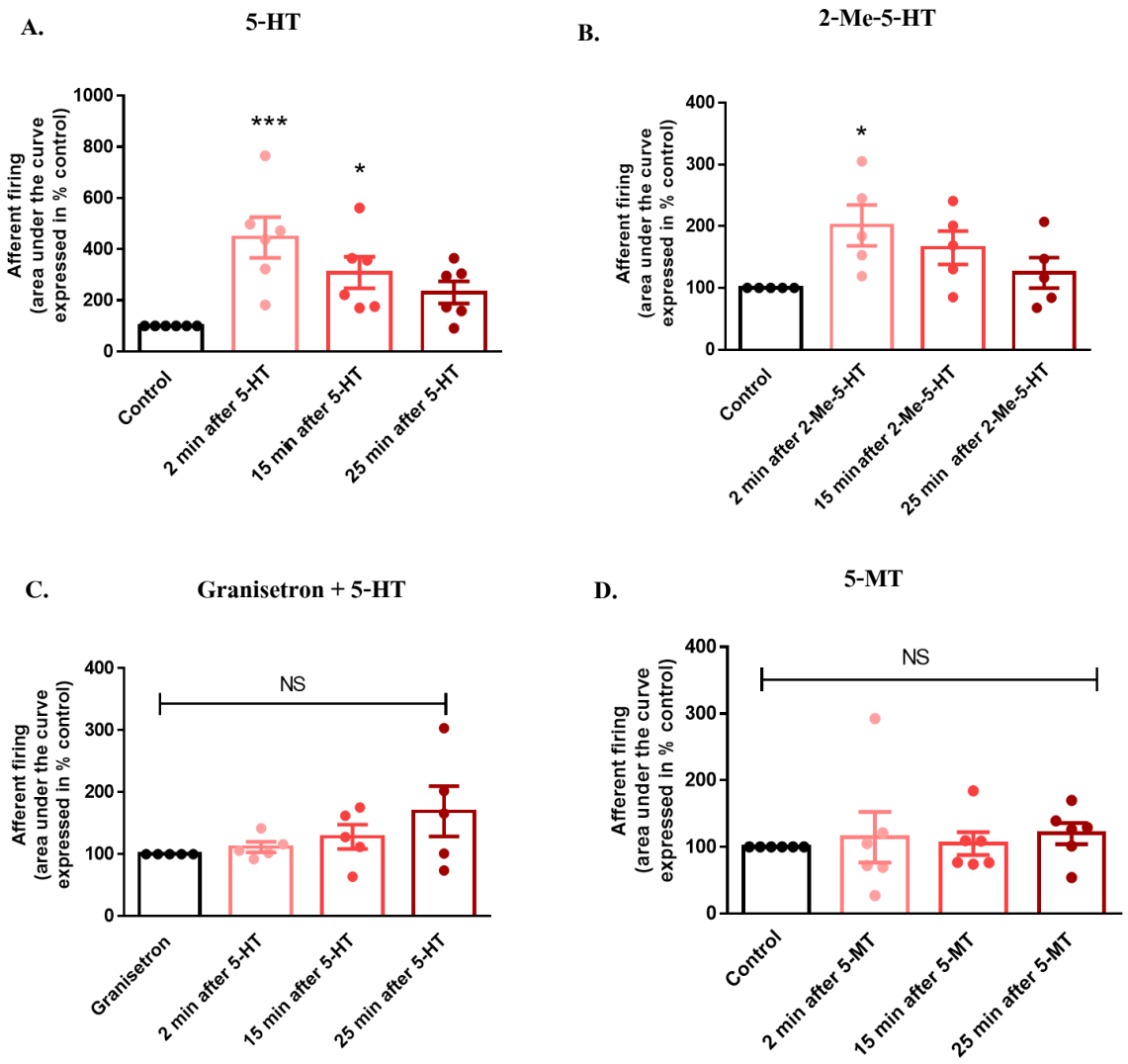


Figure 4.24 5-HT activated spontaneous afferent firing between ramp distensions mediated via 5-HT3 receptors. (A-D) Comparison of spontaneous afferent firing after 2, 15, and 25 minutes bath application of 5-HT, 2-Me-5-HT, granisetron+5-HT, and 5-MT, respectively, *P<0.05, ***P<0.001, One-way ANOVA with Dunnett's multiple comparison (N=5-6).

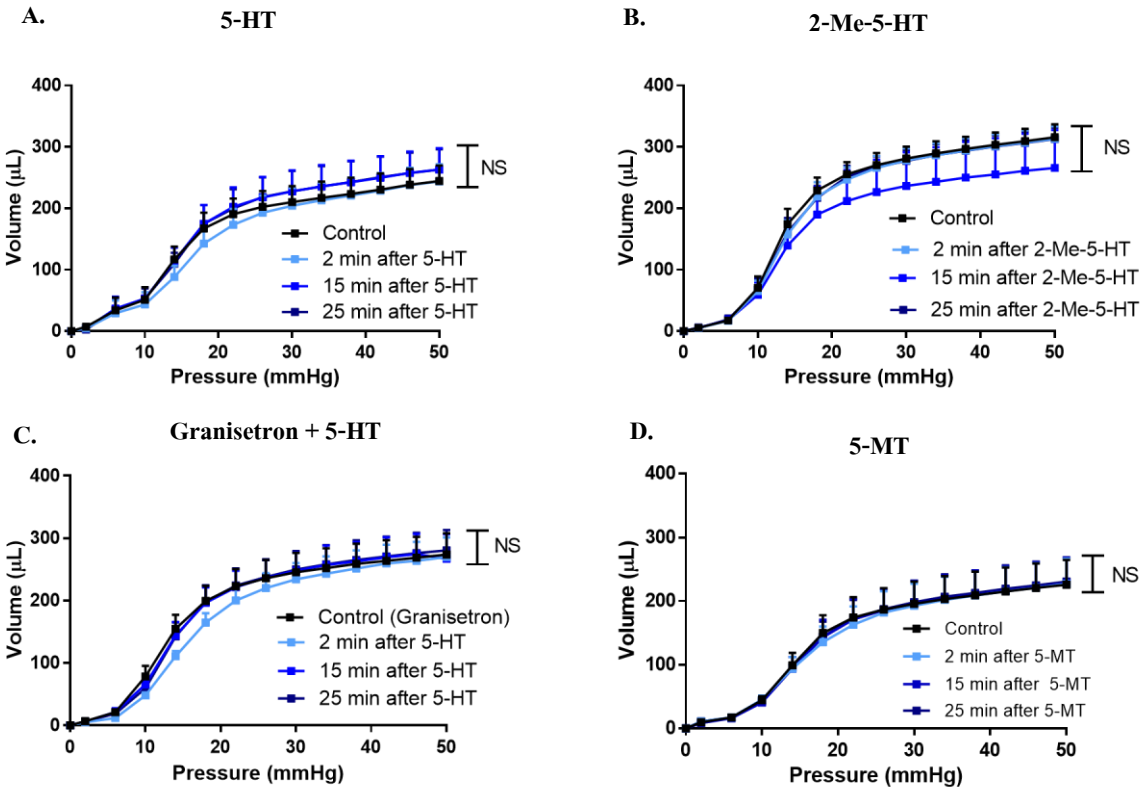


Figure 4.25 Bladder compliance was not changed following 5-HT (A), 2-ME-5-HT (B), pre-incubation with granisetron (C), and 5-MT application (D). Comparison of bladder compliance at 2, 15, and 25 minutes after 5-HT bath application, Two-way ANOVA (N=5-6).

4.5 RESULTS: EFFECT OF L-NAME ON 5-HT ATTENUATED MECHANOSENSITIVE FIRING

To determine whether 5-HT effects are mediated through NO production, the NOS inhibitor, L-NAME (1 mM) was applied 30 minutes prior to the intravesical 5-HT (100 µM) application and throughout the experiment. The experimental protocols are shown in figure 4.26.

A time control experiment was conducted to determine whether L-NAME had an effect on bladder afferent firing. As shown in figure 4.27, there was no difference in baseline and mechanosensitive firing between control and L-NAME treatment. Representative traces of intravesical 5-HT application and pre-incubation of L-NAME are shown in figure 4.28.

Similar to bath application, intravesical application of 5-HT debilitated afferent firing response to bladder at the 3rd ramp distension (*P<0.05) (Figure 29A). Pre-incubation of L-NAME prevented 5-HT-decreased afferent response to bladder distension (Figure 29B).

Intravesical 5-HT application increased spontaneous afferent firing after the 2nd ramp distension (**P<0.01) (Figure 4.30A). Interestingly, after the 2nd and 3rd ramp distensions, spontaneous firing was enhanced during pre-incubation with L-NAME (**P<0.01 and *P<0.05, respectively) (Figure 4.30B). In the presence of L-NAME, 5-HT induced an increase in spontaneous afferent firing which was augmented after the 3rd distension (5-HT, 295.10 ± 44.78% vs. L-NAME pre-incubation, 511.7 ± 88.74%) (Figure 4.30C).

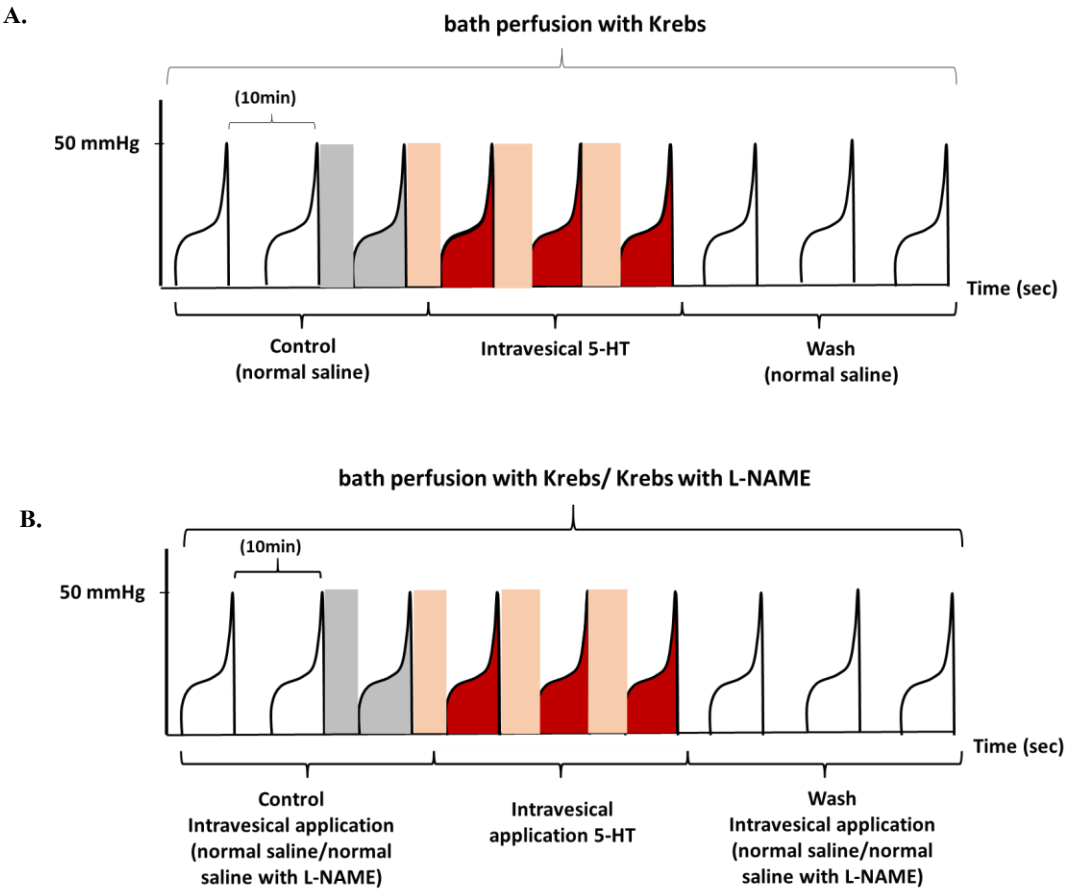


Figure 4.26 Protocol used to investigate the effect of intravesical 5-HT (A) and L-NAME prior to 5-HT application (B) on bladder afferent firing. Grey and red areas indicate where the spontaneous and mechanosensitive firing of control period and response to 5-HT application for the 1st, 2nd, and 3rd distention respectively.

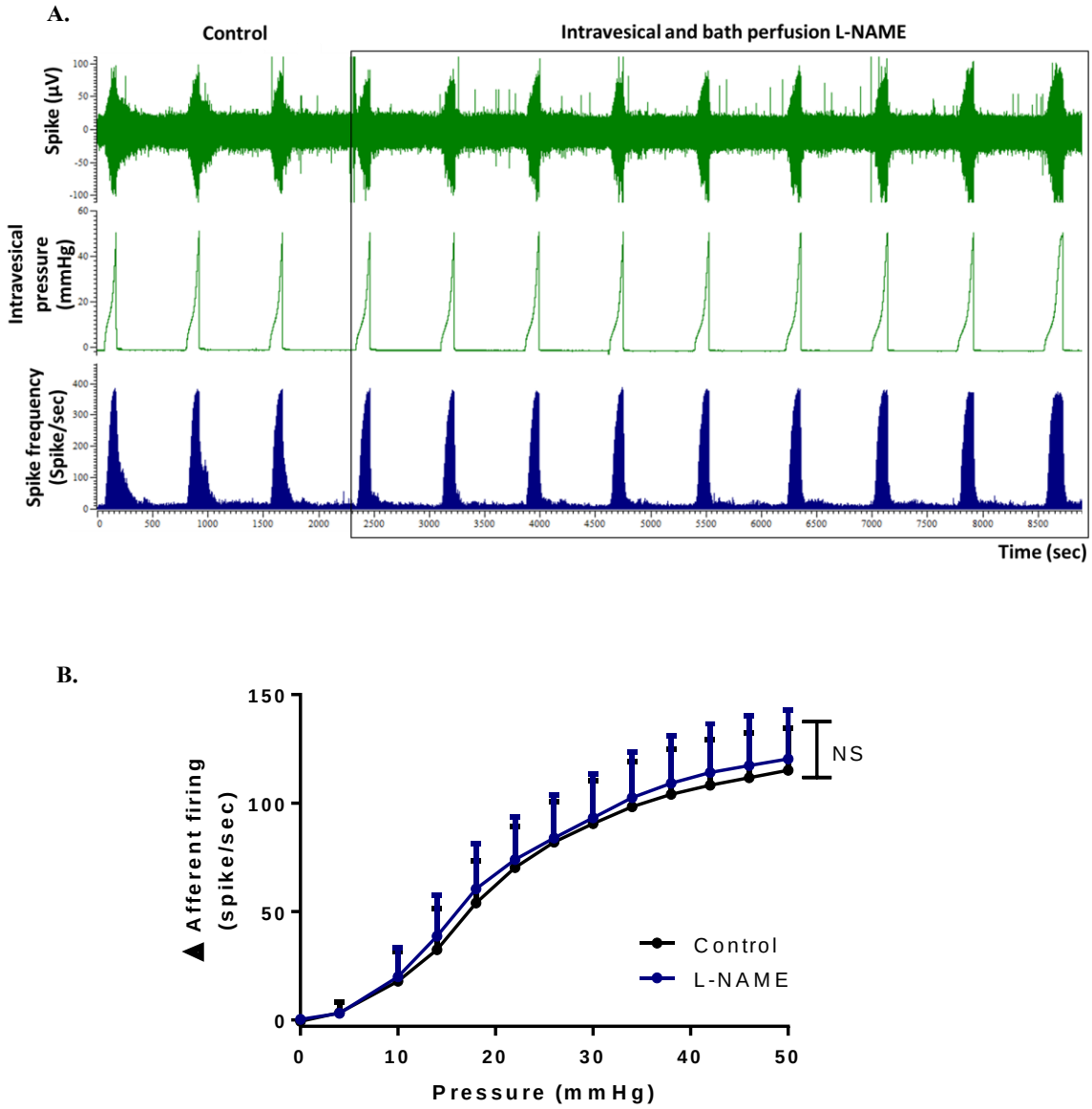


Figure 4.27 L-NAME had no effect on bladder afferent firing. (A) a representative trace of a time control experiment to determine whether L-NAME (1mM) affected bladder afferent firing. **(B)** Comparison of mechanosensitive afferent firing between the 3rd control distension and the 3rd distension of L-NAME, Two-way ANOVA (N=5).

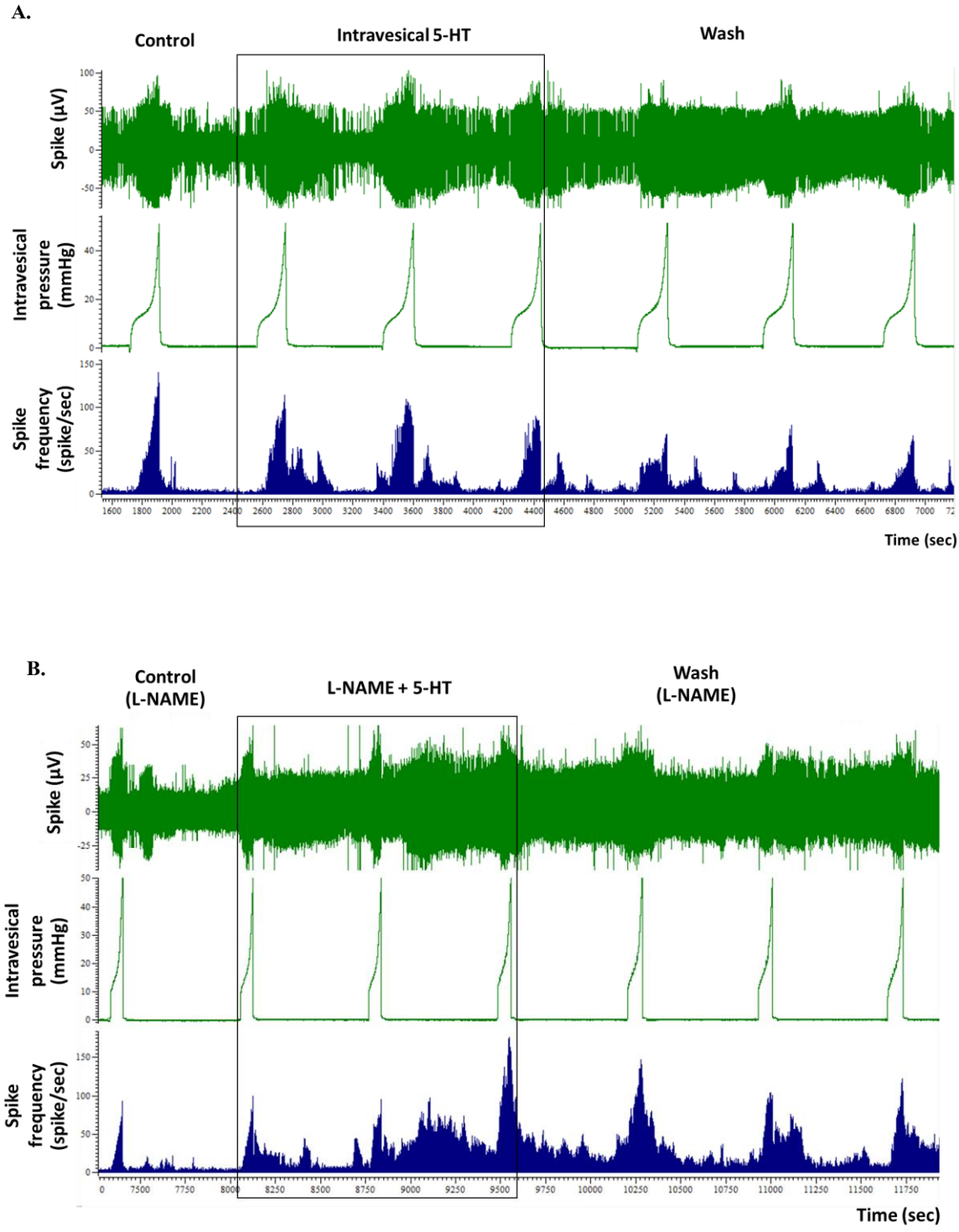


Figure 4.28 Representative traces to illustrate mechanosensitive and spontaneous afferent response to bladder ramp distentions during intravesical 5-HT (A) and intravesical 5-HT with pre-incubation of L-NAME (B).

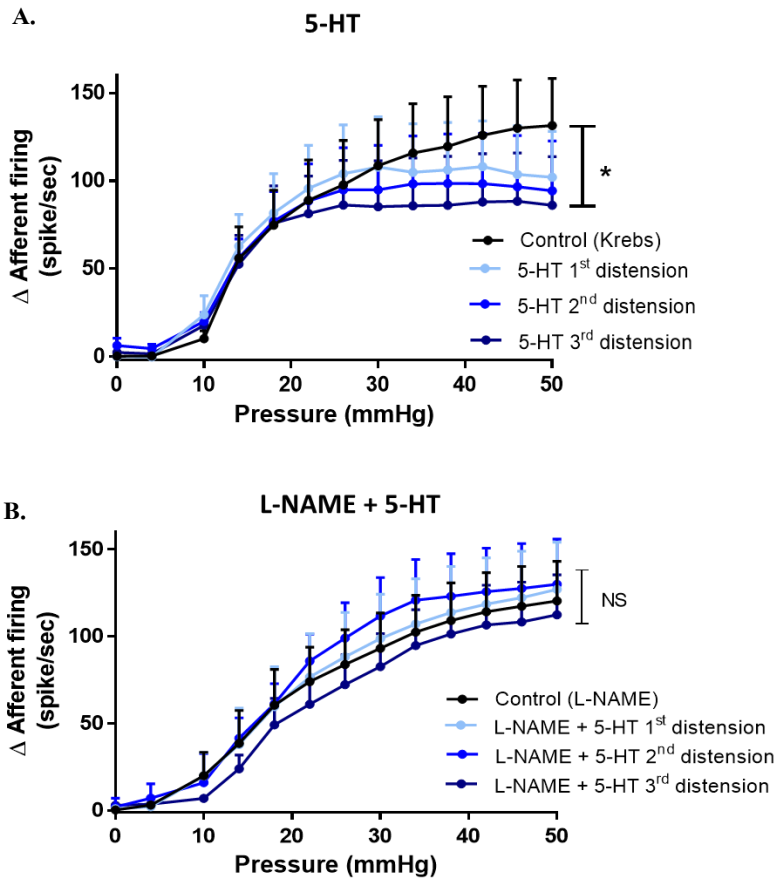


Figure 4.29 Pre-incubation with L-NAME reversed 5-HT-attenuated mechanosensitive afferent firing. (A) Comparison of bladder afferent responses to ramp distensions after intravesical application of 5-HT and **(B)** L-NAME pre-incubation with intravesical 5-HT application**, ***P<0.05, Two-way ANOVA (N=7 and 5 respectively).

