



The Role of Itch-Mediating Receptors and Channels in the Mouse Urinary Bladder

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Thesis submitted for the degree of Doctor of Philosophy

December 2021

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Abstract

Altered sensitivity of afferent signalling from the urinary bladder is implicated in chronic and debilitating conditions such as overactive bladder syndrome (OAB) and interstitial cystitis/bladder pain syndrome (IC/BPS). The range of receptors and ion channels expressed and functional on the afferent neurons drives overall afferent sensitivity. Receptors known to mediate 'itch' sensation have recently been identified in the viscera and shown to play a role in visceral hypersensitivity; where 'itch' signalling in the skin results in scratching, activation of similar signalling mechanisms in the bladder may stimulate removal of irritants via increased micturition.

In this thesis, a combination of techniques was used to identify the expression and function of histaminergic and non-histaminergic irritant-sensing G-proteins coupled receptors (GPCRs) in bladder sensory pathways. Single cell RT-PCR was used to identify expression of mRNA encoding for histamine receptor subtype 1 (H₁R; *Hrh1*), mas-related G-protein receptors A3 (*Mrgpra3*) and C11 (*Mrgprc11*), and bile-acid receptor TGR5 (*Gpbar1*) in retrogradely labelled bladder-innervating afferent neurons from the dorsal root ganglia (DRG). Co-expression of irritant-sensing GPCRs with ion channels transient receptor potential vanilloid 1 (*Trpv1*) and TRP ankyrin 1 (*Trpa1*) was also determined. Further, QRT-PCR showed *Hrh3* and *Gpbar1* expression in urothelial cells. Functional assays, including *in vitro* live cell calcium imaging and *ex vivo* bladder afferent nerve recordings in conjunction with a range of genetic knockout mouse models, were used to demonstrate that stimulation of H₁R, MrgprA3 and MrgprC11, or TGR5 in bladder-innervating afferent neurons results in increased bladder afferent sensitivity to distension. TRPV1 expression was required for H₁R-induced hypersensitivity. TGR5-induced hypersensitivity was partially mediated by TRPV1 while the absence of TRPA1 had limited impact. To further demonstrate physiological relevance of these results, *in vivo* instillation of compounds into the bladder with or without bladder distension was used in conjunction with pERK immunoreactivity assays in the lumbosacral spinal cord. Increased activation of lumbosacral spinal neurons (where peripheral afferent bladder signals input) indicated central transmission of bladder afferent signalling in response to peripheral activation of these itch-mediating GPCRs.

This thesis has identified a novel sub-class of receptors involved in mediating bladder afferent sensitivity and begun to determine the signalling pathways involved, opening a number of avenues for future research in the area of bladder sensation. Irritant-sensing mechanisms may contribute to conditions of bladder hypersensitivity such as OAB and IC/BPS and represent potentially viable future targets for treatment.

Declaration of originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Ashlee Megan Caldwell

6/12/2021

date

Acknowledgements

This work was funded by the University of Adelaide and the Visceral Pain Research Group, headed by Professor Stuart Brierley. I extend my gratitude to my co-supervisors, Dr Luke Grundy and Associate Professor Grigori Rychkov. I am grateful for the assistance and support of the Visceral Pain team over the years and of Associate Professor Leonie Heilbronn in the final stages.

I wish to acknowledge, with deepest respect, the strength, resilience, and determination of women in science around the globe. In particular, for their steadfast support and guidance: Dr Andrea Harrington, Dr Sonia Garcia-Caraballo, Dr Andelain Erickson, Dr Madeleine Archer, and soon-to-be Dr Jessica Maddern.

Finally, I wish to thank my incredible family and friends for their love and support, keeping me going through the best and the worst. I am truly humbled by the beautiful souls of my support network – it is hard to succumb to self-doubt knowing that so many incredible humans genuinely believe in your worth.

Abbreviations

ACC	Anterior cingulate cortex	HBSS	Hanks' balanced salt solution
AITC	Allyl Isothiocyanate	HPRT	Hypoxanthine Phosphoribosyltransferase
ANOVA	Analysis of variance	HRP	Horseradish peroxidase
AUC	Area under the curve	IBD	Irritable bowel disease
BAM8-22	Bovine adrenal medulla 8-22	IBS	Irritable bowel syndrome
BDNF	Brain-derived neurotrophin factor	IC/BPS	Interstitial cystitis/bladder pain syndrome
CCDC	3-(2-chlorophenyl)-N-(4- chlorophenyl)-N,5-dimethyl-4- isoxazolecarboxamide	IR	immunoreactivity
CGRP	Calcitonin gene-related peptide	IUS	Internal urethral sphincter
CK7	Cytokeratin 7	KSFM	Keratinocyte serum-free media
CNS	Central nervous system	LCA	Lithocholic acid
CQ	Chloroquine	LPS	Lipopolysaccharide
CTB	Cholera Toxin subunit-B	LS	Lumbosacral
CVH	Chronic Visceral Hypersensitivity	LSN	Lateral spinal nucleus
CYP	Cyclophosphamide	M-BAR	Membrane-bound bile acid receptor (TGR5/GPBAR1)
DAB	3,3'-Diaminobenzidine	Mrgpr	Mas-related G protein coupled receptor
DCA	Deoxycholic Acid	Na_v	Voltage-gated sodium channel
DGC	Dorsal grey commissure	ND	No distension
DMEM	Dulbecco's Modified Eagle Media	NGF	Nerve growth factor
DRG	Dorsal root ganglia	NPFF	Neuropeptide FF
ELS	Early life stress	ns	Not significant
EUS	External urethral sphincter	OAB	Overactive bladder syndrome
FXR	Farnesoid X receptor	OCT	Optimal cutting temperature
GAG	Glycosaminoglycan	PAG	Periaqueductal gray
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	PBS	Phosphate buffered saline
GIT	Gastrointestinal tract	pERK	phosphorylated-MAP-kinase ERK 1/2
GPBAR1	G-Protein Coupled Bile Acid Receptor 1 (TGR5/M-BAR)	PFA	Paraformaldehyde
GPCR	G-protein coupled receptor	PG5α	5 α -pregnan-3 β -ol-20-one sulphate
H1R-H4R	Histamine receptor subtype 1 – 4	PGE₂	Prostaglandin E ₂

Abbreviations

PMC	Pontine micturition centre	TGR5	Takeda G-protein Receptor 5 (GPBAR1/M-BAR)
PPS	Pentosane polysulphate	TL	Thoracolumbar
PRV	Pseudorabies virus	TNBS	2,4,6-trinitrobenzene sulfonic acid
QRT-PCR	Quantitative reverse transcription polymerase chain reaction	Trk	Tyrosine receptor kinase
rEGF	Recombinant epidermal growth factor	TRPA1	Transient Receptor Protein Ankyrin 1
SDH	Superficial dorsal horn	TRPV1	Transient Receptor Protein Vanilloid 1
SEM	Standard error of the mean	TRPV4	Transient Receptor Protein Vanilloid 4
SMA	Supplementary motor area	TTX	Tetrodotoxin
SP	Substance P	UTI	Urinary tract infection
SPN	Sacral parasympathetic nucleus		

Chapter 1 Introduction to the Structure and Function of the Urinary Bladder

1.1 Overview

Chronic bladder hypersensitivity disorders, including interstitial cystitis/bladder pain syndrome (IC/BPS) and overactive bladder syndrome (OAB), have a significant impact on quality of life and current treatment options are inadequate. Despite the apparent simplicity of bladder function, the gross and molecular neurophysiology underlying bladder activity is complex, with much yet to be determined. A more in-depth understanding of the cellular physiology and neurophysiology of the bladder under healthy and pathological conditions is crucial to providing safer and more efficacious treatments for bladder hypersensitivity disorders. This chapter explores the current literature covering the anatomy, function, and innervation of the urinary bladder, leading into the role of sensory networks in functional bladder pathologies (**Chapter 2**). Finally, this introduction will provide a comprehensive background on the potential role for a specific functional class of G-protein coupled receptors, known to play a role in 'itch' sensation, in regulating bladder sensation (**Chapter 3**).

1.2 Anatomy and physiology of the urinary bladder

The urinary bladder is a rounded, elastic, sac-like organ of the lower urinary tract responsible for the storage and expulsion of urine, or 'micturition'. The bladder wall consists of three main layers: the outer adventitia/serosa layer, the detrusor muscle, and the mucosa [1]. During the storage phase of micturition, urine enters the bladder from the kidneys via two ureters. Contractions of the detrusor muscle are inhibited, allowing the bladder to expand as it fills. Continence is maintained by the closure of the internal and external urethral sphincters, located at the proximal and distal ends, respectively, of the urethra. During the emptying phase, the detrusor muscle is contracted and the sphincters are opened, allowing expulsion of urine via the urethra [2, 3]. In addition to the unique anatomy of the urinary bladder, adequate storage and complete elimination of urine is achieved via integration of complex signalling mechanisms at the peripheral, spinal, and central levels. Alterations at any of these levels can result in chronic and debilitating bladder dysfunction.

1.2.1 Anatomy of the bladder wall

The anatomical structure of the bladder wall (**Figure 1.1**) enables it to stretch greatly to accommodate considerable volumes of urine (300-400 mL for adults [4]) and return to a small, folded shape on bladder emptying. A combination of adventitial and serosal tissue forms the peritoneal lining

of the bladder overlying the detrusor muscle, which is made up of a layer of circular smooth muscle between two layers of longitudinal smooth muscle [1, 5]. Rather than three distinct muscle layers, the orientation of smooth muscle bundles forming these layers is disorganised, resulting in an interlacing meshwork that allows for uniform contraction and relaxation [5-7]. The innermost layer of the bladder, the mucosa, is loosely attached to the detrusor muscle, allowing it to fold in or form trabeculae when the bladder is empty and extend as the muscle stretches to increase volume [6]. The mucosa consists of the lamina propria, basement membrane, and a specialised transitional epithelium known as the urothelium [8, 9]. The precise cellular composition and function of the lamina propria is still under debate, however current literature indicates an extracellular matrix consisting of elastic fibres, interstitial cells, adipose cells, immune cells, and fibroblasts, as well as lymphatic vessels, blood vessels, and afferent and efferent nerve fibres [1, 8-13]. Indeed, afferent and efferent nerve terminals and fibres have been identified throughout all layers of the bladder, including the detrusor muscle and into the urothelium [5, 13-17]. The composition of the mucosa suggests important roles in tissue elasticity and structural support, immune sensing, compliance and capacitance, and signal transduction and integration [9].

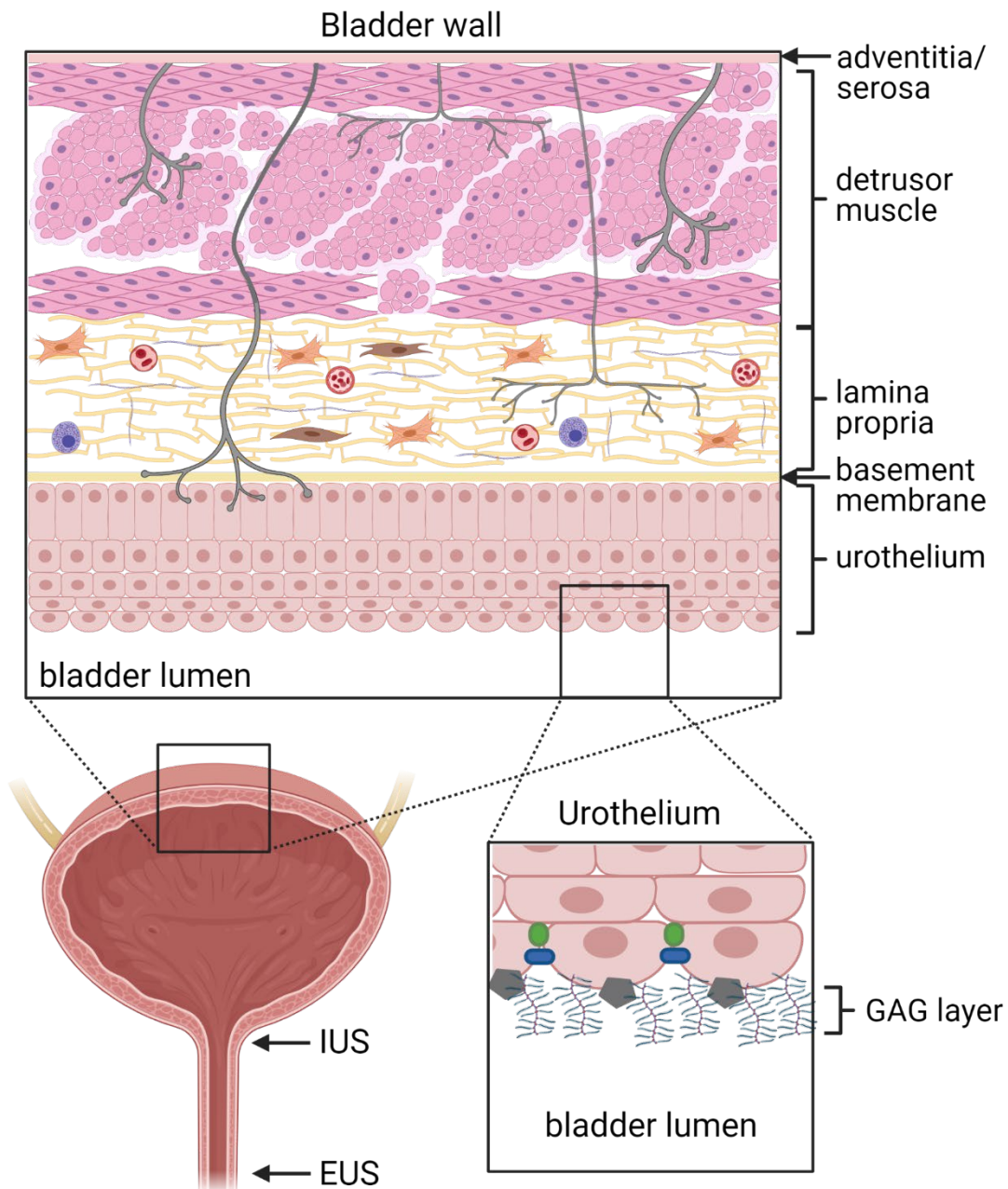


Figure 1.1: The anatomy of the bladder wall

The bladder wall consists of an outer adventitial/serosal layer overlying the detrusor muscle, made up of three moderately distinct layers: a layer of circular smooth muscle sitting between two layers of longitudinal smooth muscle. Luminal to the detrusor muscle is the mucosa, which includes the lamina propria, the basement membrane, and the urothelium. The lamina propria comprises an extracellular matrix including elastic fibres, fibroblasts, immune cells, blood and lymphatic vessels, and adipose cells. Afferent and efferent nerve fibres are present in all layers of the bladder wall. The urothelium is a specialised, stratified transitional epithelium creating an impermeable barrier between the bladder lumen and the underlying tissue. Impermeability is maintained by tight junctions between the outermost ‘umbrella’ cells of the urothelium and hydrophobic plaques on the apical surface. A considerable glycosaminoglycan (GAG) layer is thought to provide protection by physically hampering bacterial adherence. IUS: internal urethral sphincter. EUS: external urethral sphincter. *Figure created with BioRender.com*

1.2.1.1 – The urothelium: an impermeable barrier

The urothelium is the most superficial layer of the bladder mucosa, in direct contact with potentially hazardous bladder luminal contents [18, 19]. As such, urothelial cells have unique properties enabling formation of an impermeable physical barrier and protective layer, preventing access of urinary contents to the underlying interstitium and afferent nerve terminals. The stratified transitional epithelial cells of the urothelium include basal cells (the least differentiated form of urothelial cells) attached to the basement membrane, partially differentiated intermediate cells, and fully differentiated umbrella cells making up the apical or superficial layer of the urothelium [1, 20]. Urothelial impermeability is maintained by tight junctions between umbrella cells and hydrophobic uroplakin plaques on the apical surface that prevent or reduce movement of ions, solutes, and small molecules present in urine through or between cells [1, 21-24]. Additionally, a considerable glycosaminoglycan (GAG) layer on the apical surface of umbrella cells is thought to physically hamper bacterial adherence and hence protect from infection [25-27]. To maintain impermeability throughout the significant stretch incurred during bladder filling, urothelial cells undergo extensive membrane recycling via exo- and endocytosis to adapt to the stretch of the bladder during distension and recoil on emptying [28-30]. Given the importance of the urothelial barrier function, it is no surprise that following acute injury or infection the urothelium is able to quickly and efficiently undergo differentiation and repair [31]. Following induction of acute injury via *in vivo* intravesical administration of protamine sulphate, mouse urothelium was shown to recover barrier impermeability within 72 hours, with basal and intermediate cell differentiation and tight junction protein expression achieved within 5 - 10 days of injury [32].

1.3 Neuroanatomy and neurophysiology of the bladder

Inhibition and contraction of the detrusor muscle and external urethral sphincter (EUS) are controlled by efferent nerves, the stimulation of which is determined by spinal and central neural circuitry receiving input from the afferent neurons innervating the bladder. As these sensory afferent neurons terminate throughout the mucosa as well as the musculature, the impact of the mucosal and urothelial cellular environment (such as infection or injury resulting in inflammatory mediator release from resident immune cells) on the sensitivity of afferent neurons can greatly affect bladder function. Thus, a clear understanding of bladder afferent sensitivity and how this changes in response to specific stimuli or under certain conditions is crucial to developing potential therapies for bladder conditions.

While the essential function of the bladder is a simple bi-phasic process switching between storage and voiding of urine, the neural pathways underlying it are complex. Coordination of micturition is controlled via the integration of the parasympathetic, sympathetic, and somatic nervous

systems, incorporating peripheral, spinal, and central levels of circuitry [33, 34]. Afferent nerves convey sensory signals such as those relating to distension and fullness from the bladder to the spinal cord, from whence signals are transmitted to the brainstem [2, 5, 35]. From the brainstem, there are important distinctions between reflexive and conscious bladder control. Low-level firing of afferent neurons allows sympathetic and somatic efferent activation, contracting both the EUS and the internal urethral sphincter (IUS) to maintain continence and inhibiting contraction of the detrusor muscle, allowing it to stretch and thus facilitating urine storage [2, 3, 36]. This reflex component of bladder storage, which occurs below the level of consciousness, is coordinated primarily by a spinobulbospinal circuit; peripheral afferent input to the spinal cord is relayed and regulated via the brainstem, with efferent signals driving from here via the spinal cord directly into efferent output to induce detrusor muscle relaxation [3, 33, 37-41]. As the bladder fills, the level of afferent firing increases, eventually reaching a threshold of bladder fullness known as the micturition threshold. At this threshold, a conscious awareness of the desire to void is experienced [3, 33, 39, 42, 43]. Once input reaches the micturition threshold, the spinobulbospinal micturition reflex switches efferent output from sympathetic to parasympathetic signalling, initiating voiding via coordinated relaxation of the urethral sphincters and contraction of detrusor muscle to expel urine via the urethra [3, 36, 40, 44]. Critically, in the healthy system, micturition reflex activity is inhibited by cortical mechanisms and transition from storage to voiding requires conscious input from the prefrontal cortex [34, 39, 42, 45, 46]. The multifaceted neural circuitry of the urinary bladder enables conscious control over micturition. However, it also provides many opportunities for the induction or development of hypersensitivity or other sensory abnormalities. Understanding the neural pathways, both gross innervation and molecular and intracellular activity, is critical to determining pathophysiological mechanisms in order to develop effective treatment options.

1.3.1 Peripheral and spinal bladder neurophysiology

The lower urinary tract is innervated by afferent and efferent neurons from the pelvic (sacral parasympathetic), hypogastric (thoracolumbar sympathetic), and pudendal (sacral somatic) nerves [2, 3, 5, 33]. The bladder wall is innervated by both pelvic and hypogastric nerves, while the hypogastric nerve also innervates the IUS and the pudendal nerve innervates the EUS (**Figure 1.2**) [2, 3, 5, 33, 34, 47].

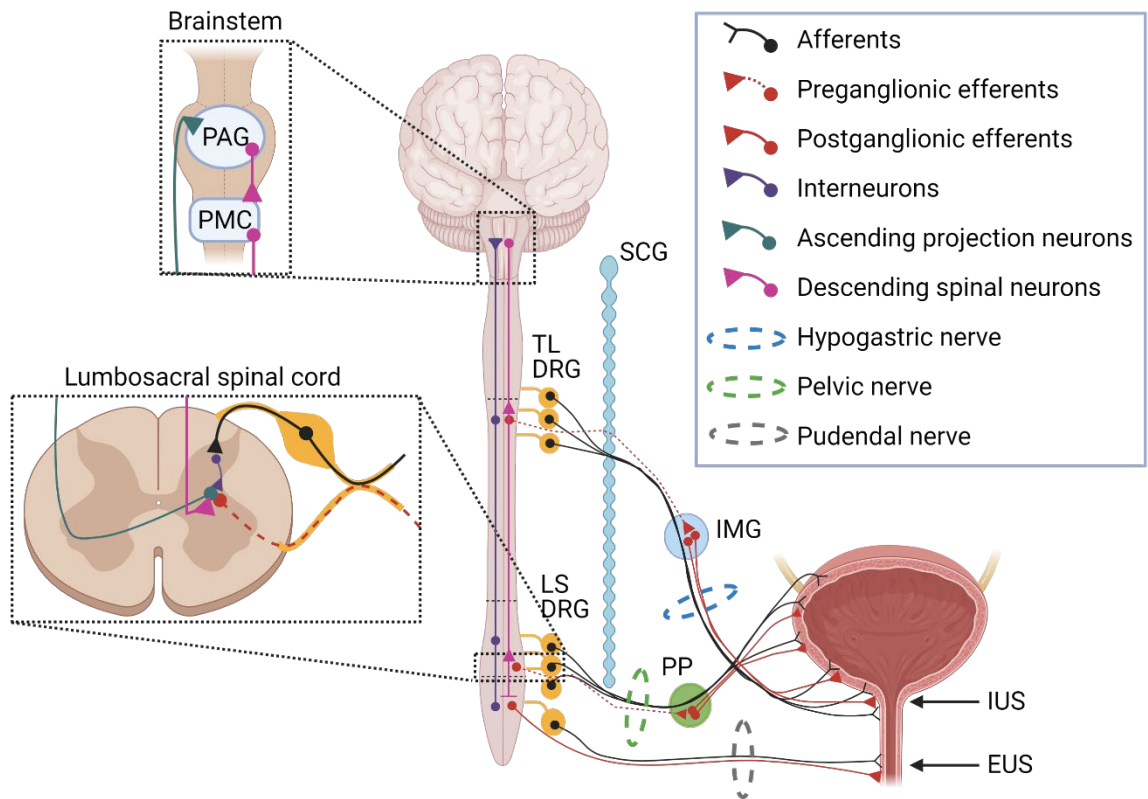


Figure 1.2: Afferent and efferent innervation of the lower urinary tract

The lower urinary tract is innervated by afferent and efferent neurons from the pelvic, hypogastric, and pudendal nerves. Cell bodies from afferent neurons sit within the dorsal root ganglia (DRG). Hypogastric afferent neurons innervate the bladder wall and the internal urethral sphincter (IUS) and enter the thoracolumbar (TL) spinal cord via the TL DRG (T11-L2). Preganglionic efferent neurons from the hypogastric nerve traverse from the TL spinal cord via the paravertebral sympathetic chain ganglia (SCG) to the inferior mesenteric ganglia (IMG), where they synapse with postganglionic sympathetic efferent neurons terminating in the bladder wall and the IUS. Pelvic afferent neurons innervate the bladder wall and enter the lumbosacral (LS) spinal cord via the LS DRG (L5-S1). Preganglionic efferent neurons from the pelvic nerve traverse from the LS spinal cord to the pelvic plexus (PP) where they synapse with postganglionic parasympathetic efferent neurons terminating in the bladder wall. The external urinary sphincter (EUS) is innervated by the pudendal nerve. Pudendal afferent neurons enter the sacral spinal cord via sacral DRG (S2-S4). Efferent neurons from the pudendal nerve are somatic, terminating directly from the sacral spinal cord onto the EUS. Lower box: Transverse section of the lumbosacral spinal cord. Afferent neurons from the DRG enter the dorsal horn of the spinal cord and synapse with interneurons. Interneurons synapse locally onto efferent neurons within the intermediolateral nucleus, which exit via the ventral horn, or ascending projection neurons, which project up to the brainstem. Upper box: Coronal view of the brainstem. Ascending projection neurons from the spinal cord terminate within the periaqueductal grey (PAG). Once the micturition reflex is activated, descending neurons from the PAG activate the pontine micturition centre (PMC), which in turn projects directly onto efferent neurons within the spinal cord to trigger voiding. *Figure created with BioRender.com*

1.3.1.1 Peripheral afferent innervation

The afferent neurons innervating the bladder represent the beginning of the neural pathway for bladder control, detecting stimuli from the periphery and projecting information to the spinal cord. Afferent neurons from the hypogastric nerve project from the base of the bladder and the IUS to the thoracolumbar (T11-L2) spinal cord segments, while the pudendal nerve innervates the EUS via sacral (S2-S4) spinal cord segments [3, 5, 33, 34, 48]. The bladder wall is innervated by sensory afferent neurons primarily from the pelvic nerve, transmitting signals from projections throughout the bladder wall to the lumbosacral (L5, L6 and S1) segments of the spinal cord [2, 33, 48, 49]. The cell bodies for bladder-innervating afferent neurons sit within the dorsal root ganglia (DRG), located next to the spinal cord [3, 49-52]. Retrograde labelling studies performed in mice and rats have shown both thoracolumbar and lumbosacral DRG innervation, with the majority of bladder-innervating DRG neurons located within L5-S1 [50-52].

Two major types of afferent fibres have been identified within the bladder. Studies utilising *ex vivo* and *in vivo* afferent recording preparations to determine conduction velocity demonstrated the presence of myelinated A- δ fibres and unmyelinated C fibres, but no A- β afferent fibres [53-55]. Early studies suggest that low-threshold myelinated A- δ fibres from the pelvic nerve act as in-series tension receptors and are responsible for sensations associated with micturition, whilst high threshold unmyelinated C fibres from the hypogastric nerve are responsible for sensations of pain [56-58]. Functional classification of sensory fibres has since proven to be more complex, with significant proportions of both high and low threshold afferents present in both A- δ and C fibre populations [53, 55, 59]. Further, both A- δ and C fibres include mechanosensitive and mechano-insensitive afferent populations, with no evident correlation between conduction velocity and activation threshold [53, 59]. In addition, high threshold, mechano-insensitive fibres can become sensitised to innocuous levels of distension following inflammation or insult [60, 61].

Several studies have attempted to differentiate bladder afferent fibres into subtypes based on receptive fields and sensitivity to multiple stimuli, identifying between them four key subtypes [62-64]. Serosal afferents responded to von Frey probing but not to stretch or mucosal stroking, while muscular mechanoreceptors responded to stretch and von Frey probing but not mucosal stroking. Muscular-mucosal mechanoreceptors are stretch-sensitive but also respond to mucosal stroking, while urothelial afferents were stretch-insensitive but responded to mucosal stroking. The lumbar splanchnic nerve, which includes the hypogastric nerve, consisted almost entirely of serosal and muscular afferents with a small proportion of muscular-mucosal [62], while the pelvic nerve included all four subtypes with stretch-sensitive muscular and muscular-mucosal afferents predominating [62-64]. These predominating, stretch-sensitive afferent subtypes were shown to act as in-series tension receptors, akin to the low-threshold pelvic afferent fibres previously associated with micturition

sensation [56]. These results therefore support the convention that pelvic afferents are primarily involved in conduction of physiological micturition signalling. However, these studies also demonstrated both high and low threshold stretch-sensitive afferents, again highlighting the need to identify mechanisms of neuronal sensitivity within the bladder.

The array of receptors and ion channels expressed on bladder afferents is key to determining neuronal excitability and activation. This in turn drives the afferent signalling input to the central nervous system and modulates bladder sensation and efferent output (bladder activity) [65]. The complete repertoire of receptors and ion channels expressed and functional on bladder afferents is unknown, however studies to date have identified roles for ion channels including voltage-gated sodium (Na_v) channels [66-72], transient receptor potential (TRP) channels TRP vanilloid 1 (TRPV1) [73-76] and TRP ankyrin 1 (TRPA1) [60, 76-79]. G-protein coupled receptors such as purinoceptors [80-84], serotonin receptors [85], and receptors for nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) [86-89] are also stipulated to impact afferent sensitivity. Indeed, sensitivity to ATP and TRPV1 agonist capsaicin in particular have been used as a means of differentiating afferent fibre subtypes, with sensitive afferents considered chemoreceptors [59, 64, 90]. Further highlighting receptor importance, activation of afferent-expressed receptors via inflammatory mediators results in increased neuronal hypersensitivity [62, 84, 85, 91, 92], while the absence or blocking of ion channel TRPV1 reduces bladder afferent sensitivity [74, 75]. Current literature exploring the role for receptors and ion channels in mediating bladder hypersensitivity is discussed further in **Chapter 2**, while putative roles for novel irritant-sensing receptors is explored in greater detail in **Chapter 3**.

1.3.1.2 Urothelial signalling

Whilst the barrier function of the urothelium is critical to healthy bladder function, there is now significant evidence demonstrating that the urothelium is also an important substructure in mediating sensory signalling, with bi-directional communication between urothelial cells and primary afferent terminals in the bladder wall [25, 93-95]. Urothelial cells express an array of 'sensory' receptors and ion channels enabling detection of mechanical and chemical stimuli, including distension-induced stretch and agonists present in the urine pertaining to changes in the external environment, as well as neurotransmitters and neuromodulators released from nearby nerve terminals [25, 94, 96-98]. While our understanding of the full spectrum of receptors and ion channels expressed in the urothelium is incomplete, studies have identified receptors for signalling molecules and inflammatory mediators, such as acetylcholine, ATP, transforming growth factor (TGF)- β , bradykinin, and prostaglandin E₂ (PGE₂), in the urothelium [99-103]. TRPV1, TRPV4, and TRPA1 have also been observed, however, results demonstrating expression of TRPV1 and TRPA1 in the urothelium have been inconsistent between groups [33, 78, 101, 104-110].

On activation, urothelial cells release a range of inhibitory and excitatory neurochemicals including ATP, nitric oxide (NO), acetylcholine, substance P, prostaglandins, NGF, and neurokinin A [2, 25, 97, 107, 108, 111, 112]. The localised release of signalling molecules from the urothelium allows interaction with and influence of neighbouring urothelial cells and, importantly, nearby afferent nerve terminals, thus mediating afferent signalling from the bladder and hence regulating micturition and bladder sensation [25, 93, 94, 97, 113].

1.3.1.3 Efferent innervation

The bladder wall also contains the terminals of efferent nerve fibres, responsible for inhibiting or stimulating the lower urinary tract musculature to facilitate storage and voiding, respectively. The efferent nerve fibres from the pudendal nerve extend directly from the sacral (S2-S4) spinal cord, whereas efferent pelvic and hypogastric nerve fibres innervate the bladder via peripheral ganglia, including the pelvic plexus, inferior mesenteric ganglia, paravertebral sympathetic chain ganglia, and intramural ganglia [2, 3, 5, 33, 114, 115]. Preganglionic efferent neurons originate from the thoracolumbar (hypogastric) or lumbosacral (pelvic) spinal cord regions and have been demonstrated to synapse with multiple postganglionic, tissue-innervating neurons within the peripheral ganglia, providing a level of divergence and amplification of signals [2, 33, 115, 116]. This ensures that the 'switch' between storage and voiding is maximal, allowing greater storage and complete bladder emptying [33].

To facilitate storage, sympathetic (hypogastric) bladder efferent neurons provide excitatory input to the urethra and IUS via α -adrenergic receptors to maintain continence, as well as inhibitory input to the detrusor muscle via β -adrenergic receptors to prevent contraction [2, 33, 117-121]. Further, somatic release of acetylcholine via the pudendal nerve triggers contraction of the EUS via nicotinic receptor activation [122-125]. To initiate voiding, activation of parasympathetic (pelvic) bladder efferent neurons results in the release of excitatory neurotransmitters acetylcholine and ATP within the bladder wall, stimulating contraction of the detrusor muscle [2, 3, 33, 126-130]. Simultaneously, sympathetic activity is suppressed, and somatic efferent release of NO relaxes the EUS [123, 125, 131].

1.3.1.4 Spinal innervation

At the level of the spinal cord, bladder-innervating afferent pelvic nerve fibres from the DRG enter the lumbosacral dorsal horn via Lissauer's tract at the apex of the dorsal horn into Lamina I [49, 132]. Within the dorsal horn, bladder afferent terminals are found in the superficial dorsal horn (SDH; laminae I and II), dorsal grey commissure (DGC), and the sacral parasympathetic nuclei (SPN) in the sacral regions [33, 49, 132-135]. The SDH is important in integrating sensory input from the periphery; with a significant number of interneurons and projection neurons, the SDH plays key roles in nociception, itch, and visceral sensation [136-138]. The DGC is also known to be important in

mediating pelvic visceral sensation and projecting sensory information to the brainstem [139]. The SPN, specific to the sacral level of the dorsal horn, contains both afferent and efferent projections innervating the bladder and other visceral organs [132, 140, 141] and as such, is important in pelvic sensation and autonomic function [132].

Viral tracing studies, which allow for detection of second order neurons, have provided insight into the spinal interneurons involved in bladder afferent signalling mechanisms. Interneurons in the lumbosacral dorsal horn relating to the bladder have been identified in the DGC, the SDH, and the SPN, similar to afferent innervation studies [142-144]. The signalling roles of these interneurons include short projection neurons connecting to spinal efferent neurons (creating a spinal reflex) and long projections leading to supraspinal centres, including the periaqueductal grey (PAG), thalamus, and pontine micturition centre (PMC) [35, 54, 145-147].

Preganglionic efferent neurons are located within the intermediolateral grey matter (laminae V-VII) of the lumbosacral dorsal horn and laminae V-VII and X of the thoracolumbar dorsal horn [5, 123, 142, 148]. Sacral somatic nerve fibres originate from lamina IX of the anterior horn in S2-S4, specifically from Onuf's nucleus [33, 149, 150]. Dendrites from pelvic preganglionic neurons are present in the SDH, DGC, and SPN, in proximity to afferent and interneurons involved in bladder sensation [132, 140, 148]. This proximity enables spinal reflexes, which are an important component of continence and micturition.

1.3.2 Continence and micturition reflexes

The storage and voiding of urine are facilitated by several signalling reflexes (**Figure 1.3**). A sacral-lumbar intersegmental reflex arc is a key mechanism for facilitating continence during the storage phase [17, 54]. Pelvic afferent signalling as the bladder fills activates interneurons within the thoracolumbar and lumbosacral spinal cord [54, 145, 151]. These, in turn, inhibit parasympathetic preganglionic neurons and activate inhibitory postganglionic efferent neurons via preganglionic sympathetic and sacral somatic efferents, inhibiting contraction of the detrusor muscle and stimulating contraction of the EUS [3, 17, 54, 152-155]. Pudendal afferent firing in response to tonic firing of EUS-innervating efferents during storage is also said to trigger spinal reflex inhibition of micturition [124].

The primary reflex involved in regulating micturition is a spinobulbospinal reflex, incorporating integral supraspinal components [35, 132, 133, 141, 154]. Afferent signalling from bladder filling is projected via ascending pathways to the PAG in the midbrain [5, 133, 156-158], which may be considered the 'control centre' for the biphasic micturition 'switch' [44]. The PAG, in turn, projects to

the PMC within the pons [159, 160], which has been shown to project directly onto parasympathetic preganglionic neurons within the intermediolateral cell groups in the lumbosacral spinal cord [161, 162]. The PMC is inactive during the storage phase, while afferent input to the PAG increases as the bladder fills [33, 44, 156]. Once the micturition threshold is reached, the 'switch' to voiding is triggered via excitatory signalling to the PMC by the PAG [163], activating parasympathetic preganglionic neurons and inhibiting sympathetic and somatic efferent pathways (including the spinal continence reflex [124, 153]) in the spinal cord, ultimately resulting in relaxation of the EUS, opening of the IUS, and contraction of the detrusor muscle [124, 133, 141, 162, 164, 165]. This spinobulbospinal micturition reflex drives bladder emptying in infants. In adults, cortical circuits are a necessary component to the 'social safety' of micturition.

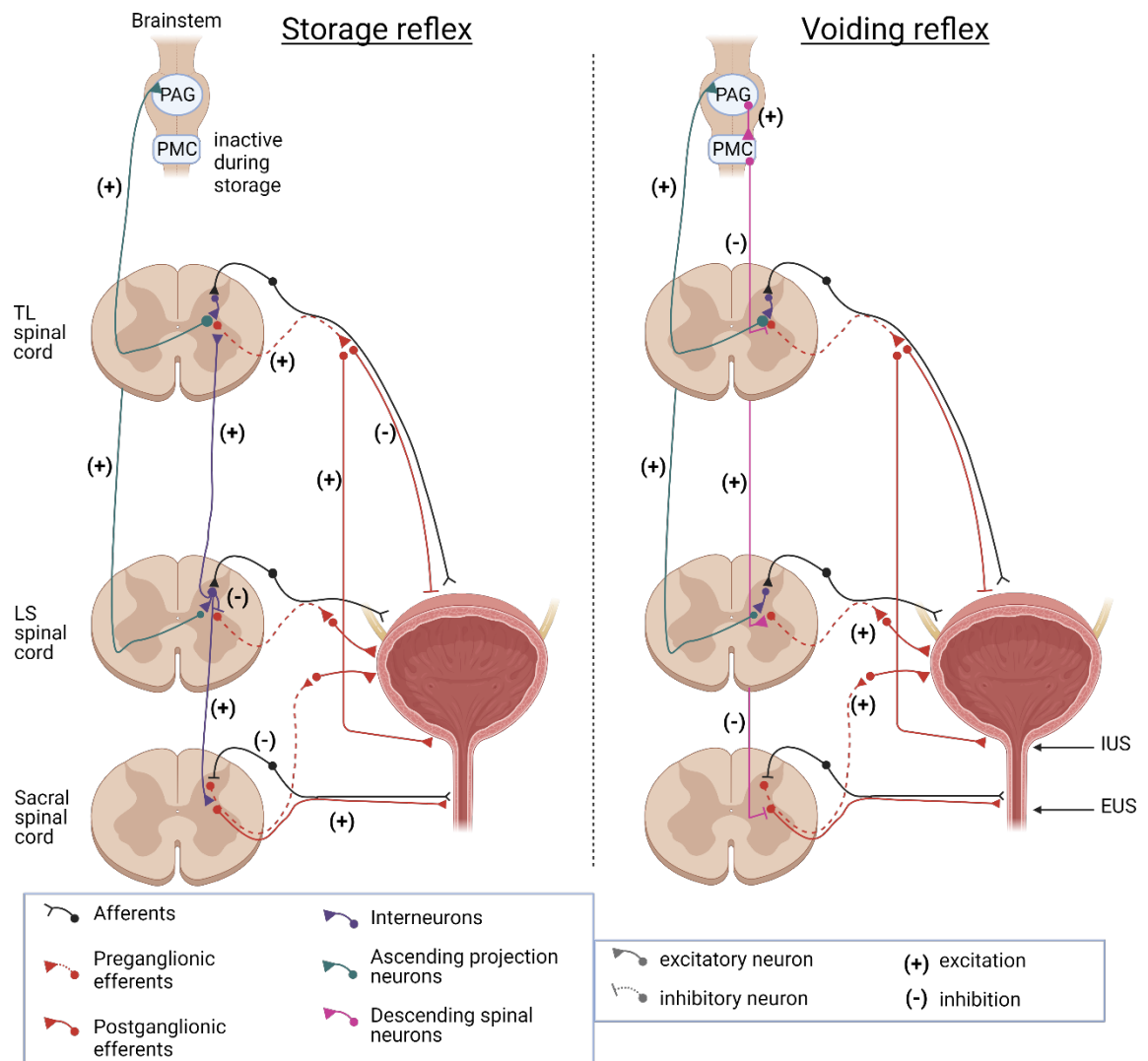


Figure 1.3: Spinal and Supraspinal micturition reflexes

Storage reflex: A sacral-lumbar reflex arc mediates urinary storage. As the bladder fills, pelvic afferent signalling excites thoracolumbar (TL) and lumbosacral (LS) interneurons. These inhibit parasympathetic (pelvic; LS) preganglionic efferent neurons and activate inhibitory postganglionic efferent fibres via preganglionic sympathetic (hypogastric; TL) efferents and somatic (pudendal; sacral) efferents. Tonic firing of pudendal (sacral) efferents innervating the external urethral sphincter (EUS) is also said to inhibit parasympathetic efferent activity via pudendal afferent firing. These reflexive mechanisms result in detrusor relaxation and sphincter contraction, mediating urinary storage.

Voiding reflex: Urinary voiding is primarily mediated by a spinobulbospinal reflex. When bladder afferent signalling to the periaqueductal grey (PAG) region of the midbrain during bladder filling reaches the micturition threshold, a reflexive 'switch' from storage to voiding is triggered. The PAG activates the pontine micturition centre (PMC) in the pons, which in turn activates parasympathetic preganglionic efferents (pelvic; LS) and inhibits sympathetic (hypogastric; TL) and somatic (pudendal; sacral) efferent pathways in the spinal cord. This reflex enables detrusor contraction and relaxation of the internal urethral sphincter (IUS) and EUS, facilitating urinary voiding. *Figure created with BioRender.com*

1.3.3 Cortical micturition control

Voluntary control over voiding is a critical component of urinary bladder function; cortical inhibition and initiation of the micturition reflex ensures voiding occurs in a safe and socially appropriate moment. The PAG is considered the primary 'relay station' in micturition, projecting not only to the PMC but also into a number of higher processing regions, identified through functional imaging and labelling studies. These regions include the thalamus, and areas within the limbic system and the cerebral cortex [45, 133, 156, 164, 166-169].

The thalamus acts as a relay station for sensory afferent information into the cortex [170], and is activated by a full bladder [171]. Within the cerebral cortex, the insula acts as the homeostatic afferent cortex, registering visceral sensations and regulating autonomic functions including bladder activity [172]. Insula activity increases with bladder filling, creating a desire to void [33, 42, 45, 167, 173]. The prefrontal cortex is involved in decision-making, particularly within a social and emotional context, and is interconnected with the limbic system as well as the insula and anterior cingulate cortex (ACC) [174, 175]. Prefrontal cortex activation is strongly associated with continence, with only weak activation demonstrated in patients with poor bladder control, and is thus suggested, together with the limbic system, to play a role in suppressing the 'switch' from storage to micturition until a socially acceptable moment [173, 176].

Key to limbic modulation of bladder function, the ACC integrates limbic and cortical input [177]. In particular, the ACC triggers emotional responses to interoception in order to elicit a physiological response. This is likely to play a more prominent role during urgency, escalating the sensation of bladder fullness [178]. Supporting this, the supplemental motor area (SMA), which is usually coactivated with the dorsal ACC, is activated during contraction of the pelvic floor and EUS, or active continence [168]. The integration of higher order emotional and cognitive processing in response to bladder sensory input results in sensations of fullness and urgency and, fundamentally, provides inhibitory input to the PAG to prevent the 'switch' from storage to micturition until a socially appropriate opportunity.

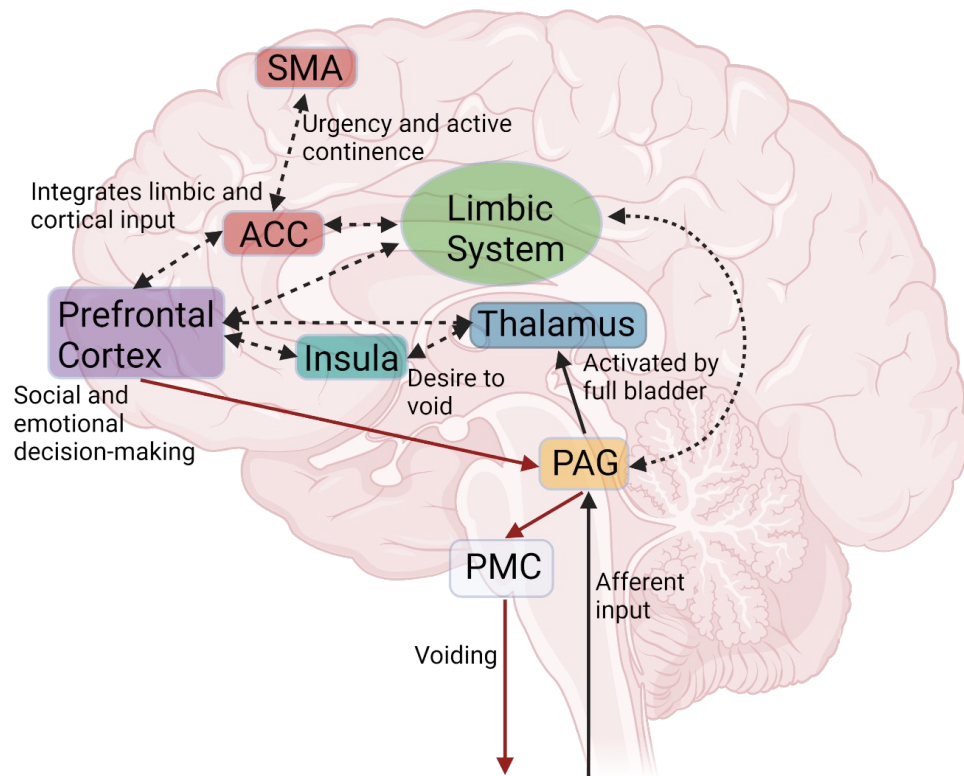


Figure 1.4: Cortical influences over the micturition reflex

Cortical control of the micturition reflex, mediated by complex integration of the thalamus, the limbic system, and the cerebral cortex, enables safe and socially acceptable voiding. The thalamus, acting as a relay centre, is activated by a full bladder by the periaqueductal grey (PAG). Insula activity in the cerebral cortex increases with bladder filling and is responsible for registering visceral sensations and the desire to void, interacting with the thalamus and the prefrontal cortex. The prefrontal cortex, responsible for social and emotional decision-making, is additionally interconnected with the thalamus, the limbic system, and the anterior cingulate cortex (ACC). The limbic system is involved in behavioural and emotional responses and, together with the prefrontal cortex, is important in suppressing the switch from storage to voiding. Limbic and cortical input is integrated by the ACC, triggering emotional responses to sensation to elicit and physiological response. The supplemental motor area (SMA) is usually coactivated with the dorsal ACC and is activated during active continence. *Figure created with BioRender.com*

1.4 Conclusion

The innervation and neurophysiology of bladder function is complex, involving peripheral, spinal, and cortical levels of control. The activity and sensitivity of peripheral sensory afferent neurons, as the start of the neural signalling pathway, is integral to both reflexive and conscious function. As such, this thesis focuses on elucidating novel receptors involved in modulating bladder afferent neuronal sensitivity. Changes to peripheral and central nervous system activity in patients with conditions of bladder hypersensitivity have been explored and are discussed in the next chapter.

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Statement of Authorship

Title of Paper	Mechanisms Underlying Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Grundy, L., A. Caldwell, and S.M. Brierley, <i>Mechanisms Underlying Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome</i> . Front Neurosci, 2018. 12 : p. 931.

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Overall percentage (%)	35%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- the candidate's stated contribution to the publication is accurate (as detailed above);
- permission is granted for the candidate to include the publication in the thesis; and
- the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 2 Mechanisms Underlying Overactive Bladder and Interstitial Cystitis/Painful Bladder Syndrome

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2.1 Abstract

The bladder is innervated by extrinsic afferents that project into the dorsal horn of the spinal cord, providing sensory input to the micturition centres within the central nervous system. Under normal conditions, the continuous activation of these neurons during bladder distension goes mostly unnoticed. However, for patients with chronic urological disorders such as overactive bladder syndrome (OAB) and interstitial cystitis/painful bladder syndrome (IC/PBS), exaggerated bladder sensation and altered bladder function are common debilitating symptoms. Whilst considered to be separate pathological entities, there is now significant clinical and pre-clinical evidence that both OAB and IC/PBS are related to structural, synaptic, or intrinsic changes in the complex signalling pathways that mediate bladder sensation. This review discusses how urothelial dysfunction, bladder permeability, inflammation, and cross-organ sensitisation between visceral organs can regulate this neuroplasticity. Furthermore, we discuss how the emotional affective component of pain processing, involving dysregulation of the HPA axis and maladaptation to stress, anxiety and depression, can exacerbate aberrant bladder sensation and urological dysfunction. This review reveals the complex nature of urological disorders, highlighting numerous interconnected mechanisms in their pathogenesis. To find appropriate therapeutic treatments for these disorders, it is first essential to understand the mechanisms responsible, incorporating research from every level of the sensory pathway, from bladder to brain.

2.2 Introduction

Overactive bladder syndrome (OAB) and interstitial cystitis/painful bladder syndrome (IC/PBS) are common, chronic, pelvic disorders affecting approximately ~16% of the western population [1-4]. Urgency, frequency, and nocturia are common symptoms of both OAB and IC. However, these

conditions may be differentiated by the presence of urge urinary incontinence in patients with OAB and pelvic pain in IC patients [5-8]. As both of these disorders are diagnosed in the absence of bacterial infection or obvious pathology, the aetiology of OAB and IC/PBS symptoms remain unknown. Accordingly, efficacious therapeutic options are limited, contributing to the significant societal and economic impact of greater than \$70 billion per annum in the United States [9, 10].

Normal bladder function requires coordination of afferent signals originating from the bladder wall with excitatory and inhibitory signals from the anterior cingulate cortex (ACC), insula, and hypothalamus to provide an overview of the appropriateness to urinate that is ultimately under conscious control by the prefrontal cortex [11, 12] (**Figure 2.1**). Bladder afferents embedded within the detrusor smooth muscle show exquisite sensitivity for mechanical distension but are also found innervating the urothelium [13-16]. This topology provides a secondary level of resolution to the transmission of sensory stimuli, including the detection of bladder infection, urothelial inflammation, or barrier breakdown (**Figure 2.1**). Accordingly, bladder sensory afferents express a range of anti- and pronociceptive receptors and ion channels [17, 18] that integrate the input from this complex signalling environment and can induce a range of sensations from fullness through to pain [19]. These afferents, whose cell bodies are located within the dorsal root ganglia (DRG), project via the pelvic, hypogastric/splanchnic nerves, synapse within the dorsal horn of the lumbosacral (LS, L5-S1) and thoracolumbar (TL, T10-L2) spinal cord [19, 20] (**Figure 2.1**), and terminate within the periaqueductal gray (PAG) [19]. The PAG acts as an integration centre for afferent signals from the spinal cord and higher brain centres [11]. A conscious “urge” to urinate is perceived when afferent activity increases beyond a pre-set threshold and, if modulating input from the brain permits, the PAG activates the pontine micturition centre (PMC) to induce efficient voiding [11, 12, 19, 21].

Clinical studies reveal that patients with OAB and IC/PBS perceive sensations of bladder fullness, urge to void, and pain at lower cystometric volumes than healthy subjects [22-24]. These observations thereby implicate the sensitisation of bladder afferent pathways to physiological stimuli as a key component in the mechanisms underlying these disorders [20, 25, 26]. Furthermore, co-morbidity of depression and anxiety is significantly higher in patients with OAB and IC/PBS, while high psychological stress levels is strongly correlated to exacerbated bladder symptoms [27-31]. As such, increased intensity of the afferent signal or modulation of the emotional affective state can have profound effects on bladder sensation. A key concept therefore, in the development of OAB and IC/PBS, is chronic sensitisation of the neuronal networks regulating bladder sensation, incorporating structural, synaptic, or intrinsic changes of peripheral or central structures that may drive subsequent changes in downstream sensory pathways [18, 32].

In this review, for brevity, we summarise both preclinical and clinical research to highlight how alterations in peripheral afferent excitability contribute to the symptoms of OAB and IC/PBS to provide insights into the mechanisms that are hypothesised to mediate these distinct disorders which have many overlapping symptoms. For an excellent review on the potential changes in central processes occurring in OAB see Reynolds et al. (2016) [33].

2.3 Urothelial Permeability

Toxic waste metabolites excreted in urine are prevented from accessing the bladder interstitium and embedded afferent endings by a tight urothelial barrier [16] (**Figure 2.1**). The urothelial barrier is maintained by tight junctions between apical urothelial cells, hydrophobic uroplakin plaques, and a considerable glycosaminoglycan (GAG) mucus layer, that block the movement of small molecules and urine [34, 35]. From a clinical point of view, numerous studies have identified that patients with IC/PBS, but not OAB, have a diminished or damaged urothelium [35-39], as well as reduced expression of the tight junction proteins zona occludens 1 (ZO-1) and E-cadherin [40]. Increased bladder permeability due to reduced urothelial integrity is thought to underlie the sensitivity of IC/PBS patients (75%) to the potassium sensitivity test (PST) compared to control patients (4%) [41]. A number of clinical studies also show that OAB patients respond to the PST, however, this may be due to overlap and misdiagnosis between IC/PBS and OAB [5, 42, 43]. Despite these observations, it remains to be determined whether bladder permeability is part of the underlying pathology of bladder hypersensitivity in IC/PBS patients or a downstream consequence of localised inflammation that further exacerbates the condition.

Glycosaminoglycan replacement therapy with pentosane polysulphate (PPS) has been shown to improve the symptoms for some, but not all, IC/PBS patients [35, 38, 44, 45]. However, PPS also induces broad anti-inflammatory actions, including the inhibition of mast cell histamine release in the bladder [46-49]. In addition, pre-clinical studies inducing urothelial permeability with protamine sulphate identified a relatively rapid recovery of the urothelial barrier and structure [50, 51], via injury-induced proliferation of basal urothelial and stromal cells [52]. A more likely scenario therefore is that bladder permeability in IC/PBS patients is secondary to localised inflammation. Continuous access of toxic urine contents combined with inflammatory mediators, such as cytokines, histamine, and proteases, can sensitise peripheral afferent endings and have the potential to trigger long term changes in neuronal function and neuroplasticity within the entire afferent network [18, 32].

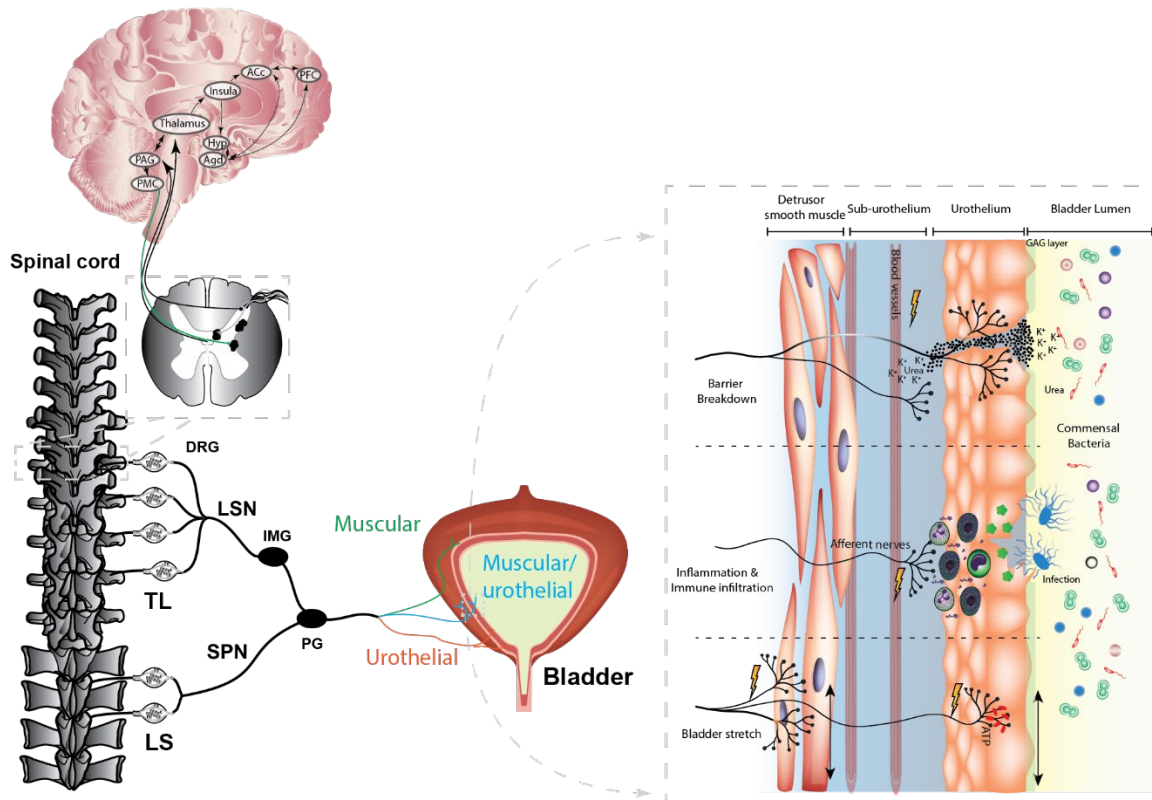


Figure 2.1: Spinal innervation of the bladder

The afferent nerves innervating the bladder wall extend into the detrusor smooth muscle to detect bladder stretch, and into the bladder urothelium to detect bladder stretch, infection, inflammation, and barrier breakdown. The bladder lumen contains numerous commensal bacteria as well as toxic contents such as urea and high potassium. Bacterial infection of the urothelium induces apoptosis of urothelial cells, the release of cytokines, and the infiltration and activation of the immune response, including mast cell degranulation and the subsequent release of histamine and cytokines that can sensitize bladder afferent neurons. During bladder stretch the urothelium releases an array of neurotransmitters, including ATP, which can activate bladder afferents. Breakdown of the urothelial barrier allows access of toxic urine contents into the underlying bladder interstitium which can activate and sensitize bladder afferents.

Bladder afferents project via the pelvic nerve or the splanchnic nerve to the dorsal horn of the thoracolumbar and lumbosacral spinal cord, where they activate second order neurons within the spinal cord synapse in the thalamus or the PAG of the midbrain. Thalamic projections provide input into limbic and cortical structures to provide the emotional affective and conscious component of the voiding reflex pathway. The thalamus relays to the PAG and the PAG feeds into the PMC to signal micturition. TL, Thoracolumbar; LS, lumbosacral; SC, spinal cord; PFC, Prefrontal Cortex; ACC, Anterior cingulate cortex; Hyp, hypothalamus; HGN, hypogastric nerve; PAG, periaqueductal gray; PMC, primary micturition center; DRG, dorsal root ganglion; DH, Dorsal horn; Agd, amygdala; LSN, lumbar splanchnic nerve; SPN, pelvic nerve; IMG, inferior mesenteric ganglion; PP, Pelvic Plexus. Figure modified from Grundy et al. (2019) [18].

2.4 Altered Release of Urothelial Factors

In addition to its role as a physical barrier, the urothelium provides bi-directional communication with underlying primary afferents [34, 53, 54] via the detection and/or release of a range of excitatory and inhibitory neurotransmitters and neuromodulators including ATP, acetylcholine, nitric oxide (NO), NGF, prostaglandin E₂ (PGE₂), neurokinin A, and inflammatory mediators as described above (and extensively reviewed by Birder et al.) [20, 34, 55-57]. Altered urothelial mediator release has been identified from OAB and IC/PBS patients in a number of studies and may be a compounding mechanism in the development of chronic neuronal hypersensitivity [58-61].

Chronic bladder inflammation enhances ATP release from the urothelium and augments purinergic signalling of bladder afferents in rats [62], whilst both stretch-mediated ATP release and its receptor P2X₃ are increased in the urothelium of OAB and IC/PBS patients [60, 63-65]. Preclinically, acetylcholine, acting via muscarinic receptors, also triggers release of ATP as well as NO and prostanoids from urothelial cells [66, 67]. In animal studies, experimentally induced cystitis upregulates muscarinic receptors and acetylcholine release, as well as subsequent cholinergic regulated urothelial NO release [68-71]. Notably, PGE₂ levels are significantly increased in OAB patients [58, 59], whilst NO and PGE₂ levels and receptor expression are also altered in IC/PBS patients with Hunner's lesions [72, 73]. Bradykinin stimulates urothelial NGF release and enhances stretch-induced ATP release in a human urothelial cell line [66, 74], whilst bradykinin 1 (B1) receptor expression is upregulated in human IC/PBS patient bladder samples [75] and in the urothelium of a rat CYP-induced cystitis model [76].

Limited causations have been determined for altered urothelial neurotransmitter release or receptor expression in OAB and IC/PBS, but there is accumulating evidence that they are a downstream consequence of inflammation, infection, or urothelial breakdown.

2.5 Inflammation

By definition, the presence of bladder inflammation precludes the clinical diagnosis of OAB. Only a small population of IC/PBS patients exhibit significant inflammation, which is characterised by the presence of Hunner's ulcers [77]. However, it is widely reported that there are increases in the amount of pro-inflammatory mediators within the bladder and urine of both OAB and IC/PBS patients [40, 65, 78-82]. These pro-inflammatory mediators, including histamine, nerve growth factor (NGF), and others released from mast cells, are known to directly sensitise afferent nerve terminals [83]. Furthermore, overexpression of pro-inflammatory genes, oedema, tissue granulation, and an increase in macrophages, chemokines, cytokines, eosinophils, as well as T and B cell markers have also been identified in IC/PBS patients [80, 84]. As the majority of cystitis-induced inflammation is localised to

the superficial mucosa, urothelial afferents are ideally placed to detect and respond to these environmental changes.

Inflammation-induced sensitisation of afferents is an essential mechanism for the induction of normal wound healing, however, chronic sensitisation of afferents can occur during prolonged inflammation or following a severe bout of inflammation [32, 85]. In support of an inflammatory-mediated pathophysiology in urological disorders, animal models of cystitis have employed a range of chemicals, including acetic acid, acrolein, cyclophosphamide (CYP), zymosan and lipopolysaccharide, that induce both acute and longer lasting bladder hyperactivity [86-89]. Whilst there are many limitations to the use of inflammatory animal models in the study of symptom defined disorders such as OAB and IC/PBS (comprehensively discussed by Fry et al., 2010 [90]), these animals show an overactive bladder phenotype, with altered cystometry and enhanced visceromotor response during bladder distension, replicating the reduced bladder capacity, plus the allodynia and hyperalgesia to bladder distension observed in humans [90-93]. Furthermore, bladder afferents show direct sensitisation to chemical and inflammatory stimuli [94], whilst retrogradely traced bladder-innervating DRG neurons from CYP-treated rats or a naturally occurring feline interstitial cystitis model exhibit lower activation thresholds and sensitisation to current injection [95, 96]. The efficacy of intraluminal therapies to treat OAB and IC/PBS [97, 98], that either (1) block bladder afferent firing (such as lidocaine and neosaxitoxin) or (2) cause peripheral nerve desensitisation (with agents such as resiniferatoxin; RTX), highlight the important role of peripheral afferents in mediating bladder hypersensitivity to distension [99].

The transient receptor potential (TRP) channel TRPV1 is upregulated in the bladders of patients with OAB and IC/PBS [100], whilst both TRPV1 and TRPA1 have consistently been implicated in mediating normal and cystitis-induced mechanical sensitivity in rodents by modulating neuronal activation thresholds and enhancing bladder afferent responses to P2X receptor activation [92, 93, 101-104]. A host of additional receptors and channels associated with nociception have also been identified upon bladder afferents that regulate neuronal sensitivity and neuronal excitability in animal models of cystitis, including voltage gated sodium (Na_v) channels [17, 105], potassium channels (K_v) [106], P2X receptors [95, 107], TRPV4 [108], and cannabinoid receptors [109-113].

It is possible that a population of patients present without active inflammation or increased bladder permeability but are in fact in remission from a preceding bladder infection or inflammation. Such a scenario could induce a protracted hypersensitive state and correspond to their enhanced sensory symptoms. Indeed, women with a clinical history of recurrent UTI as children are significantly more likely to have a diagnosis of IC/PBS as adults [114], and preclinical investigations of neonatal bladder insult in rats suggests this may be due to long term sensitisation of sensory pathways [115-

117]. Neuroplasticity of peripheral afferent circuitry following the resolution of inflammation or recovery from tissue injury has been well documented in both somatic and visceral pain models through the induction of neurogenic inflammation and neuronal sprouting [32, 94, 118]. Neonatal bladder inflammation in rats results in hypersensitive responses to inflammatory stimuli as an adult, inducing an overactive bladder phenotype [115, 116, 119], as well as enhanced spontaneous and urinary bladder distension-evoked activity of spinal visceral nociceptive neurons [117]. Alterations in spinal cord circuits responsible for bladder sensation may regulate this phenomenon, as neonatal inflammation induces a downregulation of GABA (A α -1) receptor microRNA and altered opioid peptide content in the dorsal horn [120, 121]. Furthermore, neonatal zymosan enhances bladder neuropeptide content of CGRP and Substance P compared to sham controls [119, 122].

Similarly, in pre-clinical models of adult cystitis, bladder overactivity is associated with increases in tyrosine receptor kinase (Trk) A, Trk B, and calcitonin gene-related peptide (CGRP) [123, 124], which in turn promote inflammation in the tissue where the afferent terminals reside [125]. Furthermore, patients with IC/PBS have higher elevated serum and urinary NGF levels than healthy controls [126]. NGF overexpression in mouse urothelium leads to neuronal hyper-innervation, increased mast cell counts and changes in bladder function [127]. These discoveries may explain the increased sprouting of neuronal terminals identified in the bladders of IC/PBS patients [128, 129], that has been replicated in rodent models of inflammation *in vivo* and *in vitro* [127, 130-132].

2.6 Microbiome/Urinary Tract Infection

Following the recent identification of a bladder-specific microbiome, and the role of the gut microbiome in chronic functional gastrointestinal diseases [133, 134], a link between the balance of bacteria in the bladder and the symptoms of OAB and IC/PBS has been postulated and explored [64, 135, 136]. Moreover, the traditional colony forming unit thresholds for confirming urinary tract infection (UTI) in clinical practice have been questioned, and a role for chronic UTI in the pathogenesis of OAB has been investigated [137].

