Investigating afferent mechanisms involved in bladder hypersensitivity

PhD Thesis

Natasja Barki

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Department of biomedical science
University of Sheffield
Western Bank, Sheffield, S10 2TN
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Table of contents

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Summary of thesis

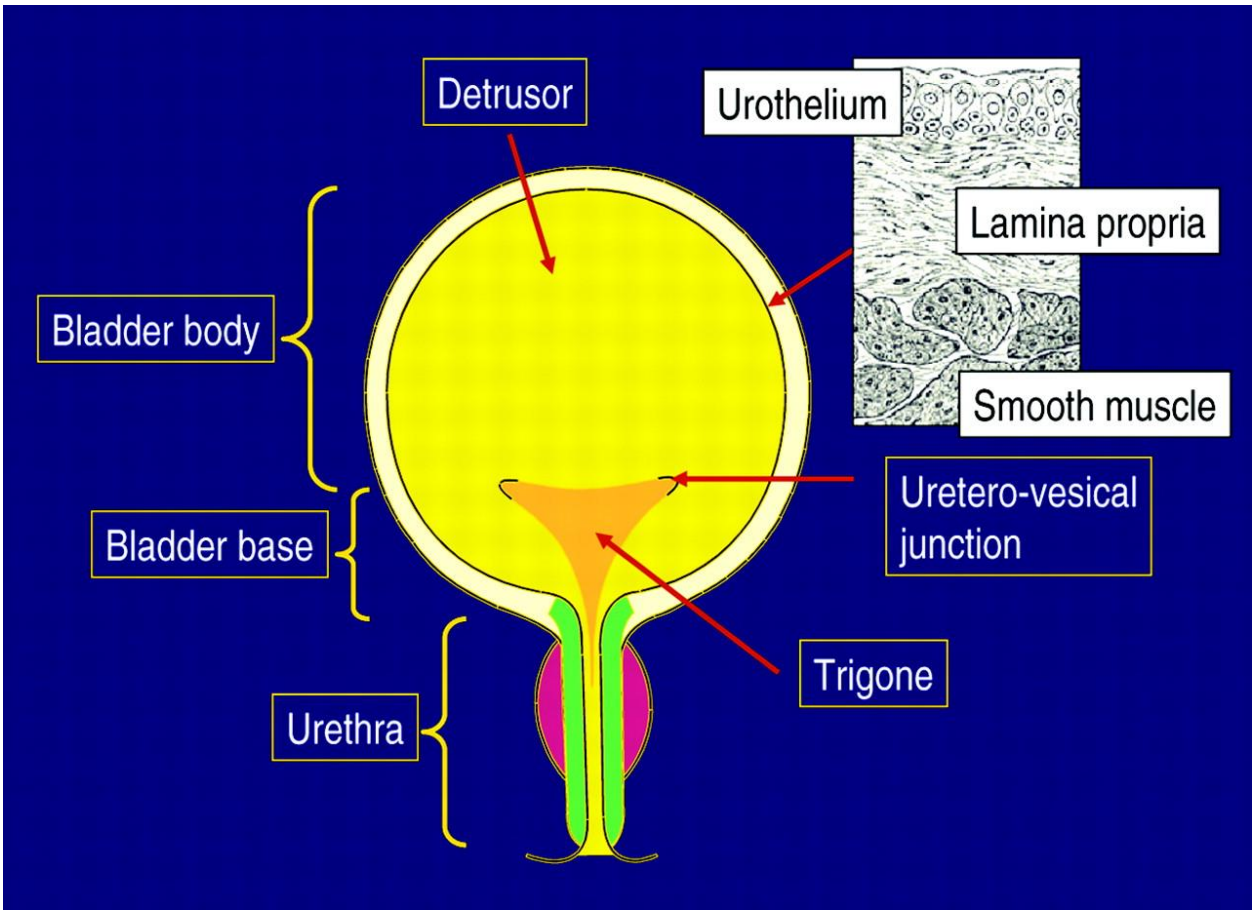
This thesis investigates the afferent mechanism that may be involved in bladder hypersensitivity. The Study primarily focuses on the role of TRPM8 on bladder sensory firing. One part of the study also aims to replicate the ice water test in an in vitro mouse model to assess the contribution of TRPM8. An interesting aspect that the study investigates is the interaction between TRPM8 and TRPV1, TRPA1 and purinergic signalling. Finally, the study investigates ERβ KO mice as a model for interstitial cystitis. Investigating these parameters may reveal more information of the sensory changes that may occur in relation to bladder hypersensitivity, hence revealing novel targets for therapy.

CHAPTER1

GENERAL INTRODUCTION

1.1 Bladder Anatomy

The bladder is a hollow organ with strong muscular walls characterised by its ability to distend. The bladder functions to store and void urine that has been excreted by the kidneys. The human bladder accommodates approximately 300ml-500ml, however has a volume capacity of 1 litre of urine. Storage of urine happens at low pressure, while emptying involves a synchronised contraction of the bladder and relaxation of the urethra. During its emptying phase a healthy bladder is able to contract and expel its contents quickly and completely (Zachoval *et al.,* 2000). When the bladder is empty it is located in the lower region of the pelvis, inferior to the peritoneum and lying slightly superior to the pubic bones. The position and shape of the bladder differ under the pressure of its own contents and neighbouring organs. Ligaments stabilise the position of the bladder (Birder *et al.,* 2007). The bladder itself can be divided into 3 distinct regions: (1) **the dome**, which is comprised of the detrusor muscle. (2) **The trigone,** which is a smooth triangular area attached by its uppermost corners to the ureteral orifices and at the base by the internal urethral orifices. (3)**The bladder neck** is the lower part of the bladder, which surrounds the urethra.

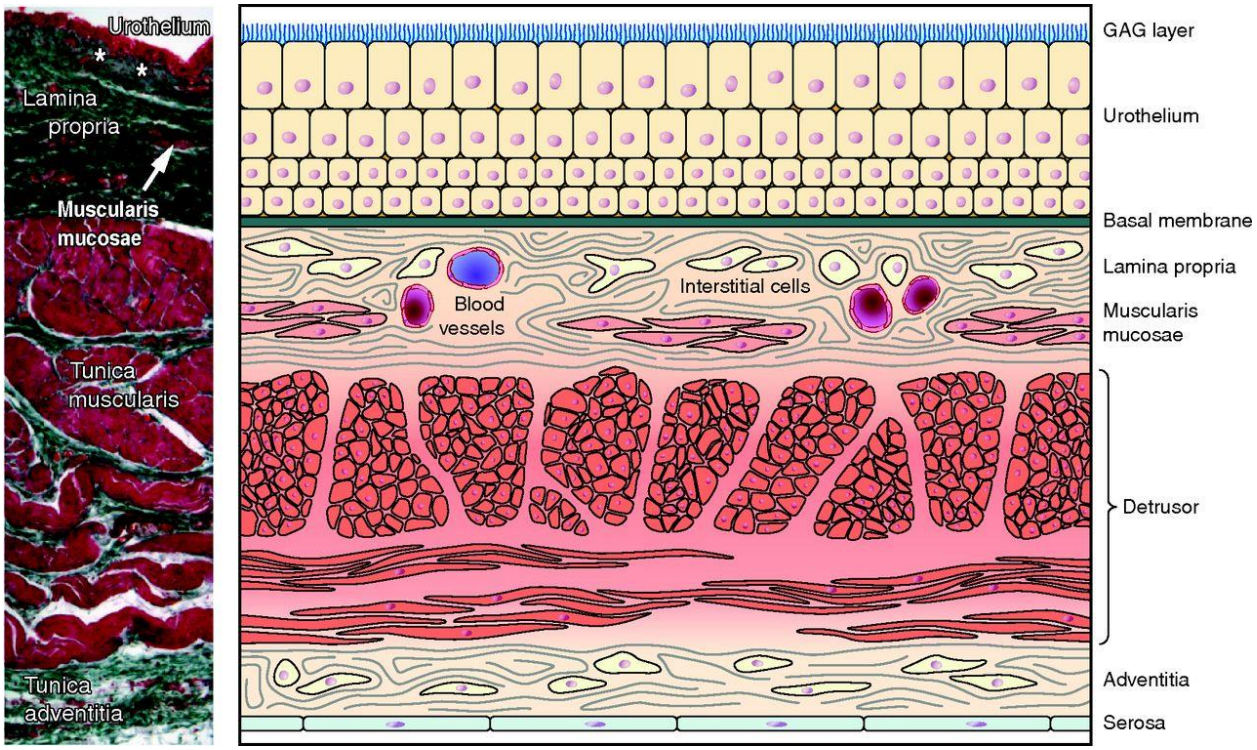


(Andersson and Arner, 2004)

Figure 1.1 Schematic diagram of the bladder showing the distinct regions.

1.1.1 Bladder histology

The mucous membrane lines the bladder and is covered partially by peritoneal serosa. Following the serosa, is the smooth muscle layer, which consists of the detrusor muscle (Gray, 1995). It is composed of three layers, (1) inner longitudinal, (2) middle circular or spiral; and (3) outer longitudinal fibres. Following the muscle layer is the lamina propria. It is a layer of loose connective tissue that connects the musculature layer with the epithelial layer known as the urothelium (Birder *et al.*, 2010).



(Birder and Andersson., 2013)

Figure 1.2: Cross section of the bladder

1.1.2 The urethra

The urethra is a tube that extends from the bladder neck to the exterior of the body. The male urethra is approximately 20cm long and is divided into four main regions pre-prostatic (intra-luminal part of the urethra), prostatic (crosses through the prostate gland), membranous (passes through the external urethral sphincter), and the penile urethra, with only the first three contributing to urinary continence. There is a small percentage of cholinergic nerves present on the urethral muscle, however it is densely innervated by noradrenergic nerves (Gosling *et al.*, 1999). Contrary to the male urethra, the female urethra is approximately 3cm long and has a much more uniform structure. It is fused with the anterior wall of the vagina and terminates between the clitoris and the vagina. The urethra of both males and females is lined by the urothelium, which plays a role in function (discussed later in detail) (Booth *et al.*, 1983; Martini, 2001). Somatic and autonomic nerves control the functions of the urethra (Brading *et al,* 1999).

1.2 Urothelium

The bladder urothelium is an epithelial lining of the lower urinary tract, between the renal pelvis and the urethra. Because the cell layers appear to change shape as the bladder stretches to accommodate the increasing volume of urine, the urothelium is also known as the ’transitional’ epithelia. The main functions of the urothelium include, providing physical protection and controlling transportation of solutes (Birder *et al*., 2010).

The urothelium consists of a minimum of three distinct layers (the exact number depends upon the species): a basal cell layer (~10µm diameter cells), which is bound to a basement membrane, an intermediate layer, (10-25µm diameter cells) and a external apical layer made from large specialised polyhedral cells also known as "umbrella cells" (25-250µm diameter). It has been suggested that the umbrella cells and intermediate cells may have projections to the basement membrane (Apodaca, 2004, Birder *et al* 2004 & Lewis, 2000).

1.2.1 Barrier function

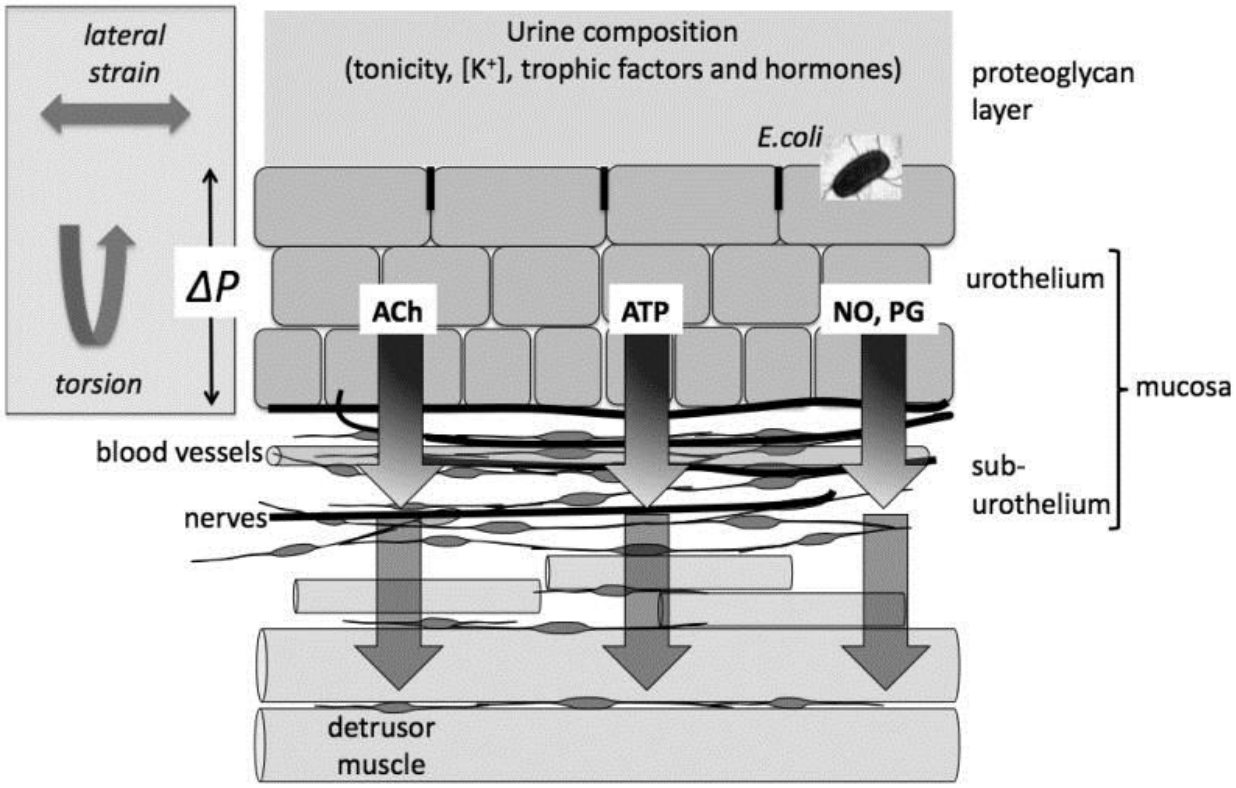
Many features of the umbrella cells allow the bladder to maintain its barrier function. These key features consist of tight junction complexes, such as, many cytoplasmic and transmembrane proteins that decrease the transfer of solutes between cells (Lewis, 2000). Moreover, specialised molecules of lipid and uroplakin proteins (cover approximately 90% of the urothelial cell surface) in the apical cell membrane decrease cellular permeability to small molecules (i.e. urea and water) (Birder *et al.,* 2010, Apodaca *et al.,* 2004). Another feature that protects the barrier function is the mucin layer that lines the surface of the urothelium. This mucin layer is composed of sulphated polysaccharide glycosaminoglycan (GAG) and is suggested to function as a non-specific anti-adherence factor, providing protection against infection (Parsons *et al.,* 1979). Moreover, during filling of the bladder when the umbrella cells change shape, they become flat and squamous and this transformation is complemented by vesicular trafficking, adding membrane to the apical surface and enlarging the overall surface area of the bladder (Balestreire *et al*., 2007 and Cheng *et al*., 2002). During

bladder filling, the urothelium accommodates by changing its morphology. It is thought that the change in shape results from the intermediate and basal cells being pushed laterally (Hicks *et al.*, 1975). The umbrella cells change from roughly cuboidal morphology to a more flat and squamous shape during bladder filling. It is thought that the change in morphology is accompanied by discoidal or fusiform vesicle exocytosis (Apodaca, 2004 and Hwang *et al*., 2005). This increases surface area of the umbrella cell, hence increasing the overall surface area of the bladder, allowing the bladder to accommodate increased volumes of urine (Truschel *et al.*, 2002 and Wang *et al.*, 2003). During emptying of the bladder, endocytosis occurs of the added apical membrane. Alternatively, it is also mentioned that the change in shape of umbrella cells is achieved by folding or unfolding of the apical plasma membrane (Koss *et al.*, 1969). These processes permit the bladder to adjust to large volume of urine during storage while maintaining the barrier function (Apodaca *et al.*, 2007 and Birder *et al.*, 2007). If the barrier function is compromised due to inflammation or injury, toxic substances can move into underlying tissue. This can result in urinary frequency, urgency and even pain during voiding, as seen in interstitial cystitis (discussed later in detail) (Apodaca, 2004 and Birder, 2004)

1.2.2 Neuron like properties of the urothelium

Many reports have reported that urothelial cells display various mechanoreceptive and nociceptive characteristics. The urothelium expresses a range of receptors and ion channels, normally found on sensory nerves. These include, bradykinin (Chopra *et al*., 2005), trkA and p75 (Murray *et al.*, 2004), purinergic receptors (Birder *et al.*, 2004,

Lee *et al.,* 2000 and Tempest *et al.,* 2004), adrenergic receptors (Birder *et al.,* 1998 and Birder *et al*., 2002), muscarinic and nicotinic receptors (Beckel *et al.,* 2006 and Chess-Williams 2002 *et al*., 2002), protease-activated receptors (D'Andrea *et al.,* 2003), Na channels (ENaC) (Carattino *et al.,* 2005, Lewis *et al*., 1985, Smith *et al.,* 1998 and Araki *et al*., 2004) and a range of transient receptor potential (TRP) channels (Birder *et al*., 2001, Birder *et al*., 2002, Birder *et al.,* 2007 Stein *et al.,* 2004 and Streng *et al.,* 2008). The urothelium has also been reported to release various neurotransmitters in response to mechanical stimulation, including ATP *(*Ferguson *et al.,* 1997), Ach (Yoshida *et al.,* 2006), nitric oxide (NO) (Birder *et al.,* 1998) and prostaglandins (Khan *et al.,* 1998; Masunaga *et al.,* 2006). These properties, together with the close proximity of the urothelium with sub- urothelial afferent nerves innervating the bladder, suggests interaction between both the structures, hence together forming a complete sensory structure of its own. It needs to be mentioned that the expression profile data varies among researchers. Moreover, the urothelium is thought to convey information about changes in intravesical pressure to the afferent nerves, resulting in the inhibition of sodium channels and the subsequent release of ATP (Ferguson *et al.,* 1997). Release of ATP causes stimulation of P2X receptors present on bladder afferents. P2X2/3 deletion studies have shown an increase in bladder capacity and a reduction in voiding frequency (Vlaskovska *et al.,* 2001, Rong *et al.,* 2002 and Cockayne *et al.,* 2005).



(Birder *et al*., 2012).

Figure 1.3 schematic diagrams showing the release of various neurotransmitters from the urothelium. The neurotransmitters may act on the afferent nerves lying in the sub-urothelial plexus or the detrusor muscle

1.2.3 Urothelium and bladder tone

In vitro studies have reported that the urothelium can interact with bladder smooth muscle cells through the release of acetylcholine and ATP from the urothelium. This release can occur in response to stretch, and results in the modulation of smooth muscle contraction. The release of NO has also been postulated to play a role in bladder tone (Birder *et al*., 1998). This is in line with the identification of NOs in the urothelium (Birder *et al.*, 2002) and on the bladder smooth muscle (James *et al.*, 1993). Moreover, bladder tone changes have also been reported after electrical field stimulation (James *et al.*, 1993) and photo induction of NO (Chung *et al*., 1996). However, evidence that NO plays a significant role in detrusor relaxation or bladder compliance is lacking.

Apart from stimulatory substances, the urothelium has also been reported to release mediators that have an inhibitory effect. These substances are thought to influence muscle contraction and may possibly influence afferent nerve signalling. Examples of such mediators include nitric oxide, prostaglandins and adenine nucleotides.

Studies in various animal species have shown that urothelium denuded muscle strips show increased agonist induced contractions (Levin *et al.*, 1995). Hawthorn *et al.*, (2000) also reported mediator release of fusible inhibitory mediators from the urothelium of the pig bladder. Augmentation has also been observed in denuded rat muscle strips in response to carbachol (Kosan *et al.*, 2005). Additionally, administration of ATP increased carbachol-induced contraction in muscle strips with intact urothelium. These responses are comparable to urothelium denuded strips, and hence indicate a role of purinergic signalling in this process (Santoso *et al.*, 2010). The factor is now known as **'the urothelium derived inhibitory factor'** (UDIF), the

release of which results in ~50% reduction in muscle contraction (Hawthorn *et al*., 2000; Templeman *et al.*, 2002). However, a role for this mediator in the physiological/pathophysiology of the bladder remains unclear.

1.2.4 Interstitial cells (ICs) in the bladder

Smett and Jonavicius first described interstitial cells of the bladder in (1996). These cells have now been reported throughout the bladder wall (Davidson and McCloskey, 2005) of the human, guinea pig, rabbit and mouse (Smet *et al.*, 1996; Klemm *et al.*, 1999; Pezzone *et al.*, 2003; Van der Aa *et al.*, 2003; Lagou *et al*., 2006). The identification of bladder ICs has acquired a lot of interest, due to similarities with ICCs (interstitial cells of Cajal) in the gut (Ward & Sanders, 2001 and Hirst & Edwards, 2004). Within the gut, ICCs function as pacemakers, driving the peristaltic movement of the gut. Studies conducted on bladder ICs have revealed certain structural and functional properties that indicate functional significance of these cell types in the bladder. However, current knowledge on the functional aspect of these cells is limited. It has been suggested that in the detrusor muscle, ICs might be involved in driving smooth muscle contraction (Sanders, 1996). However, isolated smooth muscle cells have been shown to generate spontaneous electrical activity (Montgomery & Fry, 1992, Karkanis *et al*., 2003, Hashitani *et al*., 2004 and Sui *et al.*, 2004), contradicting the above statement. In the sub-urothelium the ICs are interconnected by gap junctions, and may play a role in modulating bladder sensation. The close proximity of these ICs with the afferent terminals also suggests a possible influence of these ICs in the interaction between afferents and urothelium. The finding that ATP can stimulate

bladder ICs (Sui *et al.*, 2004) is in line with this theory, since the urothelium has been shown to release ATP in response to stretch (Ferguson *et al.*, 1997). However, evidence for a physiological role of bladder ICs is limited.

1.3 Bladder innervations

1.3.1 Generation of action potential

Investigating electrophysiological properties are important to gain a better understanding of the physiological function of neurons. The release of stimulus evoked neurotransmitters requires the generation of action potentials in many neurons. The generation of these action potentials is mainly controlled by voltage gated sodium, potassium and calcium channels. Influx of sodium into the cell via voltage gated sodium channels (down a concentration gradient) results in membrane depolarisation. Once the depolarisation reaches a threshold level an action potential is generated. This influx in turn activates more sodium channels in a positive feedback mechanism. Although the membrane is still depolarized, a separate inactivation process results in the reduction of sodium influx. Outflux of potassium ions via voltage-dependent and independent potassium channels results in the repolarisation phase. The after hyperpolarisation phase is then generated by voltage dependent potassium channels to allow the recovery of the sodium channels and reset them for the generation of the next action potential.

The function of the lower urinary tract is to store and release urine in a controlled manner and this depends on the integration of somatic and autonomic efferent and afferent mechanisms that coordinate the activity of the bladder and urethra. Sympathetic and parasympathetic nerves arise from distinct lumbar and sacral regions of the spinal cord respectively. Hypogastric and pelvic nerves convey autonomic

innervation to the bladder and somatic innervation of the external urethra is conveyed via the pudendal nerves. Postganglionic autonomic nerves fibres innervating the bladder are broadly classified as either adrenergic (sympathetic) or cholinergic (parasympathetic) fibres.

1.3.2 Efferent projection

Parasympathetic pre-ganglionic neurones innervate the intermediolateral grey matter of the sacral spinal cord (S2-S4 laminae v-vii) and travel via the pelvic nerve to the major pelvic plexus. The axons terminate at the pelvic plexus, or the ganglia located in the detrusor muscle.

(Sullivan and Yalla, 2002). Nicotinic cholinergic pathways mediate synaptic transmission in these intramural ganglia. Furthermore, the nicotinic cholinergic pathways can be influenced by various other receptors, including adrenergic, muscarinic and purinergic receptors (De Groat & Booth, 1980). The bladder dome and urethra is densely innervated by postganglionic neurons, which release neurotransmitters such as acetylcholine, hence providing excitatory input to the bladder and causing bladder contraction through muscarinic receptors.

Sympathetic preganglionic neurons pathways innervate the intermediolateral horn and dorsal gray commissure at the T11- L2 spinal level. The axons project from the spinal cord to the lumbosacral sympathetic chain ganglia and the major pelvic ganglion or to adrenergic neurones innervating the bladder and urethra (de Groat, 1997). It has been reported that some fibres from the sympathetic chain travel along with parasympathetic pre-ganglionic neurones to the pelvic nerve (de Groat & Booth,

1980). Sympathetic postganglionic nerves release noradrenaline causing the urethra to contract via the activation of α-adrenoceptors and the bladder body to relax through β-adrenoceptors (Andersson & Arner, 2004).

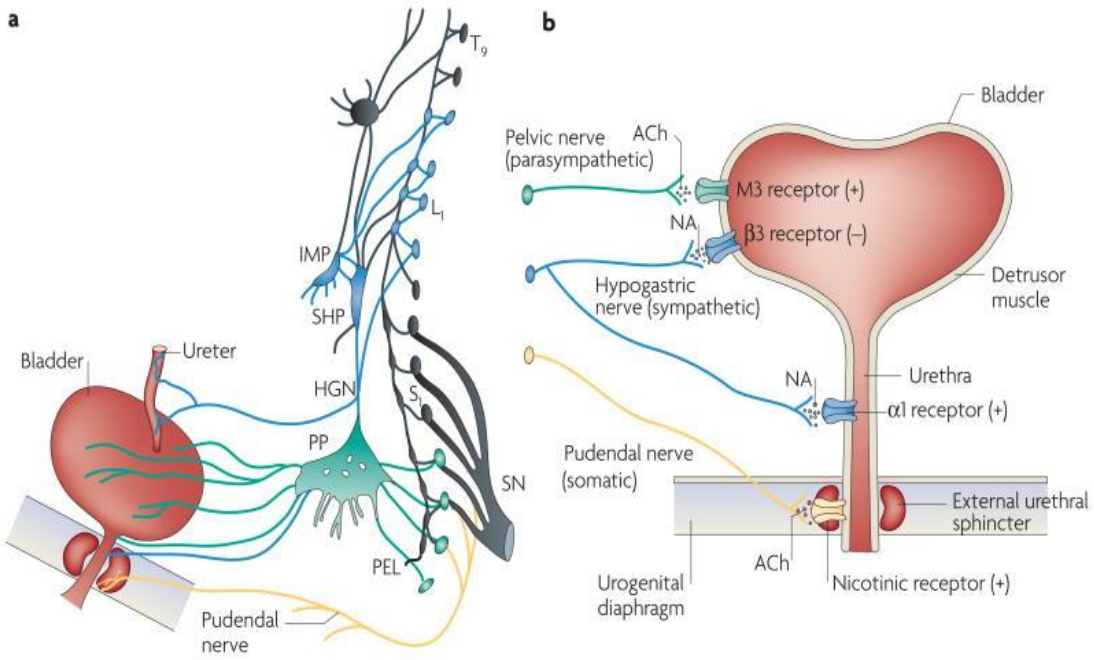
The somatic pathway is carried from Onufs nucleus in the ventral horn of the spinal cord (T11-L2) via the pudendal nerve to the urethra, resulting in muscle contraction through the release of acetylcholine (de Groat *et al*., 2001).

1.3.3 Bladder ganglions

Various intramural ganglia have been reported to be present within the bladder of human, guinea pig, rabbit, rat and mouse (Gabella, 1990, Xu *et al*., 2008 and Gillespie *et al.*, 2006). The pelvic plexus is innervated by both parasympathetic and sympathetic ganglion neurons, some of which synapse at this site (De Groat & Booth, 1993). Nicotinic receptors are present on the cell bodies of intramural nerves and mediate the transmissions between pre-ganglionic and post ganglionic fibres.

1.3.4 Afferent projections

Bladder afferents are essential in controlling voiding reflexes. They carry information from the bladder to the central nervous system about visceral sensation (Yoshimura *et al.*, 2008). Afferent projections travel from the bladder in pelvic and hypogastric nerves to lumbrosacral spinal cord (Morrison., 1999 and Weaver 1985). Sensory input from the bladder neck and urethra travels in the hypogastric and pudenal nerves (Fowler *et al*., 2008). In humans, the cell bodies of the pelvic and pudenal nerves are in dorsal root ganglia (DRGs) in the sacrum (S2-S4) and cell bodies of the hypogastric nerve in DRGs are located in the thoracolumbar region (T11-L2). From the DRGs the afferent projections travel in the posterolateral tract to second order neurons. These neurons then convey information to the pontine micturition centre, hence initiating the micturition reflex (Blok *et al*., 1997). Within the bladder the axons of these afferents have been found in the epithelium, along the muscularis, in the serosa and on blood vessels supplying the bladder (Sharkey *et al*., 1983 and Su *et al*., 1986). Innervations of the mucosa has the highest density of afferent fibres, forming a plexus in the sub-urothelial layer (Gabella, 1990). The muscle is innervated by with long axons, running parallel to the smooth muscle bundles. The density of this innervation is uniform throughout the bladder.



(Fowler *et al*., 2008)

Figure 1.4 Innervations of the lower urinary tract**.**

1.3.5 Classifying bladder afferents

The bladder afferent fibres consist of small myelinated (Aδ) and unmyelinated (C) fibres, with a conduction velocity of ~11.0m/s and ~1.7 m/s respectively in the rat (Sengupta & Gebhart, 1994) (conduction velocity less than 1.3 m second^{-1}) (Morrison *et al.*, 1999). Aδ fibres are mainly present in the detrusor muscle, and carry information regarding alteration of detrusor muscle tone, that occurs during bladder filling. Unmyelinated sensory C fibres are located mainly in the detrusor muscle, with close proximity to the urothelium in the lamina propria (Wakabayashi *et al*., 1993). It has been suggested that C fibres might transmit information regarding changes in bladder volume (Morrison *et al.*, 1999). Approximately 90% of C fibres are termed silent, since they are unresponsive in normal physiological states (Habler *et al.*, 2003). These silent receptors respond to noxious stimuli.