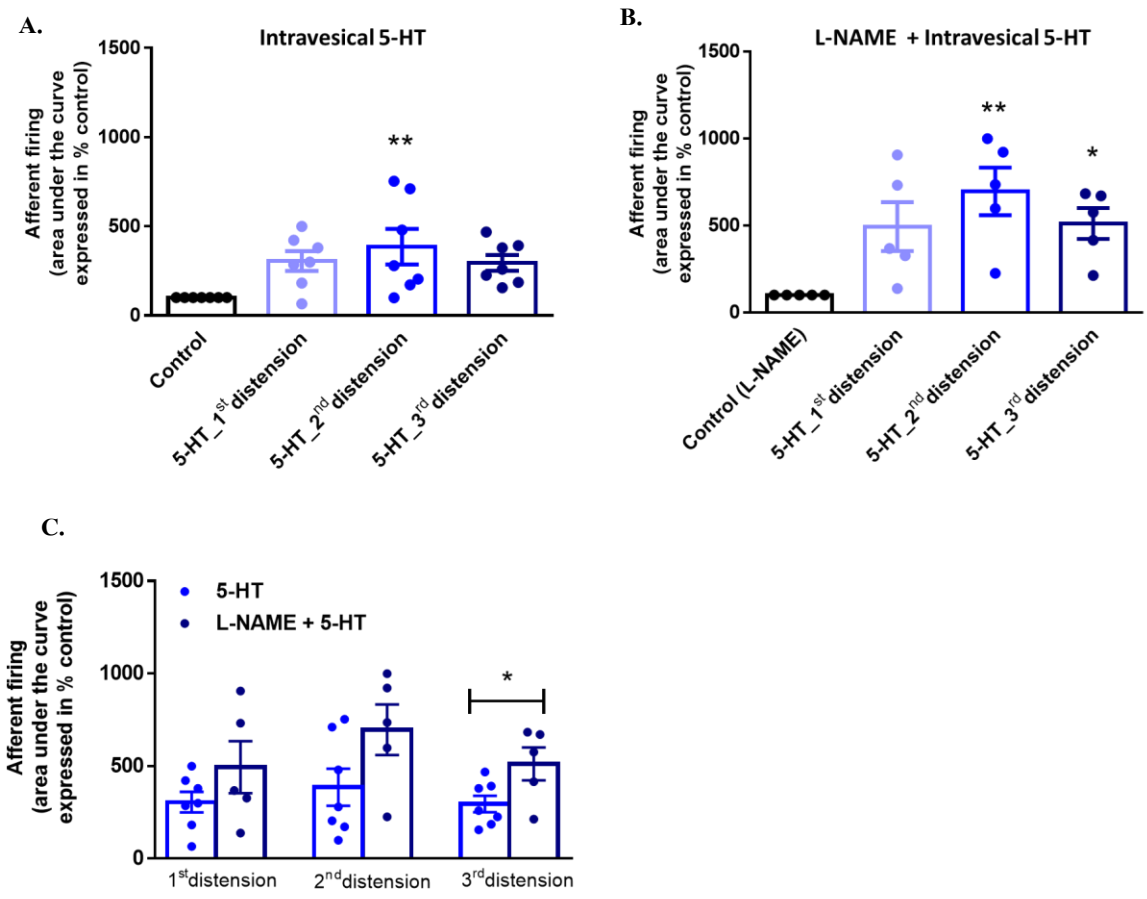


Figure 4.30 Pre-incubation of L-NAME augmented 5-HT-activated spontaneous afferent firing between ramp distensions (A) Comparison of spontaneous afferent firing responses to intravesical application of 5-HT after ramp distensions and **(B)** in the presence of L-NAME, *P<0.05, **P<0.01, One-way ANOVA with Dunnett's multiple comparison. **(C)** Comparison of spontaneous afferent firing between 5-HT and pre-incubation of L-NAME at different ramp distensions, *P<0.05 Unpaired Student's t-test (N=7 and 5 respectively).

4.6 DISCUSSION

The main findings of this chapter are

1.) 5-HT stimulation of baseline afferent firing is mainly via 5-HT3 receptors;
2.) 5-HT3 receptor activation is independent of muscle contraction;
3.) Non-5-HT3 (5-HT1, 2, 4-7) receptors activated afferent firing via potentiation of muscle contraction;
4.) 5-HT attenuated afferent firing in response to bladder ramp distension but increased spontaneous afferent discharges mediated via 5-HT3 receptors;
5.) Inhibitory effect of 5-HT on mechanosensitive firing and augmentation of spontaneous firing was related to NO signalling.

5-HT-stimulated baseline afferent firing is mainly via direct activation of 5-HT3 receptors, and independent of muscle contraction.

5-HT 10 μM and 100 μM stimulated baseline afferent firing in a concentration-dependent manner. Even though a full concentration response curve for 5-HT was not carried out in this study, a number of studies have shown the dose-response curve of 5-HT effects on detrusor potentiation. Cumulative application of 5-HT (0.01-100 μM) demonstrated to increase bladder contraction in a concentration dependent manner in rats (Sakai *et al*. 2013).

Similar to many previous studies, we also found that 5-HT triggered bladder contraction as indicated by an increase in intravesical pressure. 5-HT has been reported to potentiate detrusor contraction in many species, including rodents, rabbits, and human (Chetty *et al.* 2007; Lychkova and Pavone 2013; Candura *et al.* 1996; Messori *et al*. 1995; Corsi *et al.* 1991). Interestingly, intravesical and extraluminal (bath) 5-HT application enhanced afferent discharge with the same amplitude of the maximal responses, however, the present clearly showed that intravesical application delayed the time to peak response to 5-HT. The explanation could be that 5-HT penetrates and diffuses to bind to receptors on the nerve terminals and the detrusor layers from serosal side easier than the intravesical side due to the barrier function of the urothelial lining.

2-Me-5-HT, a selective 5-HT3 agonist also evoked baseline afferent discharge with the same peak firing response and time profile as 5-HT. As reported in chapter 3, even though 5-HT3 receptors were not expressed in mouse urothelium, Chetty and co-workers have shown that 5-HT3 receptors are expressed in the detrusor and mucosal layers of female mice (Chetty *et al.* 2007). 5-HT3A and 3B mRNA expression has also been reported in afferent terminals in spinal dorsal laminae (Kidd *et al.* 1993) and lumbosacral DRG neurons (L4-L5) (Schmitt *et al.* 2006). It is possible that 5-HT and 2-Me-5-HT activate 5-HT3 receptors in the detrusor and/or afferent terminals.

2-Me-5-HT triggered moderate bladder contraction compared to 5-HT and 5-MT, however, baseline afferent firing was still activated after pre-incubation with Y-27632 to prevent the contractile component. These data suggest that 5-HT directly activates 5-HT3 receptors in a mechanism that is independent of detrusor contraction. The key contribution of 5-HT3 receptors was also demonstrated by pre-incubation of granisetron, a 5-HT3 antagonist. As expected, granisetron attenuated 5-HT-evoked baseline afferent stimulation however, intravesical pressure was unchanged, suggesting that 5-HT3 receptors play a crucial role in 5-HT-enhanced baseline afferent discharge confirming that the main action of 5-HT3 receptors was not secondary to detrusor contraction but could be at the nerve terminals.

The pharmacological specificity of granisetron (1 μM) is one factor that needs to be considered. Several 5-HT3 antagonists are available and considered to be used to be potent 5-HT3 antagonists including granisetron, ondansetron, and tropisetron. Sanger and Nelson (1989) reported granisetron to be a potent and selective 5-HT3 receptor antagonist, in guinea-pig isolated ileum, where the drug (0.01-1μM) potently inhibited 5-HT-induced muscle contraction. The specificity of granisetron has also been confirmed, where granisetron failed to inhibit 8-OH-DPAT (a 5-HT1A full agonist)-evoked inhibitory effect of electrical induced muscle contraction (Sanger and Nelson 1989). Interestingly, granisetron on its own did not have an effect on baseline afferent activity. This result indicates that in normal conditions, there might be low concentration of extracellular 5-HT in the bladder wall. 5-HT3 may develop a more dominant role in bladder afferent firing in pathological conditions such as inflammation which 5-HT levels may be elevated (Keating *et al.* 2008; Kidd *et al.* 2009).

Our findings are correlated with a previous study that 5-HT3 receptors contribute to afferent hyperexcitability in jejunum in a post-inflammatory mouse model using in *vitro* extracellular nerve recordings (Keating *et al.* 2008). Hall and co-workers proposed a role for 5-HT3 receptors in bladder nociception in rats. They showed that systematic injection of 5-hydroxytryptophan (5-HTP), a 5-HT precursor triggered bladder hyperexcitability in a study of the visceromoter response to bladder distention; this effect was mediated by spinal 5-HT3 receptors (Hall *et al.* 2015).

The possibility that 5-HT stimulated afferent firing via regulation of neurotransmitter release in the bladder wall could not be excluded. In the CNS, 5-HT has been found to regulate neurotransmitter release, including ACh, dopamine, and noradrenaline (Fink and Göthert, 2007). 5-HT and 2-Me-5-HT reportedly facilitate dopamine release in rat striatal slices (Fink and Göthert 2008). Clear evidence that 5-HT regulates the release of other mediators was provided by Matsumoto-Miyai *et al.* (2016) who demonstrated that 5-HT mediated distension-induced ATP release from urothelium (via 5-HT4 receptors) (Matsumoto-Miyai *et al.* 2016). ATP is a key neurotransmitter which sensitizes bladder afferent nerves as shown by testing with αß-methylene-ATP, a P2X3 agonist (Rong *et al.* 2002). It is possible that 5-HT activation could mediate ATP release from urothelial cells and further sensitize afferent fibres.

In addition, in the present study 5-HT may regulate TRPV1 receptor activity, as shown in a study of colonic sensory neurons. 5-HT increased the excitability of sensory neurons innervating the colon in response to thermal ramp stimulation in wild type but not TRPV1 knock-out mice (Sugiuar 2004). It is also possible that 5-HT might decrease the threshold for the activation of TRPV1 channels expressed in afferent fibres, which in turn would contribute to excitation of 5-HT on baseline afferent firing. Grundy and co-workers (2014) showed that αß-methylene-ATP increased the hyperexcitability of bladder afferenta and this was attenuated in TRPV1 knockout mice (Grundy 2014). However, experiments supporting the role of ATP and TRPV1 in 5-HT-evoked baseline afferent firing should be studied in P2X and TRPV1 knockout mice or in the presence of specific P2X and TRPV1 antagonists.

Non-5-HT3 receptors (5-HT1, 2, 4-7) activated baseline firing via potentiation of muscle contraction.

The present study found that the 5-HT1, 2, 4-7 agonist, 5-MT, evoked baseline afferent discharge concurrent with bladder contraction. Many studies have reported expression of these receptor subtypes in detrusor muscle. 5-HT has been reported to bind to receptors on prejunctional excitatory nerves and on postjunctional detrusor smooth muscle cells (Matsumoto-Miyai *et al.* 2015). However, each subtype of non-5-HT3 receptors employs a different transduction pathway. For example, 5-HT1A, 2A, 2B, 2C activation triggers bladder contraction but in different ways: 5-HT1A activation decreases cAMP levels via $G_{i/o}$ protein, whereas 5-HT2A, 2B, 2C activation is mediated through G_q protein to increase intracellular Ca^{2+} (Mittra *et al.* 2007; Sakai *et al.* 2013). In contrast, a previous study in pig shows that 5-HT7 via Gs stimulation induces bladder neck relaxation (Recio *et al.* 2009). In addition to the detrusor, several studies have reported expression of non-5-HT3 receptors in the afferent pathway as summarised in table 3.1. 5-HT1A and 1B are found in the dorsal horn of spinal cord (Laporte *et al.* 1995). 5-HT1B, 1D, 2A are expressed in rat embryonic DRG neurons (Chen *et al.* 1998). Moreover, our PCR data (chapter 3) shows 5-HT1A, 1B, 1D, 2A, 2B, 4, 6, and 7 receptors expressed in mouse urothelial cells, exhibiting functional responses to 5-HT in the calcium imaging study.

All subtypes of non-5-HT3 are G-protien receptors. G-protein coupled receptor activation leads to activation of both Rho kinase and MLCK pathways (Liao *et al.* 2009). We found that after inhibition of detrusor contraction with Y-27632 (a Rho-kinase inhibitor) and ML-9 (MLCK inhibitor), 5-MT failed to activate baseline afferent discharge, indicating that 5-HT1, 2, 4-7 activated baseline afferent firing is due to potentiation of muscle contraction. Even though 5-HT1A, 1B, 1D, and 2A were detected in sensory nerve terminals (Matsumoto-Miyai *et al.* 2015), the contribution of these subtypes may not be essential to 5-HT signalling in bladder afferents.

5-HT3 receptors mediated an attenuation of mechanosensitive afferent firing.

In contrast to baseline afferent firing, 5-HT attenuated afferent firing in response to bladder ramp distension, 15 and 25 minutes after the drug application. This 5-HT effect was mimicked by 2-Me-5-HT. The contribution of 5-HT3 receptors was supported by granisetron's reversed of 5-HT-induced attenuation of mechanosensitive afferent firing. On the other hand, 5-MT had no effect on bladder afferent responses to bladder distension. These results indicate that 5-HT3 receptors play a major role in 5-HT-evoked attenuation of bladder mechanosensitivity.

In addition to mechanosensitive afferent firing, the key role of 5-HT3 receptors is highlighted by the finding that 2-Me-5-HT mimicked 5-HT activated spontaneous firing during and after drug application. Interestingly, effects of 5-HT and all pharmacological substances were not reversible on washout. The excitation of spontaneous firing and attenuated effects still persisted during the wash period. This may suggest that 5-HT also exerts indirect action on afferent firing via regulation of the release of other mediators.

An explanation of the 5-HT and 2-Me-5-HT evoked excitation of spontaneous firing could be due to recruitment of silent nociceptive C-fibres. The silent nociceptive receptors are induced to function only in response to noxious stimulation or pathological conditions (Yoshimura and Chancellor 2003). 5-HT also plays roles in the inflammatory response; particularly in the intestinal tract where the majority of 5-HT (95%) is produced and secreted from EC cells to regulate intestinal motility, secretion, and sensation. It has been shown that 5-HT secretion from intestinal EC cells was increased after LPS and interleukin-1ß stimulation in patients with Crohn's disease (Kidd *et al.* 2009). Much evidence has indicated the role of 5-HT3 receptors in inflammatory responses. 5-HT3 receptors are expressed in immune cells including T cells (Khan and Poisson 1999), monocytes, and dendritic cells (Fiebich *et al*. 2004). Upregulation of 5-HT3 receptors expressed in nerve fibres of colonic mucosa has been displayed in a mouse model of dextran sulfate sodium (DSS)-induced colitis (Matsumoto *et al.* 2012).

Interestingly, there was no change in spontaneous firing in response to 5-MT. Since 5-HT1, 2, 4-7 receptors exert an opposite transduction mechanism as summarised in table 4.1. 5-HT1A, 1B, 1D, and 5A receptors exert an inhibitory effect on bladder function, whereas 5-HT2A, 2B, 4, 6, and 7 have stimulatory action on detrusor contraction. It is possible that there is a

counteractive action on mechanosensitive afferent firing between different 5-HT receptors. The stimulatory effect on baseline afferent discharge by 5-MT was absent when the bladder was distended. This indicates that bladder distension may elicit inhibitory neurotransmitter release, which overcomes excitatory action of non-5HT3 receptors in baseline afferent firing.

In response to bladder distension, mechanotransduction of afferent nerves could be either a direct activation on mechanosensitive-gated ion channels in afferent terminals or an indirect chemical transduction of released mediators from non-neuronal cells (urothelial cells) (Zagorodnyuk *et al*. 2009; Burnstock 2001; Hamill and Martinac 2001). Several line of evidences support the notion that 5-HT could regulate release of other mediators which could further exert an inhibitory effect on mechanosensitive afferent fibres. 5-HT3 receptors have been reported to enhance cholinergic transmission in guinea pig isolated detrusor (Messori *et al.* 1995). Daly *et al.* has shown that muscarinic receptor activation with bethanechol or carbachol depressed the afferent response to bladder ramp distensions in mice (Daly *et al.* 2010). This raises the possibility that 5-HT3 receptor activation in the bladder wall may evoke ACh release from neuronal and non-neuronal cells and further inhibit mechanosensitive afferent firing.

One concerning point is that an inhibitory effect of 5-HT on mechanosensitive afferent firing could be due to receptor desensitization. In a previous study, Maricq and co-workers demonstrated the functional response of 5-HT3 receptors expressed in *Xenopus* oocytes by measuring a current in response to bath application of 5-HT or 5-HT3 agonists; 2-Me-5-HT, PBG and mCPBG. 5-HT and all of the 5-HT3 agonists rapidly triggered an inward current and desensitization after continuous perfusion of the drugs (Maricq *et al.* 1991). However, this is unlikely as in the present study, baseline afferents between ramp distensions were still stimulated. In this study, we did not apply the drugs directly onto the cells which expressed 5-HT3 receptors. Application of agonists to the bladder tissue have a slower penetration comparing to the direct activation into the cells. Therefore, the chance of 5-HT3 receptor desensitization should be lower.

Inhibitory effect of 5-HT on mechanosensitive firing and augmentation of spontaneous firing was related to NO pathway.

In this study, the role of NO in 5-HT mediated attenuation of mechanosensitive firing has been investigated. The NOS inhibitor, L-NAME, prevented the 5-HT-induced decrease in mechanosensitive afferent firing. This is correlated with a study which showed that L-NAME (20 mM) reversed SNAP (NO donor)-induced decrease in baseline and mechanosensitive afferent firing in CYP rats (Yu and De Groat 2013). However, they found that NO had no effect on bladder afferent firing in normal rats. Similar to this observation, L-NAME application had no effect on afferent firing in the absence of 5-HT. Rather, the effect of L-NAME was exposed only when 5-HT was presented.

Moreover, our study clearly shows that spontaneous afferent firing in response to 5-HT was augmented when NOS was inhibited. This indicates that activation of 5-HT elicits NO production which also exert an inhibitory effect on spontaneous firing. The role of NO has also been reported in rat nodose ganglion. SNAP inhibited voltage-gated Ca^{2+} channel currents and this effect involved cGMP and PKG activation (Bielefeldt *et al.* 1999). In guinea-pigs, 5-HT3 activation evoked NO release from nerve endings, leading to relaxation of the proximal colon (Sevcik *et al.* 1998). It would be interesting to study further whether 5-HT3 stimulation in the bladder wall could mediate NO release (from either urothelial cells, smooth muscle cells, or nerve ending), which then acts downstream at nerve terminals to inhibit afferent discharge. Application of 2-Me-5-HT or other 5-HT3 agonists with L-NAME should be performed to confirm if 5-HT3 receptors also exert their role through the NO pathway. However, the source of NO in the bladder wall is unclear that modulates afferent signalling, which could be released from urothelial cells (Birder *et al.* 1998), detrusor muscle (Anderson and Persson 1994), and afferent nerves (Vizzard, 1997).

5-HT evoked baseline afferent but attenuated mechanosensitive afferent firing.

The paradox phenomena of 5-HT3 receptor activation between baseline and mechanosensitive afferent firing is possibly due to activation of a different nerve fibre population which varies in receptor co-localisation. Zagorodnyuk and co-workers identified 4 major classes of mechanoreceptors in guinea-pig bladder using a number of mechanical and chemical stimulations; (i) muscle mechanoreceptor: activated by stretch and urothelial removal, this could not affect firing; (ii) muscle-mucosal mechanoreceptors: activated by stretch, mucosal stroking, α,ß methylene ATP but not capsaicin, where removal of the urothelium reduced firing; (iii) mucosal high-responding mechanoreceptors: could not be activated by stretch but mucosal stroking, α,ß methylene ATP, hypertonic normal saline and capsaicin, in which removal of the urothelium reduced the nerve firing; (iv) mucosal low-responding mechanoreceptors: could not be activated by stretch, weakly-activated by mucosal stroking, but not hypertonic saline, α,ß methylene ATP, and capsaicin, in which urothelial removal decreased the firing (Zagorodnyuk *et al.* 2007). It is possible that 5-HT activation (via 5-HT3 receptors) in detrusor muscle attenuates afferent firing, whereas mucosal 5-HT receptors which are stretch-insensitive, trigger activation of baseline afferent firing. In addition, 5-HT3 receptors have been reported to localise in both myelinated and non-myelinated afferent fibres (Zeitz *et al.* 2002), suggesting diverse roles of 5-HT3 receptors in both normal sensation and nociception in the bladder.

The schematic diagram summarises possible mechanisms involved in 5-HT actions on baseline and mechanosensitive afferent firing is depicted in figure 4.30.

In summary, this chapter demonstrates the ability of 5-HT to peripherally evoke baseline afferent firing but an inhibitory effect on mechanosensitive afferent firing in mouse urinary bladder, which is triggered mainly via 5-HT3 receptors.

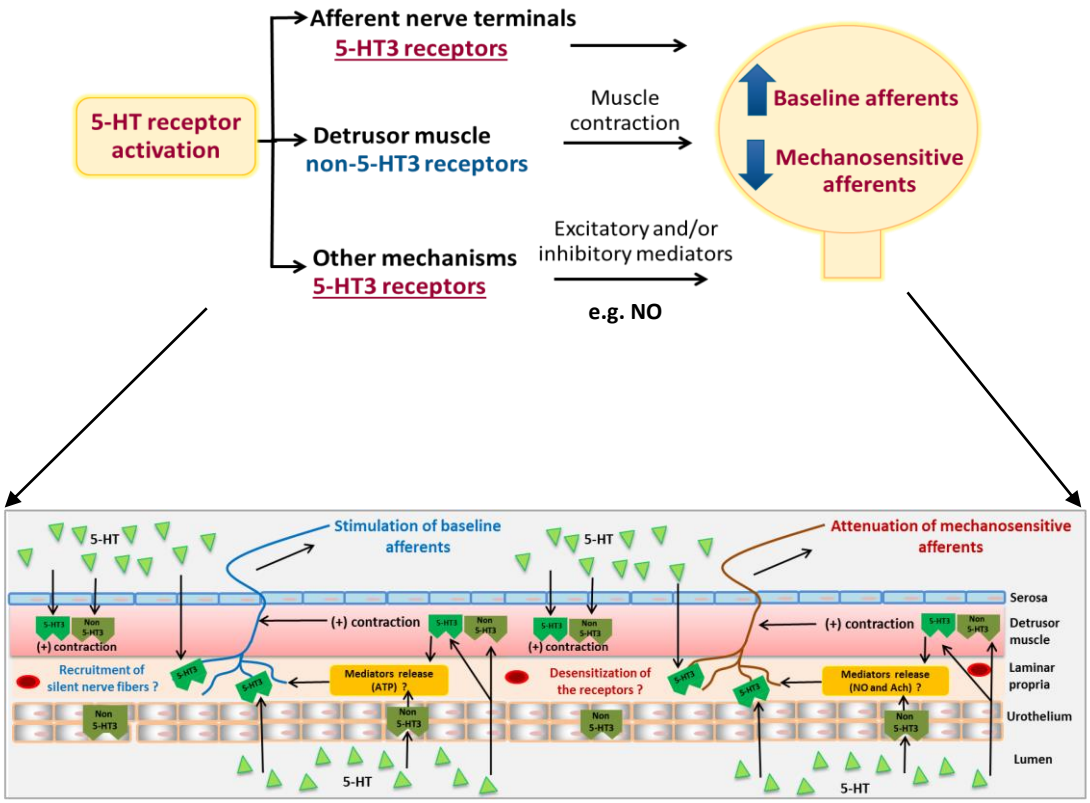


Figure 4.31 Schematic diagram summary of peripheral 5-HT actions on bladder afferent firing in this study. 5-HT stimulated baseline afferent firing but attenuated mechanosensitive afferent firing mainly via direct activation of 5-HT3 receptors on afferent nerve terminals. Non-5-HT3 receptors contribute to activation of baseline afferent firing through potentiation of detrusor contraction. 5-HT3 receptors may mediate excitatory or inhibitory neurotransmitter (NO) release and further modulate bladder afferent firing.