Patients with OAB may have genuine uropathogenic infections, and are therefore misdiagnosed, as large numbers of bacteria are undetected by routine mid-stream urine cultures [138]. Indeed, a significantly greater number of patients with refractory idiopathic detrusor overactivity show low count bacteriuria vs. controls [139]. Undiagnosed intracellular bacterial colonization of urothelial cells may also occur in OAB [140], as OAB patients exhibit significantly greater infected urothelial cell counts and microscopic pyuria than healthy subjects, which also correlates to urgency symptoms [141]. Uropathic *E. coli* infection initiates the release of multiple mediators from the urothelium, including cytokines and interleukins, as well as promoting urothelial barrier defects [142], which alert

the immune system to impending damage and initiate an immune response [85]. Immune cell infiltration and the release of pro-inflammatory cytokines are known to sensitize peripheral afferents [143], and in this way enhance bladder sensation. In support of these considerations, a recent pilot study revealed that combination antibiotic treatment of both Gram-negative and Gram-positive bacteria significantly improved OAB symptoms as well as the perception of their bladder condition [144]. Furthermore, shifts in the bacterial species that constitute the bladder microbiome have been associated with both the presence and severity of OAB and IC/PBS [64, 145-148]. For example, women with IC/PBS, but not OAB, have a less diverse microbiota than those without [84, 148-150]. Interestingly, despite significant inter-patient variability in bladder microbiome, a decrease in *Lactobacillus*, which has antimicrobial properties, in both OAB and IC/PBS patients compared to controls is a common finding [148-150]. Furthermore, the absence of *Lactobacillus acidophilus* correlates with higher pain scores and higher scores on the interstitial cystitis symptom index [84]. *Proteus*, the urinary pathogen, is also identified more commonly in patients with OAB and lower urinary tract symptoms than healthy controls [138, 148]. These data support a line of communication between the urinary microenvironment and underlying afferent nerves that is likely mediated by the urothelium.

2.7 Cross-Organ Sensitisation

Considerable clinical evidence suggests that diseases of the colon, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), can induce subsequent development of pathology in an otherwise unaffected adjacent organ, such as the bladder [105].

A mouse model of colitis induced by intra-rectal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS) induces hyperexcitability of the entire peripheral sensory pathway, from the afferent ending in the colon to the spinal cord [32]. Importantly, TNBS colitis also prompts consistent changes in bladder voiding parameters that replicate the clinical symptoms of urgency and frequency, as well as increased bladder-afferent sensitivity to bladder distention [51, 151-153]. These symptoms occur in the absence of any overt inflammation or histological damage to the bladder, highlighting the importance of altered afferent sensitivity in maintaining OAB and IC/PBS symptomology [153].

This “cross-organ sensitisation” is considered to originate within the physiological co-ordination of these pelvic organs, and persistent pathological plasticity of their shared sensory pathways within the thoracolumbar and lumbosacral DRG and spinal cord [105, 154]. Approximately 15% of colonic innervating TL and LS DRG neurons exhibit dichotomising afferents, simultaneously innervating the bladder [153, 155], whilst a similar proportion of spinal dorsal horn neurons also respond to both urinary bladder and colonic distension [105]. As such, sensitisation of colonic afferent pathways has

the potential to directly influence the excitability of bladder afferent pathways. Indeed, a very recent pre-clinical study also indicates that chronic sensitisation of colonic afferent pathways results in the subsequent sensitisation of bladder afferent pathways and the triggering of uncontrolled urinary voiding in mice. Intriguingly, these changes in bladder function can be reversed by a therapeutic treatment targeted only to the colon [156].

Additionally, neurochemical changes occur within colonic afferent pathways following colitis that indicate the development of neurogenic inflammation [105]. Multiple studies have shown that this translates to persistent upregulation of the neuromodulators NGF, BDNF, CGRP, and the high affinity receptor TrkB in bladder, bladder-innervating DRG neurons, and spinal cord [157-161]. These receptors and neuropeptides share an intimate relationship enabling the positive feedback of each other [153, 155, 162, 163], consequently inducing neuronal sensitisation and neurite outgrowth, and likely contributing to cross-organ sensitisation through paracrine actions within the ganglia to increase bladder afferent excitability [160, 164].

2.8 Cortical Regulation: Stress, Anxiety, and Depression

The sensory signals from bladder afferents converge in the PAG where they are modulated by input from the limbic system (amygdala, hypothalamus, thalamus, cingulate gyrus), insula, and prefrontal cortex [19], which can in turn modulate or be modulated by the hypothalamic pituitary adrenal (HPA) axis. As such, changes to cortical networks or modulation of the emotional affective state can have profound effects on bladder sensation and may be an underlying mechanism in the development and persistence of OAB and IC/PBS symptoms.

Patients with OAB and IC/PBS report psychological stress levels that are significantly higher than healthy controls [28], which may be a consequence of HPA axis dysregulation following chronic early life stress (ELS) [165]. Exposure of children to ELS is a significant risk factor for developing HPA abnormalities [9, 166], and shows strong correlations with the development of depression and anxiety in later life [167, 168]. To this end, a number of studies report higher incidences of ELS and trauma in IC/PBS patients than healthy controls [169], whilst clinical studies have demonstrated a strong correlation between stress, anxiety, depression, and the symptoms of bladder overactivity in patients with OAB and IC/PBS [27, 29, 30]. The increased prevalence of depression and anxiety in patients with IC/PBS occurs both following and prior to bladder symptoms, indicating a reciprocity in cause and effect, with no clear way to delineate patient cohorts to provide increased mechanistic understanding [31]. In addition to relationships with cognitive disorders, structural abnormalities within the white matter of the brain in women with IC/PBS, which facilitates the communication between and within brain regions, correlates closely to symptom severity [170]. It remains unclear whether these white

matter properties are causes or consequences of IC/PBS. It is possible that certain white matter architecture may reflect a predisposition to develop disease, but it is equally plausible that these changes are a consequence of IC/PBS disease progression and future longitudinal studies are required to test this hypothesis.

The link between stress and bladder disorders is supported by pre-clinical studies in rodents that consistently induce bladder overactivity or mechanical hyperalgesia following stress treatments [171-175]. In addition, this hypersensitivity has been found to be dependent on [173], or correlate with, significant changes in brain regions associated with emotional processing and bladder control [175]. Neonatal maternal separation in female mice, as a model of ELS, enhances visceromotor responses to urinary bladder distension accompanied by altered hippocampal input onto the HPA axis [176]. If sensitisation of peripheral afferent endings occurs in these models as a consequence of stress, or if sensitisation is arbitrated solely within the CNS has yet to be fully determined.

2.9 Conclusion

This review highlights the complex nature of both OAB and IC/PBS, incorporating evidence for changes in the dynamic signalling environment between the bladder lumen, urothelium, and afferent nerves, coordinating with adaptations to the HPA axis, and emotional affective components of sensory processing mediated within the limbic system. The bladder microbiome, bacterial infection, inflammation, and urothelial permeability contribute to the development of peripheral afferent hyperexcitability that is fundamental to the development of frequency and urgency in OAB, and pain in IC/PBS. In addition, the higher psychological stress levels, increased prevalence of anxiety and depression, as well as clinical co-morbidities with other visceral pain disorders suggests pathological plasticity within the CNS is an important component in the mechanisms underlying both OAB and IC/PBS. Determining the underlying mechanisms of bladder hypersensitivity is paramount to providing novel targets for the development of safer and more efficacious treatments.

2.10 References

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Chapter 3 Irritant sensation: itch signalling mechanisms

Irritant sensation is a powerful protective mechanism, providing an awareness of potential hazards and an innate, physiological reaction to prevent or mitigate harm. The best recognised protective mechanism is nociception or pain, which initiates removal of or withdrawal from a hazardous or painful stimulus followed by protection of an injury, allowing time for healing. Like pain, itch is a protective sensory mechanism, inducing protective scratching that removes potentially harmful irritants from the skin. Defined as “an unpleasant sensation that evokes a desire to scratch” [1], itch was initially attributed to subthreshold nociceptive signalling [2-4]. Although the neural pathways are similar, itch is now recognised as a distinct signalling mechanism dependent largely on specific receptors and channels expressed in primary sensory neurons that are activated by irritant stimuli [5-7].

Unlike pain, the sensation of itch (and hence itch-signalling mechanisms) has traditionally been considered exclusive to the skin, eyes, and nose; certainly the association of itch with the ‘scratch’ response as a means of irritant removal in the skin is not a feasible internal action. Despite this established dogma, recent studies have identified the presence of itch-sensing mechanisms in the respiratory and gastrointestinal tracts, where they mediate important physiological responses to irritants [8, 9]. These discoveries suggest that ‘itch’ and ‘scratch’ are merely the superficial consequences of a more ubiquitous sensory function for identifying and removing irritants. Aberrant irritant-sensing mechanisms are known to contribute to chronic hypersensitivity conditions in the skin, and now also the gut. Understanding the roles for these mechanisms in the bladder represents an untapped potential treatment avenue for conditions of chronic pelvic hypersensitivity.

3.1 Itch signalling mechanisms

Itch sensation is initiated by activation of primary sensory afferents terminating as free nerve endings in the epidermis of the skin [10, 11]. Activation of these afferents occurs via the interaction of itch-mediating stimuli (pruritogens) with receptors at the nerve terminals.

As with all afferent neurons, including those of the bladder, the cell bodies of ‘pruritogenic’ (itch-mediating) afferents are located within the DRG, as well as the nodose and trigeminal ganglia, from whence they project into the dorsal horn or brain stem respectively [10, 12-14]. Pruritogenic afferent neurons have been identified as predominantly unmyelinated C-fibres [15, 16], however roles for myelinated C-fibres and A δ -fibres in mediating itch sensation have also been identified [17-20]. While C-fibres and A δ -fibres also innervate the bladder, it is important to note that current evidence indicates a greater variety of neuron fibre subtypes innervating the skin compared to the viscera [21].

From the periphery, primary afferent neurons that signal itch have been shown to project into the superficial dorsal horn (SDH; lamina I and II) of the spinal cord [10, 12-14]. The SDH plays a key role in sensory signal projection as well as signal modulation via interneuron activity, mediating and projecting signals from the skin and the viscera alike [22-26]. Investigations into transmission of itch sensation from the spinal cord to the brain have primarily focused on the involvement of the spinothalamic tract [27-31], however recent studies have also identified an important role for projection neurons in the parabrachial tract [32-35]. Higher order regions of the brain into which these neurons project include the thalamus, somatosensory cortices, the periaqueductal gray (PAG), and the amygdala [36-41].

3.1.1 Itch versus pain: sensory differentiation

There is much debate over how the nervous system distinguishes between itch and pain sensation. The proposed 'labelled line' mechanism predicts the presence of distinct neuronal populations for itch and for pain, such that the activation of specific neurons in the periphery results in a given sensation independent of the nature of the stimulus (**Figure 3.1A**). Supporting this, application of typically nociceptive stimuli such as the TRPV1 agonist capsaicin to histaminergic 'itch' neurons can elicit a scratch response instead of a pain response, suggesting the neuronal subtype is 'programmed' to detect itch [11]. Despite this, pruritogenic afferent neurons appear to be polymodal, responding to nociceptive or thermal stimuli in addition to itch [42, 43], supporting the alternative 'pattern recognition' signalling mechanism (**Figure 3.1B**). This mechanism proposes distinct electrophysiological patterns from polymodal neurons dependent on unique ligand-receptor pairings that result in distinct sensory outcomes [5].

The current presiding theory for discrimination of itch sensation is a combination of the more traditional 'labelled line' and 'pattern' theories, dubbed 'population coding' (**Figure 3.1C**). This theory denotes that specific activation of afferent and spinal neurons predisposed to itch (determined by itch-mediating receptors) results in itch sensation, while additional activation of nociceptive neurons results in a 'gating' mechanism at the spinal level, inhibiting itch sensation in favour of the more urgent sensation of pain [44-48].

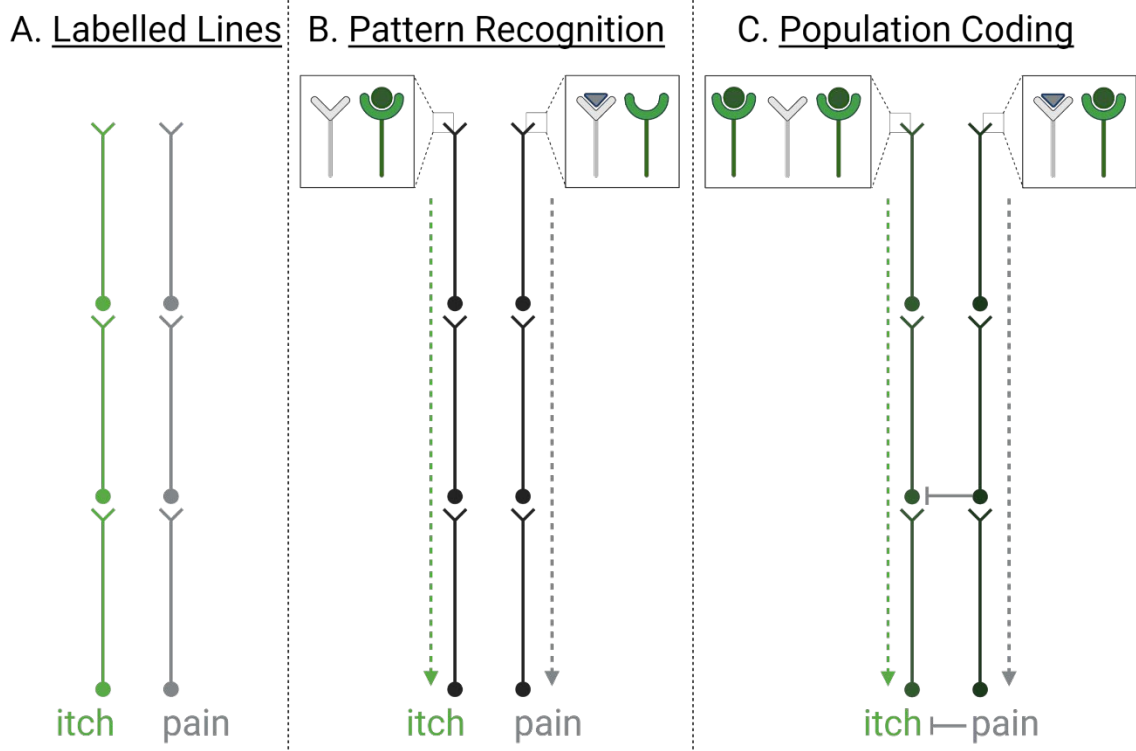


Figure 3.1: Theories underlying differentiation between itch and pain sensation

A) The labelled lines theory dictates that itch (green) and pain (grey) are mediated by distinct neural pathways from the periphery to the brain. This suggests that activation of itch or pain pathways would trigger the relevant sensation independent of the nature of the stimulus.

B) Pattern recognition postulates that the differentiation between itch and pain is determined by specific ligand-receptor interactions on polymodal (black) neurons. In this case, pruritic or nociceptive stimuli may activate any of these neurons and elicit the itch or pain sensation based on the receptor activation and not the neuronal subtype.

C) Population coding is a theory that blends labelled lines and pattern recognition theories. Polymodal neurons, predisposed to certain sensations as determined by the receptor subtypes expressed, may elicit itch or pain dependent on the specific stimulus. Importantly, this theory incorporates the notion of spinal gating, whereby painful stimuli override itch sensation by inhibiting pruritic signalling at a spinal level.

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3.2 Identification of itch-mediating receptors

Although numerous stimuli have been identified as pruritogens, itch is generally classified as histaminergic or nonhistaminergic due to the early discovery of histamine and its pruritogenic effects in the skin [2, 49-52]. Histamine, an integral mast cell mediator, elicits itch by acting on histamine receptors [20, 53, 54]. Indeed, the early development and subsequent refinement of antihistamines has proven to be an effective method for treatment of numerous itch and allergic conditions in the

past century [55, 56]. However, it was quickly noted that antihistamines were not successful in treating all forms of itch, leading to the discovery of nonhistaminergic itch mechanisms [57-59]. Several peptides, including bovine adrenal medulla 8-22 peptide (BAM8-22) and β -alanine, as well as the antimalarial compound chloroquine, have since been identified as pruritogens acting independently of histamine receptors [7, 60-63]. Instead, these pruritogens act via the recently determined 'itch-specific' Mas-related G-protein coupled receptor (Mrgpr) family [63, 64]. Additionally, non-histaminergic pruritogenic activity has been demonstrated by bile acids, acting via the bile acid receptor TGR5 [65].

3.2.1 Histaminergic itch receptors

Initial studies focusing on histamine activity hypothesised the involvement of multiple histamine receptors; to date, there are four known receptor subtypes ($H_1R - H_4R$), named in order of discovery and each encoded by the respective gene *Hrh1 - Hrh4* [66-68]. H_1R is the principle receptor involved in histaminergic itch and is the primary target for most antihistamines [55, 56, 69-72]. Although H_2R antagonists have been trialled as antipruritics, there is little to no evidence of a role for this subtype in itch mediation [73-76]. H_3R , reputedly expressed almost exclusively in neurons, is widely viewed as an inhibitory histamine receptor, with H_3R antagonists eliciting itch sensation [53, 66, 77-79]. H_4R , the most recently discovered histamine receptor, has also been implicated in itch signalling and has been the focus of a significant avenue of research towards novel antihistamines [53, 80-85]. Whilst antihistamines have proven to be an invaluable pharmacological tool, they remain ineffective in treating some forms of itch including chronic itch conditions [86-88].

3.2.2 Non-histaminergic itch receptors

The possibility of multiple itch-specific afferent neuron subtypes was presented from the findings of early microneurography work, showing that cowhage, or its active itch component mucunain, elicited itch via neurons distinct from histamine pathways [16, 89-91]. An initial study identified protease-activated receptor 2 (PAR_2) as a primary mediator for mucunain-induced itch [58]. PAR_2 is also implicated in itch induction via the activity of several other itch-inducing proteases including Cathepsin S and the PAR_2 agonist SLIGRL [92-97].

3.2.2.1 Mas-related GPCRs

The relatively recent discovery of the Mrgpr family of receptors, many of which have been demonstrated to be not only restricted to DRG neurons but specific to itch sensation, expanded the itch field further [64, 98-101]. There are approximately 24 known functional Mrgpr subtypes in mice, with significantly fewer in humans. Of these receptors, mouse MrgprA3 and MrgprC11 are orthologs of human MrgprX1, while human and mouse MrgprD are also orthologs [98]; each of these receptor subtypes have been identified as mediators of itch. Chloroquine, a common antimalarial known to

induce itch [102, 103], was discovered to act via MrgprA3 in mice, or X1 in humans [60]. Similarly BAM8-22, a preproenkephalin peptide, has been identified as an endogenous agonist for MrgprC11 and X1, resulting in non-histaminergic itch sensation [60, 63]. Finally, MrgprD has been identified as the receptor responsible for itch induced by the amino acid β -alanine, an endogenous amino acid and a common fitness supplement [62, 104-106]. The function of the Mrgpr family was not discovered until the generation of a genetic knockout mouse line which knocked out 12 functional Mrgpr genes, the *Mrgpr-cluster* $\Delta^{-/-}$ mouse line. These mice showed no behavioural deficit or abnormality except a reduction in scratching following application of specific pruritogenic Mrgpr agonists, indicating that these receptors are itch specific [60]. Since this discovery, the importance of Mrgprs in itch sensory signalling has been an area of great interest.

Non-histaminergic itch mechanisms are also believed to underlie cholestatic itch, a condition believed to be associated with the systemic build-up of bile acids [107-112]. Several non-histaminergic pruritogen and receptor pairings have been proposed, and more than likely multiple contribute [107]. Here too Mrgprs have been implicated, with recent studies demonstrating a potential role for human MrgprX4 in mediating cholestatic itch via activation by bile acids [113, 114] and by bilirubin, also shown to activate the mouse receptor MrpgrA1 [115]. MrgprC11 and precursors of the ligand BAM8-22 have both been shown to be upregulated in a cholestasis model with a concurrent increase in BAM8-22 itch responsiveness, which may indicate a role in cholestatic itch [61].

3.2.2.2 Bile acid receptor TGR5

A number of studies have identified the bile acid GPCR Takeda G-protein Receptor 5 (TGR5) as an integral mediator of cholestatic itch in mice [7, 65, 116, 117]. Several studies since have contradicted this, with one showing a reduction of TGR5 agonist deoxycholic acid (DCA)-induced itch in a mouse model of cholestasis [118]. A second study in human subjects demonstrated concentration-dependent DCA-induced itch, but no itch induced by a potent, 'non-bile acid' TGR5 agonist, implying bile acid-induced itch is TGR5-independent [114]. As with many GPCRs, the activities of TGR5 vary greatly, dependent on the tissue type and the ligand [116, 119-123]; it's role in itch is yet to be fully understood and is an area of great interest.

3.2.3 Downstream effector channels

In addition to direct activation of putative 'itch' receptors, TRP channels, particularly TRPV1 and TRPA1, have also been implicated in primary afferent pruritogenic signalling via mediation of downstream signalling from GPCR activation [14, 124-127]. Well-known for its importance in nociceptive signalling, TRPV1 has also been shown to facilitate histaminergic itch. *Trpv1* $^{-/-}$ models, targeted neuronal ablation, and specific TRPV1 antagonists are able to attenuate histamine responses

despite no direct effect of histamine on TRPV1 activation [14, 127-132]. Receptor subtypes H₁R and H₄R have both been identified as mediating itch sensation via downstream TRPV1 modulation [80, 129, 130]. Further, histamine acting on H₁R has been shown to sensitise TRPV1 responses to acid in mouse DRG neurons [133]. Interestingly, despite high receptor co-expression or co-responsiveness to relevant agonists in neurons for histamine receptors with both TRPV1 and TRPA1 [11, 43, 134], most studies suggest TRPA1 is not required for histaminergic itch [126, 132]. In an irritable bowel syndrome (IBS) model, however, H₁R activation has been shown to sensitise TRPA1 [135].

Conversely, TRPA1 has been identified as an integral downstream ion channel for transduction of nonhistaminergic itch. Mrgprs in particular appear to signal via TRPA1, with MrgprA3- and MrgprC11-mediated itch responses significantly reduced in a *Trpa1*^{-/-} but not *Trpv1*^{-/-} mouse model [11, 126, 136]. TRPA1 is also a likely downstream effector of β -alanine-induced activation of MrgprD [137]. Although nonhistaminergic signalling mechanisms have been less extensively studied, the involvement of TRPA1 as the key downstream ion channel is supported by several studies in chronic nonhistaminergic itch models [138, 139]. TGR5 has also been demonstrated to require downstream TRPA1 for itch [117], as supported by data showing a significant increase of both TGR5 and TRPA1 in a mouse cholestasis model [61].

3.2.4 'Itch-specific' afferent neurons

In attempting to identify an 'itch-specific' afferent neuron subtype, several studies have sought to characterise pruritogenic neurons based on expression and function of multiple itch receptors and neuronal markers. Early work postulated distinct populations for histaminergic and nonhistaminergic itch responses [16, 89, 91], however more recent studies suggest the reality is more complex. Mrgpr expression and function studies have shown that subtypes C11 and A3 in mice are co-expressed, while MrgprD-expressing neurons are a distinct subpopulation (**Figure 3.2**) [11, 62, 101]. MrgprA3-expressing neurons are also commonly responsive to histamine [60, 140], unlike MrgprD-expressing neurons [60, 62]. Indeed, ablation of MrgprA3-expressing DRG neurons resulted in diminished responses to histamine, chloroquine, and BAM8-22, but not β -alanine [11], whereas ablation of MrgprD-expressing neurons had no effect on itch induced by multiple pruritogens including histamine [14]. MrgprA3-expressing neurons also all expressed TRPV1 and responded to capsaicin [11], which was rarely found in MrgprD-expressing neurons, further differentiating these two pruritogenic neuronal subpopulations [10, 141].

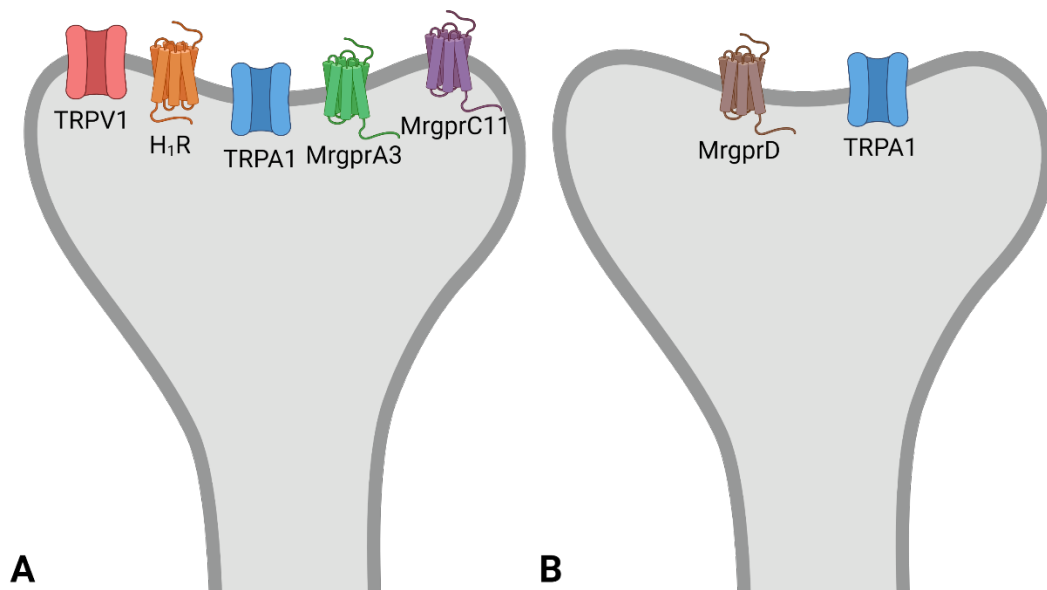


Figure 3.2: Putative itch-specific neuronal subtypes

A) Afferent neurons expressing itch-mediating receptors MrgprA3 and MrgprC11 are also commonly co-expressed with histamine receptor 1 (H₁R), transient receptor potential vanilloid 1 (TRPV1), and TRP ankyrin 1 (TRPA1).

B) Afferent neurons expressing MrgprD are distinct from those expressing MrgprA3 and MrgprC11, however both can elicit itch sensation. MrgprD neurons are also largely absent of TRPV1 and H₁R, and unresponsive to their respective agonists. Both MrgprD- and MrgprA3-expressing afferent neurons express TRPA1.

Figure created with BioRender.com

Interestingly, activation of TRPV1 exclusively in the MrgprA3-expressing population resulted in itch sensation rather than pain, while ablation of this population impacted itch but not pain responses, supporting claims of a potential ‘itch-specific’ population [11]. A large-scale single-cell RNA sequencing study corroborated this, showing distinct expression patterns of itch receptors and identifying three potential ‘itch’ neuronal subtypes [142]. Here too, MrgprD expression is distinct from MrgprA3, MrgprC11, H₁R, and TRPV1. None of these neuronal populations tested positive for H₄R or TGR5 mRNA, nor that of any other known bile acid receptor. As a large-scale study, this data did not include neurons specifically innervating the skin; an understanding of expression patterns of itch receptors within populations of afferent neurons innervating specific target tissues would be extremely informative for future studies.

3.3 ‘Itch’ as an internal irritant-sensing mechanism

Itch pathways have long been considered unique to the skin, however, several recent studies highlight the importance of itch as an irritant sensing mechanism in internal organs. Han et al.

identified the importance of vagal sensory neurons expressing MrgprC11 in mediating airway hyperresponsiveness and bronchoconstriction in mice; a physiological 'itch' in the lungs resulting in a 'cough' response to remove irritants [8]. Mast cells, responsible for releasing numerous inflammatory mediators including itch receptor agonists neuropeptide FF (NPFF) and histamine [143-145], are also known to play a critical role in airway irritation; indeed, all four histamine receptor subtypes have been implicated in asthmatic responses [146, 147].

Itch receptors have similarly been identified as important colonic afferent signalling mediators, with TGR5 and Mrgpr subtypes C11 and A3 all demonstrated to be functionally expressed in subpopulations of colon-innervating afferent DRG neurons [9]. Receptor activation triggered peripheral neuronal excitation *ex vivo* as well as increased afferent and spinal neuron responses to colonic distension *in vivo*, indicating an increase in sensitivity following itch-receptor activation [9, 148]. Further, these receptors are proposed to play a role in chronic functional gastrointestinal disorders such as IBS, as demonstrated by increased sensitivity to the itch receptor agonists in a chronic visceral hypersensitivity (CVH) mouse model [9, 148]. In this instance, an increase in motility may be perceived as a means of irritant removal via expulsion. Histaminergic mechanisms have also been implicated in IBS sensitivity in clinical studies, with histamine shown to potentiate IBS symptoms via H₁R [135, 149]. The function of itch-signalling mechanisms as a form of protection via irritant removal evidently occurs throughout the viscera and respiratory tracts in addition to the skin, and has to date been implicated in numerous chronic disease states including cholestatic pruritus [65], asthma [8, 146], and IBS [9, 135, 149].

The role of itch-signalling mechanisms in the bladder remains largely unexplored. However, given the behavioural responses in chronic bladder conditions such as IC/BPS and OAB – namely increased frequency and urgency – it is reasonable to predict an 'itch/scratch' mechanism in the bladder portrayed as increased urination as a means of irritant removal. Several parallels in the nature of afferent signalling between cutaneous itch and bladder sensation are of note: 1) the interplay between keratinocytes and neurons in the skin has been identified as a promising area of research [150, 151], not unlike the promising role of urothelial cells in bladder afferent signalling discussed in **Chapter 1** [152]. 2) While pruritogenic afferents in the skin are known in general to be polymodal, their anatomical structure and position as free nerve endings located in the epidermis of the skin is widely accepted [10, 11]; similarly, a recent study utilising antidromic tracing identified urothelial free nerve endings prominently present within the mouse bladder [153]. The recent discovery of itch mechanisms in the colon in particular strongly supports the concept of bladder 'itch', considering the evident links between bladder and gut sensory mechanisms in health and prevalent comorbidities between IBS and OAB or IC/BPS discussed in **Chapter 2** [154, 155]. While histamine as a prominent inflammatory mediator has long been postulated as a critical mediator in bladder pathology, the

expression and function of pruritogenic receptors in the bladder and bladder afferent neurons is an unexplored avenue of high potential, and hence the focus of this thesis.

This thesis identifies and explores the presence and function of histaminergic and nonhistaminergic itch-mediating receptors within the bladder. While some potential itch-specific mediating receptors have been identified in the spinal cord, the central mechanisms of itch sensation as distinct from pain and other senses is largely unknown. Present literature suggests convergence of central signalling pathways, limiting our ability to confirm the originating signal. Conversely, numerous receptors in the periphery are known to play a key role in itch-specific signal mediation. As such, this project focuses on the expression and function of itch-mediating receptors in the afferent arm of the bladder sensory system.

3.4 Itch-mediating receptors in the bladder: histaminergic mechanisms

Histamine and histamine receptors have been extensively studied, particularly regarding their roles in itch and inflammation. There are also well-known internal actions of histamine, including gastric acid secretion in the gastrointestinal tract [156, 157], pulmonary and respiratory actions [158], and central neurological processes with links to the sleep/wake cycle and migraines [159-161]. Histamine is formed via enzymatic decarboxylation of the amino acid histidine and stored in cytosolic granules of mast cells and basophils as well as gastric enterochromaffin-like cells and histaminergic neurons [67, 162, 163], although mature mast cells are the primary source of histamine [143, 144]. The potential role of histamine and mast cells in OAB and IC/BPS has also been previously explored, although interestingly, the expression and function of histamine receptor subtypes in the bladder and bladder-innervating neurons is largely unknown.

3.4.1 Histamine receptors

Histamine receptors are abundant in excitable cells and immune cells and have been shown to be relevant in a number of inflammatory disease states [67, 133]. The expression profiles and functions vary for each receptor subtype, as do the binding affinities for histamine. H₃R and H₄R, despite being the later discovered subtypes, have significantly higher binding affinities than H₁R and H₂R [158, 164]. Additionally, co-activation of multiple histamine receptor subtypes within a cell can have a synergistic effect [165, 166]. As such, it is important to consider the expression and function of each receptor subtype individually within native tissues and/or cells.

3.4.1.1 – Histamine 1 receptor

H₁R is widely expressed throughout the body, including in the brain and DRG, retina, airway, gastrointestinal tract, lymphocytes, and vascular and genitourinary smooth muscle [66, 158, 167]. In addition to its key role in histamine-dependent itch sensation [5], H₁R has been shown to initiate smooth muscle contraction [167, 168], enhance Th1 proinflammatory immune responses [169], and facilitate learning and memory [170] as well as nociception [171-175].

3.4.1.2 – Histamine 2 receptor

The concept of multiple histamine receptor subtypes was proposed based on the distinct potencies of antagonists and agonists on certain actions of histamine, particularly gastric acid secretion and uterus relaxation [176]. Similar to H₁R, H₂R is expressed in the brain, stomach, smooth muscle, endothelial and epithelial cells, and immune cells [158, 177-180], with significantly greater expression in the stomach than other organs including the brain [177]. Concordantly, the role of H₂R appears to be primarily gastrointestinal regulation, contributing to gastric acid release and gastrointestinal motility [157, 181, 182]. Overall, the actions of H₂R appear to counteract those of H₁R, initiating smooth muscle relaxation, particularly of the intestine and uterus [168, 181], and negatively regulating Th1 and Th2 immune responses [169]. H₂R does also play a neuronal role, facilitating learning and memory as well as mediating nociception [170, 183, 184], similar to H₁R.

3.4.1.3 – Histamine 3 receptor

H₃R was discovered using the H₂R antagonist burimamide, which is able to inhibit H₃R actions at far lower concentrations than required for H₂R [185, 186]. Conversely to the other histamine receptor subtypes, H₃R appears to act as an inhibitory histamine receptor [77, 185]. As with H₁R and H₂R, H₃R is widely expressed, including salivary glands, gastrointestinal mucosa, epithelial cells, heart, and kidney as well as the nervous system, where it is found in the brain, spinal ganglia, spinal cord, and peripheral neurons [187-191]. H₃R mediates a negative feedback mechanism, with activation by histamine in histaminergic neurons reducing histamine synthesis and release [186, 192]. It also triggers mucosal PGE₂ production in the stomach [193] and plays a role in nociception, although the data as to whether H₃R acts anti- or pro-nociceptively is dependent on the model, stimulus, and agonist or antagonist administration route [191, 194].

3.4.1.4 – Histamine 4 receptor

Histamine receptor 4 (H₄R) is a relatively recent discovery, with known expression in immune cells and tissue including the spleen and thymus, peripheral blood leukocytes, and the colon [195-197]. H₄R expression is also prominent in the nervous system, present in DRG sensory neurons, the spinal cord and regions of the brain [198, 199]. Since its discovery, H₄R has been shown to be a key mediator of pro-inflammatory responses, mediating mast cell and eosinophil chemotaxis [200-202], enhancing

production of inflammatory mediators in mast cells [203, 204], and promoting Th2 inflammatory responses [205, 206]. While H₄R in immune cells plays a clear pro-inflammatory role, current evidence suggests that in addition to itch, neuronal H₄R plays an anti-nociceptive role. H₄R deficient mice demonstrate increased nociceptive sensitivity to peripheral neuropathy [199], while activation of H₄R in wildtype model of neuropathic pain alleviates nociception [207].

3.4.2 Histamine receptors in the bladder

The evidence of a role for histamine and mast cells in IC/BPS and OAB has led to the use of antihistamines as a treatment option for IC/BPS, showing some efficacy [208, 209]. Despite this, the mechanism of action of histamine in the bladder and the role of histamine receptors are ill-defined, particularly in relation to bladder sensory nerve activity. Expression of all four histamine receptor subtypes has been identified in human bladder biopsies and mouse bladder tissue [210-212], but as yet expression in bladder afferents is unknown.

Administration of antihistamines as a treatment option for IC/BPS gives an indication of the role of histamine receptors. Treatment with H₁R antihistamines [208], H₂R antihistamines [213, 214], or a combination thereof [215] has shown promising but not universal improvement in IC/BPS symptoms, indicating that H₁R and H₂R likely play a role in some but not all cases. Supporting this, an increase in H₁R protein expression was identified in IC/BPS patients [216]. Another recent study identified a correlation between increases in H₁R and H₃R expression in human bladder biopsies and symptom severity, suggesting a potential role for H₃R as well as H₁R [210]. This same study was unable to identify any correlation between receptor expression and antihistamine responsiveness, however the antihistamines included in this study were H₂R antagonist cimetidine and amitriptyline, a compound exhibiting a range of pharmacological actions along with H₁R antagonism, and does not therefore give a clear indication of histamine receptor involvement.

In bladder tissue, histamine has been shown to cause contraction of isolated pig detrusor and mucosal bladder strips [217, 218]. This action was blocked by H₁R antagonist pyrilamine in both detrusor and mucosa, while application of H₂R antagonist cimetidine or H₃R/H₄R dual antagonist thioperamide enhanced histamine activity in mucosal strips, with no effect in detrusor [218]. Interestingly, the effects of H₂R and H₃R/H₄R were only present in juvenile pigs, not adults [217], suggesting a shift in histamine receptor activity or expression with aging. Histamine has also been shown to activate the vast majority (92 - 100%) of human cultured detrusor smooth muscle cells [211, 219]. Interestingly, the use of receptor-specific agonists for H₁R, H₂R, and H₃R demonstrated that histamine-induced calcium transients following H₁R and H₃R activation were significantly greater in internal sphincter smooth muscle cells compared to detrusor smooth muscle cells, with no difference

observed from H₂R activation [219]. This highlights the importance of identifying receptor subtype expression and function in specific cell types rather than the whole organ.

Other mechanistic studies have focused on the histamine receptor subtypes involved in pelvic pain associated with IC/BPS, although none have looked at the bladder-innervating neurons themselves in this context. In an autoimmune model of IC/BPS in mice, allodynia was partially reduced by the H₁R antagonist cetirizine and abolished by the H₂R antagonist ranitidine [220]. Similarly, studies in a PRV model for IC/BPS in mice showed attenuation of pelvic pain in *Hrh2*^{-/-} mice or following H₂R antagonist treatment and partial attenuation in *Hrh1*^{-/-} or with H₁R antagonist treatment, while imetit, an agonist for H₃R, had no effect [212, 221]. These studies provide important indications as to the relative importance of histamine receptors in IC/BPS. However, the use of systemic administration of antihistamines and external pain measurements via suprapubic or hindpaw skin stimulation with von Frey filaments does not provide direct evidence of histamine-receptor interactions within the bladder. A clear understanding of the roles of histamine receptor subtypes in bladder sensory signalling is necessary for providing more efficacious treatment options.

3.4.3 Mast cells and histamine in the bladder

While knowledge of the expression and function of histamine receptor subtypes in the bladder is limited, the notion of mast cells and histamine mediating IC/BPS and OAB symptoms is not novel. Early studies, with the goal of identifying a disease marker, focused on the number of mast cells present in the bladder during IC/BPS, demonstrating a significant increase [222]. The findings varied in the locations of the mast cells and in findings for ulcerative and non-ulcerative IC/BPS, likely due in part to the lack of distinction in IC/BPS definition at the time. Several studies noted significant increases in mast cells in ulcerative or ‘Hunners’ IC/BPS but not in the non-ulcerative form [223-225], while others did observe increases in non-ulcerative IC, although a greater increase was shown in Hunners IC/BPS [226, 227]. In line with the goal of using mast cell counts as a disease marker, several studies compared IC/BPS to other bladder conditions including incontinence and bacterial cystitis. While bacterial cystitis in particular also showed increased mast cell counts, IC/BPS counts were significantly higher [228-231]. Interestingly, increased mast cells in the bladder have also been observed in a cross-sensitisation model utilising colonic TNBS treatment to evoke bladder hypersensitivity in rats [232].

In addition to increased counts, studies utilising electron microscopy demonstrated significant degranulation of mast cells in IC/BPS bladders, with negligible mast cell activation observed in bacterial cystitis or healthy controls [229, 233]. Correlatively, increased levels of histamine have been identified in the bladder wall and urine of IC/BPS patients [224, 225, 234-237]. Expanding on the

observations of significant degranulation specific to IC/BPS in the bladder, several studies challenged isolated mast cells with secretagogues, finding that IC/BPS-originating mast cells were significantly more reactive in release of histamine following stimulation [238, 239]. Studies exploring mast cells and histamine in OAB are limited, however increased histamine and NGF were observed in a stress-induced OAB model [240].

The presence or absence of mast cells, and therefore histamine, is a critical factor in symptom development in bladder hypersensitivity models. In a human study, the number of mast cells in the detrusor muscle inversely correlated to bladder capacity, thus linking to frequency and likely urgency [227]. Inflammation and lower urinary tract dysfunction (frequency and urgency) were attenuated in mast cell deficient mice compared to controls in both substance P (SP)- and a lipopolysaccharide (LPS)-induced IC/BPS models and an autoimmune model [241, 242]. The importance of mast cells was confirmed in the autoimmune model with symptom onset following mast cell reconstitution [241]. Critically, symptoms in a pseudorabies virus (PRV) IC/BPS model in rats were reduced by pre-emptive degranulation of mast cells, indicating the importance of mast cell mediators such as histamine in symptomology [243].

Mast cells are also known to play a role specifically in the pelvic pain associated with IC/BPS, with significant attenuation of pelvic pain in mast cell deficient IC/BPS models [212, 244]. Conversely, neonatal capsaicin treatment to abolish sensory neurons reduces activation of mast cells via an emotional stress model of IC/BPS [245]. Electron microscopy studies have demonstrated the presence of mast cells in close proximity to nerve endings in human IC/BPS patient bladder tissue and bladders from a rat IC/BPS model [230, 246]. As such, the localised release of histamine from mast cells can interact directly with receptors on nerve terminals, triggering neuronal activation and sensitivity as well as stimulating neurogenic inflammation [247, 248]. This involves anterograde release of inflammatory neuropeptides from nerve endings, including substance P and calcitonin gene-related peptide (CGRP), which further activate mast cells and contribute to an inflammatory state, further amplifying the irritant sensitivity response [248-250]. Further, the increase in mast cell count and urinary histamine content in IC/BPS bladder tissue has been shown to correlate strongly with an increase in the density of nerve fibres, particularly small diameter, SP-expressing C-fibres [246, 251]. These data support a neuroinflammatory feedback loop between peripheral sensory neurons and mast cells in bladder hypersensitivity conditions. This has been previously postulated [252], however the role for histamine receptors in this mechanism, particularly relating to neuronal activity, is unknown.

3.5 Non-histaminergic ‘itch’ mechanisms in the bladder

In comparison to histaminergic itch, research into understanding non-histaminergic itch receptor expression and function is in its infancy, and knowledge directly relating to expression and function of these receptors in the bladder is sparse at best. This section explores current literature regarding the expression and function of Mrgprs A3, C11, and D and bile acid receptor TGR5 and how these may link to the bladder.

3.5.1 Mas-related GPCRs

Discovered by two independent groups, Mrgprs have also been termed ‘sensory neuron-specific receptors’ (SNSRs) due to the findings that a subset of these receptors are exclusively expressed within sensory DRG neurons [98, 100]. Approximately 37 coding Mrgpr genes have been identified in mice and 8 in humans [98, 101]. These genes are organised in subfamilies of MrgprA-H (mice) and MrgprX1-X4 and MrgprD-H (humans). Initial exploratory studies indicated that MrgprA subtypes, B4, B5, C11, and D, and human MrgprX1-4, are expressed exclusively in sensory DRG neurons [98, 100, 101, 253]. Mouse and human MrgprD-H are direct orthologs. Human X1-X4 and mouse A-C are more complex, however ligand specificity studies have identified similarities between mouse MrgprA3 and MrgprC11 and human MrgprX1 [60]. Whether or not there are other unpredicted orthologous pairs is yet to be determined.

Prior to their identification as key itch mediators, Mrgprs were presumed to play a role in pain sensation due to their predominant expression in small diameter, IB4-positive (nonpeptidergic), nociceptive neurons. During this time, MrgprD was highlighted as a receptor of great potential for pain research, particularly due to its translatability given the clear human and mouse orthologs. Some interest has been shown in MrgprX2, the expression profile of which varies greatly from the reputed DRG-specific receptors; MrgprX2 and MrgprB2 are expressed in mast cells as well as visceral organs, including the colon and the bladder, suggesting a potential role in visceral inflammatory conditions [254-256]. While MrgprX2/B2 may play a role in ‘itch’ irritation via mast cell degranulation and histaminergic mechanisms, there is currently no evidence of this receptor acting as a direct itch-mediator. While the majority of Mrgprs are yet to be investigated, MrgprA3 and MrgprC11 have been thoroughly explored in their capacity as irritant-sensing mediators [60, 64, 99]. As known nonhistaminergic itch-mediating receptors expressed in sensory DRG neurons, this thesis focuses on the expression and function of MrgprA3, C11, and D, and their potential role in bladder sensory signalling.

3.5.1.1 – MrgprA3 and C11

While initial studies found MrgprA3-expressing neurons innervating nowhere but the skin [11], more recent works have found expression of MrgprA3 and MrgprX1 in neurons innervating the eye conjunctiva in mice and humans respectively [136], MrgprC11 in vagal ganglia neurons innervating the airways [8], and both MrgprA3 and MrgprC11 in DRG neurons innervating the colon [9, 257].

The primary function identified for MrgprA3 and MrgprC11 to date is that of itch sensation, described above. Importantly, MrgprA3 and C11 have been implicated in chronic conditions of sensory sensitivity in the skin, with increased agonist sensitivity and receptor mRNA expression in neurons innervating the skin in several models of induced dermatitis [258-260]. Additionally, dermatitis-induced enhanced itch sensation and scratching was significantly reduced in *Mrgpr-clusterΔ^{-/-}* mice or following ablation of MrgprA3-expressing neurons [259, 260]. These studies highlight the potential for MrgprA3 and C11 to play a role in conditions of chronic hypersensitivity such as OAB and IC/BPS.

MrgprA3 and C11 have each been implicated in pain signalling as well as itch in mice and humans [11, 63]. Peripherally, an early study in humans demonstrated some pain in addition to itch following BAM8-22 administration to the skin [63]. Activation of rat MrgprC peripherally resulted in increased nociceptive responses, while central activation showed increased heat sensitivity [261]. Interestingly in mice, MrgprC11 activation inhibited high voltage (N-type) calcium channels, which are located at the central terminals of sensory neurons and are responsible for neurotransmitter release, thus their inhibition reduces central propagation of pain signalling [262]. Another study using rats transfected with human MrgprX1 corroborated the inhibition of N-type calcium channels, further demonstrating inhibition of M-type potassium channels, which are expressed in peripheral sensory nerve terminals and are responsible for raising the activation threshold [263]. Thus, peripheral activation of MrgprX1 results in a reduced activation threshold. These location-dependent response differences are an important consideration when extrapolating data to different organs.

With a focus on itch sensation and initial studies failing to identify MrgprA3-expressing neurons innervating other organs, the majority of studies looking at MrgprA3 and MrgprC11 function are based in the skin. However, these receptors are now known to play a role in neurons innervating the airways, eyes, and viscera. In the eyes, activation of MrgprA3 with chloroquine or MrgprC11 with NPFF elicits itch sensation much like the skin, as one might expect given the ability to scratch here [136]. In the airways, MrgprC11 has been identified as a key effector in initiating a ‘coughing’ response, with bronchoconstriction and increased respiratory rate induced via application of MrgprC11 agonists BAM8-22 and NPFF [8]. MrgprC11 was also shown to play a role in airway hyperresponsiveness such as that observed during respiratory infections. Hyperresponsiveness was abolished in *Mrgpr-clusterΔ^{-/-}*

$\Delta^{-/-}$ mice compared to wildtype following infection and could be replicated via MrgprC11 activation without infection in wildtype mice [8]. In the viscera, intrarectal application of chloroquine and BAM8-22 resulted in hypersensitivity to colorectal distension in mice *in vivo*, which was abolished in the *Mrgpr-clusterΔ^{-/-}* mice [9, 257]. This was reflected in *ex vivo* studies showing mechanical afferent hypersensitivity to chloroquine and BAM8-22 which was subsequently exaggerated, both *in vivo* and *ex vivo*, in a mouse model of chronic visceral hypersensitivity [9]. Taken together, these data demonstrate the various forms irritant sensation may take in organs other than the skin and the potential influence they may have in disease states, supporting further investigation into irritant-sensing mechanisms in other affected organs, such as the bladder.

3.5.1.2 – *MrgprD*

MrgprD in human and mouse genomes are orthologous, making it a valuable receptor of interest for translatability. Early Mrgpr studies identified a distinct expression profile of MrgprD, exclusive to cutaneous sensory DRG neurons innervating the stratum granulosum layer of the epidermis, which is the external-most living layer [10, 98]. Several studies have identified partial co-expression of MrgprD with TRPA1 [137, 264], however patch clamp experiments performed in mice demonstrated rare responses of MrgprD-expressing neurons to TRPA1 agonist cinnamaldehyde [265].

While the initial characterisation studies investigated MrgprD expression in numerous other organs and found none [10, 98], more recent expressional studies have identified MrgprD-expressing DRG neurons innervating dental pulp in mice and rats [266, 267] as well as the mouse colon [268, 269]. Further, MrgprD has been identified in retinal neurons, epithelial and glial cells [136, 270], as well as other non-neuronal cells, including osteocytes and cardiomyocytes [271-274]. Finally, a recent study identified expression of MrgprD throughout the gastrointestinal tract including smooth muscle, macrophages, and T lymphocytes [275]. However, this has been directly disputed in a follow up study that was able to recapitulate the results of this study in both wildtype and *MrgprD^{-/-}* mice indicating a false positive [276] and suggesting a need for further exploration.

MrgprD activation has been shown to enhance neuronal excitability, demonstrated via a reduction in the amount of current required to elicit an action potential in mouse neurons [141, 277]. Ablation of MrgprD-expressing neurons results in a reduction in mechanical pain sensitivity without impacting sensitivity to heat or cold [278-281], although extreme heat and cold have been shown to activate MrgprD-expressing neurons [280]. MrgprD has also been implicated in inflammatory and neuropathic itch and pain models. An atopic dermatitis mouse model induced neuronal excitability in MrgprD-expressing neurons, with an increase in spontaneous firing *in vivo* [260]. In a chronic constriction injury mouse model, MrgprD expression was significantly increased, and the mechanical allodynia observed following injury was less pronounced in a *MrgprD^{-/-}* model [137]. Interestingly, β -

alanine-induced pain responses were significantly increased in the chronic constriction injury model whereas itch responses were not [137]. In the viscera, MrgprD is shown to increase hypersensitivity, with application of the polyunsaturated fatty acid 5-oxoeicosatetraenoic acid (5-oxoETE), another endogenous MrgprD agonist, increasing visceromotor sensitivity in mice [268]. As levels of 5-oxoETE are increased in the colon in IBS patients, MrgprD may play a role in visceral hypersensitivity [268]. These studies demonstrate a clear role for Mrgpr in sensory sensitivity throughout the body; whether or not this may be extrapolated to the bladder is yet to be determined.

3.5.2 Bile acid receptor TGR5

TGR5, also known as membrane bile acid receptor (MBAR) or G Protein-coupled Bile Acid Receptor 1 (GPBAR1), is a membrane-bound receptor originally identified as responding to bile acids [282, 283]. Initially considered only to function as digestive detergents, bile acids are now recognised as hormone-like signalling molecules, particularly via their activity as ligands for TGR5 and the nuclear receptor farnesoid X receptor (FXR) [284, 285]. TGR5 responds to secondary and primary conjugated bile acids, with lithocholic acid (LCA) and deoxycholic acid (DCA) being two of the most potent [282, 286]. Interestingly, bile alcohols have also been identified as potent TGR5 agonists [287], as have neurosteroids [119].

Expression of TGR5 has been identified in a number of organs and cell types, with highest expression observed in the gallbladder, placenta, and spleen [283, 288]. Significant expression has also been demonstrated in skeletal muscle and cardiomyocytes [283, 289, 290], the pancreas [291, 292], liver [293, 294], adipose tissue [295], throughout the kidney [296], the gastrointestinal tract [297-299], and recently in the bladder [300]. TGR5 is observed in numerous cell types in each of these organs, however, is most commonly found in monocytes or macrophages and epithelial or outermost cells [283, 293, 297, 298, 301]. Importantly, TGR5 has also been observed in the nervous system, including neurons, astrocytes, and microglia in the brain [119, 302, 303], neurons in the enteric nervous system [297, 299], and DRG neurons and glial cells [9, 114, 117]. Neuronal tracing studies performed to date have identified TGR5 expression in cutaneous and colonic afferent DRG neurons [9, 117]. Interestingly, co-expression of TGR5 with TRP channels varies greatly between these two populations. 8% of cutaneous DRG neurons were found to express TGR5, which was 100% co-expressed with TRPA1, while only 60% of TGR5-expressing cutaneous DRG neurons co-expressed TRPV1 [117]. Contrastingly, 19% of colonic DRG neurons were positive for TGR5-expression, with 83% co-expressing TRPA1 and 78% co-expressing TRPV1 [9]. The expression of TGR5 in bladder-innervating DRG neurons, or in the different layers of the bladder, has yet to be explored.

The function of TGR5 in the bladder is also yet to be determined, however studies in other organs and cell types may give an indication. For example, TGR5 may inhibit detrusor smooth muscle contraction; in the gallbladder and gastrointestinal tract, TGR5-induced smooth muscle relaxation resulted in gallstone formation and delayed gastric emptying and intestinal transit [299, 304-307].

Several studies indicate a proinflammatory role for TGR5. Pancreatitis was significantly inhibited in *Gpbar1*^{-/-} mice [292], while activation of TGR5 in Kupffer cells in the liver with oleanoic acid increased pro-inflammatory cytokine production in wildtype but not *Gpbar1*^{-/-} mice [308]. However, in the epithelial cells of the bile duct (cholangiocytes), TGR5 activation induced opposite effects dependent on the ciliation of the cell, inhibiting proliferation in cells without primary cilia but activating proliferation in ciliated cells [309]. This highlights the potential conditional activity of TGR5, where receptor activity may depend on the agonist, cell, or tissue type.

Further supporting this, numerous studies have also identified a largely anti-inflammatory or protective function for TGR5, likely due to macrophage-expressed TGR5. TGR5-induced reduction or inhibition of inflammation has been observed in models for endometriosis [310], colitis [311], atherosclerosis [312], autoimmune encephalitis [313], and arthritis [121]. Activation of TGR5 inhibits LPS-induced inflammation by suppressing phagocytic activity and cytokine production in macrophages [283, 293, 298] and inhibiting the NF-κB inflammatory pathway [314, 315], while enhanced inflammation and chemokine expression were demonstrated in a macrophage-specific *Gpbar1*^{-/-} model [316]. A study looking at activation of TGR5 in microglia also demonstrated an anti-inflammatory effect in a model of acute neuroinflammation [303]. Finally, several studies have identified a potential role for TGR5 in epithelial integrity, with impaired gastrointestinal mucosal integrity and increased permeability in *Gpbar1*^{-/-} mice [311, 317]. *Gpbar1*^{-/-} mouse colon had significantly reduced occludin expression and discontinuously spread zonulin-1 protein, both integral proteins for maintaining barrier permeability not only in the colon but also in the bladder [311, 318]. This role for TGR5 likely further contributes to protection from inflammatory conditions, preventing damage to underlying tissue from the lumen.

These studies are in stark contrast to the few neuronal TGR5 studies, which identify TGR5 as an irritant-sensing receptor [65, 116, 117]. This indicates a unique role for TGR5 signalling in neuronal cells, particularly compared to that of immune cells. It is noteworthy, too, that an anti-inflammatory environment does not necessarily conflict with neuronal hypersensitivity. Indeed, the study demonstrating a role for TGR5 in exacerbating colonic neuronal sensitivity to distension utilises a chronic visceral hypersensitivity model designed to represent IBS [9], a disorder defined by the lack of overt structural or biochemical damage [319]. As described in **Chapter 2**, OAB is also defined via symptoms of hypersensitivity in the absence of inflammation. While little is known regarding TGR5 in

the bladder as yet, endogenous TGR5 agonists including bile acids and progesterone sulphates can be excreted via the urine [320-322], providing an opportunity for receptor-ligand interaction.

3.6 Conclusion

The notion of 'itch' as the cutaneous representation of a more ubiquitous irritant-sensing mechanism is a recently identified, vital avenue of research in determining mechanisms of neuronal sensitivity in visceral organs including the bladder. This area of research has the potential to dramatically change our understanding of the mechanisms underlying or contributing to symptoms of hypersensitivity in disorders such as OAB and IC/BPS, including urgency and frequency. The expression and function of nonhistaminergic irritant-sensing receptors TGR5, MrgprA3, MrgprC11, and MrgprD in the bladder are largely unknown, however data available from other organs strongly supports the potential for each of these receptors to play a role not only in bladder irritant sensation, but in conditions of hypersensitivity. Similarly, despite a long history of histamine and histamine receptors in the bladder in health and disease, little is known about the role of neuronal histamine receptors in bladder sensitivity. Identifying and characterising novel irritant-sensing mechanisms within the bladder is an important potential avenue for future development of novel and efficacious treatments for conditions of bladder hypersensitivity.

3.7 References

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Aims and Hypothesis

The importance and potential of 'itch' receptors in irritant sensation in organs and systems other than the skin is quickly becoming apparent, however the role that these receptors play in bladder afferent sensitivity specifically is largely unknown. To this end, this work is directed towards to following aims:

Aim 1:

To determine the expression of histaminergic (H₁R – H₄R) and nonhistaminergic (TGR5, MrgprA3, MrgprC11, MrgprD) G-Protein Coupled Receptors (GPCRs) in the urinary bladder and bladder-innervating DRG neurons of mice.

Aim 2:

To determine the function of histaminergic and nonhistaminergic GPCRs in mediating peripheral bladder afferent sensitivity *ex vivo* and *in vitro*.

Aim 3:

To determine the *in vivo* impact of peripheral activation of histaminergic and nonhistaminergic GPCRs in the bladder on central sensory signalling.

Hypothesis:

I hypothesise that an array of itch-mediating GPCRs are expressed in bladder afferent DRG neurons, and that activation of these GPCRs with their respective agonists may mediate irritant sensation from the urinary bladder in mice.

Chapter 4 Methods

4.1 Overview

In order to determine the role of 'itch'-mediating GPCRs in bladder sensation, a number of complementary methodologies were combined. Each methodology provides an additional degree of resolution in elucidating the function of itch-mediating GPCRs in bladder sensory networks *in vitro* and *in vivo* as outlaid in **Figure 4.1**. QRT-PCR was used to investigate the relative mRNA expression of itch receptors within isolated urothelial cells, separated detrusor and mucosa layers of the bladder, and lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) pairs (**Chapter 4.3**). Additionally, functional data were obtained in three distinct tissues, each with established roles in transducing bladder sensation. **1:** The urothelium; urothelial cells were isolated and cultured (**Chapter 4.6**) for live cell calcium imaging experiments to determine direct activation following application of GPCR agonists (**Chapter 4.7.3.3**). **2:** Bladder-innervating DRG neurons; retrograde tracing was used to identify bladder-innervating afferent neurons within the LS DRG (**Chapter 4.4**), which were then dissociated and cultured (**Chapter 4.5**) for live cell calcium imaging experiments to determine direct activation following application of GPCR and ion channel agonists (**Chapter 4.7**). **3:** The spinal cord; *in vivo* infusion with GPCR agonists and/or distension of the bladder was performed and activation of spinal neurons within the LS spinal dorsal horn was analysed to identify the impact of peripheral target receptor activation on bladder sensory signalling at the spinal level (**Chapter 4.8**). These techniques were combined with a number of genetic knockout mouse models to further understand the roles of target receptors as well as identify potential down-stream ion channel interactions (**Chapter 4.2**).

In addition to these methods, data were obtained via single cell RT-PCR to determine mRNA expression within individual neurons and *ex vivo* afferent recordings to identify afferent mechanosensitivity. These techniques were not performed by the author hence are not described in this chapter: methodologies for these techniques can be found in the methods sections of the results chapters, written in manuscript form (**Chapter 5**, **Chapter 6**, and **Chapter 7**).

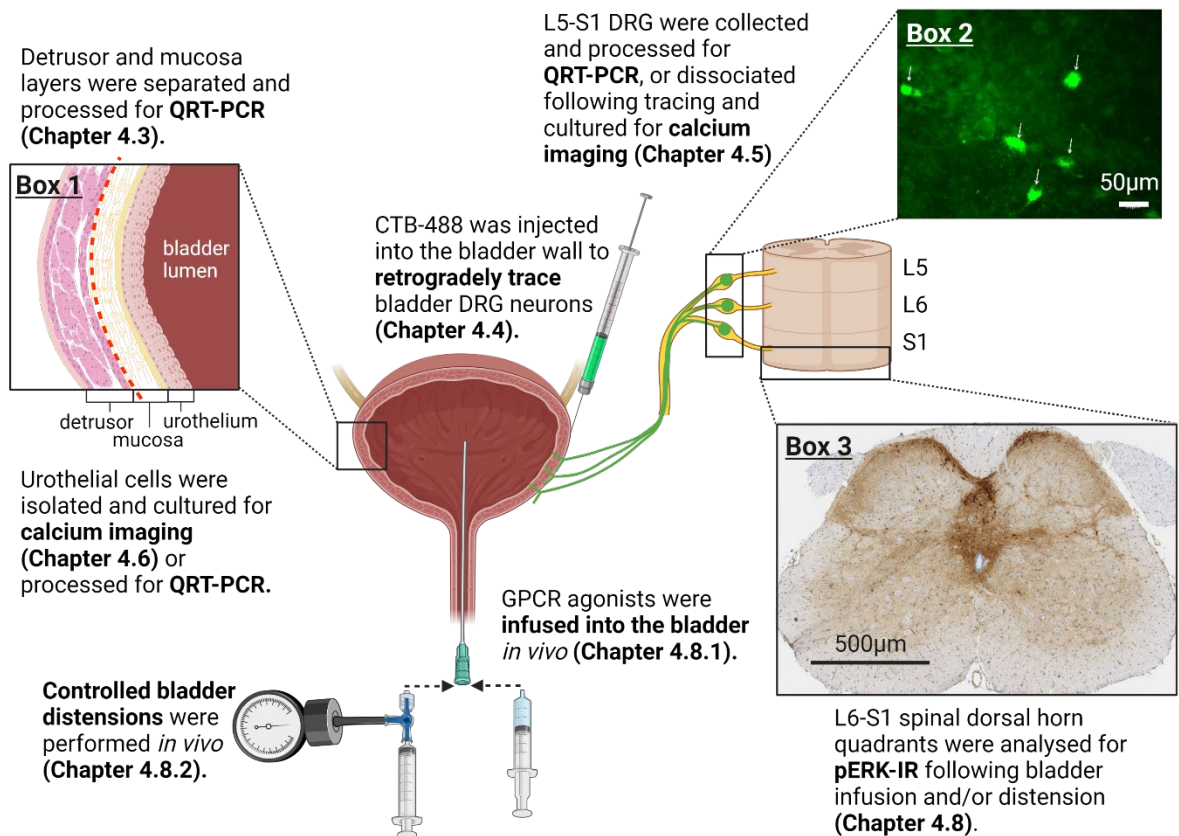


Figure 4.1: Schematic overview of tissue and techniques

To determine the role of itch-mediating GPCRs in bladder sensory signalling, expression and functional techniques were utilised at the levels of the bladder itself, the peripheral sensory neuron cell bodies within the dorsal root ganglia (DRG), and the lumbosacral spinal dorsal horn, where the bladder-innervating sensory afferents terminate. **QRT-PCR** was used to define mRNA expression of receptors in the layers of the bladder (Box 1) and in the lumbosacral (L5-S1) DRG. **Retrograde tracing** was used to identify DRG neurons specifically innervating the bladder wall, and the function of receptors in isolated bladder-innervating DRG neurons (Box 2) was evaluated using **live cell calcium imaging**, as was activation of isolated urothelial cells. Central activation relating to GPCR activity in the bladder was assessed via immunohistochemistry to analyse **pERK-IR** in the lumbosacral (L6-S1) spinal dorsal horn (Box 3) following *in vivo* **bladder infusions** and/or **bladder distensions**. Chapter sections describing each method in detail are cited within the schematic.

4.2 Ethics and animals

The studies included in this thesis were performed in, or utilised primary tissue from, adult (12- to 18-week-old) male and female C57BL/6J mice. The use of live animals (or primary tissue thereof) provides a greater insight than cell lines into the true physiological state while being safer, more ethically sound, more feasible, and better controlled (i.e. genetic and environmental factors) than experimenting on human samples. All experiments in these studies were performed in accordance with the South Australian Health and Medical Research Institute (SAHMRI) Animal Ethics Committee

approvals (SAM195, SAM190, and SAM234). Multiple genotypes of mice were utilised in this study, as detailed below; a summary of all mouse lines can be found in **Table 4.1**. Mice housed within the SAHMRI facility were group-housed (5 mice per cage) within individual ventilated cages filled with chip coarse dust-free aspen bedding (Cat#–ASPJMAEBCA; PuraBed, Niederglatt, Switzerland). Cages were stored on individual ventilated cage racks within a temperature-controlled environment of 22°C and a 12-hour light/12-hour dark cycle. Mice had free access to LabDiet JL Rat and Mouse/Auto6F chow (Cat #5K52, St. Louis, MO) and autoclaved reverse osmosis water. Sample sizes of each experiment are detailed in the corresponding figure legends.