1.3.6 Low and High threshold afferents

Studies conducted in cats, guinea pigs and rats have further classified these fibres into low and high threshold. Low threshold bladder afferents respond to intravesical pressures below ~15mmHg, whereas high threshold afferents respond to pressures above 15mmHg (Habler *et al.*, 1990, 1993[a], Sengupta and Gebhart, 1994). Both Aδ- and C fibres have been reported to respond to both high and low thresholds. This observation suggests that there is no link between conduction velocity and type of receptor (Sengupta and Gebhart, 1994c, Su *et al*., 1997 and Shea *et al*., 2000). Bladder afferents innervating the mouse and rat bladder have been reported to display

spontaneous activity (Sengupta & Gebhart, 1994 and Daly *et al.*, 2007) However, other studies have contradicted the above findings (Shea *et al*., 2000; Rong *et al.*, 2002).

1.3.7 Direct and indirect afferent mechanism

Afferent activation mechanisms have been categorized into direct and indirect. Direct activation of afferents occurs through stimulation of mechano- and osmolarity receptors present on nerve endings. The indirect activation of afferents results from mediators released from non-neuronal cells in response to mechanical stimulation. An example of indirect activation is the activation of purinergic receptors present on bladder afferents through ATP that has been released from urothelial cells in response to stretch.

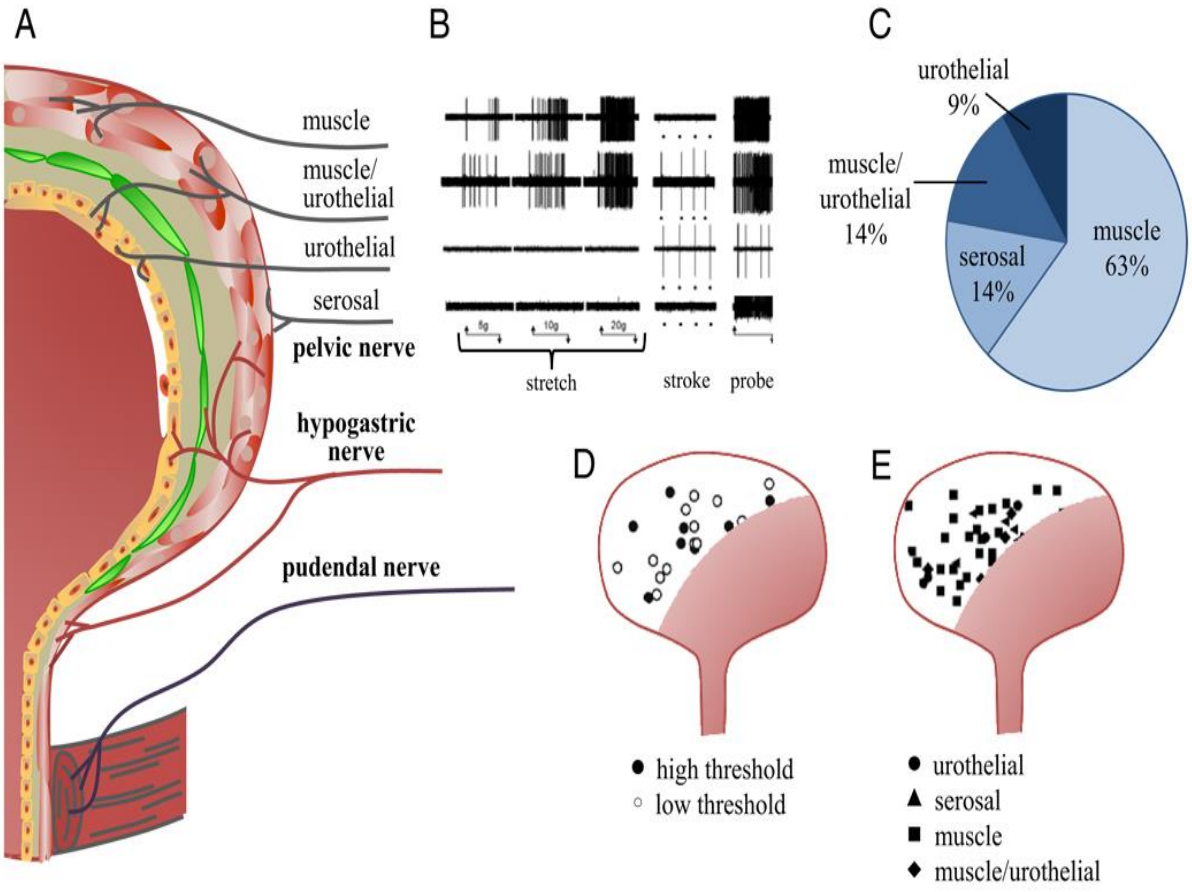
1.3.8 Stretch sensitive, stretch insensitive and silent receptors

Bladder afferents neurons have been separated into different classes. **1)** stretch-sensitive mechanoreceptors, *In vivo* studies have identified both low threshold and high threshold stretch sensitive bladder afferents (Habler *et al*., 1990, Sengupta and Gebhart, 1994 and Shea *et al.*, 2000). Low threshold fibres mainly play a role in the control of micturition, whereas high threshold fibres are thought to be involved in pain sensation (de Groat *et al,* 1997). **2)** chemo-receptors, which are stretch-insensitive afferent neurons, are activated by isotonic potassium chloride but not sodium chloride (Moss *et al.,* 1997). **3)** Around 30% of bladder afferents have been termed "silent afferents", due to lack of response to distension or chemical stimuli. These silent

afferents however could become mechanosensitive during an acute inflammation (Janig and Koltzenburg, 1993).

Two groups have further classified these receptors into either; mucosal, muscle-mucosal, muscle and serosal. *Xu et al* (2007), defined bladder afferents by their activation mechanism, using stretch, probing and stroking of mouse bladder afferents. A separate group conducted a similar study, with the addition of a chemical stimulation and also characterized these afferents into different functional groups in guinea pig, Muscle, muscle mucosal, mucosal high threshold and mucosal low threshold mechanoreceptors (Zagorodnyuk et al., 2007). There is a clear overlap in the classification of these groups, showing consistency of data. These afferents could be activated by compression of their receptive fields with von Frey hairs in the presence of nicardipine. It was reported that muscle-mucosal mechanoreceptors, could be activated both by distension and by stroking the overlying mucosa with light von Frey hairs. Mucosal high-responding mechanoreceptors were not sensitive to stretch but responded vigorously to mucosal stroking with light von Frey hairs. Mucosal low-responding mechanoreceptors were not distension sensitive but could be weakly activated by light von Frey hair stroking of their receptive field (Zagorodnyuk *et al*., 2007).

The spinal cord receives sensory information from the lower urinary tract via primary afferent neurons. The cell bodies of sensory neurons are in the DRG, which project nerve fibres (axons) to the bladder, where sensory receptors covert chemical thermal and mechanical energies they also project axons to the spinal cord, where there central endings are terminated on second-order neurons, resulting in perceptions of bladder events (Andersson, 2002 and Bielefeldt *et al.*, 2005).



(Kanai and Andersson, 2010)

Figure 1.5 Shows the different classes of afferents and their distribution throughout the bladder.

The afferent neurons of the bladder are mainly made up of myelinated (A-δ) and un-myelinated (C-fibres) axons that act in response to chemical and mechanical stimuli. It has been shown by immunochemical studies that bladder afferent neurons produce various putative neurotransmitters, including, glutamic acid, nitric oxide, aspartic acid and neuropeptides. Several receptors and ion channels are also expressed by the afferent neurons, including oestrogen receptors, transient receptor potential channels, purinergic and neurotrophic factor. Neuronal excitability has been shown to be enhanced by the activation of many of these receptors. Moreover, afferent nerves have been shown to act in response to chemicals present in urine in addition to chemicals released from the bladder wall from nerves, and epithelial cells that line the bladder lumen. Pathological conditions change the electrical and chemical properties of afferent pathways of the bladder**,** resulting in various symptoms such as urinary urgency, urinary frequency, nocturia and pain. Neurotrophic factors have been suggested to play a role in the pathophysiological mechanisms causing sensitization of bladder afferent nerves (De Groat *et al.,* 2009). Sensory neurons represent a possible target for bladder overactivity. Bladder overactivity affects around 17% of the people in USA and Europe (Milsom *et al*., 2001 and *Stewart et al.,* 2003). However, it is still unclear how many different classes of sensory neurons play a role in signalling bladder function.

1.4 The micturition reflex

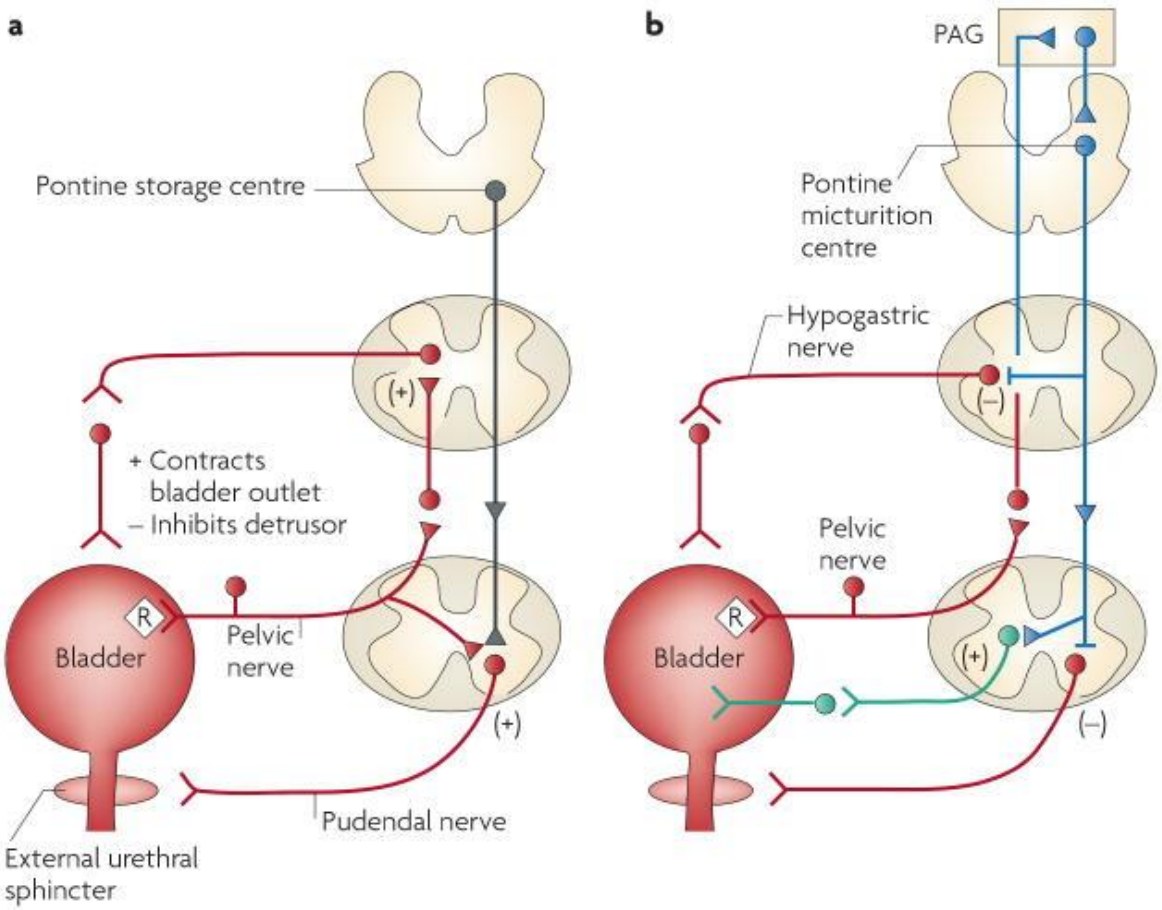
The process of micturition has two phases, a storage phase and a voiding phase. The exchanges between these two phases are coordinated and have been described as an 'on-off circuit' between the bladder (reservoir) and urethra (outlet) (Fry *et al.* 2010). These phases are under voluntary control of the central nervous system (de Groat, 1997).

1.4.1 The storage phase

During the filling phase, urine travels from the ureters into the bladder, without a significant increase in intravesical pressure. The intrinsic properties of the muscle wall (Tang & Ruch, 1955) and low-level afferent discharge have been reported to accommodate the urine in the bladder (de Groat, 2006). Although the sympathetic input to the lower urinary tract is not essential for the performance of micturition, it does contribute to the storage function of the bladder (De Groat *et al*., 1997).

The sensory afferent discharge stimulates the sympathetic pathway, leading to the release of noradrenaline and relaxation of the smooth muscle through activation of β-adrenoreceptors. Noradrenaline also results in contraction of the urethral sphincter through activation of α-adrenoreceptors on the urethral outlet (Thor and de Groat, 2010). As the bladder continuous to fill, there is a gradual increase in intravesical pressure, resulting in increased afferent firing and hence the sympathetic firing increases causing a negative feedback mechanism. After a threshold of sensory afferent firing in reached, there is inhibition of the sympathetic and somatic pathways with activation of the parasympathetic pathway, transferring the storage phase into a

voiding phase. This happens voluntarily in healthy adults and involuntarily in the infant. The input to the urethral sphincter is inhibited, resulting in relaxation of muscle via the release of nitric oxide from the parasympathetic nerves (Persson & Andersson, 1992). Simultaneously, bladder contraction occurs via the activation of muscarinic receptors through the release of Ach from the parasympathetic nerves present in the dome and body of the bladder.



(Fowler *et al.*, 2008)

Figure 1.6. The neuronal pathways controlling the micturition reflex.

1.5 Overactive bladder

Overactive bladder (OAB) is a condition characterised by symptoms of urinary urgency, with or without urinary incontinence, usually with urinary frequency and nocturia (Abrams *et al.,* 2002). In 2003, epidemiological studies have reported a prevalence of 16.9% in adults in western Europe and the USA (Stewart *et al.*, 2003). OAB is also defined as detrusor overactivity (DO). DO is a bladder filling disorder and has been defined as involuntary detrusor contractions (either spontaneous or provoked) during bladder filling (Abrams *et al.*, 2002).

Overactive bladder syndrome can be classified as neurogenic or idiopathic. Idiopathic bladder overactivity arises from no recognizable dysfunction. Approximately 90% of women with OAB fall into this category (Kleeman *et al*., 2008). Neurogenic detrusor overactivity is bladder overactivity resulting from neurological condition, such as spinal cord injury, cerebro-vascular accident and multiple sclerosis, leading to the alteration of neural pathways that control micturition. Neurogenic OAB can be further categorised into supraspinal, suprasacral or infrasacral lesions (Simpson *et al*., 1997). Supraspinal OAB results from damage above the pontine micturition centre. This results in detrusor overactivity together with normal voiding. Suprasacral results from lesion above the sacral part of the spinal cord. This results in loss of input to higher centres, causing neurogenic detrusor overactivity concurrent with bladder sphincter dyssynergia. The loss of input from the pontine micturition centre results in uncoordinated contractions of the detrusor and sphincter. Infrasacral OAB results from damage to motor or sensory nerves leading to a loss of input to the bladder.

Overactive bladder conditions are usually associated with increased detrusor contractions that are present during detected cystometry while the bladder is filling. The exact cause and mechanism responsible for detrusor overactivity remain unclear, however myogenic and neurogenic basis are implicated. Neurogenic detrusor overactivity is thought to result from alterations in peripheral and central mechanisms. For example suprapontine spinal cord damage can result in reduced central and peripheral inhibition to the bladder leading to overactivity of the muscle. In line with this theory, the cerebral cortex has been reported to have an inhibitory effect on voiding function, hence affecting these higher centers could lead to reduced inhibition and detrusor overactivity (Fowler, 2001). Changes in nerve innervations may also result due to spinal cord injury. Studies in cat have reported increased C-fibre mediated afferent input to the spinal cord (de Groat *et al*., 1990). Additionally, 'sprouting ' of afferents has also been suggested in paraplegic animal models (de Groat, 1997). These changes may result in alterations in spinal reflex mechanisms and hence lead to detrusor overactivity.

Apart from alteration in afferent mechanism, efferent mechanism could also result in an overactive detrusor. Rat studies have shown have alterations in muscarinic modulation of transmitter release in the bladder, following spinal cord injury. The M_1 receptors which are low affinity receptors were replaced by the high affinity M_3 receptors, this alteration suggests that ACh release may contribute to hyperactivity (Somogyi *et al.,* 2003). Reduced cholinergic innervations has also been observed in the bladder samples of patients with neurogenic overactivity compared to control groups (Drake *et al.,* 2003). Additionally, M2 receptors have been reported to become

the primary mediator of bladder contraction instead of m3 (Braverman *et al.,* 1999; Pontari *et al*., 2004). These alterations may contribute to detrusor overactivity.

Myogenic detrusor overactivity results from autonomous activity resulting in increased excitation and conduction of cells. *Sherrington et al.,* first reported the autonomous contraction of isolated bladder tissue in 1982. These experiments were re-visited in isolated bladder tissue of guinea pigs and mouse, as well as muscle strips from the mouse, human and pig (Sibley, 1984). Autonomous activity is described as phasic spontaneous contraction, localized stretches of the wall and increase in intra-vesical pressure. The activation of muscarinic receptors has shown to increase such activity. This might suggest that the mechanism involved in regulating such activity is separate to those involved in the excitation of the detrusor during micturition.

1.6 Interstitial cystitis

Interstitial cystitis (IC) also known as painful bladder syndrome is a chronic pelvic syndrome with no generally accepted treatment (Dell *et al*., 2009). This clinical condition is manifested by sensory hypersensitivity of the urinary bladder, leading to exaggerated pain sensation and/or pressure in response to small volume of urine. IC is characterized by the symptoms of pain, frequency, urgency, and nocturia in the absence of bacterial infection or any other identifiable pathology (Butrick, 2003) and diagnosed mainly in women. Initially, IC was considered to be a rare clinical condition, however more recent studies have shown a higher prevalence of IC than previously thought (Clemens *et al.,* 2007 and Link *et al.,* 2008). IC can affect over 700 000 women

in the US and a large percentage of men with prostatodynia and prostitis (Birder *et al.*, 2007). A comparable condition is also found in domestic cats, called feline interstitial cystitis. The persistent symptoms of IC have shown to be debilitating for many patients, and can have a marked negative influence on quality of life (Clemens *et al.*, 2007). The exact aetiology of interstitial cystitis is unclear, however urothelial abnormalities have been reported (Parsons, 2007). The intra-luminal infusion of potassium chloride caused exaggerated pain in patients with IC, suggesting impairment of barrier function (Parsons *et al.*, 2005). Alteration of ATP signalling has also been reported in IC. *Sun et al.*, reported increased ATP concentration in the urine of IC patients compared to controls (Sun *et al.*, 2001). Similarly, increase in ATP release has also been reported from cultured urothelial cells of IC patients (Sun *et al.*, 2001). Additionally, mucosal cells of these patients have been found to have increased expression of P2X3 receptors (Sun and Chai, 2004 and Tempest *et al.*, 2004). These data suggest a prominent role of purinergic signalling in IC.

Disruption of the glycosaminoglycans (GAGs) has also been reported to play a role in the pathogenesis of IC. The GAG layer lines the urothelium and acts as an anti-adherence factor (Iavazzo *et al*. 2007; Parsons, 2003). Damage to the GAG layer can lead to compromised urothelial call barrier function (Parsons *et al.*, 2007). Other bladder epithelial abnormalities observed are production of anti-proliferative factor (APF) (Keay *et al.*, 1996), reduced prostaglandin E_2 release (Rastogi *et al.*, 2006), abnormal cellular architecture and abnormal expression of uroplakins. Alteration of afferent signalling has also been reported in IC (Zeng *et al.*, 2007). For example, increased density of bladder afferents have been observed in FIC and humans with IC

(Pang e*t al.*, 1995, Buffington and Wolfe, 1998). Similarly, increased excitability has been reported in DRGs of cats with FIC (Sculptoreanu *et al.*, 2005).

1.7 ATP and the Bladder

ATP is an intracellular source of energy in cells. It exerts its effects by acting on P_2 purinergic receptors. P_2 receptors consist of the metabotropic receptors (P_2Y) and the ionotropic receptors (P_2X). Eight G-protein coupled P_2Y receptor subtypes and seven P_2X ligand gated ion channels have been identified to date (Burnstock, 2006).

Purinergic receptors have been identified in the urinary bladder, including the afferent nerve terminals, urothelial cells and detrusor muscle (Burnstock & Williams, 2000; Vlaskovska *et al.*, 2001).

P_2X_1 receptor has been found to be the dominant subtype present in the urinary bladder (Burnstock & Williams, 2000). *Valera et al.,* found mRNA expression of P_2X_1 on the detrusor muscle (Valera *et al.*, 1994; O'Reilly *et al.*, 2001). Similarly, immunohistochemical studies have shown that P_2X_1 is extensively present in the bladder (Dutton *et al.*, 1999; Lee *et al.*, 2000). Although P_2X_1 has been shown to be the predominant subtype in the bladder, Various studies have shown a role of ATP in nociception, which is mediated via the P_2X receptors containing the P_2X_2 and P_2X_3 subunits (Burnstock, 1996, Birder *et al.*, 2004, Vlaskovska *et al.*, 2001).

ATP has been shown to be released from urothelial cells in response to stretch (Ferguson *et al.*, 1997). It has been suggested that the released ATP can in turn act on

P_2X receptors present on sensory nerves lying in the suburothelial plexus and play a role in afferent nerve signalling(Burnstock, 1999).

P_2X_3 receptors have also been found on bladder nerves. Inhibition of P_2X_3 has been shown to result in reduced sensory nerve firing(Ferguson *et al.*, 1997; Bodin & Burnstock, 2001). Similar observation has been made with P_2X_3 knockout mouse, suggesting a role of ATP in mechano-sensory signalling in the bladder (Vlaskovska *et al.*, 2001; Rong *et al.*, 2002)

Intra-vesical administration of ATP has shown to cause an increase in afferent nerve firing. Additionally, the same study showed the mechanical activation of "silent fibres" after application of α, β-methylATP (Rong *et al.*, 2002). Consistent with this data, the intra-vesical administration of ATP in awake rats has been shown to induce bladder overactivity via the stimulation of C fibres (Pandita and Andersson, 2002). These data suggest that exogenous ATP can result in bladder overactivity through activation of P2X2/3 receptors.

1.8 TRP channels

Transient receptor potential channels (TRP channels) are calcium permeable non-selective ion channels. The discovery of the first TRP (TRPV1) channel in 1997 has served as a catalyst for the research of somato sensory and pain transduction, together with the identification of other members of the TRP family. TRP channels are permeable to cations and consist of six trans-membrane domain (S1–6), with a pore loop between the S5 and S6 region and a cytoplasmic N and C terminus (Vriens *et al.*, 2004 and Clapham *et al.*, 2003). TRP channels assemble a as homo- or hetero-

tetramers, forming cation-selective channels (Voets *et al.,* 2005, Schaefer, 2004 and Hellwig *et al*., 2005). In mammals, 28 TRP channels have been identified based on their sequence homology and are divided into 6 subfamilies: TRPA (ankyrin), TRPC (canonical), TRPM (melastat),TRPP (polycystin),TRPV (vanilloid) and TRP ML (mucolipin) (Clapham *et al*., 2005, Corey *et al.*, 2003, Delmas *et al.*, 2004, Montell *et al.,* 2002, Moran *et al.,* 2004). Importantly, mutations in different *TRP* genes are linked to human diseases (Nilius *et al.*, 2007). The TRPC and TRPM subfamilies contain seven and eight members, respectively (TRPC1–7 and TRPM1–8). The TRPV subfamily consists of six members (TRPV1–6). TRPA1 is the most recently identified family and contains only one member (TRPA1). Both The TRPP and TRPML subfamilies have three mammalian members.

Other than their basic membrane topology and cation permeability, TRP channels show great diversity. In general, TRP channels share a very low sequence homology, ion selectivity, and mode of activation, tissue expression and physiological function.

1.8.1 TRP channels and bladder

In recent years TRP channels have attracted a lot of attention. TRP channels are expressed in a wide variety of tissues and changes in the expression pattern have been observed in diseased condition. These channels are also expressed in the lower urinary tract. More specifically, expression has been found in the urothelium, detrusor muscle and sensory neurons of the bladder and urethra. The functional significance of these channels in the bladder remains to be elucidated, however they are thought to be involved in nociception and mechano-transduction. These TRP channels gained a lot of interest in recent years and various studies have indicated a pivotal role of TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1 in physiology and pathological bladder function (Everaerts *et al*., 2008). Further studies are required to elucidate the role of these and other TRP channels in the bladder. The expression of these TRP channels in the urothelium and their role in sensation is debatable. TRPV1 was the first heat sensitive channel to be cloned (Caterina *et al.,* 1997) and is the most investigated TRP channel within the bladder. This channel was considered as a TRP channel based on the sequence homology and similarity in hydropathy profile to a mutant channel in drosophila, which was cloned by Montell and Rubin (1989). TRPV1 is now known to be a heat sensitive channel (> 42 °C), activated by various ligands including capsaicin. The expression of TRPV1 has been found in the sensory neurons and the urothelium (Birder *et al.,* 2002), however the functional expression in the urothelium has been questioned (Everaerts *et al.,* 2008). Despite a lot of morphological and functional information, the role of TRPV1 in normal human bladder is still unclear. TRPV4 is a stretch-activated cation channel. Abundant expression of this channel has been reported in the urothelium and detrusor muscle of rat (Thorneloe et *al.,* 2008, Birder

et al., 2007 and Gevaert *et al.,* 2007). It has been suggested that TRPV4 might function as a urothelial mechanosensor for bladder distension (Yamada et *al.,* 2009). On the other hand, TRPA1 is a cold sensing TRP channel and has also been found on bladder sensory nerves. This channel is thought to be activated by noxious cold (Andrade *et al.,* 2006) and has been found on capsaicin-sensitive primary sensory neurons (Story *et al.,* 2003). The activation of TRPA1 is thought to cause a painful sensation. The exact role of this channel in the bladder is unidentified (Everaerts *et al.,* 2008).

Researchers have been long interested in the ability of the bladder to sense cold. In 2002 the first cold sensing TRP channel was named TRPM8 (McKemy *et al.,* 2002, Peier *et al.,* 2002 and Tsavaler *et al.,* 2001). TRPM8 is activated by various cooling compounds, including menthol (McKemy *et al.*, 2002 and Peier *et al*., 2002). TRPM8 have gained lot of interest, since instillation of cold water has shown to induce a painful sensation in patients with interstitial cystitis (Mukerji *et al.,* 2006). TRPM8 has been postulated to be involved in the bladder-cooling reflex, since the instillation of menthol has also shown to enhance this reflex action in both cats and humans (Peier *et al*., 2002 and Geirsson *et al.,* 1999). This reflex is seen in infants and becomes absent with maturation of the nervous system, however this reflex may be unmasked by central neuropathology. Due to the relatively low expression of TRPM8, it has been extremely difficult to investigate the functional significance of this receptor in normal bladder function. TRPM8 expression has been shown on the nerve fibres; however the finding that TRPM8 is expressed on the urothelium is debatable (Stein *et al*., 2004).

TRP channels are multi-factorial sensors that are expressed throughout the CNS and in various tissue types. Numerous reports indicate a link between bladder dysfunction and alteration of TRP channel function. However, the physiological relevance of these channels in normal bladder function remains poorly understood. This study primarily investigates the role of TRPM8 in normal bladder function, in an attempt to gain a better insight into link between TRPM8 and bladder afferent function.

1.9 TRPM8

TRPM8, also known as cold and menthol receptor (CMR-1) is a thermo-sensitive TRP channel (McKemy *et al.,* 2002). The thermo-sensitive channel gene, trp-p8 was first discovered as a prostate specific gene upregulated in malignant tissue (Tsavaler *et al*, 2001), encoding a protein with a molecular weight of ~130KDa and bearing significant homology to TRP channels. Due to a relatively high (47%) similarity to human melastin protein, it was renamed as part of the TRPM family. Using a cDNA library from rat trigeminal neurons (Mckemy *et al*, 2002) and bioinformatics (Peier *et al,* 2002), TRPM8 was identified to be a calcium permeable cation channel activated by cold and cooling compounds. TRPM8 is now known to be a non-selective voltage gated cation channel (Liu *et al.,* 2001 and Defalco *et al.,* 2011). This channel is expressed in various tissues both malignant and non-malignant (discussed later in detail) (Peier *et al.,* 2002; Tsuzuki *et al.,* 2004). However, its physiological role in these tissues remains inconclusive. TRPM8 has been considered as a potential pharmaceutical target or diagnostic biomarker for cancer.

1.9.1 Expression

TRPM8 is mainly expressed in somato-sensory neurons, where it has been shown to be involved in cooling sensation. Of high importance is the upregulation of TRPM8 in malignant tissue, although the mechanism of TRPM8 upregulation is still unclear. TRPM8 is also expressed in many non-malignant tissues, these include, colon, skeletal

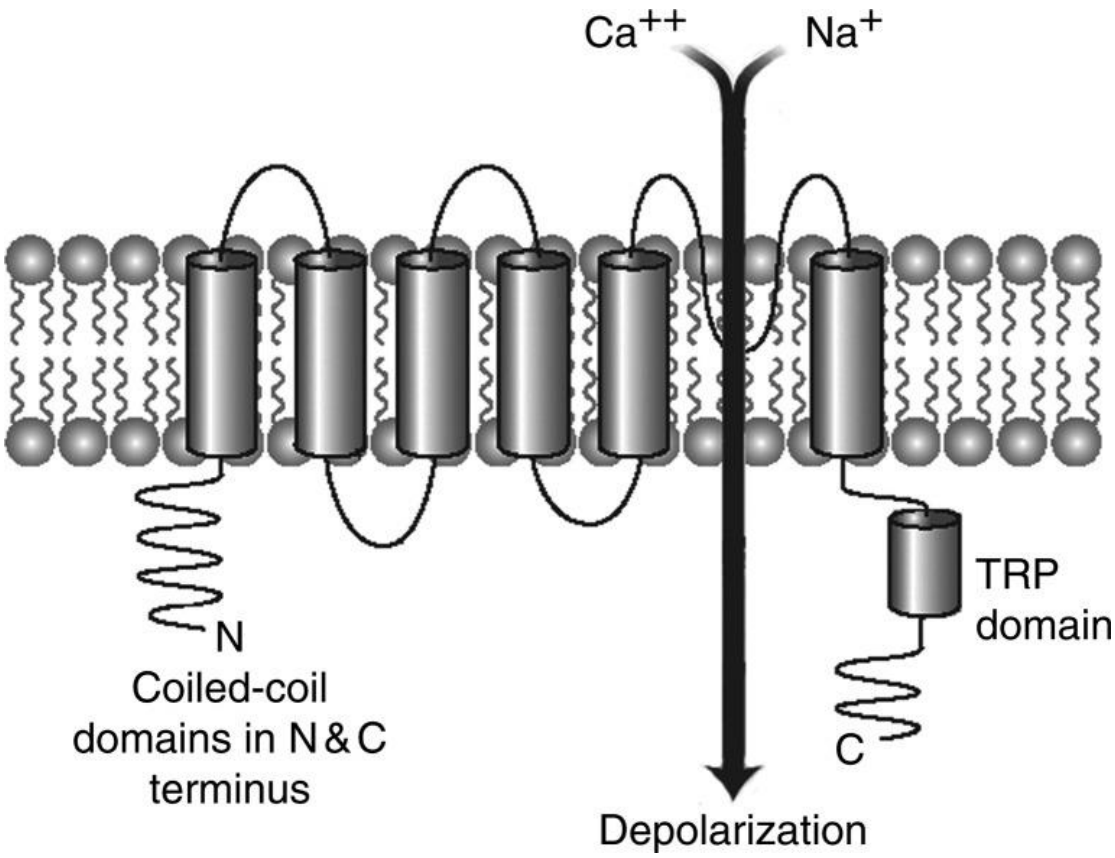
and smooth muscle, prostate, lungs and bladder. The role of this receptor within these organs remains to be identified (Harrington *et al.,* 2011, Stein *et al.,* 2004 and Mukerji *et al.,* 2006).