CHAPTER 5

EFFECT OF 5-HT ON BLADDER AFFERENT FIRING IN COLONIC TNBS-TREATED MICE

5.1 INTRODUCTION

The bowel and bladder are hollow organs that have similar functions in storing and eliminating waste from the body. The interaction of neural circuits through convergence of afferent nerves between the bowel and the bladder are essential to maintain physiological functions of these organs (McMahon and Morrison 1982). These convergent neuronal circuits allow sensitized afferents from one organ to affect the sensitivity of a second organ.

This 'cross-organ sensitization' has been reported in clinical investigations that have found that 40-60% of IBS patients to have bladder symptoms related to interstitial cystitis (IC) such as nocturia, urinary urgency, frequency, and painful urination (Jones and Nyberg 1997; Matsumoto *et al.* 2013; Novi *et al.* 2005; Prior *et al.* 1989; Zondervan *et al.* 2001). Conversely, about one-third of IC patients have been reported to exhibit IBS symptoms such as abdominal pain, discomfort, constipation, and diarrhea (Aaron and Buchwald 2001; Alagiri *et al*. 1997).

Few studies have investigated the aetiology of overlapping symptoms between these two organs. Most of the previous studies have proposed that 'cross-sensitization' between the bowel and the bladder are mainly derived from an anatomical proximity of neuronal pathway of the two organs, which leads to a convergence of sensory information. The cross-sensitization mechanism of the afferents may occur at the central (spinal/ supraspinal cord) and/or peripheral levels (antidromic axon reflexes) (Brumovsky and Gebhart, 2010; Christianson *et al.* 2008).

Interestingly, Christianson and colleagues (2008) have clearly demonstrated the existence of dichotomizing sensory neurons that innervate both organs by retrograde labelling of DRG neurons using cholera toxin subunit B (CTB) fluorescent conjugates. Around 17% in rats and 21% in mice of the total CTB positive DRG neurons from lumbosacral and thoracolumbar ganglion could be co-labelled from both organs (Christianson *et al.* 2008). A schematic diagram of cross-organ sensitization between the bowel and bladder is depicted in figure 5.1.

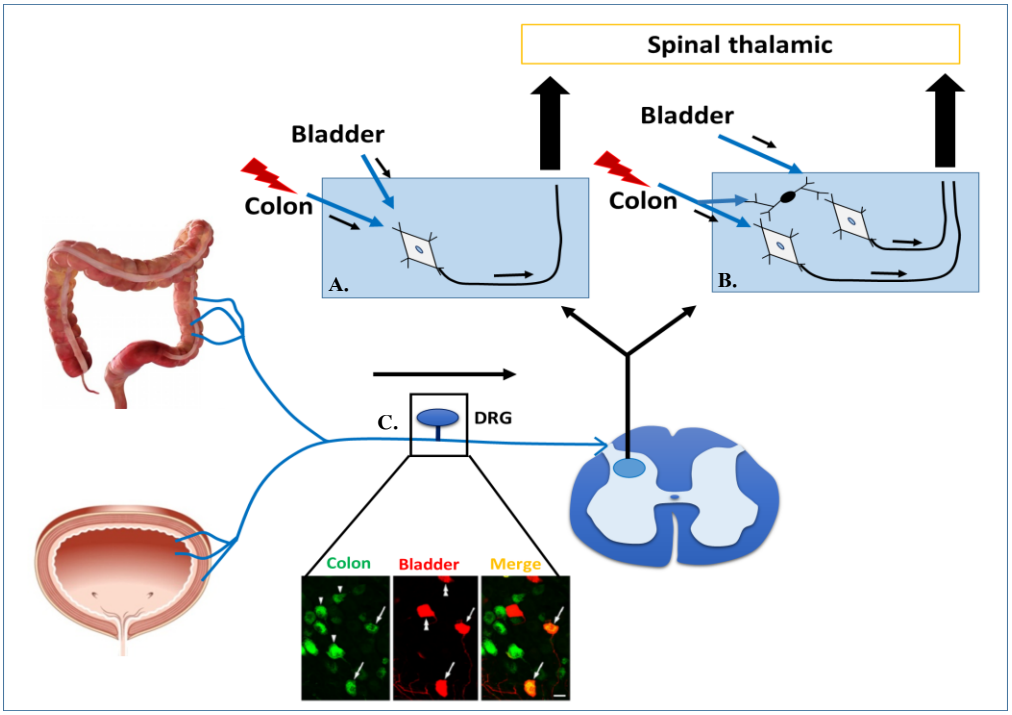


Figure 5.1 Schematic diagram depicts central (A, B) and peripheral (C) cross-sensitization mechanisms between the bowel and the bladder (modified from Brumovsky and Gebhart 2010). (A) Convergence of sensory signals from the bowel and bladder to the same second order spinal neuron. An insult in the colon (lighting symbol) increases excitability of the spinal neuron, resulting in concurrent amplification of sensory input from the bladder by the spinal neuron. **(B)** Activation from sensory input from the colon may activate spinal interneurons (filled circle), which leads to increased depolarization of primary afferents from the bladder. **(C)** Sensitized dichotomizing primary afferent neurons that innervate colon and bladder triggers an antidromic axon reflex, leading to generation of bladder inflammation. The insert immunofluorescence picture shows DRG neurons that innervate the colon (green), the bladder (red), and both organs (oranges) in merged channel as indicated by the arrows (Christianson *et al.* 2008).

In addition to IBS, clinical investigations report high prevalence of patients with inflammatory bowel diseases (IBD) which suffer from bladder symptoms (Banner, 1987; Francis *et al.* 1997). Colitis is an inflammatory condition of the intestinal epithelial layer, which is the major form of IBD. Patients with IBD have been characterized by bleeding, severe diarrhea, abdominal pain (Fuss *et al.* 1996; Papaconstantinou *et al.* 2014). In terms of tissue structure, the mucosa is thickened and has infiltration of neutrophils, macrophages, and monocytes (Ellrichmann *et al.* 2014). However, the underlying mechanisms contributing to overlapping bladder symptoms are still ambiguous.

Various chemical-induced gut inflammation animal models have been employed to investigate cross-talk between the bowel and the bladder including, 2,4,6-trinitrobenzene sulfonic acid (TNBS), dextran sulphate sodium (DSS), oxalone, and acetic acid (Randhawa *et al.* 2014). Among these intestinal inflammatory models, TNBS colonic irritation is one of the most widely used to induce colonic inflammation or colitis in both acute and post-inflammatory states (Qin *et al.* 2011; Randhawa *et al.* 2014). Acute colonic inflammation with TNBS treatment not only results in changes in the tissue structure (i.e., epithelial distortion, crypt disruption, and inflammatory cell filtration) but also hypersensitivity of colonic afferent nerves (Hughes *et al.* 2009; Motavallian *et al.* 2013).

A number of studies have shown that animals with colonic TNBS treatment have persistent colonic afferent hypersensitivity and that hyperexcitability persists after the inflammation has resolved (28 days post-treatment) (Hughes *et al.* 2009). Therefore, the TNBS model is also employed to investigate the mechanism of visceral hypersensitivity in the post-inflammatory state, which is considered to represent IBS patients recovering from intestinal inflammation but with persistent functional bowel symptoms. The aetiology of IBS is still unclear, however, hypersensitivity of afferent nerves innervating the bowels has been reported to be involved in the pathophysiology of IBS (Drossman, 1999; Hungin *et al.* 2003; Keating *et al.* 2008).

Linden and co-workers (2003) highlighted a role for 5-HT in TNBS-induced colonic inflammation. They reported an increase in 5-HT bioavailability in the colonic mucosa in guinea pigs at 6 days after TNBS colonic administration (Linden *et al.* 2003). Animals with TNBS-induced colitis showed suppression in expression and function of serotonin reuptake

transporter (SERT), a key transporter protein that transports released 5-HT back into cells in the gut wall (Linden *et al.* 2005). Decreased SERT expression levels in colonic inflammation is consistent among various species including, human (Coates *et al.* 2004), mice (Linden *et al.* 2005), and guinea pigs (Linden *et al.* 2003).

An elevated 5-HT level in colonic inflammation could also be derived from increased numbers of EC cells, which have been reported in guinea pigs (Linden *et al.* 2003) and humans with Crohn's disease (El-Salhy *et al.* 1997). In contrast, in mice, there was no change in the number of EC cells in colonic mucosa, but a higher number of mast cells that can also release 5-HT, was observed (Linden *et al.* 2005).

Fitzgerald *et al.* (2013) showed that there was a significant increase in the number of mast cells in the bladder after 12 days of colonic TNBS administration in rats. In addition, the animals had a reduced voiding interval and change in urothelial permeability (Fitzgerald *et al.* 2013). This was correlated with the finding of Grundy *et al.* (2016) who showed that in the post-inflammatory state (28 days post-treatment with TNBS) mice exhibited hypersensitivity of bladder afferents and abnormal voiding frequency (Grundy *et al.* 2016). Ustinova *et al.* reported bladder hypersensitivity after colonic TNBS treatment in both acute (1 hour after treatment) and chronic (10 days after treatment) states in female rats and reported no macroscopic or microscopic changes in the bladder wall. However, they found that both acute and chronic states showed a higher number of bladder mast cell (Ustinova *et al.* 2006; Ustinova *et al.* 2007). Mast cells are known to play a role in inflammation and they release a number of inflammatory mediators i.e., NGF, cytokines, histamine, substance P, VIP, and 5-HT. (Theoharides *et al.* 2012; Weitzman *et al.* 1985; Yu *et al.* 1999). These mediators may contribute to the development of bladder symptoms after intestinal inflammation.

Interestingly, recent studies have reported the role of 5-HT3 receptors in mediating intestinal inflammation. Tropisetron and ondansetron, 5-HT3 antagonists, reduced inflammatory signs such as epithelial damage, MPO activity, and cytokine levels in TNBS-induced colitis in rats (Motavallian-Naeini *et al.* 2012; Motavallian *et al.* 2013). The role of 5-HT3 receptors in generating post-inflammatory visceral hypersensitivity has been shown in mice infected with T. *spirallis* which is a well-characterized animal model to study post-inflammatory IBS (Mayer

and Collins 2002). T. *spirallis* infection triggers transient intestinal inflammation after which the animals develop functional abnormalities of the bowel such as change in peristalsis, hyperexcitability of afferent nerves, hypercontractility, and decreased threshold of the visceromotor response to colorectal distension (Barbara *et al.* 1997; Bercik *et al.* 2004; Akiho *et al.* 2005). Keating *et al.* (2008) showed that granisetron significantly decreased hyperexcitability of jejunal afferent nerves in response to distension in the post-inflammatory period (after 28 and 56 days). Concurrently, nodose ganglion neurons in these animals showed reduced excitability in response to 2-Me-5-HT, suggesting a decrease in 5-HT3 receptor activity in the post-inflammatory period in this animal model (Keating *et al*. 2008).

All of the above information raises the possibility that bladder hypersensitivity associated with TNBS-induced colitis may involve a 5-HT3 receptor mechanism. Therefore, this study aims;

(i) to investigate the effect of 5-HT on mechanosensitive and spontaneous firing in acute TNBS treated and chronic visceral hypersensitivity (CVH) mice;

(ii) to determine the role of 5-HT3 receptors on 5-HT mediated changes in bladder afferent firing in CVH animals;

(iii) to examine change in mRNA expression of SERT, TPH1, and TPH2 in urothelium in CVH mice.

5.2 EXPERIMENTAL PROTOCOL AND ANALYSIS

All of the experiments in this chapter were conducted in the Visceral Pain Laboratory at the South Australian Health and Medical Research Institute (SAHMRI), Adelaide, Australia. To account for any potential difference of 5-HT response in healthy mice that was previously conducted at the University of Sheffield, we firstly determined the effect of 5-HT on bladder afferent firing in healthy control animals. In addition, we used the same organ bath to ensure that there was no influence from a different experimental setup.

13-week old anesthetized mice were intracolonically administered with 2,4,6-trinitrobenzenesulfonic acid (TNBS) 0.01 mL (130 µg mL^{-1} in 30% ethanol) via a polyethylene catheter to induce colonic inflammation. Histological examination of colon and bladder was performed to monitor mucosal architecture and signs of inflammation, i.e., cellular infiltration, crypts abscesses, and goblet cell depletion. The treatment and histological study were performed by researchers in the Visceral Pain Group, SAHMRI.

A schematic diagram summarizing the time scale of treatment is shown in figure 5.2. In this study we investigated TNBS-treated mice at 2 time points; acute TNBS (3 days post-treatment) when there is an active inflammatory state and chronic visceral hypersensitivity (CVH) (28 days post-treatment) when the inflammatory signs have resolved but the bladder afferent hypersensitivity still persist.

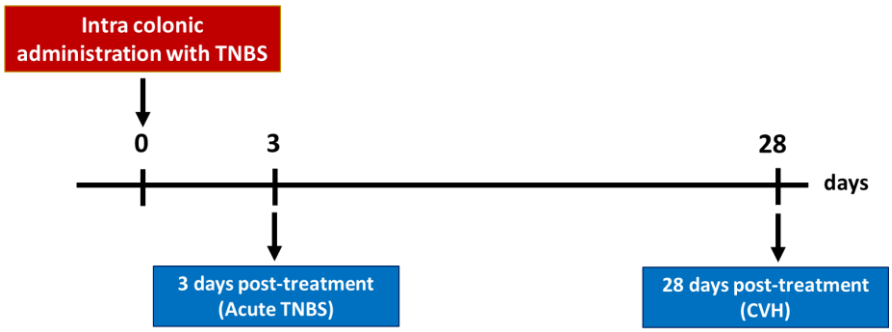


Figure 5.2 Schematic diagram of timeline for TNBS colonic administration in acute TNBS and CVH mice.

Effect of 5-HT on bladder afferent firing in acute TNBS treated and chronic visceral hypersensitivity (CVH) mice

In vitro afferent nerve recording was performed to determine the effect of 5-HT in colonic TNBS treated mice. Details of the experimental set-up are described in chapter 2.

The protocol used in this study is shown in figure 5.3. Repeated bladder distension during a control period was followed by application of 5-HT (100 μM) diluted in Krebs and constantly perfused into the recording chamber 2 minutes (5 mL minute^{-1}), before the next bladder distension. After 3 consecutive ramp distensions at 10 minute intervals, 5-HT was washed out with Krebs solution for a further 30 minutes.

To determine the contribution of 5-HT3 receptors to 5-HT mediated change in bladder afferent firing in CVH mice, granisetron (1 μM), was applied to both intraluminal and extraluminal sides of the prepatation for 30 minutes before 5-HT application and throughout thereafter.

The animals were divided into 4 groups;

(i) Healthy control (N=6)

(ii) Acute TNBS (N=5)

(iii) CVH (N=6)

(iv) CVH + granisetron (N=6)

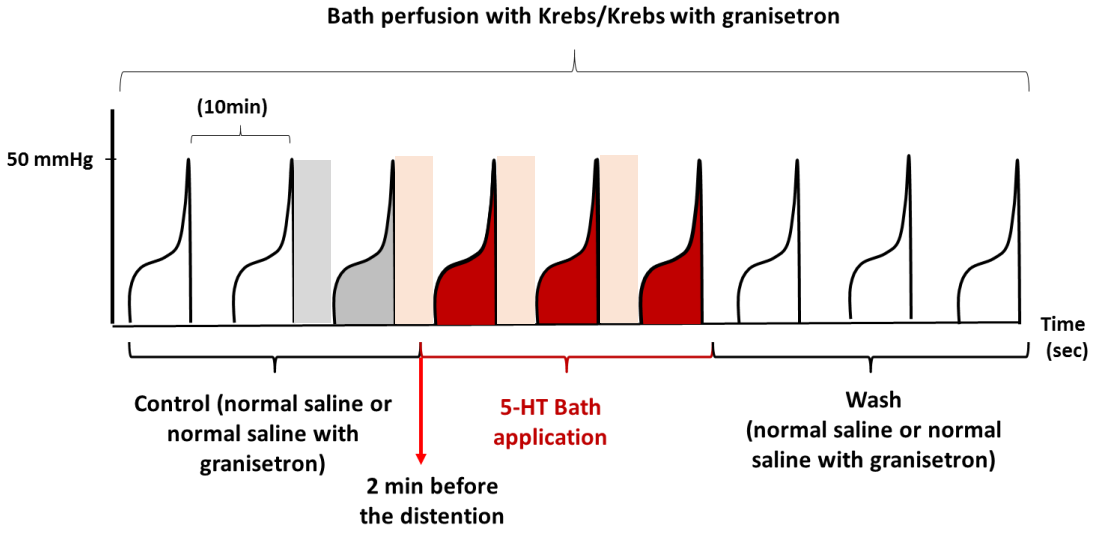


Figure 5.3 Protocol used to investigate the effect of 5-HT and 5-HT with granisetron pre-incubation in colonic TNBS-treated mice. Grey and red areas indicate where the spontaneous and mechanosensitive firing of control period and response to 5-HT application at 2, 15, and 25 minutes were analysed respectively.

The afferent firing response to bladder distension and spontaneous nerve firing between the distensions were analyzed at 2 minutes, 15 minutes, and 25 minutes after 5-HT application. The afferent nerve firing in response to bladder distension was substracted from 3 minutes baseline firing before the distension. Data are presented as mean ± SEM. Statistical analysis included Student's t-test, One-way ANOVA, and Two-way ANOVA which were tested in different experiments as described in each result figure.

Moreover, single unit analysis was performed in order to determine the proportion of low threshold, high threshold and silent fibres in the nerve bundles. Low threshold fibres are defined as those that respond at intravesical pressure 0-15 mmHg. Single units that showed increased firing between 15-50 mmHg are defined as high threshold fibres. Silent fibres are those units that responded to distension only in the presence of 5-HT. The proportion of each type of unit was analyzed using Fisher's exact test to determine significant difference of prevalence of nerve units in healthy control and CVH mice.

mRNA expression of SERT, TPH1, and TPH2 in urothelial cells of healthy control and CVH mice

Urothelial RNA samples extracted from healthy control and CVH mice were reversed transcribed. The total amount of cDNA was diluted in DNase free water (1/10 dilution). Commercial taqman probes were ordered from ThermoFisher Scientific Company and used to determine mRNA expression of SERT, TPH1, and TPH2 in urothelial cells, comparing between healthy control and CVH mice by quantitative real-time PCR. The level of gene expression was normalized to ß-actin (details of the calculation were described in section 2.5.4). The list of primers is shown in table 5.1.

Table 5.1 Summary of primers used in quantitative real-time PCR

Genes	Product code	Accession number	Sequences where probes bind
SERT	Mm00439391_m1	NM_010484.2	5' CTACCAGAATGGTGGAGGGGCCTTC 3'
TPH1	Mm01202614_m1	NM_001136084.2	5' GTGCTGAAAATCTTCCAGGAGAATC 3'
TPH2	Mm00557715_m1	NM_173391.3	5' TAGACTATTCCAGGAAAAACATGTC 3'

5.3 RESULTS: EFFECT OF 5-HT ON BLADDER MECHANOSENSITIVE FIRING AND BLADDER COMPLIANCE IN TNBS-TREATED MICE

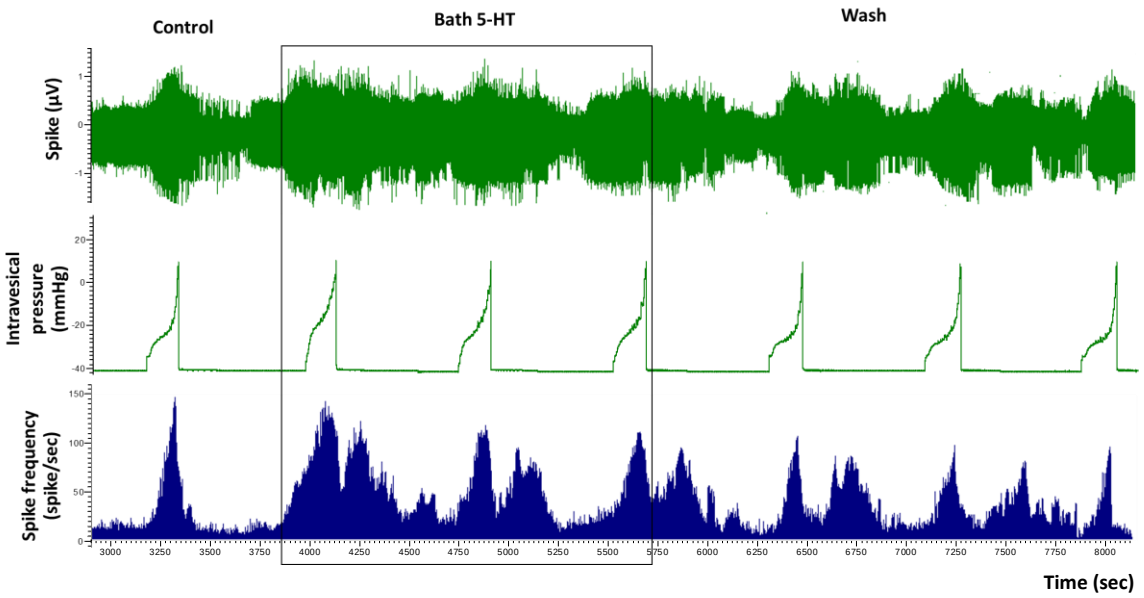
5.3.1) RESULTS: BLADDER AFFERENT FIRING IN TNBS-TREATED MICE

Representative traces of bath application of 5-HT in healthy control, acute TNBS, CVH, and CVH together with granisetron are shown in figure 5.4 and 5.5 respectively.

There was no significant difference in mechanosensitive and spontaneous afferent firing between healthy control and acute TNBS. However, acute TNBS caused a moderate (~25% from healthy control) decrease of mechanosensitive afferent firing at 50 mmHg and showed moderate increase of spontaneous firing (~21% compared with healthy controls).

In contrast, CVH mice had a significant increase in both mechanosensitive (~27%) and spontaneous afferent firing (~160%) compared to healthy control mice (*P<0.05). Granisetron reversed this hypersentivity of mechanosenstive firing in CVH mice (*P<0.05) (Figure 5.6A). Even though there was no statistical difference in spontaneous firing between CVH with or without granisetron, there was a trend to decreased (~48%) in CVH with granisetron (Figure 5.6B).