Table 4.1: Summary of mouse lines used throughout study

Mouse line	Phenotype	Origin	Projects (Chapters)
C57BL/6J	Wildtype	JAX	All (5 - 8)
<i>Trpv1</i> ^{-/-}	<i>Trpv1</i> null	JAX	Histamine (5), TGR5 (7)
<i>Trpa1</i> ^{-/-}	<i>Trpa1</i> null	David Corey	Histamine (5), TGR5 (7)
<i>Gpbar1</i> ^{-/-}	TGR5 null	Johan Auwerx and Kristina Schoonjans	TGR5 (7)
<i>Nav1.8-Gpbar1</i> ^{-/-}	TGR5 null specific to Nav1.8-expressing neurons	Daniel Poole	TGR5 (7)
<i>Mrgpr-clusterΔ</i> ^{-/-}	Lacking ~50% functional Mrgprs including A3 and C11	Xinzhong Dong	Mrgprs (6)

4.2.1 Wildtype C57BL/6J mice

The ‘wildtype’ mouse strain used in these studies was a C57BL/6J strain obtained via an in-house breeding colony at SAHMRI. These mice were originally acquired from an in-house C57BL/6J breeding program at The Jackson Laboratory (JAX strain #000664; breeding barn MP14; Bar Harbor, ME) and then bred within the specific and opportunistic pathogen-free animal care facility at SAHMRI.

4.2.2 *Trpv1*^{-/-} mice

A genetically modified mouse strain lacking a functional gene encoding for the Transient Receptor Potential Vanilloid 1 (TRPV1) ion channel (*Trpv1*^{-/-}) was used in some experiments. The *Trpv1*^{-/-} mouse strain, developed by David Julius by disrupting the *Trpv1* gene via exon deletion [1], was originally acquired from an in-house breeding program at The Jackson Laboratory (JAX stock #003770; Bar Harbor, ME) with a C57BL/6J genetic background, and then bred in-house at SAHMRI as per the wildtype mice.

4.2.3 *Trpa1*^{-/-} mice

A genetically modified mouse strain lacking a functional gene encoding for the Transient Receptor Potential Ankyrin 1 (TRPA1) ion channel (*Trpa1*^{-/-}) was used in some experiments. The *Trpa1*^{-/-} mouse strain, with a mixed C57BL/6-B6129P1/F2J background, was developed by deletion of exons transcribing the pore-forming unit of the channel and replacing them with an endoplasmic reticulum (ER) retention signal to ensure any truncated proteins produced are sequestered in the ER [2]. The *Trpa1*^{-/-} mouse strain was gifted to us by the original developer, David Corey (Harvard University, Cambridge, Massachusetts, USA), and then bred in-house at SAHMRI as per the wildtype mice.

4.2.4 *Gpbar1*^{-/-} mice

A genetically modified mouse strain lacking the *Gpbar1* gene encoding for TGR5 was used for some experiments. This mouse strain was originally gifted to us by Johan Auwerx and Kristina Schoonjans (Ecole Polytechnique de Lausanne (EPFL), Lausanne, Switzerland), who developed the line by creating a TGR5-floxed strain and removing the gene via Cre-recombinase technology [3]. The mice provided by Auwerx and Schoonjans were then bred in-house at SAHMRI as per the wildtype mice.

4.2.5 *Nav1.8-Gpbar1*^{-/-} mice

A genetically modified mouse strain with localised deletion of the *Gpbar1* gene specific to voltage-gated sodium (*Nav*)1.8-expressing neurons was also used for several experiments. These mice were generated at Monash University by crossing the above-mentioned TGR5-floxed mice [3] with a *Nav1.8-Cre* recombinase mouse line [4], and were gifted to us by Daniel Poole (Monash University, Victoria, Australia), then housed at SAHMRI's Preclinical, Imaging, and Research Laboratories (PIRL) facility for procedures.

4.2.6 *Mrgpr-clusterΔ*^{-/-} mice

A genetically modified mouse strain, with clustered deletion of genes encoding for approximately 50% of functional Mrgprs including *MrgprA3* and *MrgprC11*, was used for some experiments. This mouse strain, generated via a floxed *MrgprA1-MrgprB4* targeting vector and Cre/loxP-mediated recombination, was developed and gifted to us by Xinzhong Dong (Johns Hopkins University, Baltimore, Maryland, USA). These mice were then bred in-house at SAHMRI as per the wildtype mice.

4.3 mRNA expression analysis in DRG, cultured urothelial cells and bladder mucosa and detrusor layers

Quantitative reverse-transcription polymerase chain reaction (QRT-PCR) was performed to determine the relative abundance of mRNA for target GPCRs relating to itch sensation as well as several TRP channels in LS DRG pairs, bladder tissue layers, and isolated urothelial cells.

4.3.1 Tissue collection

Tissue was collected from 16- to 18-week-old male mice to age- and gender-match *in vitro* functional data from calcium imaging experiments (**Chapter 4.7**). Mice were humanely euthanised via CO₂ inhalation and the bladder and LS (L5-S1) DRG removed. Previous studies have identified that the majority of bladder afferent neuronal cell bodies are located within these DRG [5, 6]. LS DRG were identified using vertebrae as guides; each spinal nerve emerges from the spinal cord below the vertebra after which it is named (e.g. L5 spinal nerve exits between vertebrae L5 and L6) [7, 8], with the lower rib used as a marker for T13 (**Figure 4.2**). DRG were frozen in pairs by spinal level (L5, L6, S1) in dry ice and stored at -80°C for RNA extraction and QRT-PCR.

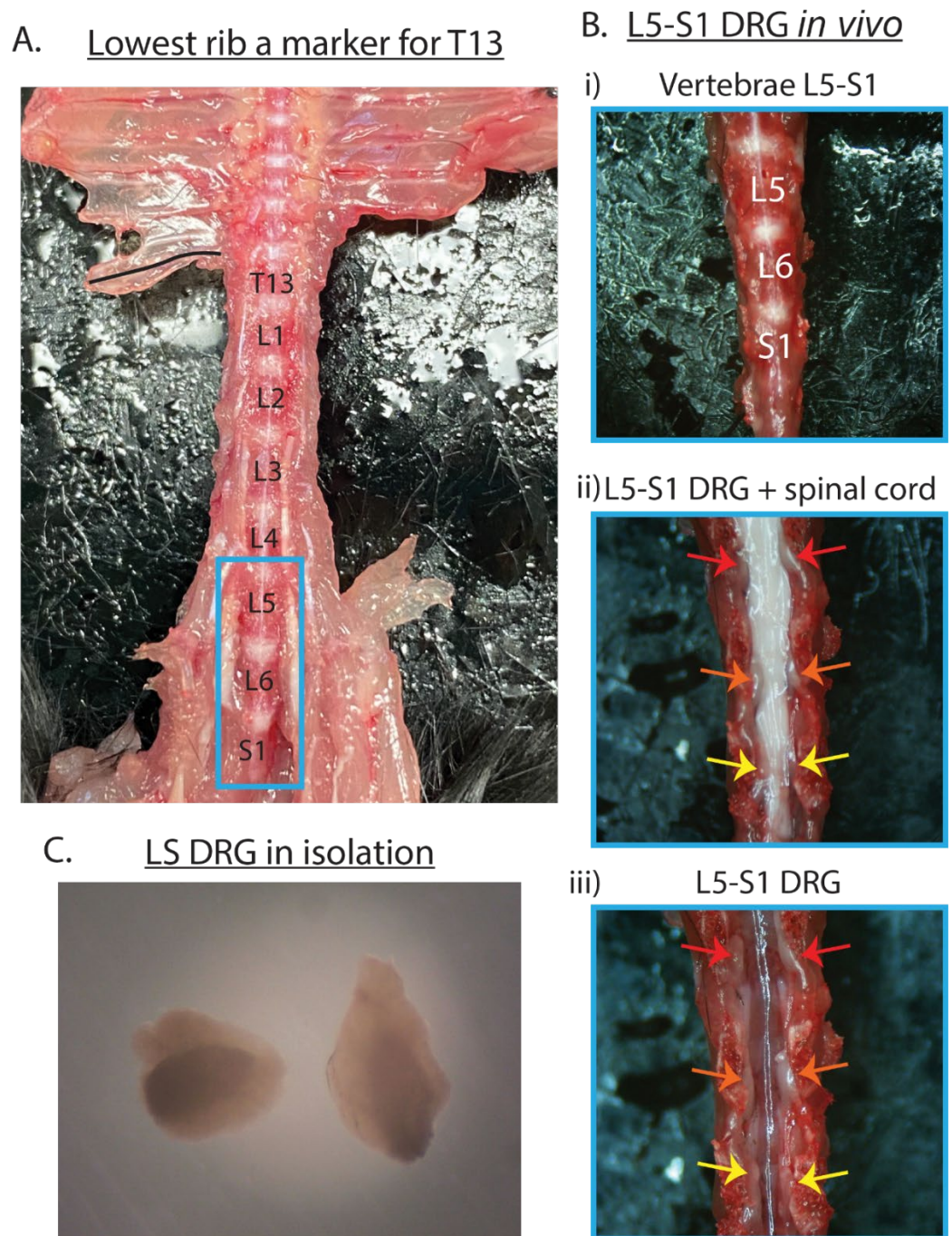


Figure 4.2: Lumbosacral dorsal root ganglia (LS DRG) sit aside the spinal cord.

LS DRG from L5, L6, and S1 were carefully extracted bilaterally and frozen in pairs for QT-PCR analysis or grouped and cultured for functional imaging assays. **(A)** DRG levels were determined using vertebrae as guides, with the bottom rib (overlaid with black line) used to identify T13. Vertebrae are labelled, with a blue box highlighting the L5-S1 region of interest. **(B)** **(i)** spinal projections and connected tissue are removed for ease of access. **(ii)** The ventral half of the spinal column is removed, revealing the spinal cord and DRG, located along each side of the spinal cord, tucked underneath the bones of the vertebrae (L5 = red arrows, L6 = orange arrows, S1 = yellow arrows). **(iii)** the spinal cord has been removed for clearer visualisation of the L5-S1 DRG *in vivo*. **(C)** The DRG are isolated and excess spinal roots and connective tissue are carefully removed *in vitro*, ready for either freezing in pairs (RT-PCR) or dissociation and culture (calcium imaging).

Due to distinct features of the different layers of the bladder, the bladder mucosa and detrusor muscle were separated for analysis. Bladders were removed and dissected in cold, sterile PBS with a single cut from the urethral opening along one side to the dome (**Figure 4.3A**), pinned and stretched out urothelial side up (**Figure 4.3B**). For tissue QRT-PCR, the mucosal and detrusor layers were gently peeled apart (**Figure 4.3C**), frozen in dry ice, and stored at -80°C for RNA extraction and QRT-PCR.



Figure 4.3: Bladder tissue layers were separated for QT-PCR analysis.

Mouse bladders were **A**) opened with a single cut from the urethral opening along one side to the dome, **B**) pinned and stretched out, urothelial side up, and **C**) mucosal and detrusor layers gently peeled apart for tissue collection.

4.3.2 Isolation of mouse urothelial cells

Primary mouse urothelial cells were also isolated for mRNA analysis. The urothelium is known to play an important role in bladder sensory signalling via an array of receptors expressed within urothelial cells, however the full spectrum is not known. Irritants can be present within the urine, and the urothelium interacts directly with the external environment (including urinary contents). Therefore, determining the expression of irritant sensing receptors in urothelial cells is an important component to identifying irritant-sensing mechanisms in the bladder. Urothelial cell isolation was performed as per previous studies [9, 10]. Following removal, stretching, and pinning as per tissue collection (**Figure 4.3A** and **B**), bladders were rinsed briefly with fresh PBS and then incubated in 2.5 mg/mL dispase (GIBCO, ThermoFisher Scientific, #17105041) dissolved in Dulbecco's Modified Eagle Media (DMEM; GIBCO, ThermoFisher Scientific, #11995065) containing 10% Foetal Calf Serum and 1 μ M/mL HEPES (Sigma, #H3375, pH 7.0) for 3 hours at room temperature. Urothelial cells were gently scraped from the bladder with a blunt scalpel and dissociated in 0.025% trypsin EDTA (GIBCO, ThermoFisher Scientific, #25200072) at 37°C in an incubator with 5% CO₂ supply for 10 min with gentle intermittent trituration. The trypsin was then deactivated by adding the cell suspension to DMEM containing 10% Foetal Calf Serum prior to centrifugation (15 min, 1500 rpm, 4°C). Cells were then resuspended in keratinocyte serum-free media (KSFM; Invitrogen, #17005042) and cell count and

viability were determined using the Countess Automated Cell Counter (Invitrogen, ThermoFisher Scientific). Average ($\pm$ SD) viability of primary urothelial cells was 81.4% ($\pm$ 12.3), with an average ($\pm$ SD) of 4.6×10^5 ($\pm$ 2.4×10^5) total cells/mL. Cells were then pelleted, frozen in dry ice, and stored at -80°C for RNA extraction and QRT-PCR.

4.3.3 RNA extraction

RNA was extracted using the PureLink RNA Micro kit (Invitrogen, Victoria, Australia, #12183-016; DRG pairs and isolated urothelial cells) or the PureLink RNA Mini kit (Invitrogen, #12183018A; bladder mucosa and detrusor layers) with DNase treatment (Life Technologies, #12185-010) according to the manufacturer's instructions. To increase yield per volume for DRG pairs and urothelial cells, final volume was halved to 22 μL by running the same elution twice through the membrane for RNA collection. A NanoDrop Lite spectrophotometer (ThermoFisher Scientific) was used to determine RNA quantity and purity, determined by ratio of RNA/DNA absorbance (A260) to protein absorbance (A280), with RNA purity ratio of ~ 2.00 (**Table 4.2**). RNA was either frozen and stored at -80°C for future reverse-transcription or immediately reverse-transcribed to cDNA using SuperScript VILO Master Mix (Invitrogen, #11755250) as per the manufacturer's instructions. cDNA was stored at -20°C for QRT-PCR.

Table 4.2: RNA concentration and purity averages

Tissue type	RNA concentration (ng/ μL ; $\pm$ SD)	RNA purity (Fluorescence ratio A260/A280; $\pm$ SD)
Urothelial cells	18.45 ± 8.53	1.90 ± 0.20
Mucosa	213.69 ± 81.77	2.10 ± 0.02
Detrusor	268.71 ± 149.37	2.09 ± 0.01
L5 DRG pairs	16.31 ± 8.40	2.03 ± 0.11
L6 DRG pairs	18.51 ± 10.10	2.10 ± 0.16
S1 DRG pairs	18.63 ± 9.46	2.05 ± 0.19

4.3.4 Quantitative reverse-transcription polymerase chain reaction (QRT-PCR)

QRT-PCR was performed using Taqman Gene Expression Master Mix (Applied Biosystems, Victoria, Australia, #4369016) with commercially available hydrolysis probes (TaqMan; Life Technologies, see **Table 4.3** for details) and RNase-free water (AMBION, Victoria, Australia, #AM9916). For each reaction, 10 μL of QRT-PCR MasterMix, 1 μL of TaqMan primer assay, 4 μL of water, and 5 μL of cDNA (diluted in RNA-free H_2O ; 1:2 for DRG pairs and urothelial cells, 1:10 for

mucosa and detrusor layers) from each sample was tested in duplicate for each target. MasterMix, water, and cDNA were premixed first for each plate or set of plates and added to wells as a 19 μ L mixture to ensure even distribution of cDNA between wells, with TaqMan primer added to individual wells. Negative controls were run for each primer in the absence of cDNA. Assays were run for 45 cycles on a 7500 Fast Real-Time PCR System (Applied Biosystems) machine, using 7500 Fast software v2.0.6.

4.3.4.1 Reference genes

mRNA quantities of target genes were determined relative to reference genes, selected for their high expression levels and consistent expression, and expressed as $2^{-\Delta C_t}$ relative to reference gene *Actb* (DRG pairs) or *Gapdh* x *Hprt* (geometric mean; mucosa, detrusor and urothelial cells). No validation assays were performed in this study, as no treatment was performed to potentially initiate altered gene expression and each reference gene is located on chromosomes distinct from any genetic knockout activity. Further, the reference genes selected are commonly used in their respective tissue types [11-14]. Comparisons were performed within tissue types and not between, thus differential expression of reference genes between tissue type is not a confounding factor in these studies.

Actb (β -actin) is a common housekeeping gene involved in cell motility, structure, and integrity, and is highly abundant. *Actb* has been demonstrated as a stable reference gene for PCR analysis of rodent DRG previously [11, 12], so was used in this study as an endogenous control for DRG pairs. A single reference gene was used for DRG pairs due to limited RNA yield, to maximise the RNA concentrations usable per target gene.

For mucosa and detrusor tissue layers and isolated urothelial cells, the geometric mean of two stable endogenous reference genes, *Gapdh* and *Hprt*, was used to determine relative target gene expression; this technique allows for increased accuracy in analysing gene expression variation, by reducing the impact of any reference gene expression variation [15]. *Gapdh*, the gene for enzyme Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is a commonly used reference gene due to its role in cellular metabolism. *Hprt* transcribes for hypoxanthine phosphoribosyltransferase, an enzyme involved in nucleotide recycling. Both *Gapdh* and *Hprt* are commonly used reference genes and have been independently identified as stable reference genes within bladder tissue and cells [13, 14].

Table 4.3: Primers used for QRT-PCR receptor expression assays

Gene Alias	Gene Target	Assay ID
β -actin (reference gene)	<i>Actb</i>	Mm00607939_s1
Hypoxanthine Phosphoribosyltransferase 1 (HPRT; reference gene)	<i>Hprt</i>	Mm00446968_m1
Glyceraldehyde 3-phosphate dehydrogenase (<i>Gapdh</i> ; reference gene)	<i>Gapdh</i>	Mm99999915_g1
Histamine Receptor subtype 1	<i>Hrh1</i>	Mm00434002_s1
Histamine Receptor subtype 2	<i>Hrh2</i>	Mm00434009_s1
Histamine Receptor subtype 3	<i>Hrh3</i>	Mm00446706_m1
Histamine Receptor subtype 4	<i>Hrh4</i>	Mm00467634_m1
Transient receptor potential vanilloid 1	<i>Trpv1</i>	Mm01246300_m1
Transient receptor potential ankyrin 1	<i>Trpa1</i>	Mm01227437_m1
Transient receptor potential vanilloid 4	<i>Trpv4</i>	Mm00499025_m1
Takeda G-protein Receptor 5 (TGR5)	<i>Gpbar1</i>	Mm04212121_s1
Mas-related G-protein Receptor A3	<i>Mrgpra3</i>	Mm02620679_s1
Mas-related G-protein Receptor C11	<i>Mrgprx1</i>	Mm02525847_g1
Mas-related G-protein Receptor D	<i>Mrgprd</i>	Mm01701850_s1

4.4 Retrograde tracing

Bladder-innervating DRG neurons were labelled for identification within a DRG culture using a retrograde tracing fluorescent dye injected into the bladder wall. A small, aseptic, abdominal incision was made in anaesthetised (2% – 4% isoflurane in oxygen) mice, and the bladder gently exposed through the incision. Cholera Toxin subunit B conjugated to AlexaFluor 488 (CTB-488, 0.5% diluted in 0.1 M phosphate buffered saline [PBS] pH 7.4; ThermoFisher Scientific) was injected at 2 – 3 sites into the bladder wall (3 μ L/injection) using a 5- μ L Hamilton syringe attached to a 30-gauge needle [16, 17]. The needle was inserted subserosally, parallel with the bladder muscle, to ensure that CTB was not injected into the bladder lumen. The abdominal incision was sutured closed and analgesic (Buprenorphine (Temvet); 0.1 mg/kg; Troy Laboratories Pty Ltd, APVMA #67612) and antibiotic (Amoxicillin; 50 mg/kg; Amoxil, AUSTR11137) administered subcutaneously as mice regained consciousness. Mice were then housed individually and allowed to recover. CTB binds to ganglioside GM1, a cell surface molecule predominant in the nervous system, resulting in endocytosis and retrograde axonal transport to the cell body [18]. As such, the fluorescent conjugate AF488 is also

retrogradely transported to the cell body. After four days recovery, during which time the retrograde tracing dye traverses from the peripheral nerve endings within the bladder wall to the cell bodies, mice were humanely euthanised for subsequent LS (L5-S1) DRG removal and the neurons isolated and cultured to visualise CTB-labelled bladder innervating neurons amongst the DRG neurons.

4.5 Cell culture of bladder-innervating DRG neurons

Following retrograde bladder neuron tracing, LS DRG were removed and digested and the neuronal cell bodies cultured for functional calcium imaging assays; bladder-innervating DRG neurons could be clearly identified within the culture via CTB-488 fluorescence for imaging experiments (see **Figure 4.4A** below). Mice were humanely euthanised via CO₂ inhalation and LS DRG were removed. DRG were digested in 2.5 mg/mL collagenase II (GIBCO, ThermoFisher Scientific, #17101015) and 4.5 mg/mL dispase (GIBCO, ThermoFisher Scientific, #17105041) dissolved in Hanks' balanced salt solution (HBSS; pH 7.4; Life Technologies, #14170161) at 37°C for 30 min. The collagenase-dispase solution was carefully aspirated and replaced with HBSS containing collagenase (4.5 mg/mL) only for 10 min at 37°C. Following aspiration of the collagenase solution and subsequent washes in HBSS (2 washes in 2 mL), DRG were gently resuspended in 600 µL complete DMEM (Dulbecco's Modified Eagle Media [DMEM; GIBCO, ThermoFisher Scientific, #11995065]; 10% Foetal Calf Serum [Invitrogen, ThermoFisher Scientific, MA, USA]; 2 mM L-glutamine [GIBCO, ThermoFisher Scientific, #25030081], 100 µM MEM non-essential amino acids [GIBCO, ThermoFisher Scientific, #11140076], 100 mg/mL penicillin/streptomycin [GIBCO, ThermoFisher Scientific, #15070063], and 96 µg/L nerve growth factor-7S [Sigma, N0513-0.1MG]), and mechanically disrupted to dissociate the cells via trituration through fire-polished Pasteur pipettes of descending diameter, and centrifuged at 50 g for 1 min [17, 19-21]. Dissociated neurons were resuspended in 360 µL complete DMEM and spot-plated (30 µL) onto 13 mm glass coverslips pre-coated with laminin (20 µg/mL; Sigma-Aldrich, #L2020) and poly-D-lysine (800 µg/mL; ThermoFisher Scientific). Coverslips were incubated at 37°C in 5% CO₂ for 2-3 hours to allow neurons to adhere before carefully flooding each well with 1.7 mL complete DMEM. Cultured neurons were maintained in an incubator at 37°C in 5% CO₂ for 18-48 hours for use in calcium imaging experiments.

4.6 Cell culture of primary mouse urothelial cells

Primary mouse urothelial cells were isolated and cultured for functional calcium imaging assays to determine the functional contribution of itch receptors in urothelial signalling, an integral component to bladder sensory signalling. Isolation of urothelial cells was performed as for mRNA

analysis (**Chapter 4.3.2**) and cultured as per previous studies [9, 10]. Following resuspension in KSM and cell counts, cells were plated onto collagen I-coated coverslips (diluted 1:20 in H₂O; Sigma; #A10483-01). Coverslips were incubated for 4 hours at 37°C in 5% CO₂ to allow the cells to settle and attach before gently flooding with complete KSM (Invitrogen, #17005042; includes provided supplements bovine pituitary extract [BPE; 50 µg/mL] and human recombinant epidermal growth factor [rEGF; 5 ng/mL]) (2 mL/well). Cells were maintained at 37°C in 5% CO₂ for 24-48 hours for calcium imaging experiments.

4.7 Calcium imaging of cultured urothelial cells and DRG neurons

Live cell calcium imaging was performed in isolated cultures to indicate activation of GPCRs or ion channels via the indirect measurement of relative changes in intracellular levels of the second messenger calcium following application of agonists. Calcium ions are integral for intracellular signalling; activation of ion channels and GPCRs results in Ca²⁺ influx, either directly (ion channels) or indirectly via signal transduction pathways (GPCRs) [22, 23]. As such, analysis of changes in intracellular Ca²⁺ concentrations is a commonly used technique to determine activation of ion channels and GPCRs.

Cultured LS DRG neurons and urothelial cells were loaded with 2 µM of the ratiometric calcium indicator Fura-2-acetoxymethyl ester (Fura-2; Invitrogen, ThermoFisher Scientific, #F1221) in 0.01% pluronic F-127 (Invitrogen, ThermoFisher Scientific, #P3000MP) for 30 min at 37°C and washed with HEPES buffer (10 mM HEPES sodium salt [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt; Sigma, #H7006-100G], 140 mM NaCl [Chem Supply, #SA046-3KG], 4 mM KCl [Chem Supply, #PA054-500G], 5 mM D-glucose anhydrous [Chem Supply, #GA018-500G], 2 mM CaCl₂ [Scharlau, #CA01951000], and 2 mM MgCl₂ [Sigma, #M8266-100G], pH 7.40) for 10 min before imaging at room temperature (~23°C). Following entry into the cell, the esters of Fura-2 are cleaved by intracellular esterases, preventing leakage of Fura-2 from the cells throughout the experiment. As a ratiometric dye, Fura-2 is excited at different wavelengths depending on whether it is bound to calcium; the resulting fluorescence ratio reduces error and fluctuation [24]. Fura-2 was excited at 340 nm, at which wavelength Ca²⁺-bound Fura-2 ions fluoresce, and 380 nm, at which wavelength the unbound or 'free anion' Fura-2 ions fluoresce. Emissions were measured at 510 nm using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, an Omega XF04 filter set for Fura-2, a Photonic Science ISIS-3 intensified CCD camera, and Universal Interface Card MetaFluor software. Fluorescence images (340 and 380 nm) were obtained using a 20x objective, every 5 s leading up to, during, and immediately following drug application, and every 20 s for washout periods. Data were recorded and further analysed offline using MetaFluor software.

After an initial baseline reading of approximately 5 min to ensure cell fluorescence was both low and stable, indicating healthy cells, DRG neurons and urothelial cells were stimulated with each agonist as per the protocols detailed below (**Chapter 4.7.3**), and changes in intracellular calcium (Ca^{2+}_i) were monitored in real time. Ionomycin (2 μM ; Sigma-Aldrich, #I0634, CAS: 6092-82-1) or KCl (40 mM) were applied as positive controls in urothelial and neuronal experiments, respectively. Ca^{2+}_i is expressed as the ratio of the fluorescence signals at 340 and 380 nm (Fura-2 (340/380)), with a low ratio indicating predominant 380 nm fluorescence and hence low Ca^{2+}_i and a high ratio indicating predominant 340 nm fluorescence and hence high Ca^{2+}_i . Baseline Ca^{2+}_i was determined by averaging 8-12 fluorescence ratio readings immediately prior to stimulation. As the resting cytoplasmic Ca^{2+} concentrations are maintained at concentrations approximately 10,000-fold lower than extracellular Ca^{2+} [23], a low fluorescence ratio is considered healthy; cells with a baseline fluorescence ratio of higher than ~ 0.8 were excluded as this indicates a disruption in the mechanisms responsible for normal intracellular calcium homeostasis. Peak response was calculated by subtracting the relevant baseline score from the maximum fluorescence ratio reading following drug application ($\Delta 340/380$). Baseline fluorescence was normalised to a ratio of 1 for example traces for clear visual comparison.

4.7.1 LS DRG cell culture imaging

LS DRG cell cultures were initially observed under light microscopy for a visual assessment of cell health, including cell body roundness, presence of dendrites, adhesion, and lack of granulation (**Figure 4.4A**). Retrogradely traced bladder DRG neurons were identified via excitation at 480 nm of the CTB-488 tracer (**Figure 4.4B**). 480 nm excitation was used as an initial identification step only and not throughout the experiment, to prevent unnecessary photobleaching. An example DRG culture experiment is shown in **Figure 4.4**: short-duration (1 minute) applications of target-specific agonists initiate transient increases in intracellular calcium in cells functionally expressing the target receptor, reflected as increases in the Fura-2 fluorescence ratio, which then return to baseline after a washout period (**Figure 4.4C**). The population of cells responding as well as the peak and shape of the responses varies depending on the characteristics of the target receptor.

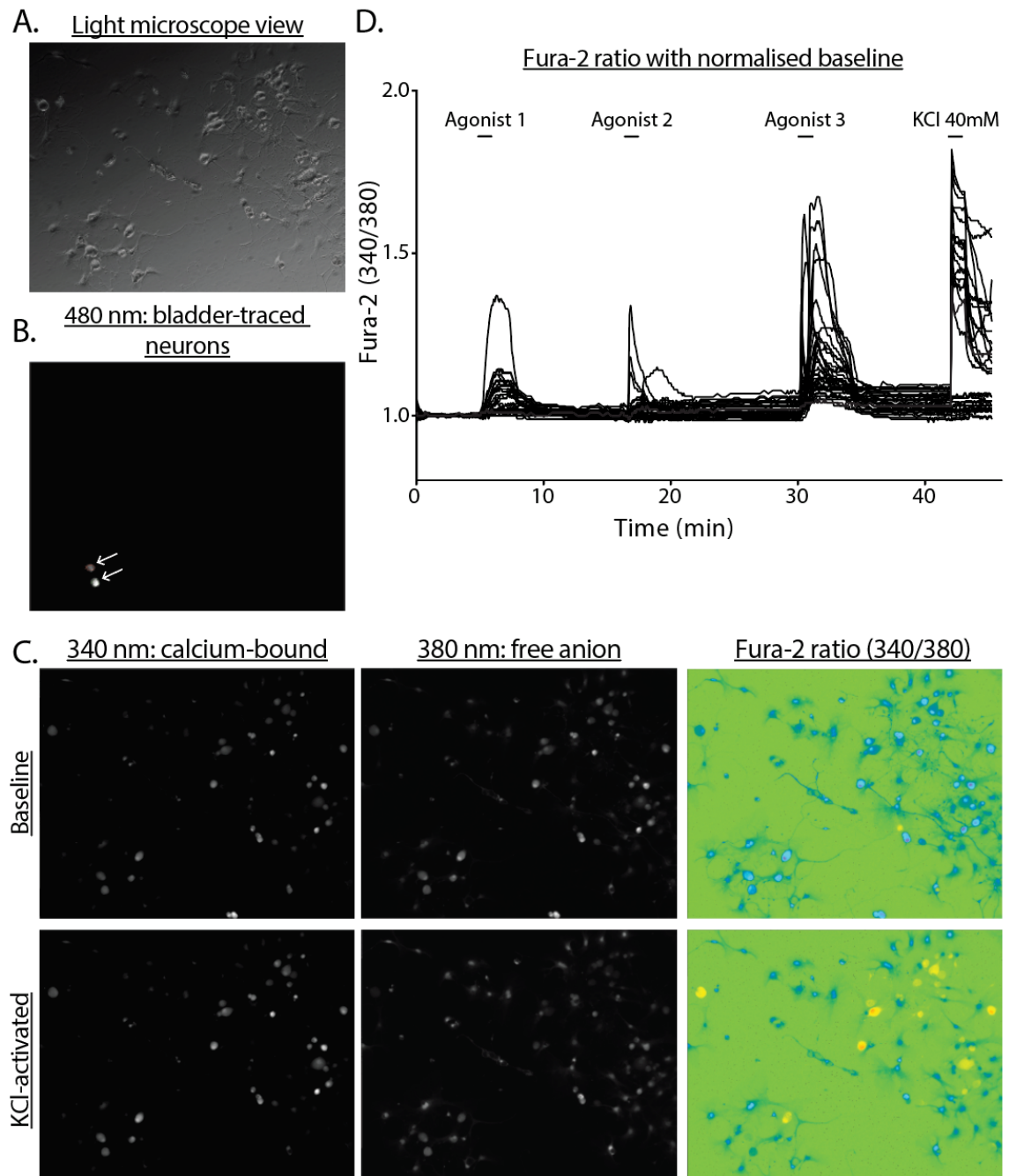


Figure 4.4: Example calcium imaging experiment in a C57BL/6J LS DRG culture

Fura-2-incubated coverslips are loaded into the microscope well-plate. **(A)** Cells can be imaged under light filter to assess cell quality and morphology. **(B)** Retrogradely labelled bladder-innervating neurons can be clearly identified when excited at 480 nm wavelength (white arrows). **(C)** Calcium-bound Fura-2 molecules fluoresce at 340 nm, whereas free Fura-2 anions fluoresce at 380 nm. Throughout the experiment, images are taken at both wavelengths and the ratio determined, indicating changes in intracellular calcium in each cell. KCl is applied at the end of each DRG experiment as a positive control for neurons; non-neuronal cells are also measured, but do not respond to KCl. **(D)** The baseline, calculated from an average of the ratio prior to application of the first agonist, is normalised to 1 for each cell and the ratio at each imaged timepoint is graphed for each cell. An increase in Fura-2 ratio indicates an increase in intracellular calcium.

4.7.2 Urothelial cell culture imaging

As with LS DRG cultures, primary urothelial cultures were initially observed under light microscopy for a visual assessment of cell health, including cell shape, adhesion, and lack of granulation (**Figure 4.5A**). To confirm the efficacy of the primary urothelial cell culture protocol, initial urothelial cell cultures were tested for intracellular calcium responses by application of ATP (10 μ M; Sigma-Aldrich, # A9187, CAS: 74804-12-9). Cultured cells exhibited consistent and timely increases in intracellular calcium levels in response to ATP application (**Figure 4.5B**), indicating physiological responsiveness of primary urothelial cells in culture.

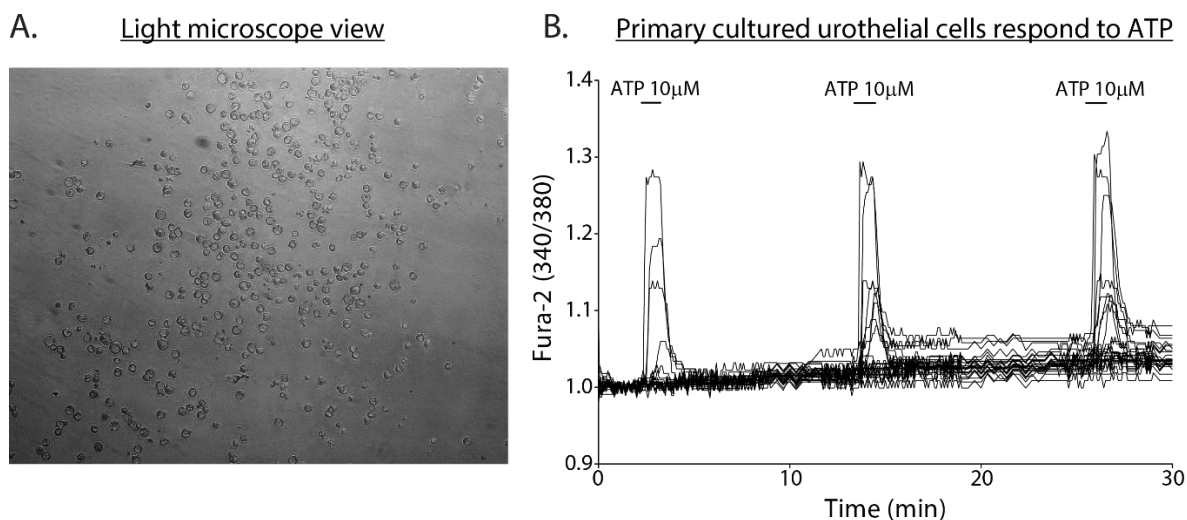


Figure 4.5: Primary cultured urothelial cells demonstrate an increase in intracellular calcium levels in response to 10 μ M ATP.

Primary mouse urothelial cells were cultured for functional calcium imaging assays. **A)** cell quality was assessed visually via light microscopy prior to each experiment. **B)** preliminary experiments assessing responsivity of cultured urothelial cells to 10 μ M ATP were performed to confirm the efficacy of the isolation and culture techniques. ATP was applied to cells via perfusion multiple times with washout periods to allow cells to return to baseline fluorescence.

4.7.3 Experimental protocols

For each project, protocol design aimed to minimise any confounding effects from desensitisation, and each protocol was standardised as much as possible to enable comparisons between cultures. To this end, all protocols started with a 5-minute baseline measurement period, with the GPCR target agonist applied first. All agonists were applied for 1 minute, deemed a sufficient timeframe to allow intracellular calcium changes in response to GPCR activation [25]. Washout periods were determined to allow intracellular calcium levels to return to baseline prior to addition of the next agonist. Where possible, agonists and wash buffer were perfused at a steady state to prevent mechanical disruption. Where volume or flow rate limitations did not allow for perfusion,

agonists were gently and gradually pipetted into the bath away from the cells to minimise mechanical disruption. Protocols for each project are detailed below and summarised in **Figure 4.6**.

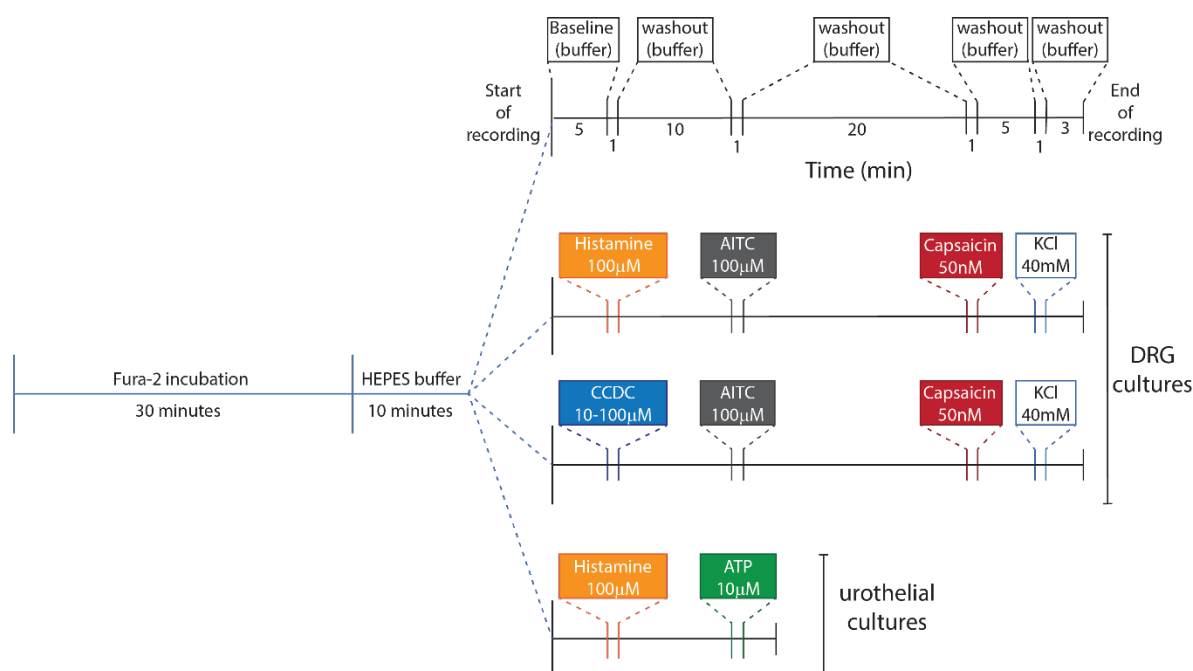


Figure 4.6: Flow chart depicting calcium imaging protocols for each project

Incubation and imaging protocols for DRG and urothelial cultures are depicted to scale for each experimental set. From the left: each coverslip was incubated in Fura-2 for 30 minutes before a 10-minute wash step in HEPES buffer. From the top: the first 5 minutes of each recording was in buffer solution to create a clear baseline. Each agonist was applied for 1 minute, with washout periods in buffer after each. DRG cultures were imaged with applications of target agonists 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N,5-dimethyl-4-isoxazolecarboxamide (CCDC; 10-100 µM) for TGR5 experiments and histamine (100 µM) for histamine receptor experiments. Allyl isothiocyanate (AITC; 100 µM) and capsaicin (50 nM) were applied to some DRG culture experiments to determine co-activation of TRPA1 or TRPV1 respectively with the target receptor(s). KCl (40 mM) was applied at the end as a positive neuronal activator. Primary urothelial cultures were imaged with histamine (100 µM) followed by ionomycin (2 µM) as a positive control.

4.8 *In vivo* spinal dorsal horn activation

To determine whether the activation of peripheral sensory neurons within the bladder translates centrally, the activation of neurons within the spinal dorsal horn was determined following peripheral stimulation of the bladder. *In vivo* intravesical infusion of compounds into the bladder with or without controlled bladder distension was performed as a means of peripheral stimulation. Each component of this procedure is discussed in detail below. In summary: under anaesthesia, female mice were catheterised for controlled bladder distension, infusion of GPCR agonists into the bladder, or a combination of the two. Immediately following *in vivo* bladder stimulation, mice were humanely killed

via Lethabarb injection and perfuse-fixed. The lumbosacral spinal cord was then removed, post-fixed and processed for immunohistochemical staining, imaging, and analysis.

Spinal activation was determined by immunolabelling for neuronal activation marker phosphorylated-MAP-kinase ERK 1/2 (pERK). While c-Fos is also a common marker for neuronal activation, ERK phosphorylation occurs far more rapidly following stimulus application, occurring in a matter of minutes compared to the hours required for c-Fos activation [26]. As the stimuli applied in this study are short duration, the rapidity of pERK activation provides a more appropriate representation, and as such quantification of pERK immunoreactivity (pERK-IR) is increasingly being used to determine spinal activation in response to peripheral stimuli [17, 19, 27, 28].

4.8.1 *In vivo* bladder infusion

In order to determine the central effect of agonist-induced receptor activation within the bladder, *in vivo* bladder infusion was performed in anaesthetised mice [17]. 12-week-old female mice were anaesthetised (isoflurane 2% - 4% in oxygen) and a catheter (PE 50 tubing) was inserted into the bladder via the urethra. To determine correct catheter placement, the catheter was inserted gently until meeting resistance (bladder dome) and retracted 2-3 mm to avoid damage to the bladder wall. A suction syringe was used to remove any urine from the bladder and urethra prior to bladder filling. Following this, a second catheter (PE 50 tubing) primed with either vehicle (saline) or target receptor compound was inserted, and 100 μ L of solution was then gently infused to fill but not overly distend the bladder and allowed to incubate for 5 minutes. Observations including time taken for and difficulty of catheterisation, and any damage to the urethra, were noted as any resulting inflammation or significant delay may impact pERK signalling. For infusion-only experiments (no distension), following removal of the compound, mice were administered an anaesthetic overdose (intraperitoneal injection, 0.125 mL/250 g Lethabarb®; Virbac Australia) followed by transcardial perfuse fixation.

4.8.2 *In vivo* bladder distension

To determine the impact that receptor activation within the bladder has on central activation in response to mechanosensitivity, controlled bladder distensions were performed post agonist infusion. Following the 5-minute incubation period described above, the compound was carefully removed from the bladder to ensure as little liquid remains as possible. A third, dry catheter was inserted, and a small length of 4-0 perma-hand silk (Ethicon, #LA53G) was knotted over the urethral opening to fix the catheter into place and minimise loss of pressure. A 5-minute wait was allowed

between tying the catheter and performing the distensions to limit false-positive pERK signalling from the act of tying. A sphygmomanometer was attached between the catheter and a 10 mL air-filled suction syringe. 5 x 20-second bladder distensions were performed at 10-second intervals, at a holding pressure of 20 or 40 mmHg, considered low or physiological distension pressures, or 60 mmHg, considered a pressure level of discomfort or pain. Observations of time between infusion and tying, visible distension via the abdomen, liquid in the distension tubing, or leaking and inconsistent pressure holding were noted, as these factors can impact the reliability of the intraluminal pressure reading and hence pERK signalling. Following the fifth 20-second distension, mice were administered an anaesthetic overdose (intraperitoneal injection, 0.125 mL/250 g Lethobarb®) followed by transcardial perfuse fixation.

4.8.3 Transcardial perfuse fixation and tissue dissection

After anaesthetic overdose, the thoracic cavity was opened and 0.5 mL heparinised saline (50 IU in 5 mL; Pfizer) was injected into the left ventricle to maximise perfusion and prevent clotting, followed by insertion of a 22-gauge needle attached to tubing and a peristaltic perfusion pump. The right atrium was cut open allowing for drainage of perfusate. Warm phosphate buffer (0.1 M; 21.7 mM NaH₂PO₄ [Chem Supply, #SA061-500G], 81 mM Na₂HPO₄ [VWR Chemicals, #102494C], pH 7.2) was perfused to clear the tissue of perfusate and allow vessels to relax. Visible clearance of blood from the liver was used as an indicator of overall clearance. Following clearance, ice-cold 4% paraformaldehyde (PFA; Sigma-Aldrich, #158127) in 0.1 M phosphate buffer was perfused. The time taken between anaesthetic overdose and initial PFA perfusion was recorded (approximately 6 – 8 minutes). After complete PFA perfusion, the bladder and the lumbosacral spinal cord (determined by the level of dorsal root ganglia (DRG) root insertion points; the lowest rib was used as an anatomical marker of DRG level T13) were removed and postfixed in 4% PFA in 0.1 M phosphate buffer at 4°C for 18 to 20 hours. After post-fixation, spinal cords and bladders were cryoprotected in 30% sucrose/phosphate buffer (Sigma-Aldrich, #S9378) for two days at 4°C, followed by a 48-hour incubation at 4°C in 50% optimal cutting temperature (OCT; VWR, #C361603E)/30% sucrose/phosphate buffer solution before freezing in 100% OCT using liquid nitrogen-cooled 2-methylbutane (Sigma-Aldrich, #M32631). Frozen sections of the spinal cord were cryosectioned (10 µm thickness) serially and distributed over 6 gelatin-coated slides for immunohistochemical processing and labelling of pERK. The number of pERK-immunoreactive (pERK-IR) neurons were counted in lumbosacral spinal cord sections, with spinal segments determined *ex vivo* by the shape of the dorsal horn (**Figure 4.7** [29]).

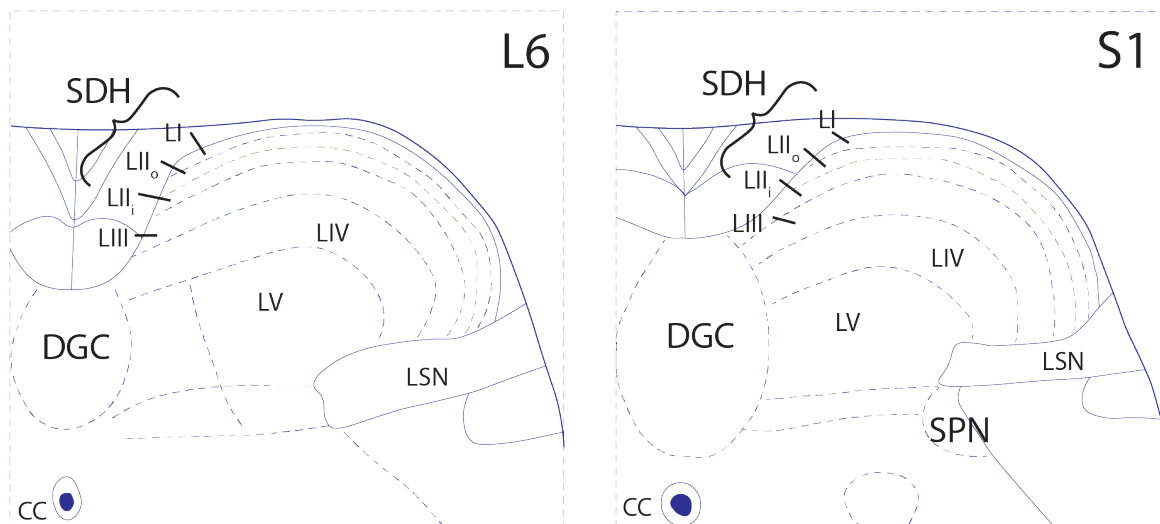


Figure 4.7: Spinal dorsal horn shape is used to determine L6 and S1 regions

Mouse spinal dorsal horn quadrant diagrams showing the dorsal horn shapes and regions of L6 and S1 adapted from the Allen Spinal Cord Atlas available at <http://mousespinal.brain-map.org> [29]. CC = central canal; DGC = dorsal grey commissure; SDH = superficial dorsal horn; LSN = lateral spinal nucleus; SPN = sacral parasympathetic nucleus; LI-LV = laminae I-V. Image credit: Allen Institute. 2008 Allen Institute for Brain Science.

4.8.4 Immunohistochemistry of phosphorylated-MAP-kinase ERK 1/2 within the spinal cord

The dorsal horn neurons activated by bladder infusion and/or controlled distension were identified by labelling for neuronal activation marker, pERK, using a well characterised method [16, 17, 19, 20, 27] that is described in detail below. The details for the primary and secondary antisera can be found in **Table 4.4**.

4.8.4.1 Fluorescence immunohistochemistry

Tissue from initial experiments, including all distension control, infusion control, histamine, and CCDC protocols (**Table 4.5**), were processed in-house via fluorescent labelling and imaging. After air drying for 1 hour, sections were washed with 0.2% Triton X-100 (Sigma-Aldrich, #T8787) in 0.1-M phosphate-buffered saline (PBS-T) to remove excess OCT. Non-specific binding of secondary antibody was blocked with 5% normal chicken serum diluted in 0.2% PBS-T. Tissue sections were incubated with pERK primary antisera diluted in PBS-T overnight (18 hours) at room temperature. Sections were then washed in PBS-T and incubated for 1 hour at room temperature with secondary antibody conjugated to AlexaFluor 594. Following secondary antibody incubation, sections were washed in PBS-T before mounting in ProLong Diamond Antifade (Life Technologies, #P-36962) and coverslipping. Negative controls were prepared as above with the primary antibody omitted. Slides were allowed to set for 24 hours before visualisation.

Table 4.4: Spinal cord immunohistochemistry primary and secondary antisera

	Species raised in	RRID/AF conjugate	Manufacturer, Catalogue No.	Dilution
Primary antisera: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP(tm) Rabbit mAb #4370	Rabbit	AB_2315112	Genesearch, #4370L	1:200
Secondary Antisera (fluorescence): anti-Rabbit IgG	Chicken	AlexaFluor 594	Life Technologies, #A-21442	1:200

4.8.4.2 DAB/HRP protocol

Tissue from all Mrgpr agonist protocols (**Table 4.5**), were processed via the University of Adelaide Health and Medical Sciences Histology services, using the DAKO Omnis autostainer (Agilent Technologies Australia, Mulgrave, Vic, AUS), with 3,3'-Diaminobenzidine (DAB) / horseradish peroxidase (HRP) secondary antibody staining. After air-drying for 1 hour, slides underwent a 1-minute post-fixation in formalin with a brief rinse in distilled water (DI) prior to target retrieval at 97°C for 30 minutes with EnVision FLEX TRS Low pH target retrieval solution (citrate buffer, pH 6.1; K8005, Agilent DAKO) followed by a wash step with wash buffer (GC807, DAKO Omnis, Agilent). Non-specific binding was prevented with a protein block step for 10 minutes with Serum-Free Protein Block (X0909, Agilent DAKO), followed by a 1-hour incubation in primary antibody (pERK; 1:800) in Antibody Diluent (S0809, Agilent DAKO). Following removal of excess primary antibody with wash buffer, a 3-minute incubation in Envision FLEX Peroxidase-blocking Reagent (GV823, Agilent DAKO) was performed to prevent endogenous peroxidase and reduce background staining. Slides were then washed twice with wash buffer before a 20-minute incubation with Envision FLEX HRP Polymer (GV823, Agilent DAKO) for HRP binding. A further 2 wash steps were performed, followed by a DI rinse and a third wash step, before a 10-minute incubation in EnVision FLEX Substrate Working Solution (DAB) for staining. Slides underwent alternate cycles of wash buffer, DI rinse, and wash buffer prior to a 3-minute incubation with Haematoxylin ready-to-use solution (K8018, Agilent DAKO). This was followed by a further DI rinse and wash step before finally being removed from the DAKO Omnis, dehydrated in alcohol, and cleared in xylene before mounting with Dibutylphthalate Polystyrene Xylene (DPX, Sigma-Aldrich) and coverslipping.

4.8.5 Microscopy

4.8.5.1 *fluorescence microscopy*

Fluorescence was visualised with an epifluorescence microscope (Olympus BX51, Tokyo, Japan) using 10× objective and 3-second exposure, with 590 nm excitation and 610/630 nm emission settings. Images were analysed using ImageJ software (NIH). Other than making moderate adjustments for contrast and brightness, the images were not manipulated in any way.

4.8.5.2 *nanozoomer microscopy*

DAB/HRP-stained slides were imaged using a nanozoomer microscope (NanoZoomer 2.0-HT, Hamamatsu), with 1 – 4 manually set focus points per section. Images of individual sections from L6 – S2 regions were taken using nanozoomer digital pathology viewing software (NDP.view2, Hamamatsu) and analysed using ImageJ software. The images were not manipulated in any way.

4.8.6 Spinal cord pERK neuronal counts and analysis

Neuronal counts were analysed from previously saved digital photomicrographs, with only neurons with intact nuclei counted. The number of pERK-IR neurons in a quadrant of the lumbosacral dorsal horn was obtained from minimum 6 sections/animal viewed at 10x magnification. The mean number of pERK-IR neurons ($\pm$ SEM) in a quadrant of dorsal horn was compared between experimental (treatment vs vehicle) groups. For histamine and Mrgpr agonist studies, the mean number of pERK-IR neurons ($\pm$ SEM) in the superficial dorsal horn (SDH; LI-II), the dorsal grey commissure (DGC), and the sacral parasympathetic nuclei (SPN) in sacral spinal segments was also compared between experimental (treatment vs vehicle) groups. These regions are known to have roles in nociceptive signalling and in autonomic reflexes [16, 30].

A summary of all *in vivo* dorsal horn pERK activation studies can be found in **Table 4.5**. Minor adaptations to the above methods, including distension pressure, compounds, catheters and staining methods, are detailed below.

Table 4.5: Summary of *in vivo* pERK activation studies

Experimental set	Mouse lines	distension	catheters	Staining	Number of mice
Distension controls	C57Bl/6J	ND vs 20 mmHg vs 60 mmHg	PE 50 tubing	fluorescence	11
Infusion controls	C57Bl/6J	60 mmHg	PE 50 tubing	fluorescence	8
Histamine vs saline	C57Bl/6J	ND	PE 50 tubing	fluorescence	10
	C57Bl/6J	20 mmHg	PE 50 tubing	fluorescence	8
TGR5 agonist CCDC vs saline	C57Bl/6J	40 mmHg	PE 50 tubing	fluorescence	10
	<i>Gpbar1</i> ^{-/-}	40mmHg	PE 50 tubing	fluorescence	7
Mrgpr agonists vs saline	C57Bl/6J	None	Metal needles	DAB/HRP	10
	<i>Mrgpr-clusterΔ</i> ^{-/-}	None	Metal needles	DAB/HRP	10

*ND = no distension

4.8.7 Distension controls

Prior to the experiments relating to bladder ‘itch’ receptors, the specificity of pERK-IR as a measure of spinal dorsal horn neuronal activation in response to bladder stimulation was validated in an initial characterisation study in which pERK-IR was determined in response to physiological (20 mmHg) and noxious (60 mmHg) distension, with no-distension controls [16]. pERK-IR was absent in no-distension controls, but clearly identified within the dorsal grey commissure (DGC), superficial dorsal horn (SDH), and sacral parasympathetic nucleus (SPN) of the LS spinal dorsal horn following distension at 20 mmHg and 60 mmHg, with pERK-IR quantification significantly increased at 60 mmHg compared to 20 mmHg distension (**Figure 3.8**). The data generated in this initial study was included as a key part of a publication by Grundy... Caldwell et al. (2018) [16] also identifying the regions of LS spinal dorsal horn projecting to the bladder via retrograde labelling and neurochemical labelling, further confirming the involvement of these regions in bladder spinal circuits and validating this technique.

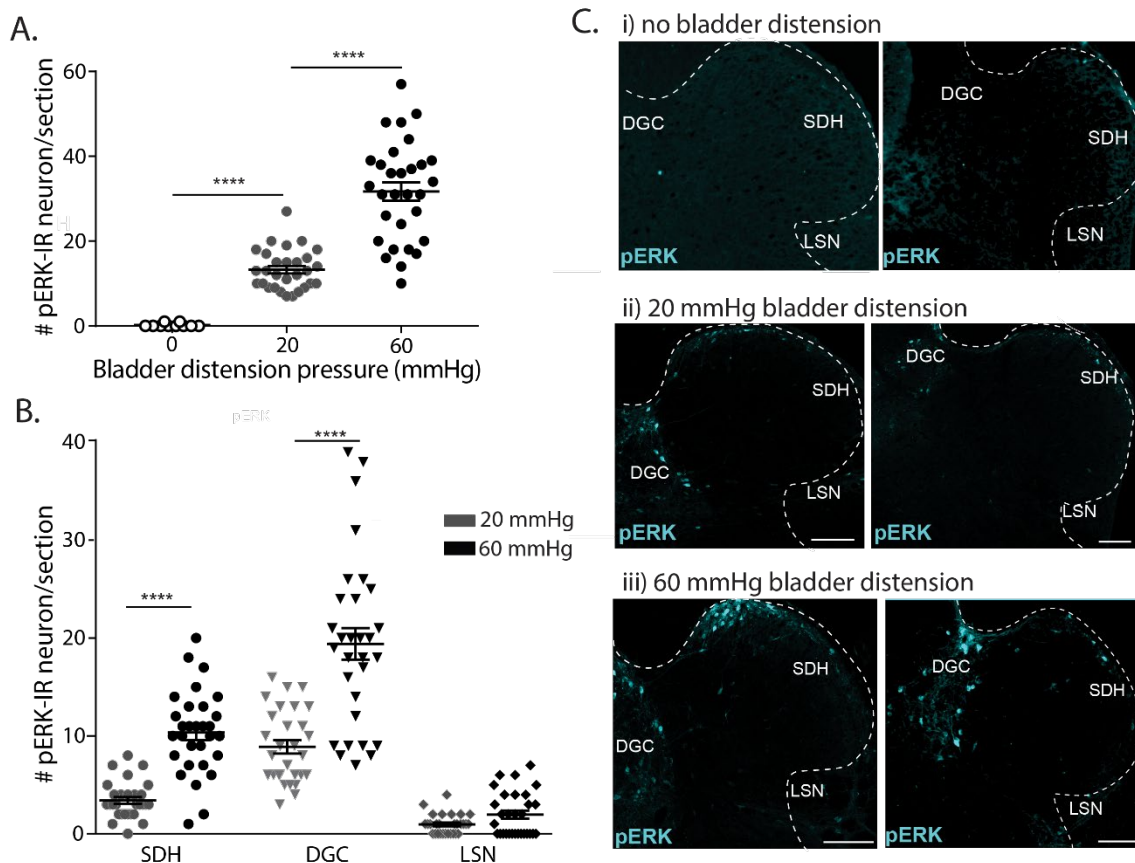


Figure 4.8: Graded *in vivo* bladder distension results in pERK immunoreactivity in the lumbosacral spinal dorsal horn.

Validation experiments were performed using bladder distension at 20 mmHg and 60 mmHg with no infusion to confirm the efficacy of pERK immunoreactivity (pERK-IR) as a measurement of bladder sensory pathway activation at the level of the lumbosacral (LS) spinal cord. **A)** pERK-IR was observed with bladder distension at 20 mmHg, with a significant increase ($p < 0.0001$) observed with 60 mmHg. No pERK-IR was observed in the absence of stimulus (no-distension controls, $p < 0.0001$ compared to 20 mmHg). **B)** pERK-IR was significantly increased in the LS spinal dorsal horn at 60 mmHg compared to 20 mmHg in the dorsal grey commissure (DGC; $p < 0.0001$) and the superficial dorsal horn (SDH; $p < 0.0001$), but not in the lateral spinal nucleus (LSN; $p > 0.05$). **C)** Example images depicting pERK-IR within the LS spinal dorsal horn following (i) no distension, (ii) 20 mmHg distension, and (iii) 60 mmHg distension. Scale bar = 100 μ m. Data were analysed using one-way ANOVA with Bonferroni post comparison tests. Figure adapted from Grundy, L., et al., *Translating peripheral bladder afferent mechanosensitivity to neuronal activation within the lumbosacral spinal cord of mice*. Pain, 2018 [16].

4.8.8 Infusion controls

A second validation study was performed to confirm the efficacy of intravesical infusion of receptor agonists/antagonists into the bladder as a means of investigating the role of a target receptor in peripheral sensory signalling and hence spinal dorsal horn pERK-IR. Tetrodotoxin (TTX), a voltage-gated sodium (Na_v) channel inhibitor selective for Na_v subtypes $\text{Na}_v1.1-1.4$, $\text{Na}_v1.6$ and $\text{Na}_v1.7$, was

used as a proof of concept, as Na_v channels are important in regulating neuronal excitability and propagation, including in the urinary bladder [17, 31-34]. 1 μM of TTX was infused in the bladder and allowed to incubate for 10 minutes prior to removal, followed by bladder distensions at 60 mmHg. Saline was infused in place of TTX as a vehicle control. Compared to saline, infusing the bladder with TTX reduced the number of pERK-IR neurons in the LS spinal dorsal horn following 60 mmHg bladder distensions (**Figure 3.9**), indicating inhibition of the bladder sensory signalling and hence confirming the efficacy of intravesical infusion of compounds into the bladder in studying the role of a peripheral target receptor in spinal dorsal horn signalling. This work was incorporated into a publication by Grundy... Caldwell et al. (2018) [17] identifying the role of TTX-sensitive Na_v channels in regulating bladder afferent responses to distension.

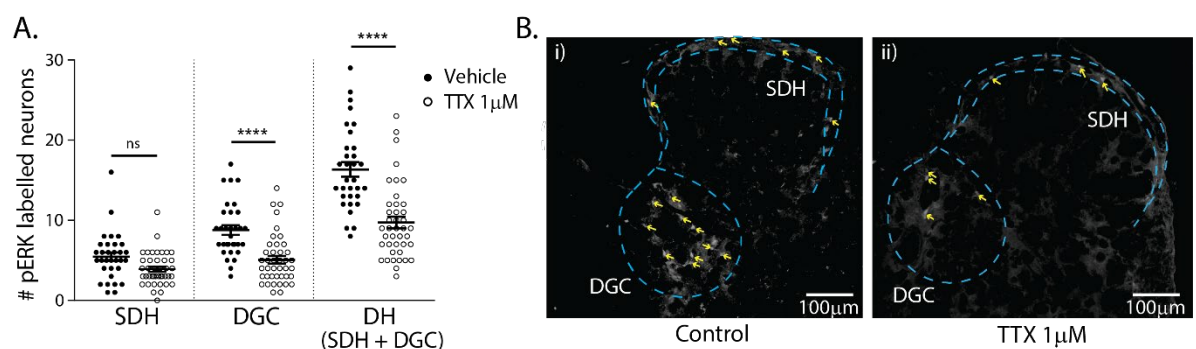


Figure 4.9: *In vivo* intravesical infusion of tetrodotoxin in the bladder reduces distension-induced pERK immunoreactivity in the lumbosacral spinal dorsal horn.

A) Noxious (60 mmHg) bladder distension-induced pERK immunoreactivity (pERK-IR) in the lumbosacral (LS) spinal dorsal horn (DH) was significantly reduced following intravesical bladder infusion of 1 μM Na_v channel antagonist tetrodotoxin (TTX; $p < 0.0001$). Reduced distension-induced pERK-IR was observed in the dorsal grey commissure (DGC; $p < 0.0001$) but not the superficial dorsal horn (SDH; $p > 0.05$). **B)** Example images depicting pERK-IR within the LS spinal dorsal horn following 60 mmHg distension after (i) control (saline) bladder infusion, and (ii) TTX bladder infusion. Scale bar = 100 μm . Data were analysed using one-way ANOVA with Bonferroni post comparison tests. Figure adapted from Grundy, L., et al., *Tetrodotoxin-sensitive voltage-gated sodium channels regulate bladder afferent responses to distension*. Pain, 2018. [17].

4.8.9 Protocol adjustments between studies

Some consistent difficulties were experienced in catheterising the mice with the PE 50 tubing. To improve the protocol, PE 50 catheters for urine removal, bladder infusions, and distensions were replaced with blunted, 51 mm, metal hub, 21-gauge needles (AS point style, Hamilton, 7751-12) for the Mrgpr experimental cohorts (performed last chronologically). These catheters provided smoother and quicker catheterisation and a more accurate pressure-hold. Post-mortem catheterisation and laparotomy of a mouse was performed to determine an accurate depth of catheterisation without damage to the bladder wall and each catheter was marked for future reference.

4.9 Data Analysis and Statistics

Data are presented as mean $\pm$ SEM or the % of neurons or cells. N indicates the number of animals, whilst n indicates the number of independent neurons, cells, or afferents. Statistical significance was reported at a level of * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data were analysed using Prism 8 (GraphPad, San Diego, CA, USA) or Stata 15 (StataCorp LLC, TX, USA). Data were tested for Gaussian distribution in order to determine the correct statistical tests. Parametric continuous data were analysed using one- or two-way analysis of variance (ANOVA) with Tukey's post hoc analyses, or student's t-tests. Non-parametric data were analysed using Kruskal-Wallis test with Dunn's multiple comparisons or Mann-Whitney analysis. The specific tests used to analyse each data set are indicated within the individual figure legends.

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Statement of Authorship

Title of Paper	Histamine induces peripheral and central hypersensitivity to bladder distension via the histamine H ₁ receptor and TRPV1
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Grundy L*, Caldwell A*, Garcia Caraballo S, Erickson A, Schober G, Castro J, Harrington AM, Brierley SM. <i>Histamine induces peripheral and central hypersensitivity to bladder distension via the histamine H₁ receptor and TRPV1</i> . Am J Physiol Renal Physiol. 2019 Dec 2. doi: 10.1152/ajprenal.00435.2019.