TRPM8 mRNA and protein has been detected at low levels in small diameter TG (10-15%) and DRG (5-10%) neurones (McKemy *et al.,* 2002, Peier *et al.,* 2002, Okazawa *et al.,* 2004, Abe *et al.,* 2005 and Sarria and Gu, 2010). Due to the low level of TRPM8 it has been very difficult to study the receptor under normal conditions. The co-expression of TRPM8 with other thermosensitive receptors is controversial (discussed later in detail in chapter 4).

1.9.2 Structure

TRPM8 encodes a protein of 1104 amino acids (Tsavaler *et al.,* 2001). The topological arrangement of TRPM8 is comparable to that of a voltage gated potassium channel. Functional TRPM8 channels exist as a tetramer, consisting of 4 subunits. The ion selectivity of the channel is contained in the ion pore region (S5-S6) (Montell *et al.,* 2002, Voets and Nilius, 2003 and Voets *et al.,* 2007). Voltage sensitivity is contained in the first four helices (S1-S4) (Zhang and barritt, 2006 and Voets *et al.,* 2007). The N-terminus comprises more than 50% of the TRPM8 sequence (Phelps *et al.,* 2007). Within The N-terminus, there are 4 regions that are homologous amongst all TRPM subfamilies. The C terminus is formed from 120 amino acids and forms a coiled structure, responsible for the assembly of the tetramer (Fleig and Penner, 2004, Tsuruda *et al.,* 2006). The C terminus also consists of a conserved TRP unit, which is important for channel activation (Rohacs *et al.,* 2005). Additionally, it also consists of

a binding site for Phosphatidylinositol 4,5-bisphosphate (PIP2), which is important for regulating channel opening.



(Bharate *et al.*, 2012)

Figure 3.1 TRPM8 channel

1.9.3 Activation mechanism

TRPM8 can be activated by temperature (28-16 °C), as well as membrane depolarization and various ligands. However, the channel is mostly recognized for its sensitivity to cold. *Mckemy et al* (2002) showed that TRPM8 causes a significant inward current at temperatures below 25°C. Temperature activation by cold also shifts the voltage dependent activation curve. The voltage dependency of TRPM8 is weak.

There are various models describing TRPM8 activation. *Voets et al* (2007), suggested a two-state model, based on its cold sensing. This model shows the additive nature of thermal and chemical stimuli. Conversely, single channel recording show rapid bursts of activity, suggesting a multi-state model. In an allosteric model of TRPM8, there is accordance between the open and closed state of the channel and the on/off transition of the temperature and voltage sensors. This model suggests that the temperature sensitivity is voltage independent. Similarly, it has been shown that the C-terminus affects the temperature sensitivity of the channel without modulating the voltage dependence. Additionally, voltage only partially activates TRPM8, but it's neither important nor sufficient for its gating.

The mechanism of TRPM8 activation is still unclear; however recent studies have characterized the importance of S4 in its activation. These include the role of Arg842 and Cys856 in the S4 helix and S4-S5 linker in voltage gating and menthol sensitivity (Voets *et al*., 2007). Moreover, further investigations have shown that depolarization results in a submaximal stimulus, whereas ligand binding results in a voltage independent activation (Matta *et al.,* 2007). The S4 helix undergoes a conformational change during TRPM8 activation. The standard α helix changes in a more elongated

3-10 helix (Villalba-Galea *et al*., 2008), this is in line with the finding that channel opening does not involve conformational change in the S5 or S6 region, but is dependent on a rigid movement of the entire module (Nilius *et al*., 2011).

1.9.4 TRPM8 ligands

Due to a growing interest in the function of TRPM8, many chemical activators of this receptor have been identified (Table 1.1). These include the cyclic terpene alcohol, menthol. Menthol, which is derived from mentha leaves is used in many commercial products and cooling agents. At low concentrations menthol induces a pleasant cooling sensation. However, higher concentrations have shown to cause a painful burning sensation (Green *et al.,* 1992). It has been shown that the latter response is due to the activation and/or sensitization of C-fibers (Wasner *et al.,* 2004). Although, menthol is an activator of TRPM8, its specificity is questionable. Various studies have shown that menthol activates TRPA1 at low concentrations and inhibits the receptor at higher concentrations (Karashima *et al.,* 2007 and Xiao *et al.,* 2008). Moreover, *Macpherson et al* (2005) showed that menthol also activates TRPV3, although only at very high concentrations. Other than TRP channels, menthol also interacts with GABA receptors found in cultured hippocampal neurons and is known to be a calcium channel blocker (Zhang *et al.,* 2008) . Some of these interactions require very high concentrations, hence out of the range of TRPM8 activation threshold.

Due to the minty and volatile side effects of menthol, Wilkinson and Sword developed another class of cooling agents known as methane carboxamides (WS compounds). These carboxamides are derived from L-menthol, and have been shown to activate

TRPM8. In a *Xenopus* oocyte expression system, *Sherkheli et al*, (2010) showed that WS-12 activates TRPM8 at low molecular concentrations (EC50 12µM), Hence being more potent and efficient than menthol (EC50 196uM). Additionally, in HEK cells the EC50 of WS-12 is in the nanomolar range (EC50 193nM) (Bodding *et al.*, 2007). CPS-128, which is an ethyl analogue of WS-12, has an EC50 of 0.5uM for TRPM8 activation (Weil *et al.,* 2005). Various other carboxylic acid ester and carboxamides have also been identified, using TRPM8 expressing cell lines. However, WS-12 and CPS-112 are probably the most potent in activating TRPM8. They are considered to be selective, as other TRP channels are not activated at low concentrations. Moreover, its efficiency has been shown to be similar to icilin (Bodding *et al.,* 2007).

Icilin, is considered as a super cooling agent, due to its higher potency and efficiency than menthol (Voets *et al*., 2004). However, icilin activates TRPM8 in manner that is different to cold and even menthol. Interestingly, icilin has been shown to activate TRPA1 currents, although with reduced potency compared to TRPM8 (McKemy *et al.*, 2002).

The specificity of any activator is debatable and very concentration dependent. Along with various activators of TRPM8, many inhibitors have also been identified (shown in table 1.2). Intracellular pH has been shown to regulate TRPM8 activity. At pH below 7 it has been shown to completely block TRPM8 currents induced by cold or icilin, but not menthol (Andersson *et al.*, 2004). Conversely, *Behrendt et al* (2004), has shown the inhibition of both menthol and icilin response at low pH (Behrendt *et al.,* 2004). These differences may be due to variation between different cell types and their activation threshold. Other compounds such as capsazepine, BCTC, thio-BCTC and

SB-452533 have also been shown as strong TRPM8 antagonists. However, many of these antagonists are also able to block TRPV1 currents. Thus, further studies are needed to establish selective TRPM8 antagonists.

Table 1.1. List of some TRPM8 activators

Chemical Inhibitor	**EC50**	**References**
(2R)-4-(3-chloro-2-pyridinyl)-2-methyl-N-[4-(trifluoromethyl)phenyl]-1-piperazinecarboxamide (CTPC)	131nM	Weil *et al*., 2005
Clotrimazole	200nM	Karashima *et al*., 2009 and Malkia *et al*., 2009
SB-452533	571nm	Weil *et al.,* 2005
N-(3-aminopropyl)-2-{[(3-methylphenyl) methyl]oxy}-*N*-(2-thienylmethyl)benzamide hydrochloride salt (AMTB)	7uM	Lashinger *et al.,* 2008
N-(4-tert.butyl-phenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2*H*)-carboxamide (BCTC)	0.5-0.8 uM	Behrendt, Germann *et al*. 2004; Malkia *et al*., 2007
SKF96365	1uM	Malkia, Madrid *et al*,. 2007
anandimide	0.13uM-3.7uM	Weil *et al.,* 2005
Thio-BCTC	3.5uM	Behrendt *et al.,* 2004
capsazepine	18uM	Behrendt *et al.,* 2004
1,10-phenanthroline	100-200uM	Malkia *et al*., 2007

Table 1.2. List of some TRPM8 antagonists

Chemical Activator	**Chemical class**	**EC50**	**References**
(-)-Menthol	p-methane-3-ol	196uM, 66.7uM , 4.1uM	McKemy *et al*., 2002, Peier *et a*l., 2002 and Sherkheli *et al.*, 2008)
(+)-Menthol	p-methane-3-ol	600uM, 14.4uM	Sherkheli *et al*., 2010 and Behrendt *et al.,* 2004
Icilin	tetrahydropyramidine-2-one	7uM, 0.2-0.36 uM	McKemy *et al*., 2002, Neuhausser *et al*. 2002; Behrendt *et al.*, 2004
WS-12	p-methane-3 carboxamide	30uM, 12uM, 193nM	Sherkheli *et al*., 2010, Bodding *et al.,* 2002, Behrendt *et al*., 2004
WS-3	p-methane-3 carboxamide	216uM, 3.7uM	Behrendt *et al.*, 2004, Sherkheli *et al.,* 2010
WS-148	phosphine oxide	4.1uM	Bodding *et al.*, 2007
WS-30	p-methane-3 carboxamide	5.6uM	Bodding *et al*., 2007
WS-23	Acyclic carboxamide	1.5mM, 44uM, 5.6uM	Behrendt *et al.*, 2004
WS-5	p-methane-3 carboxamide	26uM	Sherkheli *et al*., 2010
CPS-113	p-methane-3 carboxamide	1.2uM	Bodding *et al.*, 2007
CPS-369	p-methane-3 carboxamide	84uM, 3.6uM	Bodding *et al*., 2007 and Sherkheli *et al.,* 2010
FrescolatML	p-methane-3-ol ester	163uM, 3.3uM	Behrendt *et al.*, 2004
FrescolatMGA	p-methane-3-ol ether	184uM, 4.8uM	Behrendt *et al.*, 2004
MPD, Cooling agent 10	p-methane-3-ol ether	6.0uM	Behrendt *et al*., 2004
PMD 38	p-methane-3-ol	31uM	Behrendt *et al.*, 2004
(-)-Isopulegol (Coolact P)	p-methane-3-ol	498uM, 66uM	Behrendt *et al*., 2004 and Sherkheli *et al.,* 2010
Eucalyptol	p-methane-3-ol ether	3.4-7.7mM	McKemy, Neuhausser *et al*. 2002;Behrendt *et al*., 2004
Geraniol	Acyclic alcohol	5.9mM	Behrendt *et al.*, 2004
Linalool	Acyclic alcohol	6.7mM	Behrendt *et al*., 2004
Hydroxy-citronellal	Acyclic alcohol	19.6mM	Behrendt *et al.*, 2004
1,8-Cineole	p-methane	3.4mM, 7.6mM,	Behrendt *et al.*, 2004 and McKemy *et al.,* 2002
Cold temperature			Mc~Kemy *et al*., 2002 and Peier *et al.,* 2002
Voltage			Voets *et al*., 2004
Pip2			Rohacs *et al*., 2005

1.9.5 TRPM8 –behavioral studies

A number of studies have reported no phenotypic difference in physical appearance between wild type and TRPM8 knockout mice (Chung *et al*., 2007, Colburn *et al.*, 2007 and Daniels and McKemy., 2007). However, these knockout mice have been reported to have significant reduction in sensation to cold and menthol in the DRG and TG neurons (Chung *et al*., 2007). This suggests a role of TRPM8 in detecting cold. However, KO mice are not completely insensitive to cold, especially noxious cold (0°C) (Colburn *et al*., 2007, McKemy *et al*., 2005 and Story *et al*., 2006), indicating the involvement of TRPM8 independent mechanisms in noxious cold temperatures.

TRPM8 KO studies in mice models of neuropathic pain also suggest a possible role of this receptor in cold allodynia (Colburn *et al*., 2007 and Caspani *et al*., 2009). WT mice developed increased cold sensitivity, whereas TRPM8 KO mice showed no significant increase in sensitivity to cold (Colburn *et al*., 2007). Furthermore, TRPM8 antagonists have been shown to be effective in reversing pain sensation in visceral pain models (Lashinger *et al*., 2008).

The involvement of TRPM8 in cold and menthol induced analgesia has been studied by Dhaka *et al* (2007). Injection of formalin in mice paw, followed by mild cooling (17°C), reduced the nociceptive response in WT mice, while KO mice showed analgesia only for the second inflammatory phase (Dhaka *et al*., 2007). Similar findings were reported using rodent models of inflammatory and neuropatic pain (Proudfoot *et al*., 2006). These studies suggest a major role of TRPM8 in noxious and innocuous cold perception, cold-induced analgesia and cold hypersensitivity in pathophysiological conditions.

1.9.6 TRPM8-cancer

As previously mentioned, TRPM8 was first identified as a prostate specific TRP channel upregulated in prostate cancer tissue. However, TRPM8 protein and mRNA expression are also upregulated in many other cancers. These include, breast, lung and colon, whereas, TRPM8 expression was minimal in the corresponding non-malignant tissue (Bidaux *et al.*, 2005 and Bidaux *et al* 2007). *Yee et al* (2010) also found strong immunoreactivity for TRPM8 in cell lines of human pancreatic adenocarcinoma, compared to minimal levels of TRPM8 in normal tissue. Interestingly, TRPM8 knockdown studies, have led to cellular proliferation arrest, and also a significant increase in cell death, both non-apoptotic and apoptotic (Yee *et al*., 2010 and Zhang and Barritt 2004). TRPM8 expression and its possible function in cell survival has also been observed in the cell line of human melanoma G361 (Yamamura *et al*., 2008). Despite the various studies and observations of TRPM8 expression in malignant tissue and cell lines, the exact role of this receptor is still unknown. However, a role of androgen hormones in TRPM8 upregulation within prostate and pancreatic carcinoma has been suggested (Zhang and Barritt, 2004, Bidaux *et al*., 2005 and Konduri *et al*., 2007). Oestrogen receptors have also been implicated in TRPM8 upregulation in breast cancer cell line MCF-7 (Chodon *et al*, 2010). These observations suggest an important role of TRPM8 in the pathophysiology of epithelial cells.

1.9.7 TRPM8 and the bladder

The ability of the bladder to detect cold was first suggested by Bors and Blinn in 1957, when the bladder-cooling reflex (BCR) was reported. The BCR is an immediate

detrusor contraction in response to intravesical infusion of ice water into the bladder (Geirsson *et al*., 1999). This response has been found in patients with supraspinal neuronal lesions or idiopathic detrusor overactivity (IDO), however is absent in normal subjects (Fall and Geirsson., 1996). Because of this observation, the ice water test (IWT) has been used as a tool to investigate the correlation between urological disease symptoms and neurogenic disease. Intravesical infusion of capsaicin or resiniferatoxin, have been shown to improve overactive bladder symptoms in patients with a positive IWT (Das *et al*., 1996). Both these agents act on C fibers afferents, resulting in desensitization of TRPV1, it has been suggested that the BCR might be mediated by these afferents (Das *et al*., 1996) Various studies have suggested that TRPM8 may be involved in regulating the BCR. For example, treatment with menthol shifts the temperature threshold for the BCR upward (Geirsson ., 1993). Additionally, TRPM8 mRNA and protein have been found in the human and rat bladder (Mukerji *et al*., 2006 and Stein *et al*., 2004). In humans the localization of TRPM8 immuno-staining has been retained to the urothelium and suburothelial nerve fibers (both C and $A\delta$ fiber). A significant increase in TRPM8 expression has also been found in patients with overactive and painful bladder syndrome. Interestingly, a correlation between the relative density and the severity of disease symptom has also been reported, indicating a possible pathophysiological role of TRPM8 in these disorders (Mukerji *et al*., 2006). In anaesthetized rats, AMTB (N-(3- aminopropyl)-2-{[(3-methylphenyl) methyl] oxy}-N-(2-thienylmethyl)benzamide hydrochloride salt), a TRPM8 channel blocker reduced the number of volume-induced bladder contractions and nociceptive reflex responses to noxious bladder distension (Lashinger *et al*., 2008).

These observations suggest that TRPM8 contributes to bladder afferent sensitivity and may also play a role in the symptomatology and pathophysiology of overactive and painful bladder disorders. However, identifying the endogenous activators of TRPM8 channel in normal and diseased bladder await further studies.

1.10 Interactions between TRP channels

The thermo-sensitive ion channels TRPM8, TRPA1 and TRPV1 have been shown to play an essential role in pain sensation. Tissue injury and inflammation produce an array of pro-inflammatory mediators that can activate or sensitize the heat gated TRPV1, causing a reduction of activation threshold and resulting in hypersensitivity at the site of injury (Cesare *et al*., 1996, Numazaki *et al.*, 2002, Bhave *et al*., 2003, Zhang *et al*., 2005, Zhang *et al*., 2008). On the other hand, TRPM8 has been reported to play a role in many aspects of pain sensation; these include cold analgesia, as well as cold hypersensitivity (Proudfoot *et al*., 2006, Dhaka *et al*., 2007, Colburn *et al*., 2007 and Chung *et al*., 2007). The relatively low expression of these TRP channels has resulted in inconsistencies in findings with regards to morphological, pharmacological and physiological details. However, most of the researchers share a common agreement in the role of thermally gated transient receptor potential channels in pain signalling. In particular a role TRPM8, TRPA1 and TRPV1 has been suggested in pain signalling, since their expression levels as well as their biophysical properties are changed in chronic induced pain states. It has been suggested that these thermo-sensitive ion channels may be able to modulate the activation of each other (Harrington *et al.*, 2011) However, there is a lot of discrepancy between the expression, co-expression and hence interaction of these receptor. Many other studies have reported interaction between TRPV1 and TRPA1 (Salas *et al*., 2009, Mayur *et al*., 2010, Ruparel *et al*., 2011). However, studies for interaction with TRPM8 are lacking. Investigating the interaction between these receptors and elucidating the molecular mechanism involved in the interaction could open up new avenues for therapeutic

manipulation.

1.10.1 TRPV1

TRPV1 (Transient receptor potential 1), previously known as vanilloid receptor type 1 or the capsaicin receptor TRPV1 was cloned in 1997 (Caterina *et al*., 1997). Long before the initial cloning of TRPV1, capsaicin was shown to effect bladder sensory function, resulting in increased bladder capacity and urinary retention. Maggi *et al*, reported increased bladder contraction followed by desensitization with the administration of capsaicin (Maggi *et al*., 1985). It is now evident that capsaicin acts through TRPV1 (Liu *et al*., 2003). Other than capsaicin, TRPV1 is also activated by heat, acid and various endogenous agonists (such as, anandamide, 12-hydroxyeicosatetranoic acid) (Nagi *et al*., 2004, Hwang *et al*., 2000, Caterina *et al*., 1997; Tominaga *et al*., 2001 and Gunthorpe *et al*., 2002). Since the cloning of TRPV1, a lot of research has focused on its role within the bladder. Szallasi *et al*., first detected TRPV1 expression in the bladder of rats (Szallasi *et al.,* 1993). TRPV1 is also expressed in afferent nerve fibres (Szallasi *et al.,* 1993, Avelino *et al.,* 2002 and Lazzeri *et.,* 2004) and smooth muscle (Ost *et al.,* 2002 and Lazzeri *et al.,* 2004) of the lower urinary tract. Like other TRP channel, there is a lot of discrepancy for the expression of TRPV1 in the urothelium. Birder et al., found TRPV1 expression in the urothelium of rodents (Birder *et al.*, 2001), however, work conducted by *Everaerts et al*. (2009), has contradicted these findings. Functionally, TRPV1 has been shown to play a role in bladder inflammation and pain (Dinis *et al.*, 2004). Early functional studies revealed that capsaicin-sensitive C type bladder fibres play a role in micturition (Lecci *et al*., 2001 and Maggi *et al*., 1989). Absence of TRPV1 has shown to affect

bladder sensory firing, in particular reduced bladder reflex contraction and increased bladder capacity (Birder *et al.,* 2002). Several studies indicate an essential role of TRPV1 in bladder hypersensitivity (Apostolidis., 2005 and Brady *et al*., 2004). For example, TRPV1 -/- mice do not develop bladder over-activity during acute bladder inflammation (Silva *et al*., 2004 and Szallasi *et al*., 2006). Moreover, significant increases in TRPV1 expression have been shown in patients suffering from neurogenic detrusor over-activity (Apostolidis *et al*., 2005 and Brady *et al*., 2004). Data showing correlation between expression/sensitivity of TRPV1 and disease symptoms indicate a possible role of TRPV1 in bladder hypersensitivity.

1.10.2 TRPA1

As opposed to noxious heat receptor TRPV1, the role of TRPA1 within the bladder is poorly understood. Previously known as ANKTM1, TRPA1 was cloned by Jaquemar *et al*., in 1999 (Jaquemar *et al*., 1999). This receptor was first characterized as a thermo-receptor, which is activated by noxious cold. We know now, that a range of exogenous and endogenous ligands such as allylisothiocyanate, cinnamaldehyde and acrolein also activate TRPA1. Many parallels exist between the functions of TRPV1 and TRPA1. Both ion channels play a role in nociception, and have chemical activators that induce pain sensation. Emerging evidence suggests the functional expression of TRPA1 within the bladder. TRPA1 expression has been found in sensory nerves innervating the bladder (Nagata *et al*., 2005 and Streng *et al*., 2008), Moreover, the rat and human urothelium have also been shown to express TRPA1 (Du *et al*., 2007a and Du *et al*., 2007b). Up-regulation of TRPA1 has been found in bladder mucosa of patients with BOO (Du *et al*., 2007a). Administration of AITC and CA results in

bladder contraction mediated by TRPA1 (Andrade *et al*., 2006). Intravesical administration of TRPA1 agonists also results in bladder hyper-reflexia via C fiber-mediated pathway (Du *et al*., 2007a and Streng *et al*., 2008). The above-mentioned studies indicate that TRPA1 may play an essential role in bladder sensory processes.

1.10.3 Physiological expression of TRPM8, TRPV1 and TRPA1

In order for interaction to occur between TRPM8, TRPA1 and TRPV1, it could be said that the receptors should be expressed on the same cell, particularly if there is a direct interaction. TRPM8 expression has been shown to be restricted in a subset of small diameter neurons in the TG and DRG (Peier *et al*., 2002 and Thut *et al*., 2003). Similarly, TRPV1 and TRPA1 have also been shown to be expressed in small to medium diameter cells. Neurons that convey temperature sensitivity have been shown to have both αδ and C fibres (Patapoutian *et al*., 2003). *Abe et al*., has shown the expression of TRPM8 in both NF-200 positive and negative cells. NF-200 is a marker for αδ fibre neurons; hence they concluded that TRPM8 is expressed in both αδ and c fibre neurons (Abe *et al*., 2005). TRPV1 and TRPA1 on the other hand have only been found on C fibres within the bladder.

McKemy et al, first showed co-expresssion of TRPM8 and TRPV1 in cultured sensory neurons from rats (McKemy *et al*., 2002). Moreover, there have been reports of capsaicin responses in menthol and cold sensitive neurons, indicating co-expression (McKemy *et al*., 2002, Xing *et al*., 2006, Park *et al*., 2006, Babes *et al*., 2004 and Reid *et al*. 2002). TRPV1 expression has been found in approximately 50% of the TRPM8 expressing rat DRG neurons in culture (McKemy *et al*., 2002).

Okazawa et al, also confirmed co-expression of TRPM8 and TRPV1 in DRG sections from rats. The study was carried out using, immunohistochemical staining of TRPV1 and in situ hybridization of TRPM8 mRNA (Okazawa *et al*., 2004). In the TG, very few neurons co-express TRPM8 and TRPV1 (Abe *et al*., 2005), However, in lumbosacral DRG neurons innervating the bladder, approximately 36% of TRPM8 neurons have been reported to co-express TRPV1 (Hayashi *et al*., 2009).

Contrary to the above-mentioned studies, mice studies have refuted the co-expression of TRPM8 and TRPV1 (Peier *et al*., 2002, Story *et al*., 2003). With regards to TRPA1, most researchers found no co-expression between TRPM8 and TRPA1, either in rats or mice (Story *et al*., 2003). However, there are a lot of reports suggesting co-expression between TRPV1 and TRPA1 (Yu *et al*., 2010). Kobayashi *et al*, have also reported that TRPM8 mRNA was not expressed in the TRPV1-expressing neurons (Kobayashi *et al*., 2005). Conversely, in neuropathic pain models an increase in the co-expression of TRPM8 and TRPV1 in sensory neurons has been shown (Dhaka *et al*., 2008). Blackshaw *et al*., found TRPM8/TRPV1-immunoreactivity in approximately 27% of colonic afferent neurons in mice. Discrepancies in co-expression data may be due to an increase in TRPV1 and TRPM8 co-localisation induced by cell culture (Chuang *et al*., 2001). Alternatively, histological studies may not have been sensitive enough to detect co-localisation. Hence, it is controversial if TRPM8 and TRPV1 co-exist in situ.

Because of the controversy over expression pattern between the TRP channels, we decided to look at functional interaction. The aim of this study was to assess, any

interaction between TRPV1 and TRPA1 with TRPM8 on bladder afferents and the urothelium.

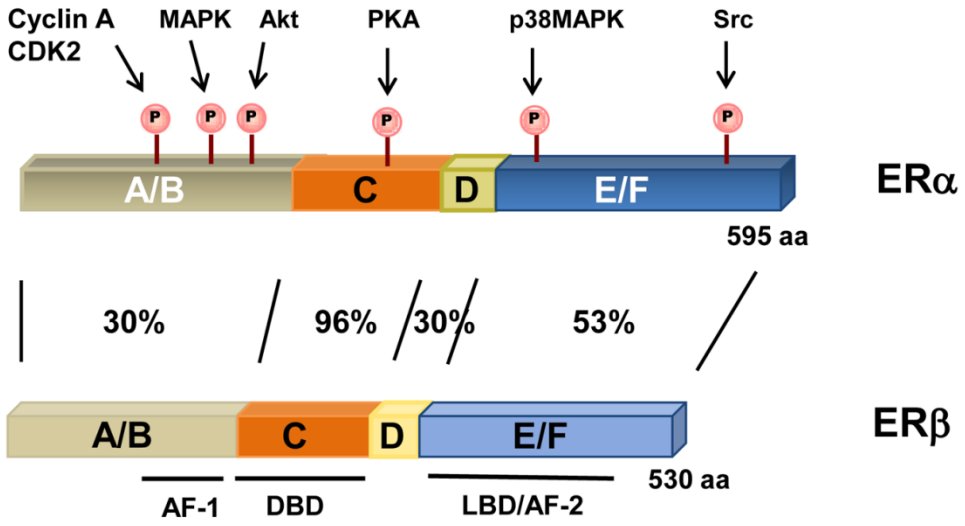
Many forms of lower urinary tract disorders exist without a clear demonstrable pathology. While these conditions can affect men and children, most of those affected are women. The available treatment options are limited, but various factors including autoimmune disorder, epithelial dysfunction and neurogenic inflammation have been reported. Regardless of the origin, most patients suffer from urothelial destruction. These functional pain disorders are commonly thought to result in an increased hypersensitivity of nociceptive pathways, such as central neurons and sensory receptors. Because interstitial cystitis is mostly seen in women with symptoms increasing postmenopausally, a role of oestrogen receptors (ER) has been implicated in the generation of such hypersensitivity, although the exact mechanism is still unclear (Sanoja *et al*., 2008)**.**

1.11 Oestrogen receptor

Three types of oestrogen are present in the circulation these are estradiol, estrone and estriol. Estradiol is the most potent activator of ER. Estradiol is the most abundantly found oestrogen type in the circulatory system and it is secreted by the ovaries. Oestrogen hormones, such as 17β-estradiol (E_2), are known to regulate cell growth in various tissues. Two sybtypes of ER exist ERα and ERβ (Beato *et al*., 1995). These are proteins found intracellularly and are members of the nuclear hormone family of intracellular receptors. The first ER was cloned in uterine cytosol in 1986 (Green *et al*., 1986). In 1996, the second ER (ER-β) was identified in rat prostate (Kuiper *et al*., 1996). The structure of ERα and ERβ is similar to all other members of the nuclear family (see figure 5.1). However, moderate homology is shared between the two

receptors in their protein sequences (58% in human and 55% in rat) (Mosselman *et al*., 1996). Interestingly, they share almost the same DNA-binding domains, which can interact with specific DNA elements (such as, estrogen-response element) and activate ER subtype-specific genes (Hyder *et al*., 1999 and). Similar to the ERα, both the receptors are activated by estradiol-17β (E2).

Oestrogen receptor is a ligand activated transcription factors. AF-2 is the hormone-binding site as well as a transcription-activating region. Upon activation, oestrogen translocates into the nucleus, resulting in the activation of transcription (DNA-binding transcription factor). AF-1, another activation site is constitutively active but weaker than AF-2. It has been reported that AF2 induces the activation of transcription even in the presence of tamoxifen, which is an oestrogen receptor antagonist. Binding of a ligand induced a conformational change within the receptor. This change may consequently result in high-affinity binding to certain Oestrogen response elements or modulate transcription at promoter elements through protein–protein interactions (Kushner *et al*., 2000). The exact role of the two oestrogen subtypes remains unclear. However ERα has been reported to play a prominent role in the regulation of reproduction, while ERβ seems to play a minor role (Warner *et al*., 1999)



(Roman-Blas *et al.*, 2009)

Figure 5.1: Schematic diagram of the structure of ERα and ERβ. Separate genes encode both receptors. ESR1 encodes ERα, while ESR2 encodes ERβ. AF1 and AF2 are the two binding sites, activation of which results in transcriptional activation. Both receptors consist of 5 domains and share significant homology.