A. Healthy control



B. Acute TNBS

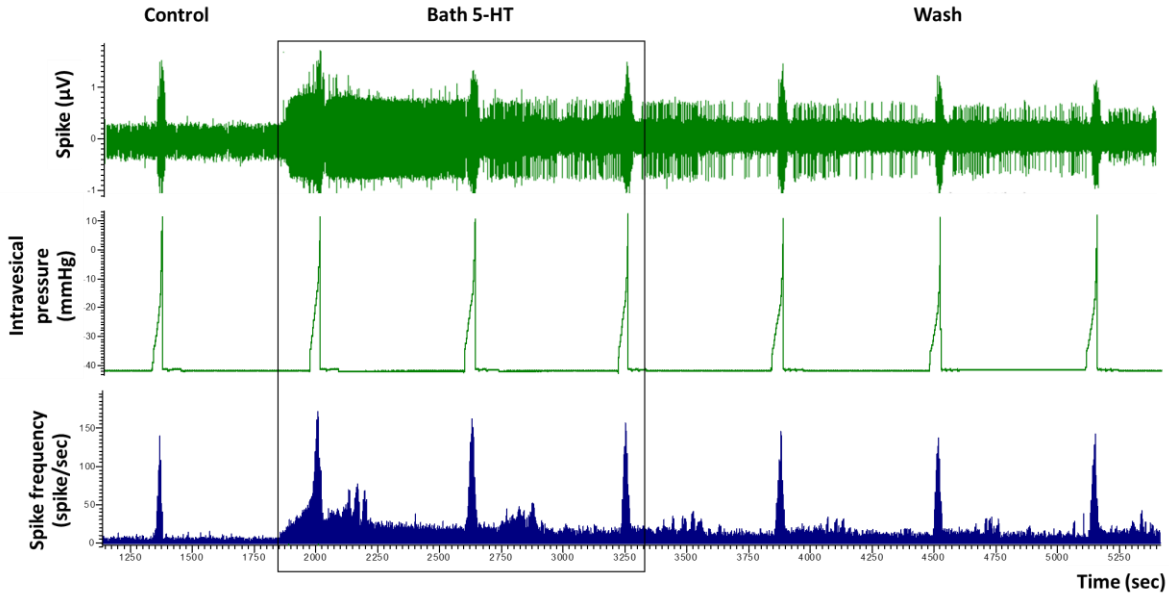
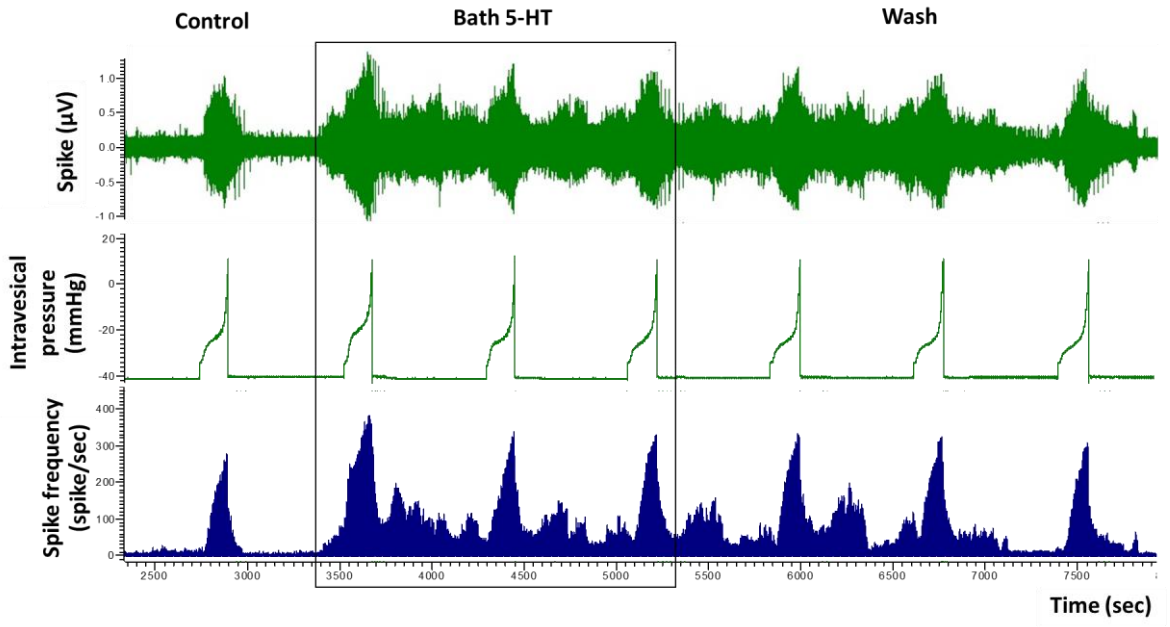


Figure 5.4 Representative traces to illustrate spontaneous and mechanosensitive afferent responses to bladder ramp distensions during 5-HT bath application in healthy control (A) and acute TNBS mice (B).

A. CVH



B. CVH+Granisetron

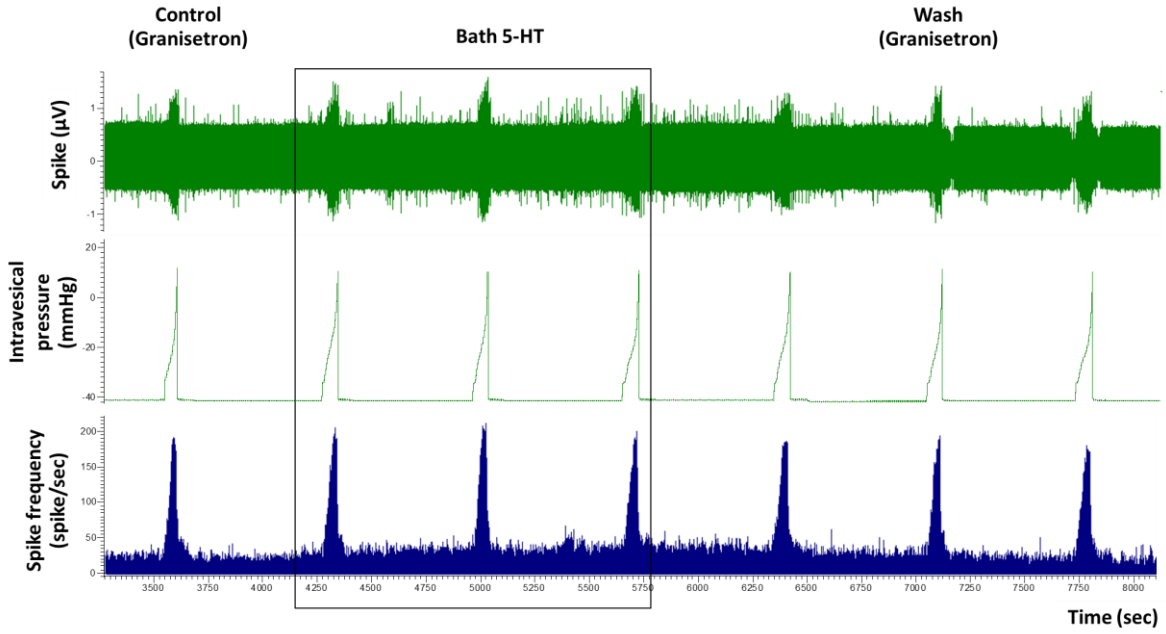


Figure 5.5 Representative traces to illustrate spontaneous and mechanosensitive afferent responses to bladder ramp distensions during 5-HT bath application in CVH (A) and CVH with granisetron pre-incubation (B).

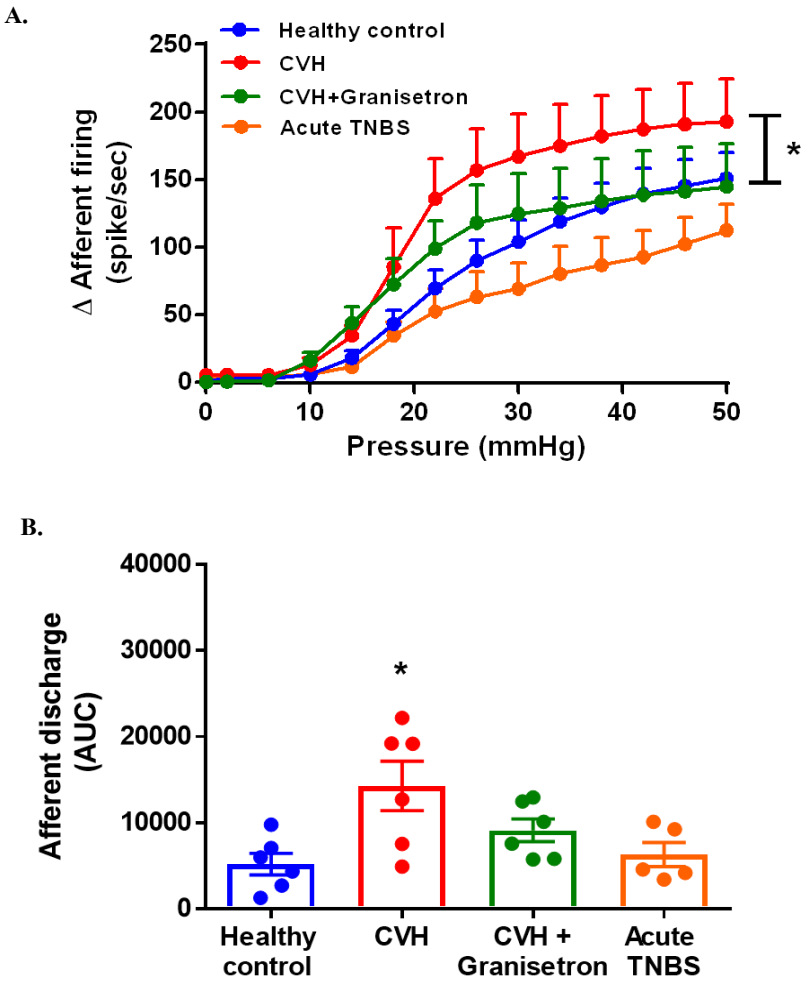


Figure 5.6 CVH mice showed mechanosensitive and spontaneous afferent hypersensitivity which were reversed by granisetron. Comparison of mechanosensitive **(A)** and spontaneous **(B)** afferent discharges before 5-HT application between healthy control, acute TNBS, CVH, and CVH with granisetron mice. *P<0.05, Two-way ANOVA and One-way ANOVA with Dunnett's multiple comparison respectively (Healthy control and CVH, N=6; acute TNBS, N=5; CVH, N=6; CVH+granisetron, N=5).

5.3.2) RESULTS: EFFECT OF 5-HT ON BLADDER MECHANOSENSITIVE AND SPONTANEOUS AFFERRENT FIRING IN TNBS-TREATED MICE

Similar that shown in chapter 4, bath application of 5-HT attenuated mechanosensitive afferent firing at 15 and 25 minutes after 5-HT application in healthy control mice (*P<0.05 and **P<0.01, respectively) (Figure 5.7A). In acute TNBS, CVH, and CVH with granisetron pre-incubation, there was no significant change in mechanosensitive afferent discharges in response to 5-HT at any time points (Figure 5.7B, C, D respectively).

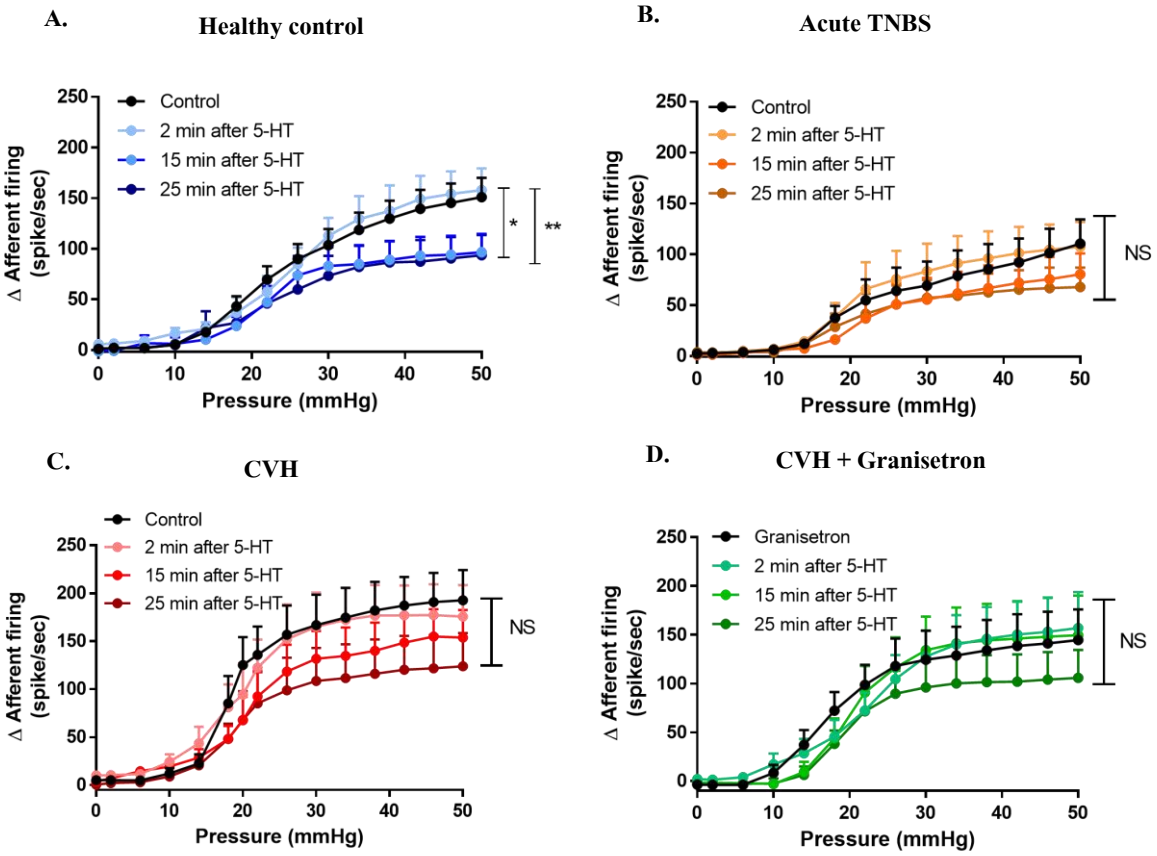


Figure 5.7 Attenuating effect of 5-HT on mechanosensitive afferent firing absent in acute, CVH, and CVH with granisetron pre-incubation. (A-D) Comparison of bladder afferent response to bladder ramp distension at 2, 15, 25 minutes after 5-HT bath application, *P<0.05, **P<0.01, Two-way ANOVA, (Healthy control, CVH, CVH + granisetron, N=6; acute TNBS, N=5).

In contrast to mechanosensitive afferent firing, 5-HT augmented spontaneous afferent firing at 2 minutes after the drug application in healthy control and acute TNBS groups (*P<0.05) (Figure 5.8A, B). However, there was no significant difference in spontaneous firing in response to 5-HT at any time points in CVH and CVH with granisetron pre-incubation (Figure 5.8C, D). The animals in all groups had no change in bladder compliance in response to 5-HT application (Figure 5.9).

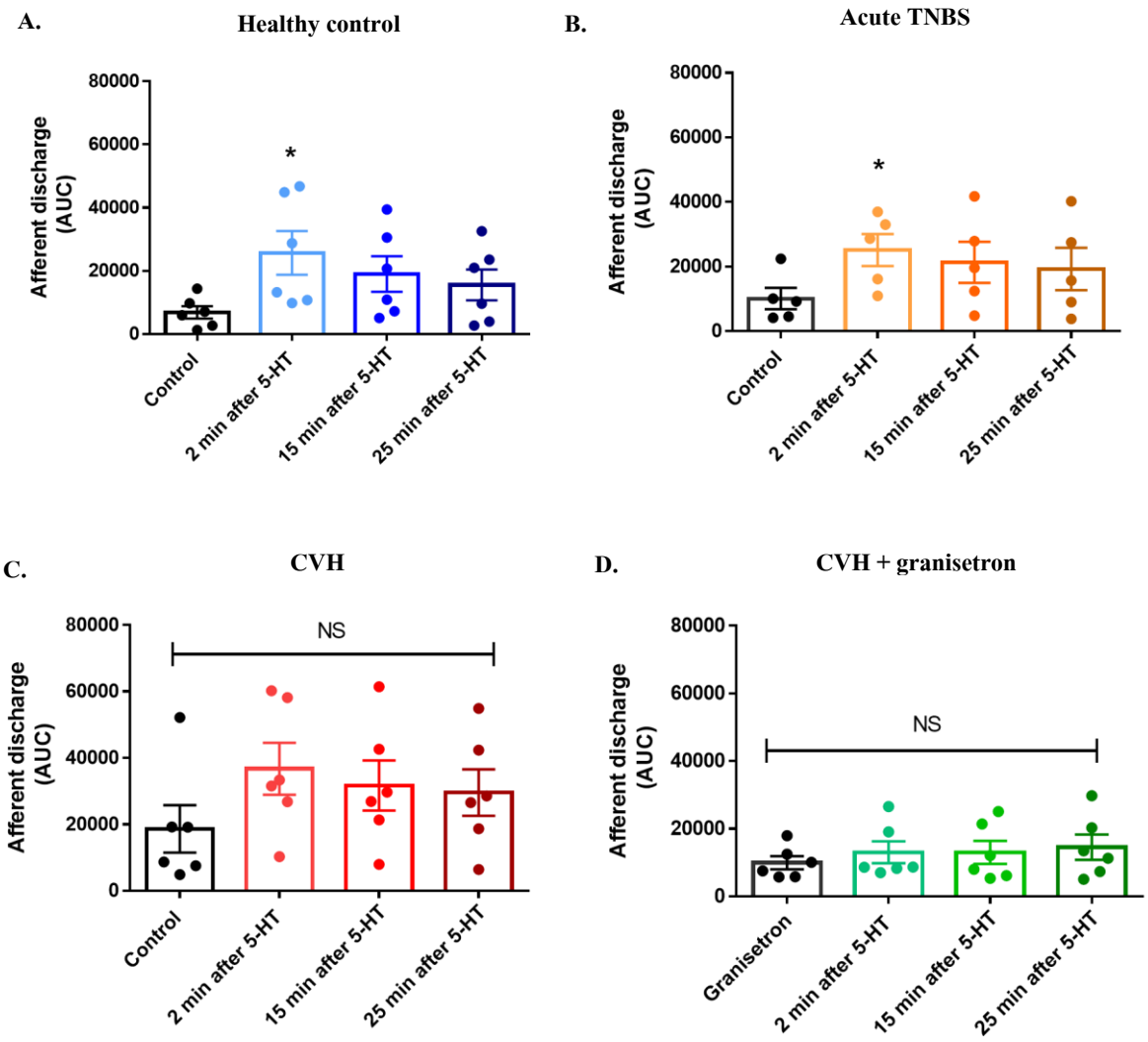


Figure 5.8 5-HT significantly increased spontaneous firing in healthy control and acute TNBS but not CVH mice. (A-D) Comparison of bladder spontaneous afferent firing at 2, 15, and 25 minutes after 5-HT bath application, *P<0.05, One-way ANOVA, (Healthy control, CVH, CVH+granisetron, N=6; acute TNBS, N=5).

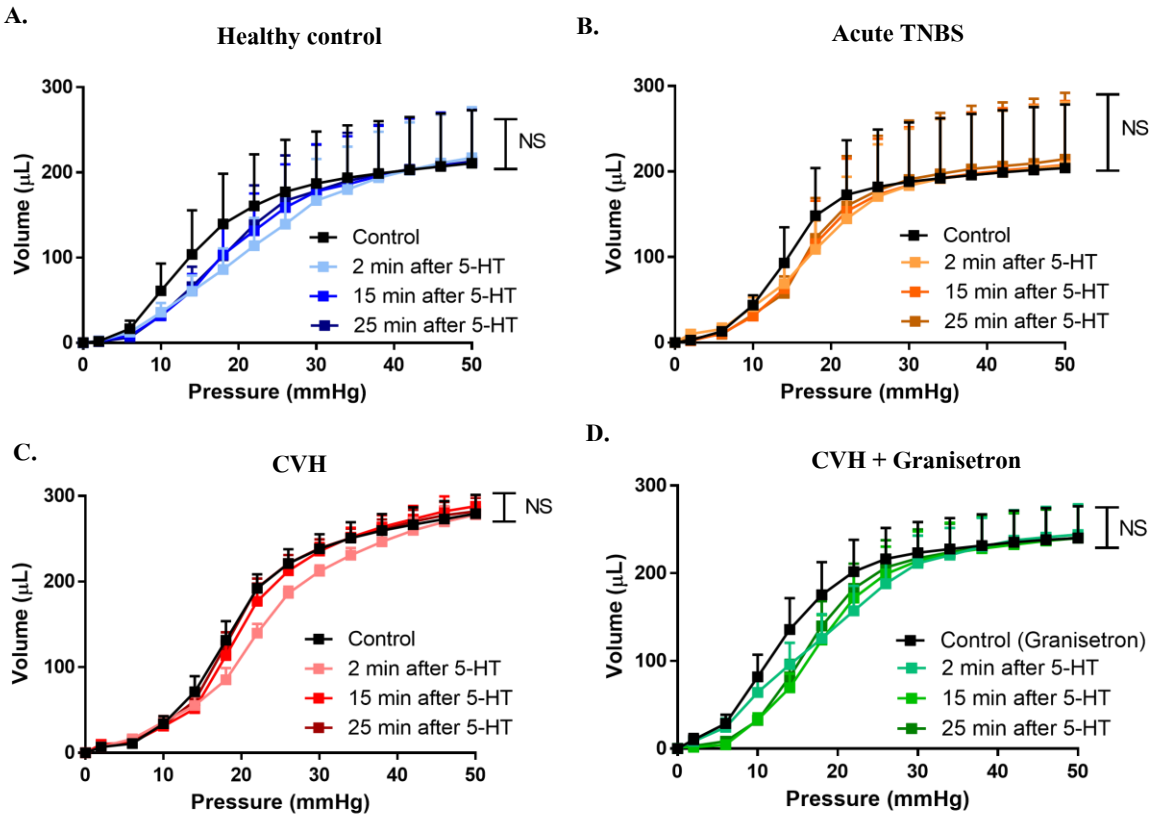


Figure 5.9 5-HT had no effect on bladder compliance in healthy control, acute TNBS, and CVH with or without granisetron pre-incubation. (A-D) Comparison of bladder compliance at 2, 15, and 25 minutes after 5-HT bath application. Two-way ANOVA, (Healthy control, CVH, CVH + granisetron, N=6; acute TNBS, N=5).