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Contribution to the Paper	Involved in planning of experiments, performed <i>in vivo</i> experiments, immunohistochemistry, retrograde tracing and cell culture, calcium imaging experiments, tissue collection and processing, QRT-PCR, data analysis and manuscript writing.		
Overall percentage (%)	40%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

- the candidate's stated contribution to the publication is accurate (as detailed above);
- permission is granted for the candidate to include the publication in the thesis; and
- the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Performed cell picking and single cell PCR, assisted with data analysis, edited manuscript		
Signature		Date	

Name of Co-Author	Dr Gudrun Schober		
Contribution to the Paper	Animal co-ordination, edited manuscript.		
Signature		Date	17/01/2020

Name of Co-Author	Dr Joel Castro		
Contribution to the Paper	Intellectual input, edited manuscript.		
Signature		Date	26.02.2020

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Contribution to the Paper	Performed imaging of urothelial cells for immunohistochemistry, assisted and advised with <i>in vivo</i> experiments and analysis, edited manuscript		
Signature		Date	10/02/2020

Name of Co-Author	Prof Stuart Brierley		
Contribution to the Paper	Experimental design, supervision and project lead. Analysis and interpretation of data. Manuscript writing, editing and figure layout.		
Signature		Date	28/2/2020

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Chapter 5 Histamine induces peripheral and central hypersensitivity to bladder distension via the histamine H1 receptor and TRPV1

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5.1 Abstract

Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS) is a common chronic pelvic disorder with sensory symptoms of urinary urgency, frequency, and pain, indicating a key role for hypersensitivity of bladder-innervating sensory neurons. The inflammatory mast cell mediator histamine has long been implicated in IC/BPS, yet the direct interactions between histamine and bladder afferents remain unclear. Here we show, using a mouse *ex vivo* bladder afferent preparation, that intravesical histamine enhanced the mechanosensitivity of sub-populations of afferents to bladder distension. Histamine also recruited ‘silent afferents’, which were previously unresponsive to bladder distension. Furthermore, *in vivo* intravesical histamine enhanced activation of dorsal horn neurons within the lumbosacral spinal cord, indicating increased afferent signaling into the central nervous system. qRT-PCR revealed significant expression of histamine receptor subtypes (*Hrh1-Hrh3*) in mouse lumbosacral dorsal root ganglia (DRG), bladder detrusor smooth muscle, mucosa, and isolated urothelial cells. In DRG, *Hrh1* was the most abundantly expressed. Acute histamine exposure evoked calcium influx in select populations of DRG neurons but did not elicit calcium transients in isolated primary urothelial cells. Histamine-induced mechanical hypersensitivity *ex vivo* was abolished in the presence of the histamine H₁ receptor (H₁R) antagonist pyrilamine and was not present in preparations from mice lacking the TRPV1 receptor. Together, these results indicate that histamine enhances the sensitivity

of bladder afferents to distension via interactions with H₁R and TRPV1. This hypersensitivity translates to increased sensory input and activation in the spinal cord, which may underlie the symptoms of bladder hypersensitivity and pain experienced in IC/BPS.

5.2 Introduction

Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS) is a common, chronic pelvic disorder that negatively impacts approximately 5% of the Western population [1, 2]. IC/BPS patients experience hypersensitivity to bladder distension, inducing symptoms of urinary urgency, frequency, and bladder allodynia (pain perception at lower distension volumes) compared to healthy subjects [3-5]. Although the etiology underlying IC/BPS remains unresolved, given the sensory nature of the symptoms [6], changes in the sensitivity of the bladder-innervating sensory afferent nerves responsible for regulating micturition likely play a key role.

Bladder afferents are located throughout the bladder wall, innervating the detrusor smooth muscle and mucosa [7]. As the bladder fills, mechanosensitive afferents transduce bladder stretch into sensory signals that are relayed into the spinal cord, where they feed into autonomic circuits responsible for maintaining continence [8, 9]. Conscious discrimination of bladder fullness and the urge to urinate occurs when afferent signaling reaches the ‘micturition threshold’, triggering a combination of reflexive and cognitive events that ultimately result in the conscious decision to urinate [8-10]. In addition to responding to mechanical stimuli or distension, the peripheral terminals of bladder afferents express an array of pro- and anti-nociceptive ion channels and receptors capable of detecting mediators released in response to bladder damage and inflammation [6, 11]. Inflammatory mediators have consistently been shown to sensitize sensory afferents innervating the bladder, such that bladder afferents become hypersensitive to distension [6, 11-14]. Befittingly, inflammation of the bladder is implicated in the pathogenesis of IC/BPS [6, 13-16].

Mast cells play an important role in the pathophysiology of a variety of inflammatory diseases. Accordingly, they are located in mucosal and epithelial tissues throughout the body, including the bladder [17-19]. Upon activation, mast cells release a number of potent inflammatory mediators, including but not limited to cytokines, proteases, and histamine [20, 21]. Importantly, mast cells are located in close proximity to nerve endings, and histamine has been shown to activate sensory afferents innervating both the skin and gastrointestinal tract [22-24]. Clinical and pre-clinical studies have shown that mast cells may be a crucial component in the pathogenesis and symptomology of chronic bladder pain. Mast cell numbers are increased in bladder tissue from IC/BPS patients compared to healthy subjects [17-19, 24], and mast cell mediators, particularly tryptase, histamine,

and methylhistamine, are also significantly upregulated in bladder tissue and urine of IC/BPS patients compared to controls [25, 26]. Induction of IC/BPS like symptoms in mast cell deficient mice, including inflammation and pain, is significantly attenuated in two separate pre-clinical models of cystitis [27, 28], while a recent study using an experimental autoimmune cystitis model demonstrated that the accumulation of mast cells in the bladder is required for IC/BPS-associated allodynia [29]. Additionally, histamine has been implicated in bladder neuroinflammation, as it is known to act as both a neuromodulator and a pro-inflammatory agent [30].

Although a number of studies have explored the action of neuropeptides on histamine release from mast cells [31, 32] and the role of histamine on detrusor smooth muscle and pain responses [30, 33, 34], a complete understanding of the direct interactions between histamine and bladder-innervating sensory afferent neurons remains lacking. In this study, we addressed this issue by investigating the effects of histamine on bladder afferent mechanosensitivity using *ex vivo* recordings, retrogradely traced bladder-innervating dorsal root ganglia (DRG) neurons, and spinal cord signaling *in vivo*. We also characterized the expression profiles of histamine receptor subtypes *Hrh1-Hrh4* in bladder muscle, mucosa, isolated urothelial cells, lumbosacral DRG, and individual retrogradely traced bladder-innervating DRG neurons. These data provide the first comprehensive analysis of bladder afferent sensitivity to histamine and provide evidence for a mechanism whereby increased histamine release from mast cells during IC/BPS contributes to bladder afferent sensitization and the sensory symptoms of urgency, frequency, and pelvic pain.

5.3 Methods

5.3.1 Ethics and animals

In this study, we used 12- to 18-week-old male and female C57BL/6J mice via an in-house breeding colony at the South Australian Health and Medical Research Institute (SAHMRI). Some experiments utilized a TRPV1 knockout (*Trpv1*^{-/-}) mouse strain with a C57BL/6J genetic background, also bred in-house at SAHMRI. All experiments were approved by and performed in accordance with the SAHMRI Animal Ethics Committee approvals (SAM195 and SAM190). Mice at SAHMRI were originally acquired from an in-house C57BL/6J breeding program (JAX strain #000664; originally purchased from The Jackson Laboratory; breeding barn MP14; Bar Harbor, ME) and then bred within the specific and opportunistic pathogen-free animal care facility at SAHMRI. Mice were group housed (5 mice per cage) within individual ventilated cages filled with chip coarse dust-free aspen bedding (Cat#-ASPJMAEBCA; PuraBed, Niederglatt, Switzerland). Cages were stored on individual ventilated cage racks within a temperature-controlled environment of 22°C and a 12-hour light/12-hour dark cycle.

Mice had free access to LabDiet JL Rat and Mouse/Auto6F chow (Cat #5K52, St. Louis, MO) and autoclaved reverse osmosis water. Sample sizes of each experiment are detailed in the corresponding figure legends.

5.3.2 *Ex vivo* bladder afferent nerve recording

Nerve recording was performed using a previously described *ex vivo* model [6, 35-37]. Mice were humanely killed via CO₂ inhalation, and the entire lower abdomen was removed and submerged in a modified organ bath under continual perfusion with gassed (95% O₂ and 5% CO₂) Krebs-bicarbonate solution (composition in mmol/L: 118.4 NaCl, 24.9 NaHCO₃, 1.9 CaCl₂, 1.2 MgSO₄, 4.7 KCl, 1.2 KH₂PO₄, 11.7 glucose) at 35°C. The bladder, urethra, and ureters were exposed by removing excess tissue. Ureters were tied with 4-0 perma-hand silk (Ethicon, #LA53G). The bladder was catheterized (PE 50 tubing) through the urethra and connected to a syringe pump (NE-1000) to allow a controlled fill rate of 30 µL/min with saline (NaCl, 0.9%). A second catheter was inserted through the dome of the bladder, secured with silk, and connected to a pressure transducer (NL108T2; Digitimer) to enable intravesical pressure recording during graded distension. Pelvic nerves, isolated from all other nerve fibers between the pelvic ganglia and the spinal cord, were dissected into fine multiunit branches and a single branch was placed within a sealed glass pipette containing a microelectrode (WPI) attached to a Neurolog headstage (NL100AK; Digitimer). Nerve activity was amplified (NL104), filtered (NL 125/126, band pass 50–5,000 Hz, Neurolog; Digitimer), and digitized (CED 1401; Cambridge Electronic Design, Cambridge, UK) to a PC for offline analysis using Spike2 software (Cambridge Electronic Design, Cambridge, UK). The number of action potentials crossing a pre-set threshold at twice the background electrical noise, was determined per second, to quantify the afferent response. Single-unit analysis was performed offline by matching individual spike waveforms through linear interpolation using Spike2 version 5.18 software. A single unit was deemed to be responsive to histamine if a greater than 20% increase in neuronal excitability was detected.

5.3.2.1 *Afferent recording experimental protocols*

At the start of each afferent recording experiment, control bladder distensions were performed with intravesical infusion of saline (NaCl, 0.9%) at a rate of 30 µL/min to a maximum pressure of 30 mmHg at 10 min intervals to assess the viability of the preparation and reproducibility of the intravesical pressure and neuronal responses to distension. The volume in the bladder was extrapolated from the known fill rate (30 µL/min) and the time taken (s) to reach maximum pressure (30 mmHg). Compliance was determined by plotting intravesical pressure against the calculated volume. We did not observe spontaneous phasic contractions during bladder distension as has been

reported by others[38]. After a stable baseline was maintained, the saline in the infusion pump was replaced by histamine (300 μ M; Sigma, #H7125-1G), with or without the histamine 1 receptor antagonist pyrilamine (100 μ M, Sigma, P5514-5G). The single nerve bundle isolated and inserted into the glass electrode during the dissection process was contained within the recording electrode for the entire experiment, allowing multiunit afferent nerve recordings to be performed and comparisons between afferent firing rates to be compared in the same nerve fibers during intra-bladder incubation with saline and histamine (300 μ M). Spontaneous baseline firing between bladder distensions was also recorded before and after intravesical histamine administration. Data were obtained immediately before and 5-15 minutes after histamine was introduced into the bladder.

5.3.3 *In vivo* spinal dorsal horn activation

5.3.3.1 *In vivo* bladder infusion

In vivo bladder infusion in anaesthetized mice was performed as previously described [37]. 12-week old female mice were anaesthetized (isoflurane 2% - 4% in oxygen) and a catheter (PE 50 tubing) was inserted into the bladder via the urethra. Correct catheter placement was determined by inserting gently until meeting resistance (bladder dome) and receding slightly (2-3 mm) to avoid damaging the bladder wall. Urine was removed using a suction syringe. Subsequently, a new catheter (PE 50 tubing) was inserted, primed with either vehicle (saline; N = 5 mice) or histamine (100 μ M; N = 5 mice), and 100 μ L of solution was then infused gently, to fill but not fully distend the bladder, and allowed to incubate for 5 minutes. Following removal of the compound, mice were administered an anesthetic overdose (intraperitoneal injection, 0.125 mL/250 g Lethobarb®; Virbac Australia) followed by transcardial perfuse fixation.

5.3.3.2 *Transcardial perfuse fixation and tissue dissection*

After anesthetic overdose, the thoracic cavity was opened and 0.5 mL heparinized saline (50 IU in 5 mL; Pfizer) was injected into the left ventricle followed by insertion of a 22-gauge needle attached to tubing and a peristaltic perfusion pump. The right atrium was cut open allowing for drainage of perfusate. Warm phosphate buffer (0.1 M; 21.7 mM NaH₂PO₄ [Chem Supply, #SA061-500G], 81 mM Na₂HPO₄ [VWR Chemicals, #102494C], pH 7.2) was then perfused, followed by ice-cold 4% paraformaldehyde (PFA; Sigma-Aldrich, #158127) in 0.1 M phosphate buffer. After complete PFA perfusion, the lumbosacral spinal cord (determined by the level of dorsal root ganglia (DRG) root insertion points; the lowest rib was used as an anatomical marker of DRG level T13) was removed and postfixed in 4% PFA in 0.1 M phosphate buffer at 4°C for 18 to 20 hours. After post-fixation, spinal cords were cryoprotected in 30% sucrose/phosphate buffer (Sigma-Aldrich, #S9378) for two days at

4°C, followed by 48-hour incubation at 4°C in 50% optimal cutting temperature (OCT; VWR, #C361603E)/30% sucrose/phosphate buffer solution before freezing in 100% OCT using liquid nitrogen-cooled 2-methylbutane (Sigma-Aldrich, #M32631). Frozen sections of the spinal cord were cryosectioned (10 µm thick) and placed onto gelatin-coated slides for immunofluorescence labelling processing. Sections were performed serially and distributed over 6 slides, which were used for immunofluorescence localization of phosphorylated-MAP-kinase ERK 1/2 (pERK). The number of pERK-immunoreactive neurons were counted in L6-S1 spinal cord sections, with spinal segments determined *ex vivo* by the shape of the dorsal horn [39].

5.3.3.3 Immunohistochemistry of phosphorylated-MAP-kinase ERK 1/2 within the spinal cord

The dorsal horn neurons activated by bladder infusion were identified by labelling for neuronal activation marker, phosphorylated-MAP-kinase ERK 1/2 (pERK), using a method described previously [6, 36, 37, 40, 41]. The details for the primary and secondary antisera can be found in **Table 5.1**.

pERK activation was determined rather than c-Fos as ERK phosphorylation is far more rapid (minutes compared to hours) [42]. As the nature of the specific peripheral stimuli applied is short duration, the rapid onset of pERK provides a better correlate. Further, spinal activation to a range of peripheral stimuli is increasingly being identified via quantification of pERK activation [36, 37, 41, 43]. After air drying for 1 hour, sections were washed with 0.2% Triton X-100 (Sigma-Aldrich, #T8787) in 0.1-M phosphate-buffered saline (PBS-T) to remove excess OCT. Non-specific binding of secondary antibody was blocked with 5% normal chicken serum diluted in 0.2% PBS-T. Tissue sections were incubated with pERK primary antisera diluted in PBS-T overnight (18 hours) at room temperature. Sections were then washed in PBS-T and incubated for 1 hour at room temperature with secondary antibody conjugated to AlexaFluor 594. Following secondary antibody incubation, sections were washed in PBS-T before mounting in ProLong Diamond Antifade (Life Technologies, #P-36962) and coverslipping. Negative controls were prepared as above with the primary antibody omitted. Slides were allowed to set for 24 hours before visualization.

Table 5.1: Spinal cord immunohistochemistry primary and secondary antisera

	Species raised in	RRID/AF conjugate	Manufacturer, Catalogue No.	Dilution
Primary antisera: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP(tm) Rabbit mAb #4370	Rabbit	AB_2315112	Genesearch, #4730L	1:200
Secondary Antisera: anti-Rabbit IgG	Chicken	AlexaFluor 594	Life Technologies, #A-21442	1:200

5.3.3.4 Microscopy

Fluorescence was visualized with an epifluorescence microscope (Olympus BX51, Tokyo, Japan) using 10× objective and 3-second exposure, with 590 nm excitation and 610/630 nm emission settings. Images were analyzed using ImageJ software (NIH). Other than making moderate adjustments for contrast and brightness, the images were not manipulated in any way.

5.3.3.5 Spinal cord pERK neuronal counts and analysis

Neuronal counts were analyzed from previously saved digital photomicrographs, with only neurons with intact nuclei counted. The number of pERK-immunoreactive (pERK-IR) neurons in a quadrant of the L6-S1 dorsal horn was obtained from minimum 6 sections/animal viewed at 10x magnification. The mean number of pERK-IR neurons ($\pm$ SEM) in the superficial dorsal horn (SDH; LI-II), the dorsal grey commissure (DGC), and the sacral parasympathetic nuclei (SPN) in sacral spinal segments was compared between vehicle- and histamine-treated mice. These regions are known to have roles in nociceptive signaling and autonomic reflexes [6, 44].

5.3.4 Retrograde tracing

A small aseptic abdominal incision was made in anaesthetized (2% – 4% isoflurane in oxygen) mice. Cholera Toxin subunit B conjugated to AlexaFluor 488 (CTB-488, 0.5% diluted in 0.1 M phosphate buffered saline [PBS] pH 7.4; ThermoFisher Scientific) was injected at three sites into the bladder wall (3 μ L/injection) using a 5 μ L Hamilton syringe attached to a 30-gauge needle [6, 37]. The needle was inserted subserosally, parallel with the bladder muscle, to ensure that CTB was not injected into the bladder lumen. The abdominal incision was sutured closed and analgesic (Buprenorphine (Temvet); 0.1 mg/kg; Troy Laboratories Pty Ltd, APVMA #67612) and antibiotic (Amoxicillin; 50 mg/kg; Amoxil, AUSTR11137) given subcutaneously as mice regained consciousness.

Mice were then housed individually and allowed to recover. After 4 days, mice were humanely euthanized for subsequent lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) removal and the neurons isolated and cultured to visualize CTB-labelled bladder innervating neurons amongst the DRG neurons.

5.3.5 Cell culture of bladder-innervating DRG neurons

Four days after bladder retrograde tracing, mice were humanely euthanized via CO₂ inhalation and lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) were removed. DRG were digested in Hanks' balanced salt solution (HBSS; pH 7.4; Life Technologies, #14170161) containing 2.5 mg/mL collagenase II (GIBCO, ThermoFisher Scientific, #17101015) and 4.5 mg/mL dispase (GIBCO, ThermoFisher Scientific, #17105041) at 37°C for 30 min. The collagenase-dispase solution was aspirated and replaced with HBSS containing collagenase (4.5 mg/mL) only for 10 min at 37°C. Following subsequent washes in HBSS, DRG were mechanically disrupted and cells dissociated in 600 µL complete DMEM (Dulbecco's Modified Eagle Media [DMEM; GIBCO, ThermoFisher Scientific, #11995065]; 10% Fetal Calf Serum [Invitrogen, ThermoFisher Scientific, MA, USA]; 2 mM L-glutamine [GIBCO, ThermoFisher Scientific, #25030081], 100 µM MEM non-essential amino acids [GIBCO, ThermoFisher Scientific, #11140076], 100 mg/mL penicillin/streptomycin [GIBCO, ThermoFisher Scientific, #15070063], and 96 µg/L nerve growth factor-75 [Sigma, N0513-0.1MG]) via trituration through fire-polished Pasteur pipettes of descending diameter, and centrifuged for 1 min at 50 g [36, 37, 40, 45]. Neurons were resuspended in 360 µL complete DMEM and spot-plated (30 µL) onto 13 mm coverslips coated with laminin (20 µg/mL; Sigma-Aldrich, #L2020) and poly-D-lysine (800 µg/mL; ThermoFisher Scientific). Coverslips were incubated at 37°C in 5% CO₂ for 2-3 hours to allow neurons to adhere before flooding with 1.7 mL complete DMEM. Cultured neurons were maintained in an incubator at 37°C in 5% CO₂ for 18-48 hours for calcium imaging or for 2 hours for cell picking for single-cell RT-PCR.

5.3.6 Isolation of mouse urothelial cells

Primary urothelial cells were isolated and cultured from mice as performed previously [35, 46, 47]. Following euthanasia by CO₂ inhalation, bladders were removed and dissected in sterile PBS with a single cut from the urethral opening along one side to the dome, pinned and stretched out urothelial side up, and incubated with DMEM containing 2.5 mg/mL dispase, 10% Fetal Calf Serum, and 1 µM/mL HEPES (Sigma, #H3375, pH 7.0) for 3 hours at room temperature. Cells were collected by gentle scraping of the urothelium with a blunt scalpel and dissociated in 0.025% trypsin EDTA (GIBCO, ThermoFisher Scientific, #25200072) at 37°C in 5% CO₂ for 10 min with gentle intermittent trituration.

The cell suspension was added to DMEM containing 10% Fetal Calf Serum to deactivate the trypsin before centrifugation (15 min, 1500 rpm, 4°C). Cells were then resuspended in keratinocyte serum-free media (KSFM; Invitrogen, #17005042) before being plated onto collagen I-coated coverslips (diluted 1:20 in H₂O; Sigma; #A10483-01). Coverslips were left for 4 hours in an incubator at 37°C in 5% CO₂ before flooding with KSFM (2 mL/well). Cells were maintained at 37°C in 5% CO₂ for 24-48 hours and used for calcium imaging.

5.3.6.1 Cytokeratin 7 immunohistochemistry

Immunohistochemical labelling for transitional epithelium was performed on isolated urothelial cells using monoclonal antibody cytokeratin 7 (CK7). The details of the primary and secondary antibodies used are in **Table 5.2**.

Coverslips were washed with 0.1 M phosphate-buffered saline (PBS) three times and fixed with ice-cold 4% PFA at 4°C for 45 minutes. Coverslips were washed with saponin 0.05% (Sigma-Aldrich; #47036) + 2% FBS in 0.1 M PBS (SF-PBS) to remove excess PFA and permeabilize cell membranes. Nonspecific binding of secondary antibodies was blocked with 3% bovine serum albumin (Sigma-Aldrich; #A9418) diluted in 0.05% SF-PBS for 1 hour. Coverslips were incubated with primary antisera diluted in SF-PBS overnight (28 hours) at 4°C. Sections were then washed 3x in PBS and incubated in the dark for 2 hours at room temperature with secondary antibody conjugated to AlexaFluor 488. Cells were then washed in SF-PBS before mounting in Prolong Gold Antifade with DAPI and coverslipping. Negative controls were prepared as above with the primary antibody omitted. Slides were allowed to set for 24 hours before visualization.

Table 5.2: Urothelial immunolabelling primary and secondary antisera details

	Species raised in	RRID/AF conjugate	Manufacturer, Catalogue No.	dilution
Primary antisera: Cytokeratin 7 Monoclonal Antibody (OV-TL 12/30)	Mouse	AB_10989596	ThermoFisher, # MA5-11986	1:50
Secondary Antisera: anti-Mouse IgG1	Goat	AlexaFluor 488	ThermoFisher, # A-21121	1:1000

5.3.6.2 Microscopy

Fluorescence was visualized with a confocal laser scanning microscope (Leica TCS SP8X; Leica Microsystems, Wetzlar, Germany). Images (1024 x 1024 pixels) were obtained using a x63 lens (software zoom x1.3) and sequential scanning (4- to 5-line average). Separation of fluorophores was

achieved using white line laser tuned to 495-nm excitation and 505- to 534-nm emission detection settings for AF-488 and 405-nm excitation and 425- to 475-nm emission detection settings for DAPI. Confocal settings were optimized to reduce background staining by adjusting the white light laser intensity, emission window (as described above), and amplifier gain [726.7 offset: x0.07 (AF488); 10 offset: x0.1 (DAPI)]. These settings were saved and used for all imaging.

5.3.7 Calcium imaging of cultured urothelial cells and DRG neurons

Cultured DRG neurons and urothelial cells (18-48 hours) were loaded with 2 μ M Fura-2-acetoxymethyl ester (Fura-2; Invitrogen, ThermoFisher Scientific, #F1221) in 0.01% pluronic F-127 (Invitrogen, ThermoFisher Scientific, #P3000MP) for 30 min at 37°C and washed with HEPES buffer (10 mM HEPES sodium salt [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt; Sigma, #H7006-100G], 140 mM NaCl [Chem Supply, #SA046-3KG], 4 mM KCl [Chem Supply, #PA054-500G], 5 mM D-glucose anhydrous [Chem Supply, #GA018-500G], 2 mM CaCl_2 [Scharlau, #CA01951000], and 2 mM MgCl_2 [Sigma, #M8266-100G], pH 7.40) for 10 min before imaging at room temperature (23°C). Fura-2 was excited at 340 and 380 nm, and emissions were measured at 510 nm using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, an Omega XF04 filter set for Fura-2, a Photonic Science ISIS-3 intensified CCD camera, and Universal Interface Card MetaFluor software. Retrogradely traced bladder DRG neurons were identified by the presence of the CTB-488 tracer, visible under excitation at 480 nm. 480 nm excitation was used as an initial bladder neuron identifier only; images were not obtained at 480 nm throughout the experiment to prevent unnecessary photobleaching. Fluorescence images were obtained every 5 s leading up to and immediately following drug application, and every 20 s for washout period, using a 20x objective. Data were recorded and further analyzed using MetaFluor software. After an initial baseline reading to ensure cell fluorescence was stable, indicating healthy cells, DRG neurons and urothelial cells were stimulated with histamine (100 μ M; Sigma, #H7125-1G), and changes in intracellular calcium (Ca^{2+}_i) were monitored in real time. Ionomycin (2 μ M) and KCl (40 mM) were applied as positive controls in urothelial and neuronal experiments, respectively. Ca^{2+}_i is expressed as the ratio between the fluorescence signals at 340- and 380-nm (Fura-2 (340/380)). Baseline Ca^{2+}_i was determined by averaging 8-12 fluorescence ratio readings immediately prior to stimulation. Peak response was calculated by subtracting the relevant baseline score from the maximum fluorescence ratio reading following drug application ($\Delta 340/380$). Baseline fluorescence was normalized to a ratio of 1 for example traces.

5.3.8 mRNA expression analysis in DRG, cultured urothelial cells and bladder mucosal and detrusor layers

5.3.8.1 Tissue collection

16- to 18-week-old mice were humanely euthanized via CO₂ inhalation and the bladder and lumbosacral (L5-S1) dorsal root ganglia (DRG) removed. DRG were frozen in dry ice in pairs by spinal level (L5, L6, S1) and stored at -80°C for RNA extraction and quantitative reverse-transcription polymerase chain reaction (QRT-PCR). The bladder was opened, stretched out and pinned flat, urothelial side up. For tissue QRT-PCR, the mucosal and detrusor layers were gently peeled apart, frozen in dry ice and stored at -80°C for RNA extraction and QRT-PCR. For urothelial cell QRT-PCR, urothelial cells were isolated as for cell culture above. Following resuspension of cells in keratinocyte serum-free media (KSFM; Invitrogen, #17005042), cell count and viability were determined using the Countess Automated Cell Counter (Invitrogen, ThermoFisher Scientific). Cells were pelleted, frozen in dry ice and stored at -80°C for RNA extraction and QRT-PCR.

5.3.8.2 RNA extraction

RNA was extracted using the PureLink RNA Micro kit (Invitrogen, Victoria, Australia, #12183-016; DRG pairs and isolated urothelial cells) or the PureLink RNA Mini kit (Invitrogen, #12183018A; bladder mucosa and detrusor) with DNase treatment (Life Technologies, #12185-010) according to the manufacturer's instructions. A NanoDrop Lite spectrophotometer (ThermoFisher Scientific) determined RNA quantity and purity. RNA was reverse transcribed to cDNA using SuperScript VILO Master Mix (Invitrogen, #11755250) as per the manufacturer's instructions. cDNA was stored at -20°C for qPCR.

5.3.8.3 Quantitative reverse-transcription polymerase chain reaction (QRT-PCR)

QRT-PCR was performed using Taqman Gene Expression Master Mix (Applied Biosystems, Victoria, Australia, #4369016) with commercially available hydrolysis probes (TaqMan; Life Technologies, see **Table 5.3** for details) and RNase-free water (AMBION, Victoria, Australia, #AM9916). For each reaction, 10 µL of qPCR MasterMix, 1 µL of TaqMan primer assay, 4 µL of water, and 5 µL of cDNA (1:2 dilution in RNA-free H₂O) from each sample was tested in duplicate for each target. *Actb* (β-actin) was used as an endogenous control for DRG pairs. *Gapdh* and *Hprt* were used as endogenous controls for mucosal and detrusor tissue layers and isolated urothelial cells. Assays were run for 45 cycles on a 7500 Fast Real-Time PCR System (Applied Biosystems) machine, using 7500 Fast software, v2.0.6. mRNA quantities are expressed as 2^{-ΔCt} relative to reference gene *Actb* (DRG pairs) or *Gapdh* x *Hprt* (geometric mean; mucosa, detrusor and urothelial cells). Using Prism 8 software (GraphPad, San Diego, CA), data were analyzed by 1-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

Table 5.3: Primers used for RT-qPCR receptor expression assays

Gene Alias	Gene Target	Assay ID
β-actin (reference gene)	<i>Actb</i>	Mm00607939_s1
HPRT (reference gene)	<i>Hprt</i>	Mm00446968_m1
Gapdh (reference gene)	<i>Gapdh</i>	Mm99999915_g1
Tubulin-3 (neuronal marker)	<i>Tubb3</i>	Mm00727586_s1
Histamine Receptor subtype 1	<i>Hrh1</i>	Mm00434002_s1
Histamine Receptor subtype 2	<i>Hrh2</i>	Mm00434009_s1
Histamine Receptor subtype 3	<i>Hrh3</i>	Mm00446706_m1
Histamine Receptor subtype 4	<i>Hrh4</i>	Mm00467634_m1
Transient receptor potential vanilloid 1	<i>Trpv1</i>	Mm01246300_m1

5.3.9 Single-cell RT-PCR of individual bladder-innervating dorsal root ganglia neurons

Under continuous perfusion of sterile and RNA-/DNase-free PBS, retrogradely traced single bladder dorsal root ganglia (DRG) neurons were identified using a fluorescence microscope and collected into the end of a fine glass capillary using a micromanipulator [37]. The glass capillary containing the cell was then broken into a sterile Eppendorf tube containing 10 µL of lysis buffer with DNase (TaqMan Gene Expression Cells-to-CT Kit; Invitrogen, #4399002). For each coverslip of cells, a bath control was also taken and analyzed together with cells. After lysis and termination of DNase treatment, samples were immediately frozen on dry ice and stored at -80°C until cDNA synthesis was performed. RNA was reverse transcribed to cDNA using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitrogen, #11766500) as per the manufacturer's instructions. cDNA was stored at -20°C for real time PCR. Tubulin-3 expression served as a neuronal marker and positive control. Expression was considered positive if a complete curve was observed before 50 cycles.

5.3.10 Data Analysis and Statistics

Data are presented as mean ± SEM or the % of neurons or afferents. N indicates the number of animals, whilst n indicates the number of independent neurons or afferents. Statistical significance was considered as P<0.05 and reported at a level of * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001. In some cases, # is used to indicate significance of at least P < 0.05 for multiple comparisons. Data were analyzed using Prism 8 (GraphPad, San Diego, CA, USA), using one- or two-way analysis of variance (ANOVA) with Tukey's or Sidak's post hoc analyses dependent on data distribution, or

student's t-tests, for parametric data. Non-parametric data were analyzed using Kruskal-Wallis test with Dunn's multiple comparisons, Mann-Whitney analysis, or Wilcoxon matched-pairs signed rank test. Data were tested for Gaussian distribution in order to determine the correct statistical tests. The specific tests used to analyze each data set is indicated within the individual figure legends.

5.4 Results

5.4.1 Histamine enhances bladder afferent sensitivity to bladder distension

In order to determine whether the presence of histamine within the bladder influences the activity of afferent sensory neurons innervating the bladder wall, we performed multiunit recordings of bladder-innervating pelvic afferents in response to graded bladder distension using an *ex vivo* preparation. Bladders were distended first with saline as a control, followed by histamine. As the pressure within the bladder increased with distension, there was a corresponding increase in afferent activity (**Figure 5.1A** and **5.1B**). We did not observe any significant effects on mechanosensitivity with 10-30 μ M histamine concentrations (**Supplementary Figure 5.1**). In contrast, 300 μ M histamine significantly enhanced the afferent firing rate at distension pressures of and above 12 mmHg compared to the saline control distensions (**Figure 5.1A** and **5.1C**). The pressure-to-volume relationship in the bladder during distension was also analyzed to ensure that muscle compliance and contractility was not altered during the protocol, thus affecting afferent firing. No difference in muscle compliance was observed between saline and histamine protocols (**Figure 5.1D**). These data suggest that the significant changes observed in bladder afferent mechanosensitivity with histamine instillation were due to a direct effect on afferents, and not secondary to changes in the ability of the bladder to accommodate an increase in volume.

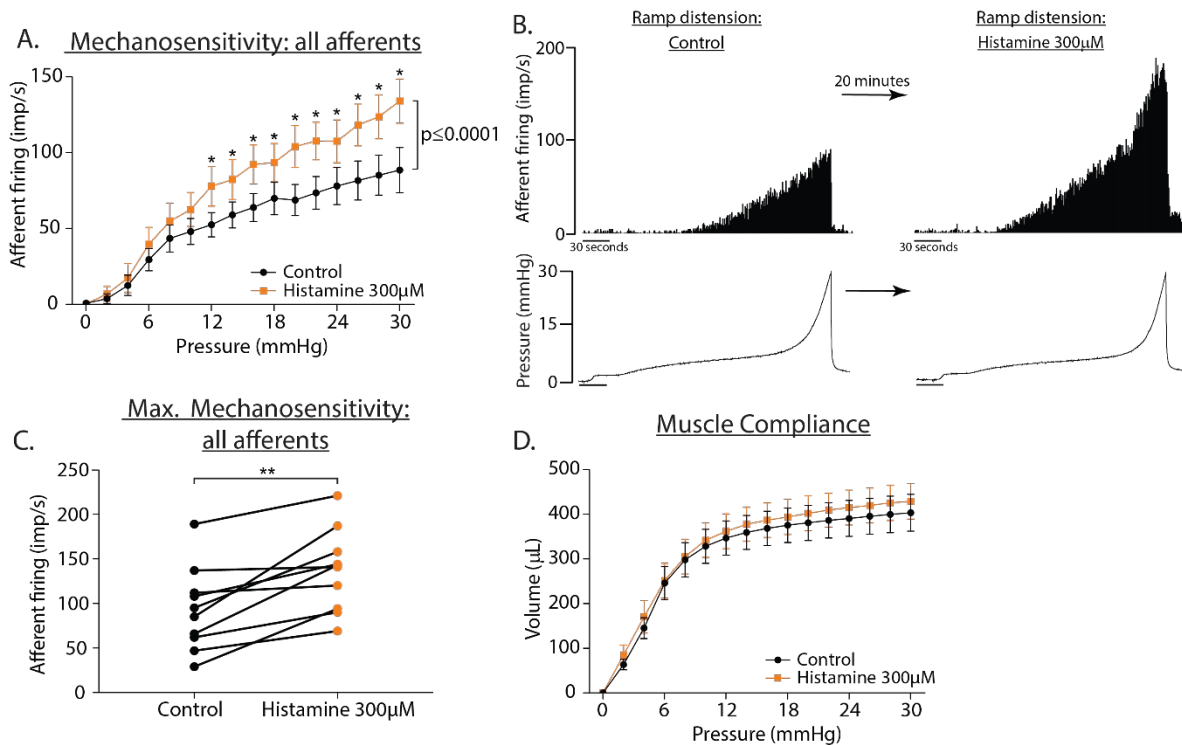


Figure 5.1: Histamine enhances bladder afferent mechanosensitivity to graded bladder distension *ex vivo*

Graded distension of the bladder in an *ex vivo* nerve recording preparation was performed with saline followed by 300 μM histamine. **(A)** Administration of 300 μM histamine resulted in an increased afferent firing rate (impulses per second; imp/s) compared to saline at distension pressures at and above 18 mmHg (N = 10; *** P < 0.001). **(B)** An example experiment showing an increase in raw afferent nerve activity in the presence of 300 μM histamine compared to saline in response to increased intraluminal pressure. **(C)** The maximum afferent firing rate (imp/s) of bladder afferents per mouse was significantly increased (** P < 0.01; $\Delta = 43.7 \pm 10.05$ imp/s) in the presence of 300 μM histamine. **(D)** No change in muscle compliance (pressure-to-volume relationship) was observed between saline and histamine protocols, suggesting that the observed effect is neuronal and not muscular. Data are represented as mean $\pm$ SEM **(A, D)** or maximum imp/s **(C)**. P values above are based on two-way ANOVA **(A, D)** with subsequent Sidak's post hoc test significance at individual data points indicated on **(A)**, or paired t-test **(C)**.

To further characterize the enhanced mechanosensitivity observed *ex vivo* in response to histamine, the single afferent units that comprise the multiunit response were analyzed (**Figure 5.2**). Overall, three distinct populations of afferent responses were observed. One, 45% of bladder afferents responded to histamine with an increase in mechanosensitivity (**Figure 5.2A, 5.2E(i)** and **5.2F**), reflecting the overall outcome observed in whole nerve recordings. Two, 50% of mechanosensitive bladder afferents were unresponsive to histamine, showing no difference in firing rates upon histamine application (**Figure 5.2B, 5.2E(ii)** and **5.2F**). Three, the remaining 5% of bladder afferents were shown to be a distinct functional subclass, identified as 'silent afferents' such that they

were non-responsive during bladder distension with saline, but accrued mechanosensitivity in the presence of histamine (**Figure 5.2C** and **5.2F**). All responding afferents showed a significant increase ($\geq 20\%$ above baseline) in afferent firing in response to bladder distension with histamine (**Figure 5.2E(i)**). These results demonstrate that histamine is able to sensitize a subset of mechanosensitive afferents in the bladder and recruit a population of 'silent afferents' to become mechanosensitive, ultimately resulting in an enhanced mechanosensory afferent response to bladder distension.

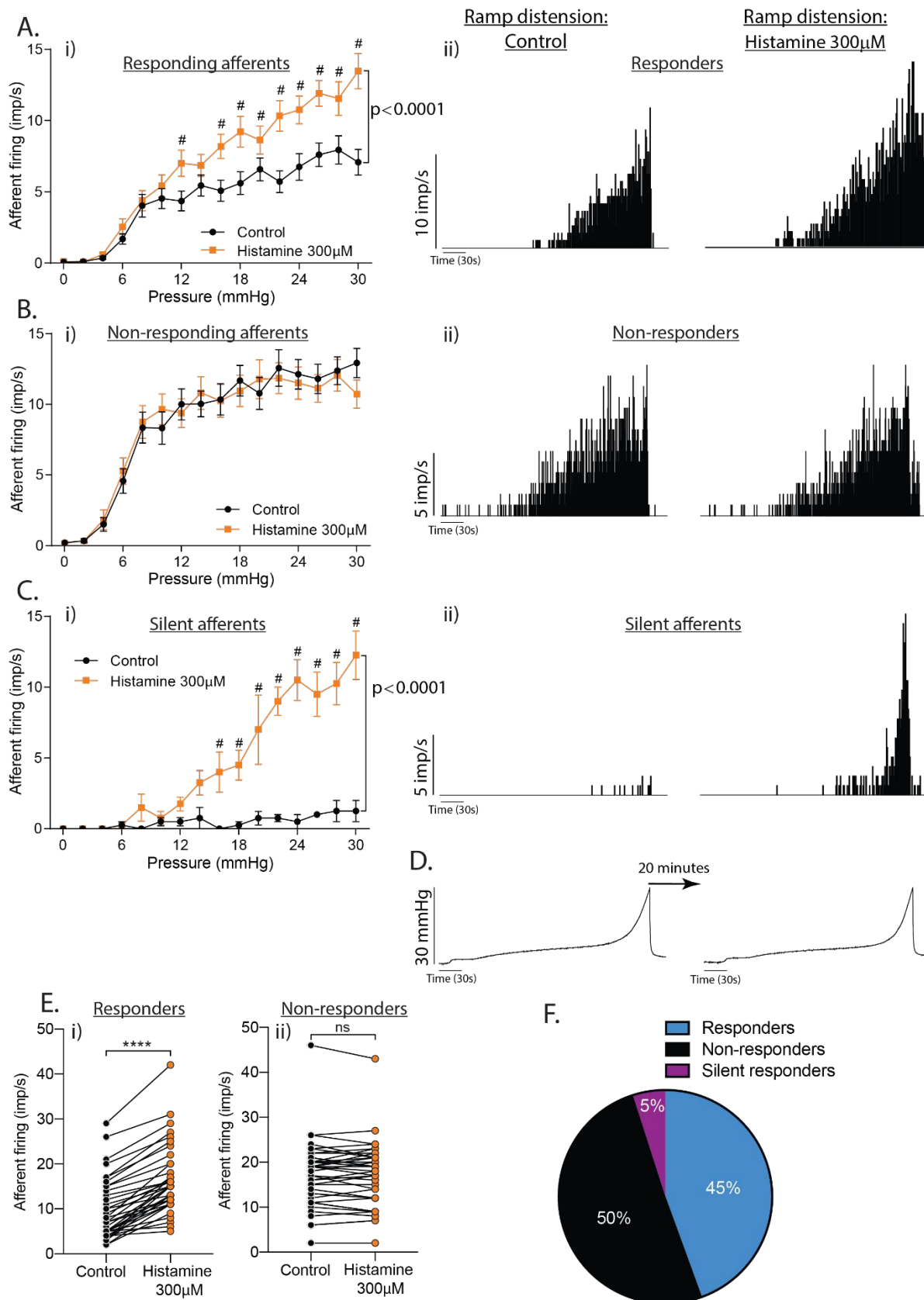


Figure 5.2: Single unit analysis of ex vivo bladder afferent mechanosensitivity in the presence of histamine

Bladder afferent nerve recordings following histamine administration in the *ex vivo* bladder afferent preparation can be separated into three distinct populations. **(A)** Responding afferents show a significant

increase in afferent firing (impulses per second; imp/s) in the presence of 300 μ M histamine compared to saline (n = 36 out of 81; **** P < 0.0001). **(B)** Non-responding afferents show no change in mechanosensitivity between saline and histamine protocols (n = 41 out of 81; P > 0.05). **(C)** 'Silent afferents' are mechanically insensitive in saline control distension but become sensitive to distension in the presence of 300 μ M histamine (n = 4 out of 81; **** P < 0.0001). **(D)** An example experiment showing graded increase in intraluminal pressure up to 30 mmHg, resulting in raw afferent nerve activity as above **(A(ii), B(ii), C(ii))**. **(E)** The peak afferent firing rate of individual bladder afferent units **(i)** responding to 300 μ M histamine (n = 40, **** P < 0.0001; Δ = 6.70 ± 0.58 imp/s), or **(ii)** not responding to 300 μ M histamine (n = 41, ns P > 0.05; Δ = -0.17 ± 0.27 imp/s). **(F)** Pie chart demonstrating the percentage of afferent units from each population (45% responding afferents, 50% non-responding afferents, 5% 'silent afferents'). Data are represented as mean $\pm$ SEM **(A-C)**, maximum imp/s **(E)**, or percentage of afferents **(F)**. P values above are based on two-way ANOVA **(A - C)** with subsequent Sidak's post hoc test significance at individual data points indicated on **(A)** and **(C)** (# represents significance of at least P < 0.05), or Wilcoxon matched-pairs signed rank test **(E)**.

5.4.2 Bladder-infused histamine activates dorsal horn neurons in the lumbosacral spinal cord

Following the identification of increased sensitivity in the bladder afferents *ex vivo*, we aimed to determine whether this hypersensitivity is relayed centrally, at the level of the spinal cord. We performed *in vivo* bladder infusion of histamine or saline (control) and analyzed the number of dorsal horn neurons with pERK-immunoreactivity (pERK-IR) as an indicator of spinal cord neuronal activation **(Figure 5.3)**. Following saline infusion, pERK-IR was observed in sections of lumbosacral spinal cord in the dorsal grey commissure (DGC), the superficial dorsal horn (SDH), and the sacral parasympathetic nucleus in sacral spinal segments (SPN; **Figure 5.3A** and **5.3B**). Infusion of histamine into the bladder resulted in significantly greater numbers of pERK-IR neurons relative to saline infusion **(Figure 5.3A** and **5.3B**) in the DGC **(Figure 5.3A(ii))** and the SDH **(Figure 5.3A(iii))**, with no significant difference in the SPN **(Figure 5.3A(iv))**. Catheter insertion alone did not evoke pERK-immunoreactivity **(Figure 5.3B(ii))**. These results indicate that the histamine-evoked hypersensitivity of bladder afferent peripheral endings is relayed into the spinal cord and enhances central signaling.

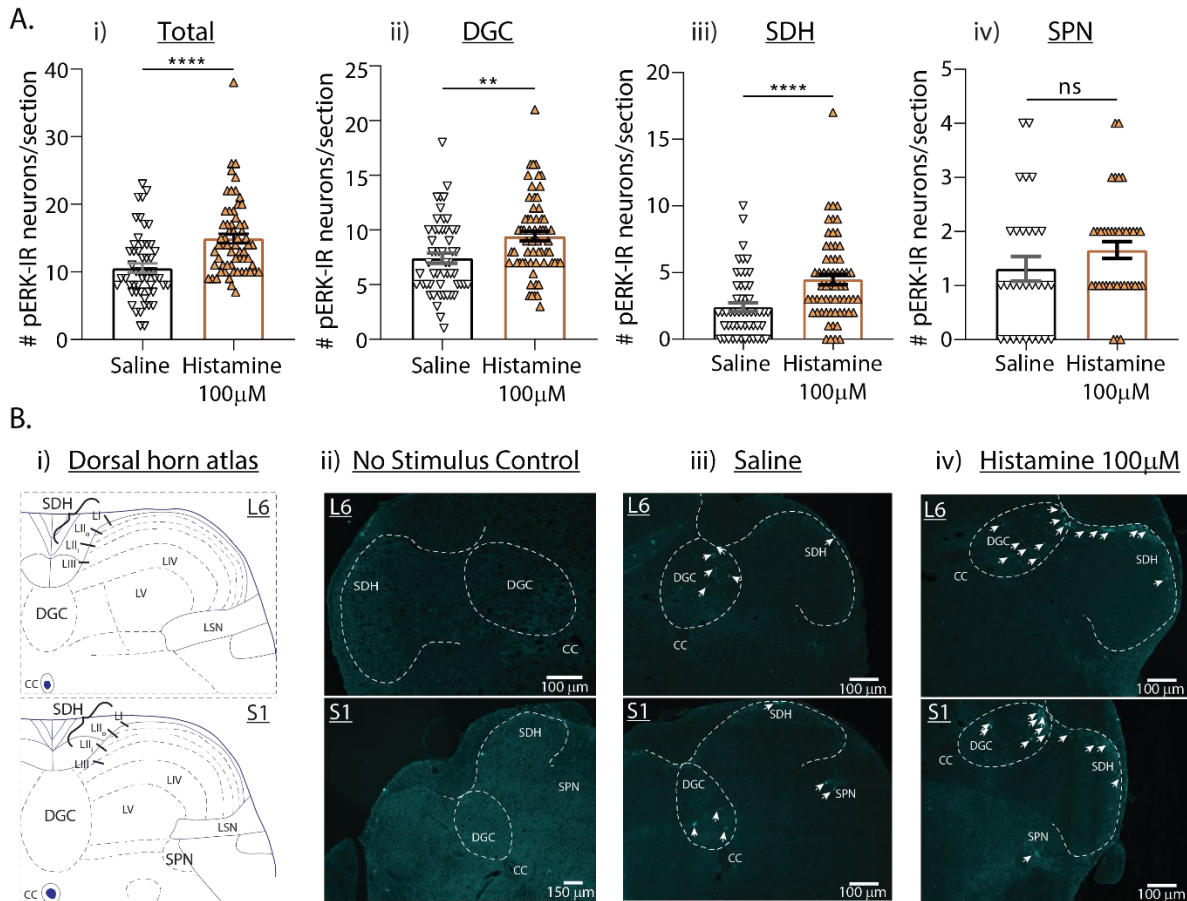


Figure 5.3: Histamine enhances bladder afferent signaling in the dorsal horn of the spinal cord *in vivo*

pERK immunoreactivity (pERK-IR) was measured to determine central signaling in the lumbosacral (LS; L5-S1) spinal cord in response to peripheral administration of saline or 100 µM histamine into the bladder. **(A)** pERK-IR was significantly increased in the LS dorsal horn **(i)** following 100 µM histamine administration (N = 5) compared to saline administration (N = 5; **** P < 0.0001). pERK-IR was observed in the dorsal grey commissure (DGC) **(ii)**, superficial dorsal horn (SDH) **(iii)**, and the sacral parasympathetic nucleus (SPN) **(iv)**, with significantly higher pERK-IR in the presence of 100 µM histamine compared to saline in the DGC (**** P < 0.0001) and the SDH (**** P < 0.0001), while no difference was observed in the SPN in sacral segments (ns, P > 0.05). **(B)** Representative pERK-labelled LS spinal cord images at each spinal level, as determined by the shape of the dorsal horn as demonstrated in **(i)** dorsal horn maps, from mice **(ii)** that received only catheter insertion, or were treated with **(iii)** saline and **(iv)** 100 µM histamine. White arrows indicate pERK-IR neurons. CC = central canal. Data are represented as the number of pERK-IR neurons per section of LS spinal cord, with a minimum of 6 sections per mouse. P values above are based on Mann Whitney tests **(A(i) - A(iii))**, or an unpaired t-test **(A(iv))**. Scale bar represents distance of 100 µm **(B)**. L6 and S1 dorsal horn atlas images **(Bi)** adapted from the Allen Spinal Cord Atlas available at <http://mousespinal.brain-map.org> [39]. Image credit: Allen Institute. 2008 Allen Institute for Brain Science.

5.4.3 Histamine receptor subtypes *Hrh1*, *Hrh2* and *Hrh3* are expressed in lumbosacral dorsal root ganglia (L5-S1), with *Hrh1* identified in ~24% of bladder innervating afferent neurons

To begin to explore the potential mechanism of action of histamine on bladder afferents, we used QRT-PCR to investigate the mRNA expression of the four histamine receptor subtypes, *Hrh1-Hrh4*, relative to the housekeeper *Actb* (β -actin) in the lumbosacral (LS; L5-S1) DRG. mRNA for *Hrh1-Hrh3*, but not *Hrh4*, which was found in spleen (positive control tissue, **Supplementary Figure 5.2**), was expressed within the LS DRG (**Figure 5.4A** and **5.4B**), with small differences observed between the DRG pairs across L5, L6 and S1 (**Figure 5.4A**). As *Hrh1* was the highest expressed receptor within LS DRG and is known to play a significant role in irritant response mechanisms, we performed single cell RT-PCR to determine *Hrh1* mRNA expression in individual bladder innervating LS DRG neurons. We found that 23.6% of bladder-innervating afferent neurons expressed mRNA for *Hrh1* (**Figure 5.4C**). In comparison, mRNA expression of transient receptor potential vanilloid 1 (*Trpv1*), which has been implicated in histamine activity in other visceral organs, was identified in 84.4% of bladder-innervating DRG neurons in an independent subset of bladder neurons (**Figure 5.4C**).

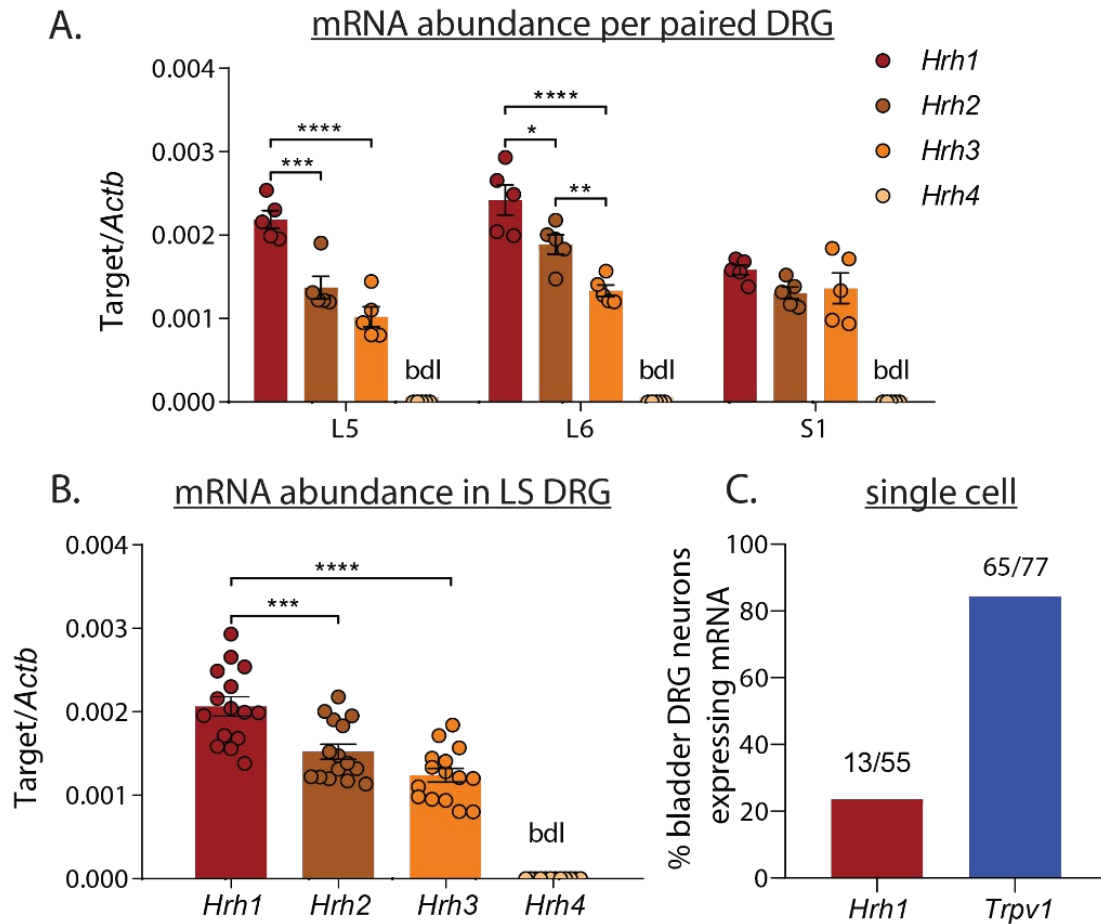


Figure 5.4: Neuronal mRNA expression of histamine receptor subtypes

(A) QRT-PCR was used to determine how mRNA expression of histamine receptor subtypes *Hrh1*, *Hrh2*, *Hrh3*, *Hrh4* relative to *Actb* differs between lumbosacral (LS; L5, L6, S1) dorsal root ganglia (DRG; N = 5). Each dot represents paired DRG from each level. *Hrh1* mRNA expression was significantly more abundant than *Hrh2* (***) and *Hrh3* (****) at L5 and L6 (* P < 0.05, and **** P < 0.0001 respectively). *Hrh2* and *Hrh3* were also significantly differently expressed at L6 (** P < 0.01), however no differences between *Hrh1*, *Hrh2* and *Hrh3* were observed in S1. *Hrh4* mRNA expression was not detected in all LS DRG (below detectable limit; bdl). **(B)** Global expression of histamine receptor subtypes *Hrh1*, *Hrh2*, *Hrh3* and *Hrh4* relative to *Actb* in pooled lumbosacral (LS; L5, L6 and S1) dorsal root ganglia (DRG; N = 5). Each sample contained one DRG pair (at L5, L6 or S1) and was run in duplicate for each target and mean target Ct relative to mean *Actb* Ct determined ($2^{-\Delta Ct}$). *Hrh1* was significantly more abundant than *Hrh2* (***) and *Hrh3* (****) ($P < 0.0001$). *Hrh4* mRNA expression was not detected (below detectable limit; bdl). **(C)** The percentage of retrogradely labelled bladder-innervating DRG neurons expressing *Hrh1* and *Trpv1* was determined using single cell RT-PCR. Overall, 23.6% of bladder-innervating DRG neurons were positive for *Hrh1* mRNA expression (13 out of 55 neurons). 84.4% of an independent subset of retrogradely labelled bladder-innervating DRG neurons were positive for *Trpv1* mRNA expression (65 out of 77 neurons). Data are represented as mean $\pm$ SEM ($2^{-\Delta Ct}$) on a linear scale (**A-B**), or percentage of neurons (**C**). P values above are based on one-way ANOVA with Tukey's multiple comparison test, comparing *Hrh1*, *Hrh2* and *Hrh3* (**A-B**).

5.4.4 Histamine evokes direct activation in a small subpopulation of bladder DRG neurons

Having identified that histamine has a significant effect on the afferent sensitivity of the bladder both peripherally and at the level of the spinal cord, we aimed to determine whether this effect was as a result of direct neuronal activation. Retrogradely traced bladder afferent cell bodies from LS DRG were dissected, dissociated, and cultured for live cell calcium imaging to measure intracellular calcium transients in response to short (1 minute) incubations in histamine. Direct application of histamine evoked calcium transients in a population of LS DRG neurons, including a small population of bladder-innervating neurons (2 out of 63; **Figure 5.5A** and **5.5B**). While these numbers do not equate to the percentage of cells expressing histamine receptors, this data demonstrates that histamine can activate bladder neurons directly.

The direct action of histamine on bladder afferents was also examined in the *ex vivo* bladder afferent preparation by measuring direct firing, in response to histamine between bladder distensions. Interestingly, whilst histamine evoked marked increases in afferent firing to bladder distension (**Figure 5.1**), in the same *ex vivo* preparations we did not observe action potential firing in response to histamine compared to saline application (**Figure 5.5C** and **5.5D**). Taken together, these results indicate that the observed increase in peripheral and central afferent signaling in response to histamine *ex vivo* and *in vivo* are not simply a result of direct, independent activation of the neurons.

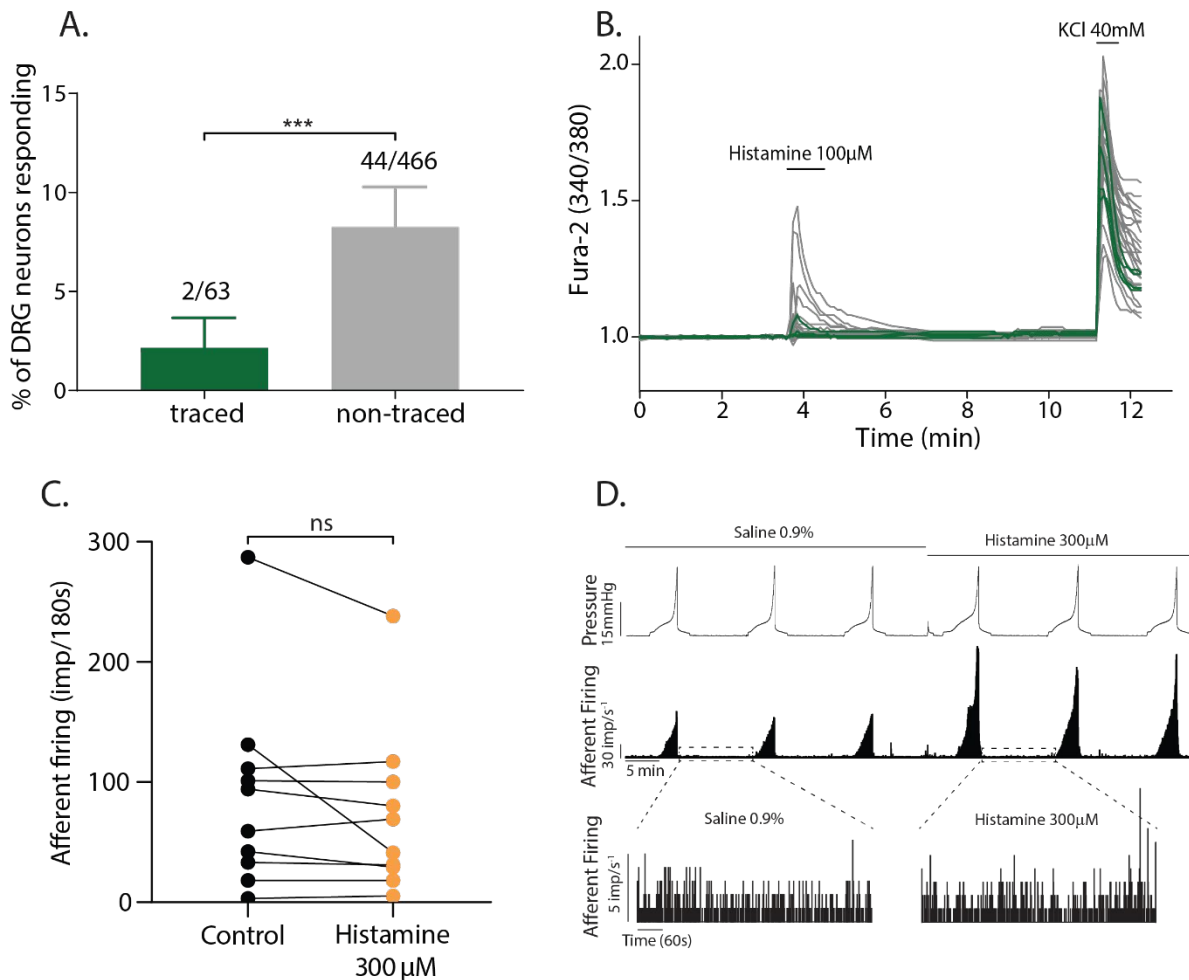


Figure 5.5: Direct effect of histamine on bladder afferents *in vitro* and *ex vivo*

Direct responses of bladder afferent neurons to histamine were determined using live cell calcium imaging *in vitro* and afferent nerve recordings without bladder distension in the *ex vivo* model. **(A)** A small population of isolated and cultured primary bladder afferent neurons from lumbosacral (LS) dorsal root ganglia (DRG) responded to 100 μM histamine (3%; 2 out of 63 neurons), compared to 9.4% (44 out of 466 neurons) of untraced LS DRG neurons (** $P < 0.001$; $N = 10$). **(B)** Example calcium imaging experiment. Traced neurons are shown in green (1 out of 4 responding), untraced neurons are shown in grey (10 out of 28 responding). Fura-2 fluorescence ratio (340/380) indicates relative intracellular calcium, baseline fluorescence normalized to ratio of 1. 40 mM KCl was used as a positive control for neuronal responsiveness. **(C)** Afferent firing (impulses per 180 seconds (imp/180s) in response to instillation of 300 μM histamine (5-15 minutes) in the bladder is not significantly different to baseline control (saline) in the *ex vivo* model without distension (ns; $P > 0.05$). **(D)** An example experiment showing very low levels of multiunit raw afferent nerve activity in the presence of saline (control) or 300 μM histamine before bladder distension (afferent firing in direct response to the compound) measured in 5 impulses per second (5 imp/s). Data are represented as mean percentage of neurons per experiment $\pm$ SEM (total 27 experiments) **(A)** or mean $\pm$ SEM **(C)**. P values above are based on a Mann Whitney test **(A)** or a Wilcoxon matched-pairs signed rank test **(C)**.

5.4.5 Histamine does not evoke calcium transients in isolated primary urothelial cells

As the effects observed during our *ex vivo* and *in vivo* experiments resulted from histamine infused into the bladder lumen and the urothelium has been shown to exert a sensory role within the bladder by responding to and releasing a number of neuromodulators, we sought to determine whether urothelial cells play a role in mediating histamine-induced sensitivity in the bladder. To do this, we assessed the expression and function of histamine receptors in isolated primary urothelial cells via QRT-PCR and live cell calcium imaging (**Figure 5.6**). We confirmed that the isolation process was generating pure cultures of urothelial cells through positive immunolabelling of CK7 in isolated urothelial cells (**Figure 5.6A**). Similar to what we observed in the LS DRG, primary urothelial cells demonstrated expression of *Hrh1-Hrh3* mRNA, with *Hrh4* mRNA expression levels below reliable detection limits (**Figure 5.6B**). Interestingly, relative expression of *Hrh3* was significantly higher than that of *Hrh1* and *Hrh2* in primary urothelial cells. Relative expression was also determined in the whole mucosa (including urothelium and underlying interstitial tissue with the detrusor smooth muscle removed) and the whole detrusor smooth muscle separately (**Figure 5.6C**). Expression of each of the histamine receptor subtypes relative to housekeepers *Gapdh* and *Hprt* was higher in the mucosa than the isolated urothelial cells but of a similar ratio, with *Hrh3* the highest expressed receptor. Expression in the detrusor muscle was highest for *Hrh2* and *Hrh1*, with significantly lower expression of *Hrh3*. As with isolated urothelial cells, *Hrh4* mRNA was below the detectable limits for both bladder mucosa and detrusor (**Figure 5.6C**).