1.11.1 Oestrogen in the bladder

Oestrogen receptors are expressed in various tissues, including: prostate, intestine, lung and the bladder. In these tissues ER has been shown to be involved in terminal differentiation of the epithelium (Forster *et al*., 2002, Morani *et al*., 2006 and Imamov *et al*., 2004). Within the bladder, ERβ is thought to be the most predominant isoform expressed with very little ERα expression **(**Saunders *et al*., 1997**).** ERs are present in the trigone, but are absent in the dome of the bladder. Both ER subtypes have also been found in the urothelium.

It has been reported that in interstitial cystitis (IC), disruption of the urothelial barrier may initiate a cascade of events in the bladder, leading to symptoms and disease. Specifically, epithelial dysfunction leads to the migration of urinary solutes, in particular, potassium, that depolarizes nerves and muscles and cause tissue injury. It has also been suggested that ER may be involved in mediating proliferation of urothelial cells (Teng *et al*., 2008). Impairment of the mucosal barrier function and changes in prostaglandin levels has been reported in oestrogen deficient mice (Hass *et al*., 2009). Thus, changes in ER signaling may induce changes in the urothelial structure, resulting in impairment of the barrier function. An extensive study was carried out by *Imamov et al* (2007), investigating the role of ERs in modulating urothelial cell structure and function. Female $ER\beta^{-/-}$ mice were found to have urothelial atrophy, ulceration and shredding of the bladder urothelium and increased bladder permeability in comparison to their wild type littermates. As a result of urothelial impairment, invasion of immune cells were observed in the stroma and the epithelium. γδ T cells were found to be more concentrated in areas of atrophy and

urothelial shredding in ERβ$^{-/-}$ mice. High infiltration with γδ T cells and macrophages were shown in the bladder of ERβ$^{-/-}$ female mice (Imamov *et al*, 2007). These morphological changes seen in female ERβ$^{-/-}$ mouse bladders resembles those seen in IC. However, there is no evidence if these changes lead to changes in bladder afferent signaling, and hence modulate pain sensation, which is a characteristic of IC.

Interestingly, Schroder *et al*., conducted studies with oestrogen receptor knockout mice and found no changes in the voiding behavior of these mice (Schroder *et al*., 2003). No changes were also reported in contraction of isolated muscle strips obtained from these mice compared to their wild type littermates (Schroder *et al*., 2003). However, an interesting observation during this study was the absence of any overactive cystometry pattern induced by the intravesical administration of capsaicin in ERα KO mice (Schroder *et al*., 2003). Capsaicin is a well-known TRPV1 agonist, whose receptors play a role in pain sensation (Wang *et al*., 2008). The absence of this response in ERα mice, suggests an interaction between oestrogen and TRPV1. This indicates a possible role of oestrogen in the alteration of bladder afferent signaling. This may possibly explain the increase occurrence of IC in postmenopausal women. However, the overactive cystometric pattern was present in the ERβ mice. These results are contradictory to *Imamov et al* data, where the role of ERβ was reported to be predominant in the pathogenesis of IC. However, these results do provide a possible role of oestrogen in bladder afferent signaling.

Bennet *et al*., reported expression of both ER subtypes in adult female rat lumbosacral DRGs (Bennett *et al*., 2003). Similarly, mRNA of ERs have been identified within

small and medium sized lumbosacral dorsal root ganglion DRGs (Papka *et al*., 2003 and Papka *et al*., 2001). Moreover, coexpression between both ERs and TRPV1 has been reported in more than 30% of these neurons, suggesting direct interaction by which steroid modulation could affect TRPV1 activation, hence influence pain modulation.

In addition, ERs have been found on mast cells in women with interstitial cystitis (Pang *et al*., 1995). It has been suggested that estrogen can directly influence (non-genomic) the function of the detrusor muscle through the modulation of muscarinic receptors (Batra *et al*., 1989) and by inhibition of calcium influx into muscle cells (Wang *et al*., 2008). Subsequently, it has been reported that estradiol attenuates both the amplitude frequency of spontaneous contractions of the detrusor muscle (Acar *et al.,* 2006) increased bladder sensation in some women has also been reported (Fantl *et al*., 1988). Additionally, a reduction in the ERβ expression has been shown in bladders of rats with chemically induced cystitis (Acar *et al.,* 2006)

The above mentioned studies indicate that oestrogen receptor β may play a role in the pathogenesis of IC; hence ERβ KO mice may present a useful animal model for this condition. However, there is some discrepancy in the voiding pattern reported previously and there is no direct evidence for the functional role of ERβ in bladder sensation.

ERβ null female mice offer an opportunity to investigate the mechanisms of sensory hypersensitivity that might results due to changes in the expression of estrogen

receptor in females. This study with the use of ERβ null mice investigates the role of this receptor in bladder afferent signaling.

CHAPTER 2

MATERIALS AND METHODS

2.1 Recording of intra-vesical pressure and afferent nerve firing

Animals

All experiments were performed using adult male mice with a C57 black background (25-30g). $TRPM8^{+/+}$ and $TRPM8^{-/-}$ mice with a genetic background of C57/BL6 were generated by Pfizer. There were no overt differences in feeding behaviour, litter size, growth rate and body weight between WT and KO groups. Studies with knockout strains were compared to wild type littermates of similar age and weight.

All animals were allowed free access to food and water and were humanly killed by cervical dislocation in accordance with UK Home Office regulations covering schedule one procedure.

Afferent nerve preparation

The animal was sacrificed by cervical dislocation. The abdomen was opened and the pelvic region was removed. The spinal cord was severed at the level of L1-L2 and all proximal tissue discarded leaving the whole pelvic region (containing ureters, kidneys, bladder, vas deferens, seminal vesicles, testes and urethra) intact. The pelvic region was placed in a recording chamber that was continually perfused with oxygenated (95% O_2 and 5% CO_2) Krebs-bicarbonate solution (composition, mM: NaCl 118.4, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7) at 35 °C (pH: 7.4). The bladder was visualized using a dissection microscope (Nikon, SMZ645) to enable the introduction of luminal catheters and identification of the nerve bundles from which afferent recordings were to be made. The ureters were tied with silk suture (US7/0) to prevent back flow. The pubic symphysis was cut centrally to expose the

underlying urethra. A polythene catheter (0.28mm) connected to a syringe pump (Genie, Kent,multi-phaser™ model NE-1000) was inserted into the bladder via the urethra. An incision was made at the dome of the bladder using a syringe needle (BD microlance™, 19G 2"). A double lumen catheter was inserted into the incision point and tied with sutures. One port was connected to a pressure transducer (DTX™ plus DT-XX, Becton Dickinson, Singapore) to allow recording of intravesical pressure both at rest and during distension while the second port was connected to a two-way tap. Opening and closing the tap allowed emptying and filling of the bladder. Bladder nerve bundles, containing a mix population of hypogastric and pelvic afferent nerves are located at the base of the bladder. These nerve bundles were carefully dissected into individual branches. One nerve branch was inserted into a recording electrode (tip diameter 50-100µm) attached to neurology headstage (NL100, Digitimer Ltd, UK), AC amplifier (NL104) and filter (NL125, band pass 300–4000 Hz) and captured by a computer via a Power 1401 interface and Spike2 software (version 5.14, Cambridge Electronic Design, UK).

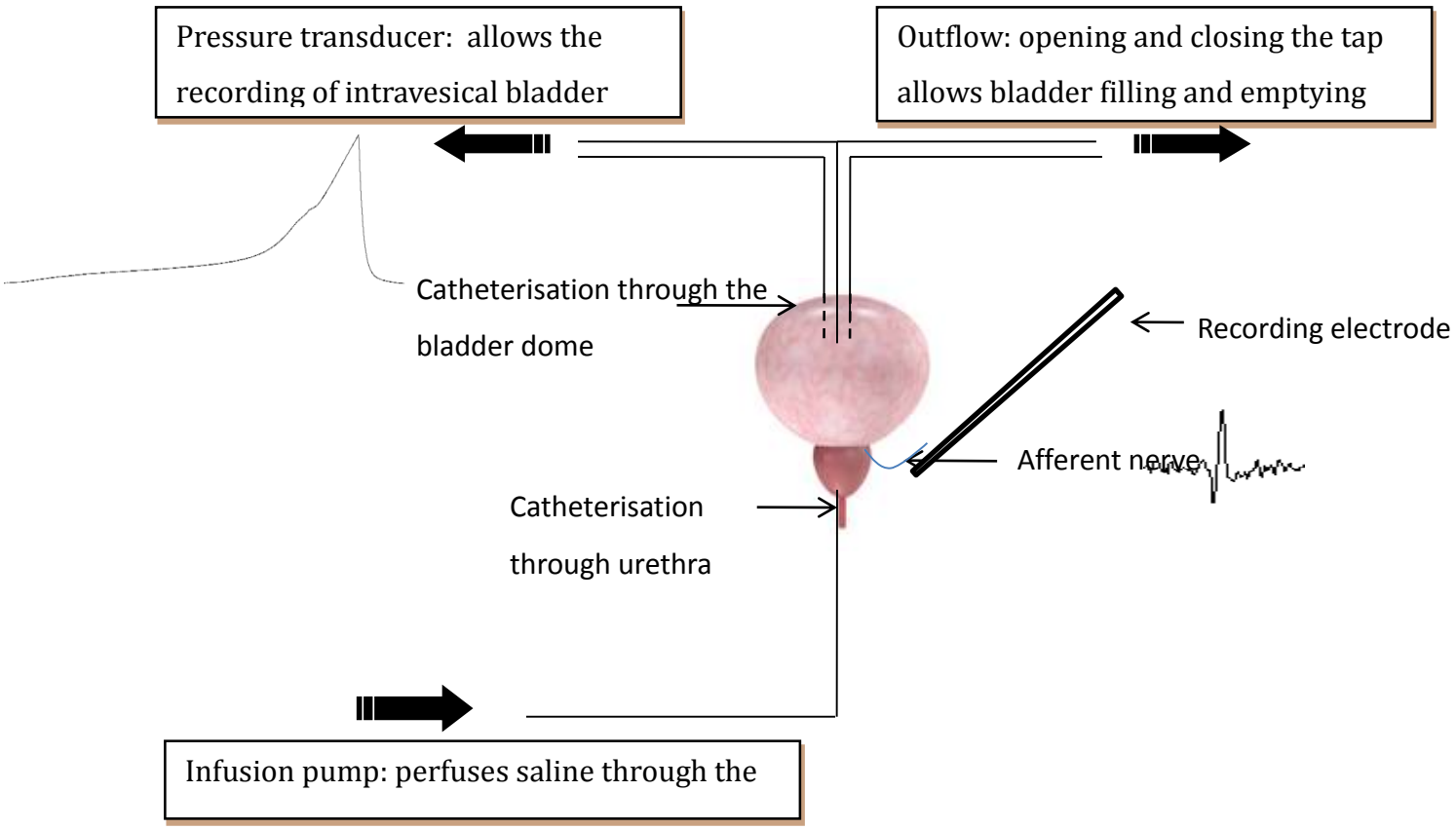


Figure 2.1. Schematic diagram representing an *In vitro* model for recording of intravesical pressure and record afferent nerve firing. The urethral catheter was attached to pump allowing infusion of saline/drugs through the lumen of the bladder. The catheter inserted into the bladder dome was attached to a pressure transducer to allow monitoring of intravesical pressure). Mix population of bladder afferents were identified, dissected and inserted in a recording electrode. Action potential generated were recorded using computer software.

2.2 Experimental protocol

Multiunit nerve recording were performed both at baseline and during bladder distension. The preparation was allowed to stabilize for 30 minutes, before starting the protocol. Bladder distension was performed using isotonic saline (NaCl 0.9%) at a rate of 100µl min^{-1} to a maximum intravesical pressure of 40mmHg or 50mmHg after which the bladder was emptied by opening the outflow tap of the two-way catheter attached to the bladder dome. This was repeated every 10 minutes until a stable pressure and afferent responses to bladder distensions were obtained.

Extra-luminal application of pharmacological agents

Following stabilisation of afferent nerve recording, drugs were applied to the extra-luminal Krebs solution. The Krebs solution containing the drug was perfused for 30 minutes (unless otherwise stated). During this extra-luminal application distensions were carried out every 10 minutes. After 30 minutes the extra-luminal Krebs solution containing the drug was replaced with fresh Krebs solution (washout). Afferent and intravesical responses to drug application were compared to a 30-minute control and washout period. Afferent responses were investigated at baseline and during distension.

Intra-luminal application of pharmacological agents

Drugs were applied into the lumen of the bladder through the urethral catheter. Drugs were diluted in isotonic saline to the appropriate concentration. The drugs were

continuously perfused into the bladder using a perfusion pump, with the out-flow tap open to avoid distending the bladder afferent and intravesical responses to drug application were compared to a 30 minute control and washout period. Afferent responses were investigated at baseline and during distension.

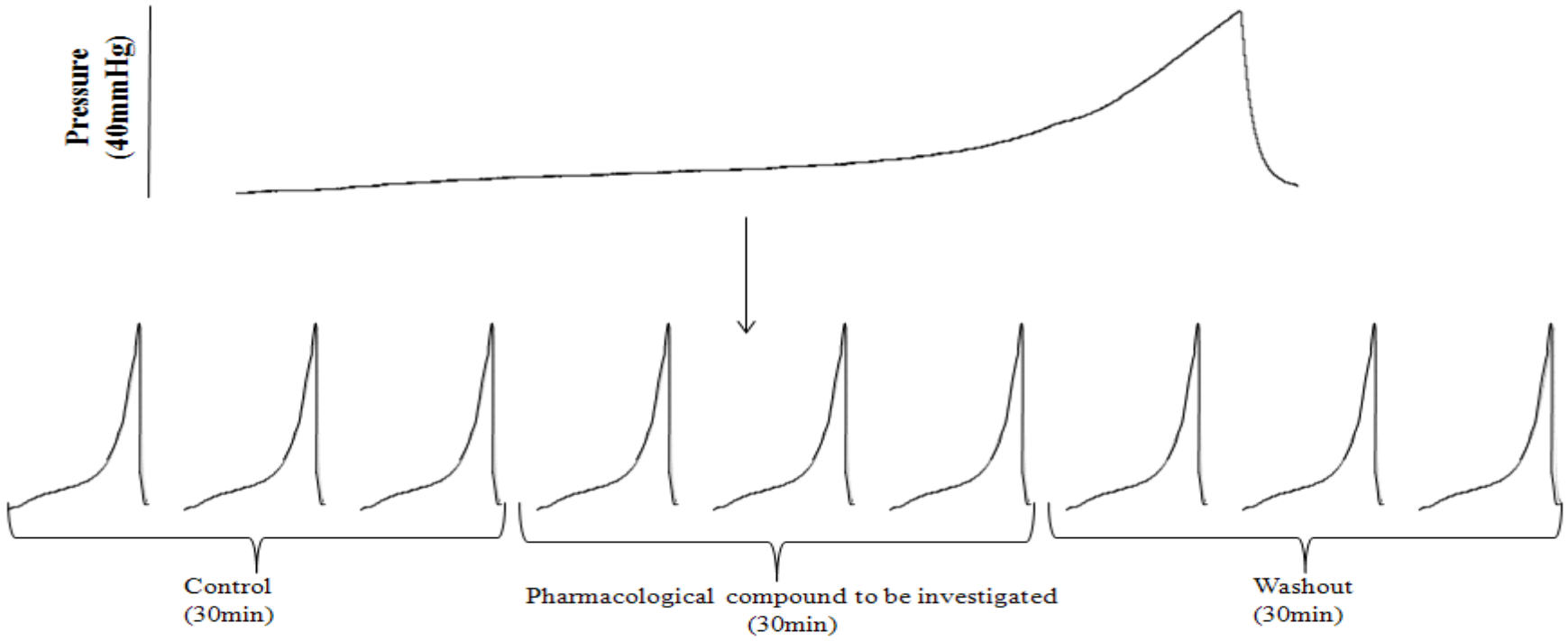


Figure 2.2: Schematic diagram representing the protocol used to record the intravesical pressure and afferent nerve firing. The first three reproducible afferent responses were used as the control (at 10 min interval). The nerves were then stimulated with the chosen drug and three distensions were performed. Finally, the drug was replaced with Krebs (washout) and three distensions were performed. This order was used to investigate the change afferent nerve firing induced by the application of various pharmacological agents (unless otherwise stated)

2.3 Data analysis

Distension

Multi-unit nerve activity was quantified using a spike processor, which counted the number of spikes that cross a pre-set threshold; with the threshold level for spike counting set at the peak of the smallest identifiable spike. This was further quantified in a sequential rate histogram, which was used to define the stimulus-response function. Baseline afferent activity was obtained by averaging the discharge in the 100s period prior to the distension. The afferent response during distension was calculated by measuring the afferent discharge per second during various intravesical pressures (0, 5, 10, 15, 20, 25, 30, 35, 40 mmHg). The baseline firing was then subtracted from the given value to give a measure of change in afferent response during distension. The baseline firing was calculated as 100 seconds before the start of the distension.

Investigating the effect of drug on baseline firing

The effect of a drug on baseline firing was calculated by measuring the mean peak response after drug application. The baseline firing was then subtracted from the mean peak response. The time of the response was calculated by measuring the time it took from the application of the drug to the mean peak response (unless otherwise stated).

Bladder compliance

The bladder compliance was calculated from the increase in bladder volume as a function of intravesical pressure

Volume (µl) = Rate (µl min^{-1}) x Time(s)

The pressure and volume relationship was calculated at various intravesical pressures (0, 5, 10, 15, 20, 25, 30, 35, 40mmHg) during bladder filling and determined from the rate of infusion (100µl/min) and the time (s) from the start of the infusion. This calculation was used to construct a pressure-volume xy plot, representing the compliance.

Single unit analysis

To identify and sort individual single units from the multi-unit nerve activity in an experiment, off-line analysis (single unit analysis) using Spike 2 software version 5.14 was performed. Spike templates were formed using a 2.5 m sec^{-1} sampling period, with the cursors set to encompass both the positive and negative ends of the spike. Individual spikes were assigned to a specific waveform template generated from the raw nerve trace. The classification of individual spikes was based on approximately 10% or less differences in amplitude and above 60% of data points within the template boundary.

Afferent nerve discharge is expressed as impulses per second (imp s^{-1}). Data are expressed as means ± S.E.M. Statistical analysis was carried out using a either a 2-way

ANOVA followed by a Bonferroni post-test where necessary or one–way ANOVA/paired Student's t test, followed by Bonferroni post-test and significance was set at $P< 0.05$. (All graphical and statistical analysis used in this thesis was performed using Graph Pad Prism (Version 5.00 for Windows, Graph Pad Software, San Diego California USA, www.graphpad.com).

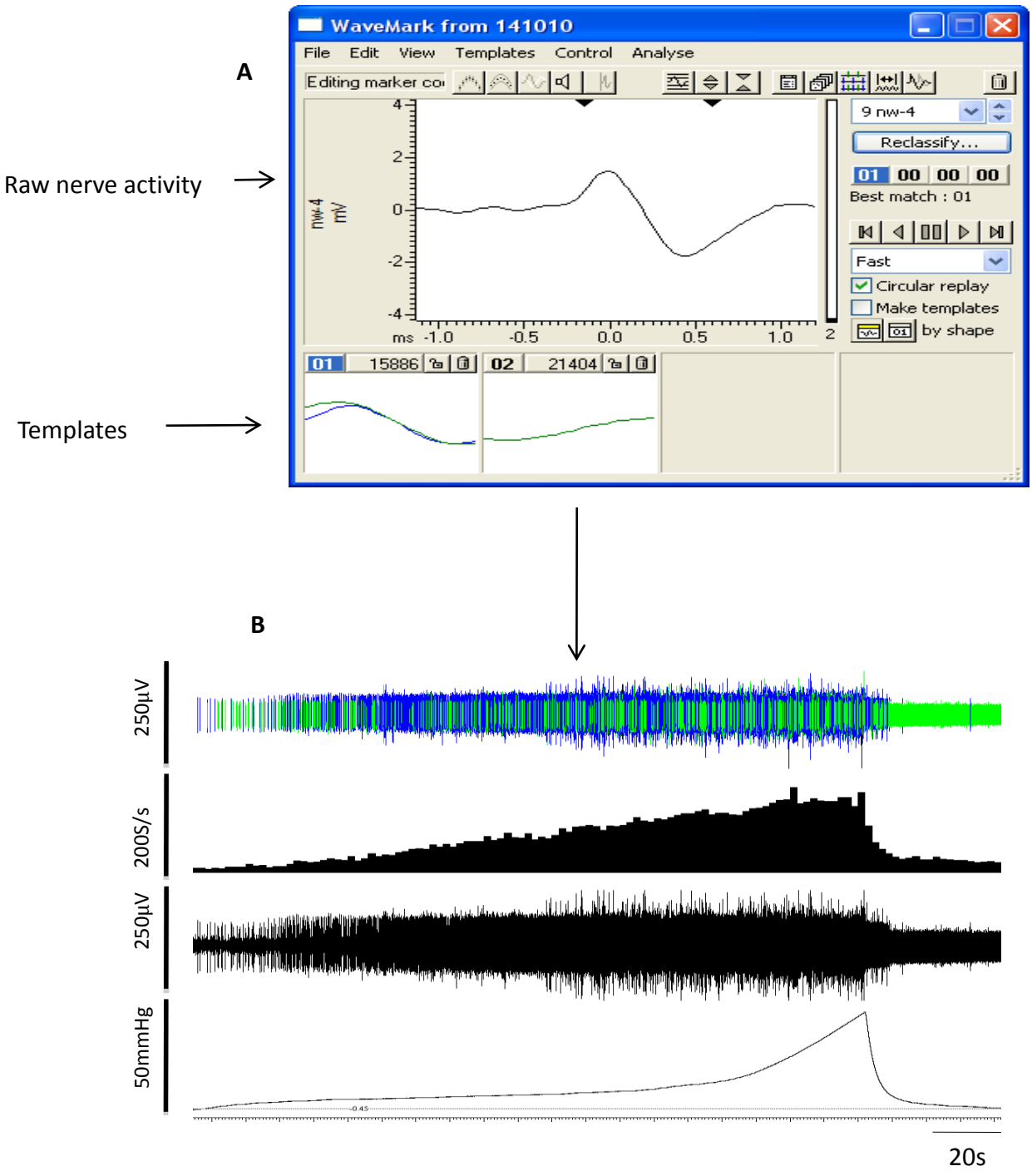


Figure 2.3 Example of single unit analysis. A; example of templates generated from scanning the raw nerve trace using Spike 2 software. B; example of an afferent response to bladder distension and the wavemark generated by spike.

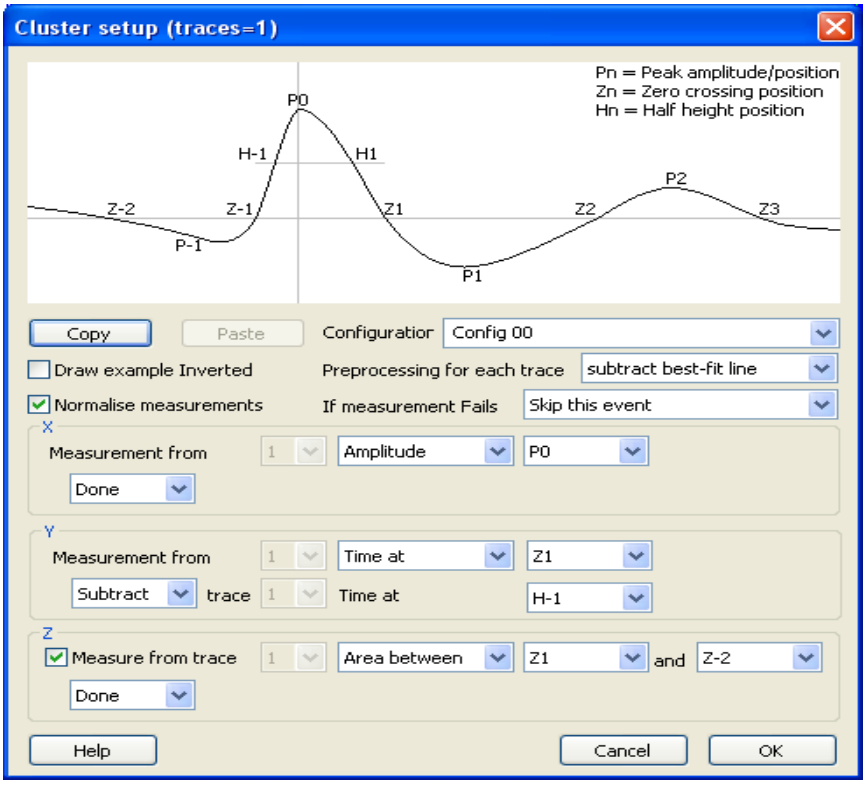
Principal component analysis

Principal component analysis (PCA) was used to differentiate between the waveforms that make up the different spikes. This method was used to analyse whether the single units identified are different enough to be characterised as separate nerve fibres. Principal component analysis extracts the features that contribute towards the differences observed between the waveforms that produce the spike. These include amplitude, latency, area and slope. These data are normalised and scaled as x, y, and z, producing a 3-dimensional display of clusters (Figure 2.4, the clusters are shown in a 2 dimensional display). The different coloured clusters represent different templates. Overlapping clusters were classified as the same nerve fibres. Clusters that could be confidently differentiated as separate groups were defined as distinct nerve fibres. However, since the clusters are evaluated by eye and classification is mainly dependent on individual opinion, these data may be influenced by personal bias.

Low and high threshold nerve fibres

After individual single units were sorted, they were further classified into low and high threshold, depending on their activation threshold. The wavemark was separated based on the distinct nerve fibres and converted into a rate histogram where the firing/sec was plotted at each pressure to a maximal pressure of 40mmHg. Single units firing below a pressure of 15mmHg were defined as low threshold; whereas above 15mmHg were defined as high threshold.

A



B

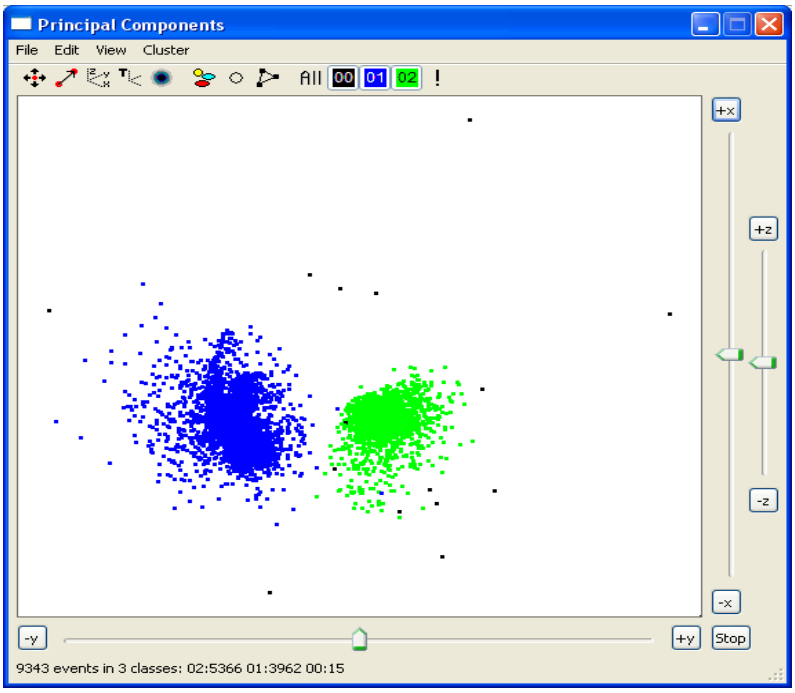


Figure 2.4 example of a two-dimensional figure showing distinct single units. A, Parameters used by Spike 2 to analyse the difference between the templates generated. B, Outcome of the principle component analysis, showing the distinct clusters of fibres.

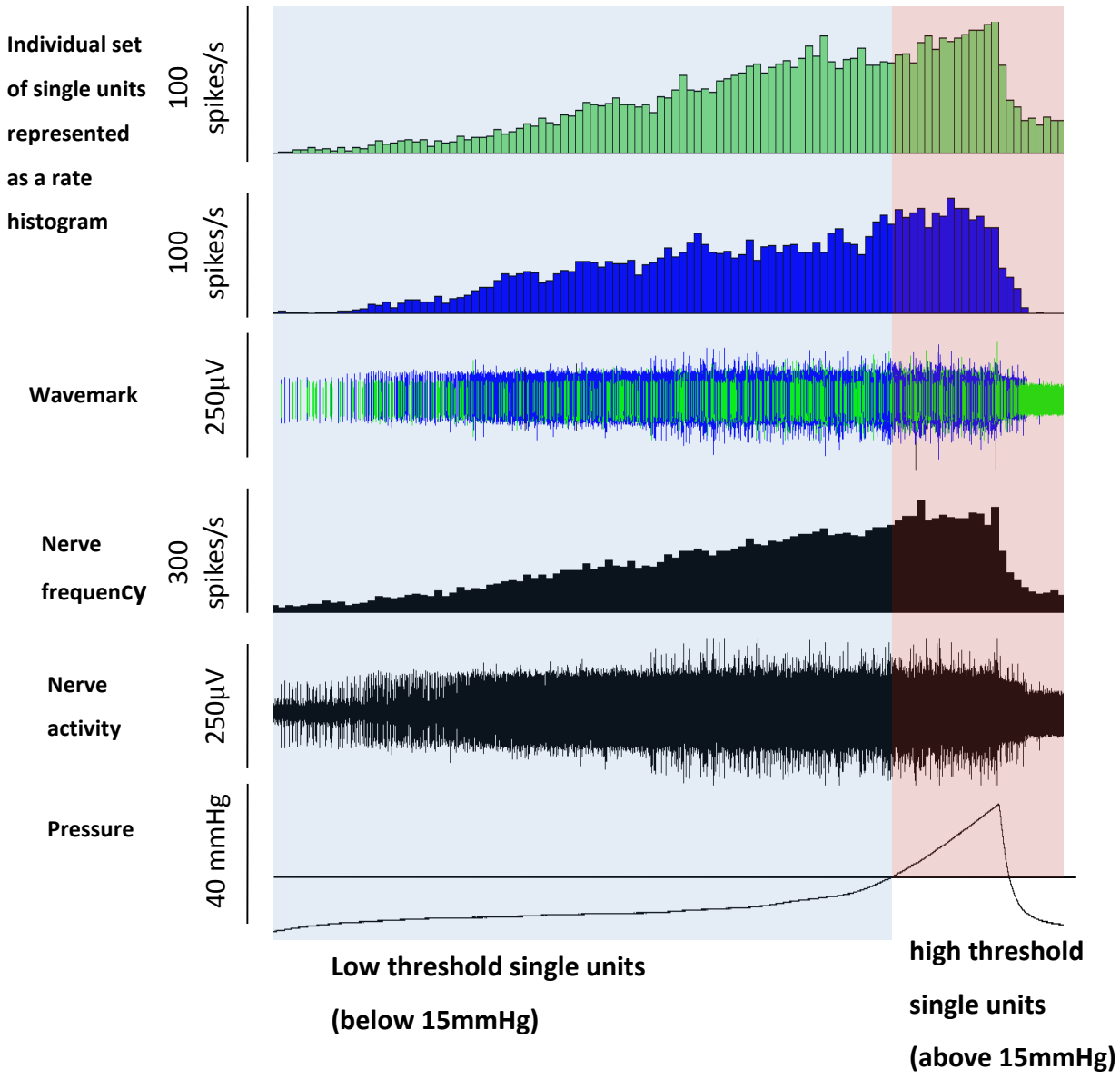


Figure 2.5: Classification of low and high threshold nerve fibres. After the initial single unit analysis, a wavemark was generated. The wavemark allows differentiation between the distinct nerve fibres and can be represented as a histogram (as shown above). The low and high threshold units were calculated by measuring the frequency of the single units responding below an intravesical pressure of 15mmHg and high threshold after 15mmHg.