Single unit analysis of bladder afferent nerve in healthy control and CVH mice is shown in figure 5.10. In the healthy control group, the proportion of high threshold unit was 55.6%. Low threshold and silent units were 37 % and 7.4%, respectively. In the CVH group, the proportion of high threshold units was increased to 78%. This was concurrent with a decrease in low threshold and silent unit proportion, 19.5% and 2.4% respectively. In the CVH with granisetron group, the proportion of high threshold, low threshold and silent units was 66.7%, 27.8%, and 5.6%, respectively.

The afferent subunit composition of the CVH group was significantly changed compared to the healthy control (P=0.0024). In contrast, there was no significant difference in nerve unit types between CVH with and without granisetron (P=0.159).

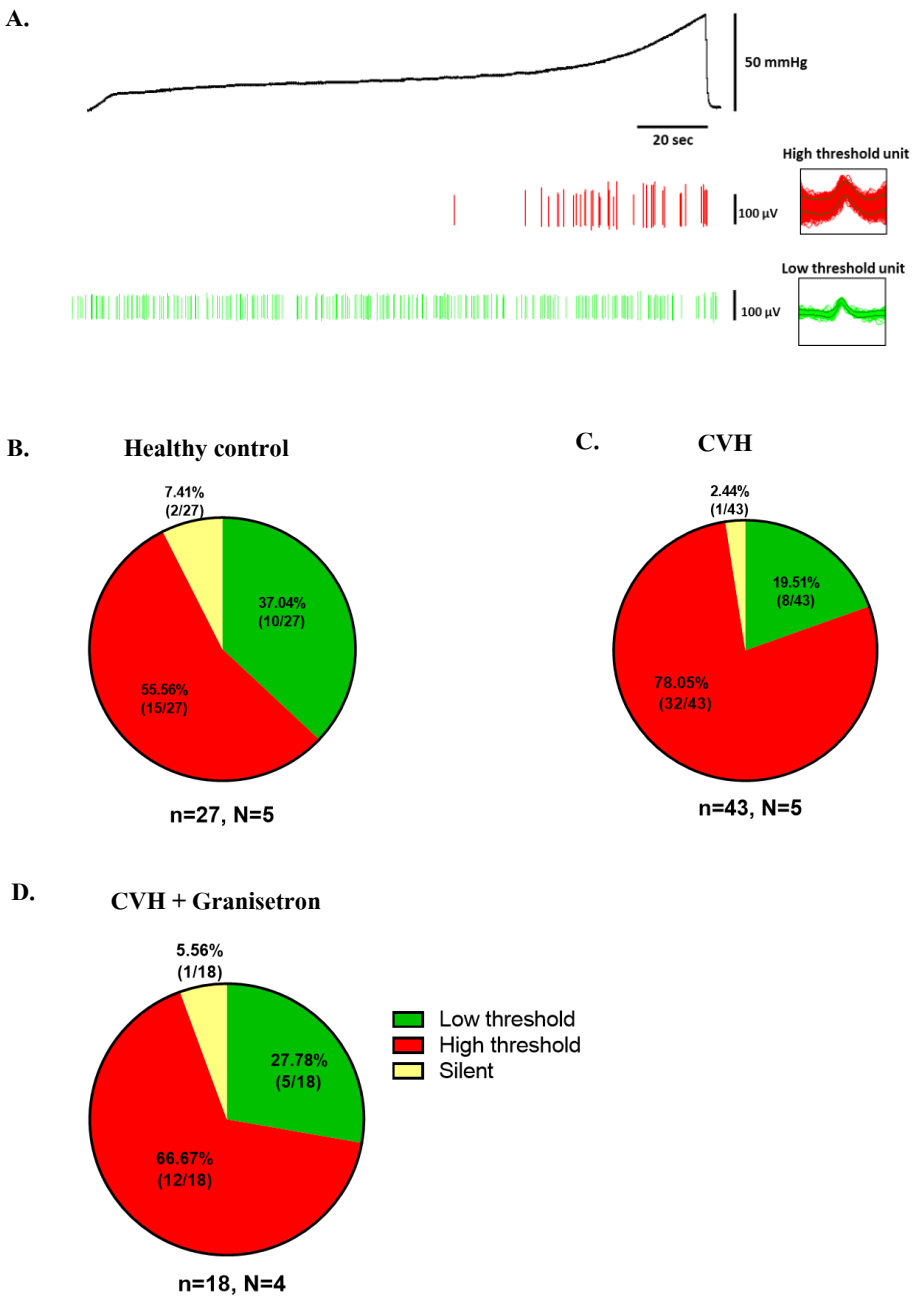


Figure 5.10 High threshold fibres were increased in CVH mice (A) Sample trace of waveform analysis of low threshold and high threshold fibres in bladder afferent in response to ramp distensions. Percentages of unit subtypes in healthy control **(B)**, CVH mice **(C)**, and CVH mice with pre-incubation of granisetron **(D)**.

5.4 RESULTS: mRNA EXPRESSION OF SERT, TPH1 AND TPH2 IN MOUSE UROTHELIAL CELLS FROM HEALTHY CONTROL AND CVH MICE

As shown in figure 5.11, the level of SERT expression was significantly downregulated in CVH urothelial cells (*P<0.05). There was no significant difference in TPH1 and TPH2 mRNA expression between healthy control and CVH.

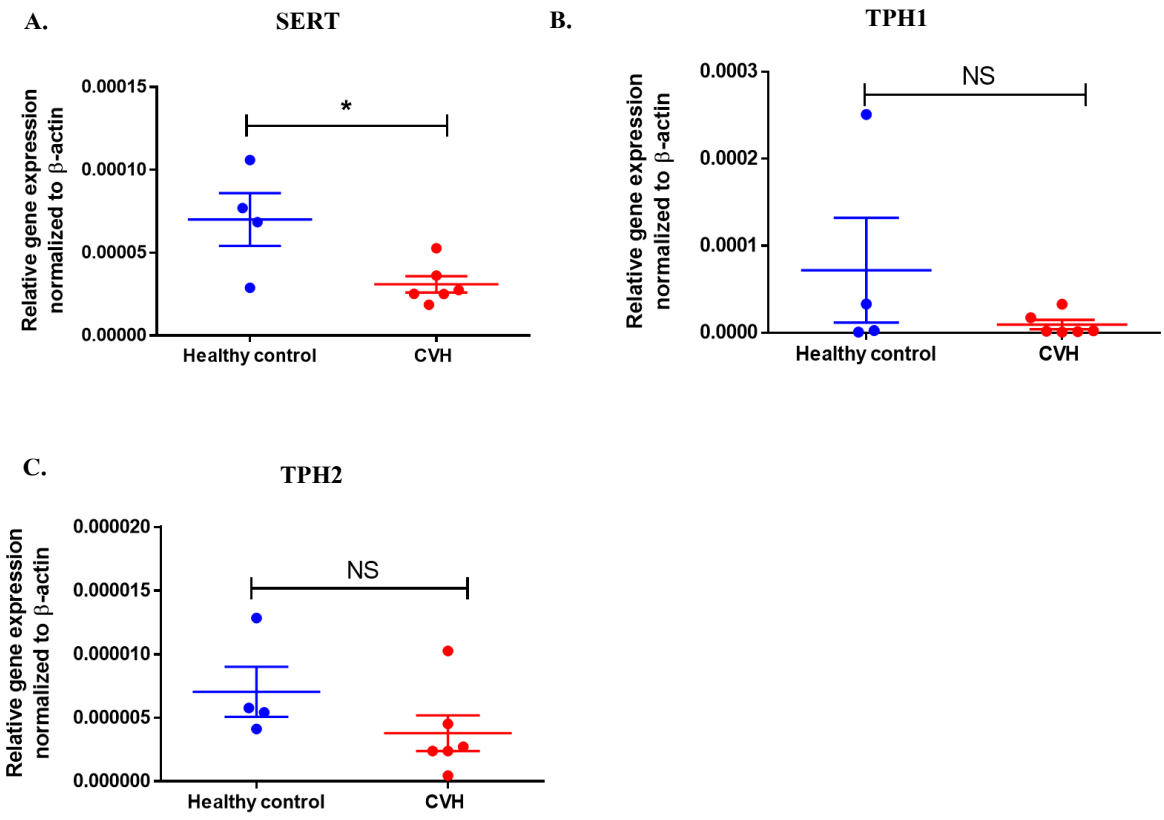


Figure 5.11 SERT mRNA expression was downregulated in urothelial cells of CVH mice. **(A)** SERT, **(B)** TPH1, **(C)** TPH2 mRNA expression in urothelial cells comparing between healthy control and CVH mice (healthy control; N=4, CVH; N=6).

5.5 DISCUSSION

The main findings in this chapter are

1.) There was an increase in bladder mechanosensitive and spontaneous firing in CVH mice but not acute TNBS (if anything decreased) and this was reversed by granisetron;

2.) An attenuation effect of 5-HT on mechanosensitive firing was decreased in acute TNBS, CVH, and CVH with granisetron mice;

3.) 5-HT-evoked spontaneous firing was blunted in CVH and CVH with granisetron pre-incubation but not acute TNBS mice;

4. Bladder compliance did not change following 5-HT application in acute TNBS, CVH, and CVH with granisetron groups;

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

Bladder afferent activity was changed in CVH but not acute TNBS mice.

In the present study, we showed that CVH but not acute TNBS mice exhibited mechanosensitive and spontaneous afferent hypersensitivity. This is consistent with a study by Grundy *et al.* showing that TNBS treated mice in the post-inflammatory period exhibited bladder hypersensitivity and showed abnormal voiding patterns (Grundy *et al.* 2016). There was no change in bladder compliance in any of the animal groups. This indicates that the change in mechanosensitivity was not secondary to changes in muscle tone but mainly through a direct afferent nerve component.

There is still uncertainty about the underlying mechanisms contributing to the bladder afferent hypersensitivity in this animal model. A study by Ustinova *et al.* suggested that neurotrophic factors from the muscle layer or colonic DRG neurons possibly influenced axonal sprouting and neurite outgrowth in the spinal cord. This could lead to changes in sensory and/or motor function in the bladder (Ustinova *et al.* 2007). Unpublished histological data from the Visceral Pain Group, SAHMRI showed that colonic tissues from acute inflammation (from day 3 to day 7 post-treatment) animals showed active inflammatory signs in colon, i.e., mucosa disruption, cellular infiltration, and crypt abscesses. These inflammatory signs were resolved by day 28 after TNBS treatment, which are consistent with an investigation by Hughes *et al.* 2009. There

were no inflammatory signs observed in the bladder wall of the treated animals in any states (3, 7, and 28 days post treatment).

After 3 days of colonic TNBS treatment, bladder afferent activity (although attenuated) was not significantly different from the healthy control group. Similar findings were reported by Keating *et al.* (2008). They found an initial hyposensitivity (14-16 days post-infection) but post-infectious hypersensitivity (28 and 56 days post-infection) of afferent nerves in the jejunum of T. *spirallis* infected mice (Keating *et al.* 2008). We also found moderate attenuation in mechanosensitive responses in acute TNBS groups (~25%) compared to healthy control groups. This may be explained by previous findings showing that mediators from immune cells could suppress colonic afferent activity (Hughes *et al.* 2009a; Labuz, 2006).

Ustinova *et al.* (2007) showed that systemic capsaicin injections 3 days before TNBS treatment, which desensitizes sensory neurons and subsequently blocks neurotransmission of mediators, significantly reversed hyperactivity of bladder afferent and normalized the number of mast cells. This suggests that neuropeptides from C-fibres may play a significant role in developing bladder afferent hypersensitivity (Ustinova *et al.* 2007). Various neuropeptides have been reported in the bladder including VIP, CGRP, substance P, bradykinin (BK) (Arms and Vizzard 2011). A study reported that BK via activation of BK2 receptor in the urothelium plays a key role in cyclophosphamide-induced bladder inflammation in rats (Chopra *et al.* 2005). Interestingly, Mense (1981) reported that under an elevated level of 5-HT, BK had increased ability to sensitize muscle receptors of unmyelinated fibres of gastrocnemious-soleus muscle in cats (Mense, 1981).

We performed offline single unit analysis in order to determine changes in nerve fiber population between healthy control and CVH mice. Interestingly, we found an increase in the proportion of high threshold fiber in CVH mice, while the proportion of low threshold and silent nerves were decreased. Our finding is consistent with a study by Keating *et al.* (2008) which reported an increase of high threshold units, while the number of low threshold units was reduced in post-inflammatory state of mice infected with T. *spirallis* in jejunum. High threshold

fibres that sense a noxious range of intravesical pressure (>15 mmHg) could be C-fibres which are responsible for nociception. An increase in high threshold unit composition in the post-inflammatory state may suggest an ability of low threshold and silent nerve units to develop to high threshold fibres as an adaptive mechanism to detect noxious stimuli. However, more N numbers in each group should be increased to corroborate this finding.

Bladder afferent firing in response to 5-HT in CVH and acute TNBS mice was decreased.

The present study showed that the attenuation of mechanosensitive discharge by 5-HT was absent in both acute TNBS and CVH mice. The excitatory effect of 5-HT on spontaneous firing was preserved in the acute TNBS groups at 2 minutes after 5-HT application. In contrast, in CVH mice the excitatory effect of 5-HT on spontaneous firing was absent despite an elevation in baseline firing. The reason we did not observe an excitation of spontaneous firing after 5-HT application in CVH mice could be that the baseline of spontaneous firing in CVH mice was already elevated. In addition, the basal level of mechanosensitive and spontaneous firing was attenuated by granisetron, suggesting that in CVH mice there might be an elevation in the endogenous level of 5-HT.

A study by Linden and colleagues reported that 5-HT levels in the intestinal mucosa of TNBS-induced colitis is increased in guinea pigs (Linden *et al.* 2003). Keating *et al*. (2008) reported mice infected with T. *spiralis* had an increased 5-HT release and contents in the jejunum in both acute inflammation and the post-infection recovery period. Moreover, nodose ganglion neurons that innervate the jejunum showed decreased excitability to the 5-HT3 agonist (2-Me-5-HT). This data indicates functional downregulation of 5-HT3 receptors of these neurons in post-inflammatory animals (Keating *et al.* 2008). This raises the possibility that the reduction in mechanosensitive response to 5-HT observed in acute TNBS and CVH animals may arise from an adaptive response mechanism that decrease 5-HT3 receptor sensitivity in order to limit hyperexcitation of afferent nerves.

Unpublished data from the Visceral Pain Group, SAHMRI using retrograde labelling from the urinary bladder found there was no significant difference in traced DRG neurons that expressed 5-HT3 receptors between healthy control mice (90%) and CVH mice (94%). This data may suggest that in the post-inflammatory state, animals adjust 5-HT3 receptor sensitivity rather than the level of the receptor expression. However, further experiments i.e., calcium imaging or patch-clamp are needed to confirm 5-HT3 receptor sensitivity in DRG neurons innervating the bladder in CVH animals. It would be also interesting to investigate further if there are changes in 5-HT3 receptor expression in the afferent terminals and other sites in the bladder wall i.e., detrusor muscle.

Recent evidence has shown the contribution of 5-HT3 receptors to symptoms in IBS patients. Cremon and co-workers (2011) reported that 5-HT release and EC immunopositive cells in colonic mucosa were increased in IBS patients. An excitation of afferent nerves in rats by supernatants from IBS patients was attenuated by granisetron (Cremon *et al.* 2011). Motavalian and colleagues (2013) have reported an anti-inflammatory effect of tropisetron, a 5-HT3 antagonist, in TNBS-induced colonic inflammation in rats. Tropisetron and ondansetron reduced colonic damage, MPO activity, and inflammatory cytokine levels (Motavallian-Naeini *et al.* 2012; Motavallian *et al.* 2013). However, whether 5-HT3 receptors could ameliorate bladder symptoms derived from intestinal inflammation would require further study.

SERT mRNA expression was downregulated in CVH mice.

This study, for the first time demonstrated that SERT expression in the bladder along with SERT in the gut is reduced in the post-inflammatory state after colonic inflammation. Our investigation is in agreement with studies reporting a decrease in function and expression of SERT after TNBS-induced colitis in mice (Linden *et al.* 2005) and guinea pigs (Linden *et al.* 2003). Decreased SERT expression has been shown to be associated with impaired 5-HT reuptake in colonic inflammation (Linden *et al.* 2005).

5-HT producing enzymes, TPH1 and TPH2 mRNA expression were not significantly different in urothelial cells between healthy control and CVH mice. Kerckhoffs and co-workers (2012) have shown that TPH1 mRNA expression is downregulated in the rectum of IBS patients

irrespective of their visceral hypersensitivity status (Kerckhoffs *et al.* 2012). This may suggest an adaptive mechanism in response to high levels of 5-HT. However, while our findings are consistent with altered 5-HT bioavailability, future studies are needed to determine actual bladder 5-HT content and 5-HT reuptake in CVH mice in order to quantify changes in 5-HT metabolism in the bladder. A schematic diagram depicting the axon-reflex mechanism hypothesized to contribute to changes in 5-HT metabolism in the colon and bladder of CVH mice is shown in figure 5.12.

With limited time to conduct experiments at SARMRI, Australia, we could not conduct further experiments to confirm the findings. Afferent nerve recordings in TNBS-treated groups showed higher variability compared to healthy control animals which may have contributed to a lack of statistical power. This variability could be derived from variation in the immune responses of each animal that could also influence the level of bladder hypersensitivity. Therefore, further studies are required to increase N numbers in both afferent nerve recordings and the quantification of mRNA expression to confirm our investigation.

In summary, this current investigation has provided initial information about the role of 5-HT in bladder afferent activity of TNBS-induced colonic inflammation animals. However, further investigation is required to elucidate the mechanisms of 5-HT involved in this model, which would help to identify a new therapeutic drug target to treat bladder symptoms i.e., frequency, urgency, and incontinence derived from neuronal cross-talk between the bowel and the bladder.

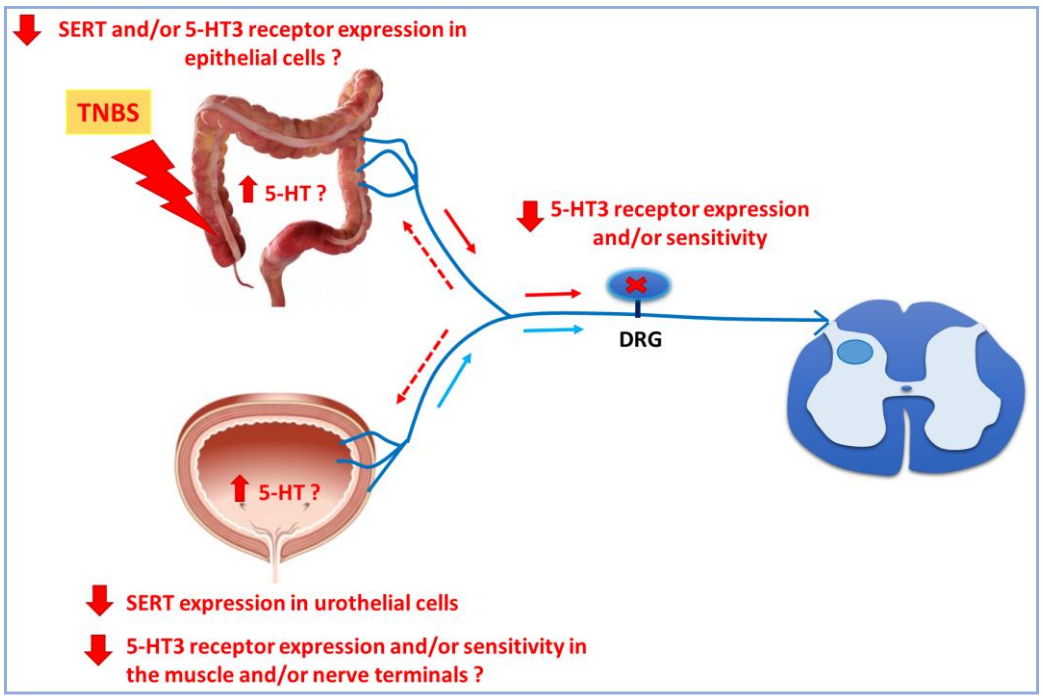


Figure 5.12 Diagram depicting the axon-reflex mechanism hypothesized to contribute to changes in 5-HT metabolism in the colon and bladder of CVH mice. In the post-inflammatory period of colonic TNBS treatment, colonic afferents are sensitized due to inflammation. Hypersensitized colonic afferents convey sensory signals to the dichotomizing neurons in DRG that also receive afferent signals from the bladder (dotted arrows). The dichotomizing neurons generate axon-reflexes to downregulate expression of SERT in the colon and bladder, leading to an increase of 5-HT bioavailability in both the colon and the bladder. Following an increase in local 5-HT levels in both organs, 5-HT3 receptor excitability and/or expression may be decreased, which contributes to reduce sensitivity to 5-HT application.

CHAPTER 6

IS ENDOGENOUS 5-HT PRESENT IN THE MOUSE URINARY BLADDER?

6.1 INTRODUCTION

Regulation of 5-HT homeostasis is essential for many physiological processes in both central and peripheral organs. In the brain, 5-HT dysregulation involves many psychiatric and neurological symptoms including, anxiety, depression, motor control, sleep, and addiction (Roth 1994; Roth and Xia 2004). In the periphery, 5-HT imbalance leads to diverse pathological states, i.e., irritable bowel syndrome (Stasi *et al.* 2014), cardiovascular diseases (Ramage and Villalón 2008), metabolic disorders (Merahbi *et al.* 2015), and osteoporosis (Ducy and Karsenty 2010). In order to maintain balance of 5-HT levels, 2 processes are required to be regulated; 5-HT production and 5-HT reuptake into the cells.

The major source of 5-HT (95%) is produced in the gastrointestinal tract by EC cells, but mast cells and myenteric neurons in the gut wall also produce 5-HT (Gershon and Tack 2007), while about 5% is centrally produced from serotonergic neurons originating from raphe nuclei in the brainstem. Both central and peripheral 5-HT is synthesized from the substrate amino acid, tryptophan. 5-HT synthesis requires a rate limiting enzyme, tryptophan hydroxylase (TPH). TPH has 2 isoforms; TPH1 and TPH2. TPH2 is the pre-dominant isoform in neuronal tissues, while TPH1 is broadly expressed and produces 5-HT in non-neuronal tissues (Lovenberg *et al.* 1967; Walther *et al.* 2003).

The role of TPH enzymes in peripheral tissues has been studied in knockout animals. Li *et al.* found that TPH1 knockout mice exhibit normal gut functions (gastric empting, colonic motility, and intestinal transit), while TPH2 knockout mice showed abnormality in all of the above gastrointestinal functions. Double knockout mice of TPH1 and TPH2 exhibited changes in gut functions similar to TPH2 knockout mice (Li *et al.* 2011). However, TPH1 knockout animals showed a delayed onset and less severity to dextran sulphate sodium (DSS) induced colonic inflammation including, disease activity index (weight loss and frequent stool), histological damage score, and inflammatory cytokine levels (Ghia *et al.* 2009). According to this information, TPH1 which mainly produces peripheral 5-HT (90%) seems to play a crucial role in pathological conditions, i.e., colitis of the gastrointestinal tract, while neuronal TPH2 which is a minor proportion (~10%) exerts a major role in regulation of gastrointestinal motility (Amireault *et al.* 2013).