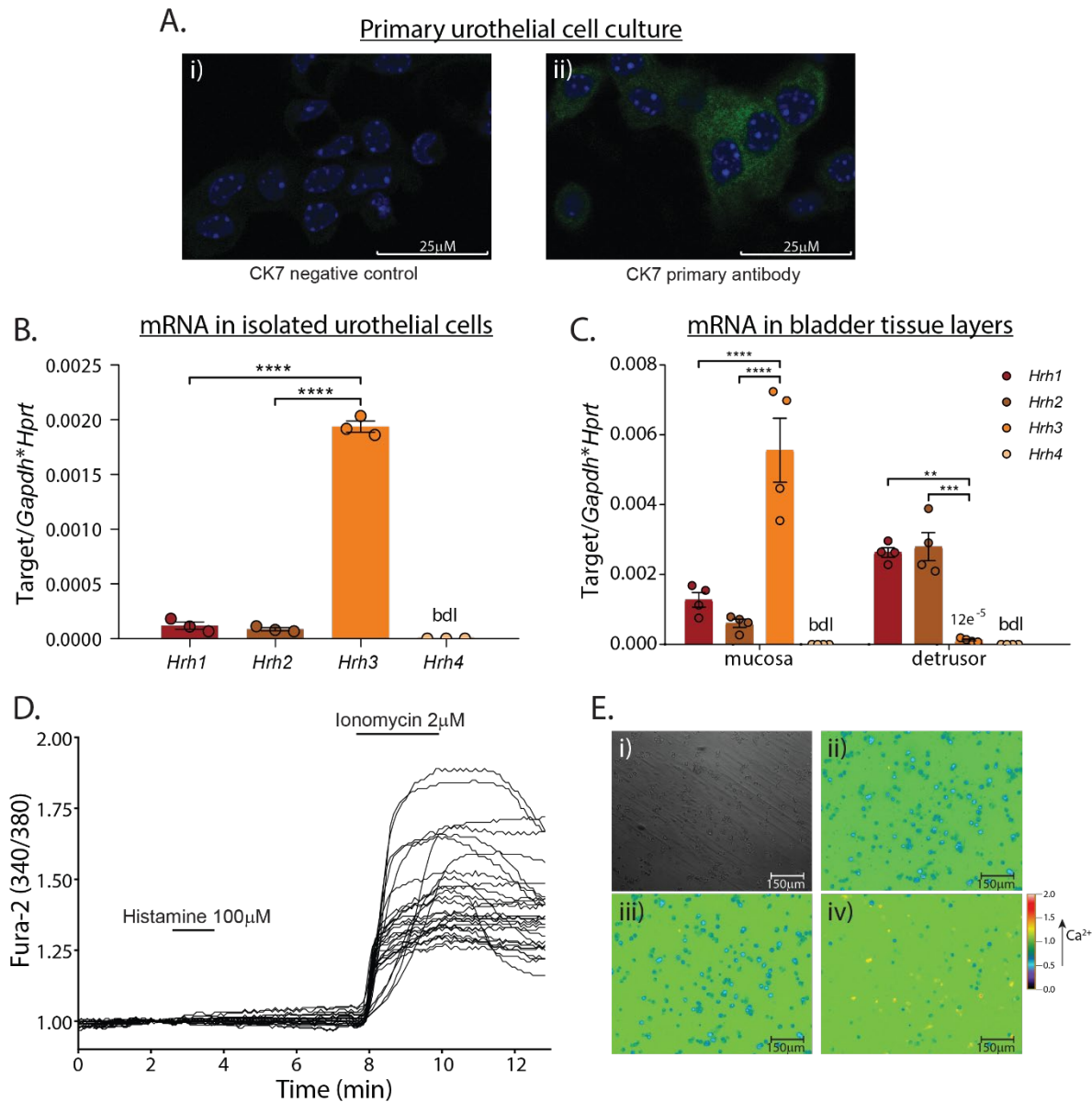


Figure 5.6: Histamine does not evoke calcium transients in isolated primary urothelial cells
(A) Cytokeratin 7 (CK7) staining (green) was used to confirm cell type. Cell nuclei were identified by DAPI staining (blue). **(B)** Histamine receptor subtypes *Hrh1*, *Hrh2* and *Hrh3* were all expressed in isolated urothelial cells (N = 3), while *Hrh4* expression was below the detectable limit (bdl). Expression of target receptors is relative to the geomean of *Gapdh* and *Hprt*. All receptors were measured in duplicates. *Hrh3* showed significantly higher relative expression compared to *Hrh1* (**** P < 0.0001) and *Hrh2* (**** P < 0.0001). **(C)** mRNA expression profiles of histamine receptor subtypes *Hrh1*, *Hrh2*, *Hrh3*, *Hrh4* relative to genes *Gapdh* and *Hprt* (*Gapdh***Hprt*) differ between mucosal and detrusor smooth muscle layers of the bladder (N = 4). Each sample was run in duplicate for each target and mean target Ct relative to geomean of mean housekeeper Ct values was determined ($2^{-\Delta Ct}$). *Hrh3* expression was significantly higher than *Hrh1* (**** P < 0.0001) and *Hrh2* (**** P < 0.0001) in the mucosa, corresponding to the expression profiles observed in isolated urothelial cells in **(B)**. Conversely, *Hrh1* (** P < 0.01) and *Hrh2* (** P < 0.001) were both significantly more abundant than *Hrh3* in the detrusor smooth muscle. Between the tissue types, *Hrh2* expression was

significantly higher in the detrusor smooth muscle than in the mucosa (** $P < 0.01$, represented as '+'), while *Hrh3* expression was significantly higher in the mucosa than in the detrusor layer (***) $P < 0.001$, represented as '^'). *Hrh4* mRNA expression was not detected (below detectable limit; bdl). **(D)** An example calcium imaging experiment demonstrating that application of 100 μ M histamine did not evoke calcium transients in Fura-2 loaded urothelial cells. Application of 2 μ M ionomycin was used as a positive control for calcium influx (represented as an increase in Fura-2 340/380 fluorescence ratio) in calcium imaging experiments. **(E)** Example images of urothelial cells from a calcium imaging experiment **(i)** prior to experiment imaged via light filter, and imaged as 340/380 fluorescence ratio to determine changes in intracellular calcium **(ii)** at the start of the experiment, **(iii)** following addition of 100 μ M histamine, and **(iv)** following addition of 2 μ M ionomycin. An increase in 340/380 fluorescence ratio and hence intracellular calcium is depicted by color change on a heat scale from blue (baseline intracellular calcium) to yellow (high intracellular calcium) or red (very high intracellular calcium). Data are represented as mean $\pm$ SEM ($2^{-\Delta Ct}$) on a linear scale. P values above are based on one-way ANOVA with Tukey's multiple comparison test, comparing *Hrh1*, *Hrh2* and *Hrh3*.

Given the relative abundance of histamine receptors in urothelial cells, we used live-cell calcium imaging to determine whether isolated urothelial cells are directly activated in the presence of histamine, as a potential mechanism contributing to the enhancement of bladder mechanosensitivity we observed *ex vivo* and *in vivo*. Histamine application was unable to evoke calcium transients in any urothelial cells across multiple independent experiments (**Figure 5.6D** and **5.6E**). The administration of 2 μ M ionomycin following wash-out of histamine as a control for urothelial cellular responsiveness demonstrated that the cultured cells were healthy and able to respond with increases in intracellular calcium. These results suggest that while histamine receptors are present in the bladder and specifically in urothelial cells, histamine is unable to directly trigger calcium influx in an isolated urothelial culture.

5.4.6 Histamine H1 receptor (H1R) mediates histamine-evoked enhanced mechanosensitivity to bladder distension *ex vivo*

Given that *Hrh1* was the most abundantly expressed histamine receptor subtype in LS DRG, and the significant proportion of bladder-innervating afferent neurons expressing *Hrh1* mRNA, we sought to determine what role this receptor subtype plays in mediating histamine activity in the bladder. Application of pyrilamine, a H₁R antagonist, blocked histamine-evoked mechanical hypersensitivity in an *ex vivo* bladder afferent preparation (**Figure 5.7A** and **5.7B**). Furthermore, pyrilamine had no effect on baseline mechanosensitivity, indicating H₁R is not an essential component of bladder mechanosensitivity under normal conditions. Pylamine also had no effect on bladder muscle compliance (**Figure 5.7C**), indicating that, as with the histamine-induced mechanosensitivity without

pyrilamine, the inhibition of this response is at the afferent level rather than a secondary effect from altered bladder capacity. These results suggest that the enhanced mechanosensitivity observed in response to histamine is mediated by H₁R.

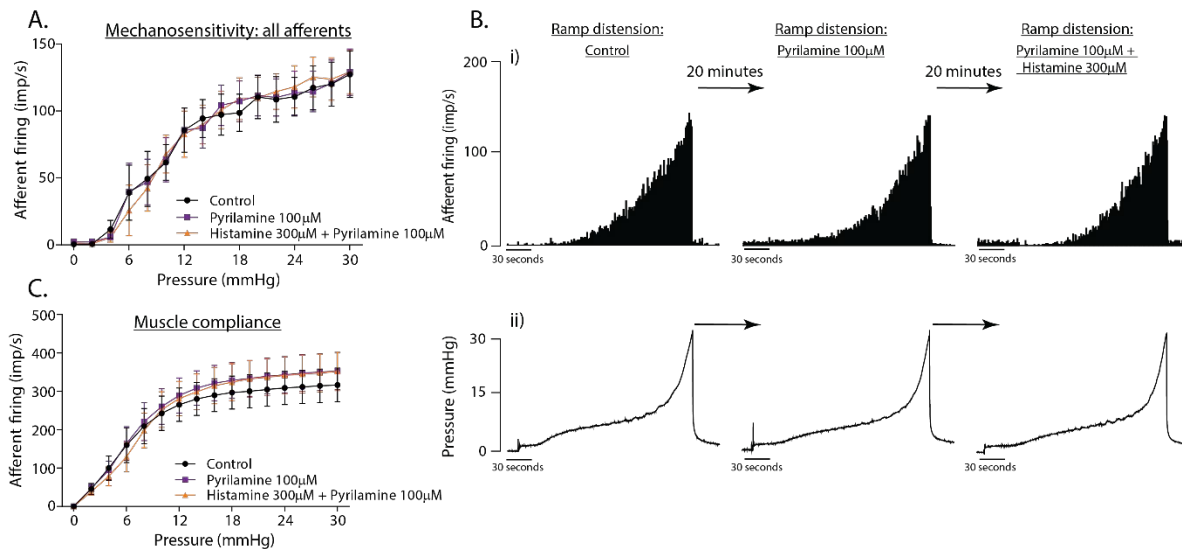


Figure 5.7: Histamine H₁ receptor (H₁R) antagonist pyrilamine blocks histamine-evoked increase in bladder mechanosensitivity

Graded distension of the bladder in an *ex vivo* nerve recording preparation was performed with saline and 300 µM histamine in the presence of H₁R antagonist pyrilamine (100 µM). **(A)** No changes were observed in afferent firing rate (impulses per second; imp/s) following administration of 100 µM of pyrilamine alone or 100 µM pyrilamine with 300 µM histamine compared to saline at any distension pressure (0 – 30 mmHg; N = 6; ns P > 0.05). **(B)** An example experiment showing raw afferent nerve activity in the presence of **(i)** saline, 100 µM pyrilamine, and 100 µM pyrilamine with 300 µM histamine in succession, increasing in response to **(ii)** graded increase in intraluminal pressure. **(C)** No change in muscle compliance (pressure-to-volume relationship) was observed between saline, pyrilamine, or pyrilamine and histamine protocols, suggesting afferent responses were not affected by changes in musculature. Data are represented as mean ± SEM. P values above are based on two-way ANOVA with subsequent Sidak's post hoc test significance at individual data points (ns, P > 0.05).

5.4.7 TRPV1 expression is required for histamine-evoked hypersensitivity to bladder distension *ex vivo*

TRPV1 has previously been implicated in H₁R-mediated histamine responses in other organs [48, 49]. As our results show a high proportion of bladder-innervating afferent neurons expressing *Trpv1* mRNA and a critical role for H₁R in the histamine-induced mechanosensitization *ex vivo*, we aimed to

determine whether the effect of histamine was lost in preparations from *Trpv1*^{-/-} mice (**Figure 5.8**). Supporting previous studies, when we compared control (saline) bladder distensions between *Trpv1*^{-/-} and wildtype mice in the *ex vivo* bladder preparation we found a significant reduction in mechanosensitivity in the *Trpv1*^{-/-} mice (**Supplementary Figure 5.3**). Importantly, while the overall magnitude of the response was blunted compared to the wildtype mice, the afferent response to distension in the *Trpv1*^{-/-} mice demonstrated a graded increase in firing in response to increased intraluminal pressure in the bladder, of an equivalent pattern to the wildtype preparation. In the *Trpv1*^{-/-} mice, histamine failed to elicit a change in overall bladder afferent mechanosensitivity compared to saline control responses (**Figure 5.8A**). Consistent with healthy control mice, no changes in muscle compliance were observed (**Figure 5.8B**). Interestingly, analysis of the single units in the *Trpv1*^{-/-} preparations revealed that while the overall histamine response was abolished, a small proportion (20%) of single units maintained histamine sensitivity (**Figure 5.8D and 5.8E**). These histamine-sensitive afferents demonstrated a significant increase in mechanosensitivity following histamine administration, as with histamine-sensitive afferents in the wildtype mice. We also did not observe the recruitment of silent afferents in the presence of histamine in the *Trpv1*^{-/-} experiments. Furthermore, histamine failed to evoke calcium transients in dissociated bladder-innervating LS DRG from *Trpv1*^{-/-} mice (**Figure 5.8F**), whilst the percentage of non-traced LS DRG neurons responding to histamine in *Trpv1*^{-/-} mice (4.1%) was reduced compared to wild type mice (9.4%; see **Figure 5.5A and Figure 5.8F**). Taken together, these results suggest that TRPV1 is important in mediating histamine-induced mechanosensitivity to bladder distension, however a component of the histamine response is also TRPV1-independent.

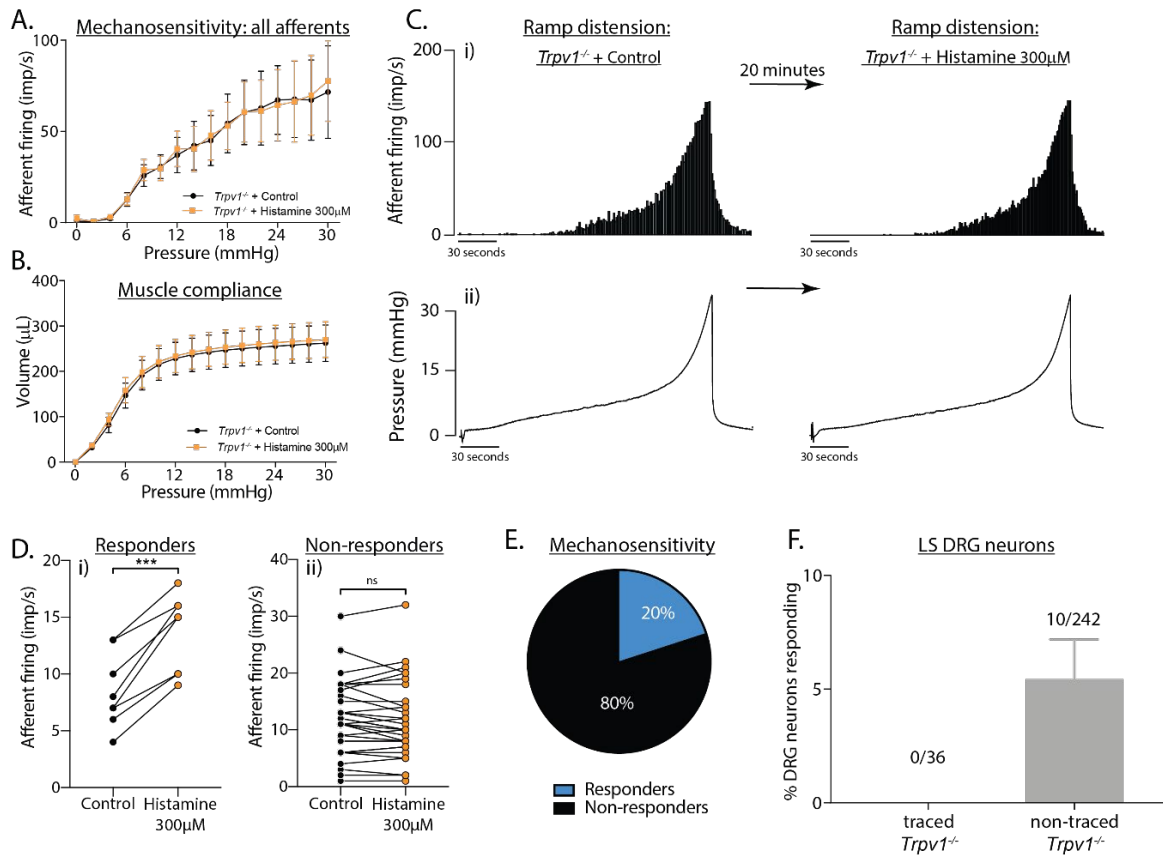


Figure 5.8: Histamine-evoked increase in bladder afferent mechanosensitivity *ex vivo* is severely reduced in *Trpv1^{-/-}* mice

Graded distension of the bladder in a *Trpv1^{-/-}* *ex vivo* nerve recording preparation was performed with saline followed by 300 μM histamine. **(A)** No change in overall afferent firing rate (impulses per second; imp/s) was observed following administration of 300 μM histamine compared to saline at any distension pressure (0 – 30 mmHg; N = 5; P > 0.05). **(B)** No change in muscle compliance (pressure-to-volume relationship) was observed between saline and histamine protocols, suggesting afferent responses were not affected by changes in musculature. **(C)** An example experiment showing **(i)** an equivalent increase in raw afferent nerve activity in the presence of 300 μM histamine compared to saline in response to **(ii)** increased intraluminal pressure. **(D)** Single unit analysis showed that the majority of *Trpv1^{-/-}* afferents (n = 32 out of 40; ns P > 0.05; $\Delta = -0.33 \pm 0.34$ imp/s) were histamine insensitive **(ii)**, with no change in overall afferent firing rate (imp/s) between saline and 300 μM histamine protocols, while a small proportion of *Trpv1^{-/-}* single afferent units **(i)** showed a significant increase in overall afferent firing rate (imp/s) between saline and 300 μM histamine protocols (n = 8 out of 40 afferents; *** P < 0.001; $\Delta = 5.125 \pm 3.60$ imp/s). **(E)** Pie chart depicting the proportion of histamine responsive (20%) and histamine insensitive (80%) single unit afferents. **(F)** Isolated bladder-innervating lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) neurons from *Trpv1^{-/-}* mice (N = 6) were unresponsive to 100 μM histamine (0 out of 36 neurons). A small percentage of untraced LS DRG neurons from *Trpv1^{-/-}* mice responded to 100 μM histamine (10 out of 242 neurons, 4.1%). Data are represented as mean $\pm$ SEM **(A, B, F)**, maximum imp/s **(D)**, or mean percentage of neurons per experiment $\pm$ SEM (total 21 experiments) **(E)**. P values above are based on two-way ANOVA **(A, B)** with subsequent Sidak's post hoc test

significance at individual data points (ns, $P > 0.05$), or Wilcoxon matched-pairs signed rank test or paired t-test (**D**).

5.5 Discussion

The chronic sensory symptoms of urgency, frequency, and pelvic pain experienced by IC/BPS patients implicate altered bladder afferent activity as a key component in the pathophysiology of this disorder. Despite this, the specific mechanisms underlying a switch from normosensitivity (healthy state) to hypersensitivity in IC/BPS are still unclear, and thus effective treatments are currently lacking [6]. Previous studies have indicated that a relationship exists between the release of histamine from mast cells and the sensitization of bladder afferent neurons in IC/BPS that contributes to the symptoms of hypersensitivity, including pelvic pain [27-34]. In this study, for the first time, we show that histamine is able to directly sensitize mechanosensitive bladder afferent neurons via mechanisms regulated by the histamine H₁ receptor (H₁R) and transient receptor potential vanilloid 1 (TRPV1).

Our results demonstrate that a population of primary afferent neurons innervating the bladder exhibit enhanced mechanosensitivity in the presence of histamine. Furthermore, we identified a sub-population of 'silent afferents' that became mechanosensitive only in the presence of histamine. These neurons represent a distinct functional subclass previously reported to be responsible for signaling 'high-threshold' or 'nociceptive' stimuli, responding only to noxious levels of distension, that can be sensitized by chemical mediators [50-52]. Together, these data suggest that if an increase in histamine occurs in the bladder wall, it has the ability to sensitize bladder afferents to mechanical distension, such that they fire more frequently in response to a range of bladder distension pressures, potentially leading to exaggerated sensation. Our data is supported by previous studies showing a role for histamine in the activation and sensitization of sensory afferents innervating both the skin and gastrointestinal tract [22-24, 53, 54], as well as intracranial nociceptors [55]. Lower concentrations of histamine had no effect on bladder mechanosensitivity *ex vivo*. The primary role of the urothelium is to provide a barrier to the toxic solutes in the urine, however, we and others utilizing these techniques have shown that by using relatively high concentrations of experimental compound we are able to overcome this barrier to target the underlying mechanosensory afferent nerves [37, 56]. Whilst this provides the limitation that the exact concentrations at the afferent terminals is unknown, and prevents meaningful washout of the agonist in question, it provides significant value in proof of concept studies such as this. It is also important to consider that the concentrations of histamine that bladder nerve terminals are exposed to in health and disease are unknown, but likely exceed those found in the urine. Mast cells are the primary source of histamine [20, 21], and a number

of previous studies have identified increased activated mast cell counts in IC/BPS patients [17-19, 24, 57] as well as increased urinary levels of histamine in these patients [25, 26]. Mast cells have been identified in extremely close proximity to nerve endings [24, 58-61], allowing direct mast cell nerve interactions that likely involve high localized concentrations of histamine and which may promote the underlying hypersensitivity associated with IC/BPS that contributes to urinary frequency and urgency.

Bladder-innervating primary afferents project into the lumbosacral spinal dorsal horn, where subpopulations of neurons within the DGC, SDH, and the SPN have been previously identified within the bladder sensory pathways [6, 8, 14, 62-65]. Such pathways are known to project up to the brain as well as form local connections to spinal reflex arcs, feeding into networks which are important for both micturition and nociception [6, 8, 44, 66]. Using pERK-IR to examine dorsal horn neuronal activation we confirmed that the exaggerated peripheral afferent signal stimulated by histamine evokes enhanced neuronal activation within these dorsal horn spinal cord regions [8, 44, 62, 63]. We have previously demonstrated that during graded bladder distension from physiological to noxious bladder distension pressures, similar regions of the spinal cord are activated, with nociceptive signaling identified by an increase in the number of pERK-IR neurons within those same regions, rather than activation of a distinct nociceptive pathway [6]. Similarly, the presence of histamine in the bladder *in vivo* increases activation in these same regions, rather than activating distinct irritant pathways.

Having established a direct involvement for histamine in mediating both peripheral and central sensitivity to bladder distension, we sought to understand the specific mechanism of this interaction. We show that histamine sensitizes bladder afferents via a H₁R-dependent mechanism, as histamine-evoked hypersensitivity to distension was completely abolished in the presence of the H₁R antagonist pyrilamine. This is an interesting finding as afferents innervating the colon are also activated by histamine via a H₁R-dependent mechanism [48]. Similarly, histamine also activates cutaneous DRG neurons, although this effect appears more promiscuous, by acting on H₁R, H₃R and H₄R [30, 67]. The down-stream mechanisms of H₁R have also been identified in these populations of DRG neurons. Histamine sensitizes TRPV1 channels via H₁R in irritable bowel syndrome (IBS), a chronic visceral sensitivity disorder [48]. In cutaneous DRG neurons, TRPV1 also mediates H₁R-dependent histamine-sensitization [48, 49], with coupling between histamine receptors and TRPV1 occurring via activation of phospholipase C and phospholipase A2 [68].

Our QRT-PCR data show *Hrh1* is the most abundant of the histamine receptors in LS DRG, and we confirmed *Hrh1* mRNA expression in ~24% of bladder-innervating LS DRG neurons with single cell RT-PCR. We also identified that TRPV1 is an essential integrator of histamine-induced mechanosensitivity in the bladder. No overall increase in mechanosensitivity was observed in the presence of histamine

in *Trpv1*^{-/-} mice and nor were calcium transients to histamine observed in dissociated *Trpv1*^{-/-} mouse bladder DRG neurons. While the overall effect of histamine on mechanosensitivity is absent in *Trpv1*^{-/-} mice, a sub-population of bladder afferents are still histamine sensitive, albeit a reduced percentage compared to wild-type mice. As with many GPCRs, the activation of histamine receptors results in several intracellular processes [68-70]; evidently a subset of histamine-sensitive bladder afferents respond in a TRPV1-independent manner. Along these lines, histamine also sensitizes colonic neurons via TRPV1[48] and a related TRP channel, TRPV4 [71]. In the current study, we found that ~84% of bladder-innervating LS DRG neurons express TRPV1. This is consistent with a number of previous studies from both rat and mouse bladder innervating DRG showing between 48-90% express TRPV1 or show functional responses to the TRPV1 agonist capsaicin [72-74]. Although TRPV1 is generally considered a marker of polymodal nociceptors or C-fibers, we and others have found *Trpv1*^{-/-} mice exhibit alterations in afferent firing at physiological distension pressures [75], building on a wealth of research suggesting traditional nomenclature and characterizations of 'C-fibers' as nociceptors should not be relied upon as a proxy for functional characterization of sensory neurons in the bladder [73, 76, 77].

The question remains as to whether the histamine-dependent sensitivity of bladder afferents also involved non-neuronal mechanisms. This is important as the urothelium is known to express various ion channels and receptors that enable it to mediate sensory signaling by responding to chemical and mechanical stimuli [78-80]. Given the demonstrated increase of histamine and metabolites of histamine in the urine of IC/BPS patients [25, 26], we explored the potential role of urothelial cells in mediating histamine-enhanced bladder afferent mechanosensitivity. Here we confirmed the expression of histamine receptors *Hrh1-Hrh3* in all layers of the mouse bladder but did not detect *Hrh4*. These findings differ from previously reported results in mice but are comparable to results from human tissue [28, 81, 82]. *Hrh4* is predominantly expressed in granulocytes and we validated this probe against mRNA isolated from the spleen. Importantly, our mRNA expression data demonstrated expression of *Hrh1*, *Hrh2* and *Hrh3* for the first time in isolated urothelial cells, highlighting the potential for urothelial cells in mediating histamine-induced modulation of mechanosensitivity. Notably, in mucosa and primary urothelial cells *Hrh3* displayed the highest abundance of all histamine receptors, which differs to our data in DRG, where *Hrh1* is most abundant. Furthermore, in detrusor muscle mRNA *Hrh2* and *Hrh1* were significantly more abundant than *Hrh3*, demonstrating clear differences with expression profiles in DRG, detrusor muscle, mucosa and primary urothelial cells. Despite the relative expression of histamine receptor subtypes in isolated urothelial cell culture, application of histamine did not induce calcium influx in these cells. Our primary mouse urothelial cell culture was confirmed to be of urothelial origin, but the proportion of umbrella,

intermediate, and basal cells remains undetermined. Previous studies have indicated that whilst this type of primary culture isolates a heterogeneous urothelial cell population [83], the majority of these cells are of basal and intermediate origin and exhibit functional responses to a range of ion channel and GPCR agonists [46, 83-87]. Whilst this does not preclude an effect of histamine on bladder urothelial cells, as we saw no responses to histamine in any of the cells from this study, our data indicates that intracellular calcium responses are not directly initiated by short duration exposure to histamine. Further dissection of this mechanism following the development of techniques to isolate individual populations of urothelial cells or *in vitro* imaging of the mucosa would provide valuable insight into these interactions. Two considerations remain unanswered here. The first, that *Hrh3* as the highest expressed receptor in urothelial cells may be contributing in its capacity to function as an inhibitory receptor in healthy states. Second, while short term application allows us to explore the direct activity of histamine on cells via receptors, IC/BPS is a chronic condition, with diagnoses often occurring several months or even years after symptom onset [88]. As such, the expression of histamine receptors in the urothelial cells may be important in the long-term potentiation of symptoms, if not acute activation.

The findings of the current study raise an interesting question as to role of histaminergic mechanisms within the bladder. We can draw parallels from other systems, whereby histamine evokes somatic itch via activation or sensitization of peripheral afferents innervating the skin [30, 53, 67, 89-92]. As the physiological response to cutaneous itch is scratching to remove harmful irritants, the corresponding physiological response in the bladder would be achieved via micturition-induced irritant expulsion. This would involve histamine sensitizing bladder afferents to mechanical stimuli and triggering increases in the frequency of micturition, and thus experiencing sensations of urgency at lower distension volumes via an increase in mechanosensitivity may be a fundamental protective mechanism.

In conclusion, this study has demonstrated for the first time that histamine in the urinary bladder causes enhanced mechanosensitivity to bladder distension as well as recruitment of silent afferents, which translates to increased neuronal activation in the spinal cord dorsal horn. As such, in conditions such as IC/BPS, where mast cells and urinary histamine concentrations are increased, we propose that histamine contributes to the sensitization of mechanosensitive bladder afferent pathways resulting in symptoms of urgency, frequency and suprapubic pain at physiological distension pressures.

5.6 References

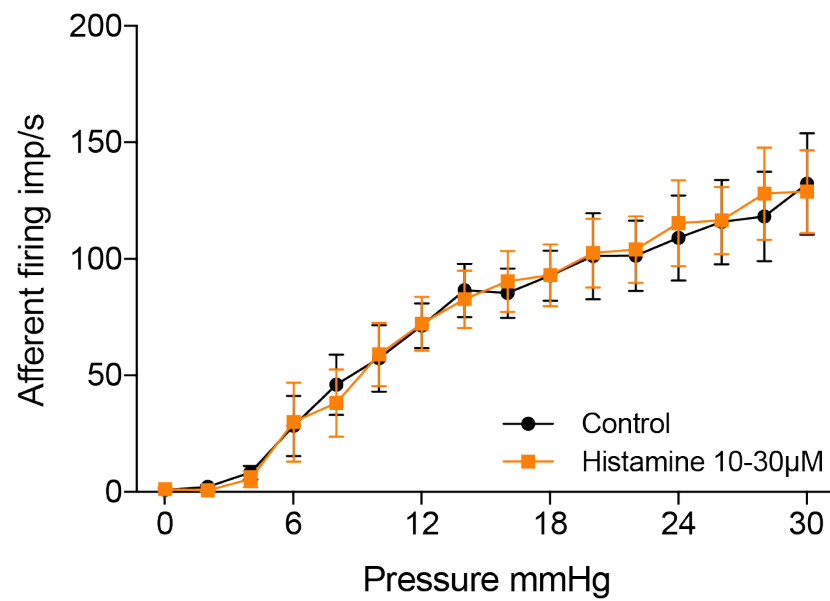
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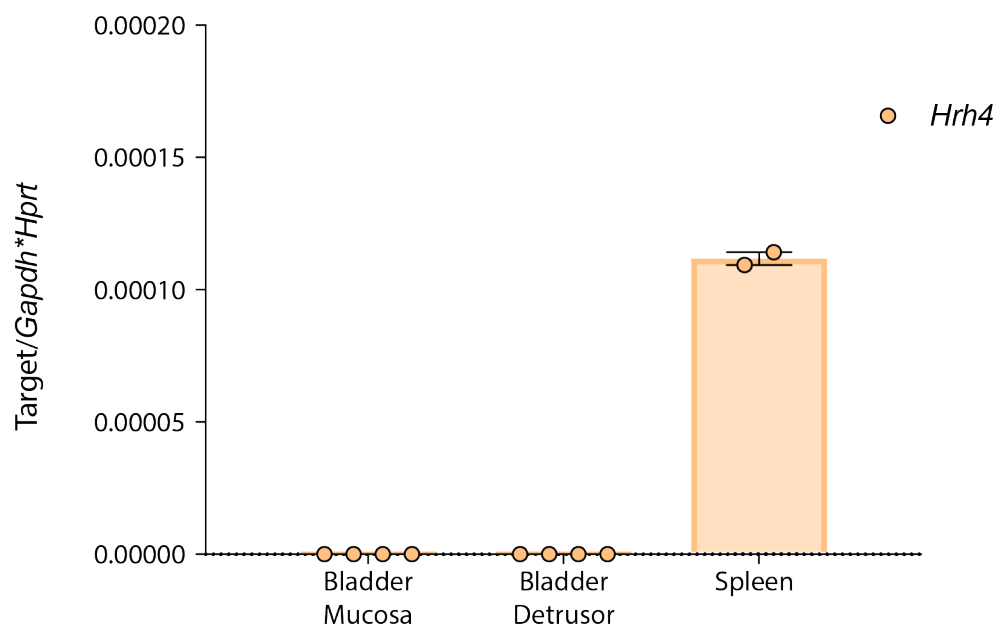
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5.7 Supplementary Figures

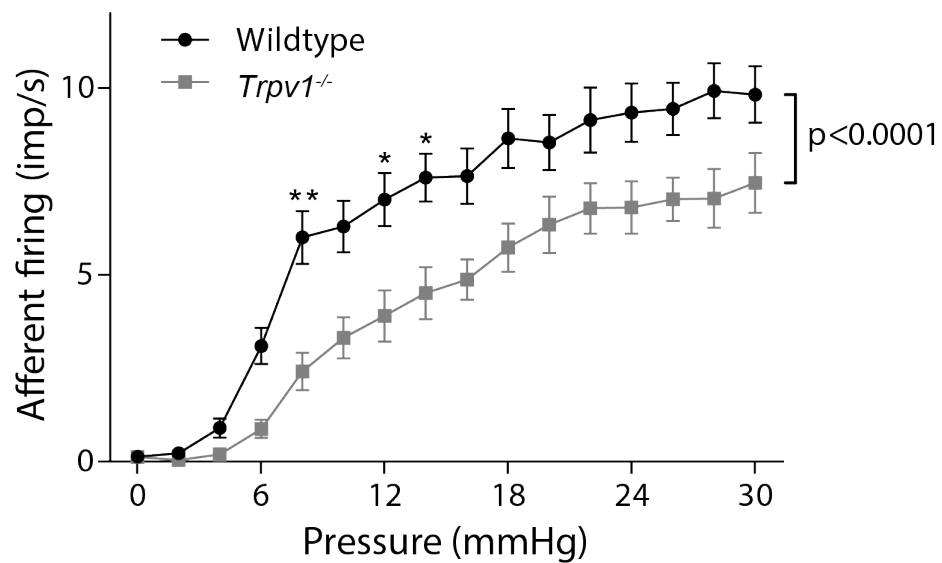


Supplementary Figure 5.1: Afferent firing in response to graded bladder distension *ex vivo* was unchanged following intrabladder infusion of histamine (10-30 μM, N=6)



Supplementary Figure 5.2: Validation of histamine receptor 4 (*Hrh4*) PCR primer in mouse spleen. *Hrh4* was expressed in mouse spleen but not expressed in bladder mucosa or bladder detrusor.

Mechanosensitivity: saline



Supplementary Figure 5.3: Mechanosensitivity to bladder distension is reduced in *Trpv1*^{-/-} mice compared to C57/Bl6J wildtype mice. Afferent firing in response to graded bladder distension *ex vivo* was compared between *Trpv1*^{-/-} mice (N=5) and healthy control mice (C57/Bl6J; N=10).

Statement of Authorship

Title of Paper	Activation of MrgprA3 and MrgprC11 on bladder-innervating afferents induces peripheral and central hypersensitivity to bladder distension
Publication Status	☒ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Grundy L, Caldwell A, Garcia-Caraballo S, Grundy D, Spencer NJ, Dong X, Castro J, Harrington AM, Brierley SM. <i>Activation of MrgprA3 and MrgprC11 on bladder-innervating afferents induces peripheral and central hypersensitivity to bladder distension</i> . J Neurosci. 2021 Mar 16;JN-RM-0033-21. doi: 10.1523/JNEUROSCI.0033-21.2021. Epub ahead of print. PMID: 33727332.

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Chapter 6 Activation of MrgprA3 and MrgprC11 on bladder-innervating afferents induces peripheral and central hypersensitivity to bladder distension

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6.1 Abstract

Understanding the sensory mechanisms innervating the bladder is paramount to developing efficacious treatments for chronic bladder hypersensitivity conditions. The contribution of Mas gene-related G protein-coupled receptors (Mrgpr) to bladder signalling is currently unknown. Here we show in mice with single-cell RT-PCR that sub-populations of dorsal root ganglion (DRG) neurons innervating the mouse bladder express *Mrgpra3* (14%) and *Mrgprc11* (38%), either individually or in combination, with high levels of co-expression with *Trpv1* (81-89%). Calcium imaging studies demonstrated MrgprA3 and MrgprC11 agonists (chloroquine, BAM8-22 and neuropeptide FF) activated sub-populations of bladder-innervating DRG neurons, showing functional evidence of co-expression between MrgprA3, MrgprC11 and TRPV1. In *ex vivo* bladder-nerve preparations chloroquine, BAM8-22 and neuropeptide FF all evoked mechanical hypersensitivity in subpopulations (20-41%) of bladder afferents. These effects were absent in recordings from *Mrgpr-clusterΔ^{-/-}* mice. *In vitro* whole-cell

patch clamp recordings showed that application of an MrgprA3/C11 agonist cocktail induced neuronal hyper-excitability in 44% of bladder-innervating DRG neurons. Finally, *in vivo* instillation of an MrgprA3/C11 agonist cocktail into the bladder of wildtype mice induced a significant activation of dorsal horn neurons within the lumbosacral spinal cord, as quantified by pERK-immunoreactivity. This MrgprA3/C11 agonist-induced activation was particularly apparent within the superficial dorsal horn and the sacral parasympathetic nuclei of wild-type but not *Mrgpr-clusterΔ^{-/-}* mice. This study demonstrates, for the first time, functional expression of MrgprA3 and MrgprC11 in bladder afferents. Activation of these receptors is not required for normal bladder function but does trigger hypersensitivity to distension, a critically valuable factor for therapeutic target development.

6.2 Introduction

Patients suffering from common urological disorders such as overactive bladder (OAB) and interstitial cystitis/bladder pain syndrome (IC/BPS) experience chronic urinary urgency, frequency, and discomfort or pain [1, 2]. Hypersensitivity of the peripheral afferents innervating the bladder are considered a key component in the underlying symptomology of these conditions [3-5]. Numerous nociceptive ion channels and receptors, including TRPV1 and P2X3, have been identified as contributing to bladder afferent hypersensitivity, and have been subsequently explored in order to develop novel treatments for bladder disorders [3, 6-11]. However, other mechanisms are also likely involved. In this regard, cues can be taken from sensations that originate from the skin, whereby itch and pain are commonly experienced protective sensations that occur in response to irritant or noxious stimuli [12]. Both of these processes require activation and sensitisation of sensory afferents that project from the skin into the dorsal horn of the spinal cord where they provide input to the central nervous system [13-16]. Interestingly, itch can be mediated by histamine release from immune cells, which activate histamine receptors on sensory afferents [17], whilst several histamine-independent itch mechanisms have also been described. These include those dependent on Mas related G-protein coupled receptors (Mrgprs) [18, 19], a large subfamily of GPCRs predominantly expressed in the dorsal root ganglia (DRG) [20, 21]. In particular, MrgprA3 has been identified as the receptor responsible for itch associated with the antimalarial drug chloroquine [22], whilst MrgprC11 activation via the endogenous peptide agonist bovine adrenal medulla 8-22 (BAM8-22) or mast cell mediator neuropeptide FF (NPFF), a dual MrgprC11 and A4 agonist [22] also initiate pruritus and scratching [23, 24]. Traditionally these mechanisms have been considered unique to the skin, however, pruritogenic-like mechanisms are being increasingly identified in the viscera. The same histamine receptors that mediate pruritus have been explored in a number of visceral organs, including the gastrointestinal tract and bladder, where histamine has been found to activate and induce hypersensitivity of visceral

afferents [25-27]. More recently, MrgprC11 and MrgprA3 have been localised in subpopulations of colon-innervating afferents [28, 29], as well as vagal afferents innervating the lungs [30]. Activation of MrgprA3 and MrgprC11 expressed on colonic afferents results in colonic hypersensitivity, enhanced abdominal pain and altered animal behaviours [28, 29], effects that were significantly increased in an animal model of chronic visceral hypersensitivity [28]. Given the relative similarities in the extrinsic sensory afferent innervation of the colon and bladder, including the ability to encode a variety of nociceptive and non-nociceptive stimuli [31-34], we sought to explore the expression of key Mrgprs in the bladder and their role in mediating neuronal excitability and bladder sensory signalling. Here, we show that MrgprC11, MrgprA3, and to a much smaller extent MrgprD are expressed in sub-populations of bladder-innervating DRG neurons. Agonists of MrgprC11 and MrgprA3 cause pronounced afferent hypersensitivity to bladder distension *ex vivo*, activate and evoke hyperexcitability of bladder-innervating DRG neurons *in vitro*, as well as enhancing activation of neurons within the dorsal horn of the spinal cord *in vivo*.

6.3 Methods

6.3.1 Ethics and animals

In this study, 12- to 18-week-old male and female C57BL/6J mice from an in-house breeding colony at the South Australian Health and Medical Research Institute (SAHMRI) were used. Mice were acquired from an in-house C57BL/6J breeding programme (JAX strain #000664; originally purchased from The Jackson Laboratory; breeding barn MP14; Bar Harbor, ME) and then bred within the specific and opportunistic pathogen-free animal care facility at SAHMRI. A genetically modified mouse strain lacking a cluster of MRGPR genes (*Mrgpr-cluster* $\Delta^{-/-}$), including *Mrgpra3* and *Mrgprc11*, was utilised for some experiments [22]. The *Mrgpr-cluster* $\Delta^{-/-}$ mice were a generous gift from Prof Xinzhong Dong (Howard Hughes Medical Institute, Johns Hopkins University, Baltimore, Maryland, USA) then bred in-house at SAHMRI as per the C57BL/6J mice. All experiments were approved by and performed in accordance with the approval of the SAHMRI Animal Ethics Committee (SAM195 and SAM190). Mice were group housed (up to 5 mice per cage) within individual ventilated cages filled with chip coarse dust-free aspen bedding (Cat#– ASPJMAEBCA; PuraBed, Niederglatt, Switzerland), with free access to LabDiet JL Rat and Mouse/Auto6F chow (Cat #5K52, St. Louis, MO) and autoclaved reverse osmosis water. Cages were stored within a temperature-controlled environment of 22°C and a 12-hour light/12-hour dark cycle on individual ventilated cage racks. Experiment sample sizes are detailed in the corresponding figure legends.

6.3.2 *Ex vivo* bladder afferent nerve recordings

Nerve recording was performed using a previously described *ex vivo* model [9, 10, 27, 35-37]. Mice were humanely killed via CO₂ inhalation, and the entire lower abdomen was removed and submerged in a modified organ bath under continual perfusion with gassed (95% O₂ and 5% CO₂) Krebs-bicarbonate solution (composition in mmol/L: 118.4 NaCl, 24.9 NaHCO₃, 1.9 CaCl₂, 1.2 MgSO₄, 4.7 KCl, 1.2 KH₂PO₄, 11.7 glucose) at 35°C. The bladder, urethra, and ureters were exposed by removing excess tissue. Ureters were tied with 4-0 perma-hand silk (Ethicon, #LA53G). The bladder was catheterised (PE 50 tubing) through the urethra and connected to a syringe pump (NE-1000) to allow a controlled fill rate of 30 µL/min with saline (NaCl, 0.9%). A second catheter was inserted through the dome of the bladder, secured with silk, and connected to a pressure transducer (NL108T2; Digitimer) to enable intravesical pressure recording during graded distension. Pelvic nerves, isolated from all other nerve fibres between the pelvic ganglia and the spinal cord, were dissected into fine multiunit branches and a single branch was placed within a sealed glass pipette containing a microelectrode (WPI) attached to a Neurolog headstage (NL100AK; Digitimer). Nerve activity was amplified (NL104), filtered (NL 125/126, bandpass 50–5,000 Hz, Neurolog; Digitimer), and digitised (CED 1401; Cambridge Electronic Design, Cambridge, UK) to a PC for offline analysis using Spike2 software (Cambridge Electronic Design, Cambridge, UK). The number of action potentials crossing a pre-set threshold at twice the background electrical noise, was determined per second to quantify the afferent response. Single-unit analysis was performed offline by matching individual spike waveforms through linear interpolation using Spike2 version 5.18 software. A single unit was deemed to be responsive to an agonist if a greater than 20% increase in neuronal excitability (impulses/second across all distension pressures 0-30mmHg (AUC)) was detected.

6.3.2.1 *Afferent recording experimental protocols*

At the start of each afferent recording experiment, control bladder distensions were performed with intravesical infusion of saline (NaCl, 0.9%) at a rate of 30 µL/min to a maximum pressure of 30 mmHg at 10 min intervals to assess the viability of the preparation and reproducibility of the intravesical pressure and neuronal responses to distension. The volume in the bladder was extrapolated from the known fill rate (30 µL/min) and the time taken (s) to reach maximum pressure (30 mmHg). Compliance was determined by plotting intravesical pressure against the calculated volume. After a stable baseline was maintained, the saline in the infusion pump was replaced by either BAM8-22 (20 µM; Tocris Bioscience; #1763), Chloroquine (100 µM; Sigma, #PHR1258-1G), Neuropeptide FF (NPFF: 50 µM; Tocris Bioscience; #3137), or an 'itch cocktail' containing all three agonists combined (BAM8-22: 20 µM, Chloroquine: 100 µM and NPFF: 50 µM). All experimental compounds were dissolved in dH₂O to form stock solutions which were frozen at -80°C and defrosted

immediately before being used for each experiment. The single nerve bundle isolated and inserted into the glass electrode during the dissection process was contained within the recording electrode for the entire experiment, allowing multiunit afferent nerve recordings to be performed and comparisons between afferent firing rates to be compared in the same nerve fibres during intra-bladder incubation with saline and Mrgpr agonists.

6.3.3 Retrograde tracing from the bladder

A small, aseptic, abdominal incision was made in anaesthetised (2% – 4% isoflurane in oxygen) mice. A 5µL Hamilton syringe attached to a 30-gauge needle was used to inject Cholera Toxin subunit B conjugated to AlexaFluor 488 (CTB-488, 0.5 % diluted in 0.1 M phosphate buffer saline [PBS] pH 7.4; ThermoFisher Scientific) at three sites into the bladder wall (3 µL/injection) [27, 35, 36]. To prevent injection of CTB into the bladder lumen, the needle was inserted subserosally, parallel with the bladder muscle. The abdominal incision was then sutured closed and analgesic (Buprenorphine (Temvet); 0.1 mg/kg; Troy Laboratories Pty Ltd, APVMA #67612) and antibiotic (Amoxicillin; 50 mg/kg; Amoxil, AUSTR11137) administered subcutaneously as mice regained consciousness. Following laparotomy, mice were individually housed and allowed to recover. After 4 days, mice were humanely euthanised and the lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) removed for subsequent isolation and culture of the neurons to visualise CTB-labelled bladder innervating neurons amongst the DRG neurons.

6.3.4 mRNA expression analysis in DRG, isolated urothelial cells and bladder mucosal and detrusor layers

6.3.4.1 Tissue collection and urothelial cell isolation

16- to 18-week-old mice were humanely euthanised via CO₂ inhalation and the bladder and lumbosacral (L5-S1) dorsal root ganglia (DRG) removed. DRG were frozen in dry ice in pairs by spinal level (L5, L6, S1) and stored at -80°C for RNA extraction and quantitative reverse-transcription polymerase chain reaction (QRT-PCR). The bladder was opened, stretched out and pinned flat, urothelial side up. For tissue QRT-PCR, the mucosal and detrusor layers were gently peeled apart, frozen in dry ice and stored at -80°C for RNA extraction and QRT-PCR. For urothelial cell QRT-PCR, urothelial cells were isolated as performed previously [9, 10, 27]. Following a rinse in sterile PBS, the bladder was incubated for 3 hours at room temperature in DMEM containing 2.5 mg/mL dispase (GIBCO, ThermoFisher Scientific, #17105041), 10% foetal calf serum, and 1 µM/mL HEPES (Sigma, #H3375, pH 7.0). A blunt scalpel was used to collect cells by gentle scraping of the urothelium. Cells

were dissociated in 0.025% trypsin EDTA (GIBCO, ThermoFisher Scientific, #25200072) at 37°C in 5% CO₂ for 10 min with gentle intermittent trituration. The cell suspension was then added to DMEM containing 10% foetal calf serum to deactivate the trypsin before centrifugation (15 min, 1500 rpm, 4°C). Following aspiration of supernatant, cells were resuspended in keratinocyte serum-free media (KSFM; Invitrogen, #17005042) and cell count and viability were determined using the Countess Automated Cell Counter (Invitrogen, ThermoFisher Scientific). Cells were pelleted, frozen in dry ice and stored at -80°C for RNA extraction and QRT-PCR.

6.3.4.2 RNA extraction

RNA was extracted using the PureLink RNA Micro kit (Invitrogen, Victoria, Australia, #12183-016; DRG pairs and isolated urothelial cells) or the PureLink RNA Mini kit (Invitrogen, #12183018A; bladder mucosa and detrusor) with DNase treatment (Life Technologies, #12185-010) according to the manufacturer's instructions. A NanoDrop Lite spectrophotometer (ThermoFisher Scientific) was used to determine RNA purity and quantity. Using SuperScript VILO Master Mix (Invitrogen, #11755250), RNA was reverse transcribed to cDNA as per the manufacturer's instructions. cDNA was then stored at -20°C for QRT-PCR. Quantitative reverse-transcription polymerase chain reaction (QRT-PCR) QRT-PCR was performed using Taqman Gene Expression Master Mix (Applied Biosystems, Victoria, Australia, #4369016) with commercially available hydrolysis probes (TaqMan; Life Technologies, see **Table 6.1** for details) and RNase-free water (AMBION, Victoria, Australia, #AM9916). For each reaction, 10 µL of qPCR MasterMix, 1 µL of TaqMan primer assay, 4 µL of water, and 5 µL of cDNA (1:2 dilution in RNA-free H₂O) from each sample was tested in duplicate for each target. Housekeeping genes *Actb* (β-actin; DRG pairs) and *Gapdh* and *Hprt* (glyceraldehyde 3- phosphate dehydrogenase and hypoxanthine phosphoribosyltransferase 1; mucosal and detrusor tissue layers and isolated urothelial cells) were used as endogenous controls. Assays were run for 45 cycles on a 7500 Fast Real-Time PCR System (Applied Biosystems) machine, using 7500 Fast software, v2.0.6. mRNA quantities are expressed as $2^{-\Delta Ct}$ relative to reference gene *Actb* (DRG pairs) or *Gapdh* x *Hprt* (geometric mean; mucosa, detrusor, and urothelial cells) [28, 35, 38].

Table 6.1: Primers used for QRT-PCR and RT-PCR receptor expression assays

Gene Alias	Gene Target	Assay ID
B-actin (reference gene)	<i>Actb</i>	Mm00607939_s1
HPRT (reference gene)	<i>Hprt</i>	Mm00446968_m1
Gapdh (reference gene)	<i>Gapdh</i>	Mm99999915_g1
Tubulin-3 (neuronal marker)	<i>Tubb3</i>	Mm00727586_s1
Mas-related G-protein Receptor A3	<i>Mrgpra3</i>	Mm02620679_s1
Mas-related G-protein Receptor C11	<i>Mrgprx1</i>	Mm02525847_g1
Mas-related G-protein Receptor D	<i>Mrgprd</i>	Mm01701850_s1
Transient receptor potential vanilloid 1	<i>Trpv1</i>	Mm01246300_m1
Transient receptor potential ankyrin 1	<i>Trpa1</i>	Mm01227437_m1
Transient receptor potential vanilloid 4	<i>Trpv4</i>	Mm00499025_m1

6.3.5 Cell culture of bladder-innervating DRG neurons

Four days following retrograde tracing, mice were humanely euthanised via CO₂ inhalation and lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) were removed. DRG were digested in Hanks' balanced salt solution (HBSS; pH 7.4; Life Technologies, #14170161) containing 2.5 mg/mL collagenase II (GIBCO, ThermoFisher Scientific, #17101015) and 4.5 mg/mL dispase (GIBCO, ThermoFisher Scientific, #17105041) at 37°C for 30 min. Following aspiration of the collagenase-dispase solution, DRG were incubated in HBSS containing collagenase (4.5 mg/mL) only for 10 min at 37°C. After subsequent washes in HBSS, trituration through fire-polished Pasteur pipettes of descending diameter in 600 µL complete DMEM (Dulbecco's Modified Eagle Media [DMEM; GIBCO, ThermoFisher Scientific, #11995065]; 10% foetal calf serum [Invitrogen, ThermoFisher Scientific, MA, USA]; 2 mM L-glutamine [GIBCO, ThermoFisher Scientific, #25030081], 100 µM MEM nonessential amino acids [GIBCO, ThermoFisher Scientific, #11140076], 100 mg/mL penicillin/streptomycin [GIBCO, ThermoFisher Scientific, #15070063], and 96 µg/L nerve growth factor-7S [Sigma, N0513-0.1MG]) mechanically disrupted DRG and dissociated cells, which were then centrifuged for 1 min at 50 g [35, 36, 39, 40]. Supernatant was gently aspirated, and neurons resuspended in 360 µL complete DMEM and spot-plated (30 µL) onto laminin (20µg/mL; Sigma-Aldrich, #L2020) and poly-D-lysine (800 µg/mL; ThermoFisher Scientific) coated 13 mm coverslips. Coverslips were incubated for 2-3 hours at 37°C in 5% CO₂ to allow adherence of neurons before flooding with 1.7 mL complete DMEM. Cultured neurons were then maintained in an incubator at 37°C in 5% CO₂ for 18-48 hours for calcium imaging, patch clamp recordings, or cell picking for single-cell RT-PCR [28, 35].

6.3.6 Single-cell RT-PCR of individual bladder-innervating dorsal root ganglia neurons

Under continuous perfusion of sterile and RNA-/DNase-free PBS, single retrogradely traced bladder DRG neurons were identified using a fluorescence microscope and collected into the end of a fine glass capillary using a micromanipulator [35]. The glass capillary containing the cell was then broken into a sterile Eppendorf tube containing 10 μ L of lysis buffer with DNase (TaqMan Gene Expression Cells-to-CT Kit; Invitrogen, #4399002). A bath control was also taken for each coverslip of cells and analysed concurrently with cells. After lysis and DNase treatment, samples were immediately frozen on dry ice and stored at -80°C until cDNA synthesis was performed. RNA was reverse transcribed to cDNA using SuperScript™ IV VIL0™ Master Mix with ezDNase™ Enzyme (Invitrogen, #11766500) as per the manufacturer's instructions. cDNA was then stored at -20°C for real time PCR. Tubulin-3 expression was used as a neuronal marker and positive control. Expression of each target gene within a single cell was determined by looking at the log (ΔR_n) curve against cycle number. Only amplification curves that exhibited an exponential phase that rose steadily and eventually plateaued within 50 cycles were counted.

6.3.7 Calcium imaging of cultured DRG neurons

Cultured DRG neurons (18-48 hours) were loaded with 2.5 μ M Fura-2-acetoxymethyl ester (Fura-2; Invitrogen, ThermoFisher Scientific, #F1221) in 0.01% pluronic F-127 (Invitrogen, ThermoFisher Scientific, #P3000MP) at 37°C for 30 min followed by a 10 min wash with HEPES buffer (10 mM HEPES sodium salt [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt; Sigma, #H7006-100G], 140 mM NaCl [Chem Supply, #SA046-3KG], 4 mM KCl [Chem Supply, #PA054-500G], 5 mM D-glucose anhydrous [Chem Supply, #GA018-500G], 2 mM CaCl₂ [Scharlau, #CA01951000], and 2 mM MgCl₂ [Sigma, #M8266-100G], pH 7.40) before imaging at room temperature (23°C) [27, 35, 41]. Emissions for Fura-2 were measured at 510 nm, following excitation at 340 and 380 nm, using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, an Omega XF04 filter set for Fura-2, a Photonic Science ISIS-3 intensified CCD camera, and Universal Interface Card MetaFluor software. Retrogradely traced bladder DRG neurons were identified by the presence of the CTB-488, visible with excitation at 480 nm. Excitation at 480 nm was used only as an initial bladder neuron identifier; to prevent unnecessary photobleaching, images were not obtained at 480 nm throughout the experiment. Fluorescence images were obtained every 2 seconds using a 20x objective. Data were recorded and further analysed using MetaFluor software.

After an initial baseline reading to ensure cell fluorescence was stable, indicating healthy cells, DRG neurons were stimulated with chloroquine (1 μ M; Sigma-Aldrich, #PHR1258-1G), BAM8- 22 (2 μ M; Tocris, #1763) and NPFF (5 μ M; Tocris, #3137) with changes in intracellular calcium (Ca^{2+}_i) were monitored in real time. The TRPV1 receptor agonist capsaicin (3 μ M; Sigma-Aldrich, M2028) was applied at the end of experiments. Ca^{2+}_i is expressed as the ratio between the fluorescence signals at 340- and 380-nm (Fura-2 (340/380)).

6.3.8 Patch-clamp electrophysiology of bladder-innervating dorsal root ganglia neurons

6.3.8.1 Current-clamp recordings

Whole cell patch-clamp recordings in current clamp mode were made from retrogradely traced fluorescently labelled lumbosacral (L5-S1) bladder-innervating DRG neurons using borosilicate glass pipettes (WPI) fire-polished to 3 to 7 MW and back-filled with an intracellular solution consisting of (in mM): 135 KCl; 2 $MgCl_2$; 2 $MgATP$; 5 EGTA-Na; 10 HEPES-Na; adjusted to pH 7.3, 275 mOsm. Rheobase (amount of current required to fire an action potential) was determined in normal extracellular bath solution ((in mM): 140 NaCl; 4 KCl; 2 $MgCl_2$; 2 $CaCl_2$; 10 HEPES; 5 glucose, adjusted to pH 7.4, 285 mOsm) by application of a series of depolarising pulses from a holding potential of -65 mV (10 or 25 pA increments [480ms]) followed by hold at -65 mV (100 ms). Cells were continually perfused with bath solution by a gravity driven multi-barrel perfusion system positioned within 1 mm of the neuron under investigation [35, 38, 39]. After baseline recordings in external bath solution, an Mrgpr agonist 'itch cocktail' (MrgprA3 agonist, Chloroquine: 10 μ M, Sigma-Aldrich, #PHR1258-1G; MrgprC11 agonist, BAM8-22: 2 μ M, Tocris, #1763; MrgprC11/A4 agonist, Neuropeptide FF (NPFF): 5 μ M, Tocris, #3137) was applied for a 2-minute incubation period and a second recording was made.

6.3.9 *In vivo* spinal dorsal horn activation

6.3.9.1 *In vivo* bladder infusion

In vivo bladder infusion in anaesthetised mice was performed as previously described [27, 35]. 12-week-old female mice were anaesthetised (isoflurane 2% - 4% in oxygen) and a catheter (AS point style, 51 mm, metal hub, 21-gauge needle [Hamilton, 7751-12]) was inserted into the bladder via the urethra. Correct catheter placement was determined by inserting gently until meeting resistance (bladder dome) and receding slightly (2-3 mm) to avoid damaging the bladder wall. Urine was removed using a suction syringe. Subsequently, a new catheter (AS point style, 51 mm, metal hub, 21-gauge needle) was inserted, primed with either vehicle (saline) or an Mrgpr agonist cocktail (BAM8-22: 20 μ M; NPFF: 50 μ M; and Chloroquine: 100 μ M), and 100 μ L of solution was then gently infused,

without fully distending the bladder, and allowed to incubate for 5 minutes. Immediately following removal of the compound, mice were administered an anaesthetic overdose (intraperitoneal injection of pentobarbitone, 1.1 mg/g body weight; Lethabarb®; Virbac Australia) followed by 4% buffered paraformaldehyde transcardial perfuse fixation.

6.3.9.2 Transcardial perfuse fixation and tissue dissection

After anaesthetic overdose, the thoracic cavity was opened and 0.5 mL heparinised saline (50 IU in 5 mL; Pfizer) was injected into the left ventricle. A 22-gauge needle attached to tubing and a peristaltic perfusion pump was then inserted into the injection site. The right atrium was cut open, allowing for perfusate drainage. Warm phosphate buffer (0.1 M; 21.7 mM NaH₂PO₄ [Chem Supply, #SA061-500G], 81 mM Na₂HPO₄ [VWR Chemicals, #102494C], pH 7.2) was perfused, followed by ice-cold 4% paraformaldehyde (PFA; Sigma-Aldrich, #158127) in 0.1 M phosphate buffer. Following complete PFA perfusion, the lumbosacral spinal cord (determined by the level of dorsal root ganglia (DRG) root insertion points; the lowest rib was used as an anatomical marker of DRG level T13) was removed and post-fixed in 4% PFA in 0.1 M phosphate buffer at 4°C for 18 to 20 hours. After post-fixation, spinal cord segments were cryoprotected in 30% sucrose/phosphate buffer (Sigma-Aldrich, #S9378) for 48 hours at 4°C, followed by a 48-hour incubation at 4°C in 50% optimal cutting temperature (OCT; VWR, #C361603E)/30% sucrose/phosphate buffer solution prior to freezing in 100% OCT using liquid nitrogen-cooled 2-methylbutane (Sigma-Aldrich, #M32631). Frozen spinal cord segments were cryosectioned (10 µm thick) and placed onto gelatin-coated slides for immunostaining. Sections were performed serially and distributed over 6 slides, which were used for immunohistochemical localisation of phosphorylated-MAP-kinase ERK 1/2 (pERK) [27, 35, 36, 42]. The number of pERK-immunoreactive neurons were counted in L6-S2 spinal cord sections, with spinal segments determined *ex vivo* by the shape of the dorsal horn [43].

6.3.9.3 Immunohistochemistry of phosphorylated-MAP-kinase ERK 1/2 within the spinal cord

The dorsal horn neurons activated by bladder infusion were identified by labelling for neuronal activation marker, phosphorylated-MAP-kinase ERK 1/2 (pERK), using the DAKO Omnis autostainer (Agilent Technologies Australia, Mulgrave, Vic, AUS), with 3,3'-Diaminobenzidine (DAB) / horseradish peroxidase (HRP) secondary antibody staining. Sections were processed via the University of Adelaide Health and Medical Sciences Histology services. pERK activation was determined rather than c-Fos due to the rapidity of ERK phosphorylation (minutes compared to hours) [44]. Given the short-duration nature of the specific peripheral stimuli applied, the rapid onset of pERK provides a more appropriate correlate. Further, quantification of pERK activation is increasingly being utilised to identify spinal activation to a range of peripheral stimuli [27, 35, 36, 45, 46]. After air drying for 1 hour, sections were post-fixed in formalin with a brief rinse in distilled water (dH₂O) prior to target retrieval

at 97°C for 30 minutes with EnVision FLEX TRS Low pH target retrieval solution (citrate buffer, pH 6.1; K8005, Agilent DAKO), followed by a wash step with wash buffer (GC807, DAKO Omnis, Agilent). A 10-minute protein block step with Serum-Free Protein Block (X0909, Agilent DAKO) to prevent non-specific binding was followed by a 1-hour incubation in primary antibody (pERK; 1:800) in Antibody Diluent (S0809, Agilent DAKO). Following removal of excess primary antibody with wash buffer, a 3-minute incubation in Envision FLEX Peroxidaseblocking Reagent (GV823, Agilent DAKO) was performed. Slides were then washed twice with wash buffer before a 20-minute incubation with Envision FLEX HRP Polymer (GV823, Agilent DAKO) for HRP binding. A further 2 wash steps were performed, followed by a DI rinse and a third wash step, before a 10-minute incubation in EnVision FLEX Substrate Working Solution (DAB) for staining. Slides underwent alternate cycles of wash buffer, DI rinse and wash buffer prior to a 3-minute incubation with Haematoxylin ready-to-use solution (K8018, Agilent DAKO) and a further DI rinse and wash step. Slides were removed from the DAKO Omnis, dehydrated in alcohol, and cleared in xylene before mounting with dibutylphthalate polystyrene xylene (DPX, Sigma-Aldrich) and cover slipping.

6.3.9.4 Microscopy

DAB/HRP-stained slides were imaged using a NanoZoomer microscope (NanoZoomer 2.0- HT, Hamamatsu), with 1 – 4 manually set focus points per section. Images of individual sections from L6-S2 regions were taken using NanoZoomer digital pathology viewing software (NDP.view2, Hamamatsu) and analysed using ImageJ software. The images were not manipulated in any way.

6.3.9.5 Spinal cord pERK neuronal counts and analysis

Neuronal counts were analysed from previously saved digital photomicrographs, with only neurons with intact nuclei counted. The number of pERK-immunoreactive (pERK-IR) neurons in a quadrant of the L6-S2 dorsal horn was obtained from minimum 6 sections/animal viewed at 10x magnification. The mean number of pERK-IR neurons ($\pm$ SEM) in the superficial dorsal horn (SDH; LI-II), the dorsal grey commissure (DGC), and the sacral parasympathetic nuclei (SPN) in sacral spinal segments were compared between vehicle- and Mrgpr agonist cocktail-treated mice. These regions are known to have roles in nociceptive signalling and autonomic reflexes [47] and are known to be activated by bladder afferent output [5, 34].

6.3.10 Experimental design and statistical analyses

Data are presented as Mean $\pm$ SEM or the % of afferents or neurons. Within the specific figure legends, N indicates the number of animals, whilst n indicates the number of independent afferents or neurons. Sample size was based on historical data and the use of power calculations. Statistical

significance was reported at levels of * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. In some cases, due to space limitation, # is used to indicate significance of $P < 0.0001$ for multiple comparisons. Data were tested for Gaussian distribution using Prism 8 (GraphPad, San Diego, CA, USA) in order to determine if they were normally distributed or not and therefore the correct statistical tests to be used. Data were then analysed accordingly using Prism 8 (GraphPad, San Diego, CA, USA), via one- or two-way analysis of variance (ANOVA) with Tukey's or Sidak's post hoc analyses dependent on data distribution, or student's t-tests, for parametric data. Non-parametric data were analysed using Kruskal-Wallis test with Dunn's multiple comparisons, Mann-Whitney analysis, or Wilcoxon matched-pairs signed rank test. The specific tests used for analysis of each data set is indicated within the individual figure legends.

6.4 Results

6.4.1 Mrgpr agonists induce distension evoked mechanical hypersensitivity of bladder afferents

In order to determine if pruritogenic receptors have a functional role in bladder sensory signalling, we performed *ex vivo* bladder afferent recordings and determined their mechanosensitivity to bladder distension before and during bladder instillation with Mrgpr agonists. Intravesical application of the MrgprA3 agonist, chloroquine, evoked bladder afferent hypersensitivity to bladder distension in whole nerve recordings as determined by the total area under the curve of the response (**Figure 6.1A, 6.1B**). Analysis of the pressure/volume relationship in the bladder during repetitive graded bladder distensions before and during chloroquine revealed no effect on bladder compliance (**Figure 6.1C**). This indicates that the mechanical hypersensitivity induced by bladder infusion of chloroquine is not secondary to changes in bladder muscle function, which can indirectly influence bladder afferent responsiveness. To further investigate the afferent response, we performed post-hoc single unit analysis, which revealed that distinct populations of bladder afferents became hypersensitive following chloroquine application (called responders; **Figure 6.1Di, 6.1Dii**), whilst other afferents were unaffected (called non-responders; **Figure 6.1Ei, 6.1Eii**). Of the afferents that were affected by chloroquine, the predominant effects were apparent throughout most distension pressures from 14-30 mmHg (**Figure 6.1Di, 6.1F**). Overall, 36% of bladder afferents were responsive to chloroquine application (**Figure 6.1G**).