2.4 Investigating voiding parameters of male and female ERβ$^{-/-}$ and ERβ$^{+/+}$ mice

ERβ$^{+/+}$ and ERβ$^{-/-}$ mice were provided by Pfizer, UK. Maintenance and killing of the animals followed principles of good laboratory practice in compliance with UK national laws and regulations. The mice were killed humanely by CO_2 inhalation followed by cervical dislocation.

Male and female ERβ$^{+/+}$ and ERβ$^{-/-}$ mice were singly housed and maintained under standard laboratory conditions under 12:12 hour reversed dark: light cycle (07:00 lights off) with food and water offered *ad libitum*. There were no overt differences in feeding behaviour and body weight between ERβ$^{+/+}$ and ERβ$^{-/-}$ mice groups. Mice were tail marked and weighed before randomisation. In each experiment two animals were selected from each of group (male ERβ+/+, male ERβ-/-, female ERβ+/+, and female ERβ-/-).

The voiding pattern was assessed by measuring the voiding frequency and volume. For this purpose, animals were placed in metabolic cages for 3hrs to acclimatise for three consecutive days. The total amount of urine voided was measured for 6 hours on the fourth day. Recording was also performed on the 5th day; the mice were water-loaded, by intraperitoneal injection (20/ml per kg) of distilled water. Two mice from each group were randomly placed in metabolic cages with free access to distilled water. The urine was collected on a sponge placed on a balance (Mettler PM 100 or AJ 100, Mettler-Toledo AG, Greifensee, Switzerland) connected to a PC running Notocord data acquisition software. Voiding frequencies and volumes were recorded for 6 hrs.

2.5 Isolation and culturing of primary urothelial cells

After cervical dislocation, the whole bladder was removed and placed in Phosphate buffered saline solution *(*PBS). The bladder was cut from the urethra upwards and pinned flat in a Sylgard coated dish, with the urothelium facing upwards. The bladder was incubated with Minimum Essential Media (MEM) (Invitrogen) containing 2.5 mg/ml of dispase II enzyme (Sigma) for 3 hours at room temperature. The urothelial cells were then gently scraped from the underlying muscle layer and treated with trypsin-EDTA (Invitrogen) for 5-10 minutes and resuspended in Keratinocyte media (Invitrogen). Urothelial cells were cultured as previously described (Everaerts *et al.* 2009). The cell suspension was counted and plated on collagen (IV) coated coverslips and incubated overnight at 37°C in an atmosphere of 5% CO_2- 95% O_2.

2.6 Isolation and culturing of DRGs

Following cervical dislocation the DRGs were removed from the T11-L2 and L6-S2 region of the spinal cord. The isolated DRGs were placed in Hank's Balanced Salt Solution HBSS (pH7.4) (Invitrogen) and treated with 0.7mg/ml L-Cystein (Sigma- Aldrich (Poole,UK) and 4mg/ml papain (Sigma- Aldrich (Poole,UK) for 20 minutes at 37^{o}C and then 4mg/ml collagenase (Sigma- Aldrich (Poole,UK) and 4.7mg/ml dispase (Sigma- Aldrich (Poole,UK) for 20 min at 37^{o}C. Following treatment, the DRGs were washed with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 **(**DMEM/F12) (Invitrogen) culture media, containing 1 in 10ml fetal calf serum (FCS) (Invitrogen). The enzymatically treated DRG were gently triturated using a Pasteur pipette and then distributed onto Matrigel coated glass coverslips (6x16mm). Cells were then incubated in a 5% CO_2-95% O_2 incubator at 37°C for 2 hours.

Following incubation, the cells were flooded with PenStrepto media (Invitrogen) and incubated overnight at 37C in a 5% CO_2-95% O_2 incubator.

2.7 Calcium imaging

The measurement of intracellular calcium concentrations was performed by loading the cells with 2 μM fura-2 acetoxymethyl ester (fura-2AM) (Sigma- Aldrich (Poole,UK) 0 for 30 min at 37°C in the dark. The ratiometric fluorescent dye diffuses into the cell and binds to free intracellular calcium. After 30 min the cells were transferred into a perfusion chamber and mounted onto an fluorescence microscope and perfused with HEPES buffer (150mM NaCl, 6mM CsCl, 1mM $MgCl_2$, 5mM $CaCl_2$, 10mM glucose, and 10mM HEPES, buffered to pH 7.4 with NaOH) for 30 minutes. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as the ratio between the fluorescence signal measured at 350 nm and at 380 nm normalized to baseline. Regions of interest were chosen, based on the number and type of cells present. After 30 minutes of perfusion with HEPES buffer, cells were stimulated for 2 minutes with HEPES containing the drug to be investigated. The drug was then washed out by switching the perfusion back to HEPES buffer, finally the cells were stimulated with the calcium ionophore ionomycin (5μM) (Sigma- Aldrich (Poole,UK). Ionomycin was applied as positive control and only the responding cells were included in the analysis. Results are expressed as relative fluorescence (RF). All the drugs were prepared in HEPES buffer.

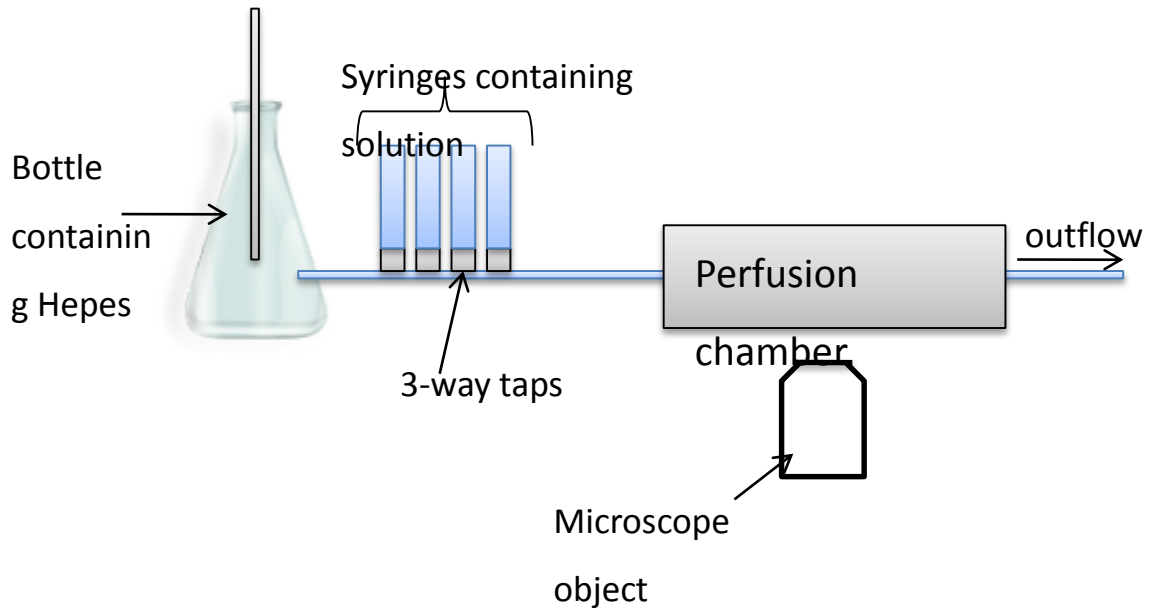


Figure 2.6. Schematic diagram of the Ca2+ imaging perfusion system
Standard imaging solution in a 1 litre bottle continually superfused cells on a coverslip in the recording chamber. Syringes on a perfusion rack were used to apply additional solutions to cells; the outflow tube was connected to a peristaltic pump.

2.8 Drugs and Solubility

All the drugs used in this thesis are listed in table 2.1. The data from the drug manufacturer was used to determine the solubility of a drug. All the drugs were dissolved in either ethanol (Sigma-Aldrich (Poole,UK) or DMSO (Sigma- Aldrich (Poole,UK) to make a stock solution. The stock was then finally diluted in either saline (for intra-luminal application) or Krebs (extra-luminal application) to the appropriate final concentration. In each case a vehicle control was performed to account for any effect of the solvent. The purchase information of the drugs are listed in table 2.1

Table 2.1 Summary of the drugs used in this study

Compound	Main action	concentration	solubility	Purchased from
17β-estradiol	Oestrogen receptor agonist	1μM, 30μM, 100μM	DMSO	Sigma-Aldrich (Poole,UK)
Allylisothiocyanate (AITC)	TRPA1 agonist	1μM, 3μM, 30μM, 100μM	Ethanol	Sigma-Aldrich (Poole,UK)
Capsaicin	TRPV1 agonist	1μM, 30μM, 100μM	Ethanol	Sigma-Aldrich (Poole,UK)
Capsazepine	TRPV1 antagonist	10μM	Ethanol	Sigma-Aldrich (Poole,UK)
Cinnamaldehyde	TRPA1 agonist	10μM, 100μM	DMSO	Sigma-Aldrich (Poole,UK)
Forskolin	Protein kinase A activator	10μM	DMSO	Sigma-Aldrich (Poole,UK)
GÖ- 6983	Protein kinase C inhibitor	1μM	Ethanol	Tocris Cookson (Bristol, UK)
H-89 dihydrochloride	Protein kinase A inhibitor	1μM	DMSO	Sigma-Aldrich (Poole,UK)
Hydrogen sulphide	TRPA1 agonist	100μM	Distilled water	Sigma-Aldrich (Poole,UK)
Menthol	TRPM8 agonist	1μM, 3μM, 10μM, 30μM, 100μM, 150μM, 2mM	Ethanol	Sigma-Aldrich (Poole,UK)
PF-05105679	TRPM8 antagonist	10μM	DMSO	Pfizer, UK
Phorbol 12-myristate 13-acetate (PMA)	Protein kinase C activator	10μM	DMSO	Sigma-Aldrich (Poole,UK)
U-73122	Phospholipase C inhibitor	10μM	Ethanol	Sigma-Aldrich (Poole,UK)
WS-12	TRPM8 agonist	10μM, 30μM 100μM	DMSO	Sigma-Aldrich (Poole,UK)
α,β-Methyleneadenosine 5'triphosphate (αβMethATP)	P2X agonist	3μM, 10μM, 30μM, 100μM	DMSO	Sigma-Aldrich (Poole,UK)

CHAPTER 3

THE EFFECT OF TRPM8 ACTIVATION ON BLADDER AFFERENT FIRING

3.1. Aim of the study

Fundamental questions regarding the role of TRPM8 in sensory signalling from the normal bladder and any changes that might occur in disease. The current study combines a transgenic approach utilizing the TRPM8 knockout mouse and TRPM8 selective compounds to investigate the role of TRPM8 in normal bladder afferent signalling.

3.2 Experimental protocol and analysis of data

The afferent nerve recording technique was performed as described in chapter 2

Measurement of afferent nerve activity

Bladder afferent responses were investigated both at rest and during distension. The mechanical afferent response to distension was assessed, by filling the bladder to a maximum pressure of 40mmHg. Bladders were filled using isotonic saline at a rate of 100µl min^{-1}. Several distensions (typically 3 or 4) were carried out every 10 minutes, until stable responses were achieved. The various protocols described below were undertaken only once a stable response to distension had been obtained. The afferent response to distension was analysed as the afferent activity per unit of pressure and is expressed as impulses per second (imp sec^{-1}). Baseline afferent nerve firing was assessed as the mean afferent discharge in a period of 100s before bladder distension.

Afferent response to Menthol and WS-12

To investigate the role of TRPM8 in bladder afferent firing, a cumulative dose response with the TRPM8 agonists Menthol (1µM, 3µM, 10µM, 30µ M, 100µM, 150µM, 300µM, 1mM and 2mm) and WS-12 (1µM, 3µM, 10µM and 30µM) were performed. These compounds were administered intraluminally into the bladder of TRPM8 knockout mice and their wild type littermates to compare effects on baseline firing and responses to distension. After each drug application a washout period of 30 minutes was carried out using intraluminal infusion of isotonic saline (NaCl, 0.9%).

The effect of cold (12°C) on the afferent response to distension

To investigate the contribution of TRPM8 in sensing cold within the bladder, cold saline at a temperature of 12°C was administered into the bladder. In preliminary experiments the response to cold was seen to desensitize with repeat application. To avoid this alternating application of both cold and warm saline (12°C and 38°C) were used, followed by a recovery period of 30 minutes using intraluminal infusion of isotonic saline (NaCl, 0.9%, 36°C).

The effect of PF-05105679 on **the afferent response to distension**

The effect of the TRPM8 antagonist PF-05105679 on the afferent response to bladder distension, baseline firing and compliance was assessed. PF-05105679 was applied intraluminally at a maximum concentration of 10μM. In separate experiments PF was applied either alone or in combination with WS-12.

The single unit response to distension

Spike 2 software was used to analyse the multiunit afferent nerve recordings in order to quantify activity in individual single units at baseline and in response to distension. Fibres responding at intraluminal pressure below 15mmHg were characterized as low threshold units, whereas fibres responding above 15mmHg were characterized as high threshold units. Principle component analysis for each experiment was carried out to verify the accuracy of single unit discrimination.

Statistical significance

Where appropriate data is always displayed as means ± S.E.M. Statistical analysis was carried out using a either a 2-way ANOVA with a Bonferroni post-test or one-way ANOVA.
Significance was assumed at $P<0.05$.

3.3 Results

3.3.1 Comparison of sensory nerve response between TRPM8 WT and KO mice

We saw no obvious bladder phenotype between the TRPM8 knockout animals and their wild type littermates. This was confirmed by the lack of any difference in either bladder compliance or afferent response at baseline and during distension. These data are summarized in Figure 3.1, which shows the afferent response to distension, baseline firing and bladder compliance. No differences were also found when the afferent responses were further analysed with single unit analysis (Figure 3.2 and 3.3).

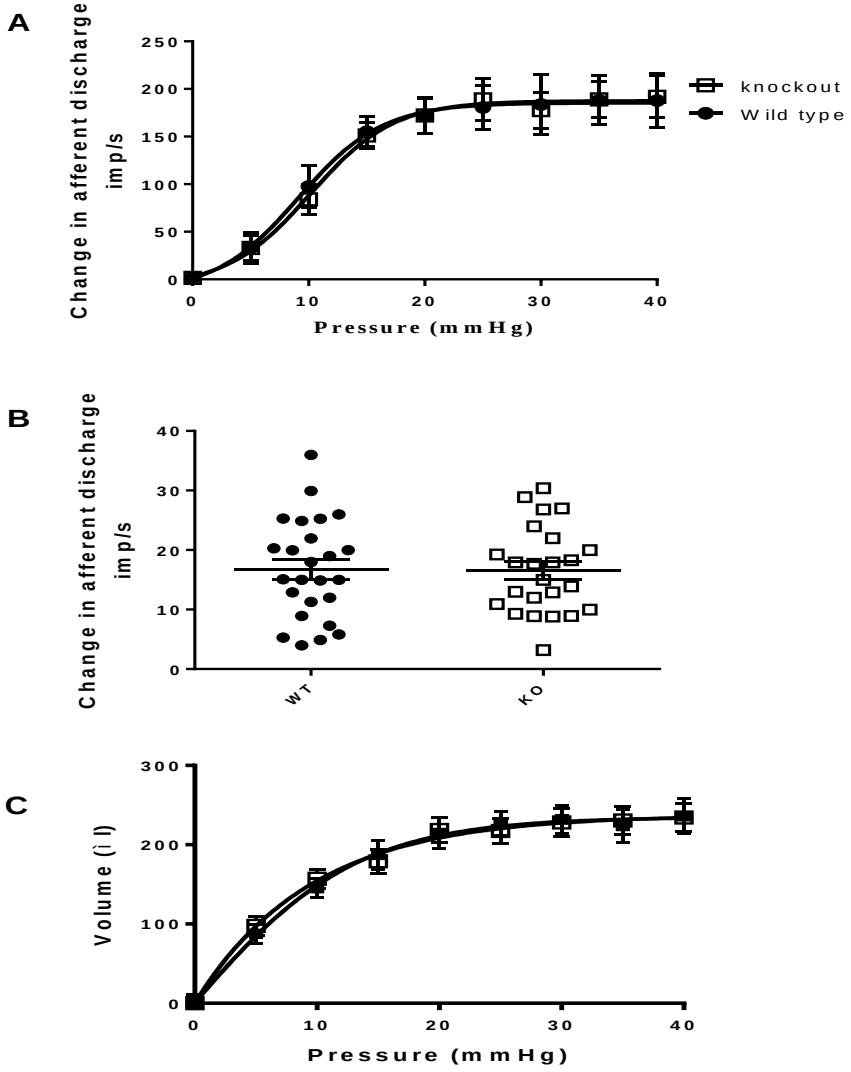


Figure 3.1: comparison of afferent response between TRPM8$^{+/+}$ and TRPM8$^{-/-}$

A) The afferent response to an increase in intravesical pressure during ramp distension with isotonic saline (0.9%) to a maximal intravesical pressure of 40mmHg. **B)** No significant difference in the spontaneous firing between WT and KO mice. **C)** There was no change (P>0.05) in bladder compliance. Results are shown as mean ± SEM (n = WT: 24, KO: 22).

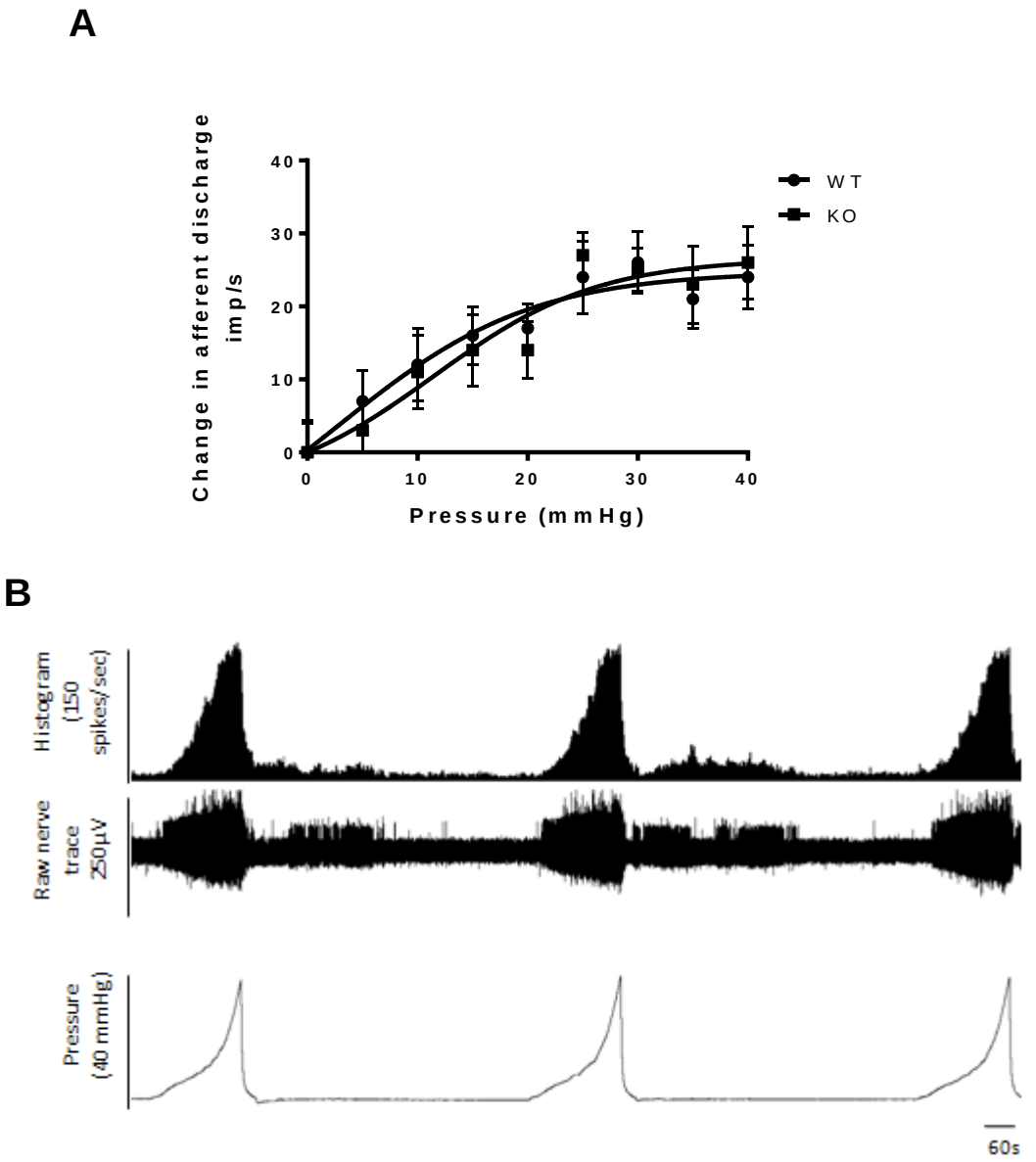


Figure 3.2: The response to bladder distension in KO mice.

A) Fibre averaging analysis reveals no change in the response between TRPM8$^{-/-}$ mice and TRPM8$^{+/+}$ mice in response to a control ramp distension **B)** Three reproducible control distensions in KO mice.

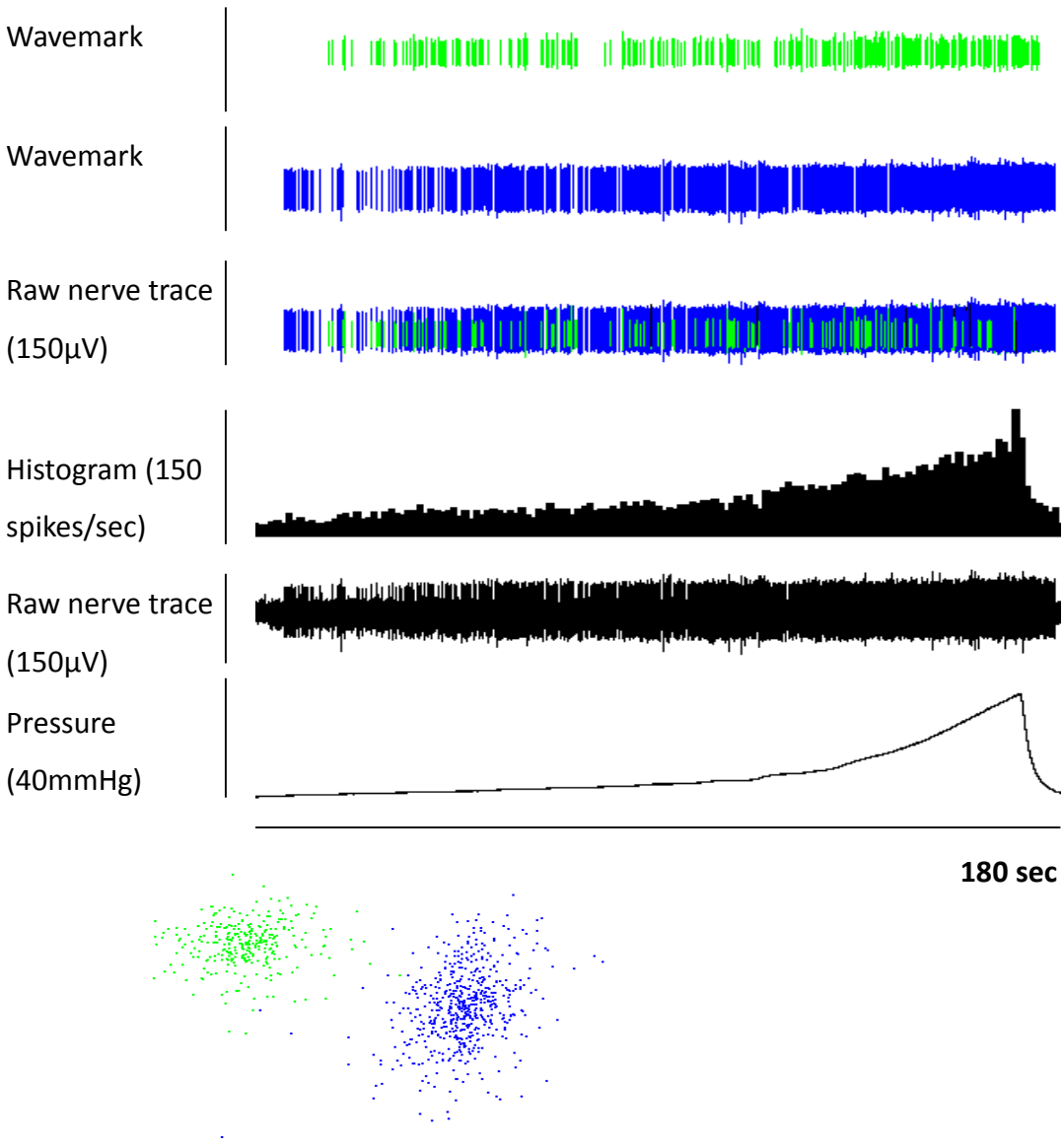


Figure 3.3: Single unit analysis of a distension response in KO mice. Single unit analysis, producing two distinct wavemarks. Principle component analysis showing district clusters of fibres.

3.3.2 The effect of menthol on bladder afferent firing

To investigate the effect of activating TRPM8 directly on bladder afferents, menthol was used. A cumulative concentration curve of menthol was produced, showing both excitatory and inhibitory effects (Figure 3.4). 1μM, 3μM, 10μM, 30μM, 100μM, 150μM, 300μM and 1mM menthol was intraluminal applied into the bladder. 150μM menthol was also applied on its own, resulting in a significant increase in firing both at distension and baseline in the WT (Figure 3.5). The excitatory response was absent in the KO mice (Figure 3.6). This suggests a TRPM8 dependent effect of menthol at this concentration. At a concentration of 2mM menthol resulted in significant inhibition, both in the WT and KO mice (Figure 3.7-3.9).

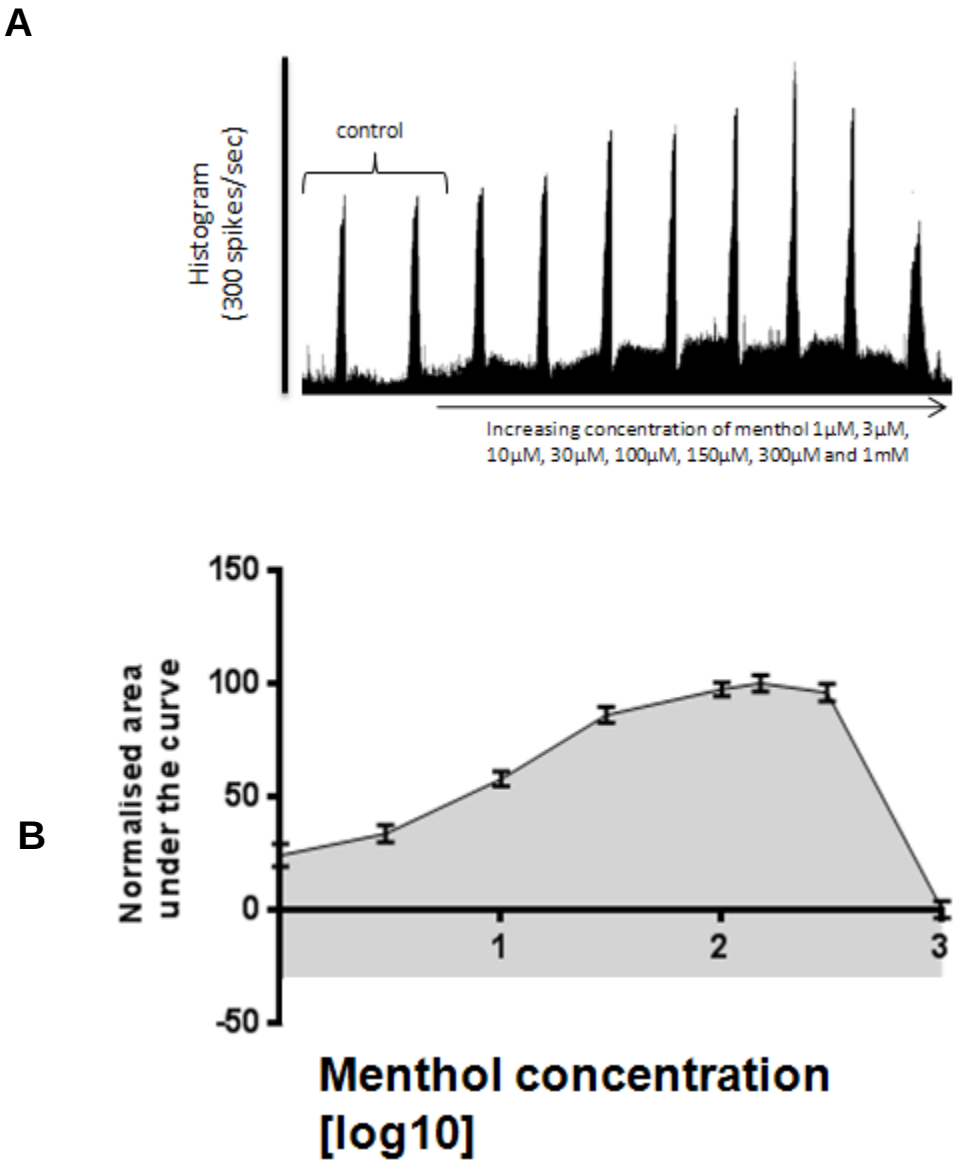


Figure 3.4: effect of menthol on the afferent response to bladder distension in wild type mice.
A; Nerve histogram showing response to the infusion of 1µM, 3µM, 10µM, 30µM, 100µM, 150µM, 300µM and 1mM menthol. **B;** Menthol caused a significant ($P<0.01$ one way ANOVA) increase of spontaneous activity. Results are shown as mean ± SEM ($n = 6$).