Excessive 5-HT is recycled into serotonergic neurons (in the brain) or into enterocytes (in the bowel) via the function of the serotonin reuptake transporter (SERT). SERT is encoded by the solute carrier family 6, member 4 (SLC6A4) which is a member of the Na^+ /Cl^- dependent transport family. SERT is expressed in many organs and the brain (Quick, 2003). Neuronal SERT has been cloned in many species including, human (Ramamoorthy *et al.* 1993), rats (Takayanag *et al.* 1995), mice (Chang *et al.* 1996), guinea pigs (Chen *et al.* 1998), and cows (Mortensen *et al.* 1999). In peripheral organs, SERT has been detected in platelets (Brenner *et al.* 2007), carotid bodies (Yokoyama *et al.* 2013), the pulmonary vascular endothelium (Lee and Fanburg 1986), and the gastrointestinal tract (Gill *et al.* 2008). SERT knockout mice showed abnormal bowel functions, i.e., alternate diarrhea and constipation due to overactivity and inadequate colorectal movements (Chen *et al.* 2001) and increased sensitivity to colonic nociception (Coates *et al.* 2006).

Inhibition of SERT function is a target for antidepressant drugs. Serotonin reuptake inhibitors (SSRIs) is one group of antidepressant drugs, which work via inhibiting 5-HT reuptake from the synaptic cleft to the pre-synaptic neurons. The mechanisms of SERT and SSRI action are depicted in figure 6.2.

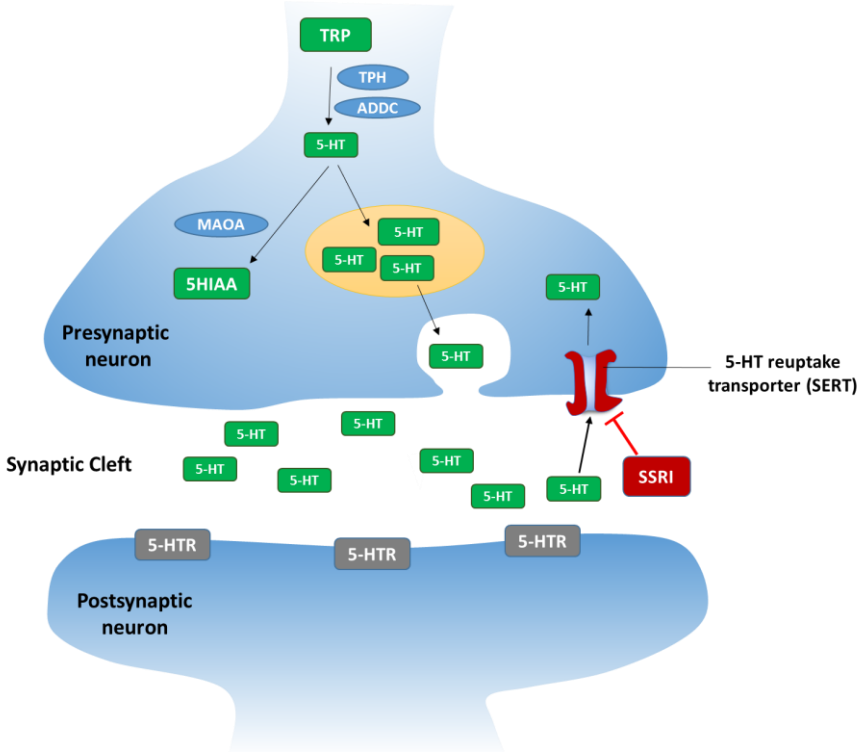


Figure 6.2 Schematic diagram of serotonin reuptake inhibitors (SSRIs) action (adapted from Sangkuhl *et al.* 2009). In the presynaptic neuron, synthesized 5-HT is stored in the synaptic vesicles. 5-HT that is not stored in the synaptic vesicles is metabolized into 5-hydroxyindoleacetic acid by monoamine oxidase enzyme. An action potential stimulates Ca^{2+}-dependent exocytosis to release 5-HT from the vesicles into the synaptic cleft. 5-HT subsequently binds to 5-HT receptors. Excessive 5-HT in the synaptic cleft is taken back up into the pre-synaptic neuron via SERT. SSRI inhibits SERT to prevent 5-HT reuptake, which results in accumulation of 5-HT level in the synaptic cleft. (ADDC = L-amino acid decarboxylase, 5-HIAA = 5-hydroxyindoleacetic acid, 5-HTR = 5-hydroxytryptamine receptors, MAOA = monoamine oxidase enzyme, TPH = tryptophan hydroxylase, TRP = tryptophan).

Currently, five different SSRIs are used therapeutically; fluoxetine, fluvoxamine, paroxetine, sertraline, and citalopram (Hiemke and Härtter 2000; Cipriani *et al.* 2010). SSRI users report adverse side effects including nausea, vomiting, sexual dysfunction, agitation (Mitchell 1994). Interestingly, a retrospective follow-up study showed that SSRI users report increased urinary incontinence (Movig *et al.* 2002). Citalopram (Celexa^{TM}) is a highly selective SSRIs; in clinical treatment, reported side effects include, dizziness, nausea, tremor, erectile dysfunction, fatigue,

abdominal pain. Interestingly, citalopram users are reported to have bladder adverse effects including, polyuria, micturition frequency, urinary incontinence, and urinary retention (Forest Laboratories Ireland, 2002), symptoms that might reflect alter sensory signalling. However, the mechanism related to bladder symptoms remains elusive.

A recent study by Matsumoti-Miyai and co-workers (2016) demonstrated that application of citalopram to mouse bladder strips inhibited urothelium-released ATP induced by bladder distension, which mimicked the action of exogenous 5-HT. However, whether citalopram directly affects bladder afferent nerve firing remains unknown. In addition, TPH1 transcripts are expressed in mouse bladder (Matsumoto-Miyai *et al.* 2016). This information suggests that the bladder may have the potential to produce 5-HT.

Recently, immunoreactive expression of 5-HT has been found in the prostate and urethra in close proximity with CGRP immunoreactive nerve fiber in rats (Yokoyama *et al.* 2017). All of the above information raises the possibility that 5-HT may be produced within the lower urinary tract and SERT inhibition by citalopram may affect bladder afferent nerve firing.

To determine whether the urinary bladder has an endogenous source of 5-HT which can affect bladder afferent signalling,

(i) to study the effect of citalopram on bladder afferent firing

(ii) to examine mRNA expression of 5-HT producing enzymes (TPH1 and TPH2) and SERT in mouse urothelial cells using RT-PCR

(iii) to investigate 5-HT and SERT expression in the bladder wall using immunohistochemistry.

6.2 EXPERIMENTAL PROTOCOL AND ANALYSIS

Effect of citalopram on baseline and mechanosensitive afferent firing

In vitro afferent nerve recording was performed to investigate the effect of the selective 5-HT reuptake inhibitor, citalopram on bladder afferent nerve activity. The details of the bladder preparation were outlined in chapter 2.

Citalopram (1 μM) was applied to the bath and intravesically for 60 minutes and then washed out for a further 60 minutes. The dose was designed according to a previous investigation by Chiba *et al*. who showed that local application of citalopram (1 μM) increased extracellular 5-HT (Chiba *et al*. 2016). The bladder was distended to 50 mmHg at 10 minutes interval throughout the experimental protocol. In order to determine the role of 5-HT3 receptors in citalopram mediated changes in bladder afferent firing, the 5-HT3 antagonist granisetron (1 μM) was applied to the bath and intravesically for 30 minutes prior to citalopram application. The summarised protocol is shown in figure 6.3.

Mechanosensitive afferent firing at 30, 60, 90, and 120 minutes after the drug application was investigated and compared to the last control distension response. The spontaneous firing between the ramp distension at each time point was determined by peak afferent discharges according to the same time points as mechanosensitive firing. All the afferent discharge was subtracted from the baseline firing 3 minutes before drug application. Data was presented as mean ± SEM. Statistical analysis includes One-way ANOVA, and Two-way ANOVA as appropriate.

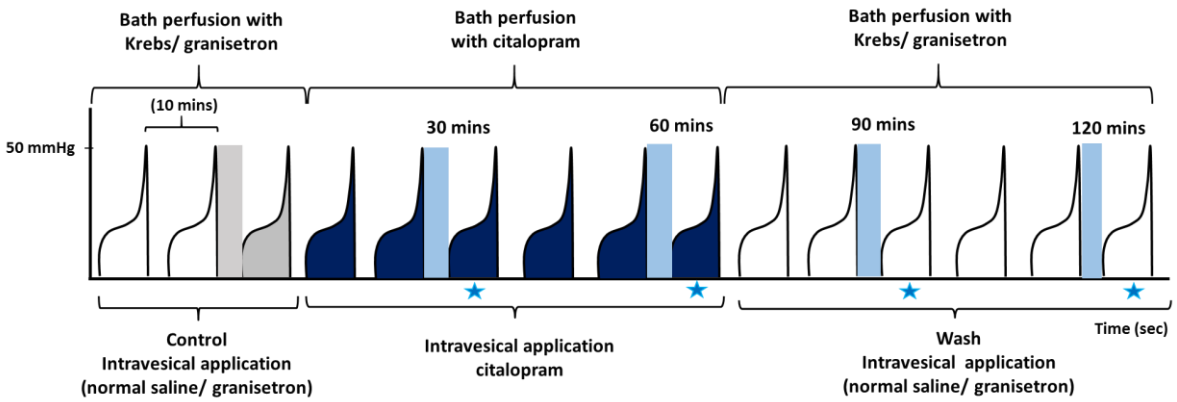


Figure 6.3 Protocol used to study the effect of citalopram on bladder mechanosensitive and spontaneous afferent firing. Grey colour indicates area where the control data was analyzed and blue colour indicates the area of spontaneous afferents obtained for each time point after citalopram application (★ = time points where data was analyzed).

mRNA expression of TPH1, TPH2, and SERT in mouse urothelial cells

RT-PCR was performed to examine mRNA expression of TPH1, TPH2, and SERT in mouse urothelial cells as described in chapter 2 (section 2.5). Duodenum was used as a positive control for TPH1 and SERT. Brain was used as a positive control for TPH2. Negative control was prepared in a similar fashion but cDNA was omitted (N=3).

Table 6.1 Summary of primer sequences used in RT-PCR

Genes	Accession number	Primer sequences	Product size (bps)	Positive control
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'		

Immunohistochemistry of 5-HT and SERT in the urinary bladder

5-HT and SERT protein expression was examined using immunohistochemistry. The detailed protocol is described in chapter 2 (section 2.6). Bladder and jejunum specimens were fixed in 4% PFA, embedded in OCT and sectioned with a cryostat at 10 µm. The lists of primary and secondary antibodies are summarised in table 6.2 and 6.3.

The jejunum was used as positive control for both genes to validate the antibodies and optimise the staining protocol. All the slides were counterstained with DAPI to determine nucleus of the cells. Blank control was performed using the same protocol but the primary antibody was omitted.

Table 6.2 Summary of primary antibodies used in this study

Antigen	Company	Catalogue number	Source species	Target species	Dilution
5-HT	Abcam	AB66047	Goat	Mouse, Rat, Human	1/400
SERT	Abcam	AB44520	Rabbit	Mouse, Human	1/500

Table 6.3 Summary of secondary antibodies used in this study

Species	Company	Catalogue number	Fluorophore	Dilution
Rabbit anti-Goat	Santa Cruz	SC-2777	FITC	1/400
Goat anti-Rabbit	Santa Cruz	SC-2012	Alexa Fluor 594	1/500

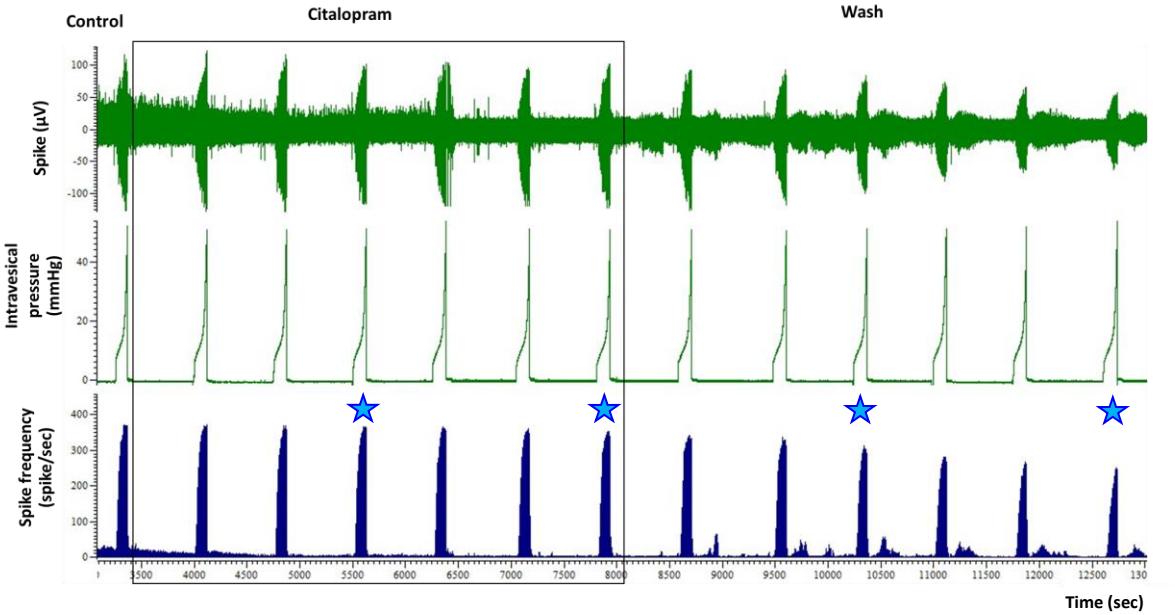
6.3 RESULTS: EFFECT OF CITALOPRAM ON BLADDER AFFERENT FIRING

A representative trace of bladder afferent responses after treatment with citalopram is shown in figure 6.4. Citalopram gradually attenuated mechanosensitive afferent firing and it reached significance at 120 minutes after the drug application (**P<0.01) (Figure 6.5A). Bladder compliance was increased at 90 and 120 minutes after drug application (*P<0.05) (Figure 6.5B).

The inhibitory effect of citalopram was reduced by pre-incubation with granisetron (Figure 6.5B). There was a trend to increase bladder compliance similar to ciatalopram, however, there was no statistical difference in bladder compliance at any of these time points after granisetron (Figure 6 6.5D).

Citalopram had no significant effect on spontaneous firing. At 30-60 minutes post application, there was a trend towards a decrease and at 90-120 minutes there was a trend towards an increase in spontaneous firing (Figure 6.6A). Application of granisetron prior to citalopram increased spontaneous firing 60 minutes following citalopram application (*P<0.05) (Figure 6.6B).

A.



B.

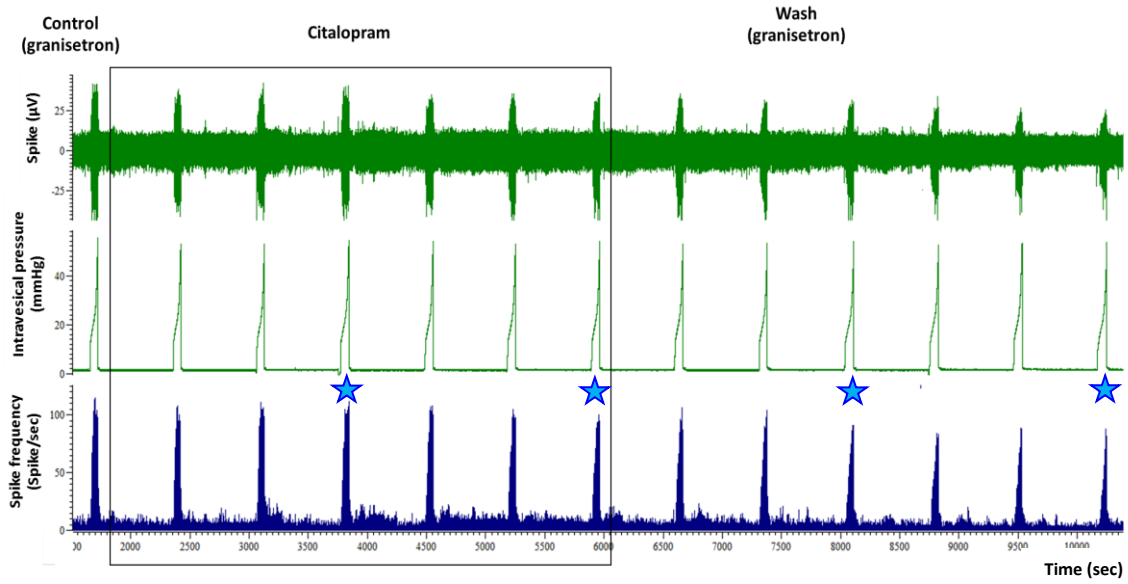


Figure 6.4 Representative traces to illustrate bladder afferent firing in response to bladder distension in the presence and after citalopram application (A) and with pre-incubation with granisetron (B) (★ = time points that data was analyzed).

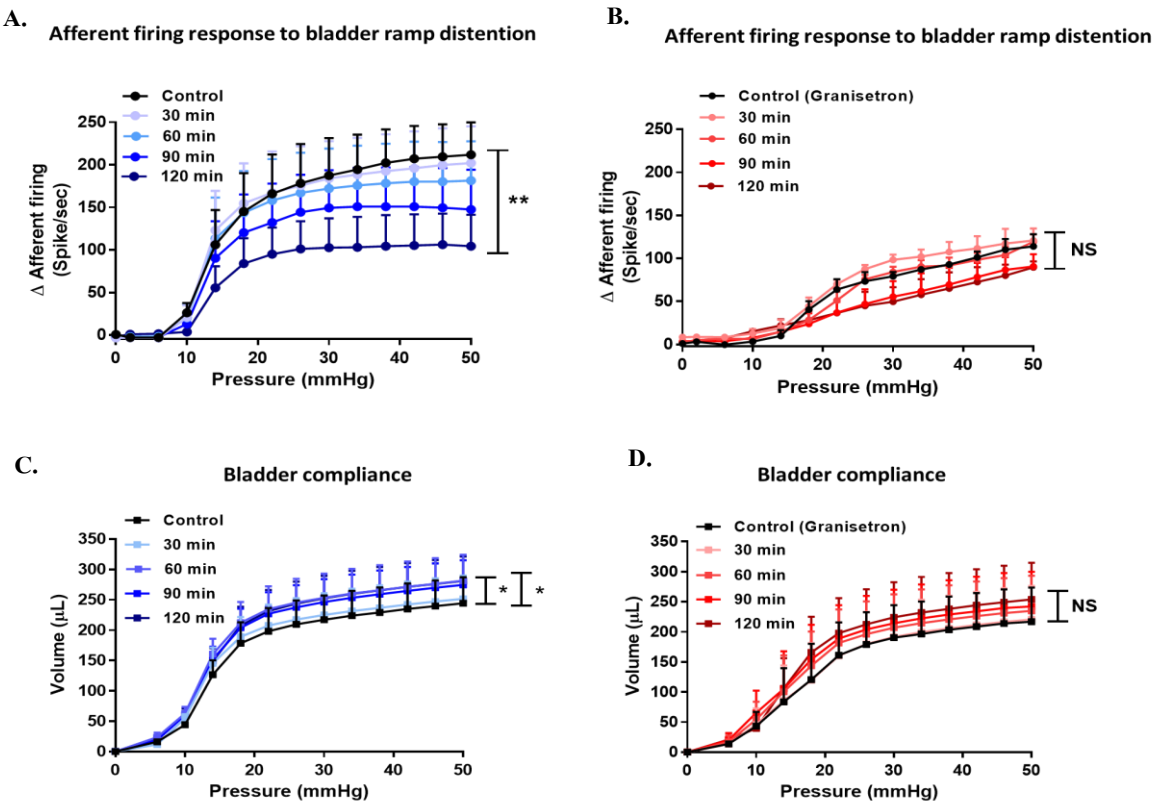


Figure 6.5 Citalopram and citalopram with granisetron pre-incubation decreased mechanosensitive afferent firing. (A and B) Comparison of bladder afferent responses to ramp distension at 30, 60, 90, and 120 minutes after citalopram application and pre-incubation with granisetron, respectively. **(C and D)** Comparison of bladder compliance at 30, 60, 90, and 120 minutes after citalopram application and pre-incubation with granisetron before citalopram, respectively, *P<0.05, **P<0.01, ***P<0.001 vs. control, Two-way ANOVA with Dunnett's multiple comparison (citalopram experiment, N=6 and granisetron+citalopram, N=3).

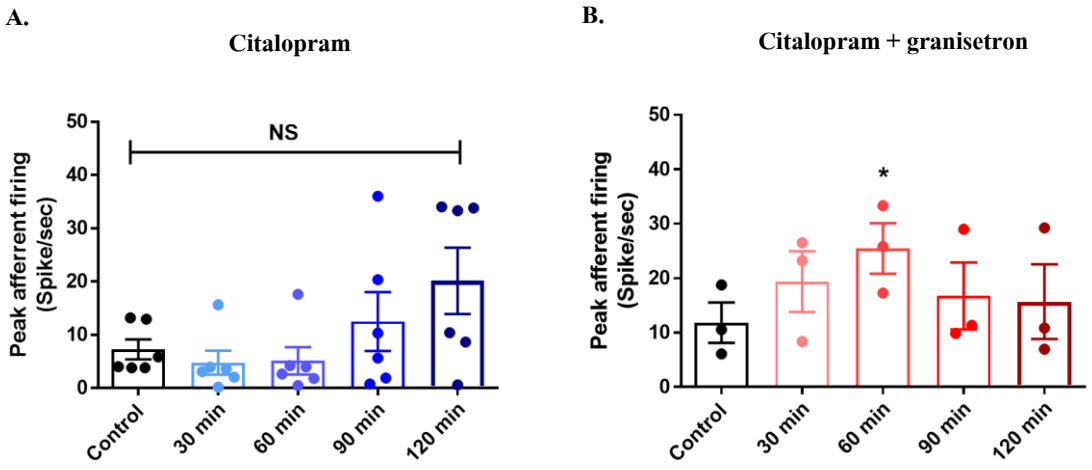


Figure 6.6 There was no significant difference in spontaneous firing after citalopram application but pre-incubation with granisetron increased spontaneous firing. (A) Comparison of bladder spontaneous afferent responses to ramp distensions at 30, 60, 90, and 120 minutes after citalopram application (N=6) and **(B)** pre-incubation with granisetron prior to citalopram (N=3). *P<0.05, One-way ANOVA with Dunnett's multiple comparison.

6.4 RESULTS: mRNA EXPRESSION OF 5-HT PRODUCING ENZYMES (TPH1, TPH2) AND 5-HT-REUPTAKE TRANSPORTER (SERT) IN MOUSE UROTHELIAL CELLS

As shown in figure 6.7, mRNA expression of 5-HT producing enzymes, TPH1 and TPH2, and SERT were detected in mouse urothelial cells (N=3).