Activation of MrgprA3 and MrgprC11 on bladder-innervating afferents induces peripheral and central hypersensitivity to bladder distension

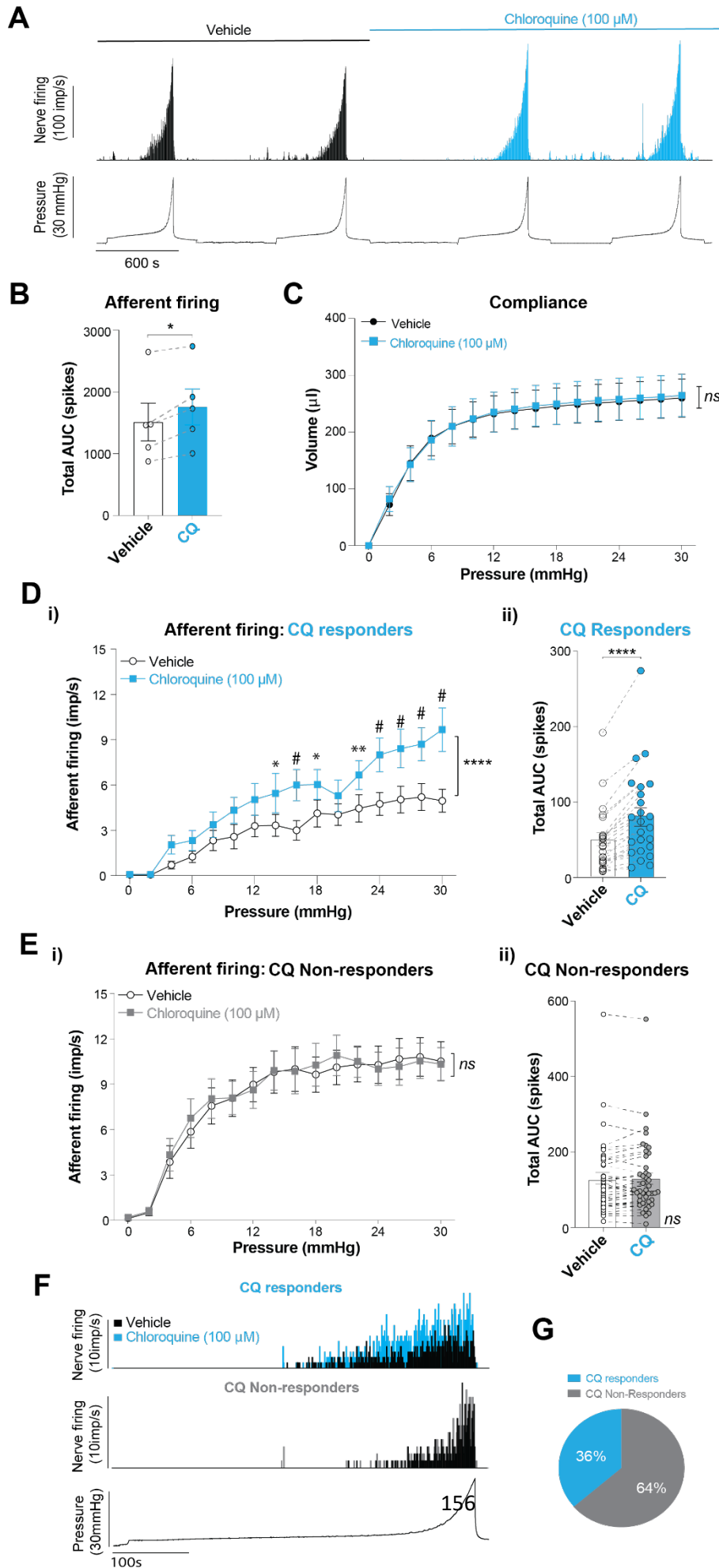


Figure 6.1: The MrgprA3 agonist chloroquine (CQ) evokes mechanical hypersensitivity in a cohort of bladder afferents.

A) Original recordings from an *ex vivo* nerve recording preparation showing bladder afferent firing and intravesical pressure. Recordings show exaggerated whole nerve bladder afferent responses to distension following intravesical infusion with chloroquine (100 μ M). **B)** The sum total of afferent firing (Total Area Under Curve; 0-30 mmHg) for each individual experiment showing bladder instillation with chloroquine (100 μ M) significantly increased bladder afferent firing to distension (* $P < 0.05$, paired t-test, $N = 5$ mice). Dots indicate whole nerve responses from individual mice before and after the itch cocktail. **C)** The relationship between intra-bladder pressure and volume (compliance) during graded bladder distension (30 μ L/min from 0-30 mmHg) is unaffected by intravesical administration of chloroquine (100 μ M, *ns*, $P > 0.05$, repeated-measures two-way ANOVA, $N = 5$). **D) i)** Post-hoc single unit analysis of the multiunit *ex vivo* bladder recordings shown in panel **A** identified bladder afferents that were responsive to chloroquine (showing a $>20\%$ increase in afferent firing) in the presence of chloroquine (*** $P < 0.001$, two-way ANOVA with * $P < 0.05$, ** $P < 0.01$, # $P < 0.0001$, Sidak's multiple comparisons test, $n = 24$ afferents, $N = 5$ mice). **ii)** Chloroquine responsive bladder afferents showing enhanced firing as determined by the total number of action potentials fired (**** $P < 0.0001$, Wilcoxon matched-pairs signed rank test, $n = 24$ afferents, $N = 5$ mice). **E)** Post-hoc single unit analysis also identified bladder afferents unaffected by chloroquine when analysed **i)** across the entire distension range (*ns*, $P > 0.05$, repeated-measures two-way ANOVA, $n = 42$ afferents, $N = 5$ mice) or as **ii)** the total number of action potentials fired (*ns*, $P > 0.05$, Wilcoxon matched-pairs signed rank test, $n = 42$ afferents, $N = 5$ mice). **F)** Original recordings of chloroquine (100 μ M) responding (upper panel) and non-responding (middle panel) bladder afferents during controlled bladder distension from 0-30 mmHg (lower panel). **G)** Overall, 36% of single units (24 of 66) bladder afferents showed a $>20\%$ increase in afferent firing to chloroquine. Data represent Mean $\pm$ SEM. Chloroquine: CQ. Area Under Curve: AUC. Impulses per second: imp/s⁻¹.

To follow up on these findings, we also used the MrgprC11 agonist BAM8-22 and found that it also significantly enhanced bladder afferent responses to bladder distension (**Figure 6.2A, 6.2B**), without affecting muscle compliance (**Figure 6.2C**). Single unit analysis of bladder afferents revealed that BAM8-22 also sensitised a sub-population of bladder afferents (**Figure 6.2Di, 6.2Dii**), whilst another population was unaffected (**Figure 6.2Ei, 6.2Eii**). Responsive afferents were particularly affected at distension pressures at or above 18mmHg (**Figure 6.2Di, 6.2F**) and constituted 20% of the bladder afferents recorded (**Figure 6.2G**). We also found that the MrgprC11/MrgprA4 agonist neuropeptide FF (NPFF) evoked significant bladder afferent hypersensitivity (**Figure 6.3A, 6.3B**), that was independent of changes in muscle compliance (**Figure 6.3C**) and affected the responsiveness of 41% of bladder afferents recorded (**Figure 6.3D, 6.3E, 6.3F, 6.3G**). Neither chloroquine, BAM8-22, nor NPFF recruited 'silent' afferents to become subsequently mechanosensitive. Overall, these findings show that chloroquine, BAM8-22, and NPFF all evoke hypersensitivity to distension in subpopulations

of bladder afferents, suggesting the Mrgpr receptors they activate are expressed on specific subpopulations of bladder-innervating DRG neurons.

Activation of *MrgprA3* and *MrgprC11* on bladder-innervating afferents induces peripheral and central hypersensitivity to bladder distension

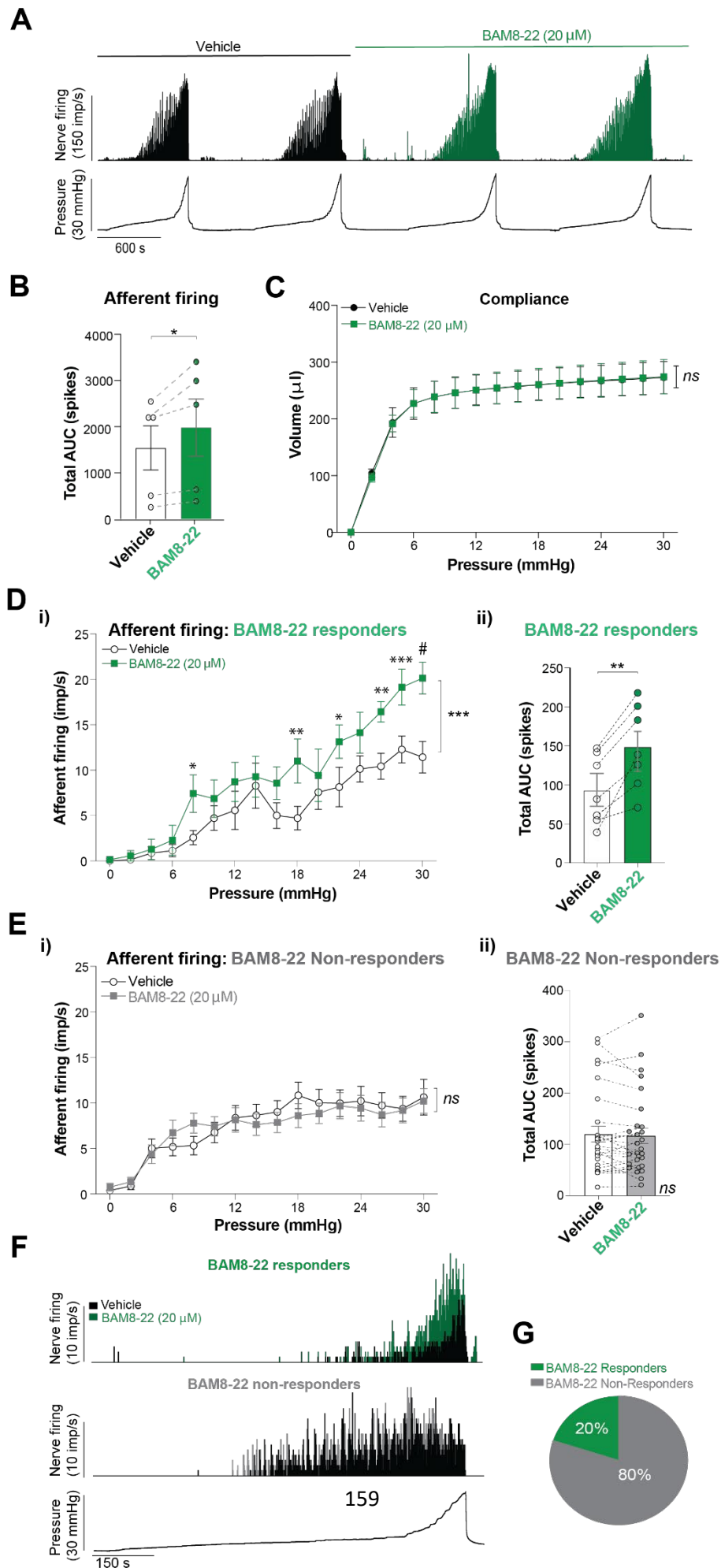


Figure 6.2: The MrgprC11 agonist BAM8-22 evokes mechanical hypersensitivity in a cohort of bladder afferents.

A) Original *ex vivo* recordings showing exaggerated bladder afferent responses to distension following intravesical infusion with BAM8-22 (20 μ M). **B)** Total bladder afferent firing was significantly increased following bladder instillation with BAM8-22 (20 μ M, * $P < 0.05$, paired t-test, $N = 5$ mice). **C)** Bladder compliance during graded bladder distension was unaffected by intravesical administration of BAM8-22 (20 μ M, *ns*, $P > 0.05$, repeated-measures two-way ANOVA, $N = 5$). **D)** Post-hoc single unit analysis demonstrated a population of BAM8-22 responsive bladder afferents showing enhanced firing as determined **i)** across the distension range (** $P < 0.001$, repeated-measures two-way ANOVA, with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # $P < 0.0001$ based on Sidak's multiple comparisons test $n = 7$ afferents, $N = 5$ mice) or as **ii)** the total number of action potentials fired (** $P < 0.01$, paired t-test, $n = 7$ afferents, $N = 5$ mice). **E)** Post-hoc single unit analysis also identified bladder afferents unaffected by BAM8-22 when analysed **i)** across the entire distension range (*ns*, $P > 0.05$, repeated-measures two-way ANOVA, $n = 28$ afferents, $N = 5$ mice) or as **ii)** the total number of action potentials fired (*ns*, $P > 0.05$, Wilcoxon matched-pairs signed rank test, $n = 28$ afferents, $N = 5$ mice). **F)** Original recordings of BAM8-22 responding and non-responding bladder afferents during bladder distension from 0-30 mmHg. **G)** Overall, 20% (7 of 35) of bladder afferents showed a $>20\%$ increase in afferent firing to BAM8-22. Data represent Mean $\pm$ SEM. Impulses per second: imp/s⁻¹.

Activation of MrgprA3 and MrgprC11 on bladder-innervating afferents induces peripheral and central hypersensitivity to bladder distension

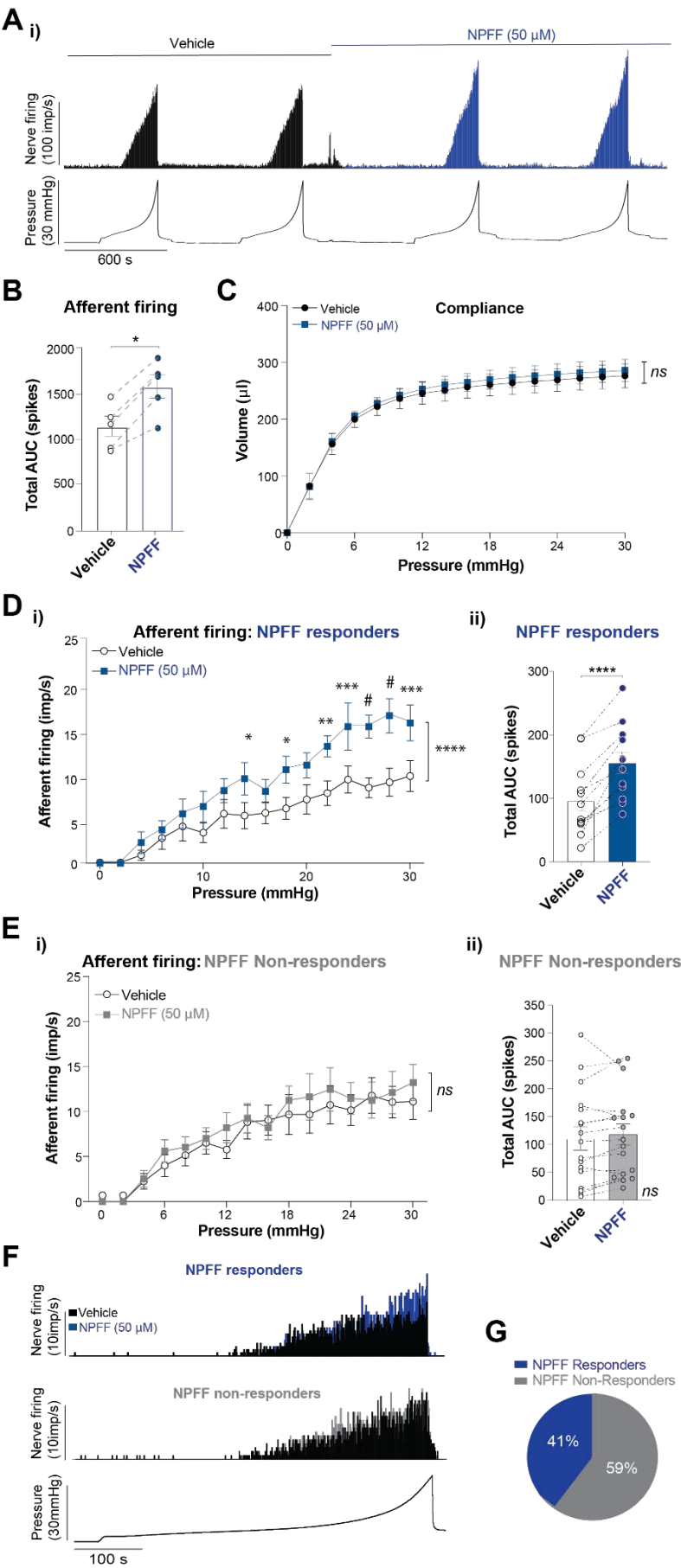


Figure 6.3: The *MrgprC11/A4* agonist NPFF evokes mechanical hypersensitivity in a cohort of bladder afferents.

A) i) *Ex vivo* recordings showing an enhanced afferent response to distension following intravesical infusion with NPFF (50 μ M). **B)** Total bladder afferent firing was significantly increased following bladder instillation with NPFF (50 μ M, * $P < 0.05$, paired t-test, $N = 5$ mice). **C)** Bladder compliance is unaffected by intravesical administration NPFF (5 μ M, *ns*, $P > 0.05$, repeated-measures two-way ANOVA, $N = 5$ for each group). **D)** Post-hoc single unit analysis showed a population of NPFF responsive bladder afferents showing enhanced firing **i)** across distension pressures (***) $P < 0.001$, repeated-measures two-way ANOVA, with # $P < 0.0001$ based on Sidak's multiple comparisons test $n = 12$ afferents, $N = 5$ mice) or as **ii)** the total number of action potentials fired (**** $P < 0.0001$, paired t-test, $n = 12$ afferents, $N = 5$ mice). **E)** A population of bladder afferents were unresponsive to NPFF based on **i)** responses across the distension range (*ns*, $P > 0.05$, repeated-measures two-way ANOVA, $n = 17$ afferents, $N = 5$) or **ii)** the total number of action potentials fired (*ns*, $P > 0.05$, paired t-test, $n = 17$ afferents, $N = 5$ mice). **F)** Example traces of responding and non-responding bladder afferents to NPFF (50 μ M) during bladder distension. **G)** Overall, 41% of single units (12 of 29) bladder afferents showed a $>20\%$ increase in afferent firing to NPFF. Data represent Mean $\pm$ SEM. Impulses per second: imp/s⁻¹.

In order to confirm that these effects were mediated via *Mrgpr* activation we performed additional *ex vivo* bladder afferent recordings with tissue from *Mrgpr-cluster* $\Delta^{-/-}$ mice (**Figure 6.4**). In our previous studies focusing on colonic sensitivity, we have shown that a combined application of *Mrgpr* agonists, as an 'itch cocktail', causes pronounced colonic mechanical hypersensitivity, an effect which is absent in *Mrgpr-cluster* $\Delta^{-/-}$ mice [28]. In the current study we found that *ex vivo* bladder afferent responses to graded distension in *Mrgpr-cluster* $\Delta^{-/-}$ mice were identical when comparing between the application of vehicle or the 'itch cocktail' of co-applied chloroquine, BAM8-22, and NPFF (**Figure 6.4A, 6.4Bi, 6.4Bii**). Additionally, these studies also showed that bladder compliance was unaltered in vehicle or 'itch cocktail' treated bladders from *Mrgpr-cluster* $\Delta^{-/-}$ mice (**Figure 6.4C**). These findings confirm the role of *Mrgprs* in chloroquine, BAM8-22, and NPFF induced bladder afferent mechanical hypersensitivity.

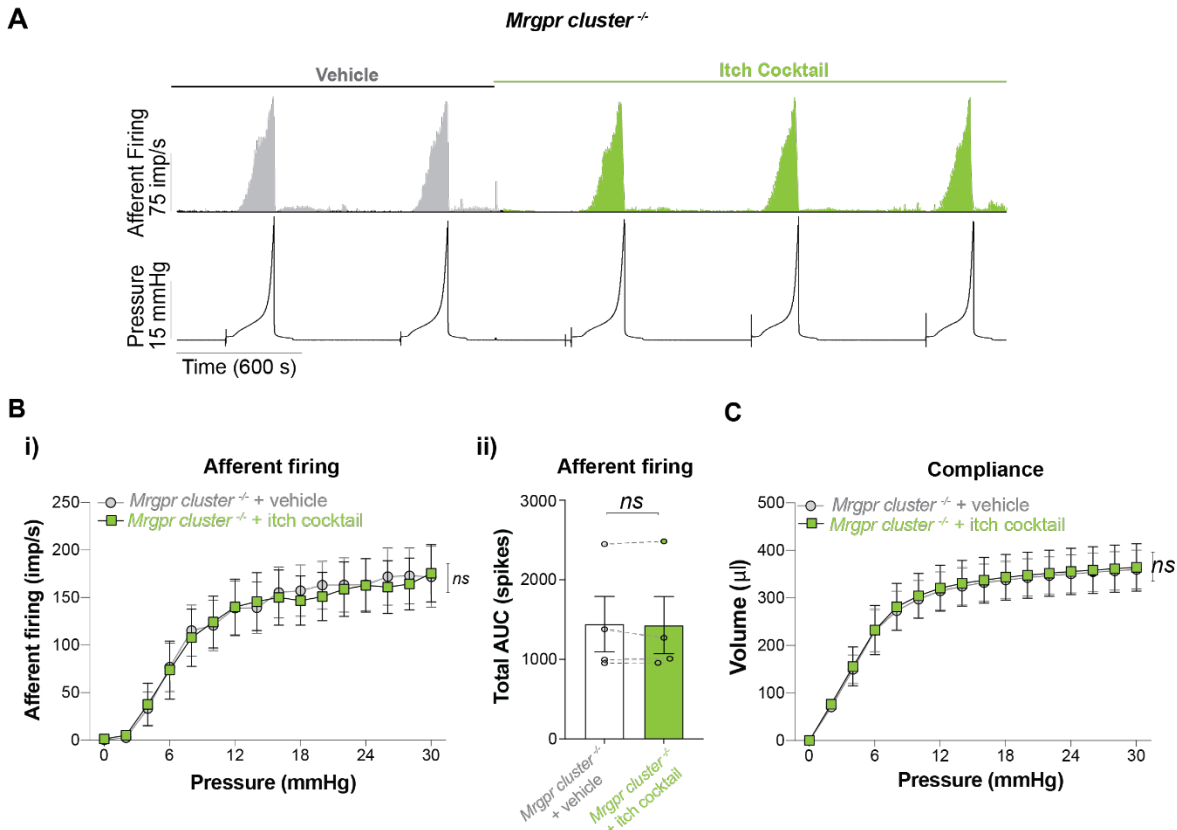


Figure 6.4: Bladder afferent responses to an 'itch cocktail' are absent in *Mrgpr-cluster*^{Δ⁻} mice.

A) Original recordings of bladder afferent firing and intravesical pressure during consecutive bladder distensions (0-30 mmHg) with saline followed by the 'itch cocktail' containing the Mrgpr agonists chloroquine (100 μM), BAM8-22 (20 μM) and NPFF (50 μM). Afferent responses were stable during saline infusion and unchanged after administration of the Mrgpr agonists. **B) i)** Group data from *ex vivo* afferent recordings using *Mrgpr-cluster*^{Δ⁻} mice showing almost identical responses to bladder instillation of vehicle (saline) or an 'itch cocktail' (ns, $P > 0.05$, repeated-measures two-way ANOVA, $N = 4$ mice). **ii)** Total bladder afferent firing was unchanged following bladder instillation of an 'itch cocktail' (ns, $P > 0.05$, paired t-test, $N = 4$ mice). Dots indicate whole nerve responses from individual mice before and after the itch cocktail. **C)** Bladder compliance during graded bladder distension (30 μL/min, 0-30 mmHg) was unaffected by intravesical administration of the Mrgpr agonist itch cocktail in *Mrgpr-cluster*^{Δ⁻} mice (ns, $P > 0.05$, repeated-measures two-way ANOVA, $N = 4$). Data represent Mean ± SEM.

6.4.2 *MrgprA3* and *MrgprC11* are expressed in sub-populations of bladder-innervating DRG neurons

To determine the mechanisms by which Mrgpr agonists induce distension-evoked bladder afferent hypersensitivity, we determined Mrgpr expression in DRG as well as in bladder tissue. Lumbosacral (LS) DRG contain the cell bodies of sensory neurons innervating a number of different

pelvic organs, including >30% of which innervate the mouse bladder [38, 48]. Therefore, we performed retrograde tracing from the bladder wall in order to isolate those that innervate the bladder. Single-cell RT-PCR from retrogradely traced bladder-innervating DRG neurons revealed that of the 77 individual neurons studied, 14% expressed *Mrgpra3*, whilst 38% expressed *Mrgprc11* (**Figure 6.5A, 6.5B**). In comparison, mRNA transcripts for *Trpv1* and *Trpa1*, key channels involved in bladder afferent function [6, 7], were detected in 84% and 15% of bladder-innervating DRG neurons respectively. Interestingly, another pruritogenic Mrgpr, *Mrgprd*, was only expressed in 6% of bladder-innervating DRG neurons, so was not investigated further with functional studies (**Figure 6.5A, 6.5B**). Co-expression analysis revealed that the majority of bladder-innervating neurons that express *Mrgpra3* co-express *Mrgprc11* (90%) and *Trpv1* (81%), with a smaller proportion co-expressing *Trpa1* (36%) or *Mrgprd* (18%) (**Figure 6.5B, 6.5C**). Bladder-innervating *Mrgprc11* expressing DRG neurons commonly co-expressed with *Trpv1* (89%), but less so with *Mrgpra3* (34%), *Trpa1* (25%) or *Mrgprd* (7%) (**Figure 6.5B, 6.5D**). Of the few cells that expressed *Mrgprd*, 40% co-expressed with either *Mrgpra3*, *Mrgprc11*, *Trpv1* or *Trpa1* (**Figure 6.5B, 6.5E**). Overall, no bladder-innervating DRG neuron expressed the full repertoire of receptors investigated. Interestingly, the Mrgpr expression profile in bladder-innervating DRG neurons contrasts starkly with that observed in the whole LS DRG, whereby *Mrgprd* mRNA is expressed in the highest abundance (**Figure 6.5F**). In terms of bladder tissue, *Mrgpra3*, *Mrgprc11* and *Mrgprd* were either expressed below the detection limit or were poorly expressed in the detrusor (**Figure 6.5G**), the mucosa (**Figure 6.5H**), or primary urothelial cells (**Figure 6.5I**), particularly when compared with an abundantly expressed target such as *Trpv4* [49, 50]. Mrgpr mRNA expression was approximately 1000-100,000x lower than expression of *Trpv4*. Overall, these findings indicate that the pruritogenic receptors MrgprC11 and MrgprA3 are expressed on sensory neurons innervating the bladder, and their expression profiles correlate well with our observations that subpopulations of bladder afferents display mechanical hypersensitivity following application of the respective Mrgpr agonists. The relatively low expression of Mrgprs in bladder tissue correlates well with our findings that Mrgpr agonists do not affect bladder compliance.

Activation of *Mrgpra3* and *Mrgprc11* on bladder-innervating afferents induces peripheral and central hypersensitivity to bladder distension

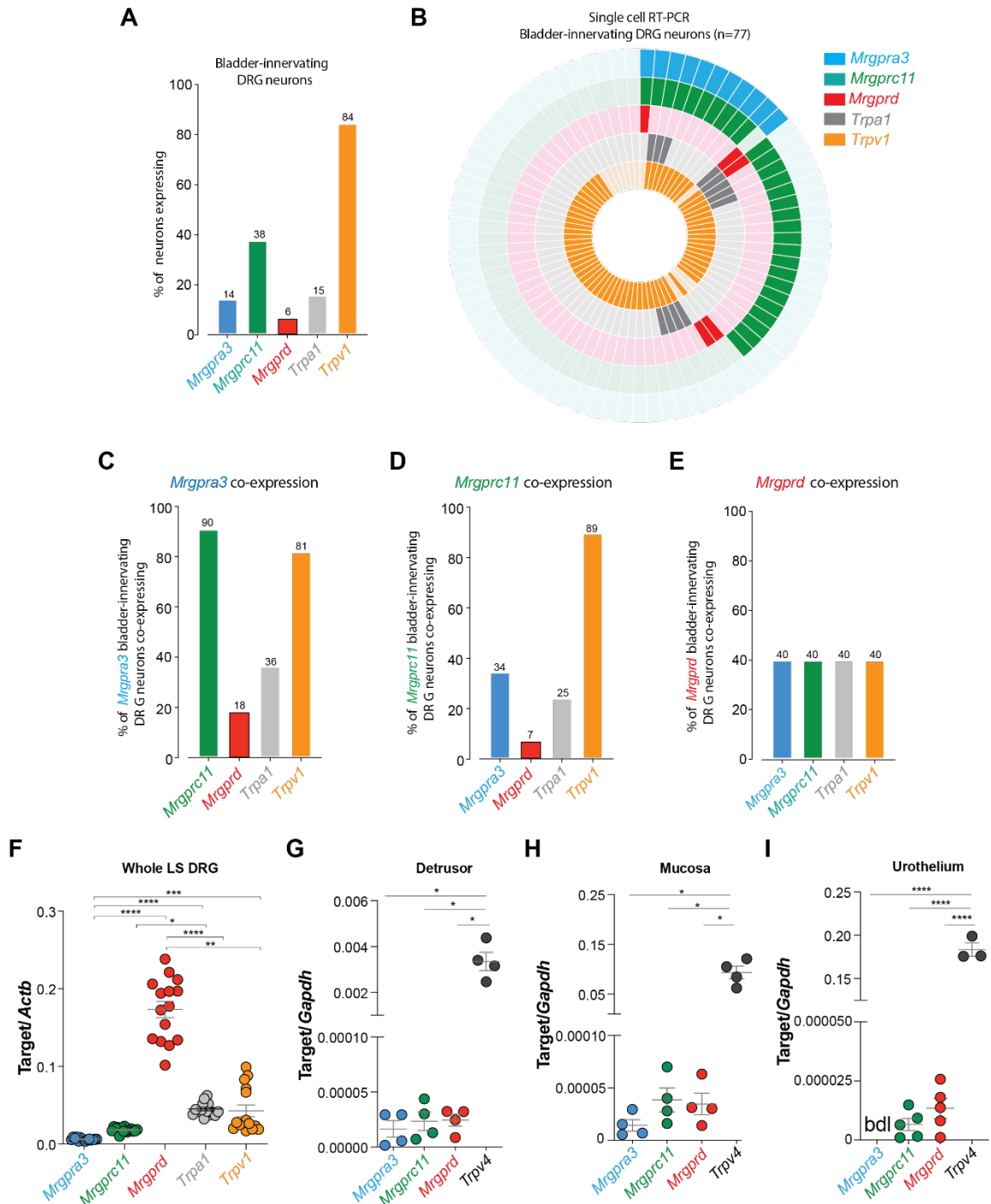


Figure 6.5: Mrgpr receptors are expressed by bladder-innervating DRG neurons.

A) Single-cell RT-PCR of retrogradely traced bladder-innervating DRG neurons reveals the percentage of neurons expressing and co-expressing genes encoding Mrgprs and TRP channels (n = 77 single cells from N = 6 mice). mRNA for *Mrgpra3* and *Mrgprc11* were expressed in 14% and 38% of bladder-innervating DRG neurons respectively. In contrast, *Mrgprd* was expressed in only 6% of bladder-innervating DRG neurons. *Trpa1* and *Trpv1* mRNA transcripts were detected in 15% and 84% of bladder-innervating DRG neurons. **B)** Donut plot showing expression and coexpression of mRNA encoding *Mrgprc11*, *Mrgpra3*, *Mrgprd*, *Trpa1*, and *Trpv1* in 77 individual retrogradely traced bladder-innervating DRG neurons. Each colour represents an individual gene with expression marked by bold colouring. *Mrgpra3* is represented in the outer ring with

Trpv1 in the inner ring. Individual neurons are arranged radially, such that co-expression of genes in a single neuron can be easily identified running from outside to inside. **C)** The majority of bladder-innervating DRG neurons that express *MrgprA3* co-express *MrgprC11* (90%) and *Trpv1* (81%). In contrast, a minor proportion of *MrgprA3*-expressing bladder DRG neurons also co-express *MrgprD* (18%) or *Trpa1* (36%). **D)** *MrgprC11* is commonly co-expressed with *Trpv1* (89%), but less so with *MrgprA3* (34%), *MrgprD* (7%), and *Trpa1* (25%). **E)** Of the very few cells that express *MrgprD* (5 of 77 neurons), 40% (2 of 5 neurons) co-express with either *MrgprA3*, *MrgprC11*, *Trpv1* or *Trpa1*. **F)** mRNA expression of *MrgprA3*, *MrgprC11*, *MrgprD*, *Trpv1*, and *Trpa1* in whole LS (L5-S1) DRG. Expression of *MrgprD* mRNA is significantly higher than *MrgprC11*, *MrgprA3*, *Trpv1* and *Trpa1*. *MrgprC11* and *MrgprA3* are significantly less abundant than *Trpv1* and *Trpa1* (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Kruskal-Wallis test with Dunn's multiple comparisons post hoc test, $n = 15$ pairs of DRG from $N = 5$ mice). **G-I)** mRNA expression of *MrgprA3*, *MrgprC11*, *MrgprD* (and the abundantly expressed *Trpv4* as a comparator) in bladder **(G)** detrusor smooth muscle, **(H)** mucosa, and **(I)** primary urothelial cells. Expression of all *Mrgprs* were similar between the detrusor, urothelial cells, and mucosa (tissue samples from $N = 3 - 5$ mice). All data represent Mean $\pm$ SEM.

6.4.3 Mrgpr agonists activate sub-populations of bladder-innervating DRG neurons

In order to confirm the results of our single-cell RT-PCR at a functional level, and to investigate the roles of Mrgpr agonists in activating bladder-innervating DRG neurons, we measured intracellular calcium ($[Ca^{2+}]_i$) using Fura-2AM in response to application of chloroquine, BAM8-22, and NPFF. Overall, calcium imaging of dissociated bladder-innervating DRG neurons revealed that individual application of chloroquine (**Figure 6.6A**), BAM8-22 (**Figure 6.6B**) or NPFF (**Figure 6.6C**) resulted in activation of sub-populations of bladder-innervating DRG neurons. 8.1%, 22.9%, and 16.2% of bladder-innervating LS DRG neurons were activated by chloroquine, BAM8-22, and NPFF respectively (**Figure 6.6D, 6.6E**). In-series application of multiple Mrgpr agonists demonstrated functional co-expression of *MrgprA3* and *MrgprC11* in populations of bladder-innervating DRG neurons (**Figure 6.6E – 6.6J**), supporting our single cell RT-PCR data suggesting colocalisation of *MrgprC11* and *MrgprA3* in bladder-innervating DRG neurons.

Activation of *MrgprA3* and *MrgprC11* on bladder-innervating afferents induces peripheral and central hypersensitivity to bladder distension

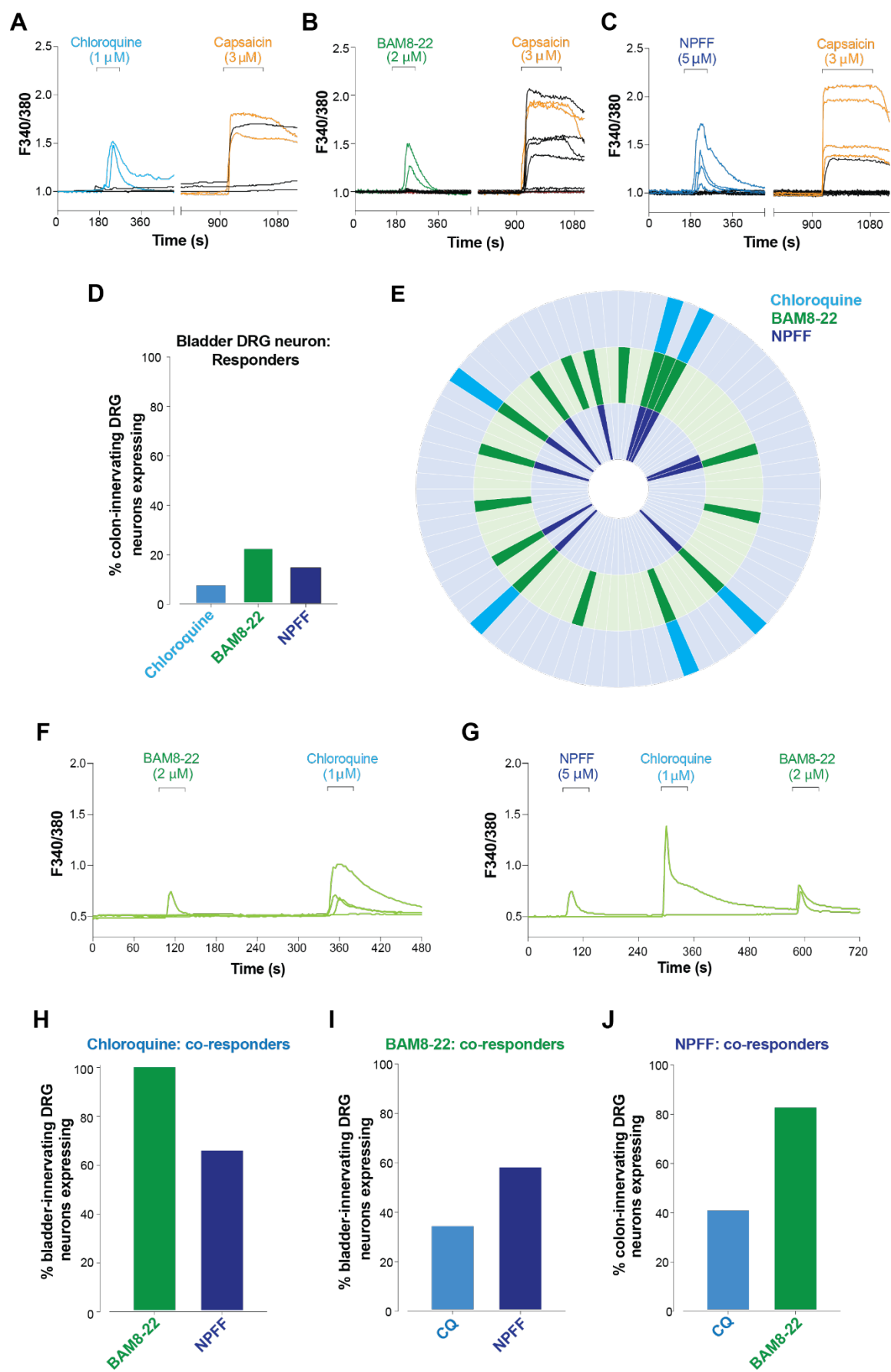


Figure 6.6: A cohort of bladder-innervating DRG neurons respond to Mrgpr agonists.

A-C) Calcium responses elicited by **(A)** chloroquine (CQ; 1 μ M) **(B)** BAM8-22 (2 μ M) and **(C)** NPFF (5 μ M), in isolated cultured retrogradely traced bladder-innervating LS DRG neurons loaded with Fura-2. Capsaicin was added at the end of each experiment to investigate the functional co-expression of TRPV1 with the Mrgprs. Each line represents an individual neuron, coloured lines indicate bladder DRG neurons responding to Mrgpr agonists. Orange capsaicin traces indicate bladder neurons that responded to either BAM8-22, chloroquine or NPFF. **D)** Percentage of bladder-innervating DRG neurons responding to chloroquine (8.1%: 6 of 74 neurons), BAM8-22 (22.9%: 17 of 74 neurons) or NPFF (16.2%: 12 of 74 neurons). **E)** Donut plot showing functional responsiveness of individual neurons to chloroquine, BAM8-22 and NPFF. Bold colouring represents a functional response to an Mrgpr agonist. Chloroquine is represented in the outer ring with NPFF in the inner ring. Individual neurons are arranged radially, such that functional responsiveness of a single neuron to the Mrgpr agonists can be easily identified running from outside to inside. **F-G)** Example traces showing bladder-innervating DRG neurons can respond to more than one Mrgpr agonist. **H)** All bladder-innervating DRG neurons responding to chloroquine also responded to BAM8-22 (100%: 6 of 6 neurons), with 66.6% of chloroquine-responding neurons also responding to NPFF (4 of 6 neurons). **I)** 35.2% of BAM8-22 responding neurons also responded to chloroquine (6 of 17 neurons), with 58.8% responding to NPFF (10 of 17 neurons). **J)** Of the NPFF responding neurons, 41.6% (5 of 12 neurons) also responded to chloroquine, with 83.3% (10 of 12 neurons) also responding to BAM8-22.

To further investigate the role of Mrgprs in modulating bladder sensation we used whole-cell patch-clamp electrophysiology of retrogradely traced bladder-innervating DRG neurons. In these studies, we co-applied the Mrgpr agonists chloroquine, BAM8-22, and NPFF at the same time in the form of an 'itch cocktail', as used in some of our bladder afferent recording studies (**Figure 6.7**). We found that this cocktail caused significant hyperexcitability in a sub-population (44%) of bladder-innervating DRG neurons, as indicated by a significant decrease in the rheobase, or amount of current required to fire an action potential (**Figure 6.7Ai, 6.7Aii, 6.7Aiii**). These changes were also accompanied by a significant increase in the number of action potentials fired at 2x rheobase (**Figure 6.7Bi, 6.7Bii, 6.7Biii**). In comparison, the neuronal excitability of another population of bladder-innervating DRG neurons was unaffected by the Mrgpr agonist cocktail (**Figure 6.7A, 6.7B**).

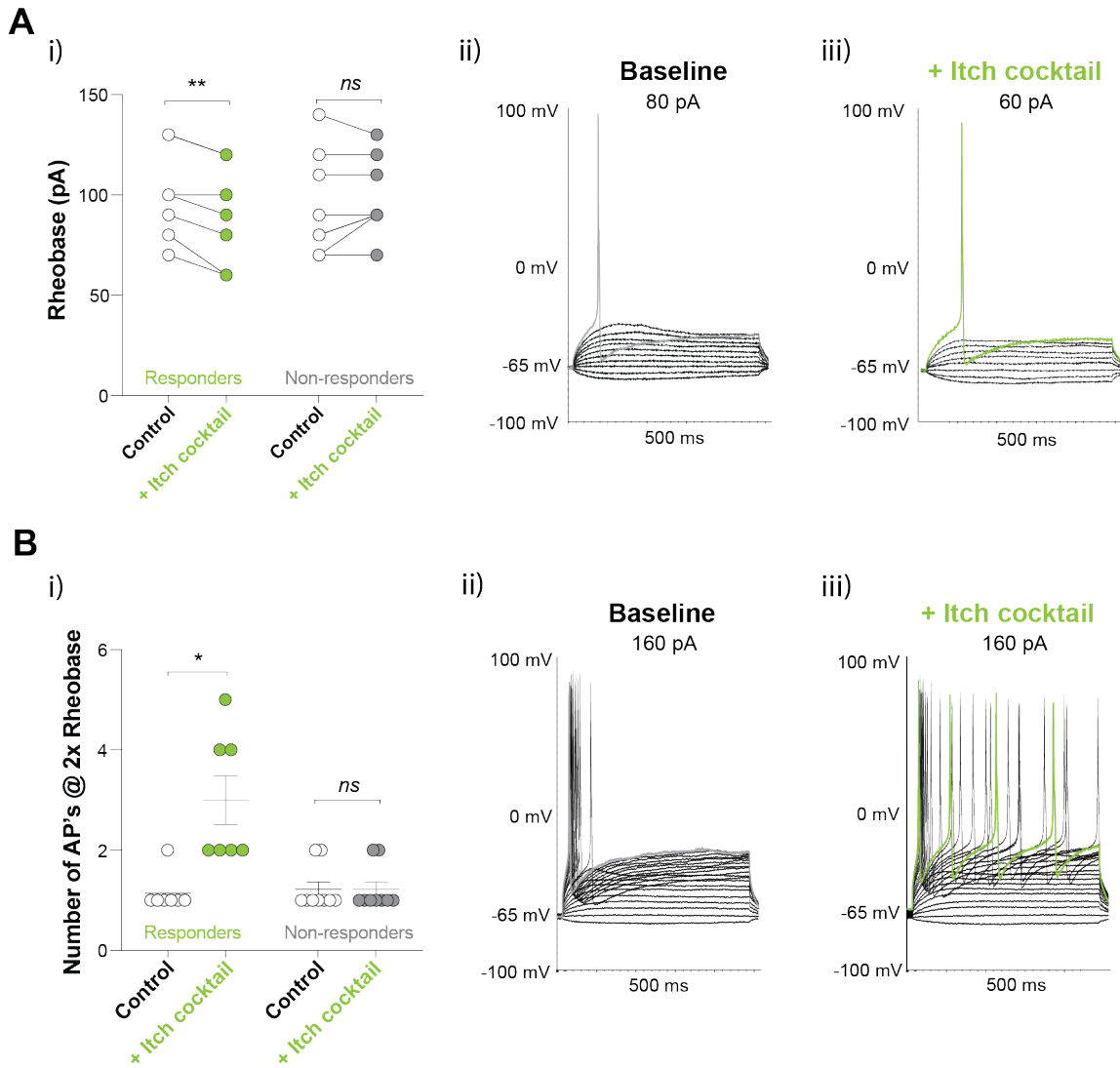


Figure 6.7: Mrgpr agonists induce neuronal hyperexcitability in a cohort of bladder-innervating DRG neurons.

A) Whole-cell patch clamp recordings of retrogradely traced bladder-innervating LS DRG neurons. **(i)** The Mrgpr agonist itch cocktail significantly decreases the amount of current required to elicit an action potential in a subset (7 of 16) of bladder-innervating DRG neurons (rheobase; ** $P < 0.01$, paired t-test, $n = 16$ neurons from $N = 5$ mice). Another population of bladder-innervating DRG neurons (9 of 16) were unaffected by the itch cocktail (ns, $P > 0.05$; paired Student t-test, $n = 9$ neurons from $N = 5$ mice). **(ii)** Original recording of membrane voltage from a single bladder-innervating DRG neuron during current ramp protocols. Each line represents a 10pA increase in current. In this example, an 80pA current was required to elicit an action potential under control conditions (grey trace). **(iii)** Perfusion with the 'itch cocktail' consisting of chloroquine (1 μ M), NPFF (5 μ M) and BAM8-22 (2 μ M) for 2 minutes onto the same DRG neuron reduced the amount current required to elicit an action potential (green trace), indicating increased neuronal excitability in this 'responding' neuron. **B)** **(i)** Application of the itch cocktail significantly increased the number of action potentials fired at a current twice (2x) the rheobase in a subset (7 of 16; 44%) of bladder-innervating DRG neurons (* $P < 0.05$, Wilcoxon Matched-pairs signed rank test, $n = 7$ neurons from $N = 5$ mice). Other bladder-innervating DRG neurons were unaffected by the itch cocktail (9 of 16; 56%, ns $P > 0.05$, paired t-test, $n = 16$

neurons from N = 5 mice). **(ii)** Original whole-cell current-clamp recordings from a single bladder-innervating DRG neuron during current clamp protocols. Each line represents a 10pA increase in current. In this example an 80 pA current was required to elicit an action potential under control conditions, with 160 pA representing 2x rheobase (grey trace). **(iii)** Following a 2-minute perfusion with the itch cocktail, the same DRG neuron dramatically increased the number of action potentials at 2x rheobase (green trace), indicating an increase in neuronal excitability. Data in **Bi** represent Mean $\pm$ SEM. DRG: dorsal root ganglia; LS: lumbosacral; AP's: Action potentials.

6.4.4 Peripheral activation of Mrgprs in the bladder results in activation of dorsal horn neurons within the lumbosacral spinal cord

Based on our findings that activation of MrgprA3 and MrgprC11 sensitised the response of a proportion of bladder afferents *ex vivo*, we hypothesised that *in vivo* intra-bladder infusion of Mrgpr agonists should correspondingly enhance bladder signalling into the dorsal horn of the spinal cord. Infusion of saline into the bladder resulted in pERK-immunoreactivity (pERK-IR) in neurons within the LS spinal cord dorsal horn, due to low-level bladder distension (**Figure 6.8A, 6.8B**). In LS spinal segments pERK-IR was seen in the superficial dorsal horn (SDH; **Figure 6.8Aii, 6.8B**), the sacral parasympathetic nucleus (SPN; **Figure 6.8Aiii, 6.8B**), and the dorsal grey commissure (DGC; **Figure 6.8Aiv, 6.8B**). Infusion of the 'itch cocktail' by co-applying chloroquine, BAM8-22, and NPFF into the bladder resulted in significantly greater total numbers of pERK-IR neurons in the LS spinal cord relative to saline infusion (**Figure 6.8Ai, 6.8B**). Furthermore, significant increases in pERK-IR neurons were observed across the SDH (**Figure 6.8Aii, 6.8B**), SPN (**Figure 6.8Aiii, 6.8B**) and the DGC (**Figure 6.8Aiv, 6.8B**), indicating that Mrgpr-evoked bladder afferent hypersensitivity is relayed into the spinal cord and enhances sensory signalling. To confirm that the effects we observed with Mrgpr agonists are mediated via Mrgpr activation we performed the same *in vivo* experiments with *Mrgpr-cluster* $\Delta^{-/-}$ mice. Comparing Mrgpr agonist- and vehicle-treated *Mrgpr-cluster* $\Delta^{-/-}$ mice, we found no differences in the total number of pERK-IR neurons in the LS spinal cord (**Figure 6.8Ci, 6.8D**), the SDH (**Figure 6.8Cii, 6.8D**), the SPN (**Figure 6.8Ciii, 6.8D**), with a small but significant increase in the DGC (**Figure 6.8Civ, 6.8D**).

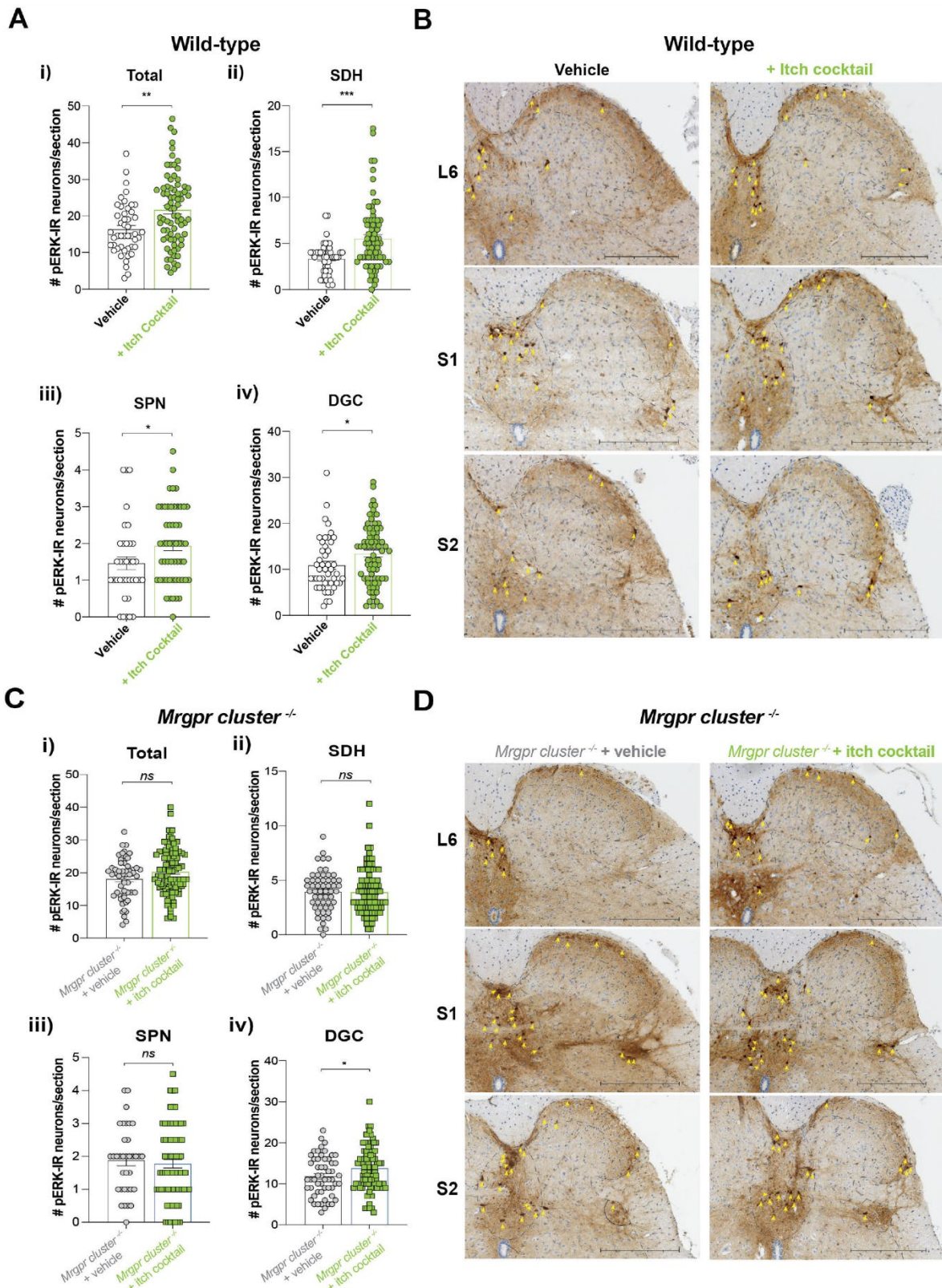


Figure 6.8: *In vivo* intra-bladder administration of an *Mrgpr* agonist ‘itch cocktail’ results in enhanced activation of dorsal horn neurons within the lumbosacral spinal cord.

A) Phosphorylated-MAP-kinase ERK 1/2 immunoreactivity (pERK-IR) was measured to determine signalling within the lumbosacral (LS; L6–S2) spinal cord in response to intra-bladder administration of saline or an ‘itch cocktail’ of *Mrgpr* agonists (chloroquine: 10 μ M, BAM8-22: 20 μ M, NPFF: 5 μ M). **i)** pERK-IR was significantly

increased in the LS dorsal horn of wild-type mice administered the itch cocktail compared with those administered saline (** $P < 0.01$, Mann Whitney test, $n = 47-76$ sections from spinal regions L6-S2; $N = 5$ mice). **ii-iv)** Wild-type mice administered the intra-bladder itch cocktail displayed significantly increased pERK-IR in specific regions of the dorsal horn including the **ii)** superficial dorsal horn (SDH), **iii)** sacral parasympathetic nucleus (SPN) and the **iv)** dorsal gray commissure (DGC) (* $P < 0.05$, *** $P < 0.001$, Mann Whitney tests). Data are presented as numbers of pERK-IR neurons/section of the LS spinal cord, with a minimum of 6 sections/mouse. **B)** Representative pERK-labelled LS spinal cord images at each spinal level (L6-S2) from mice that had bladders infused with either saline or an Mrgpr agonist 'itch cocktail'. Yellow arrows indicate pERK-IR neurons. Scale bar: 250 μm . **C) i)** In contrast to wild-type mice, pERK-IR was not significantly different across the whole LS dorsal horn of *Mrgpr-cluster $\Delta^{-/-}$* mice administered either intra-bladder saline or the Mrgpr agonist 'itch cocktail' (*ns*, $P > 0.05$, Mann Whitney test, $n = 55-91$ sections from $N = 5$ mice). **(ii-iii)** pERK-IR was not significantly altered in the **ii)** SDH, nor the **(iii)** SPN (*ns*, $P > 0.05$, Mann Whitney tests, $N = 5$ mice). **iv)** Following the 'itch cocktail' infusion there was a small but significant increase in pERK-IR in the DGC that remained in *Mrgpr-cluster $\Delta^{-/-}$* mice (* $P < 0.05$; Mann Whitney test, $N = 5$ mice). Data are presented as numbers of pERK-IR neurons/section of the LS spinal cord, with a minimum of 6 sections/mouse. **D)** Representative pERK-labelled LS spinal cord images at each spinal level (L6-S2) from *Mrgpr-cluster $\Delta^{-/-}$* mice that had bladders infused with either saline or the Mrgpr agonist 'itch cocktail'. Yellow arrows indicate pERK-IR neurons. Scale bars: 250 μm . Data represent Mean $\pm$ SEM.

6.4.5 Baseline bladder afferent responses and signalling within the spinal cord to bladder distension are unaltered in *Mrgpr-cluster $\Delta^{-/-}$* mice

In order to determine if Mrgprs contribute to bladder afferent responses to distension at baseline, we compared responses in wildtype and *Mrgpr-cluster $\Delta^{-/-}$* littermate mice. *Ex vivo* bladder afferent firing to bladder distension with saline showed that responses were indistinguishable between wildtype and *Mrgpr-cluster $\Delta^{-/-}$* mice (**Figure 6.9A**). Similarly, following saline bladder distension *in vivo* the numbers of pERK-IR neurons within the LS spinal cord were not significantly different between wildtype and *Mrgpr-cluster $\Delta^{-/-}$* mice (**Figure 6.9B, 6.9C, 6.9D, 6.9E**). Overall, these findings suggest Mrgprs are not responsible for bladder afferent mechanosensitivity per se, rather have a prominent role in sensitising bladder afferent responsiveness to distension following activation by pruritogens.

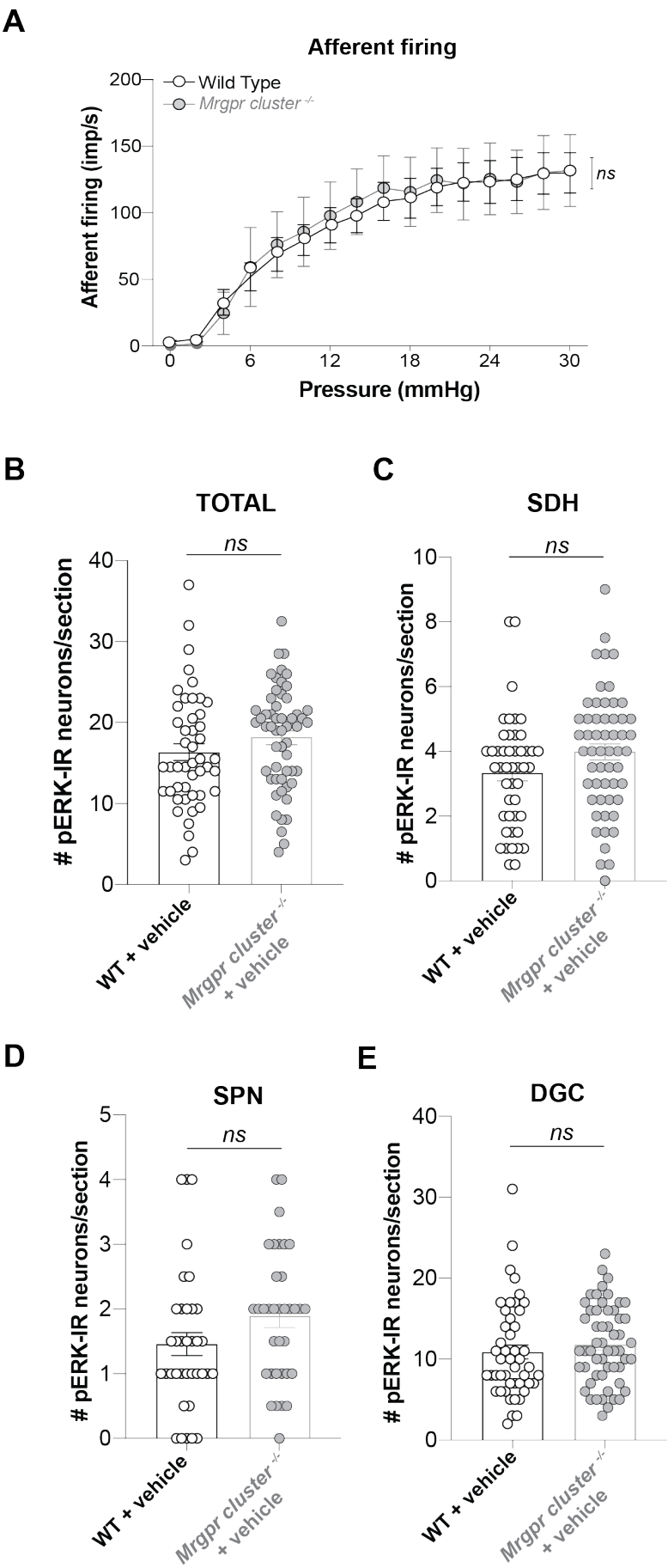


Figure 6.9: *Mrgpr-clusterΔ^{-/-}* mice do not display altered baseline responses to bladder distension.

A) Group data from *ex vivo* recordings from wild-type mice and age matched *Mrgpr-clusterΔ^{-/-}* mice. There was no significant difference in the mechanosensitivity of bladder afferents in response to graded bladder distension (0-30 mmHg) between wild-type and *Mrgpr-clusterΔ^{-/-}* mice (*ns*, $P > 0.05$, two-way ANOVA, $N = 4-15$ mice). **B-E)** Compared with wild-type mice *Mrgpr-clusterΔ^{-/-}* mice do not display altered neuronal activation within the dorsal horn of LS spinal cord following intra-bladder vehicle administration. Phosphorylated-MAP-kinase ERK 1/2 immunoreactivity (pERK-IR) was measured to determine signalling in the lumbosacral (LS; L6–S2) spinal cord in response to saline instillation in wildtype and age matched *Mrgpr-clusterΔ^{-/-}* mice. There was no difference in the **(B)** total number of pERK-IR neurons identified within the LS dorsal horn in response to saline infusion into the bladder between wildtype and *Mrgpr-clusterΔ^{-/-}* mice. In terms of specific regions of the LS dorsal horn, there were no significant differences between wild-type and *Mrgpr-clusterΔ^{-/-}* mice in the number of pERK-IR neurons following intra-bladder administration within the **(C)** superficial dorsal horn (SDH), **(D)** sacral parasympathetic nucleus (SPN) or **(E)** dorsal grey commissure (DGC) regions of the dorsal horn (*ns*, $P > 0.05$, Mann Whitney tests, $N = 5$ mice/group). Data represent Mean $\pm$ SEM.

6.5 Discussion

The ability of the somatosensory nervous system to easily differentiate stimuli such as pruritogens or algogens is essential as it evokes a tailored response, such as scratching or withdrawal of a limb. As a consequence, there is significant functional and anatomical diversity of somatosensory afferent nerves. In contrast, sensory discrimination in the bladder is more limited in scope, ranging from fullness to urge and finally pain [4]. The differentiation of distinct sensory stimuli (inflammation, infection, irritation) is less important if the overarching physiological response remains the same – increased voiding to rapidly remove irritants from the bladder. As such, mechanosensitive afferents innervating the bladder are considerably less diverse than cutaneous afferents. The majority of bladder neurons are characterised as peptidergic C-fibres that express *Trpv1* [4, 36], are embedded within the detrusor smooth muscle [4, 34, 36], and are broadly tuned to allow the detection of a variety of mechanical and chemical stimuli [32, 51, 52]. Therefore, the excitability of these afferents is key in providing appropriate sensory information in response to stimuli, including bladder filling and emptying. Understanding the mechanisms responsible for regulating bladder afferent mechanosensitivity is thus crucial to the development of novel treatments for the chronic sensory symptoms associated with bladder hypersensitivity and dysfunction, including urinary urgency, frequency, and pelvic pain. In this study we discovered a novel role in bladder sensory afferent pathways for irritant sensing mechanisms mediated via *MrgprC11* and *MrgprA3*.

Consistent with previous reports that Mrgpr agonists are able to excite isolated peripheral sensory neurons [22, 28, 53] we showed, using patch clamp recordings, that chloroquine, BAM8-22, and NPFF activated and induced neuronal hyperexcitability in sub-populations (44%) of bladder-innervating DRG neurons. In calcium imaging experiments, we observed some functional overlap between responses to chloroquine and BAM8-22, which has been previously described in afferents innervating the skin [22] and colon [28]. Employing single-cell RT-PCR of bladder DRG neurons, we revealed expression and co-expression of Mrgprs in bladder-innervating neurons. The majority of neurons expressing *Mrgprc11* also contained *Mrgpra3*, consistent with previous reports of colocalisation in other sensory pathways [20, 28, 54, 55].

High colocalisation of *Mrgpra3* and *Mrgprc11* was also observed with *Trpv1* in bladder DRG neurons, either on an mRNA level with single cell RT-PCR, or functionally with bladder neurons exhibiting calcium transients in response to chloroquine, BAM8-22, NPFF, and the TRPV1 agonist capsaicin. This is expected as the majority of bladder-innervating DRG neurons express *Trpv1* [56-58], whilst TRPV1 is a key integrator of histamine and P2X3 dependent bladder afferent activation [10, 27]. These findings are consistent with the literature suggesting *Mrgpra3*-positive neurons are in a subpopulation of *Trpv1*-positive neurons [55, 59, 60]. However, the signalling mechanisms in the bladder, where only 15% of bladder neurons express *Trpa1*, appear to contrast with those in the colon and skin. For example, Mrgpr agonist-induced mechanical hypersensitivity to colorectal distension is not evoked in *Trpa1*^{-/-} mice [28]. In the skin TRPA1 has been identified as the downstream target of both MrgprA3 and MrgprC11, whereby *Trpa1*^{-/-} mice display little to no scratching in response to chloroquine or BAM8-22, with functional coupling of MrgprA3 or MrgprC11 to TRPA1 occurring via Gβγ and PLC signalling respectively [61].