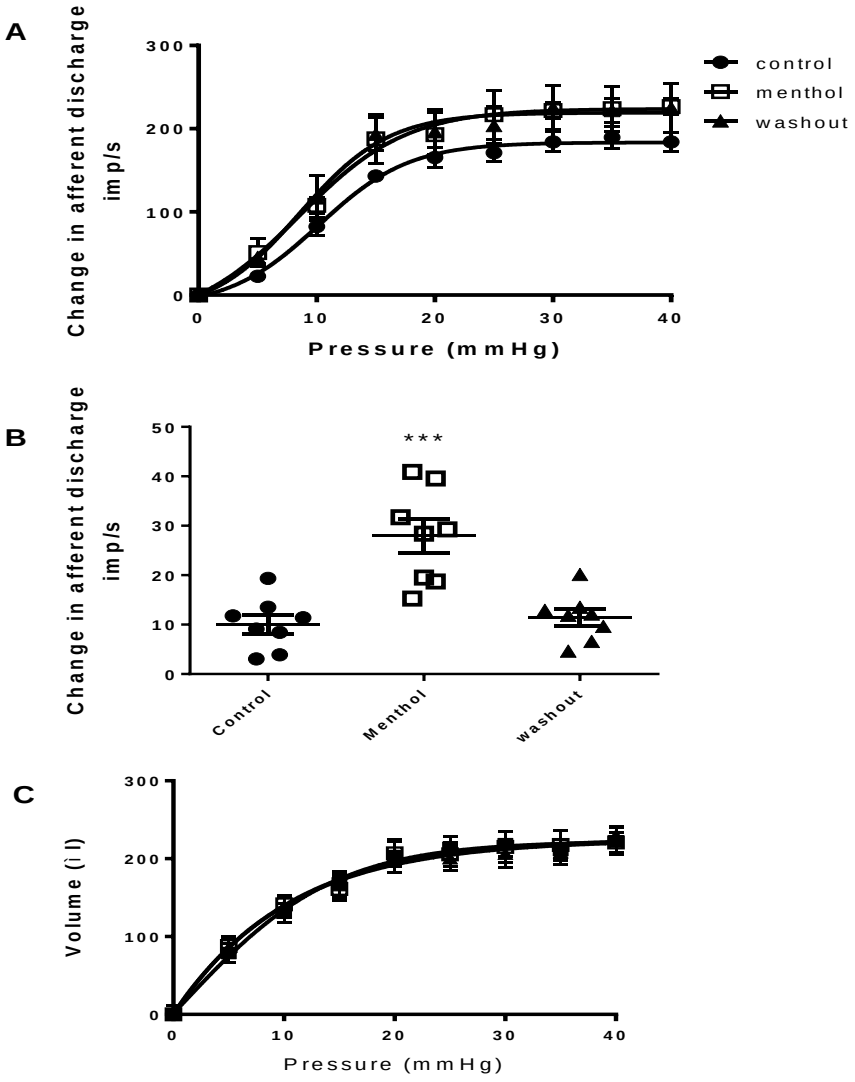


Figure 3.5: The effect of 150µM menthol on the afferent response to bladder distension in wild type mice.
A; Menthol caused an increase ($P < 0.05$ two way ANOVA) of the afferent response to bladder distension, followed by a 30 minute washout **B;** Menthol caused a significant (P<0.01 one way ANOVA) increase of spontaneous activity, followed by a complete washout. **C;** there was no change ($P > 0.05$) of bladder compliance. Results are shown as mean ± SEM ($n = 6$). ***$P < 0.001$ compared to control.

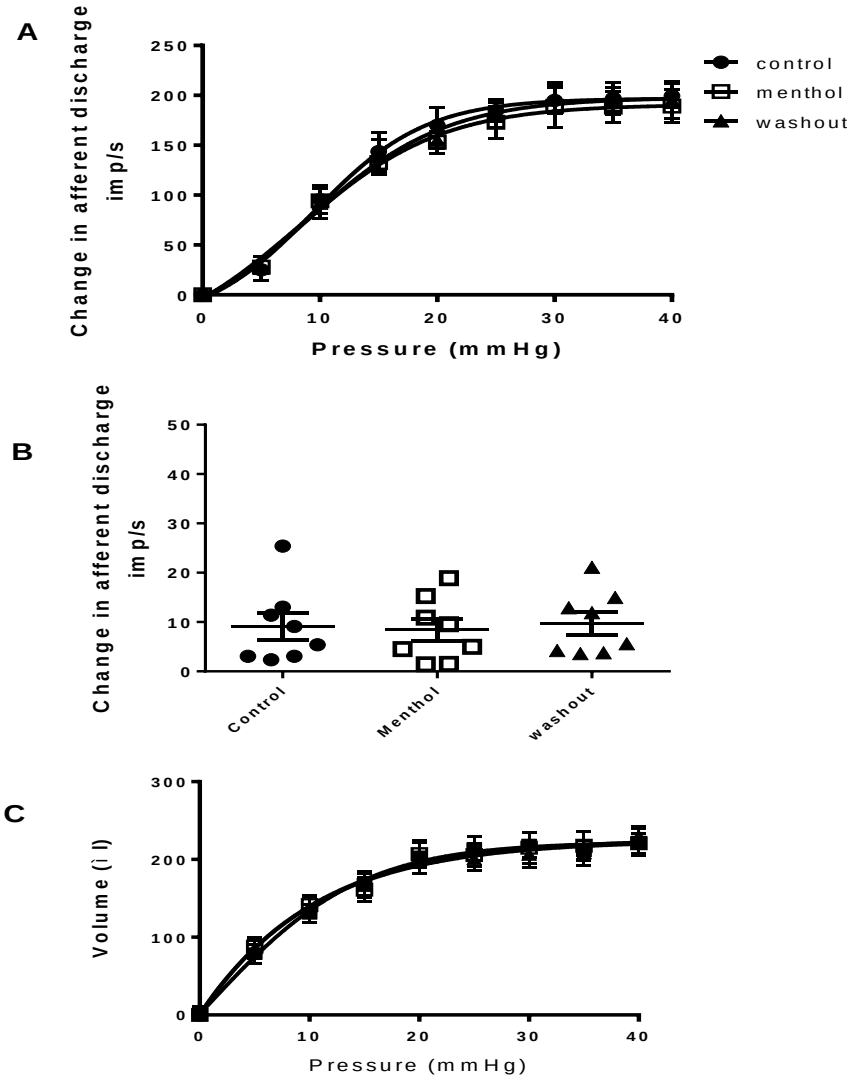


Figure 3.6: The effect of 150µM menthol on the afferent response to bladder distension in TRPM8$^{-/-}$.
A; Menthol did not change (P > 0.05 two way ANOVA) the afferent response to bladder distension **B;** No alteration (P > 0.05 one way ANOVA) of spontaneous activity was observed. **C;** There was no change (P>0.05) in bladder compliance. Results are shown as mean ± SEM (n = 6).

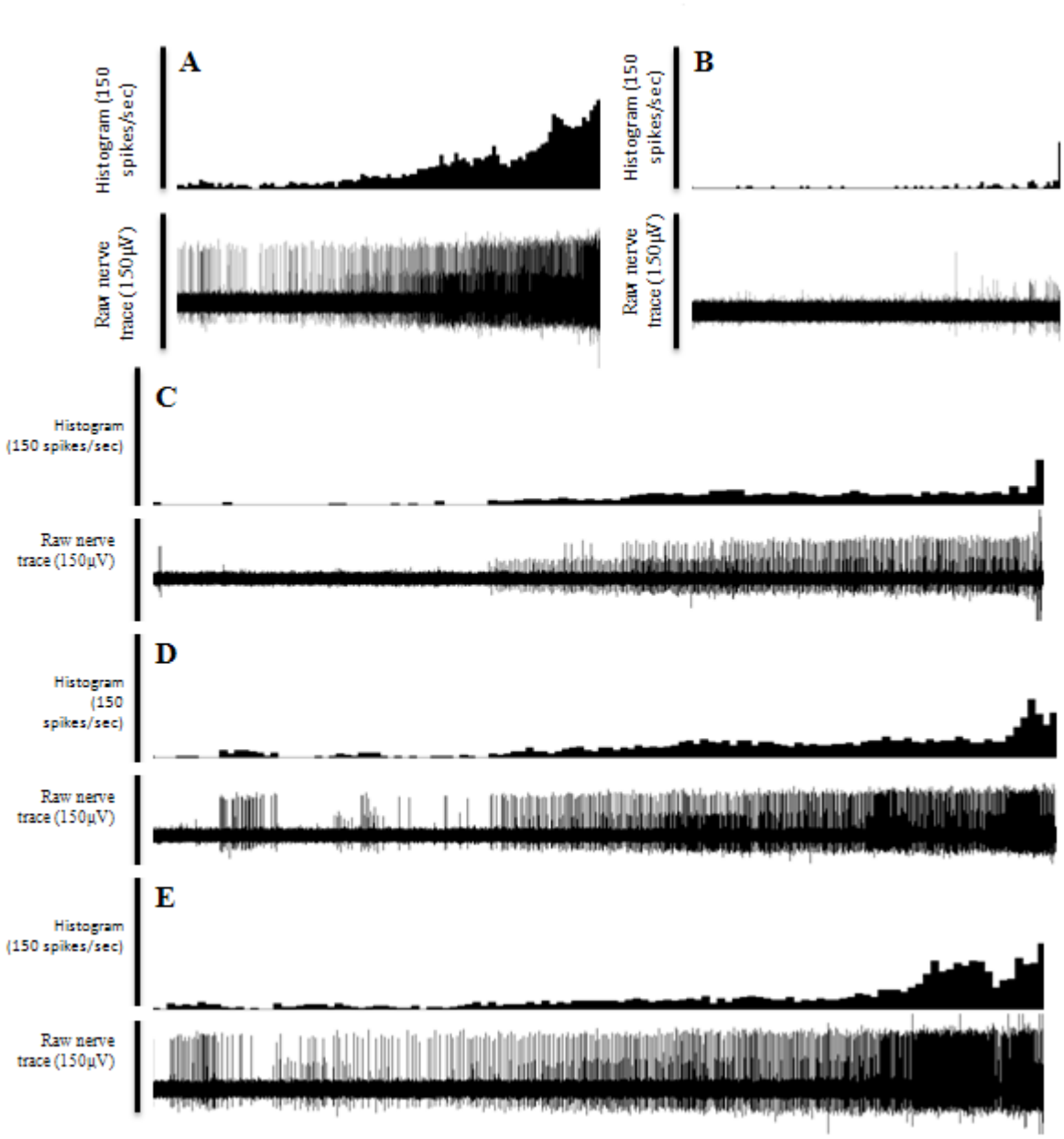


Figure 3.7: The effect of 2mM menthol on the afferent response to bladder distension in wild type mice.
A; Raw nerve trace and the corresponding histogram in response to a control distension in WT mouse. **B;** Response to distension after application of 2mM menthol. **C;** response to distension during first washout. **D;** 2nd washout **E**; third washout.

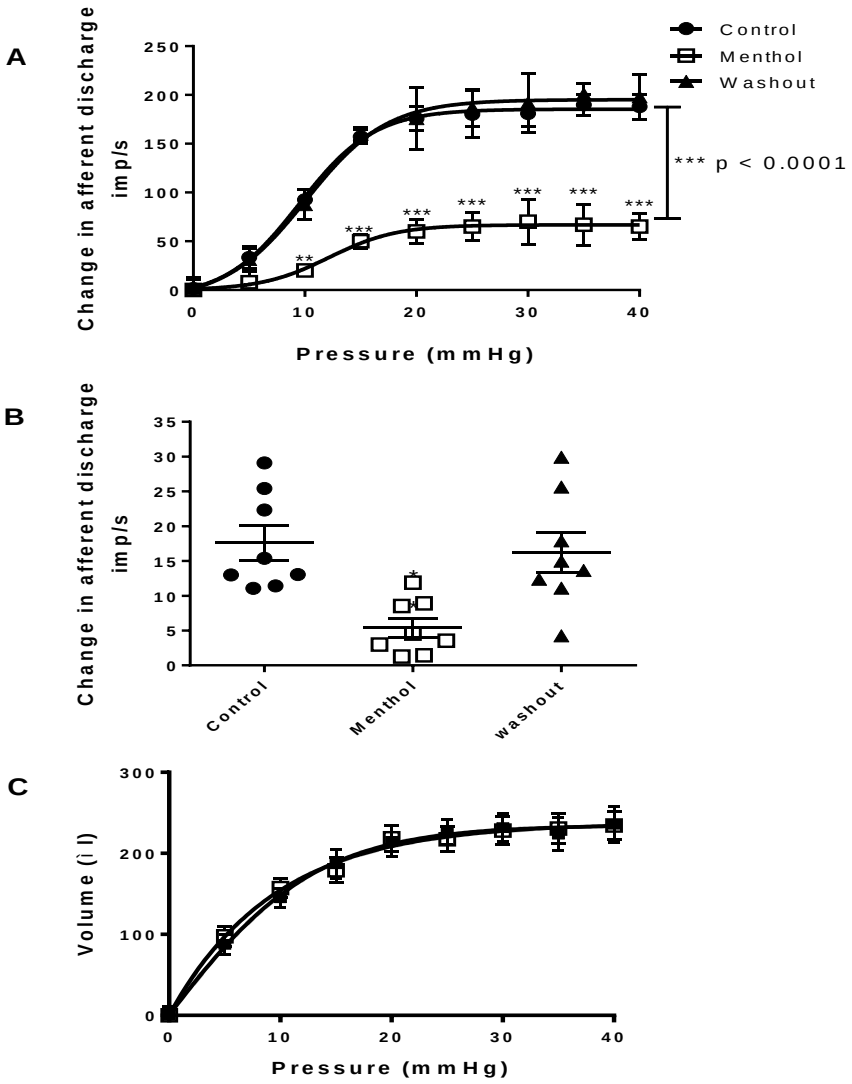


Figure 3.8: The effect of 2mM menthol on the afferent response to bladder distension in wild type mice.

A; Menthol caused a profound attenuation (***P* < 0.01 ****P* < 0.001 two way ANOVA) of the afferent response to bladder distension, followed by a washout. **B;** Menthol caused a significant (**P<0.01 one way ANOVA) decrease of baseline firing. **C;** There was no significant difference (*P*>0.05) in the bladder compliance. Results are shown as mean ± SEM (*n* = 6).

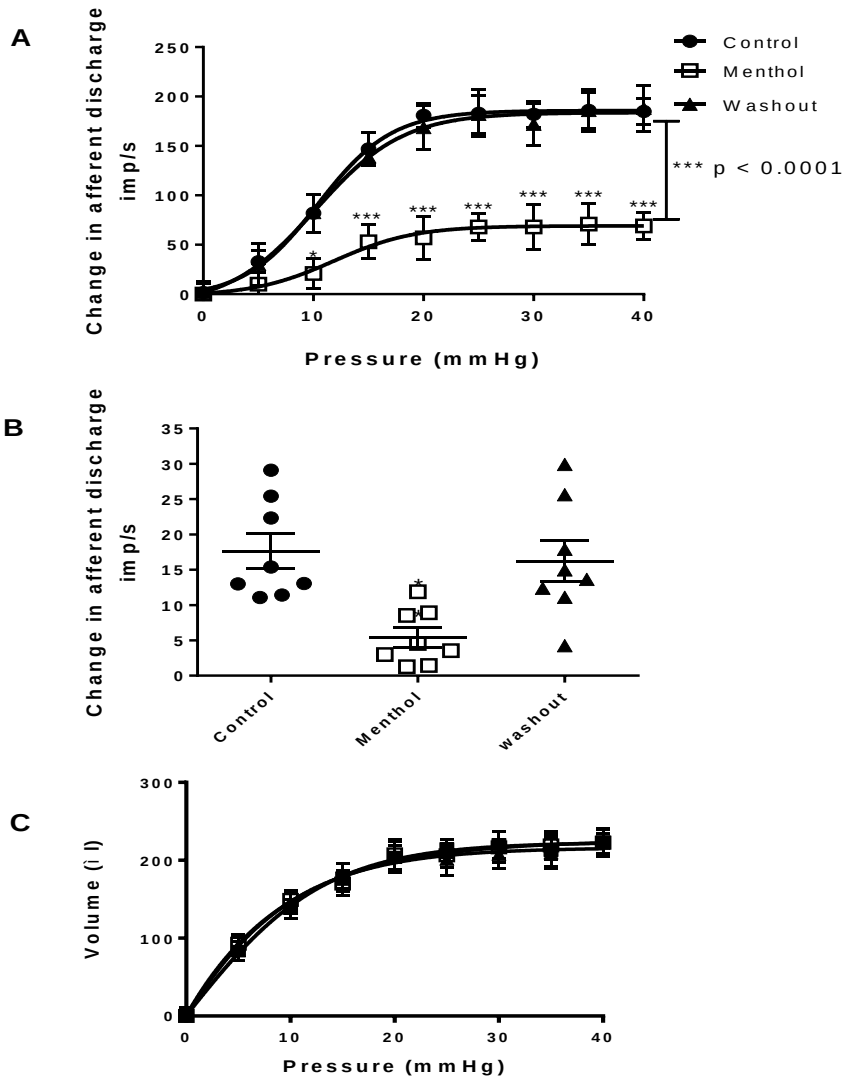


Figure 3.9: The effect of 2mM menthol on the afferent response to bladder distension in TRPM8$^{-/-}$ mice.

A; Menthol caused a profound attenuation (*P < 0.05 **P < 0.01***P < 0.001 two way ANOVA) of the afferent response to bladder distension, followed by a complete washout. **B;** Menthol caused a significant (**P<0.01 one way ANOVA) decrease of baseline firing. **C;** There was no significant difference (P>0.05) in the bladder compliance. Results are shown as mean ± SEM (n = 6).

3.3.3 The effect of WS-12 on bladder afferent firing

Because menthol appeared to have non-specific effect on bladder sensory afferent, we used a more specific agonist WS-12. The intraluminal and extraluminal application of 10μM WS-12 caused a significant increase of the afferent response to distension, although there was no recovery after 30 minutes of washout. No changes were observed at baseline firing. The compliance was also unchanged in the WT mice (Figure 3.10 and 3.11). In the KO mice WS-12 did not induce any changes in the response to distension, baseline firing and compliance, suggesting that the increase in afferent response observed during distension is TRPM8 specific (Figure 3.13).

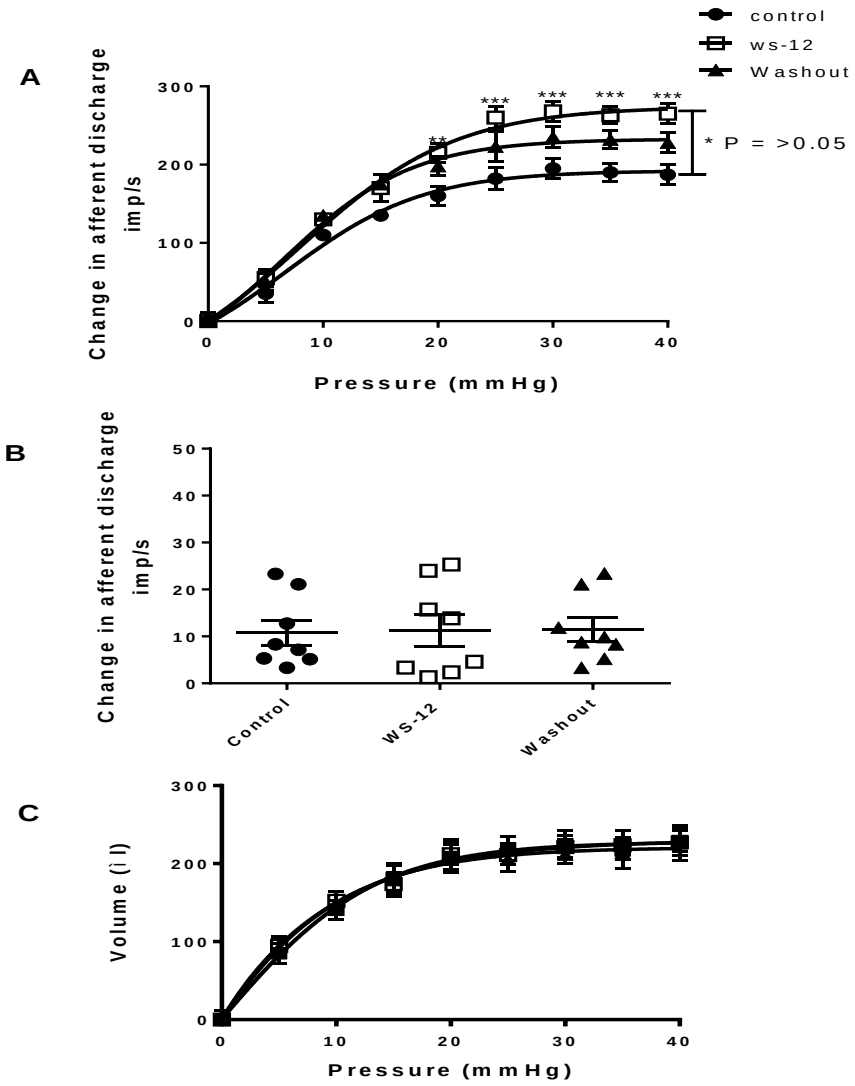


Figure 3.10: The effect of extra-luminal application of 10µM WS-12 on the afferent response to bladder distension in wild type mice.
A; WS-12 caused a profound increase (**$P < 0.01$ ***$P < 0.001$ two way ANOVA) of the afferent response to bladder distension. **B;** There was no significant alteration ($P>0.05$) of spontaneous activity. **C;** There was no significant difference ($P>0.05$) in bladder compliance. Results are shown as mean ± SEM ($n = 6$)

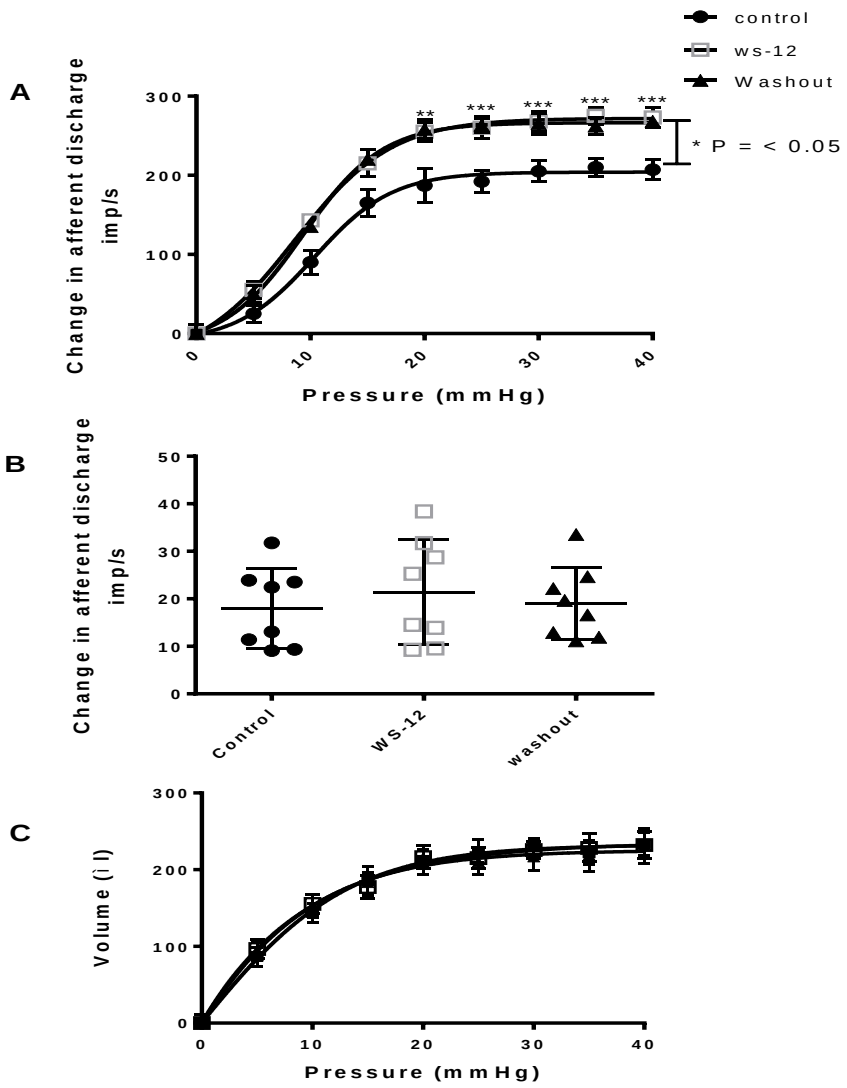


Figure 3.11: The effect of intraluminal infusion of 10µM WS-12 on the afferent response to bladder distension in wild type mice.
A; WS-12 caused a profound increase (**$P < 0.01$ ***$P < 0.001$ two way ANOVA) of the afferent response to bladder distension. **B;** There was no significant alteration ($P>0.05$) of spontaneous activity. **C;** There was no significant difference ($P>0.05$) in bladder compliance. Results are shown as mean ± SEM ($n = 6$)

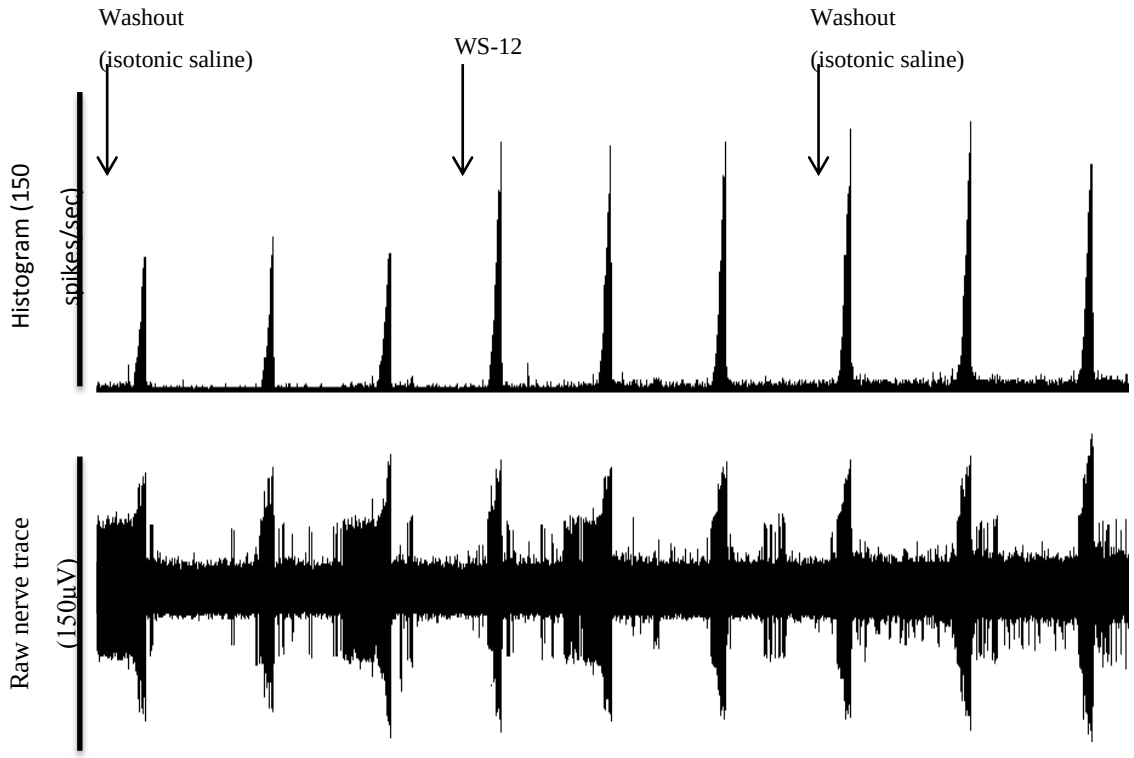


Figure 3.12: The effect of 10µM WS-12 on the afferent response to bladder distension in wild type mice.
Raw nerve trace and histogram showing response to extra-luminal infusion of WS-12.