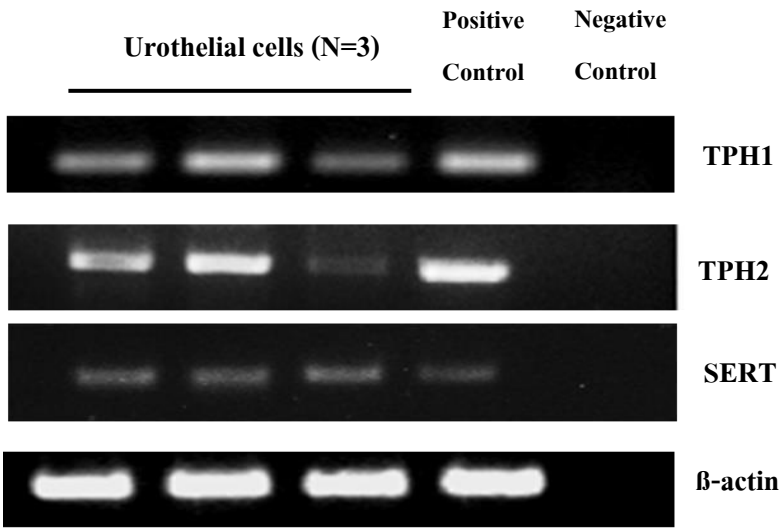


Figure 6.7 mRNA expression of 5-HT producing enzymes (TPH1 and TPH2) and SERT in mouse urothelial cells. TPH1, TPH2, and SERT were detected in mouse urothelial cells. ß-actin was used as a house keeping gene. Duodenum was used as a positive control of TPH1 and SERT. Brain was used as a positive control for TPH2 (N=3).

6.5 RESULTS: IMMUNOHISTOCHEMISTRY TO IDENTIFY SERT AND 5-HT IN THE URINARY BLADDER

SERT staining from three bladders is shown in figure 6.8. SERT expression in jejunum was shown as a positive control. In all three bladder samples, positive SERT immunoreactivity was detected. The density of staining was greater in the urothelium but there was also some positive detection in the lamina propria.

The expression of 5-HT was also examined, in these experiments, jejunum sections were labelled with an anti-5-HT antibody as a positive control (Figure 6.9A). The schematic diagram (Figure 6.9B) shows the regions from which positive 5-HT staining was detected.

Some positive cells were observed in the prostatic urethra (Figure 6.9C, E, F). 5-HT immunopositive cells had various morphologies and were distributed mainly in the prostatic urethra wall (Figure 6.9C, E, F) and around the glandular structures (Figure 6.9G-I). Most of the cells projected cytoplasmic processes either to apical or basolateral sides of the lumen. A few cells were thin and spindle shaped. Some of the cells projected their cytoplasmic processes in two opposite directions. Higher magnification of a 5-HT immunopositive cell is showed in figure 6.9J.

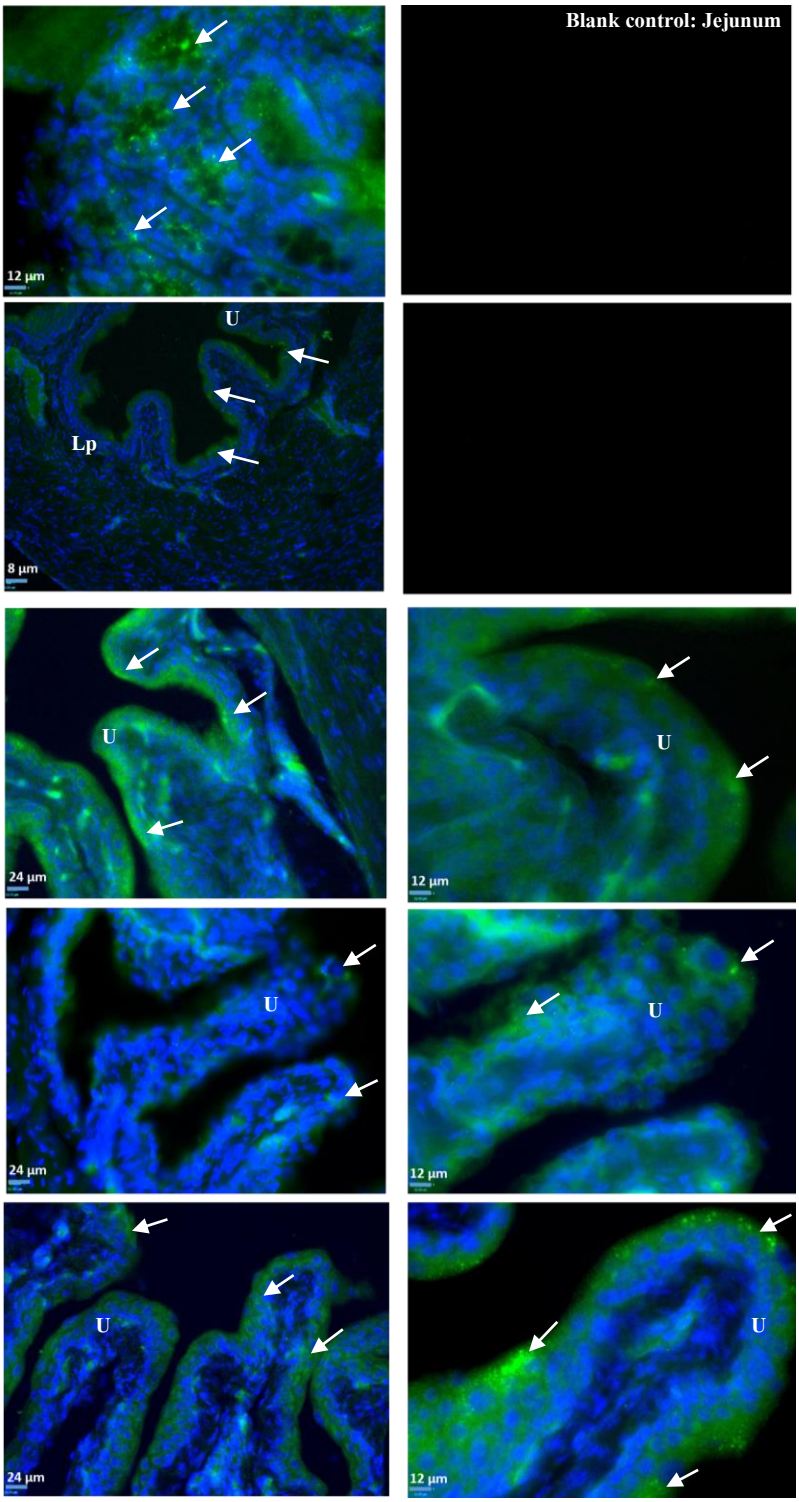


Figure 6.8 Representative images of mouse jejunum and bladder labelled with anti-SERT. **(A)** SERT positive staining in jejunum **(B)** Blank control for jejunum **(C)** SERT immunopositive staining was distributed in urothelium and lamina propria. **(D)** Blank control for bladder. **(E-F, G-H, and I-J)** SERT immunopositive staining in the urothelium from the three bladders, respectively. Green=SERT, U= urothelial layers, Lp= lamina propria, arrows indicate SERT immunopositive staining.

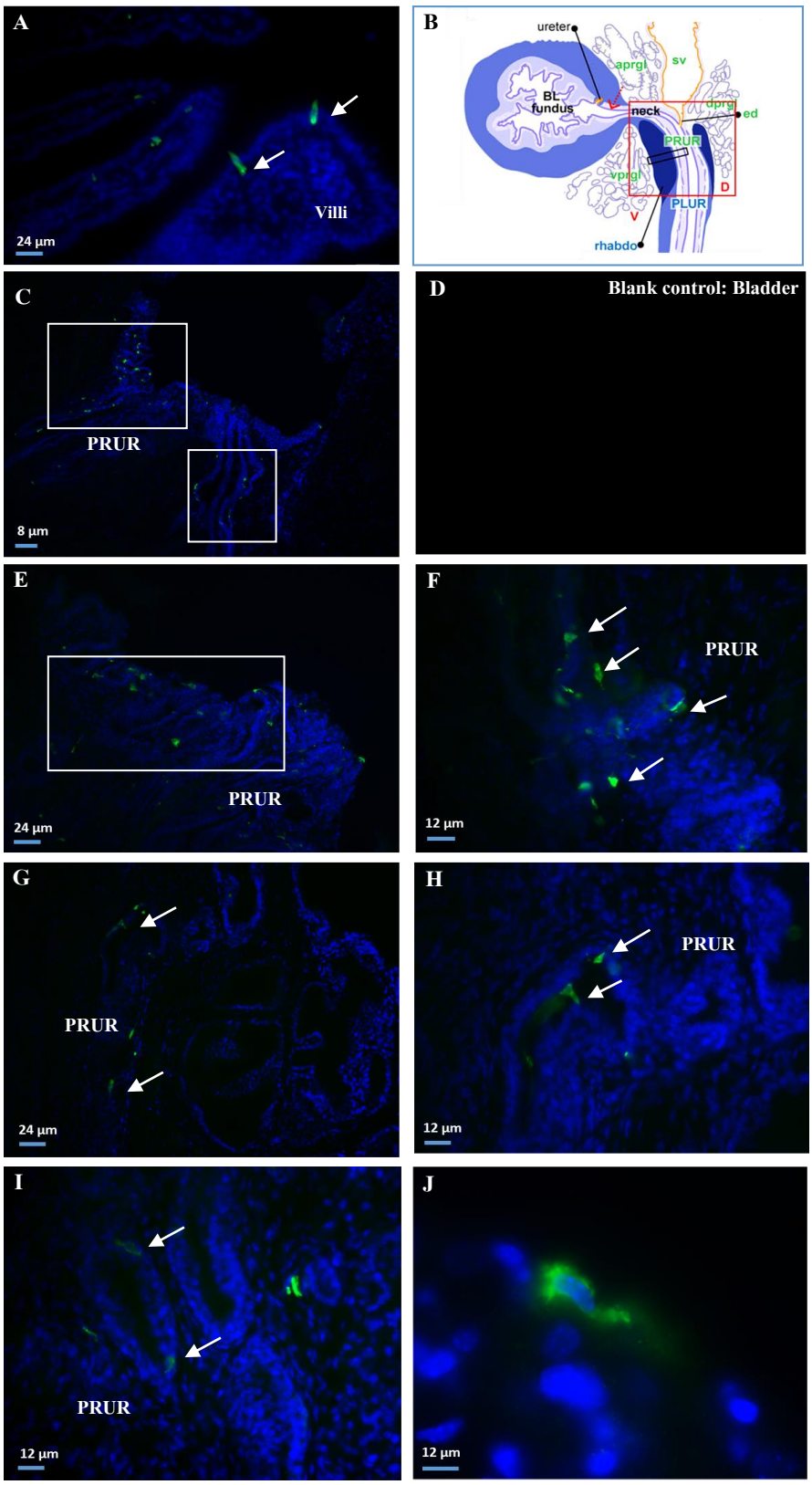


Figure 6.9 Representative images of mouse jejunum and urethra labelled with anti-5-HT. **(A)** EC cells were labelled with anti-5-HT in villi of jejunum, **(B)** Schematic illustrates sagittal section of male mouse bladder, red box indicates the prostatic urethra (PRUR) where 5-HT

immunopositive cells were detected (Georgas *et al*. 2015), **(C, E, F)** 5-HT immunopositive staining was distributed in prostatic urethra wall, **(D)** Blank control for bladder, **(G-I)** 5-HT immunopositive cells distributed around glandular structures in the prostatic urethra, **(J)** Higher magnification of 5-HT immunopositive cell that projected cytoplasmic process to luminal side of the urethra. Green=5-HT, blue=DAPI, PRUR=prostatic urethra, arrows indicate 5-HT immunopositive cells.

6.6 DISCUSSION

The main findings of this chapter are

1.) Blocking SERT with citalopram attenuated mechanosensitive afferent firing but there was no effect on spontaneous firing;
2.) Granisetron application prior to citalopram reversed an attenuation effect of citalopram on mechanosensitive firing;
3.) Citalopram and granisetron application prior to citalopram increased bladder compliance;
4.) TPH1 and TPH2 mRNA expression was found in mouse urothelium;
5.) mRNA expression and protein expression of SERT were detected in mouse urothelium;
6.) 5-HT positive cells were detected in the urethra.

Citalopram attenuated mechanosensitive firing but tended to increase spontaneous firing over time.

For the first time, the data in this chapter show the effect of citalopram on bladder afferent firing. Citalopram attenuated mechanosensitive firing at 120 minutes after the drug application, which was a similar effect to exogenous 5-HT application that was shown in chapter 4. The spontaneous firing tended to increase over time, however, there was no significant difference to that of the control period.

Chiba and colleagues (2016) demonstrated that local perfusion of citalopram (1 μM) in the prefrontal cortex increased 5-HT concentration, measured by in vivo microdialysis, by 600% from baseline level after 60 minutes. They also showed that increased brain 5-HT level was associated with a decrease in intercontraction interval of the bladder measured by cystometrography (Chiba *et al.* 2016) - this suggests that changes in 5-HT centrally can drive alteration is bladder voiding although a peripheral effect cannot be ruled out.

In the present study, citalopram showed an effect that only became significant at 120 minutes. This is longer than the effect observed by Chiba and colleagues and suggests that in the brain

there may be a higher concentration of 5-HT following SERT inhibition than in the periphery. It might be that in the periphery, less 5-HT and lower SERT levels mean a longer period is needed before 5-HT levels in the extracellular space reach a concentration sufficient to influence afferent firing. Matsumoto-Miyai also reported the efficacy of citalopram to increase the level of extracellular 5-HT in the mouse bladder. They showed that application of citalopram (10 μM) for 30 minutes prior to bladder distension mimicked the inhibitory effect of exogenous 5-HT in bladder distention-induced ATP release (Matsumoto-Miyai *et al.* 2016).

In chapter 4, the attenuation of mechanosensitivity in response to exogenous 5-HT is described as being mediated by 5-HT3 receptors and blocked by granisetron. We found that pre-incubation of granisetron prior to citalopram prevented an attenuation of mechanosensitive discharge following citalopram application, suggesting that there might be an endogenous 5-HT released after citalopram application. However, we observed an increase in bladder compliance in response to citalopram, which in chapter 4 had not been observed. Could this mean that citalopram also exerted independent effects compared with SERT and 5-HT? Citalopram is known to affect a variety of ion channels including inhibition of L-type calcium channels. Hamplová-Peichlová showed that citalopram decreased L-type calcium current (I_{Ca}) in rat cardiomyocytes in a dose dependent manner (3 and 10 μM) (Hamplová-Peichlová *et al.* 2002). In this respect it is interesting that in the current study there was an increase in compliance reflecting a decrease in bladder detrusor tone. This persisted to a similar extent after granisetron treatment although because of low N numbers this failed to reach significance. It is therefore possible that the attenuated mechanosensitivity after citalopram is secondary to changes in muscle tone rather than resulting from a direct effect of 5-HT on the afferent endings. Additional studies would be necessary to determine if this is the case perhaps using muscle blockers.

Even though citalopram has been proposed to be one of the most selective SSRI, it has been reported to have some slight affinity to α_1-adrenergic receptors and histamine H1 receptors (Owens *et al.* 1997). A previous study reported that activation of α-adrenergic receptors in urothelial cells could enhance NO release (Birder *et al.* 1998). This NO released from urothelial

cells may further affect afferent nerves in the suburothelial layer and the detrusor muscle. Further experiments would be necessary to determine the contribution of NO to the response.

Our finding is inconsistent with the side effect profile reported by citalopram users, who have polyuria, frequency and incontinence. However, this inconsistency may be derived from differences in methodology and doses of citalopram administered. In our investigation, we directly applied citalopram into the bladder. Another key difference is we excluded the afferent nerves from the influence of central effects, which are the main target for citalopram to treat their psychiatric conditions.

Our investigation employed an indirect method to detect 5-HT release from the bladder via using SSRI drug to block SERT function and determine bladder afferent discharge. This has the disadvantage that other pharmacological issues could influence the results, e.g., the off-target action of citalopram described above. Measuring 5-HT levels in supernatants following bladder distension by ELISA could be an alternative method. However, the sensitivity of the 5-HT detection methods may be a limitation in tissue with limited sources of 5-HT (see below). Generally, the sensitivity of commercially-available 5-HT detection kits is in the range 5 ng/mL-100 ng/mL (5-HT ELISA kit, abcam133053) and therefore might not sensitive enough to detect small 5-HT release in volumes collected from bladder emptying in mice which are also small volumes (~100-120 μL). Other detection methods might include western blot or immunohistochemistry. Therefore, we performed immunohistochemistry in an attempt to identify an endogenous source of 5-HT in the bladder.

TPH1, TPH2, and SERT mRNA expression was detected in mouse urothelial cells.

It has been reported that TPH1 transcript but not TPH2 is found in mouse bladder using nested PCR. Matsumoto-Miyai and colleagues reported a small level of TPH1 mRNA expression in the whole bladder tissue (Matsumoto-Miyai *et al.* 2016). However, in our study, both TPH1 and TPH2 mRNA expression was detected in mouse urothelial cells. This discrepancy in TPH2 expression could partly be due to different methodology. Our study used urothelial cell lysates, whereas the study of Matsumoto-Miyai, collected the whole bladder. If TPH2 expression was mainly in the urothelium, the level for detection might be far lower in the samples from the whole bladder making detection of TPH2 more difficult.

TPH1 protein expression is found in EC cells, mast cells, nerve fibres, and cell bodies both in rats and human in the gastrointestinal tract (Yu *et al.* 1999). Even though TPH2 is defined to be a major TPH isoform in neuronal tissues, many studies have shown that TPH2 expresses in the peripheral organs. Ortiz-Alvarado and co-workers (2006) have reported TPH2 expression in enteric neurons in the gut and neuroepithelial cells in taste buds in mice (Ortíz-Alvarado *et al.* 2006). In rat, TPH2 has been found in retinal pigment epithelial cells (Zmijewski *et al.* 2009). In catfish, TPH2 has been detected in muscle, heart, spleen, kidney, liver, and gill (Raghuveer *et al.* 2011).

Our study, for the first time, showed that SERT mRNA is expressed in urothelial cells of the mouse. Moreover, the immunohistochemistry experiment also suggests that SERT may also be expressed at protein level in urothelial and submucosal layers. More investigations of SERT expression have been described in the intestinal tissues. SERT mRNA and protein expression have been shown in mucosal epithelial cells in rats, guinea pigs, and human (Wade *et al.* 1996; Chen *et al.* 1998; Gill *et al.* 2008). The expression patterns are scattered in apical membrane of enterocytes, intracellular part, and some nerve fibres in submucosal layers (Gill *et al.* 2008). Interestingly, co-expression of SERT and TPH1 has been reported in glomus cells in the carotid body (Yokoyama *et al.* 2013). However, our study did not perform double staining SERT with TPH enzymes. It would be interesting to examine if SERT and TPH are express in the same cells or arrange in proximity. This would provide more information about any potential role of SERT in the urinary bladder.

5-HT expression was detected in mouse urethra.

Our study showed that 5-HT immunoreactive cells were distributed in the urethra wall. This is correlated with a recent study by Yokoyama and co-workers (2017) in rat urethra. They showed that 5-HT immunoreactive cells are densely distributed either in the proximal part of the prostate urethra, epithelial cells of coagulating glands, prostatic glands, and seminal vesicles (Yokoyama *et al.* 2017). A functional role for 5-HT-positive endocrine cells in the urethra has also been studied. Fan and co-workers (2014) demonstrated that intravenous injection of 5-HT1A agonist, 8-OH-DPAT decreased the flow rate of urine by potentiating urethra smooth muscle and also decreased residual volume in anesthetized male rats. This suggests that urethral 5-HT may exert a fine tuning function during micturition by controlling smooth muscle contraction in the urethra (Fan *et al.* 2014).

Various morphologies of 5-HT positive cells were observed in our study. Some of the cells had a triangular shape which is a typical morphology for EC cells in the gut. Interestingly, most of the cells located in the epithelial layers and extended their cytoplasmic processes either to the apical or basolateral sides of the lumen. Some of the cells projected cytoplasmic processes to both apical and basolateral sides. This raises an interesting possibility that 5-HT positive cells in the urethra may exert sensory function to detect luminal change and convey sensory signals to other nearby cells via 5-HT secretion.

Notably, from their oval nuclear shaped with spindle and elongated cytoplasmic processes, this brings us to speculate that some of 5-HT containing cells might be ICC. ICC are thin spindle shapes cells which, have been demonstrated in the bladder dome and the urethra (Lyons *et al.* 2007; McCloskey and Gurney 2002). Using c-Kit and vimentin immunostaining for ICC cell makers, Davidson and McCloskey (2005) showed that several types of ICC distribute in the bladder wall of guinea pigs either the lamina propria which form proximity to the urothelial cells or the detrusor muscle which are found to line on the muscle edge, between the muscle bundle, and close to cholinergic nerves (Davidson and McCloskey 2005). Expression of 5-HT receptors on ICC in the gut have also been reported (Wouters *et al.* 2007a; Wouters *et al.* 2007b). Nevertheless, the higher relative number of ICC in the urethra than our 5-HT positive staining is one point to consider. It may be possible that only a sub-population of ICC expressed 5-HT. However, future study is required to rule out this speculation. Using

immunohistochemistry with co-staining of ICC markers (c-kit and vimentin) and 5-HT could rule out this hypothesis.

In the present study, we found TPH1, TPH2, and SERT expression in mouse urothelial cells and 5-HT positive cells in the urethra. The functional study of citalopram showed similar effects to exogenous 5-HT although the pharmacology is unclear. All of this information may suggest that 5-HT may possibly be produced in the urinary bladder. Even though we could not detect 5-HT expression in bladder dome, the explanation could be that in the normal state, the urinary bladder may not produce and store 5-HT in the bladder wall. 5-HT production might be enhanced in response to mechanical stimulation (bladder distension) or in pathological states, i.e., inflammation or interstitial cystitis. In addition, 5-HT is a small labile peptide molecule, we could not exclude the possibility that 5-HT may be degraded during sample preparation.

The other candidate sources of 5-HT in the bladder wall are mast cells and platelets. Mast cells synthesize 5-HT from 5-hydroxytryptophan and they have been reported to express TPH1 in rat and human gastrointestinal tracts (Weitzman *et al.* 1985; Yu *et al.* 1999). Mast cells are distributed in the lamina propria, adventitial blood vessels, and the detrusor muscle. However, in healthy rats, there is a low density of mast cells compared to rats with colonic TNBS-treated group (Fitzgerald *et al.* 2013b).