Differences are also apparent between bladder and cutaneous neurons when interrogating the role of MrgprD. Within cutaneous populations two distinct pruritogenic sub-populations of DRG neurons have been identified; those expressing *Mrgpra3*, *Mrgprc11* and histamine receptor 1 (*Hrh1*), and those expressing *Mrgprd* [22, 59, 62]. MrgprD, initially postulated to play an important nociceptive role, is now known to mediate itch induced by the amino acid β-alanine [62, 63]. Despite high *Mrgprd* expression in whole LS DRG, only 6% of bladder-innervating DRG neurons expressed *Mrgprd*, which showed equal co-expression (40%) with *Mrgprc11* and *Mrgpra3*. This suggests a far more prominent role for MrgprC11 and MrgprA3 than MrgprD in bladder sensory function. In keeping with this reasoning, we show that chloroquine, BAM8-22, and NPFF enhanced the mechanosensitivity of subpopulations (20-41%) of wildtype bladder afferents to bladder distension, which did not occur in experiments with *Mrgpr-clusterΔ*^{-/-} mice. The proportion of wildtype afferents responding to these Mrgpr agonists correlated well with the proportion of bladder neurons expressing *Mrgpra3* (14%) and

Mrgprc11 (38%). Mrgpr mRNA expression in bladder tissue was extremely low or below the detection limit, explaining why intra-bladder instillation of Mrgpr agonists did not affect bladder compliance. These findings are in keeping with a number of studies showing that these Mrgprs are expressed almost exclusively in sensory structures, including cell bodies of sensory neurons located in the DRG, trigeminal, and jugular ganglia [20, 28, 30, 55, 60]. We found that Mrgpr agonists evoked bladder afferent mechanical hypersensitivity across a broad range of distension pressures. Unlike other irritant and inflammatory stimuli, such as histamine or inflammatory cytokines, Mrgpr agonist incubation did not recruit silent afferents to become mechanosensitive [27, 64]. This is an important distinction as silent afferents represent a discrete functional subclass of bladder afferents that, after becoming sensitised, acquire an ability to respond to 'high-threshold' or 'nociceptive' mechanosensory stimuli [64-66]. These findings are consistent with MrgprA3- and MrgprC11-positive neurons that have previously been reported as being responsive to mechanical stimuli, including probing of cutaneous receptive fields [55]. Furthermore, a recent study investigating the role of Mrgprs in colonic afferent mechanosensitivity found that MrgprA3- and MrgprC11-positive fibres are also sensitive to mechanical stimulation [28]. Mrgpr-positive cutaneous nerve endings have been reported to exclusively innervate the epidermis [55], however this does not appear to be the case in the bladder. Whilst some mechanosensitive afferents innervate the bladder mucosa, an equivalent to the epidermis, the majority of mechanosensitive bladder afferents are found innervating the bladder detrusor [52, 67], and we observed significant hypersensitivity of these distension sensitive afferents to Mrgpr agonists.

The homogeneity of bladder sensory pathways is further reflected in their activation of spinal cord circuits in response to bladder distension, which unlike the distinct organisation of primary afferent circuits that occurs in the spinal cord for processing specific somatosensory stimuli [47], appears graded only to the level of peripheral stimuli [36]. As such, an increase in the intensity of the peripheral afferent signal corresponds to increased activation of second-order neurons that project within multiple visceral sensory pathways to regulate networks responsible for both micturition and sensation, rather than any obvious sensation-specific activation. Accordingly, we found Mrgpr activation of peripheral afferents in the bladder *in vivo* resulted in significant increases in the number of pERK-immunoreactive neurons in all regions of the lumbosacral dorsal horn that receive input from mechanosensitive afferents [36]. This included increased numbers of neurons within the superficial dorsal horn, dorsal grey commissure and sacral parasympathetic nucleus (SPN). As such, we would expect that the physiological consequence of an Mrgpr-induced increase in afferent firing would be similar to other stimuli that evoke an increase in primary afferent excitability from the bladder - urinary urgency and urinary frequency.

Interestingly, we observed no difference in bladder afferent mechanosensitivity nor activation of central pathways in the spinal cord to distension with saline between *Mrgpr-clusterΔ^{-/-}* mice and wildtype mice. Similar observations have been made before in other sensory pathways, where *Mrgpr-clusterΔ^{-/-}* mice do not show deficits in cutaneous mechanosensation [22]. Furthermore, in the colon *Mrgpr-clusterΔ^{-/-}* mice had similar visceromotor responses to wildtype mice [28]. Overall, these data suggest that endogenous activators of MrgprC11 and MrgprA3 are not tonically released from the bladder to set the baseline tone of bladder afferent mechanosensitivity. Whether or not these mechanisms are up-regulated in conditions of bladder pathophysiology is the subject of future research.

In conclusion, we have shown that MrgprC11 and MrgprA3 are present and functional on mechanosensitive bladder afferents and their activation induces mechanical hypersensitivity and increased activation of sensory circuits within the spinal cord. Enhanced sensation via an increase in mechanosensitivity may be a fundamental protective mechanism and relate more specifically to bladder disorders associated with urgency and frequency in the absence of pain, such as OAB.

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Statement of Authorship

Title of Paper	Bile acid receptor TGR5 mediates mouse bladder afferent mechanosensitivity <i>ex vivo</i> and <i>in vivo</i>
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☒ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Ashlee Caldwell		
Contribution to the Paper	Involved in planning experiments, performing <i>in vivo</i> experiments, retrograde tracing, cell culture, calcium imaging, QRT-PCR, data analysis, manuscript writing and editing		
Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
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Chapter 7 Bile acid receptor TGR5 mediates mouse bladder afferent mechanosensitivity *ex vivo* and *in vivo*

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7.1 Abstract

The symptoms of chronic bladder conditions such as interstitial cystitis/bladder pain syndrome (IC/BPS) and overactive bladder syndrome (OAB), including frequency, urgency, and discomfort, suggest a hypersensitivity within bladder sensory pathways. The underlying mechanisms for these conditions are incompletely understood, however targeting specific receptors mediating neuronal sensitivity to specific stimuli is a key pharmacological treatment method for numerous conditions. Irritant-sensing receptors that mediate pruritus, such as the bile acid receptor TGR5, have been identified within the viscera and are thought to play a role in neuronal sensitivity. Here, in mice, we identify mRNA expression of TGR5 (*Gpbar1*) in all layers of the bladder wall as well as in the lumbosacral dorsal root ganglia (DRG) and in isolated bladder-innervating DRG neurons using QRT-PCR. Further, *Gpbar1* mRNA was 100% co-expressed with *Trpv1* and 30% co-expressed with *Trpa1* in bladder-innervating DRG neurons. Using a mouse *ex vivo* bladder afferent preparation, we show that intravesical administration of TGR5 agonists Pg5 α and CCDC enhanced afferent mechanosensitivity to bladder distension, while *in vivo* intravesical administration of CCDC enhanced spinal dorsal horn neuron activation in response to bladder distension. In *Gpbar1*^{-/-} mice, *ex vivo* and *in vivo* CCDC-induced enhanced mechanosensitivity is abolished. Finally, we performed live-cell calcium imaging of bladder-innervating DRG neurons *in vitro* to demonstrate direct activation of a sub-population of bladder neurons with CCDC, which was diminished in DRG neurons from *Trpv1*^{-/-} mice but not *Trpa1*^{-/-} mice. Together, these results indicate a role for the irritant-sensing receptor TGR5 in mediating bladder afferent sensitivity to distension, and thus may be important in symptoms of hypersensitivity such as those experienced in IC/BPS and OAB.

7.2 Introduction

Chronic bladder pathologies such as interstitial cystitis/bladder pain syndrome (IC/BPS) and overactive bladder syndrome (OAB) affect up to 16% of the population, significantly decreasing quality of life for sufferers [1-4]. Although there are important differences between these conditions, the common symptoms of frequency, urgency, and discomfort [1, 5] are strongly indicative of neuronal hypersensitivity within bladder sensory pathways. Treatment options for each of these conditions are limited, suggesting a more complete understanding of the mechanisms underlying bladder sensory signalling is required.

The primary sensory afferents innervating the urinary bladder have cell bodies within the lumbosacral (LS) dorsal root ganglia (DRG) [6, 7]. Sensory nerve endings for these afferent neurons are located throughout all layers of the bladder wall including the detrusor smooth muscle, the mucosal/interstitial tissue, and the urothelium [8, 9]. Activation of these sensory nerve endings by various mechanical, chemical, or inflammatory stimuli results in sensory input into the dorsal horn of the LS spinal cord, where the bladder afferent neurons terminate [6, 10, 11]. From here, signals are relayed into autonomic and central pathways via activation of second order neurons, facilitating bladder relaxation and conscious sensory perception respectively [11, 12]. The specificity of the sensory input into central pathways is driven by the combination of receptors and ion channels expressed on the sensory terminals, allowing for the differentiation of distinct stimuli. Specific targeting of receptors on sensory nerve endings therefore presents an opportunity to modulate the intensity of bladder sensory signals. Pharmacological modulation of receptors is an invaluable method of treatment development for a variety of conditions; G-protein coupled receptors (GPCRs) in particular constitute the majority of current and potential pharmaceutical receptor targets [13].

GPCRs mediating 'itch' or pruritus, including histamine receptor 1 (H₁R), mas-related GPCRs (Mrgprs), and the bile acid receptor TGR5, have recently been highlighted as potential pharmacological targets in visceral hypersensitivity conditions [14]. We have recently demonstrated that activation of H₁R or Mrgpr subtypes A3 and C11 from within the bladder modulates the sensitivity of bladder afferents to distension [15, 16], indicating roles for both histaminergic and non-histaminergic itch-mediating GPCRs in mediating bladder afferent sensitivity.

Takeda G-Protein Receptor 5 (TGR5), also known as Membrane-Bile Acid Receptor (M-BAR) or G-protein-coupled Bile Acid Receptor 1 (GPBAR1), is a membrane-bound G-protein coupled bile acid receptor [17, 18]. TGR5 is expressed throughout the viscera and the nervous system, including the gastrointestinal tract [19-21], the bladder [22], and neurons and glial cells in the DRG [14, 23, 24]. TGR5 has been recently proposed as the integral receptor required for pruritus associated with chronic and pregnancy-related liver diseases, with TGR5 activation in DRG neurons resulting in

neuronal hyperexcitability and the release of neurotransmitters in the dorsal horn [14, 19, 23]. Moreover, TGR5 has been implicated in the pathology of irritable bowel syndrome (IBS), a common co-morbidity of OAB and IC/BPS [14]. In addition to bile acids, TGR5 can be activated by neurosteroids such as progesterone and progesterone metabolites [25, 26] to evoke itch sensations via a TGR5-dependent mechanism [26, 27]. Progesterone metabolites are conjugated and excreted via the urine, while bile acids have also been identified in the urine [28-32]. However, a role for TGR5 in regulating bladder sensation has yet to be explored.

Given the presence of endogenous TGR5 agonists in the bladder and the implicated roles for TGR5 in irritant sensation, we sought to investigate the expression and function of TGR5 in bladder afferent sensitivity. We performed *ex vivo* bladder afferent recordings and dorsal horn neuronal activation following *in vivo* bladder distensions with TGR5 agonists in wildtype and *Gpbar1*^{-/-} mice to investigate the physiological role of TGR5 in peripheral and central afferent signalling. To explore the mechanism of action we used QRT-PCR, single cell RT-PCR, and live cell calcium imaging of retrogradely traced bladder-innervating DRG neurons. We have defined TGR5 responses in bladder neurons from wild-type, as well as *Trpa1*^{-/-} and *Trpv1*^{-/-} mice to assess downstream signalling. This study has characterised, for the first time, the ability of TGR5 to regulate bladder afferent mechanosensitivity.

7.3 Methods

7.3.1 Ethics and animals

In this study, we used 12- to 18-week-old male and female C57BL/6J mice provided via an in-house breeding colony at the South Australian Health and Medical Research Institute (SAHMRI). Mice at SAHMRI were originally acquired from an in-house C57BL/6J breeding programme (JAX strain #000664; originally purchased from The Jackson Laboratory; breeding barn MP14; Bar Harbor, ME) and then bred within the specific and opportunistic pathogen-free animal care facility at SAHMRI. Some experiments utilised TRPV1 knockout (*Trpv1*^{-/-}), TRPA1 knockout (*Trpa1*^{-/-}), or TGR5 knockout (*Gpbar1*^{-/-}) mouse strains with a C57BL/6J genetic background, also bred in-house at SAHMRI. *Trpv1*^{-/-} mice were originally acquired from an in-house breeding program at The Jackson Laboratory (JAX stock #003770; Bar Harbor, ME), *Trpa1*^{-/-} mice were gifted to us by the original developer, David Corey (Harvard University, Cambridge, Massachusetts, USA), and *Gpbar1*^{-/-} mice were gifted to use by Johan Auwerx and Kristina Schoonjans, Ecole Polytechnique de Lausanne (Lausanne, Switzerland). These genetically modified mouse lines were all then bred in-house at SAHMRI as per the wildtype mice. *Nav1.8Cre-Gpbar1*^{-/-} mice were gifted to us by Daniel Poole (Monash University, Victoria, Australia), then housed at SAHMRI's Preclinical, Imaging, and Research Laboratories (PIRL) facility for procedures.

All experiments were approved by and performed in accordance with the SAHMRI Animal Ethics Committee approvals (SAM195 and SAM190). Mice were group housed (5 mice per cage) within individual ventilated cages filled with chip coarse dust-free aspen bedding (Cat#-ASPJMAEBCA; PuraBed, Niederglatt, Switzerland). Cages were stored on individual ventilated cage racks within a temperature-controlled environment of 22°C and a 12-hour light/12-hour dark cycle. Mice had free access to LabDiet JL Rat and Mouse/Auto6F chow (Cat #5K52, St. Louis, MO) and autoclaved reverse osmosis water. Sample sizes of each experiment are detailed in the corresponding figure legends.

7.3.2 *Ex vivo* bladder afferent nerve recording

Nerve recording was performed in an *ex vivo* model as previously described [15, 33-35]. Mice were humanely killed via CO₂ inhalation, and the entire lower abdomen was removed and submerged in a modified organ bath under continual perfusion with gassed (95% O₂ and 5% CO₂) Krebs-bicarbonate solution (composition in mmol/L: 118.4 NaCl, 24.9 NaHCO₃, 1.9 CaCl₂, 1.2 MgSO₄, 4.7 KCl, 1.2 KH₂PO₄, 11.7 glucose) at 35°C. The bladder, urethra, and ureters were exposed by removing excess tissue. Ureters were tied with 4-0 perma-hand silk (Ethicon, #LA53G). The bladder was catheterised (PE 50 tubing) via the urethra and connected to a syringe pump (NE-1000) to allow a controlled fill rate of 30 µL/min with saline (NaCl, 0.9%). To enable intravesical pressure recording during graded

distension, a second catheter was inserted through the dome of the bladder, secured with silk, and connected to a pressure transducer (NL108T2; Digitimer). Pelvic nerves, isolated from all other nerve fibres between the pelvic ganglia and the spinal cord, were dissected into fine multiunit branches and a single branch was placed within a sealed glass pipette containing a microelectrode (WPI) attached to a Neurolog headstage (NL100AK; Digitimer). Nerve activity was amplified (NL104), filtered (NL 125/126, band pass 50–5,000 Hz, Neurolog; Digitimer), and digitised (CED 1401; Cambridge Electronic Design, Cambridge, UK) to a PC for offline analysis using Spike2 software (Cambridge Electronic Design, Cambridge, UK). The number of action potentials crossing a pre-set threshold at twice the background electrical noise was determined per second to quantify the afferent response. Single-unit analysis was performed offline using Spike2 software by matching individual spike waveforms through linear interpolation. A single unit was deemed to be responsive if a greater than 25% increase in neuronal excitability was detected.

7.3.2.1 Afferent recording experimental protocols

At the start of each afferent recording experiment, control bladder distensions were performed with intravesical infusion of saline (NaCl, 0.9%) at a rate of 30 μ L/min to a maximum pressure of 30 mmHg at 10 min intervals to assess the viability of the preparation and reproducibility of the intravesical pressure and neuronal responses to distension. The volume in the bladder was extrapolated from the known fill rate (30 μ L/min) and the time taken (s) to reach maximum pressure (30 mmHg). Compliance was determined by plotting intravesical pressure against the calculated volume. After a stable baseline was maintained, the saline in the infusion pump was replaced by 5 α -pregnan-3 β -ol-20-one sulphate (Pg5 α ; 300 μ M; Steraloids, #P3865-000) or 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N,5-dimethyl-4-isoxazolecarboxamide (CCDC; 300 μ M; Biovision, #1722-5, CAS: 1197300-24-5). The single nerve bundle isolated and inserted into the glass electrode during the dissection process was contained within the recording electrode for the entire experiment, allowing afferent nerve responses to be compared in the same nerve fibres during intra-bladder incubation with saline and Pg5 α (300 μ M) or CCDC (300 μ M).

7.3.3 *In vivo* spinal dorsal horn activation

7.3.3.1 *In vivo* bladder distension

In vivo bladder infusion in anaesthetised mice was performed as previously described [15, 35]. 12-week old female mice were anaesthetised (isoflurane 2% - 4% in oxygen) and a catheter (PE 50 tubing) was inserted into the bladder via the urethra. Correct catheter placement was determined by inserting the catheter gently until meeting resistance (bladder dome) and receding slightly (2-3 mm) to avoid damaging the bladder wall. Urine was removed using a suction syringe and the catheter

removed. A new catheter (PE 50 tubing), primed with either vehicle (saline; N = 5 mice) or CCDC (100 μ M; N = 5 mice), was inserted and 100 μ L of solution infused gently, to fill but not fully distend the bladder, and allowed to incubate for 5 minutes. The solution was then carefully removed from the bladder to ensure minimum liquid remained in the bladder. A third, dry catheter (PE 50 tubing) was inserted, and a small length of 4-0 perma-hand silk (Ethicon, #LA53G) was knotted over the urethral opening to fix the catheter into place and minimise loss of pressure. A 5-minute stabilisation period was allowed between tying the catheter and performing the distensions to limit false-positive pERK signalling from the act of tying. A sphygmomanometer was attached between the catheter and a 10 mL air-filled suction syringe. 5 x 20-second bladder distensions were performed at 10-second intervals, at a holding pressure of 40 mmHg. Following the fifth 20-second distension, mice were administered an anaesthetic overdose (intraperitoneal injection, 0.125 mL/250 g Lethobarb®; Virbac Australia) followed by transcardial perfuse fixation.

7.3.3.2 Transcardial perfuse fixation and tissue dissection

Following anaesthetic overdose, the thoracic cavity was opened and 0.5 mL heparinised saline (50 IU in 5 mL; Pfizer) was injected into the left ventricle. A 22-gauge needle attached to tubing and a peristaltic perfusion pump was then inserted in or near the injection site in the left ventricle. The right atrium was cut open to allow for perfusate drainage. Warm phosphate buffer (0.1 M; 21.7 mM NaH_2PO_4 [Chem Supply, #SA061-500G], 81 mM Na_2HPO_4 [VWR Chemicals, #102494C], pH 7.2) was then perfused, followed by ice-cold 4% paraformaldehyde (PFA; Sigma-Aldrich, #158127) in 0.1 M phosphate buffer. After complete PFA perfusion, the lumbosacral spinal cord was removed and postfixed in 4% PFA in 0.1 M phosphate buffer at 4°C for 18 to 20 hours. Spinal cords were then cryoprotected in 30% sucrose/phosphate buffer (Sigma-Aldrich, #S9378) for two days at 4°C, followed by a 48-hour incubation at 4°C in 50% optimal cutting temperature (OCT; VWR, #C361603E)/30% sucrose/phosphate buffer solution before freezing in 100% OCT using liquid nitrogen-cooled 2-methylbutane Sigma-Aldrich, #M32631). The frozen spinal cord segments were cryosectioned (10 μ m thickness) and placed onto gelatin-coated slides for processing of immunofluorescence labelling. Sectioning was performed serially and distributed over 6 slides, which were used for immunofluorescence localisation of phosphorylated-MAP-kinase ERK 1/2 (pERK). The number of pERK-immunoreactive neurons were counted in spinal cord sections from L6-S1, with spinal segments determined *ex vivo* by the shape of the dorsal horn [36].

7.3.3.3 Immunohistochemistry of phosphorylated-MAP-kinase ERK 1/2 within the spinal cord

The dorsal horn neurons activated by bladder distension were identified by immunofluorescent labelling for neuronal activation marker, phosphorylated-MAP-kinase ERK 1/2 (pERK), using a method described previously [15, 34, 35, 37, 38]. The details for the primary and secondary antisera can be found in **Table 7.1**.

Table 7.1: Spinal cord immunohistochemistry primary and secondary antisera

	Species raised in	RRID/AF conjugate	Manufacturer, Catalogue No.	Dilution
Primary antisera: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP(tm) Rabbit mAb #4370	Rabbit	AB_2315112	Genesearch, #4730L	1:200
Secondary Antisera: anti-Rabbit IgG	Chicken	AlexaFluor 594	Life Technologies, #A-21442	1:200

pERK activation was determined rather than c-Fos as ERK phosphorylation occurs within minutes of the peripheral stimuli and thus correlates better to the short-duration peripheral stimuli applied [39]. Further, spinal neuronal activation in response to a range of peripheral stimuli is increasingly being identified via pERK activation quantification [34, 35, 38, 40]. After air drying for one hour, excess OCT was removed from sections by washing with 0.2% Triton X-100 (Sigma-Aldrich, #T8787) in 0.1-M phosphate-buffered saline (PBS-T). Non-specific secondary antibody binding was blocked with 5% normal chicken serum diluted in 0.2% PBS-T. Tissue sections were then incubated with pERK primary antisera diluted in PBS-T at room temperature overnight (18 hours). Sections were washed in PBS-T and incubated with secondary antibody conjugated to AlexaFluor 594 for 1 hour at room temperature, followed by a PBS-T wash before mounting in ProLong Diamond Antifade (Life Technologies, #P-36962) and coverslipping. Negative controls were prepared as above with omission of the primary antibody. Slides were allowed to set for 24 hours prior to visualisation.

7.3.3.4 Microscopy

Fluorescence was visualised with an epifluorescence microscope (Olympus, Tokyo, Japan) using a 10× objective and 3-second exposure. ImageJ software (NIH) was used for image analysis. Aside from making moderate adjustments for brightness and contrast, the images were not manipulated in any way.

7.3.3.5 Spinal cord pERK neuronal counts and analysis

Neuronal counts were analysed from previously saved digital photomicrographs, with only neurons with intact nuclei counted. The number of pERK-immunoreactive (pERK-IR) neurons in a quadrant of the L6-S1 dorsal horn was obtained from a minimum of 6 sections/animal viewed at 10× magnification. The mean number of pERK-IR neurons ($\pm$ SEM) was compared between vehicle- and CCDC-treated mice.

7.3.4 Retrograde tracing

A small, aseptic, abdominal incision was made in anaesthetised (2% – 4% isoflurane in oxygen) mice. Cholera Toxin subunit B conjugated to AlexaFluor 488 (CTB-488, 0.5% diluted in 0.1 M phosphate buffered saline [PBS] pH 7.4; ThermoFisher Scientific) was injected into the bladder wall at three sites (3 μ L/injection) using a 5 μ L Hamilton syringe attached to a 30-gauge needle [34, 35]. The needle was inserted subserosally, parallel with the bladder muscle, to prevent injection of CTB into the bladder lumen. The abdominal incision was sutured closed and antibiotic (Amoxicillin; 50 mg/kg; Amoxil, AUSTRI1137) and analgesic (Buprenorphine (Temvet); 0.1mg/kg; Troy Laboratories Pty Ltd, APVMA #67612) given subcutaneously as mice regained consciousness. Mice were then individually housed and allowed to recover. 4 days post-surgery, mice were humanely killed for subsequent removal of the lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) and isolation and culture of the neurons to visualise CTB-labelled bladder-innervating neurons within the DRG population.

7.3.5 Cell culture of bladder-innervating DRG neurons

Four days after retrograde tracing, mice were humanely euthanised via CO₂ inhalation and lumbosacral (LS; L5-S1) dorsal root ganglia (DRG) were removed. DRG were digested in Hanks' balanced salt solution (HBSS; pH 7.4; Life Technologies, #14170161) containing 4 mg/mL dispase (GIBCO, ThermoFisher Scientific, #17105041) and 3 mg/mL collagenase II (GIBCO, ThermoFisher Scientific, #17101015) at 37°C for 30 min. The collagenase-dispase solution was aspirated and replaced with HBSS containing collagenase (3 mg/mL) only for 10 min at 37°C, followed by washes in HBSS. DRG were then mechanically disrupted and cells dissociated in 600 μ L complete DMEM (Dulbecco's Modified Eagle Media [DMEM; GIBCO, ThermoFisher Scientific, #11995065]; 10% Foetal Calf Serum [Invitrogen, ThermoFisher Scientific, MA, USA]; 2 mM L-glutamine [GIBCO, ThermoFisher Scientific, #25030081], 100 μ M MEM non-essential amino acids [GIBCO, ThermoFisher Scientific, #11140076], 100 mg/mL penicillin/streptomycin [GIBCO, ThermoFisher Scientific, #15070063], and 96 μ g/L nerve growth factor-7S [Sigma, N0513-0.1MG]) via trituration through fire-polished Pasteur pipettes of descending diameter, and centrifuged at 50 $\times$ g for 1 min [34, 35, 37, 41]. Neurons were resuspended in complete DMEM (360 μ L) and spot-plated onto 13 mm coverslips (30 μ L/coverslip) coated with laminin (20 μ g/mL; Sigma-Aldrich, #L2020) and poly-D-lysine (800 μ g/mL; ThermoFisher Scientific) in a 12-well plate. Coverslips were incubated at 37°C in 5% CO₂ for 2-3 hours to allow adherence of neurons before gently flooding each well with 1.7 mL complete DMEM. Cultured neurons were maintained in an incubator at 37°C in 5% CO₂ for 18-48 hours for calcium imaging or cell picking for single-cell RT-PCR.

7.3.6 Calcium imaging of cultured LS DRG neurons

Cultured LS DRG neurons (18-48 hours post-plating) were loaded with 2 μ M Fura-2-acetoxymethyl ester (Fura-2; Invitrogen, ThermoFisher Scientific, #F1221) in pluronic F-127 (Invitrogen, ThermoFisher Scientific, #P3000MP) and incubated for 30 min at 37°C before washing with HEPES buffer (10 mM HEPES sodium salt [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt; Sigma, #H7006-100G], 140 mM NaCl [Chem Supply, #SA046-3KG], 4 mM KCl [Chem Supply, #PA054-500G], 5 mM D-glucose anhydrous [Chem Supply, #GA018-500G], 2 mM CaCl_2 [Scharlau, #CA01951000], and 2 mM MgCl_2 [Sigma, #M8266-100G], pH 7.40) for 10 min and imaging at room temperature (23°C). Fura-2 was excited at 340 and 380 nm and emissions were measured at 510 nm using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, an Omega XF04 filter set for Fura-2, a Photonic Science ISIS-3 intensified CCD camera, and Universal Interface Card MetaFluor software. Retrogradely traced bladder-innervating DRG neurons were identified by the presence of the CTB-488 tracer, visible under excitation at 480 nm. 480 nm excitation was used for initial bladder neuron identification only; to prevent unnecessary photobleaching, images were not obtained at 480 nm during the experiment. Fluorescence images were obtained every 5 s leading up to and immediately following drug application, and every 20 s for washout period, using a 20x objective. Data were recorded and further analysed using MetaFluor software.

After an initial baseline reading to ensure cell fluorescence was stable, indicating healthy cells, DRG neurons were stimulated with CCDC (100 μ M; Biovision, #1722-5, CAS: 1197300-24-5), and changes in intracellular calcium $[\text{Ca}^{2+}]_i$ were monitored in real time. For experiments assessing downstream TRP channel signalling, application and washout of CCDC was followed by application of TRPA1 agonist allyl isothiocyanate (AITC; 100 μ M; Aldrich, #377430, CAS: 57-06-7) then TRPV1 agonist Capsaicin (50 nM; Sigma, #M2028, CAS: 404-86-4). KCl (40 mM; Chem Supply, #PA054-500G) was applied as a neuronal positive control. $[\text{Ca}^{2+}]_i$ is expressed as the ratio between the fluorescence signals at 340 and 380 nm. Baseline $[\text{Ca}^{2+}]_i$ was determined by averaging 8-12 fluorescence ratio readings immediately prior to stimulation. Maximum peak response was calculated by subtracting the relevant baseline score from the maximum fluorescence ratio measurement following compound application. Baseline fluorescence was normalised to a ratio of 1 for example traces.

7.3.7 Analysis of mRNA expression in LS DRG, isolated urothelial cells, and bladder mucosal and detrusor layers

7.3.7.1 Tissue collection

16- to 18-week-old mice were humanely euthanised via CO_2 inhalation and the bladder and lumbosacral (L5-S1) dorsal root ganglia (DRG) removed. DRG were frozen in dry ice in pairs by spinal

level (L5, L6, S1) and stored at -80°C for RNA extraction and quantitative reverse-transcription polymerase chain reaction (QRT-PCR). The bladder was dissected in sterile PBS with a single cut from the urethral opening along one side to the dome, stretched out, and pinned flat, urothelial side up. For QRT-PCR of the bladder layers, the mucosa and detrusor were gently peeled apart, frozen in dry ice, and stored at -80°C for RNA extraction and QRT-PCR.

7.3.7.2 Isolation of mouse urothelial cells

For urothelial cell QRT-PCR, urothelial cells were isolated as performed previously [15, 33, 42, 43]. Following stretching and pinning of the opened bladder as per bladder layer separation, the bladder was incubated with DMEM containing 2.5 mg/mL dispase, 10% Foetal Calf Serum, and 1 µM/mL HEPES (Sigma, #H3375, pH 7.0) at room temperature for 3 hours. Cells were collected by gentle scraping of the urothelium with a blunt scalpel and dissociated in 0.025% trypsin EDTA (GIBCO, ThermoFisher Scientific, #25200072) at 37°C in 5% CO₂ for 15 min with gentle intermittent trituration. The trypsin was deactivated by adding the cell suspension to DMEM containing 10% Foetal Calf Serum before centrifugation (15 min, 1500 rpm, 4°C). Cells were then resuspended in keratinocyte serum-free media (KSFM; Invitrogen, #17005042). Cell count and viability were determined using the Countess Automated Cell Counter (Invitrogen, ThermoFisher Scientific). Cells were then pelleted, frozen in dry ice, and stored at -80°C for RNA extraction and QRT-PCR.

7.3.7.3 RNA extraction

RNA was extracted using the PureLink RNA Micro kit (Invitrogen, Victoria, Australia, #12183-016; DRG pairs and isolated urothelial cells) or the PureLink RNA Mini kit (Invitrogen, #12183018A; bladder mucosa and detrusor) with DNase treatment (Life Technologies, #12185-010) according to the manufacturer's instructions. A NanoDrop Lite spectrophotometer (ThermoFisher Scientific) determined RNA quantity and purity. RNA was reverse transcribed to cDNA using SuperScript VILO Master Mix (Invitrogen, #11755250) as per the manufacturer's instructions. The cDNA was stored at -20°C for QRT-PCR.

7.3.7.4 Quantitative reverse-transcription polymerase chain reaction (QRT-PCR)

QRT-PCR was performed using Taqman Gene Expression Master Mix (Applied Biosystems, Victoria, Australia, #4369016) with commercially available hydrolysis probes (TaqMan; Life Technologies, see **Table 7.2** for details) and RNase-free water (AMBION, Victoria, Australia, #AM9916). For each reaction, 10 µL of MasterMix, 1 µL of TaqMan primer assay, 4 µL of water, and 5 µL of cDNA (1:2 dilution in RNA-free H₂O) from each sample was tested in duplicate for each target. Reference genes *Actb* (DRG pairs) and *Gapdh* (mucosa, detrusor, and urothelial cells) were used as endogenous controls. Assays were run for 45 cycles on a 7500 Fast Real-Time PCR System (Applied

Biosystems) machine, using 7500 Fast software, v2.0.6. mRNA quantities are expressed as $2^{-\Delta Ct}$ relative to reference gene.

Table 7.2 - Primers used for QRT-PCR receptor expression assays

Gene Alias	Gene Target	Assay ID
β -actin (reference gene)	<i>Actb</i>	Mm00607939_s1
GAPDH (reference gene)	<i>Gapdh</i>	Mm99999915_g1
Tubulin-3 (neuronal marker)	<i>Tubb3</i>	Mm00727586_s1
Glial fibrillary acidic protein (glial marker)	<i>Gfap</i>	Mm01253033_m1
Takeda G-protein Receptor 5 (TGR5)	<i>Gpbar1</i>	Mm04212121_s1
Transient receptor potential vanilloid 1	<i>Trpv1</i>	Mm01246300_m1
Transient receptor potential ankyrin 1	<i>Trpa1</i>	Mm01227437_m1

7.3.8 Single-cell RT-PCR of individual bladder-innervating dorsal root ganglia neurons

Under continuous perfusion of sterile and RNA-/DNase-free PBS, single retrogradely traced bladder dorsal root ganglia (DRG) neurons were identified using a fluorescence microscope and collected into the end of a fine glass capillary using a micromanipulator [35]. The glass capillary was then broken into a sterile Eppendorf tube containing 10 μ L of lysis buffer with DNase (TaqMan Gene Expression Cells-to-CT Kit; Invitrogen, #4399002). For each coverslip of cultured DRG neurons, a bath control was also taken and analysed together with cells. Following lysis and termination of DNase treatment, samples were immediately frozen on dry ice and stored at -80°C until cDNA synthesis was performed. RNA was reverse transcribed to cDNA using SuperScript™ IV VIL0™ Master Mix (Invitrogen, #11766500) as per the manufacturer's instructions. cDNA was stored at -20°C for RT-PCR. *Tubb3* (tubulin-3) expression served as a neuronal marker and positive control, and *Gfap* (glial fibrillary acidic protein) expression served as a glial cell marker for exclusion. Expression was considered positive if a complete curve was observed before 50 cycles.

7.3.9 Experimental design, data analysis and statistics

Data are presented as mean $\pm$ SEM or the % of neurons or afferents. N indicates the number of animals, whilst n indicates the number of independent neurons or afferents. Statistical significance was reported at a level of * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. In some cases, # is used to indicate significance of at least $P < 0.05$ for multiple comparisons. Data were tested for Gaussian

distribution using the D'Agostino and Pearson omnibus normality test to determine the correct statistical tests. Data were analysed using Prism 8 (GraphPad, San Diego, CA, USA), using one- or two-way analysis of variance (ANOVA) with Tukey's or Sidak's post hoc analyses dependent on data distribution, or student's t-tests, for parametric data. Non-parametric data were analysed using Kruskal-Wallis test with Dunn's multiple comparisons, Mann-Whitney analysis, or Wilcoxon matched-pairs signed rank test. Fisher's Exact test was used to determine significance of contingency datasets. The specific tests used to analyse each data set are indicated within the individual figure legends.

7.4 Results

7.4.1 TGR5 agonist PG5 α causes bladder afferent mechanosensitivity

To first determine if activation of the TGR5 receptor could influence bladder sensation, we investigated the impact of localised TGR5 activation on afferent sensitivity to bladder distension using an *ex vivo* bladder afferent preparation. Intravesical application of Pg5 α , a progesterone sulphate previously identified as an endogenous TGR5 agonist [26], resulted in a significant increase in bladder afferent firing in response to graded bladder distension compared to saline (**Figure 7.1A**), indicating increased mechanosensitivity following TGR5 activation. No change was observed in bladder compliance (pressure/volume relationship) between saline and Pg5 α experiments (**Figure 7.1B(i)** and **7.1B(ii)**), indicating that afferent hypersensitivity was not caused by changes in detrusor muscle tone.

Single-unit analysis of the bladder afferent nerve fibres revealed that 44.4% (20/45) of the mechanosensitive bladder afferents demonstrated an increase in mechanosensitivity following instillation of Pg5 α ('responders'), whereas 55.5% (25/45) of mechanosensitive afferents were unresponsive to Pg5 α ('non-responders') (**Figure 7.1C**). Responding afferents showed a significant increase in the maximum peak of afferent firing in response to distension following Pg5 α instillation compared to saline (**Figure 7.1D(i)**). Further, the afferent firing rate of Pg5 α -responsive afferents was significantly increased at pressures of 20 mmHg and above (**Figure 7.1D(ii)** and **7.2D(iii)**). In comparison, no change in mechanosensitivity was observed between saline and Pg5 α experiments for non-responding afferents, neither in the maximum peak (**Figure 7.1E(i)**) nor at any particular pressures (**Figure 7.1E(ii)** and **7.1E(iii)**). These data demonstrate that the progesterone metabolite Pg5 α causes enhanced mechanosensitivity of bladder afferent nerve fibres in response to graded bladder distension.

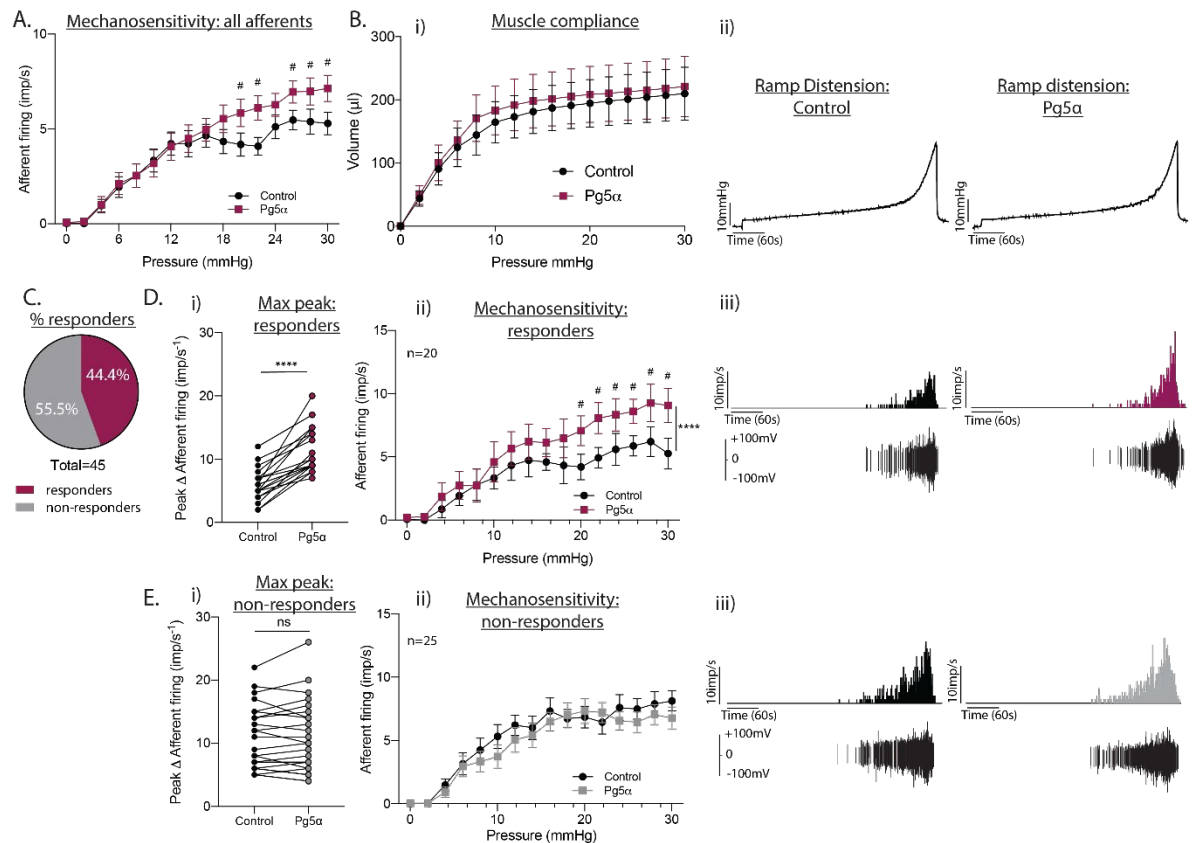


Figure 7.1: Pg5α enhances mechanosensitivity in bladder afferent neurons *ex vivo*.

Afferent nerve firing was measured in response to graded bladder distension in an *ex vivo* preparation in the presence of progesterone sulphate '5α-pregnan-3β-ol-20-one sulphate' (Pg5α; 300 μM) compared to saline (control). **(A)** Application of Pg5α increased the afferent firing rate (impulses per second; imp/s) of overall bladder afferents in response to bladder distension compared to saline ($n = 45$ single units from $N = 5$ mice; *** $P < 0.001$). **(B)** The intraluminal pressure following increased volume during bladder distension does not differ between saline and Pg5α (ns, $P > 0.05$), **(i)** indicating no change in muscle compliance. **(ii)** An experimental example demonstrating the graded increase in pressure with volume over time in the presence of saline followed by Pg5α. **(C)** Of the 45 mechanosensitive bladder afferents recorded, 44.4% (20 out of 45) responded to Pg5α with an increase in mechanosensitivity, while 55.5% (25 out of 45) were unchanged in mechanosensitivity between saline and Pg5α. **(D)** Responding afferents had **(i)** a significantly higher maximum peak in afferent firing in the presence of Pg5α compared to saline (**** $P < 0.0001$), and **(ii)** significantly increased afferent firing rates at pressures of 20 mmHg and above (# $P < 0.05$). **(iii)** An experimental histogram example of a Pg5α-responsive afferent during bladder distension in the presence of saline followed by Pg5α. **(E)** Non-responding mechanosensitive afferents had **(i)** no significant difference in the maximum peak of afferent firing between distensions with saline and with Pg5α (ns, $P > 0.05$), and **(ii)** no significant difference in afferent firing at any distension pressure. **(iii)** An experimental histogram example of non-responsive mechanosensitive afferents during bladder distension in the presence of saline followed by Pg5α. Data are represented as mean $\pm$ SEM (**A**, **B(i)**, **D(ii)** and **E(ii)**), percentage of afferents (**C**), or maximum imp/s (**D(i)** and **E(i)**). 'Responders' were defined by a greater than 25% increase in neuronal excitability (**C**, **D**, and **E**). P values above are based on two-way ANOVA (**A**, **B(i)**, **D(ii)** and **E(ii)**), with subsequent Sidak's post

hoc test significance at individual data points indicated on **(A)** and **(D(ii))** (# represents significance of at least $P < 0.05$), or Wilcoxon matched-pairs signed rank test **(D(i))** and **E(i)**.

7.4.2 Synthetic TGR5 agonist CCDC replicates Pg5 α -induced increase in bladder afferent mechanosensitivity

We next aimed to further define the specific involvement of the TGR5 receptor in mediating bladder afferent mechanosensitivity. To this end, we used a potent, synthetic TGR5 agonist, 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N,5-dimethyl-4-isoxazolecarboxamide (CCDC), reported to be TGR5-specific [44]. Similar to Pg5 α , on applying CCDC to the *ex vivo* bladder afferent preparation we observed an increase in afferent mechanosensitivity to bladder distension compared to saline (**Figure 7.2A**). No change to muscle compliance was observed between saline and CCDC intravesical instillation (**Figure 7.2B(i)** and **7.2B(ii)**), demonstrating that the effect of CCDC on mechanosensitivity is a direct afferent effect and not secondary to muscular changes.

Single unit analysis of the mechanosensitive bladder afferents showed 50% (20/40) of afferents responded to CCDC with an increase in mechanosensitivity to bladder distension compared to saline ('responders'), while 50% (20/40) showed no change in mechanosensitivity following CCDC instillation ('non-responders') compared to saline (**Figure 7.2C**). CCDC-responsiveness was indicated by a significant increase in the maximum peak of afferent firing (**Figure 7.2D(i)**) following CCDC instillation compared to saline. Interestingly, responding neurons showed an increase in mechanosensitivity compared to saline at pressures of 10 mmHg and above (**Figure 7.2D(ii)**). No changes in afferent firing maximum peak or at any individual pressures were observed in the non-responding afferents (**Figure 7.2E(i)** and **7.2E(ii)**).

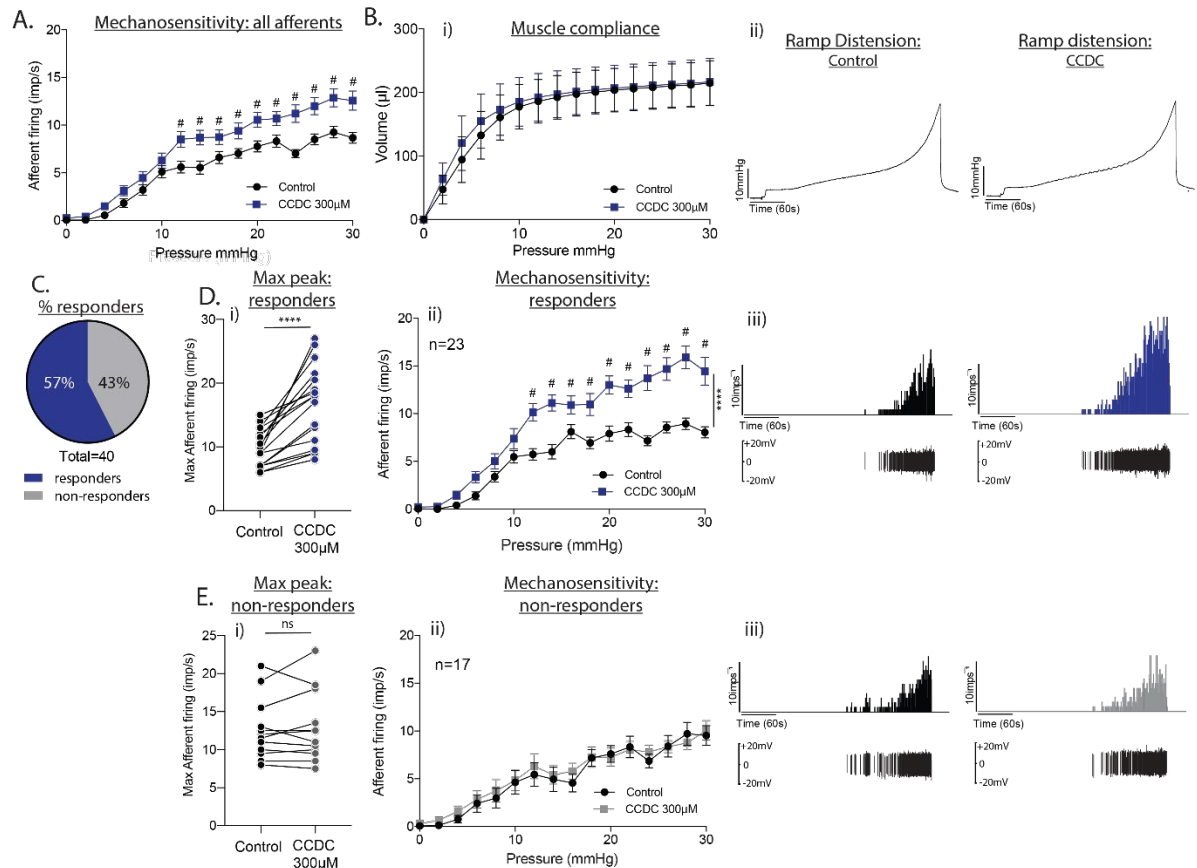


Figure 7.2: Synthetic TGR5 agonist CCDC enhances bladder afferent mechanosensitivity.

Afferent firing was recorded in response to graded bladder distension in an *ex vivo* preparation, comparing saline (control) responses to synthetic TGR5 agonist CCDC responses. **(A)** Application of CCDC caused a significant increase in the overall afferent firing rate (impulses per second; imp/s) at distension pressures at and above 12 mmHg (n = 40 single units from N = 4; **** P < 0.0001). **(B)** No difference was observed in the pressure to volume relationship following increased volume between saline and CCDC, indicating **(i)** no change in muscle compliance. **(ii)** An experimental example demonstrating the graded increase in pressure with steady increased volume over time with saline, followed by CCDC. **(C)** Of the 40 mechanosensitive bladder afferents recorded, 50% (20 out of 40) responded to CCDC with an increase in mechanosensitivity and 50% (20 out of 40) were unchanged in mechanosensitivity between saline and CCDC. **(D)** CCDC-responsive afferents (responders; n = 20) demonstrated a significant increase in mechanosensitivity following CCDC administration. **(i)** The maximum peak of afferent firing with CCDC was significantly higher than that with saline (**** P < 0.0001), as was **(ii)** Responders showed significantly increased mechanosensitivity to distension at pressures at and above 10 mmHg. **(iii)** An experimental histogram example demonstrating graded increase in afferent firing in response to bladder distension, with a visible increase in imp/s following administration of CCDC. **(E)** CCDC-unresponsive mechanosensitive afferents (non-responders; n = 20) showed no difference in mechanosensitivity between saline and CCDC. **(i)** No change was observed in the maximum imp/s, and **(ii)** there was no difference in imp/s at any distension pressure. **(iii)** An experimental histogram example demonstrating graded increase in afferent firing in response to bladder distension, with no evident change in response between saline and CCDC administrations. Data are represented as mean ± SEM **(A, B(i), D(ii) and E(ii))**, percentage of afferents **(C)**, or maximum imp/s **(D(i) and E(i))**. ‘Responders’ were defined by

a greater than 25% increase in neuronal excitability (**C**, **D**, and **E**). P values above are based on two-way ANOVA (**A**, **B(i)**, **D(ii)** and **E(ii)**), with subsequent Sidak's post hoc test significance at individual data points indicated on (**A**) and (**D(ii)**) (# represents significance of at least $P < 0.05$), or Wilcoxon matched-pairs signed rank test (**D(i)** and **E(i)**).

7.4.3 Intravesical administration of TGR5 agonist CCDC increases the number of bladder distension-induced pERK-immunoreactive spinal dorsal horn neurons

Our results thus far have established a role for TGR5 agonists in peripheral bladder afferent sensitivity. To determine whether or not this increased mechanosensitivity is conveyed to the central nervous system, we assessed dorsal horn neuronal activation in response to *in vivo* bladder distension in the presence of TGR5 agonist CCDC. Following intravesical incubation in saline, controlled bladder distension at 40 mmHg induced pERK-immunoreactivity in LS dorsal horn neurons (**Figure 7.3A**). The pERK-immunoreactive (IR) neurons were identified predominantly in the regions of the dorsal grey commissure and the superficial dorsal horn, with some pERK-IR neurons in the lateral spinal nuclei and the sacral parasympathetic nucleus in sacral spinal segments (**Figure 7.3B(i)**). Compared to saline, *in vivo* intravesical incubation in CCDC induced a significant increase in the number of LS dorsal horn pERK-IR neurons in response to bladder distension at 40 mmHg (**Figure 7.3A**). The pERK-IR LS dorsal horn neurons following CCDC incubation were distributed in the same regions of the dorsal horn as seen in the saline experiments (**Figure 7.3B(ii)**). As these regions are known to play a role in bladder afferent input, integration, and projection of autonomic and nociceptive visceral signalling [34, 45], an increase in pERK-IR neurons indicates an increase in central sensitivity to bladder distension and hence projection of the peripheral mechanosensitivity observed *ex vivo*.

increase in mechanosensitivity was observed in 17.1% (6/35) of mechanosensitive afferent fibres (**Figure 7.4C**). These CCDC-responsive neurons demonstrated an increase in the maximum peak of afferent firing (**Figure 7.4D(i)**), although multiple comparison analysis showed that the increased mechanosensitivity in CCDC-responsive *Gpbar1*^{-/-} afferents occurred specifically at 10 mmHg (**Figure 7.4D(ii)**). In line with the overall bladder afferent data, the vast majority of mechanosensitive single units (82.9%; 29/35) showed no difference in mechanosensitivity following CCDC instillation compared to saline (**Figure 7.4E(i-ii)**).

LS spinal dorsal horn neuronal pERK-IR induced by *in vivo* bladder distensions following intravesical incubation with CCDC or saline was also determined in *Gpbar1*^{-/-} mice. As with the *ex vivo* bladder afferent preparation, the CCDC-induced enhanced spinal dorsal horn neuronal response to bladder distension was abolished in the *Gpbar1*^{-/-} model, with no difference in the number of LS dorsal horn pERK-IR neurons between CCDC and saline in these mice (**Figure 7.4F**). The pERK-IR neurons activated by bladder distension following intravesical instillation of both saline and CCDC were observed in the same dorsal horn regions as seen in the wildtype, suggesting no differences in spinal bladder afferent signalling pathways in the *Gpbar1*^{-/-} mice (**Figure 7.4G(i-ii)**). Taken together, these results demonstrate that activation of the TGR5 receptor is able to mediate an increase in peripheral and spinal sensitivity to bladder distension indicative of sensory neuron activation.

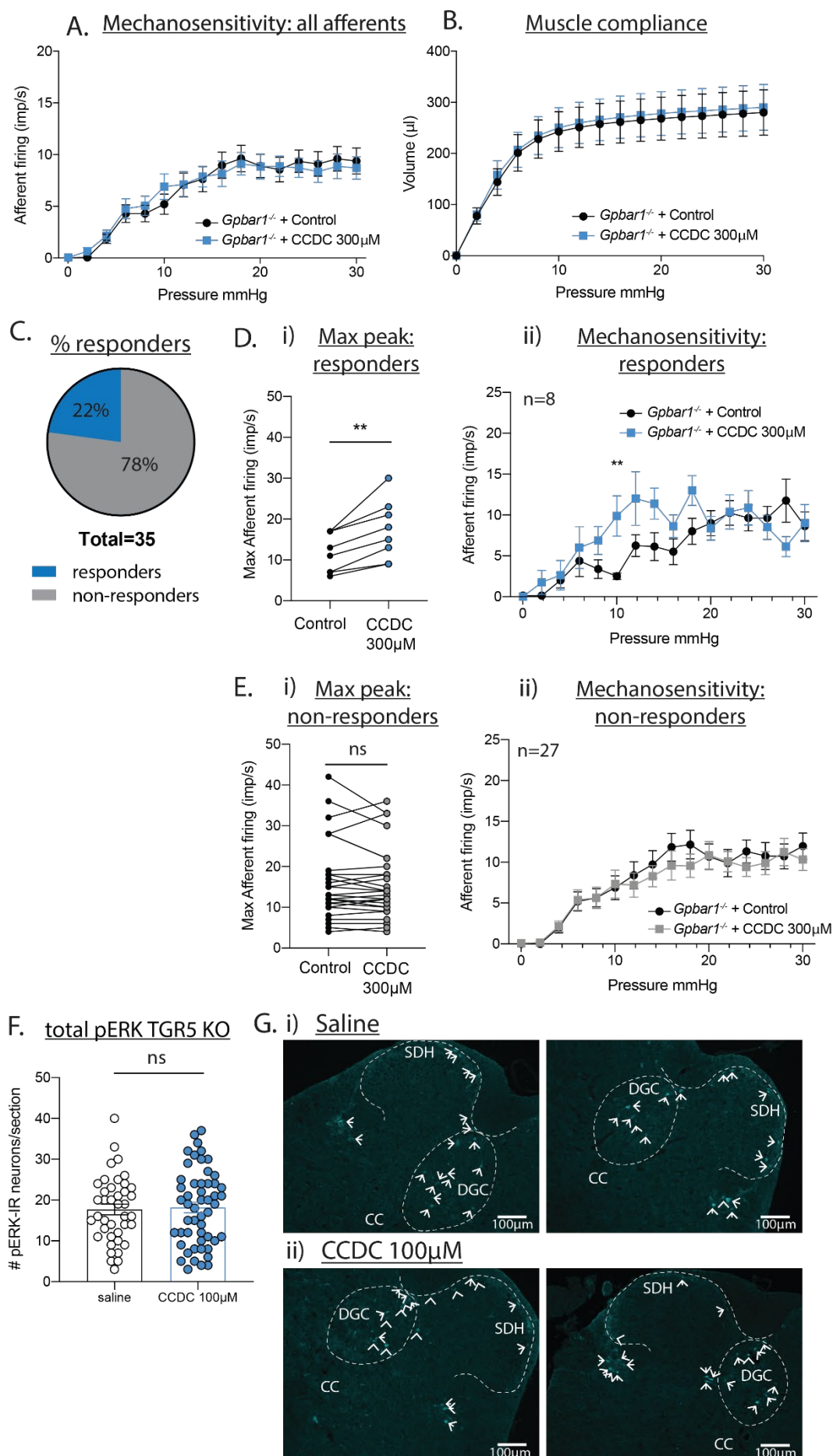


Figure 7.4: CCDC-induced hypersensitivity to distension is abolished in *Gpbar1*^{-/-} mice.

Bladder distensions were performed *in vivo* and *ex vivo* with saline (control) and CCDC in *Gpbar1*^{-/-} mice. *Ex vivo* bladder afferent recordings were analysed and the number of pERK-immunoreactive (pERK-IR) neurons in 10 µm sections of the lumbosacral (LS; L6 and S1) spinal dorsal horn was determined from *in vivo* studies.

(A) No difference in afferent firing (impulses per second; imp/s) was observed following administration of CCDC compared to saline in the *Gpbar1*^{-/-} mice (n = 35 single units from N = 4; ns, P > 0.05). **(B)** The pressure to volume relationship during bladder distension is unchanged following CCDC administration compared to saline, indicating no change in muscle compliance (ns, P > 0.05). **(C)** While the majority of afferents reflected the overall results with no change in afferent firing (29 out of 35; 82.9%; non-responders), 6 out of 35 (17.1%) afferents responded to CCDC with an increase in mechanosensitivity (responders). **(D)** In CCDC-responsive afferents, **(i)** the maximum imp/s following CCDC administration was significantly increased compared to saline in responders (** P < 0.01), and **(ii)** a significant increase in imp/s was observed following CCDC administration overall (** P < 0.01) and specifically at a distension pressure of 10 mmHg (** P < 0.01). **(E)** In non-responding afferents, **(i)** no significant difference was observed between the maximum imp/s of non-responders following administration of CCDC compared to saline (ns, P > 0.05), and **(ii)** no change in imp/s at any distension pressure was observed in non-responders between CCDC and saline distensions (ns, P > 0.05). **(F)** No difference was observed in the number of pERK-IR neurons in the LS dorsal horn per section following bladder distension following intravesical administration of either saline (N = 3) or CCDC (N = 4; ns, P > 0.05). **(G)** Images are representative dorsal horn sections from the L6 and S1 regions from **(i)** saline-treated and **(ii)** CCDC-treated *Gpbar1*^{-/-} mice, with dashed lines to delineate the superficial dorsal horn (SDH) and dorsal grey commissure (DGC), and white arrows pointing to pERK-IR neurons. Data are represented as mean ± SEM **(A, B, D(ii), E(ii), and (F))**, percentage of afferents **(C)**, or maximum imp/s **(D(i) and E(i))**, or total AUC **(C(iii) and D(iii))**. Each dot in **(F)** represents the total number of pERK-IR neurons within a single dorsal horn quadrant from one section. P values above are based on two-way ANOVA **(A, B, D(ii), and E(ii))**, with subsequent Sidak's post hoc test significance at individual data points indicated on **(D(ii))**, Wilcoxon matched-pairs signed rank test **(D(i) and E(i))**, or unpaired t test **(F)**. CC = central canal, SPN = sacral parasympathetic nucleus, LSN = lateral spinal nucleus.

7.4.5 TGR5 is expressed in the bladder and bladder innervating DRG neurons

To begin unravelling the mechanisms through which TGR5 agonists CCDC and Pg5α exert their influence on bladder afferent mechanosensitivity we determined the relative mRNA expression of the TGR5 gene *Gpbar1* within the bladder and bladder innervating sensory neurons. *Gpbar1* mRNA expression was identified throughout the bladder wall; in isolated urothelial cells, the mucosa, and the detrusor smooth muscle (**Figure 7.5A**), as well as in isolated LS (L5-S1) DRG (**Figure 7.5B**). *Gpbar1* expression was abolished in all tissues from *Gpbar1*^{-/-} mice (**Figure 7.5A and 7.5B**), while the relative

abundance of neither *Trpv1* nor *Trpa1* in LS DRG from *Gpbar1*^{-/-} mice was altered compared to wildtype (**Figure 7.5B**).

Transient receptor potential (TRP) channels are common downstream targets for G-protein coupled receptor (GPCR) signalling cascades. Previous studies have identified downstream coupling of irritant-sensing receptors with TRP vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) channels [14, 15, 23, 46, 47]. To assess potential TRP channel coupling with TGR5 in bladder neurons, we used single cell RT-PCR to determine the co-expression of *Gpbar1* with *Trpv1* and *Trpa1*. Single cell QRT-PCR identified *Gpbar1* expression in 13.0% (10/77) of retrogradely traced bladder afferent neurons (**Figure 7.5C(i)**). 84.4% (65/77) and 18.5% (12/77) of bladder-innervating DRG neurons expressed *Trpv1* and *Trpa1* mRNA respectively (**Figure 7.5C(i)**). Of the bladder DRG neurons expressing *Gpbar1* mRNA, 100% co-expressed *Trpv1* and only 30% co-expressed *Trpa1* (**Figure 7.5C(ii)** and **7.5C(iii)**), despite previous functional data in DRG showing distinct coupling of TGR5 with TRPA1 [23]. These data show that mRNA for the TGR5 receptor is present in bladder-innervating DRG neurons and may act uniquely to previously reported DRG neuronal pathways.

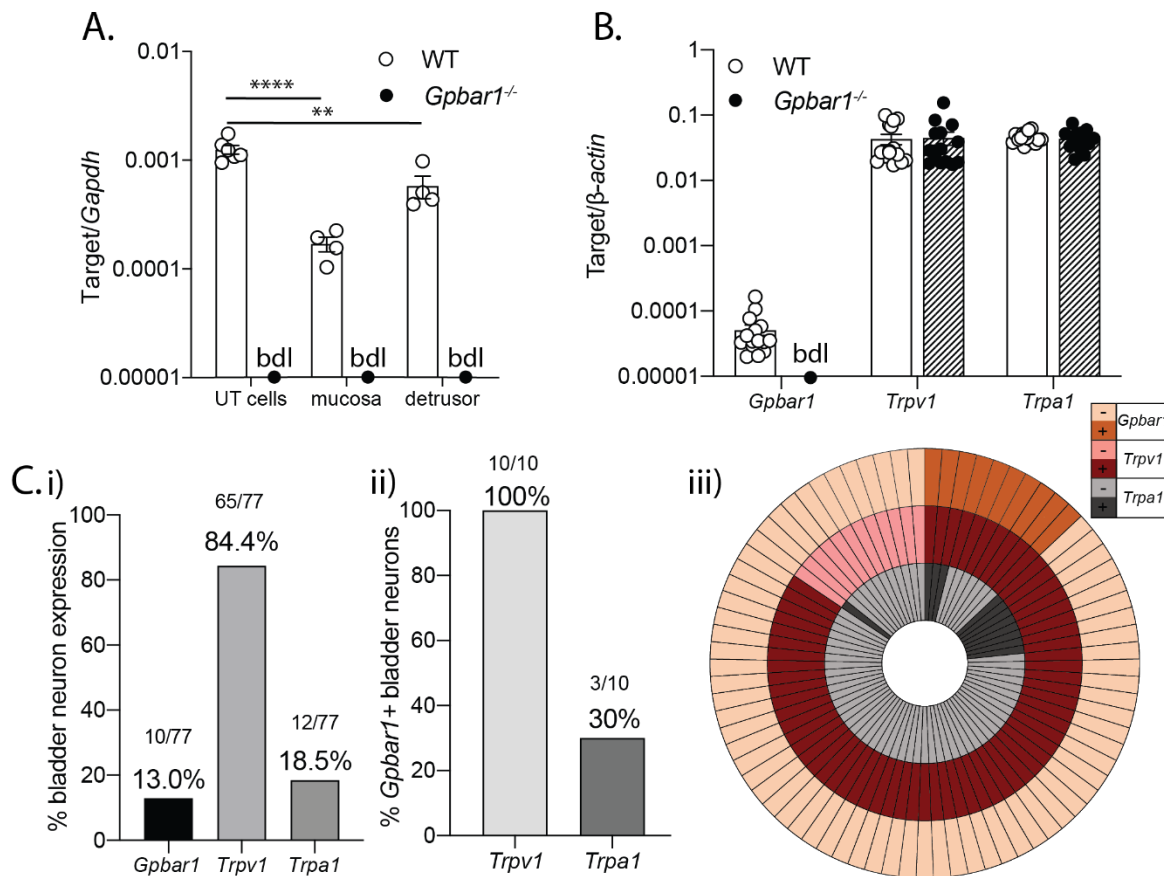


Figure 7.5: Relative mRNA expression in LS DRG, isolated bladder DRG neurons, and bladder tissue layers.

(A) QRT-PCR was used to determine relative abundance of the TGR5 gene *Gpbar1* in urothelial cells, mucosal tissue, and detrusor smooth muscle of the bladder in wildtype and *Gpbar1*^{-/-} mice. *Gpbar1* was expressed in all layers of the wildtype bladder, with relative abundance in the isolated urothelial cells (UT cells; N = 6 mice) significantly higher than in mucosa (N = 4 mice; **** P < 0.0001) and detrusor (N = 4 mice; ** P < 0.01). No significant difference was observed between mucosa and detrusor layers (ns, P = 0.0833). *Gpbar1* mRNA levels were below detectable limits at all layers in the *Gpbar1*^{-/-} mice. (B) Relative mRNA expression of *Gpbar1*, *Trpv1*, and *Trpa1* in lumbosacral (LS) dorsal root ganglia (DRG) in wildtype (WT) and *Gpbar1*^{-/-} mice. *Gpbar1* is expressed in LS DRG from WT mice. *Gpbar1* levels were below the detectable limit (bdl) in *Gpbar1*^{-/-} mice. No difference was observed between wildtype and *Gpbar1*^{-/-} mice for highly expressed neuronal genes *Trpv1* or *Trpa1*. (C) *Gpbar1*, *Trpv1*, and *Trpa1* mRNA expression in isolated, retrogradely traced bladder DRG neurons was determined with single cell RT-PCR. (i) 13% of bladder neurons (10 out of 77 neurons, N = 3) expressed *Gpbar1*, while 84.4% (65 out of 77 neurons) expressed *Trpv1* and 18.5% (12 out of 77 neurons) expressed *Trpa1*. (ii) Of the 10 bladder DRG neurons expressing *Gpbar1*, 100% (10 out of 10) co-expressed *Trpv1*, while 30% (3 out of 10) co-expressed *Trpa1*. (iii) Polar plot depicting the expression profile (positive, dark; negative, pale) of each bladder DRG neuron with respect to *Gpbar1* (outer ring), *Trpv1* (middle ring), and *Trpa1* (inner ring). Data are represented as mean ± SEM (2^{-ΔCt}) on a log10 scale (A, C and D), or percentage of neurons (B(i) and B(ii)). P values above are based on one-way ANOVA with Tukey's multiple comparison test, with *Gpbar1*^{-/-} excluded from analysis.