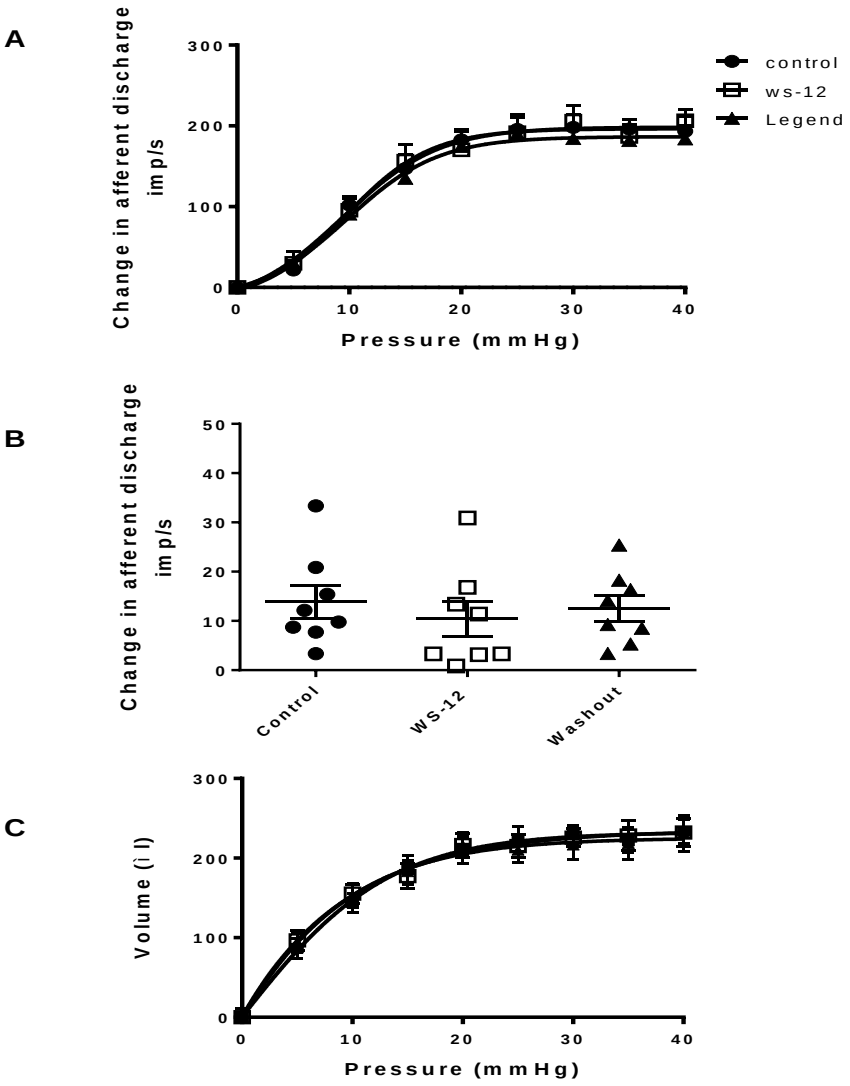


Figure 3.13: The effect of 10μM WS-12 on the afferent response to bladder distension in TRPM8$^{-/-}$ mice.
A; WS-12 did not alter the afferent response to mechanical stimulation. **B;** There was no change (P>0.05) of spontaneous activity. **C;** There was no significant difference (P>0.05) in bladder compliance. Results are shown as mean ± SEM (n = 6)

3.3.4 Effect of cold saline on bladder afferents

We tried to investigate the contribution of TRPM8 on the ice water test by infusing cold saline into the bladder of WT and TRPM8 KO mice. The infusion of cold saline (12°C) caused a significant increase in the afferent response to distension in the WT mice. This increase was only seen in the first distension. No changes were observed at baseline firing. The infusion of cold saline had no effect on either the afferent response to distension or baseline firing in the KO mice (Figure 3.14). In an attempt to avoid the desensitisation, seen in the subsequent distension in the WT mice, we used alternating infusion of cold (12°C) and warm (38°C) saline. This caused a significant increase in all the distension responses in the WT. No changes were observed in at baseline firing. Neither the distension response nor baseline firing was affected by this protocol (Figure 3.15).

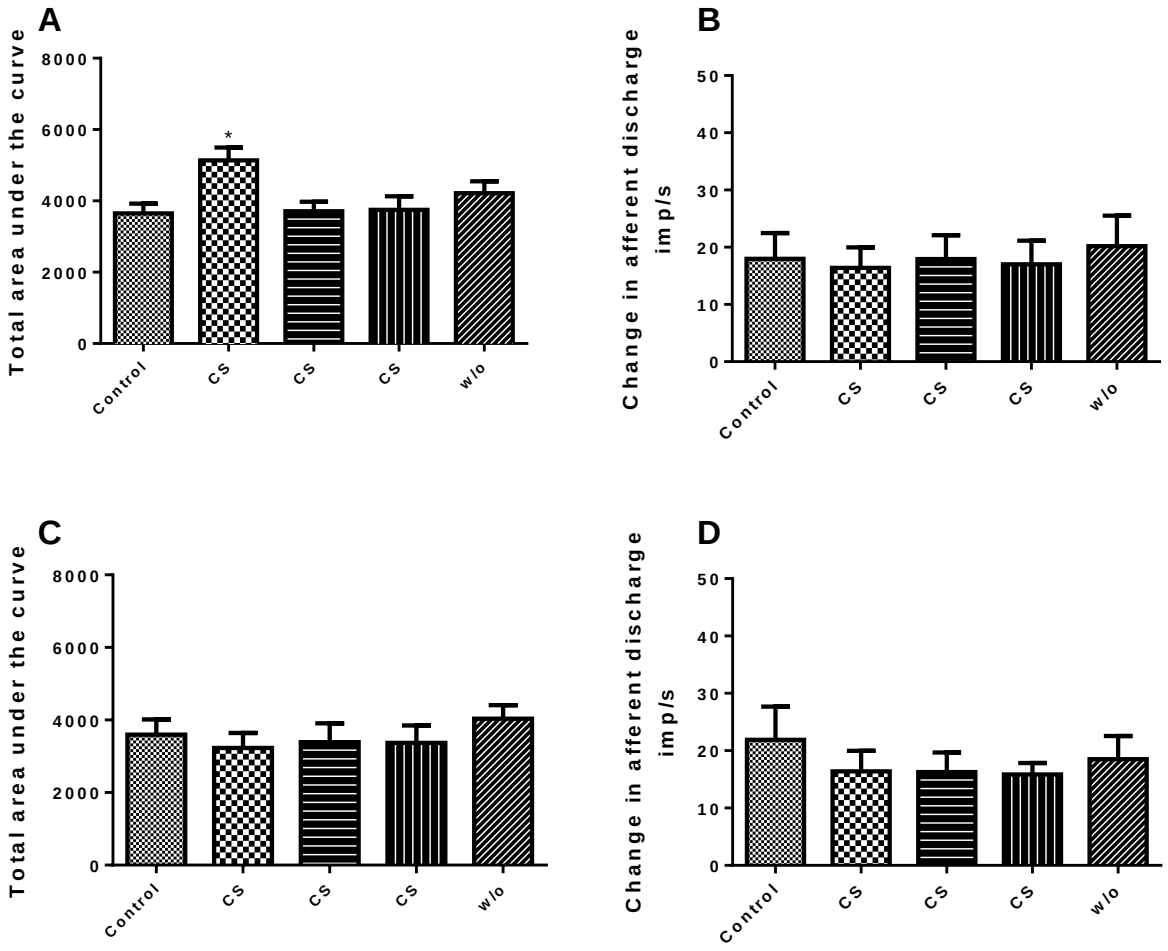


Figure 3.14: The effect of cold saline (12 °C) on the afferent response to bladder distension in TRPM8$^{+/+}$ and TRPM8$^{-/-}$ mice.
A; infusion of cold saline significantly increased (*P < 0.05 one way ANOVA) the afferent response to mechanical stimulation in WT mice. **B;** There was no change (P>0.05) of spontaneous activity in WT mice. **C;** infusion of cold saline did not alter the afferent response to mechanical stimulation in KO mice. **D;** There was no significant difference (P>0.05) in spontaneous activity in KO mice. Results are shown as mean ± SEM (n = 6)

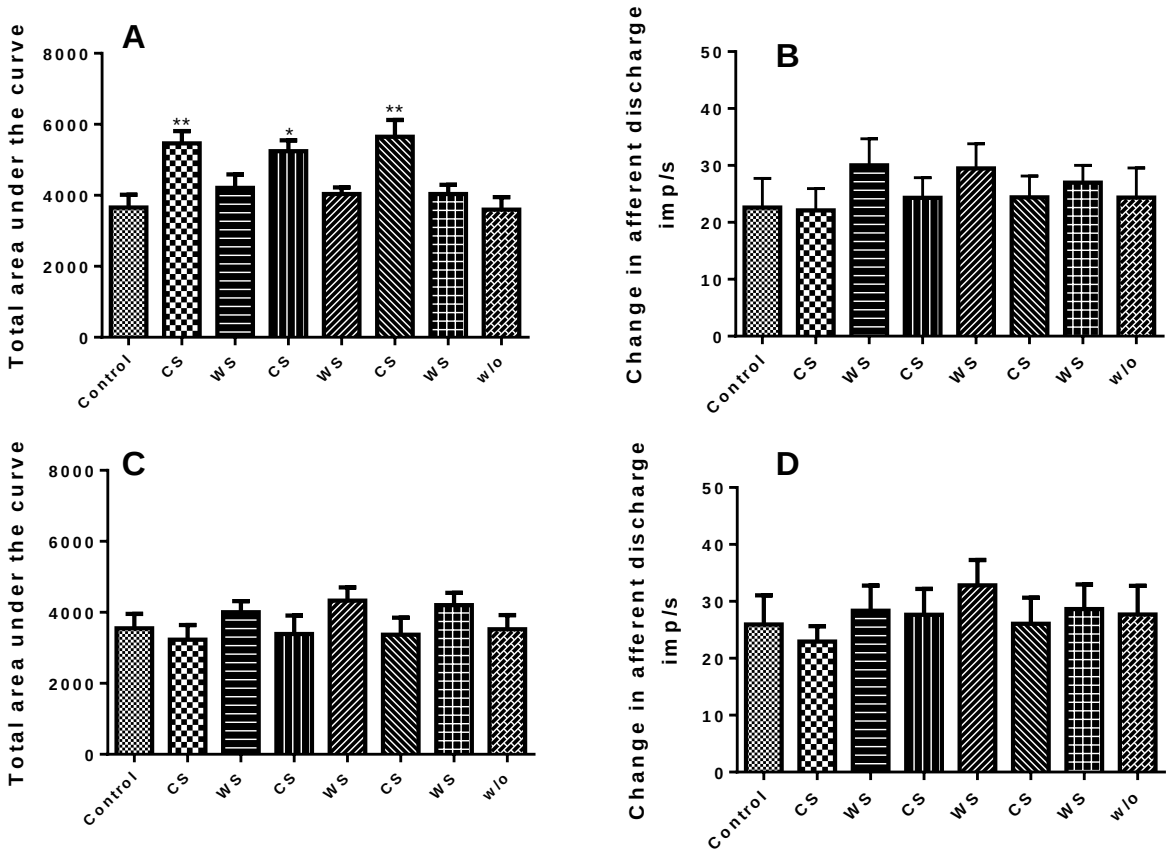


Figure 3.15: The effect of alternating infusion of cold (CS) (12 °C) and warm saline (WS) (38°C) on the afferent response to bladder distension in TRPM8$^{+/+}$ and TRPM8$^{-/-}$ mice.

A; infusion of cold saline significantly increased (**P* < 0.05 one way ANOVA) the afferent response to mechanical stimulation in WT mice. No change was observed with warm saline **B;** There was no change (*P*>0.05) in the spontaneous activity in WT mice. **C;** infusion of cold and warm saline did not alter the afferent response to mechanical stimulation in KO mice. **D;** There was no significant difference (*P*>0.05) in spontaneous activity in KO mice. Results are shown as mean ± SEM (*n* = 6)

3.3.5 The effect of PF-05105679 on bladder sensory nerves.

We also investigated the effect of PF-05105679 on bladder afferents. PF-05105679 is a TRPM8 antagonist. The application of PF-05105679 on its own had no effect on distension, baseline firing or compliance. The combined application of 10μM WS-12 with PF-05105679, blocked the augmentation observed with the application of WS-12 on its own in the WT mice, confirming specificity to TRPM8 (Figure 3.16).

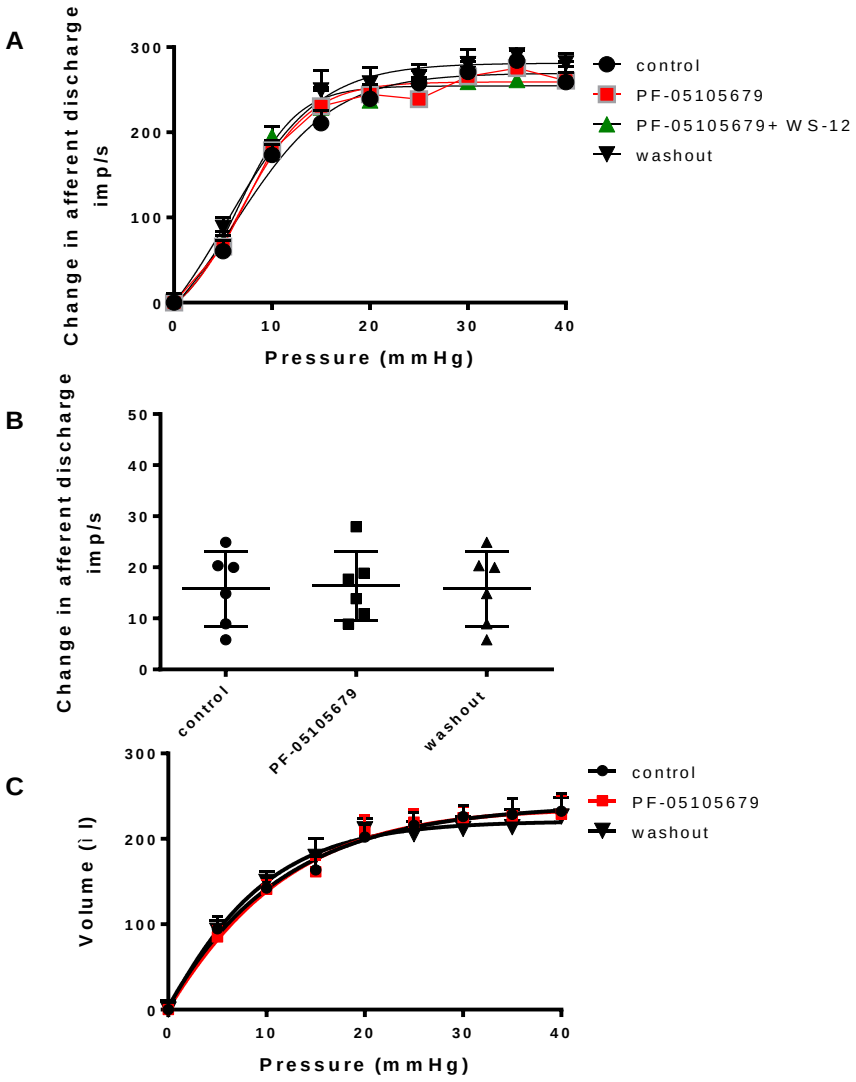


Figure 3.16: The effect of PF-05105679 on the afferent response to bladder distension in wild type mice.
A; PF-05105679 did not alter the afferent response to bladder distension. **B;** PF-05105679 had no significant effect on spontaneous activity **C;** There was no change ($P > 0.05$) of bladder compliance. Results are shown as mean ± SEM ($n = 6$).

3.3.6 Summary

- This chapter provides an extensive study investigating the role of TRPM8 in mouse bladder afferents. It also provides further insight into the effect of menthol, WS-12, cold and PF-05105679 on these afferents.
- No changes were observed in the afferent response and compliance between the WT and TRPM8 KO mice.
- At low concentrations, menthol caused a TRPM8 dependent excitation of the afferent response to distension.
- At high concentration menthol caused a TRPM8 independent inhibition of afferent firing both during distension and at baseline.
- Administration of WS-12 resulted in a TRPM8 dependent excitation of the afferent response to distension. The specificity of the drug was confirmed with the use of a KO and TRPM8 channel blocker (PF-05105679)
- In an attempt to replicate the ice water test, cold saline was infused into the bladder lumen causing an increase in afferent response to distension. This increase was absent in the KO, confirming a TRPM8 dependent pathway.
- TRPM8 may not have a physiological role in normal bladder function; however, knowing that TRPM8 is present on bladder afferents and understanding the effect of modulating this receptor through specific activators and inhibitors may provide new potential for therapy during diseases conditions.

CHAPTER 4

THE INTERACTION OF TRPM8 WITH OTHER THERMOSENSITIVE CHANNELS

4.1 Aim of study

The aim of this study was to assess, any interaction between TRPM8 and the heat activated TRPV1. The study also looked at interaction with TRPA1, a TRP channel thought to be involved in inflammatory pain and noxious cold perception. To assess whether the interaction of TRPM8 extends beyond the TRP channel family, we further investigated if there is any interaction between purinergic signaling and TRPM8 activation.

4.2 Experimental protocol

In vitro nerve recording and single unit analysis were carried out as previously described in section 2.5 and 3.2.4

Afferent nerve responses to capsaicin, AITC, CA, H2S, methylene ATP, U-73122 (PLC inhibitor), H-89 (PKA inhibitor), GÖ-6983 (PKC inhibitor), forskolin (indirectly activated PKA) and phorbol 12-myristate 13-acetate (PMA) (activates PKC)

All the above-mentioned drugs were applied into the lumen of the bladder with the outflow tap open to avoid desensitisation. After the initial application of capsaicin a one hour washout period was required to see a response to the second application of capsaicin, hence the same washout period was used for all the drug combinations. (See figure 4.1).

The combined applications of the drugs were alternated from first to second application. 10mM stock of capsaicin was dissolved in saline to a final concentration of 1μM, 10μM, 30μM and 100μM (0.01% ethanol). 10mM stock of WS-12 was dissolved in saline to a final concentration of 10μM (0.1%DMSO). AITC (300μM), CA (10μM) and αβmethylene ATP (10μm 30μm and 100μm) were dissolved in (0.01%) ethanol to the appropriate concentration.

Bladder innervating DRGs were isolated, cultured and imaged as discussed in section 2.9

10mM stock of WS-12 (0.1% DMSO) was dissolved in HEPES to a concentration of 1μM. A stock of 10mM capsaicin (0.1% ethanol) was dissolved in HEPES to a final concentration of 1μM and 100μM for and capsaicin.

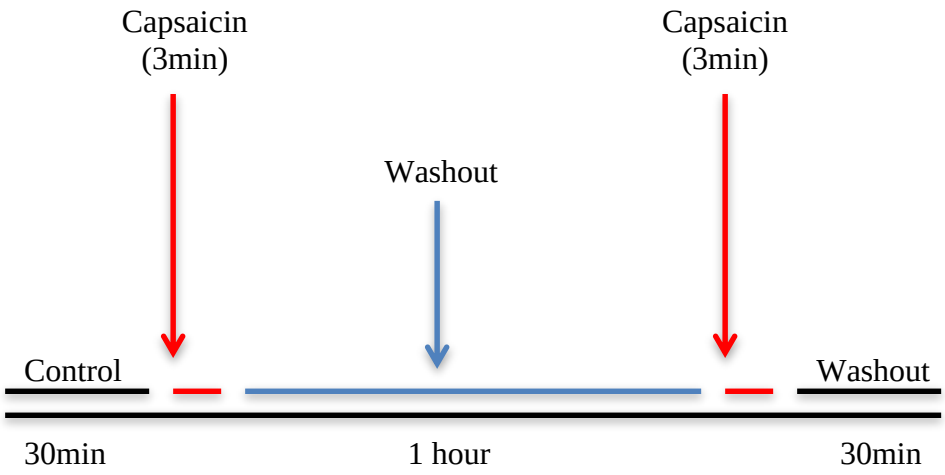


Figure 4.1: schematic diagram representing the protocol used to investigate the effect of capsaicin on bladder afferent firing. The intraluminal infusion of isotonic saline (0.9%), followed by 3min application of capsaicin. After 3 min capsaicin was changed for the continuous perfusion of isotonic saline for 1 hour (representing the washout). 2nd application of capsaicin was applied followed by 30min washout. Time matched control experiments were also carried out to see if the vehicles had any effect on the nerve recording.

4.3 Results

4.3.1. Functional interaction between TRPM8 and TRPV1

To investigate the interaction between TRPM8 and TRPV1, the effect of activating TRPV1 on its own and in combination with WS-12 was investigated on bladder. The response of capsaicin on bladder afferents was previously investigated (Daly *et al*., 2006). Using a similar protocol, the effect of intraluminally applying 1µM, 10µM, 30µM and 100µM capsaicin was assessed. The responses are shown in figure 4.2. We observed no significant effect ($P>0.05$) with the intraluminal application 1, 3, and 30µM capsaicin on baseline firing. A 100µM capsaicin concentration resulted in significant excitation of bladder afferents followed by desensitization. To investigate the interaction between TRPM8 and TRPV1, both WS-12 (10µM) and capsaicin (100µM) were combined intraluminally into the bladder. In contrast to 1µM capsaicin alone, which was a powerful stimulus for afferent firing, it had no significant effect when applied in combination with WS-12. In other words WS-12 inhibited the response to 100µM capsaicin. In contrast, when WS-12 (10µM) was applied in combination with lower concentrations of capsaicin (1, 3, and 10µM), it caused augmentation in both the magnitude and duration of the afferent response. The extent of this augmentation in afferent discharge was increased with reducing concentration of capsaicin. Examples of these afferent responses are shown in figure 4.1, 2 and.

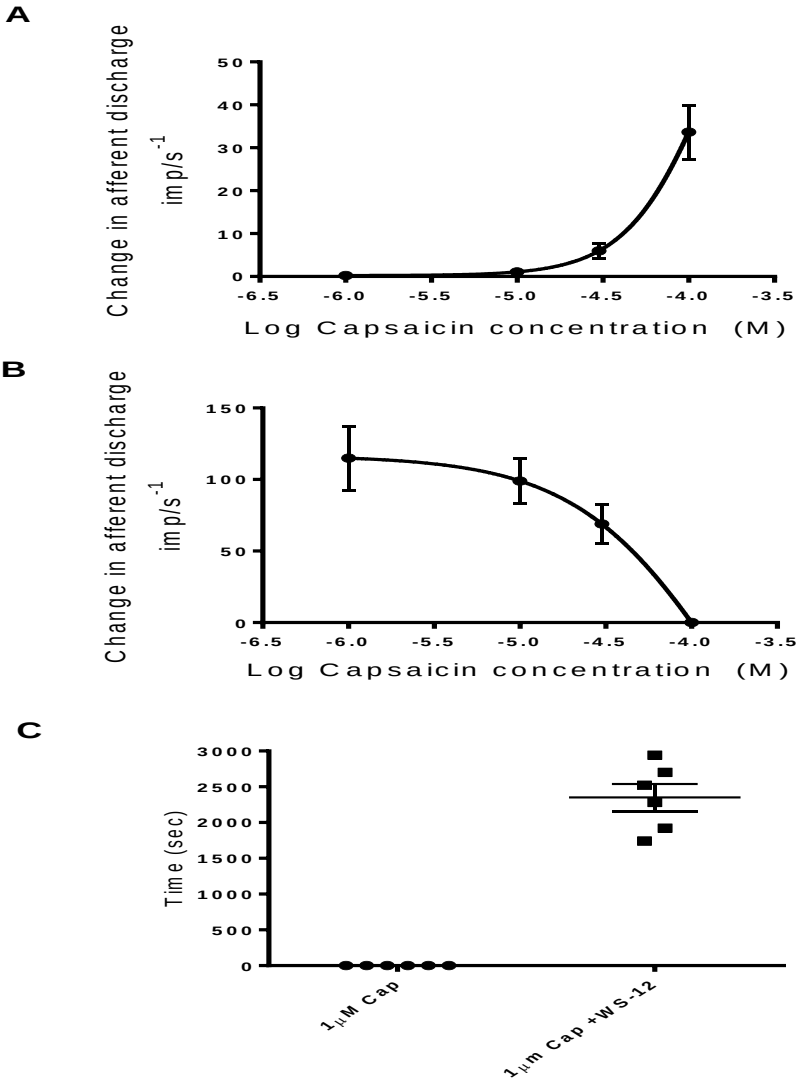


Figure 4.1: Functional interaction between capsaicin and WS-12 on bladder afferent discharge. **A**; Cummulative log concentration response curve of capsaicin on bladder sensory firing. 100µM capsaicin induced a significant response on baseline firing (n=6, ****P<0.0001one way ANOVA, followed by Bonferroni test). **B**; Cummulative concentration response curve, showing afferent response to dual application of WS-12 and capsaicin. (n=6, ****P=0.0001 one way ANOVA, followed by Bonferroni test) **C;** Comparison of duration between the baseline response induced by a 1µM capsaicin and 1µM capsaicin +WS-12 (n=6).

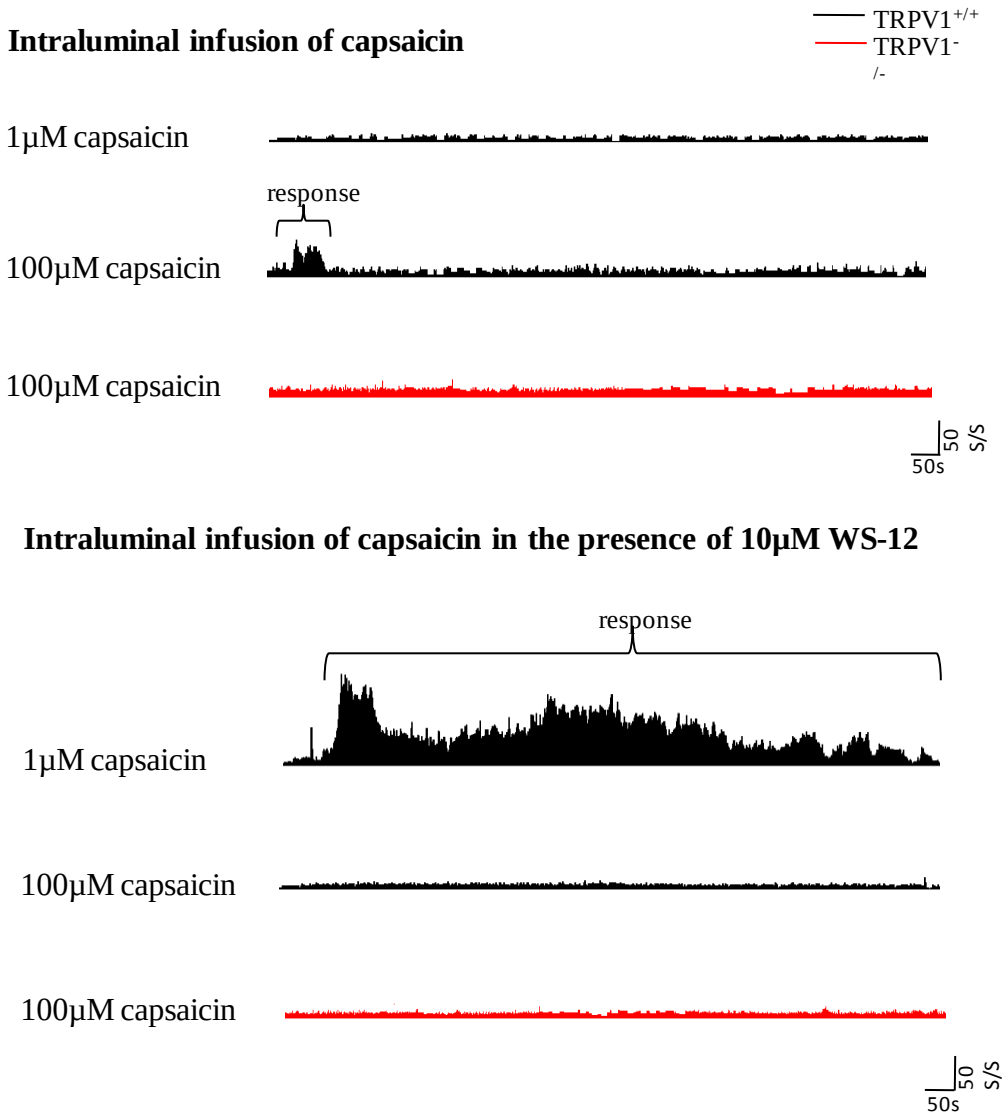


Figure 4.2: examples of histogram traces representing nerve responses to application of capsaicin (1µM and 100µM) and capsaicin with WS-12 Control experiments were performed with the intraluminal infusion of isotonic saline (0.9%), followed by 3min application of capsaicin. The intraluminal application of 100µM capsaicin induced a significant increase in bladder sensory firing. 100µM capsaicin with WS-12 did not alter sensory nerve firing. The infusion of 1µM capsaicin on its own did not alter baseline firing. The combined application of 1µM capsaicin with WS-12 significantly increased duration and frequency of spikes firing at baseline.

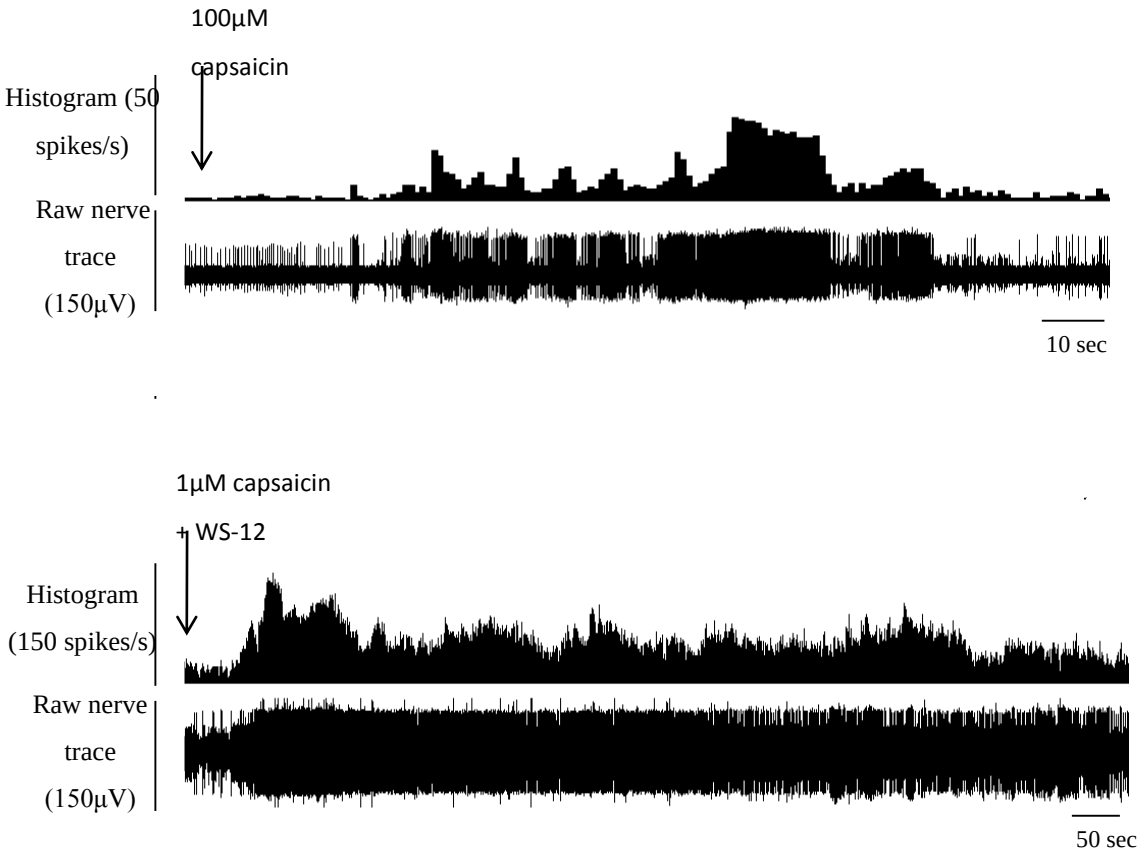


Figure 4.3: afferent response induced by the application of a 100µM capsaicin and 1µM capsaicin with WS-12. 100µM capsaicin induced a robust increase the frequency of spikes firing at baseline. Desensitization was followed straight after. There was no change in the intraluminal pressure. 1µM capsaicin and WS-12 induced a robust increase in the frequency of spikes firing at baseline. Unlike, the response to 100µM capsaicin on its own, 1µM capsaicin response was not followed by desensitization.