Our study provides only preliminary information about SERT and 5-HT expression in the urinary bladder. To ensure the consistency and pattern of the expression, greater N numbers are required. SERT and 5-HT immunopositive cells distribution needs to be systematically quantified in each regions of the bladder. Double staining of 5-HT with other cell markers including, mast cells (CD34), urothelial markers (uroplakin), platelet markers (CD41), and ICC (c-Kit and vimentin) would also help to clarify an endogenous source of 5-HT in the bladder. Therefore, all of the conclusions are derived from immunohistochemistry study in this chapter require further investigation.

CHAPTER 7 GENERAL DISCUSSION

This thesis describes a modulatory role for 5-HT on bladder afferent firing in healthy animals and in a model of colon-bladder cross organ sensitization. Afferent nerves are the first key component that sense mechanical (stretch and volume), chemical, and noxious stimuli and convey the signal to the CNS in order to regulate the micturition reflex and mediate sensation. Dysregulation of afferent nerve activity therefore leads to bladder pathological conditions such as OAB and IC, in which patients have urinary frequency, urgency, nocturia, and pain during urination (Klein 1988; Andersson 2002; Yoshimura and Chancellor 2003). IC has been reported to affect people of all ages and both sexes (Held *et al*. 1990). Bladder disorders impact quality of life including decreased emotional well-being and impairment in sexual function (Coyne *et al*. 2009; Irwin *et al*. 2006). 45% of the worldwide population has been estimated to have at least one bladder symptom (Irwin *et al*. 2011) and this causes a high amount of economic burden for the health care system (Klotz *et al*. 2007; Ganz *et al*. 2010).

5-HT is widely known to be a key neurotransmitter/hormone regulating many physiological functions both centrally and in the periphery. Especially in the bowel, 5-HT exerts crucial roles in digestion, secretion, and motor functions (Berger *et al*. 2009; Grundy 2008). In addition to providing beneficial roles, 5-HT has been reported to be an inflammatory mediator playing a role in developing afferent hypersensitivity in the bowel, which is associated with pathological symptoms such as abdominal pain, discomfort, diarrhoea, and constipation (Gershon and Tack

2007; Grundy 2008; Sikander *et al*. 2009). Compared to the bowel, less information about the modulatory role of 5-HT on bladder sensation and inflammation has been established. Most of the previous investigations have focused on its roles in central brain mechanisms involved in micturition and detrusor contraction. However, there is still little evidence for a modulatory role of 5-HT on bladder afferent signalling and related to bladder storage symptoms (i.e., urgency and frequency). Therefore, we were interested in investigating the role of 5-HT in bladder afferent signalling. Importantly, 5-HT has been shown to be involved in intestinal inflammation, so we hypothesized that 5-HT may play a role in bladder hypersensitivity derived from neuronal cross-talk between the bowel and the bladder, to account for the overlapping symptoms recorded in IBS and IC patients.

➢ Endogenous 5-HT in the urinary bladder

It has become widely accepted that the urothelium is not just a passive barrier to protect the bladder but also exerts plastic and essential roles in immune response, permeability and cellular communication in response to stimuli via secretion of various mediators such as ATP, NO, ACh, substance P, bradykinin and others (Birder *et al.* 1998; Birder and Andersson 2013; Birder *et al.* 2003; Ferguson *et al.* 1997). These urothelial mediators could activate the urothelium itself in an autocrine manner and/or convey chemical signal to other nearby cells (i.e., afferent nerve fibres, ICC and smooth muscle cells) in order to control bladder responses to certain stimuli (Birder and Andersson 2013; Birder *et al*. 2003). However, since we have identified a number of 5-HT receptor subtypes expressed in the urothelium, we hypothesized that the urothelium may also be source of 5-HT in the bladder. In this respect we detected TPH1 and TPH2 mRNA expression in the mouse urothelium. In addition to 5-HT producing enzymes, we found SERT mRNA and protein expression. These data suggest that urothelial cells may be a potential source of endogenous 5-HT. Our study is in line with Matsumoto-Miyai and colleagues who showed that TPH1 transcripts are expressed in the mouse bladder (Matsumoto-Miyai *et al.* 2016).

Our functional study on bladder afferent firing using the selective 5-HT reuptake inhibitor citalopram showed an attenuation in afferent firing in response bladder distension. This mimics the response to exogenous 5-HT except that the effect of citalopram takes a longer time to

develop. This suggests that 5-HT may be endogenously produced by the bladder. Even though pre-incubation with granisetron reduced the attenuation effect of citalopram consistent with an action of endogenous 5-HT at 5-HT3 receptors, off-target actions of citalopram on other receptors/channels cannot be ruled out as discussed in chapter 6. An indication of such off target effects of citalopram may be the increased bladder compliance seen with citalopram but was not observed with 5-HT administration. It is possible that one mechanism of citalopram to regulate bladder afferents is via modulation of muscle tone. Therefore, in order to determine that 5-HT is released from the bladder, other functional assays with higher accuracy and sensitivity should be considered i.e., electrochemical detection using carbon fiber electrode. This method has been used to directly detect 5-HT secretion from the gastrointestinal epithelial layer in real-time and determine concentration following mechanical stimulation in the bowel (Bertrand and Bertrand 2010).

It is possible that endogenous 5-HT in the bladder may be metabolized into other mediators, for example melatonin. In addition to the pineal gland, it has been shown that 5-HT can also be converted to melatonin in the bowel, which in turn regulates many intestinal functions i.e., motility, pain, and inflammation (see review by Chen *et al.* 2011). However, melatonin synthesis requires two key enzymes; N-acetytransferase and hydroxyindole-O-methyltransferase (Hong and Pang 1995). However, there is still no clear evidence that these enzymes are present in the bladder wall, and future study will be required to prove if this is the case.

Interestingly, patients on citalopram medication have been recorded as reporting bladder symptoms such as urinary incontinence, frequency, and urgency. However, in our nerve recording experiments we did not observe hyperexcitability of mechanosensitive afferents, but a tendency for the spontaneous firing to increase over time. It is plausible that citalopram may require a longer time period for the 5-HT concentration to reach a sufficient level to activate afferent nerves. It may be possible that any adverse effect of citalopram on bladder functions may be mediated through indirect mechanism that alter other signalling pathways. One possible candidate for this could be ATP since recent evidence has shown that 5-HT potentiates distension-induced ATP release from the urothelium (Matsumoto-Miyai *et al.* 2016). It is important to recognize that in our study any influence of efferent and central nervous control has been excluded. Therefore, it is reasonable to postulate that citalopram's effect on the bladder

may primarily be mediated through central mechanisms. Even though the mechanisms underlying citalopram side effect the bladder, it may be implied from the other side effects e.g., appetite change, sleep deprivation, nausea, and headache (Ferguson, 2001), that CNS may be a major site involving these adverse symptoms.

We detected TPH mRNA expression in the urothelial layer suggesting an endogenous source of 5-HT. However, we could not detect 5-HT expression in the bladder dome using immunocytochemistry. As briefly discussed in chapter 6, one of the possible explanations for this discrepancy could be that in a resting state (without mechanical or chemical stimulation) condition; relatively small amounts of 5-HT are produced by the urothelial cells, which are below the detection threshold for antibody staining. There might be an increase in 5-HT production from the bladder following pathological insults such as inflammation as occurs with interstitial cystitis giving 5-HT a prominent role in modulating the afferent response to inflammation. An increase of 5-HT production following inflammation has been extensively shown in the intestine (Linden *et al.* 2003; Keathing *et al.* 2008; Rapalli *et al.* 2016). However, we did not determine whether 5-HT expression or 5-HT levels are increased in our cross-organ sensitivity TNBS model. With more time, 5-HT expression/levels in the bladder could be measured in the inflammatory model and compared to the healthy state using immunohistochemistry, HPLC, or electrochemical detection. Further investigations using an array of inflammatory models would also be needed to explore this further.

Interestingly, we found 5-HT immunopositive straining of cells in the urethra, which corroborates a recent study by Yokoyama and co-workers (2017). They showed that 5-HT positive cells in proximity to CGRP positive nerve fibres (Yokoyama *et al.* 2017). In addition, 5-HT expression was detected in rat urethral epithelial cells and some of these formed a close relationship with NOS positive cells in the suburothelial layer (Eggermont *et al*. 2016). Since urethral afferents are conveyed through pudendal and hypogastric nerves, we cannot exclude the possibility that some of the afferent nerves that we recorded (mixture of pelvic and hypogastric nerves) may also originate from the urethra with close proximity to 5-HT positive cells. (Janig and Morrison 1986).

It is still unclear which types of cell express 5-HT in the urethra. Notably, from the location of 5-HT positive cells that are distributed in the urethra wall, around the glandular structure close to the lumen, this may suggest various functions of 5-HT in the urethra. Possible candidate cells could be urothelial cells, suggested by our RT-PCR study that found TPH1 and TPH2 mRNA expression in the urothelium. Moreover, various cell types in the suburothelial layers such as mast cells, platelets, and nerve terminals, which have been suggested to produce and/or store 5-HT may be potential candidate cells for 5-HT expression in the urethra. However, double staining with other cell markers i.e., uroplakin (for urothelial cells), tryptase (for mast cells), and CD61 (for platelets) would allow a clearer interpretation of cell types and its role from relationship with the nearby cells.

- **Opposing actions of 5-HT on spontaneous and mechanosensitive afferents**

We have shown that 5-HT exerts opposite effects on mechanosensitive and spontaneous afferent firing, both predominantly mediated via 5-HT3 receptors. Endogenous or exogenous 5-HT could bind to receptors at multiple sites in the bladder wall; (*i*) urothelial cells, (*ii*) detrusor muscle, (*iii*) afferent nerve terminals and (*iv*) other cells in the suburothelial and muscularis layers.

In the urothelium we found various subtypes of non-HT3 receptor transcripts expressed on the urothelial cells. In calcium imaging experiments, we found that 5-HT triggered an increase in intracellular Ca^{2+} via binding to non-5-H3 receptors (possibly 5-HT1A, 1B, 1D, 2A, 2B, 4, 6, or 7) expressed on urothelial cells. It is possible that 5-HT may activate these receptor on the urothelial cells directly conveying sensory signals to nerve terminals and/or nearby cells in the suburothelium (i.e., ICC and blood vessels). We therefore cannot exclude the possibility that 5-HT results in mediator release from the urothelium that then results in an indirect effect on afferent firing. Several studies have reported a role of 5-HT in the release of other mediators such as ATP and ACh (Fink and Göthert 2008; Matsumoto-Miyai *et al.* 2016). These mediators could be released from the urothelium in response to mechanical and/or chemical stimulation. However, further experiments are required to prove such interactions. For example, pre-incubation with the P2 purinoreceptors antagonist (suramin) before 5-HT

application could rule out if 5-HT exert indirect mechanism on afferent firing through mediated ATP release.

As discussed in chapter 3, various 5-HT receptor subtypes including 5-HT3 have been found in mouse detrusor muscle (Chetty *et al.* 2007). Our investigation also showed that 5-HT and 5-MT (a full agonist of non-5-HT3 receptors) triggered bladder contraction concurrently to sensitization of baseline afferent firing. The bladder compliance in response to all of agonists was unchanged. Using Y-27632 and ML-9 to block detrusor contraction prior to application of 5-MT abolished the effect of 5-MT on baseline afferent firing. These data clearly demonstrate that non-5-HT3 receptors induced activation of baseline afferent firing is dependent on detrusor contraction. To determine whether 5-HT3 receptors-stimulated baseline afferent firing is secondary to any induced detrusor contraction, bladders were treated with Y-27632 prior to application of 2-Me-5-HT, a selective 5-HT3 agonist. We found no significant difference in peak afferent discharge in response to 2-Me-5-HT after blocking the contractile components, indicating that 5-HT3 modulate bladder afferent firing is independent of muscle contraction.

Since bladder distension could also lead to various types of mediator release including NO, we explored the possibility using pharmacological tools. Our finding that a NOS inhibitor (L-NAME) prevented 5-HT-induced attenuation of mechanosensitive afferent firing while augmented excitation of spontaneous firing suggests that 5-HT may regulate NO production. Our data is correlated with a study by Yu and de Groat (2013) who found that application of SNAP (NO donor) and L-arginine (NO substrate) depressed bladder afferent firing in response to bladder distension. The inhibitory effect of L-arginine was blocked by application of L-NAME in cyclophosphamide (CYP) treated rats. These finding indicate that NO has a depressive effect on hyperexcitability of bladder afferents in pathological conditions and may suggest an anti-nociceptive role of NO in response to bladder inflammation (Yu and De Groat 2013). Our data showing the interaction of 5-HT and NO pathway supports the hypothesis that NO may reflect an adaptive mechanism to counteract hypersensitivity of bladder afferents following sensitization of bladder afferent nerves by 5-HT. However, there is still uncertainty about the source of NO that could contribute to afferent modulatory effects. NOS has been shown to be expressed and distributed in the bladder wall including, urothelium (Birder 1998, 2008), smooth muscles (Andersson and Persson 1993, 1995; Birder, 1998), parasympathetic efferent nerves (Andersson and Persson 1995), and afferent nerves (Vizzard *et al.* 1995, 1996).

It is possible that any of these candidate sources may act in a concerted way to regulate bladder afferents. However, future study should be conducted to measure NO release in response to bladder distension comparing with and without 5-HT.

One possible explanation for the different effect of 5-HT on spontaneous and mechanosensitive afferent firing could be that 5-HT has a differential effect on different populations of bladder afferent fibres. As discussed in chapter 1 and 4, Xu and Gebhart classified mouse pelvic afferent nerves based on their response to various stimulus into 4 major classes; urothelial (9%), muscle/urothelial (14%), muscle (63%), and serosal (14%) afferents. Each class of afferent may include different proportion of Aδ- and C-fibres. For instance, urothelial afferents are not responsive to stretch but are activated in response to chemical stimuli, whereas muscle afferents primarily response to stretch during bladder distension. It has been reported that the majority of C-fibres innervate urothelial and suburothelial layers and some of these could be volume receptors which do not response to intravesical pressure, while Aδ-fibres innervate the detrusor layer (Gabella and Davies 1998; Birder 2013; Kanai and Andersson 2010). These polymodal properties (response to multiple stimuli) are similar to the mechanosensitive afferent innervating the colon (Su and Gebhart 1998). It is possible that 5-HT potentiates other mediator release (e.g. NO) that further modulates stretch sensitive afferent fibres. However, one experiment which could rule out the different response to 5-HT among the different population of afferent fibres is to use electrical stimulation of pelvic nerves to discriminate Aδ- and C- fibres according to their conduction velocity. In addition, measuring bladder afferent firing in response to 5-HT in urothelium denuded tissues would eliminate contribution of mucosal stretch-insentitive fibers.

The opposing action of 5-HT on spontaneous afferent and mechanosensitive or distension sensitive afferents may suggest two distinct roles of its modulatory actions; (i) excitatory mediator of bladder sensation to convey chemical stimulation or noxious stimuli (ii) inhibitory mediator to attenuate afferent firing when there is an over distension or bladder accommodation is limited.

➢ Reduced sensitivity of bladder afferent firing to 5-HT in TNBS-induced colonic inflammation

We employed TNBS-induced colonic inflammation to generate bladder hypersensitivity. Experimental TNBS induction is a well characterized model of colonic inflammation via activation of Th1 cells (Kiesler *et al.* 2015) which resulting in release of proinflammatory cytokines such as IL-1ß, IL-6, and TNF-α (Dohi *et al.* 2006). In addition, T cell activation further recruits macrophages which also release various pro-inflammatory cytokines i.e., IL-6, IL-8, IL-1ß, IL-12, TNF-α, NO and reactive oxygen species (Cloez-Tayarani and Changeux, 2007). This information is in line with investigations in IBD patients that have shown an increase in pro-inflammatory cytokines i.e, TNF-α, IL-6, and IL-1ß release from macrophages, neutrophils, and endothelial cells (Rahimi *et al.* 2007).

A number of studies successfully used TNBS-induced colonic inflammation to investigate cross-organ sensitization mechanism between the bowel and the bladder. However, one point that still needs to be considered is the extent to which this chemical injury model mimics physiological phenomena in intestinal inflammation. Other models that could be more relevant to physiological responses include nematode infection i.e., T.*spirallis* and infection with pathogenic bacteria, which should be consider as alternative methods in order to generate cross-talk mechanism. Various strain of bacteria have been reported to generate colonic inflammation such as *Salmonella, Campylobacter, Shigella,* and *Escherichia* (Papaconstantinou and Thomas 2007). Ibeakanma *et al*. studied *Citrobacter rodentium* infection mouse model to mimic E.*coli* infection in human. They showed that *Citrobacter rodentium* generated post-infectious hyperexciatability of colonic DRG neurons (Ibeakanma *et al.* 2009). However, one challenge of these physiological-induced model is the variability of mouse immunity which may be more difficult to control in order to provide consistent inflammation levels for each animal.

CVH animals developed hypersensitivity in both spontaneous and mechanosensitive bladder afferent firing. We found that the inhibitory effect of 5-HT on bladder mechanosensitive firing was blunted in acute TNBS and CVH animals while there was no significant change in spontaneous firing in the CVH group. This might be that in the post-inflammatory state, bladder afferents are already sensitized by inflammatory mediators i.e., TNF-α, IL-1ß, IL-6, and 5-HT

released from immune cells in the blood circulation, therefore the effect of 5-HT on bladder afferent discharge may be masked. Nevertheless, there might also be an increase in 5-HT release in CVH animals. This is suggested from the experiment in which granisetron reduced hypersensitivity of spontaneous and mechanosensitive firing. A previous study has suggested an increase in gastrointestinal 5-HT production in TNBS-treated animals. Linden *et al.* 2003 showed increased number of EC-cells which associated with 5-HT availability in colonic mucosa of guinea pigs (Linden *et al.* 2003). However, future studies are required to determine whether there is a change in 5-HT level in the bladder following TNBS treatment.

We observed a downregulation of SERT mRNA expression in urothelial cells of CVH mice. This may indicate an impairment in 5-HT metabolism in the bladder in the post-inflammatory state. Our study did not show change in TPH1 and TPH2 mRNA expression, however, the urothelium may not be the only potential source of 5-HT. As discussed earlier, 5-HT could be secreted from other cell types in the bladder wall i.e., mast cells and platelets, although these were not evident from immunohistochemistry. Future study should address TPH1, TPH2, and 5-HT expression in the bladder wall in CVH mice to examine if there is a change in 5-HT production in the bladder following this post-inflammatory state. Decreased SERT expression in the urothelium may result in an increase of 5-HT level in the bladder wall, which would potentially activate 5-HT receptors on the bladder afferents. Importantly, ligand-gated 5-HT3 subtype may become desensitized in the continued presence of 5-HT or there may be a downregulation of 5-HT3 expression and/or sensitivity after chronic exposure to endogenous 5-HT.

Our data suggests that 5-HT signalling in the bladder of CVH animals is altered. One possible mechanism could be that some colonic afferent fibres that were sensitized following TNBS treatment may also have branching to innervate the bladder, leading to a reduction in 5-HT3 sensitivity and/or receptor expression in bladder afferents. This may reflect an adaptive mechanism following chronic exposure of 5-HT. Further studies are needed to determine if 5-HT3 receptor expression in the bladder wall and DRG neurons is altered in CVH animals.

Functional studies using patch clamp could determine whether DRG neurons from CVH mice have altered excitability following 5-HT3 agonist application.

Alternatively, it is also possible that other 5-HT receptor subtypes become dominant under pathological conditions. As discussed in chapter 5, Rapalli *et al*. recently showed that 5-HT2A receptors exert a major action in development of colonic and systemic inflammation in the TNBS model with an additional moderate role of 5-HT4 receptors. In contrast, 5-HT1A receptors have an opposite effect to delay and prevent colitis progression. In addition, they reported an increase in plasma nitrites, stable metabolites of NO in TNBS mice and administration of 5-HT1A antagonist (WAY100135) augmented nitrite level (Rapalli *et al*. 2016). Such information raises an interesting point for future study to determine the role of other 5-HT receptor subtypes including 5-HT2A, 5-HT4, and 5-HT1A in hypersensitivity of bladder afferent nerves. However, the present study did not determine the contribution of non-5-HT3 receptors in the TNBS model, future experiments are required to determine if this is indeed the case.

The bladder, like the bowel, develops hypersensitivity in response to inflammation. Hughes and colleagues (2009) studied colonic afferent sensitization in colonic TNBS-treated mice and showed colonic afferent hypersensitivity in both acute inflammation (7 days post-treatment) and in the recovery period (28 days post-treatment). However, in our study we found there was no change in spontaneous and afferent firing in response to bladder distension in the acute phase of TNBS colitis. This difference could be due to variation in experimental design and in particular the timing of the investigation post-treatment. We selected 3 days post TNBS for our acute assessment of bladder hypersensitivity while the colon was investigated after 7 days. In CVH mice at day 28 we observed similar hypersensitivity in the bladder to that seen in the colon. This would support the hypothesis that there is bladder and bowel neuronal convergence such that sensitization of colonic leads to sensitization of bladder afferent via central and/or peripheral cross-sensitization as discussed in chapter 5. Decreased SERT expression and function have been shown in the colon following acute inflammation (6 days after TNBS administration) (Linden *et al.* 2005). Bischoff and co-workers reported that SERT knockout

mice had more severe colonic inflammation, which indicates an important role of 5-HT in generating colonic inflammation (Bischoff *et al.* 2009). SERT downregulation in the urothelium could be driven through antidromic activation from sensitized colonic afferents. Our investigation relates to clinical evidence indicates that IBS patients still report bladder symptoms in the recovery state of inflammation. However, we provided an initial information about the role of 5-HT in bladder afferent firing in colon-bladder cross-organ sensitization. Further investigation are required to better understand the underlying mechanism.

➢ Limitation and Future direction

With a limitation of time, many interesting questions still require future investigation to clarify the role of 5-HT in bladder afferent signalling in health and disease. One important question that we have been unable to answer definitively is the site of action for 5-HT for its modulatory effect on bladder afferents. One approach might be to use pre-incubation with protamine sulphate to eliminate the urothelial layer. This would help to determine if 5-HT receptors on the urothelium contribute to the afferent effect. The endogenous source of 5-HT in the bladder is an intriguing question that requires further investigation. Identifying the source of 5-HT in the bladder is essential to understand the significance of 5-HT signalling in the bladder. Moreover, since 5-HT bioavailability is increased in the bowel after inflammation, it would be interesting to determine if this is also the case for the bladder. Using specific agonists/antagonists of non-5-HT3 receptors would allow a clearer understanding of the mechanism by which 5-HT contributes to bladder hypersensitivity following colon-bladder cross-organ sensitization.

Finally, we have clearly shown that 5-HT exerts a modulatory role in the bladder afferent activity which is mainly mediated through 5-HT3 receptors. The next step in this study would be to investigate changes in bladder afferent firing in 5-HT3 receptors or SERT conditional knockout mice. This would help to confirm the role of 5-HT signalling in bladder afferent activity in both normal and TNBS-induced colonic inflammation model. An understanding of 5-HT signalling in bladder afferents would be essential to provide information for developing new therapeutic drug target to treat bladder symptoms i.e., urinary frequency, urgency, incontinence, and pain during urination, which may derive from neuronal cross-talk between the bowel and the bladder.

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