7.4.6 *In vitro* application of CCDC to bladder afferent DRG neurons increases intracellular Ca^{2+}

To investigate the mechanism of action of neuronal TGR5 in mediating bladder afferent sensitivity, we performed live cell calcium imaging in isolated LS DRG neurons from retrogradely bladder-traced mice. Following direct application of CCDC to the neurons, a significant increase in intracellular Ca^{2+} , indicated by an increase in the Fura-2 fluorescence ratio (**Figure 7.6A(i)** and **7.6A(ii)**), was observed in 58.82% (40/68) of bladder-traced DRG neurons (**Figure 7.6B(i)**). Interestingly, the percentage of bladder-traced neurons responding to CCDC was substantially higher than the percentage on non-traced neurons, of which only 24.65% (126/511) responded (**Figure 7.6B(i)**). The average magnitude of CCDC-induced increases in intracellular Ca^{2+} was also greater in bladder-traced compared to non-traced neurons, as indicated by a significantly higher maximum peak value and area under the curve of response (**Figure 7.6B(ii-iii)**). These results demonstrate the TGR5 agonist CCDC is able to cause direct activation of LS DRG neurons and indicate the possibility that bladder neurons are tuned to evoke greater responses to TGR5 agonists.

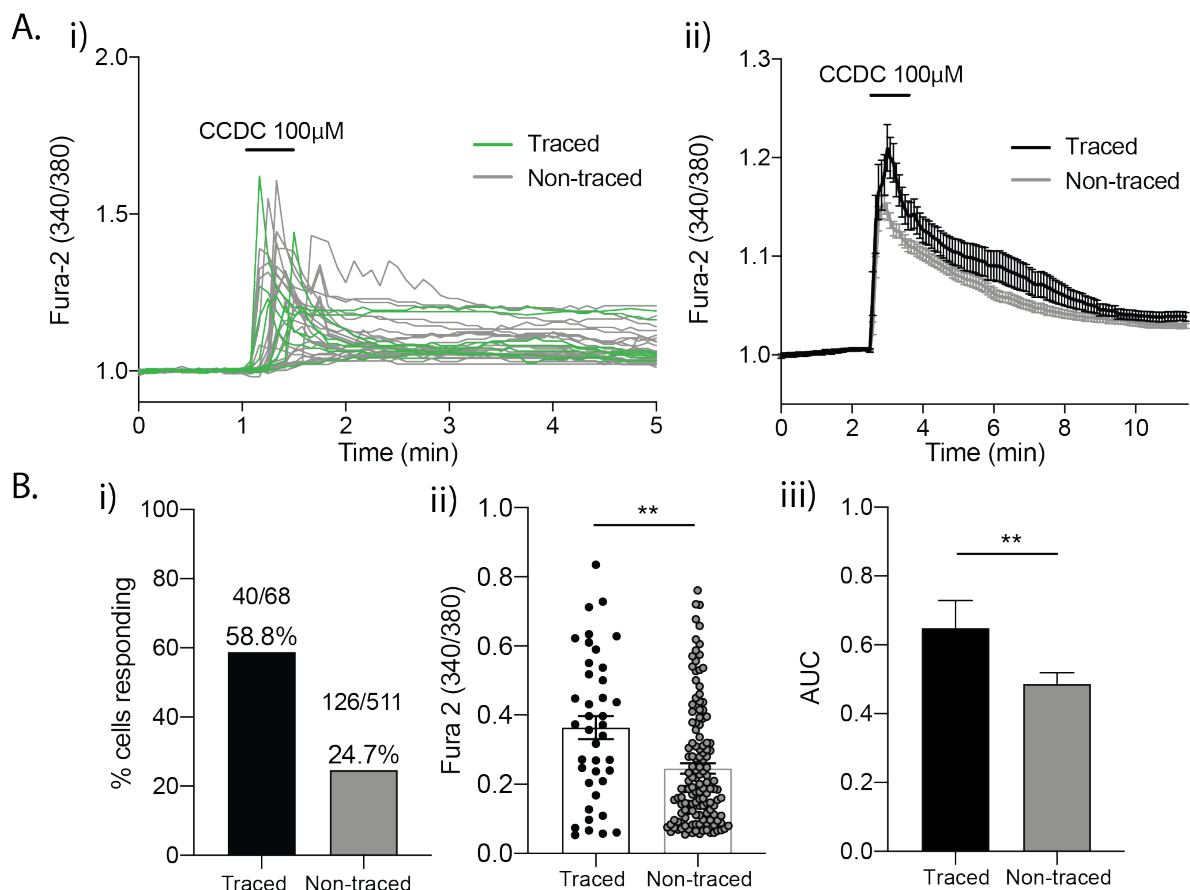


Figure 7.6: *In vitro* activation of TGR5 in primary DRG neurons results in increased intracellular Ca^{2+} .

Live cell calcium imaging was performed on retrogradely traced, isolated LS DRG neurons from wildtype mice to measure intracellular Ca^{2+} changes in response to direct CCDC administration. **(A) (i)** An example experiment showing bladder traced (green) and non-traced (grey) neurons from a single coverslip, with each line representing a single neuron over time and an increase in the Fura-2 fluorescence ratio (340/380) indicating an increase in intracellular Ca^{2+} from baseline normalised to a ratio of 1. **(ii)** Mean $\pm$ SEM of intracellular Ca^{2+} responses to CCDC (100 μ M) in traced ($n = 40$) and non-traced neurons ($n = 126$). **(B) (i)** 58.82% of bladder traced neurons (40 out of 68; $N = 7$) responded to CCDC administration with an increase in intracellular Ca^{2+} , compared to only 24.65% of non-traced neurons (126 out of 511). The response to CCDC for traced and non-traced neurons showed **(ii)** a significantly higher maximum peak (**** $P < 0.0001$) and **(iii)** A significantly larger area under the curve (AUC) (** $P < 0.01$) in traced neurons compared to non-traced.

7.4.7 *In vitro* CCDC-induced increases in intracellular Ca^{2+} in bladder-innervating DRG neurons is partially mediated by TRPV1

Coupling between TGR5 and TRPA1 has been previously demonstrated in cutaneous neurons [23], while our single cell mRNA expression data demonstrated 100% co-expression of *Trpv1* with *Gpbar1* compared to only 30% co-expression of *Trpa1* with *Gpbar1* (**Figure 7.5C(ii)** and **7.5C(iii)**), suggesting a

distinct intracellular signalling pathway in bladder-innervating DRG neurons. To functionally explore potential coupling between TGR5 and TRP channels in bladder afferent DRG neurons *in vitro*, we determined co-activation of TGR5, TRPA1, and TRPV1 in individual neurons with successive administration of CCDC, TRPA1 agonist AITC, and TRPV1 agonist capsaicin using live cell calcium imaging (**Figure 7.7A** and **7.7B**). In line with our single cell co-expression data, 87.5% (21/24) of bladder traced neurons that responded to CCDC also responded to capsaicin. 33.3% (8/24) of CCDC-responsive bladder-traced neurons also responded to AITC (**Figure 7.7B**). Only 16.7% (4/24) of CCDC-responsive bladder-traced neurons were unresponsive to both AITC and capsaicin.

To further explore the potential functional relationships between TGR5 and TRP channels TRPV1 and TRPA1, we measured bladder-traced DRG neuronal responses to CCDC *in vitro* in *Trpv1*^{-/-} and *Trpa1*^{-/-} mouse lines. The percentage of bladder-traced DRG neurons responding to CCDC was reduced by approximately 50% in *Trpv1*^{-/-} mice (27.3%) compared to wildtype mice (58.8%), with no obvious change observed in the *Trpa1*^{-/-} mice (60.9%) compared to wildtype mice (**Figure 7.7C**). The quality of CCDC responses between wildtype and both TRP channel knockout models was also examined (**Figure 7.7D(i-iii)**). While there were no statistical differences in average maximum peak and area under the curve of CCDC responses (**Figure 7.7D(ii)** and **7.7D(iii)**), the few *Trpv1*^{-/-} neurons responding show a trend towards reduced response amplitudes. Overall, these data implicate a role for TRPV1 in mediating TGR5 activity in bladder-innervating DRG neurons.

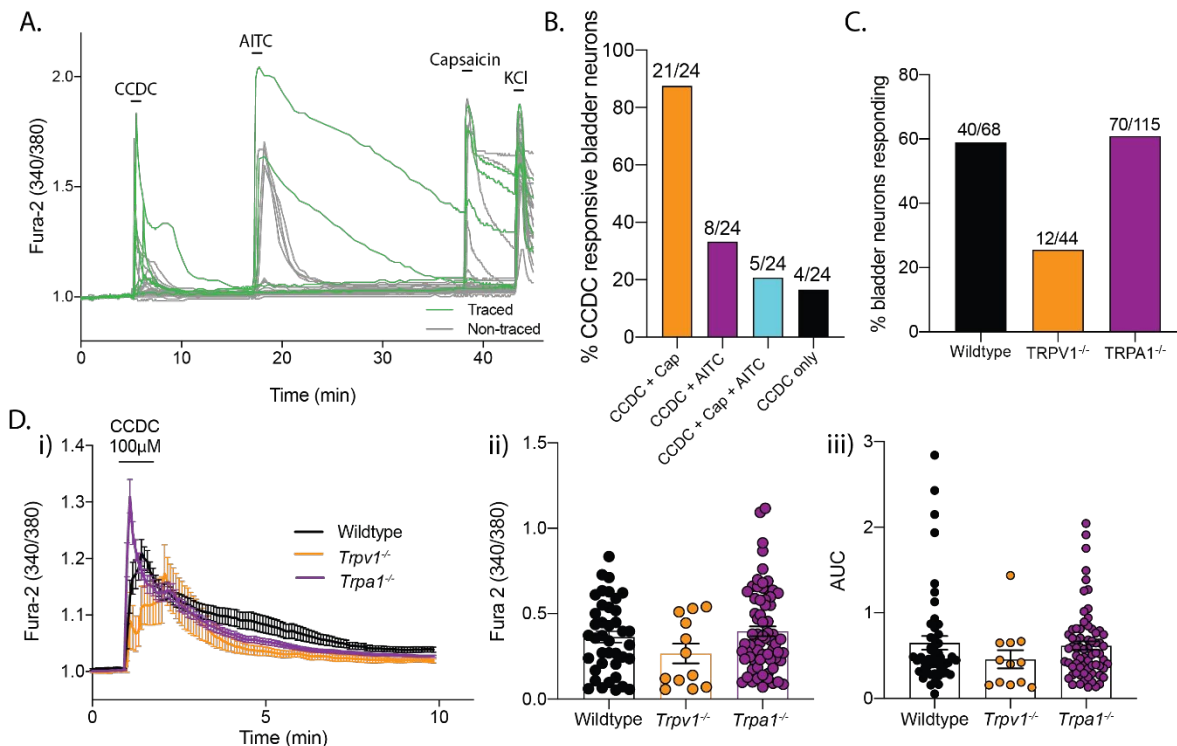


Figure 7.7: Intracellular calcium responses to CCDC in *Trpv1*^{-/-} and *Trpa1*^{-/-} mice.

Live cell calcium imaging was performed on isolated, bladder-traced DRG neurons from WT, *Trpv1*^{-/-} and *Trpa1*^{-/-} mice to determine changes in intracellular Ca²⁺ in response to direct application of CCDC. **(A)** An example experiment showing bladder traced (green) and non-traced (grey) neurons from a single coverslip, with each line representing a single neuron over time and an increase in the Fura-2 fluorescence ratio (340/380) indicating an increase in intracellular Ca²⁺, from baseline normalised to a ratio of 1. TRPA1 agonist AITC and TRPV1 agonist capsaicin were applied in succession following CCDC and washout periods to determine co-activation of neurons. KCl was added at the end of each experiment as a positive neuronal control. **(B)** The percentage of neurons co-responding with CCDC to each compound was determined. Of the CCDC-responsive neurons, (87.5% (21/24)) also responded to capsaicin. 33.3% (8/24) of CCDC-responsive bladder-traced neurons also responded to AITC. Only 16.7% (4/24) of CCDC-responsive bladder-traced neurons were unresponsive to both AITC and Capsaicin. **(C)** The percentage of bladder-traced neurons responding to CCDC in WT, *Trpv1*^{-/-} and *Trpa1*^{-/-} mice. Compared to 58.8% observed in wildtype neurons, only 27.3% of *Trpv1*^{-/-} bladder-traced neurons (12 out of 44; N = 4) responded to CCDC. 60.9% of bladder-traced neurons from *Trpa1*^{-/-} mice responded to CCDC (70 out of 115; N = 5). **(D)** (i) An average response curve for each population was determined from the CCDC-responsive bladder DRG neurons, to visually compare the neuronal responses between wildtype (black), *Trpv1*^{-/-} (orange), and *Trpa1*^{-/-} (purple) bladder neurons. (ii) maximum peak fluorescence and (iii) area under the curve (AUC) of fluorescence were compared across neurons from wildtype (black), *Trpv1*^{-/-} (orange), and *Trpa1*^{-/-} (purple) mice. No significant differences between either *Trpv1*^{-/-} or *Trpa1*^{-/-} neurons and wildtype neurons were observed. Data are represented as percentage of neurons **(A)** or mean ± SEM **(B and C)**. P values were determined using Kruskal-Wallis non-parametric ANOVA tests with subsequent Dunn's post hoc multiple comparisons tests **(C)**.

7.4.8 *In vitro* CCDC-induced increases in intracellular Ca^{2+} in bladder-innervating DRG neurons remain in *Gpbar1*^{-/-} mice

In order to confirm that the *in vitro* effects of CCDC on isolated bladder-innervating DRG neurons were due to activation of the TGR5 receptor we examined CCDC responses firstly in cells isolated from *Gpbar1*^{-/-} mice and subsequently in *Nav1.8Cre-Gpbar1*^{-/-} mice. To our surprise, responses to CCDC remained in neurons from these mice (**Figure 7.8**). The percentage of cells responding to CCDC from *Gpbar1*^{-/-} and *Nav1.8Cre-Gpbar1*^{-/-} mice was roughly equivalent to that from wildtype mice (**Figure 7.8Aiii, 7.8Bi, ii**). The total fluorescent response to CCDC, depicted as the area under the response curve (AUC), was significantly reduced in cells from *Nav1.8Cre-Gpbar1*^{-/-} but not *Gpbar1*^{-/-} mice (**Figure 7.8Aiii, 7.8C**), however, a robust response remained. No differences in the maximum fluorescence in response to CCDC were observed in cells from wildtype, *Gpbar1*^{-/-} and *Nav1.8Cre-Gpbar1*^{-/-} mice (**Figure 7.8D**).

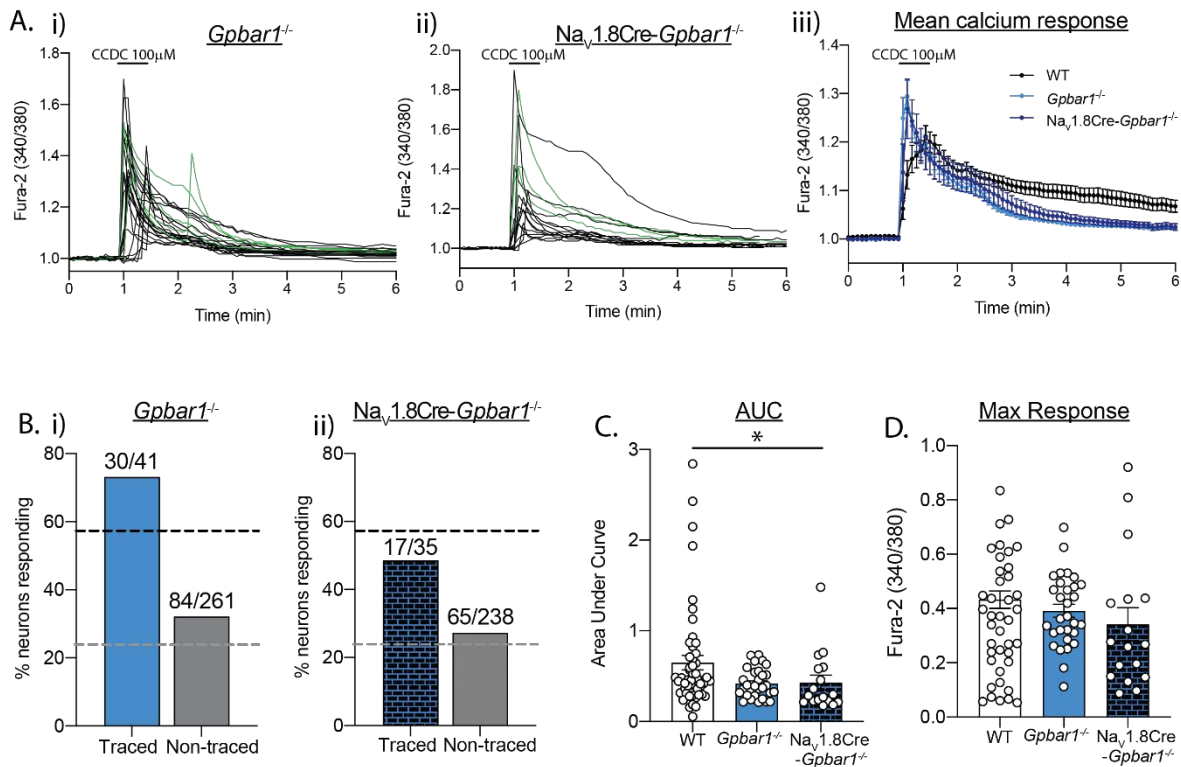


Figure 7.8: Intracellular calcium responses to CCDC in neuronal cultures from *Gpbar1*^{-/-} mice. Intracellular calcium changes were measured in bladder-traced (coloured) and untraced (grey) lumbosacral dorsal root ganglia (LS DRG) neurons from *Gpbar1*^{-/-} and *Nav1.8Cre-Gpbar1*^{-/-} mice in response CCDC. **(A)** Example experiments from **(i)** *Gpbar1*^{-/-} and **(ii)** *Nav1.8Cre-Gpbar1*^{-/-} mice showing bladder traced (green) and non-traced (grey) neurons from a single coverslip responding to CCDC (100 μ M). Each line represents a single neuron and an increase in the Fura-2 fluorescence ratio (340/380) indicates an increase in intracellular Ca^{2+} . **(iii)** Mean $\pm$ SEM of intracellular Ca^{2+} responses to CCDC (100 μ M) in bladder-innervating neurons from Wildtype, *Gpbar1*^{-/-} and *Nav1.8Cre-Gpbar1*^{-/-} mice. **(B)** The percentage (%) of neurons responding to 100 μ M CCDC in *Gpbar1*^{-/-} and *Nav1.8Cre-Gpbar1*^{-/-} mice. **(i)** 73.2% (30 out of 41) of bladder-traced *Gpbar1*^{-/-} neurons, and **(ii)** 48.6% (17 out of 35) of bladder-traced *Nav1.8Cre-Gpbar1*^{-/-} neurons responded to CCDC compared. Dashed black and grey lines represent % bladder-innervating neurons from wildtype mice responding to CCDC. **(C)** The average intracellular calcium change over time (AUC) in response to 100 μ M CCDC was determined for *Gpbar1*^{-/-} (light blue) and *Nav1.8Cre-Gpbar1*^{-/-} (dark blue) bladder-traced LS DRG neurons and compared to wildtype (clear). No statistical difference in the area under the curve (AUC) was observed between wildtype and *Gpbar1*^{-/-} responses (ns; $P > 0.05$), whereas *Nav1.8Cre-Gpbar1*^{-/-} bladder-traced responses had a significantly reduced AUC compared to wildtype (* $P < 0.05$). No significant difference was observed in AUC between *Gpbar1*^{-/-} and *Nav1.8Cre-Gpbar1*^{-/-} responses. **(D)** Maximum fluorescent response to 100 μ M CCDC was not significantly different between wildtype, *Gpbar1*^{-/-}, and *Nav1.8Cre-Gpbar1*^{-/-} bladder-traced neurons ($P > 0.05$). Data are represented as percentage of cells **(B)** or mean $\pm$ SEM **(C, D)** with individual data points in **C** and **D** representing values from individual cells. P values above were determined by Kruskal-Wallis non-parametric analysis of variance with Dunn's multiple comparisons tests **(C, D)**.

7.4.9 CCDC evokes increases in intracellular Ca^{2+} in a small proportion of non-neuronal cells

We have shown that the effects of CCDC in the intact bladder, namely mechanical hypersensitivity of pelvic afferents and spinal cord activation, is prevented in *Gpbar1*^{-/-} mice. Therefore, we questioned whether the *in vitro* effects we observed with CCDC in both *Nav1.8Cre-Gpbar1*^{-/-} and *Gpbar1*^{-/-} mice resulted from additional cell types responding to CCDC that are not present in the bladder (**Figure 7.9**). A small population (3%) of non-neuronal cells from wildtype mice responded to CCDC (**Figure 7.9A, 7.9Bi**). Non-neuronal responses to CCDC were retained in cells from *Gpbar1*^{-/-} and *Nav1.8Cre-Gpbar1*^{-/-} mice (**Figure 7.9Bi**). Furthermore, the area under the response curve (AUC) and the maximum fluorescence in response to CCDC were not significantly reduced in non-neuronal cells from *Gpbar1*^{-/-} or *Nav1.8Cre-Gpbar1*^{-/-} mice (**Figure 7.9Bii, 7.9Biii**).

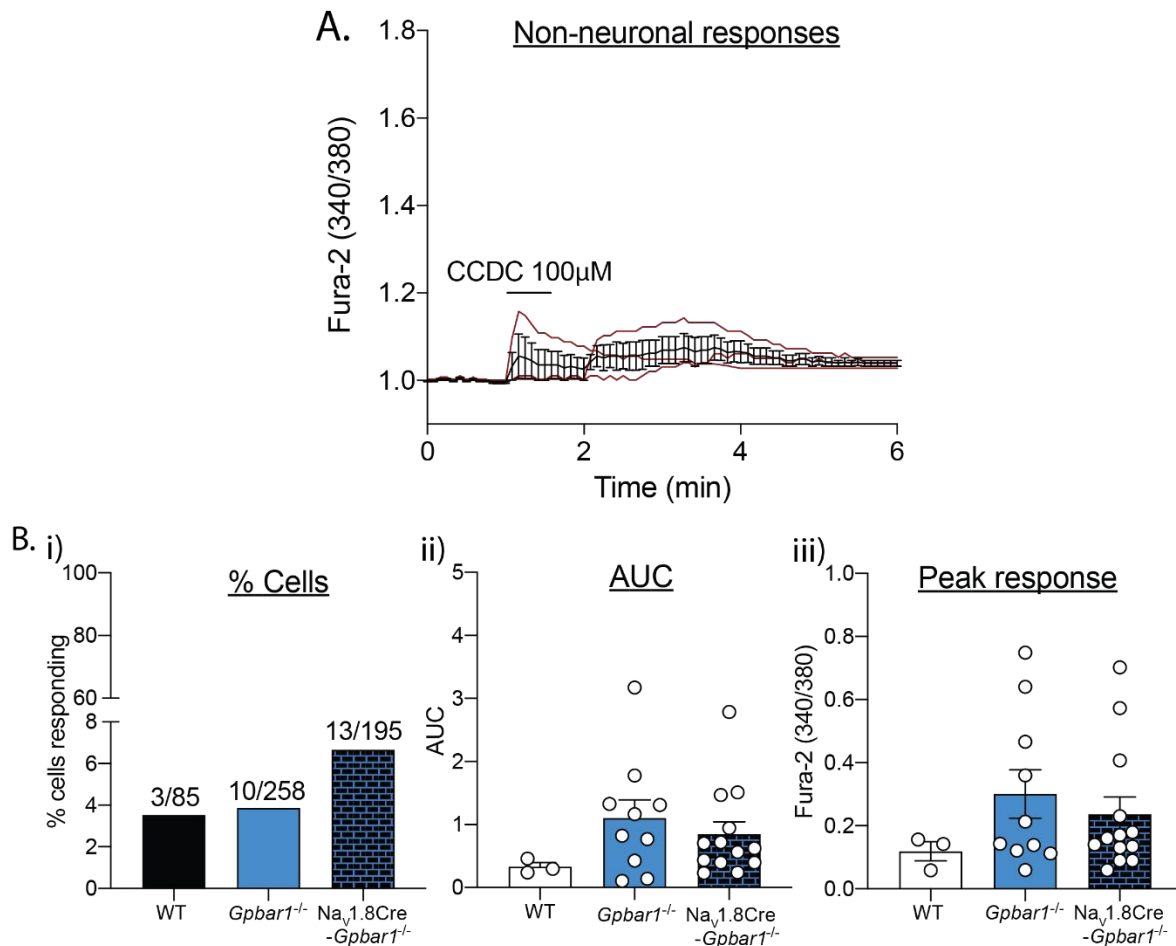


Figure 7.9: Intracellular calcium responses to CCDC in non-neuronal cells from Wildtype, *Gpbar1*^{-/-}, and *Nav1.8Cre-Gpbar1*^{-/-} mice.

Intracellular calcium changes were measured in non-neuronal cells from wildtype (WT; clear) *Gpbar1*^{-/-} (light blue) and *Nav1.8Cre-Gpbar1*^{-/-} (dark blue) mice in response to CCDC (100 μM). **(A)** Mean ± SEM of intracellular Ca²⁺ responses to CCDC (100 μM) in non-neuronal cells from wildtype mice. Red lines represent the three individual responding cells (3 out of 85). **(B) (i)** The percentage (%) of non-neuronal cells responding to 100 μM CCDC. 3.5% (3 out of 85), 3.88% (10 out of 258), and 6.67% (13 out of 195) of non-neuronal cells from wildtype, *Gpbar1*^{-/-}, and *Nav1.8Cre-Gpbar1*^{-/-} respectively responded to CCDC. No significant difference was observed in either **(ii)** the average intracellular calcium response over time or **(iii)** the peak fluorescent response to 100μM CCDC between non-neuronal cells from wildtype, *Gpbar1*^{-/-} (light blue) and *Nav1.8Cre-Gpbar1*^{-/-} (dark blue) mice. Data are represented as percentage of cells **(B(i))** or mean ± SEM **(B(ii)-(iii))**, with individual data points representing values from individual cells. Significance was determined by Kruskal-Wallis non-parametric analysis of variance with Dunn's multiple comparisons tests.

7.5 Discussion

The overlapping symptoms of OAB and IC/BPS, including frequency, urgency, and discomfort, indicate an increase in the sensitivity of bladder afferent neurons to bladder filling. Identifying the receptors and channels involved in bladder afferent neuronal activation, and the impact these have on behavioural responses in states of health and hypersensitivity, is a crucial step to developing efficacious treatment options for patients suffering bladder hypersensitivity disorders. Here, we have identified a role for the bile acid receptor TGR5 in mediating bladder afferent mechanosensitivity *in vivo* and *ex vivo* via intravesically administered progesterone metabolite Pg5 α and the synthetic agonist CCDC.

Intravesical administration of TGR5 agonists resulted in increased afferent sensitivity to bladder distension at both peripheral and spinal levels, a response which we have shown to be TGR5-dependent. The use of an *ex vivo* bladder afferent nerve preparation allowed for identification of mechanosensitive afferents activated by graded distension of the bladder. Endogenous TGR5 agonists, including bile acids and progesterone metabolites, are present at low levels in the urine even in healthy subjects [31, 32]. As such, the increased sensitivity of these mechanosensitive bladder afferents following intravesical administration of the progesterone metabolite Pg5 α demonstrates a potential physiological role for TGR5 agonists in mediating afferent sensitivity. However, in addition to TGR5 [26], progesterone metabolites act on progesterone receptors and GABA_A receptors [48, 49]. CCDC is a synthetic agonist developed to be TGR5-specific [44]. The increase in bladder afferent mechanosensitivity induced by CCDC largely mirrored that of Pg5 α , although the sensitisation was observable from an earlier distension pressure following CCDC administration compared to Pg5 α . Crucially, CCDC-induced bladder afferent hypersensitivity to distension was abolished in *Gpbar1*^{-/-} mice, confirming that CCDC is acting via TGR5 and is therefore a practicable TGR5 agonist. The higher percentage of responding afferents and the increased mechanosensitivity at lower pressures compared to Pg5 α suggests a higher potency, supporting the notion that CCDC is a potent TGR5 agonist. The similar effects seen in response to CCDC compared to Pg5 α suggest that the TGR5 receptor is responsible for the peripheral increase in mechanosensitivity in response to bladder distension induced by the progesterone sulphate, supporting the notion of TGR5 as an irritant-sensing receptor in the bladder.

TGR5 is known to play an important role in smooth muscle relaxation, particularly in the gallbladder and the stomach [50-52]. It is particularly important to note, therefore, that bladder muscle compliance was unchanged following intravesical administration of either Pg5 α or CCDC. In addition to identifying TGR5 expression in bladder-innervating afferent DRG neurons, our mRNA expression results confirm *Gpbar1* expression in all layers of the mouse urinary bladder, building on

a previous study showing expression of TGR5 gene *Gpbar1* mRNA in whole rat bladder [22]. Whether direct application of TGR5 agonists to the detrusor smooth muscle impacts afferent signalling is not measured here. Intravesical administration of agonists emulates the presence of compounds in the urine, which, due to the impermeability of the urothelium, are unable to interact with the detrusor. These results therefore indicate that high levels of progesterone metabolites or other endogenous TGR5 agonists in the urine are able to directly influence bladder afferent mechanosensitivity.

Projections from primary bladder afferents terminate within the lumbosacral spinal dorsal horn [10, 53]. Following peripheral activation, these neurons release neurotransmitters within the dorsal horn which act on second order neurons. Sub-populations of second order spinal neurons involved in bladder sensory pathways have been identified within the superficial dorsal horn, the dorsal grey commissure, and the sacral parasympathetic nuclei [6, 15, 34, 53-57]. These second order neurons relay peripheral bladder sensory input to the brain, thus resulting in autonomic micturition reflexes and cognitive perception [6, 34, 45, 58]. Previously, we have demonstrated that increased peripheral stimulation, via increased bladder distension pressures or intravesical irritant administration, results in increased neuronal activation within the dorsal horn spinal cord regions associated with micturition and nociception [15, 34]. Here, we demonstrate an increase in the number of pERK-IR neurons as a measure of spinal dorsal horn neuronal activation in response to bladder distension following intravesical administration of TGR5 agonist CCDC. This indicates central translation of peripheral afferent hypersensitivity induced by TGR5 agonism.

Our *ex vivo* and *in vivo* data have indicated a physiological role for TGR5 agonists and demonstrated a TGR5-dependent mechanism. We used live cell imaging and gene expression studies in individual bladder-traced DRG neurons to explore the cellular mechanism of TGR5 activity. Our expression data, showing *Gpbar1* mRNA expression in 13% of bladder DRG neurons, is comparable to recent studies in cutaneous and colonic DRG neurons showing 8% and 19% positive for *Gpbar1* mRNA expression respectively [14, 23]. Both studies further analysed co-expression with TRP channels *Trpa1* and *Trpv1*. TRPA1 and TRPV1 ion channels are common downstream targets for GPCR signalling and have both been shown to play a role in mediating itch and pain sensation. Early studies have indicated TRPA1 as the key downstream effector coupled to neuronal TGR5, with both cutaneous and colonic afferent sensitivity to CCDC abolished in a *Trpa1*^{-/-} mouse model [14, 23]. Previous expression data largely supports this, as cutaneous and colonic DRG neurons show high co-expression with both *Trpa1* (100% in cutaneous and 83% in colonic *Gpbar1*-positive neurons) and *Trpv1* (60% in cutaneous and 78% in colonic *Gpbar1*-positive neurons) [14, 23]. Interestingly, we have shown that bladder-innervating *Gpbar1*-expressing DRG neurons are 100% co-expressed with *Trpv1* but only 30% co-expressed with *Trpa1*. Another study assessing *Gpbar1* mRNA expression in DRG neurons selected for small diameter, thus reputedly nociceptive, showed lower associations with both *Trpa1* (41% of

Gpbar1-positive small diameter DRG neurons) and *Trpv1* (32% of *Gpbar1*-positive small diameter DRG neurons) [27]. The high percentage of bladder-innervating DRG neurons expressing *Trpv1* is similar to single cell data in the colon and in small-diameter DRG neurons, while the bladder-innervating *Trpa1* neuronal population is significantly smaller [14, 27].

Our *in vitro* functional data indicate calcium influx associated with TGR5 activation in a population of bladder afferent neurons, supporting previous studies in colonic and cutaneous DRG neurons [14, 23]. Unlike previous studies, significant functional co-expression, as determined by activation of individual neurons by multiple agonists, was observed between TGR5 and TRPV1 with less interaction observed between TGR5 and TRPA1. These data suggest an alternative downstream coupling mechanism for TGR5 to that seen in colonic or cutaneous neurons. The potential associations of *in vitro* CCDC activity with TRPA1 and TRPV1 were further dissected using *Trpa1*^{-/-} and *Trpv1*^{-/-} mouse lines, respectively. Data from these models demonstrate that CCDC-induced activation of bladder afferent neurons is greatly but incompletely inhibited by TRPV1, while no difference was observed in the absence of TRPA1. Together, these data indicate that TGR5 signalling in bladder afferent neurons is partly mediated by TRPV1, a potential downstream effector.

Interestingly, the proportion of bladder neurons responding to CCDC *in vitro* is greater than the single cell gene expression data predicts. Similarly, CCDC responses were retained in neurons from *Gpbar1*^{-/-} mice, a global knockout model, and *Nav1.8Cre-Gpbar1*^{-/-} mice, wherein TGR5 is specifically absent in *Nav1.8*-expressing (sensory) neurons. We additionally observed non-neuronal DRG cells responding to CCDC. It may be that CCDC is able to act via a second receptor *in vitro*, likely absent in the cells of the bladder influencing afferent sensitivity, is as yet undetermined; non-histaminergic irritant-sensing receptors remain a growing class, indeed MRGPRX4 was recently identified as a novel potential bile acid receptor [59]. However, the evident absence of CCDC-induced responses *in vivo* and *ex vivo* presented in this study strongly indicate that the physiological actions of both the synthetic and the endogenous TGR5 agonists are due to activation of TGR5. While *in vitro* studies can offer mechanistic clarity, the physiological relevance of the *ex vivo* and *in vivo* data should not be understated.

Acute application of endogenous and synthetic TGR5 agonists stimulates neuronal hypersensitivity in healthy mice. While concentrations of known endogenous TGR5 agonists are low in the urine in healthy subjects [31, 32] [31, 32], they have been known to increase dramatically under different conditions, including pregnancy and liver disease [60, 61]. How the bile acid or progesterone metabolite urinary content is altered during bladder hypersensitivity is not yet known.

Another important consideration in determining the role for TGR5 in OAB and IC/BPS is the permeability of the urothelium. Increased urothelial permeability is a common finding in IC/BPS in

particular, allowing increased access of urinary contents, including bile acids and progesterone metabolites, to the underlying tissue and nerve endings within the bladder wall [1, 62]. Further studies are required to determine the impact of TGR5 signalling in bladder afferents during states of hypersensitivity, and any changes specific to this pathway including receptor expression and agonist concentrations in the urine and tissue.

In conclusion, this study has identified expression and function of bile acid receptor TGR5 in bladder-innervating afferent DRG neurons. Intravesical administration of TGR5 agonists in the urinary bladder results in TGR5-dependent enhanced mechanosensitivity to bladder distension and increased spinal dorsal horn neuronal activity, indicating a role for TGR5 in mediating the sensitivity of bladder sensory signalling pathways. Further, we have shown that TGR5 activation in bladder-innervating neurons is partially mediated by TRPV1. As an irritant-sensing GPCR now known to mediate bladder mechanosensitivity, TGR5 may represent a potential target for future treatment in conditions of bladder hypersensitivity.

7.6 References

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Chapter 8 Discussion

Chronic bladder conditions such as interstitial cystitis/bladder pain syndrome (IC/BPS) and overactive bladder syndrome (OAB) affect approximately 16% of the western population [1]. The common symptoms of these conditions, including urgency, frequency, and pain or discomfort associated with bladder filling, suggest a heightened sensitivity to bladder distension [1, 2]. Whilst the innervation of the urinary bladder is complex, incorporating spinal and supraspinal reflexes as well as cortical modulation, it is evident that the sensitivity of the peripheral afferent neurons – as the start of the micturition pathway – is critical in determining both sensory and motor responses to bladder filling [2]. The sensitivity of these peripheral bladder afferents is driven by the range of receptors and ion channels expressed that respond to various stimuli [3]. This thesis has identified potential roles for a group of G-protein Coupled Receptors (GPCRs) known to play a role in ‘itch’ or irritant sensation as potential mediators of bladder afferent sensitivity, summarised in **Figure 8.1**.

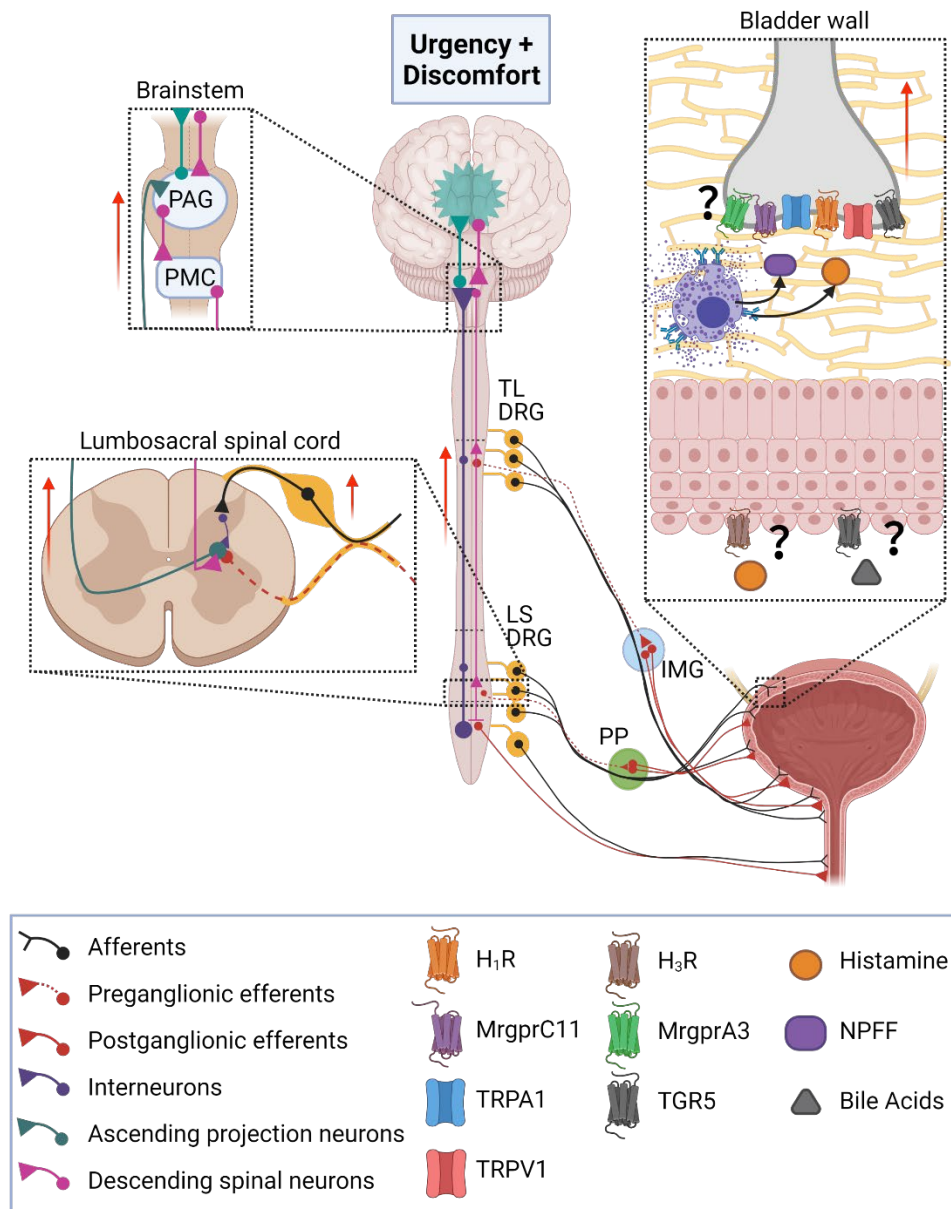


Figure 8.1: Summary of the role of itch-mediating receptors and channels in urinary bladder sensory mechanisms

Histaminergic and non-histaminergic itch-mediating receptors mediate bladder afferent sensitivity. Histamine Receptor subtype 1 (H₁R), Mas-related G-protein couple receptors MrgprA3 and MrgprC11, and bile acid receptor TGR5 are all expressed in bladder afferent neurons. Ion channels transient receptor potential vanilloid 1 (TRPV1) and TRP ankyrin 1 (TRPA1) are also present. Activation of itch-mediating receptors on bladder afferents increases afferent sensitivity to distension (red arrow), resulting in increased signalling to the brainstem and brain via the lumbosacral spinal cord (red arrows), leading to sensations of urgency and fullness, and potentially discomfort. Activated mast cells release histamine and Neuropeptide factor FF (NPFF) which act on histamine receptors and MrgprC11 respectively. H₃R and TGR5 are expressed in urothelial cells; their function here remains unknown however agonists histamine and bile acids are present in the urine. Endogenous agonists for MrgprA3 are currently unknown. PAG: periaqueductal gray. PMC: pontine micturition centre. TL: thoracolumbar. DRG: dorsal root ganglia. LS: lumbosacral. IMG: inferior mesenteric ganglia. PP: pelvic plexus.

Figure created with BioRender.com

8.1 Irritant-sensing receptors play a role in bladder afferent sensitivity

The mechanism of itch, and subsequent scratch reaction, is a form of irritant sensation similar to but distinct from nociception, functioning to remove potentially harmful stimuli [4]. Itch signalling occurs via the interaction of itch-mediating ligands with specific receptors located on sensory nerve terminals in the periphery [5, 6]. In the skin, these nerve terminals are predominantly located within the epidermis, enabling interaction with surface stimuli as well as itch mediators released internally from immune cells [7]. Comparatively in the bladder, nerve terminals present within the mucosa are proximal to both the bladder lumen (via the urothelial cells) and immune cells, although immune cells have also been identified in the detrusor and can therefore interact with both muscular and mucosal bladder afferents [8]. An urge to ‘scratch’ within the bladder seems nonsensical, however an increased micturition frequency may equally function to rapidly remove potentially harmful irritants.

The key aim of this thesis was to determine whether irritant-sensing receptors known to play a role in cutaneous itch are involved in bladder afferent sensitivity. Using a range of expression and functional techniques, we have demonstrated not only expression of ‘itch’ receptors H_1R , $MrgprA3$ and $MrgprC11$, and $TGR5$ in bladder-innervating neurons, but also an increase in peripheral and spinal sensitivity to bladder distension following activation of both histaminergic and non-histaminergic irritant-sensing receptors. Cumulative afferent input into the periaqueductal gray (PAG) of the brainstem leads to both cognitive awareness of bladder sensation and the ‘switch’ from storage to voiding [9]. As such, an increase in afferent sensitivity to distension, consequently increasing sensory signals into the PAG, may lead to sensations of bladder fullness and micturition at lower distension pressures [1], resulting in increased micturition frequency and ultimately removing the ‘irritant’ from the bladder lumen via urinary voiding (**Figure 8.1**).

Supporting this concept, histaminergic and non-histaminergic irritant-sensing receptors have been implicated in airway irritation, with a ‘cough’ response acting to remove irritants in lieu of an itch-scratch response [10, 11], and in the gastrointestinal tract (GIT) [12-14], where an increase in motility is thought to facilitate irritant removal. Within the GIT, these receptors have been linked to irritable bowel syndrome (IBS), a gastrointestinal disorder presenting with symptoms of chronic afferent hypersensitivity [12-14]. The neurological mechanisms initiating the urinary equivalent to the itch-scratch response may underlie or contribute to the symptoms of hypersensitivity observed in OAB and IC/BPS.

Applied in context, irritant-sensing mechanisms would not be expected to play a role in physiological micturition but rather only in the presence of an irritant. Our results support this; the absence or antagonism of the receptors investigated did not significantly alter afferent sensitivity to bladder distension compared to control. Indeed, *Mrgpr-cluster $\Delta^{-/-}$* mice demonstrate no behavioural

deficits or abnormalities aside from altered scratching behaviour reported in previous studies [14, 15]. This suggests that targeting irritant receptors is unlikely to impact physiological activity, thus reducing risk of unwanted side-effects. Additionally, this demonstrates a level of ligand-receptor specificity; the irritant must be present in the bladder to mediate hypersensitivity. Although bladder inflammation precludes OAB diagnosis and is present only in a small cohort of IC/BPS patients [1], the literature discussed in **Chapter 2** and **Chapter 3** supports the presence of irritants, particularly histamine, in the bladder and the urine in conditions of chronic hypersensitivity [16, 17]. Mast cells, as the primary source of histamine in addition to other irritants including MrgprC11 agonist Neuropeptide FF (NPFF), are also present in increased numbers in the bladder of IC/BPS patients [18].

Increased bladder afferent sensitivity has been demonstrated in animal models resulting from long-term neuroplasticity triggered following early life damage, infection, or stress [19, 20] and causing symptoms of increased urinary frequency and bladder sensitivity. This has also been observed in a clinical setting, with IC/BPS diagnoses correlating with a history of recurrent childhood UTIs [21]. This increased neuronal sensitivity equates to a reduced activation threshold, thus resulting in exaggerated responses to stimuli. The reduced activation threshold of bladder afferent neurons in conjunction with the presence of irritants and irritant-releasing immune cells local to the afferents may therefore result in exaggerated activation of irritant-sensing mechanisms. Additionally, alterations to neuronal receptor expression profiles and neuronal sprouting due to neuroinflammation and neuroplasticity [2, 22, 23] may cause an imbalance of irritant-sensing receptor expression and activity, resulting in increased sensitivity and symptoms of urgency, frequency, and discomfort. Certainly, in cutaneous hypersensitivity conditions such as atopic dermatitis, enhanced expression and activation of histaminergic and non-histaminergic itch-mediating receptors has been linked to disease symptoms [24, 25]. In either instance, pharmacological targeting of these receptors presents a potential avenue for reducing bladder activation and therefore mitigating the debilitating symptoms of diseases such as OAB and IC/BPS. To this end, this thesis assessing the impact that each of these irritant-sensing receptors has on bladder afferent sensitivity in the healthy state is a critical step towards determining their contributions to hypersensitivity and their potential as treatment targets.

8.2 Histaminergic irritant signalling in the urinary bladder

Our findings that histamine and histamine receptors are relevant to bladder function are supported by an already extensive body of literature. However, our work uniquely focused on the irritant-signalling function of histamine and histamine receptors in the afferent system of the urinary bladder. The impact of histamine receptor activation on afferent neurons has been identified in other

organs including the skin, the gastrointestinal tract, and the brain [12, 26, 27]. In addition to physiological and pathological itch sensation, histaminergic afferent signalling has been implicated in migraine symptoms and in gastrointestinal hypersensitivity [12, 13, 26]. In demonstrating afferent hypersensitivity to bladder distension following administration of histamine in the bladder, we present a potential mechanism underlying the symptoms of hypersensitivity in IC/BPS and OAB.

8.2.1 Histamine in the bladder results in symptoms of frequency, urgency, and pain

Our results demonstrate that the presence of histamine in the bladder lumen of an otherwise healthy mouse significantly increases afferent sensitivity to distension. Physiologically, this heightened sensitivity to bladder filling implies increased micturition frequency and urgency, thus imitating the symptoms of chronic hypersensitivity conditions. Fully understanding the ramifications of histamine-mediated bladder afferent sensitivity in disease states requires the use of an appropriate disease model. However, the relevance of the present work is strongly supported by the literature, which demonstrates an increase in mast cells and histamine in the bladder, as well as histamine and metabolites in the urine, of IC/BPS patients [16, 18]. The proximity of these mast cells to nerve endings in the bladder [28, 29] highlights the significant exposure of neuronally-expressed receptors to histamine within the disease setting. Taken together, these findings indicate a clear mechanism for exposure of bladder afferents to histamine in the bladder during chronic bladder hypersensitivity, supporting the clinical relevance of our results.

Additional studies support the inferences drawn linking the observed histamine-induced hypersensitivity to symptomology. Mast cell-deficient mice demonstrated attenuated symptoms of frequency, urgency, and pain following induction of IC/BPS [30-33], while pre-emptive degranulation (and hence reduction of available mediators including histamine) also reduced symptoms [34]. Clinically, bladder capacity in humans was inversely correlated to the number of mast cells within the detrusor, indicating a correlation between mast cell numbers and urinary frequency [35].

8.2.2 Histamine receptor subtype 1 (H₁R) mediates bladder afferent hypersensitivity

Using the H₁R antagonist pyrilamine, we were able to demonstrate that the observed histamine-induced afferent hypersensitivity to bladder distension is mediated by H₁R, the principal histamine receptor involved in irritant sensation [5]. Previous studies have also implicated H₁R in bladder hypersensitivity, with increased protein expression of H₁R in IC/BPS patients which, crucially, correlated to symptom severity [36, 37]. A similar mechanism of hypersensitivity has been observed in IBS, whereby H₁R mediated histamine-induced visceral afferent sensitivity, antagonism of which led to a reduction in symptoms [12]. As with IC/BPS, increased mast cell numbers and degranulation, particularly in proximity to nerve endings in the colon, have been identified in IBS and correlated to pain symptoms [38]. Overall, these studies support our results, indicating that increased histamine in

the bladder and the urine during bladder hypersensitivity results in H₁R-mediated afferent sensitivity to distension, leading to symptoms of frequency, urgency, and pain. Whether the increased expression of H₁R in bladder described above reflects any changes to expression specifically within afferent neurons is currently unknown, however this too may contribute to irritant hypersensitivity induced symptoms and is a valuable question for future studies.

8.2.3 Multiple histamine receptor subtypes may be involved in bladder sensation

Whilst H₁R is the primary receptor involved in histaminergic irritant sensation, expression of *Hrh2* and *Hrh3* was also identified in the bladder and the DRG. While our work clearly demonstrates the importance of H₁R, it does not rule out roles for H₂R or H₃R in mediating bladder afferent sensation. Indeed, H₁R and H₂R have been shown to function via cooperative activation, whereby activation of both receptors creates a synergistic rather than additive effect [39, 40]. It is therefore possible that although antagonism of H₁R abolished the histamine-induced hypersensitivity, H₂R and H₃R may yet be involved. Both receptors, along with H₁R, have been previously identified in the bladder [30, 36, 41] and have both been implicated in bladder hypersensitivity symptoms. Antagonism of H₂R or use of a *Hrh2*^{-/-} model attenuated pelvic pain in IC/BPS models, directly implicating H₂R in bladder sensation [30, 42, 43]. Interestingly, neither antagonism nor agonism of H₃R were shown to have any impact on pelvic pain [43], despite increased expression of H₃R in the bladder correlating to symptom severity in human IC/BPS patients [36]. Our results identified significant *Hrh3* expression in the mucosa and urothelial cells compared to the other histamine receptor subtypes. In the brain, H₃R commonly mediates negative feedback, inhibiting release of histamine from neurons [44], whereas H₃R activation in the stomach triggers mucosal production of prostaglandin E₂ (PGE₂) [45], an inflammatory mediator previously linked to frequency and urgency symptoms in the bladder [46]. How H₃R impacts bladder sensation in general, and how either of H₂R and H₃R impact bladder afferent sensitivity, has yet to be determined.

8.3 Non-histaminergic irritant signalling in the urinary bladder

In the skin, both histaminergic and non-histaminergic irritant signalling pathways mediate itch sensation. A role for non-histaminergic irritant signalling in the bladder was identified in this thesis. The presence of Mrgpr-innervating neurons in organs other than the skin in general is quite novel, with only a handful of studies previously identifying non-cutaneous roles [11, 14]. Similarly, the initial identification of TGR5 as an irritant-sensing receptor is recent [47]. As such, our work identifying non-histaminergic irritant signalling mechanisms in the urinary bladder exposes an entirely novel avenue of potential pharmacological targets in the bladder.

8.3.1 Non-histaminergic irritant mediators in the bladder sensitise afferents to distension

The effects observed following intrabladder administration of non-histaminergic receptor agonists, whether targeting MrgprA3 and MrgprC11 or TGR5, were similar to those following addition of histamine, reflecting an increase in afferent sensitivity to bladder distension and thus implicating a role in increased micturition frequency and urgency. Unlike histamine, little is known regarding the endogenous agonists of these non-histaminergic irritant receptors and their presence in the bladder, nor how these may be affected during chronic bladder hypersensitivity conditions.

In addition to a key role in histaminergic signalling, mast cells may be important in non-histaminergic signalling, as MrgprC11 agonist NPFF is an inflammatory mediator released from mast cells [48]. TGR5 may be activated by agonists within the urine, as bile acids as well as pregnenolone metabolites, which may include neurosteroidal TGR5 agonists, are excreted via the urine [49-52]. While endogenous agonists for MrgprA3 are currently unknown, exogenous A3 agonist Chloroquine is also excreted via the urine [53]. Further studies investigating non-histaminergic irritants in the bladder, particularly during conditions of chronic hypersensitivity, may be key to determining the underlying mechanisms, given the impact of receptor activation.

8.3.2 MrgprA3 and MrgprC11 are neuron-specific irritant sensing receptors

Previous studies have demonstrated sensory neuron-specific expression of MrgprA3 and MrgprC11 [54, 55]. Our results effectively support this; while mRNA for *Mrgpra3* and *Mrgprc11* were detected in the bladder, the level of expression is indicative of a single strand of mRNA, likely offering no physiological relevance. Additionally, we and others have demonstrated functional localisation of Mrgprs via direct activation of isolated DRG neurons by Mrgpr agonists [14, 15]. Our work furthers these findings by demonstrating that subsets of DRG neurons innervating the urinary bladder not only express MrgprA3 and MrgprC11, but also respond to MrgprA3 and MrgprC11 agonists. This specific localisation not only confirms that the agonists instilled into the bladder in our studies were acting directly on nerve terminals, but also highlights the potential for these receptors as a targeted treatment option going forward, given that a) physiological activity appears not to be impacted, and b) off-target effects such as excitation or inhibition of the musculature are less likely.

Interestingly, one other study looking at the effects of an Mrgpr agonist in the bladder showed an increase in micturition interval (therefore a decrease in urinary frequency and likely decreased afferent signalling) following administration of BAM8-22 in a bladder hypersensitivity model in rats [56]. This work contrasts our own findings in a healthy mouse model. Importantly, in addition to utilising an alternative animal model, this study focused on measuring inter-contraction intervals and threshold pressure of the bladder wall rather than directly measuring afferent signalling. Whether this conflicting finding results from the use of alternative methodologies and model, or whether

Mrgpr signalling is effectively reversed following instillation of an inflammatory disease model, is unclear. Further investigation is warranted to confirm the effect of Mrgpr activation in the bladder, both in healthy as we have demonstrated, and in disease models.

Species-specific differences are certainly an important consideration for Mrgpr research translatability. Mrgpr subtypes themselves differ between species, and while MrgprD has clear orthologues between mice and humans [55], human Mrgprs are overall less diverse, with a single family (MrgprX) encompassing the MrgprA, MrgprB, and MrgprC rodent subfamilies [57]. Importantly, the agonists utilised in this study, MrgprA3 agonist chloroquine and MrgprC11 agonists NPFF and BAM8-22, have all been shown to also activate human MrgprX1 [15]. As such, the results obtained may be extrapolated to human function in principle, however specific studies investigating MrgprX1 expression and function in human bladder signalling pathways are needed to confirm this.

The activation of MrgprA3 and MrgprC11 in the bladder, as sensory-neuron-specific receptors, results in afferent hypersensitivity to distension likely contributing to increased frequency and urgency in mice. As a novel sensory mechanism in the bladder, much is yet to be determined in this pathway, including how Mrgpr-mediated afferent signalling is altered in disease models and whether this mechanism is replicated following activation of human MrgprX1. The localisation of these receptors to sensory neurons and the limited impact on physiological signalling highlights the potential as treatment targets and thus warrants further investigation.

8.3.3 TGR5 functions as an irritant-sensing receptor in the bladder

To date, data describing TGR5 function is complex. In addition to an irritant-sensing role, both pro- and anti-inflammatory roles have been shown [58-61]. Unlike Mrgprs, TGR5 is broadly expressed across cell types and systems [62-64]; it is likely that TGR5 function is dependent on the cell type and system involved [65]. With respect to the irritant-sensing function of TGR5, our results are largely supported by the literature. TGR5 expressed on DRG neurons was shown to mediate somatic itch (irritant sensation) induced by bile acids [47]. Similarly, colonic itch data demonstrates an irritant-sensing function for TGR5 which results in heightened afferent sensitivity to colonic distention [14]. These data clearly match our own which show a TGR5-dependent mechanism of bladder afferent hypersensitivity, which may underlie sensations of urgency and increased frequency.

The complexity of TGR5 lies in the range of non-neuronal actions. In epithelial cells of the bile duct, activation of TGR5 may activate or inhibit proliferation depending on whether or not the cell is ciliated [66]. This may begin to explain why epithelial TGR5 activation has been shown to delay wound healing in the colon in one mouse study [67] yet induce intestinal proliferation and reduce severity of

colitis in another [68] and also protect the liver from cholestatic-induced injury [69]. We have shown that TGR5 is expressed in the urothelium, however TGR5 function in these epithelial cells has not been determined. While our *ex vivo* and *in vivo* studies suggest any TGR5-induced urothelial activity does not counteract irritant sensation, understanding the effect of TGR5 activation or inhibition in urothelial cells may be a key component to recognising TGR5 action as a whole in bladder function.

TGR5 is also expressed in immune cells including macrophages and glial cells [70-72]. Interestingly, the literature describing immunological TGR5 activity demonstrates a predominantly anti-inflammatory role [60, 73-75]. This includes in a number of chronic inflammatory disorders such as endometriosis, colitis, and arthritis [75-77]. In the brain, TGR5 activation on microglia promoted anti-inflammatory responses, thus acting neuroprotectively and preventing or mitigating neuroinflammation [71, 78, 79]. Whether TGR5 activation in the bladder may mediate distinct actions under inflammatory conditions compared to healthy or even chronic non-inflammatory hypersensitivity conditions is a point of great interest for future research.

The apparent diversity in TGR5 activity adds a layer of complexity to data interpretation. Future studies investigating how inflammatory conditions or urothelial damage affect TGR5-induced bladder afferent signalling are certainly warranted.

8.4 “Pruritogenic” afferent neurons in the bladder

Researchers investigating mechanisms of itch sensation have sought to identify “itch-specific” afferent neuron subtypes. While current evidence does not support such simplistic characterisation, co-expression studies have identified “pruritogenic” polymodal afferent subtypes that express a range of itch-mediating receptors and are therefore predisposed to itch signalling [6, 80]. H₁R, MrgprA3, and MrgprC11 are commonly co-expressed in one neuronal subtype, while MrgprD is expressed in a distinct subset of pruritogenic afferent neurons [7, 15, 57, 80-82]. Where TGR5 fits in these subtypes is largely unknown, although a study of colon-innervating DRG neurons demonstrates significant co-expression (approximately 40%) with both MrgprA3 and MrgprC11 in colon-innervating DRG neurons [14]. Preliminary data from our group investigating co-expression of Mrgprs and TGR5 in bladder-innervating neurons support that of the colonic itch study, demonstrating moderate co-expression of TGR5 with MrgprC11 and MrgprA3, but no co-expression with MrgprD. H₁R co-expression with non-histaminergic itch receptors in bladder neurons cannot currently be confirmed, however we can speculate based on previous co-expression data for “pruritogenic” neurons that TGR5-expression overlaps with the histaminergic subset and not with the MrgprD subset of neurons.

The characterisation of afferent neuronal subtypes according to the range of receptors expressed gives insight into relative excitability and responsivity to a variety of stimuli. Neuronal excitability is determined not by a single receptor-ligand pairing, but by the range of receptors and ion channels expressed. The specificity of the receptors determines what stimuli the neurons respond to, while the interaction and activity of these receptors and channels determine the activation threshold. In the case of bladder afferents, the vast majority are mechanosensitive and therefore respond to bladder distention [83-86]. Modulating the overall sensitivity of these afferents affects how they respond to distension (their activation threshold); a higher threshold may require higher distension pressures to activate the neuron, while lowering the threshold results in neuronal activation at lower distension pressures, as observed during chronic bladder hypersensitivity conditions [1]. Our data, concurrent with the literature, suggests that irritant receptors MrgprA3, MrgprC11, H₁R, and TGR5 are all moderately co-expressed in mechanosensitive “pruritogenic” bladder afferent neurons. Therefore, targeting one or a combination of these receptors in bladder afferent neurons may be of therapeutic benefit in conditions of hypersensitivity, whereby modulating overall neuronal sensitivity may prevent hypersensitivity to bladder distension.

It is noteworthy, however, that our evidence suggests overlap but not complete co-expression. First, the percentages of bladder-innervating neurons expressing each of the receptor subtypes varies, and even MrgprA3 and MrgprC11 are incompletely co-expressed. Second, crucially, application of histamine recruited “silent” afferents, which were previously mechano-insensitive. This effect was not observed in response to non-histaminergic agonists, suggesting that either a) histamine activates receptors not responsive to the non-histaminergic irritants investigated, or b) histamine-induced sensitivity of bladder afferents occurs in a mechanism different to that of non-histaminergic activity. Future studies investigating what role these silent afferents play in chronic hypersensitivity conditions will be of great interest.

8.4.1 Downstream effectors for irritant-sensing receptors in bladder afferents

The development of pharmacological treatments targeting receptors relies on a thorough understanding of the intracellular signalling mechanisms involved. For this reason, we investigated the roles of TRPA1 and TRPV1 as likely downstream ion channels in irritant receptor-mediated bladder afferent signalling. In cutaneous and colonic itch studies, H₁R acts via downstream TRPV1 activity and not TRPA1 [12, 87], whereas nonhistaminergic receptors MrgprA3, MrgprC11, and TGR5 all act via TRPA1 and not TRPV1 [14, 88, 89]. In comparison, we identified TRPV1 as the primary downstream effector in both histaminergic and non-histaminergic irritant signalling in bladder afferents. The uniformity in downstream signalling of irritant receptors may be attributed to the biphasic

requirements of the urinary bladder; sensory variability from the bladder is limited, therefore greater nuanced signalling is unnecessary. We also identified that the vast majority (84%) of bladder afferents expressed TRPV1, supporting previous literature [90-92], whereas TRPA1 was far less abundant. Further, we, along with others previously [93], showed that mechanosensitivity of bladder afferents is reduced in *Trpv1*^{-/-} compared to Wildtype mice, highlighting the importance of TRPV1 signalling in bladder activity.

8.5 Future directions

The discovery of histaminergic and non-histaminergic irritant-sensing afferent mechanisms in the bladder is novel and opens the field to a wealth of potential opportunity. A natural and critical progression is to determine the changes in expression and function of irritant receptors in bladder afferent neurons in a model of chronic hypersensitivity. This has already been investigated in the colon, utilising a chronic visceral hypersensitivity (CVH) model to emulate irritable bowel syndrome [14]. In this CVH model, increased expression of *Mrgprc11* in colon-innervating DRG neurons was observed as well as a greater increase in mechanosensitivity in response to MrgprA3 and MrgprC11 or TGR5 agonists. Further, TGR5 activation in CVH mice resulted in recruitment of silent afferents, which we observed only following histamine application in the bladder.

The importance of the urothelium in mediating bladder afferent signalling is clear. We have determined expression of irritant receptors, particularly *Hrh3* but also *Gpbar1*, in urothelial cells. Both H₃R and TGR5 have demonstrated complex activation profiles depending on the model and state of activation [65, 94, 95]; their respective roles in the urothelium may be important in regulating irritant-sensing mechanisms. These, too, may be impacted by disease models. In particular, the concept of increased urothelial permeability as a potential mechanism underlying OAB and IC/BPS suggests important urothelial changes, and this may include altered urothelial signalling.

At the central end of the peripheral afferent signalling arm, the role of satellite and glial cells in modulating afferent activity has recently come to light, particularly in relation to chronic pain and itch [96-98]. TGR5 has been identified in satellite and glial cells [71, 72, 99], and we too observed TGR5-mediated activation of non-neuronal DRG cells. Although an interesting finding, this work has not identified the purpose for TGR5 activation in these non-neuronal cells nor how this may impact bladder afferent sensitivity. Plasticity in glial cells and neuro-glial interactions during inflammatory states have been linked to chronic pain [98], and therefore investigating glial changes in the irritant-sensing afferent pathways may provide further clues as to bladder afferent hypersensitivity in OAB and IC/BPS.

8.6 Concluding remarks

To conclude; in this work we have focused on a range of GPCRs hitherto unknown to influence bladder afferent sensitivity. Histaminergic and non-histaminergic irritant-sensing or “itch” receptors are expressed and functional in the afferent pathways innervating the urinary bladder. Activation of these receptors in bladder afferent neurons results in enhanced mechanosensitivity which may cause sensations of urinary urgency and frequency at lower distension pressures. Physiologically, this response feasibly acts to remove potentially harmful irritants from the bladder in lieu of a “scratch” response. Further, we have shown that targeting these receptors is unlikely to impact physiological micturition reflexes, altering afferent sensitivity only in the presence of the irritant. As a result, these newly identified bladder irritant-sensing receptors represent a novel family of potential pharmaceutical targets for chronic bladder hypersensitivity conditions including OAB and IC/BPS.

8.7 References

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