4.3.2 Specificity of interaction between TRPV1 and TRPM8

In order to determine the contribution of TRPV1 in the augmentation observed with the combined application of capsaicin and WS-12, the protocol was repeated in TRPV1$^{-/-}$ mice. The excitation followed by desensitization previously observed with the application 100μM capsaicin was absent in TRPV1$^{-/-}$ (Figure 4.4A). Similarly, afferent responses to combined application of 1μM capsaicin and 10μM WS-12 were also absent in TRPV1$^{-/-}$ (Figure 4.4B). The same protocol was also repeated in the presence of the TRPV1 antagonist capsazepine (10μM). Surprisingly, capsazepine, was unable to inhibit the response to 1μM capsaicin with WS-12 in TRPV1$^{+/+}$ (Figure 4.4C).

In the previous chapter, using both TRPM8 null mice and PF-05105679 we have determined the specificity of 10μM WS-12 to TRPM8. In order to confirm the contribution of TRPM8 in the augmentation observed, we applied both WS-12 and 1μM capsaicin in the presence of PF-05105679 (TRPM8 antagonist). Significant inhibition was observed in the augmented afferent discharge (Figure 4.5). It should be noted that the PF-05105679 did not completely block the augmentation.

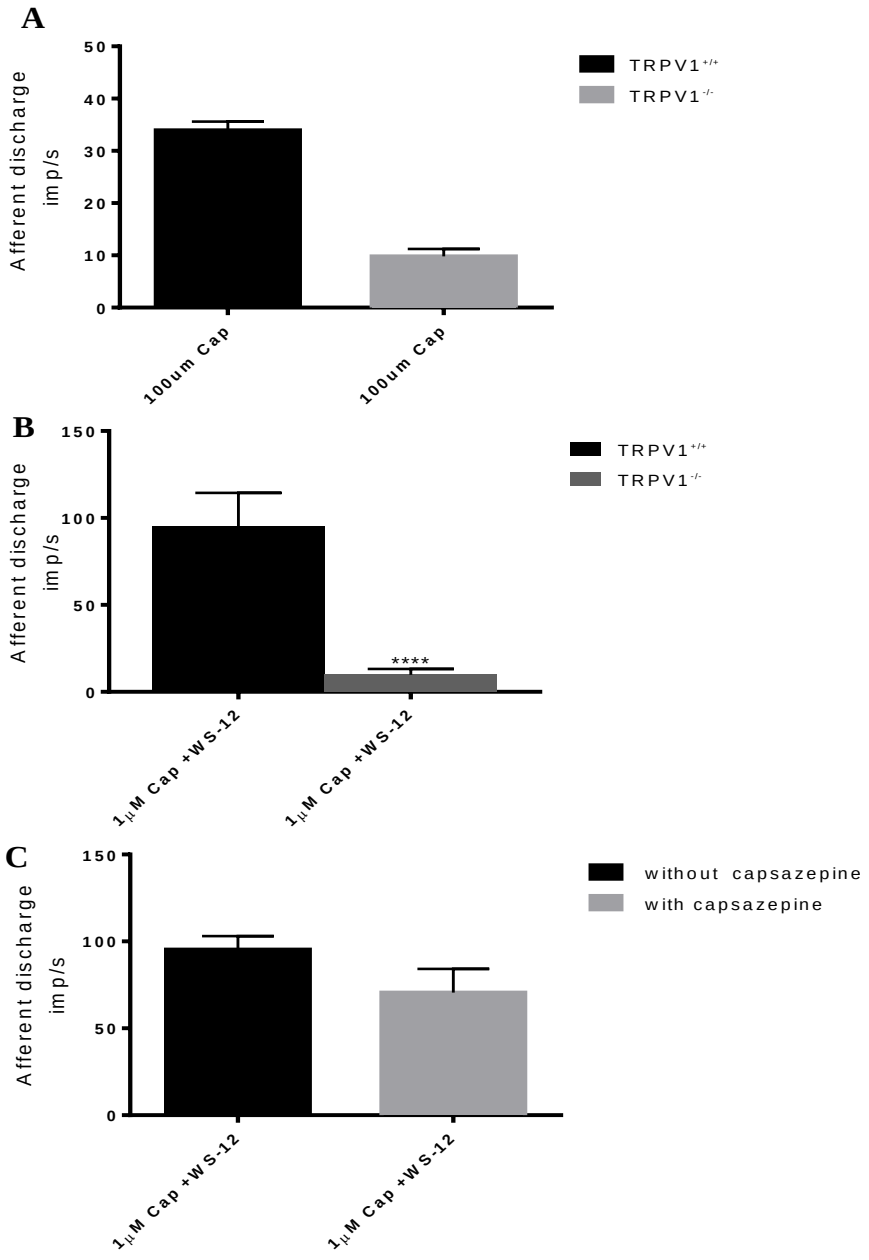


Figure 4.4: The afferent response to capsaicin and WS-12 IN TRPV1$^{-/-}$ mice and in the presence of capsazepine. No change in afferent response was observed with either the application of **A;** 100µM capsaicin or **B;** a combined application of 1µM capsaicin with WS-12 in the TRPV1$^{-/-}$(P>0.05, student t test, n=6). **C;** Capsazepine (TRPV1 antagonist), did not significantly, inhibit the response to 1µM capsaicin and WS-12 in TRPV1$^{+/+}$ mice (P>0.05, student t test, n=6).

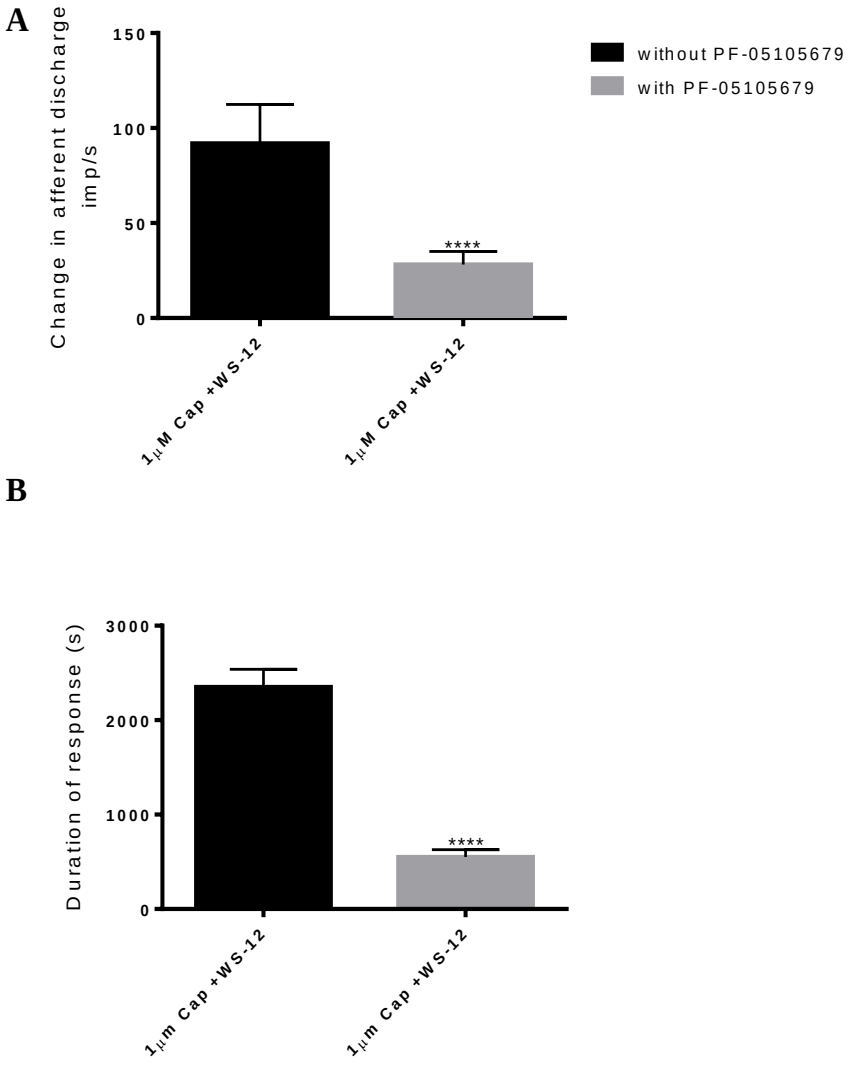


Figure 4.5: The afferent response to capsaicin and WS-12 in the presence of PF-05105679. Combined application of 1μM capsaicin with WS-12 in the presence of PF-05105679, **A**; significantly reduced sensory afferent firing (****P<0.0001 student t test followed by bonferroni correction, n=6) and **B;** duration of response (****P<0.0001 student t test, n=6).

4.3.3. Intracellular mechanism involved in the interaction between TRPM8 and TRPV1

PLC, PKA and PKA pathways are intracellular signalling pathways that have been postulated to play a role in TRPV1 sensitization. To investigate the potential involvement of a PLC, PKA and PKC dependent pathway, various inhibitors were applied together with WS-12 (10µM) and capsaicin (1µM). U-73122 (PLC inhibitor) did not result in inhibition of the augmented response seen with WS-12 and capsaicin. However, 1µM H-89 (PKA inhibitor) significantly reduced (****P<0.0001, n=6) inhibition, 1µM GÖ- 6983 (PKC inhibitor) almost completely abolished (****P<0.0001, n=6) the response to WS-12 and capsaicin (Figure 4.8B). This suggests that the excitatory response seen with the combined application of low dose capsaicin and WS-12 may involve both PKA and PKC pathways.

To investigate the primary contributor responsible for the augmentation, both WS-12 and capsaicin were either separately applied with 10µM forskolin (PKA activator) and 10µM PMA (PKC activator) or with a combination of both forskolin and PMA. Forskolin is an indirect activator of PKA. Forskolin raises the levels of cyclic AMP (cAMP). The increase in levels of cAMP activates cAMP sensitive pathways, including PKA. Surprisingly, no significant potentiating of the WS-12 and capsaicin activated sensory responses was observed with the infusion of PMA and forskolin (P>0.05. n=6) (Figure 4.6C).

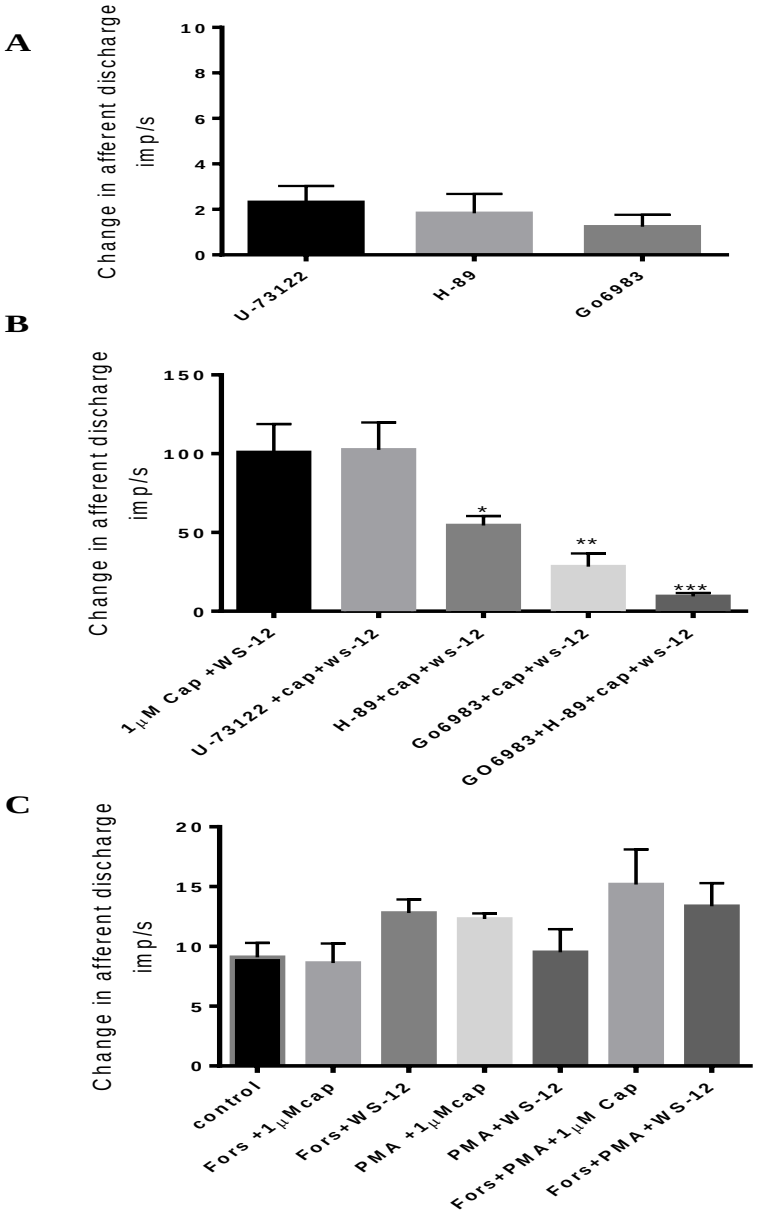


Figure 4.6: Intracellular signalling pathways involved in the interaction between TRPM8 and TRPV1. A; U-73122 did not alter the response to combined application of WS-12 and 1μM capsaicin. Significant reduction was observed in the presence of H-89 and G6983. The augmentation was almost completely abolished in the presence of both H-89 and GÖ6983 (****P=0.0001, n=6). B; No change in afferent firing was observed with forskolin or PMA (P>0.05 one way ANOVA followed by Bonferroni test, n=6)

4.3.4. Functional interaction between TRPA1 and TRPM8

Functional interaction between TRPA1 and TRPM8 were also investigated. Previous studies have reported excitatory effects of TRPA1 agonists on bladder sensation. In the current study, the effect of AITC, CA and H2S were investigated on bladder afferents. Contrary to previous results (Figure 4.1), the intraluminal application of 10µM AITC did not alter bladder sensory firing. However 30µM, 100µM and 300µM AITC significantly inhibited baseline firing (*P<0.05, ****P<0.0001, n=6). No significant effect was observed on mechano-sensitive responses to AITC. Similarly, 10 and 30µM CA did alter either baseline or distension responses, whereas 100µM CA significantly attenuated (****P<0.0001, n=6) baseline firing, although no effect was observed on distension response (Figure 4.7B). Interestingly, Hydrogen sulphide significantly increased baseline firing at a concentration of 100µM and 300µM (*P<0.05, ****P<0.0001, n=6) (Figure 4.7C).

It has been previously reported that AITC is the most specific agonist of TRPA1; hence AITC was further utilized to investigate interaction between TRPA1 and TRPM8. Combined application of 300µM AITC and WS-12 induced excitation of sensory response (****P<0.0001, n=6). 10µM AITC with WS-12 did not alter bladder afferent firing (Figure 4.9A). This is contrary to the responses observed with capsaicin, since highest dose of AITC investigated yielded the most significant augmentation, when combined with WS-12. The increase observed with 300µM AITC and WS-12 was inhibited in the presence of 1µM GÖ-6983 and 1µM H-89 (**P<0.01, n=6) (Figure 4.9B).

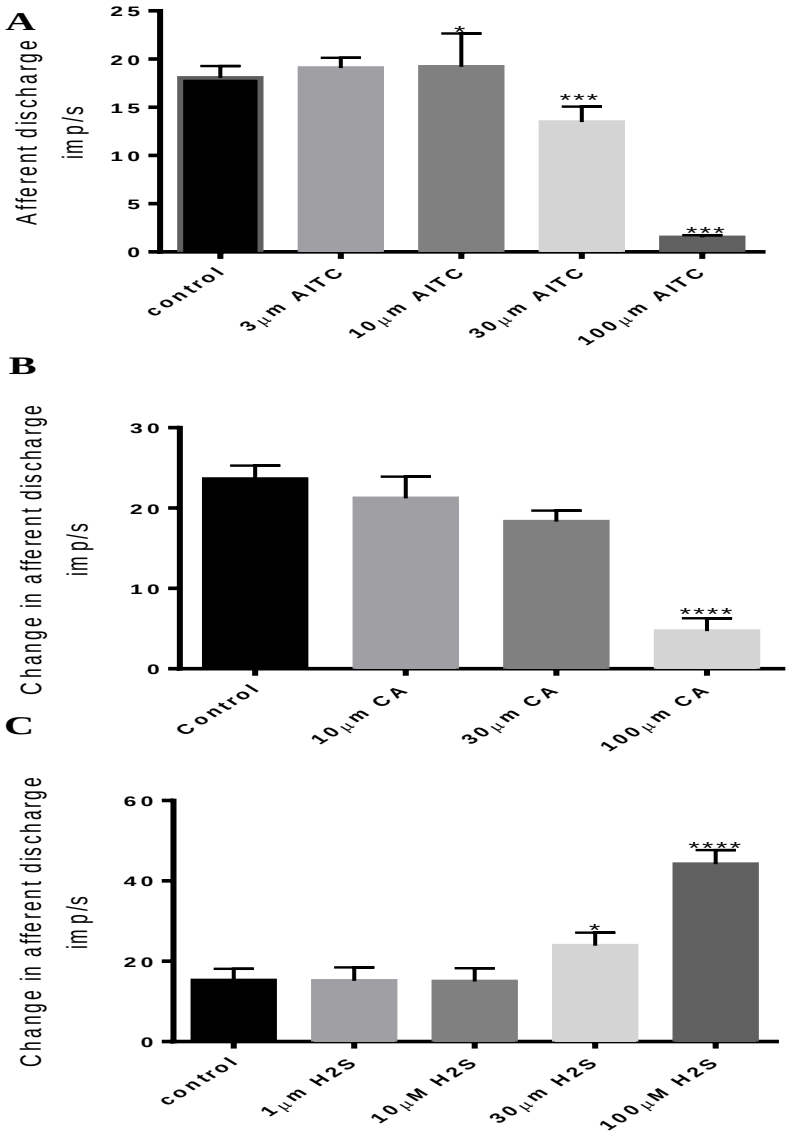


Figure 4.7: sensory afferent response to the intraluminal application of TRPA1 agonist. **A**; Bar graph, showing afferent response to the application of 10, 30, 100 and 300µM AITC. 100 and 300µM AITC significantly inhibited baseline firing (*P<0.05, ****P<0.0001, n=9). B; Application of 100µM CA significantly inhibited sensory function (****P<0.0001, n=6), whereas 10M and 30µM CA did not alter afferent nerve firing. C; 30µM and 100µM H_2S significantly increased baseline firing (*P<0.05, ****P<0.0001, n=6). No changes were observed with 1 and 10µM H2S. All analyses were performed with One-way ANOVA, followed by Bonferroni test for multiple comparisons.

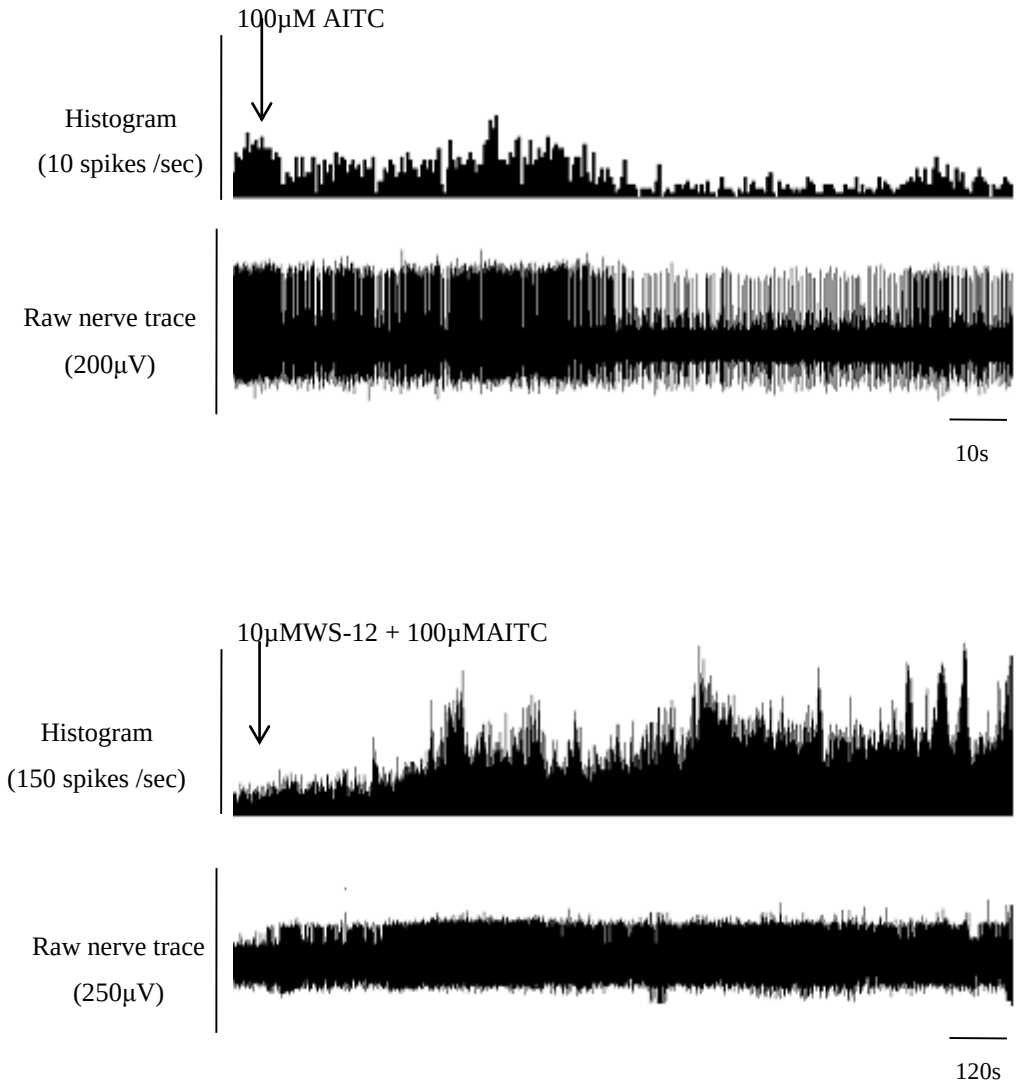


Figure 4.8: The afferent response to AITC and AITC in the presence of WS-12. A; Sensory response to the intraluminal infusion of 300µM AITC. **B;** augmented sensory response with the combined application of AITC (300µM) and WS-12.

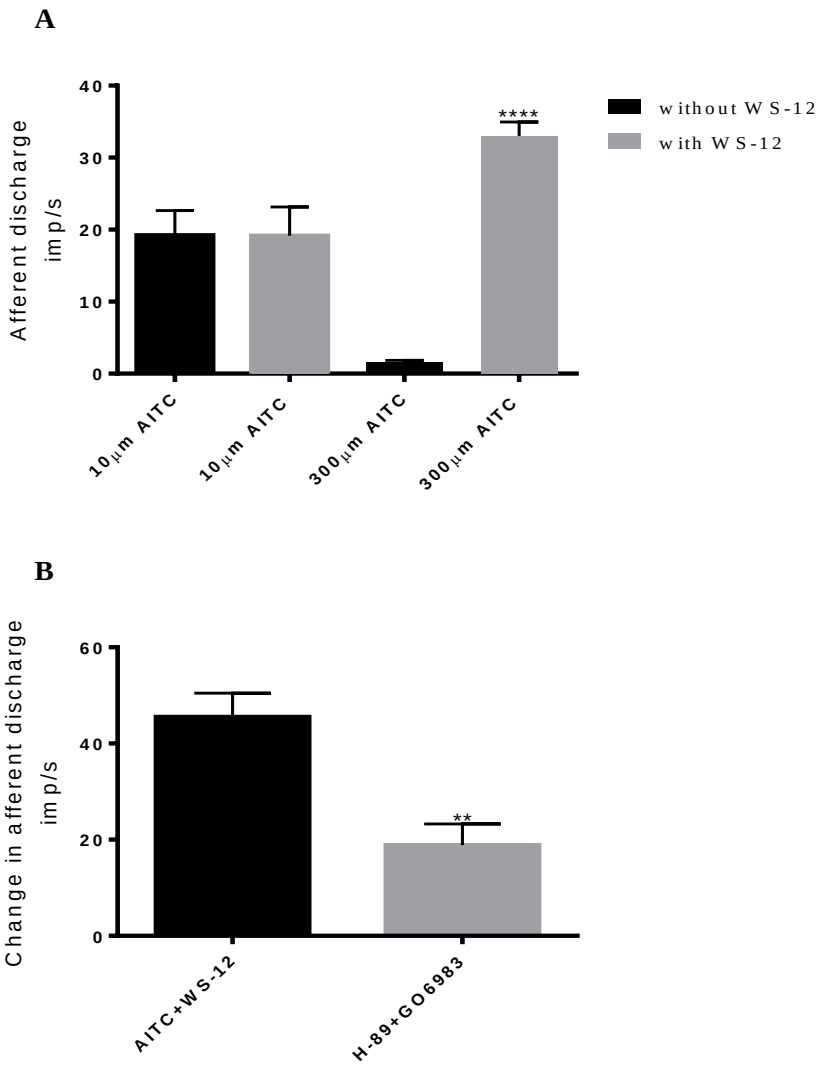


Figure 4.9: functional interaction between TRPA1 and TRPM8. A; The afferent response to 10µM AITC and WS-12 was comparable to the afferent response induced by application of 10µM AITC on its own. 300µM AITC with WS-12 significantly increased afferent response at baseline firing (****P=0.0001, n=6). B; In the presence of H-89 and GÖ6983 the augmented response was inhibited (**P=0.01, n=6 Student t test).

4.3.5. Interaction between TRPM8 activation and ATP signalling

To investigate whether, WS-12 induced sensitization is limited to or extends beyond TRP channels; the same interaction was investigated with purinergic signalling. Previous studies have characterized the effect of ATP stimulation on bladder afferents. To investigate, the effect of activating P2X receptors in the current set-up, a series of experiments were performed. Figure 4.13 shows a typical response to intraluminal application of 3, 10, 30, and 100µM methylene ATP. Purinergic stimulation resulted in robust excitation of sensory afferents followed by a period of desensitization. The magnitude of sensory activation increased with increasing concentration of methylene ATP. This resulted in significant increases in afferent activity with the application of 30 and 100µM αβmethylene ATP. The augmented afferent discharge was presumably due to the activation of P2X (P_2X_1 and P_2X_3) receptors, which are present on bladder afferents. Similar to previously described preparation a period of 1hr washout was applied, resulting in reproducible responses to αβmethylene ATP (n=6) (Figure 4.10A).

To investigate the interaction between P_2X receptor signalling and the activation of TRPM8, both 10µM WS12 and 10µM αβmethylene ATP were applied intraluminally for 3 min into the bladder. Figure 4.11 show representative traces of the response. Following application of both the compounds together a significant increase was seen in the sensory afferents response at baseline firing compared to the application of αβmethylene ATP on its own.

The combined response of WS-12 and αβmethylene ATP was significantly inhibited by the application of 1µM H-89 and 1µM GÖ6983 (Figure 4.12).

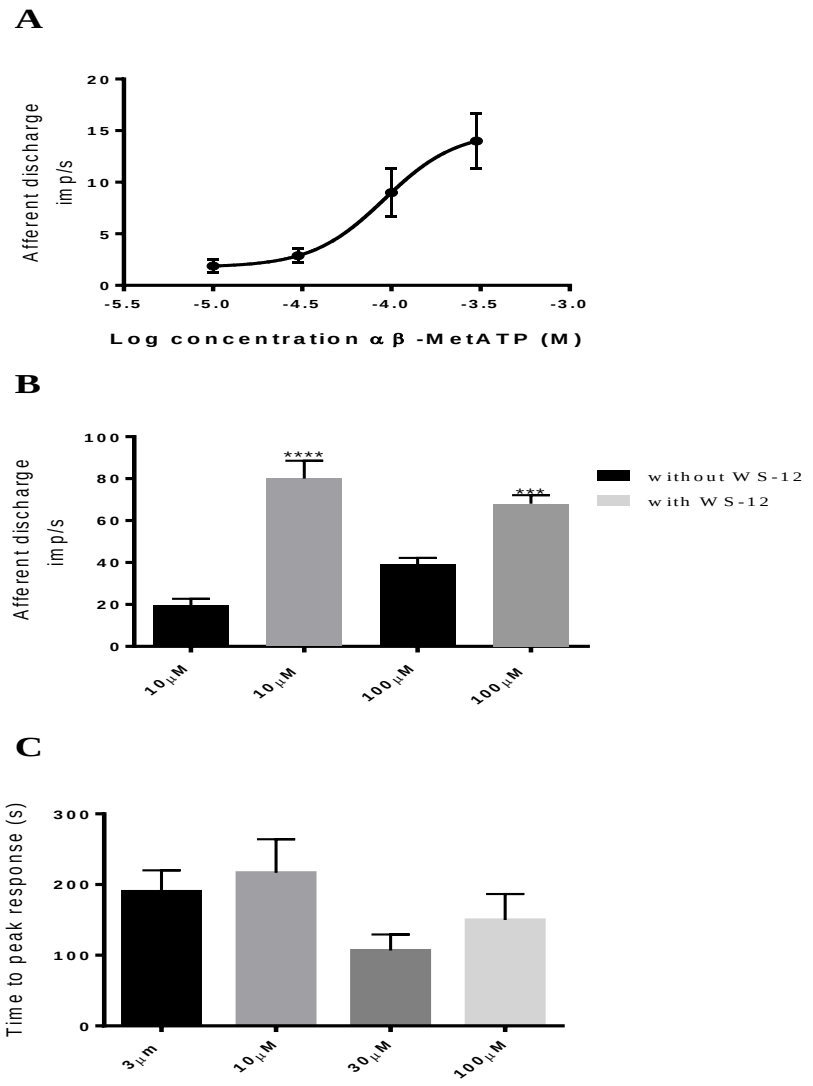


Figure 4.10: Functional interaction between αβmethyleneATP and WS-12 on bladder afferent discharge. **A**; Cummulative log concentration response curve of αβmethyATP on bladder sensory firing. In the presence of WS-12, both B; the frequency of afferent response and C; Time to peak response after administration of αβmethyleneATP.

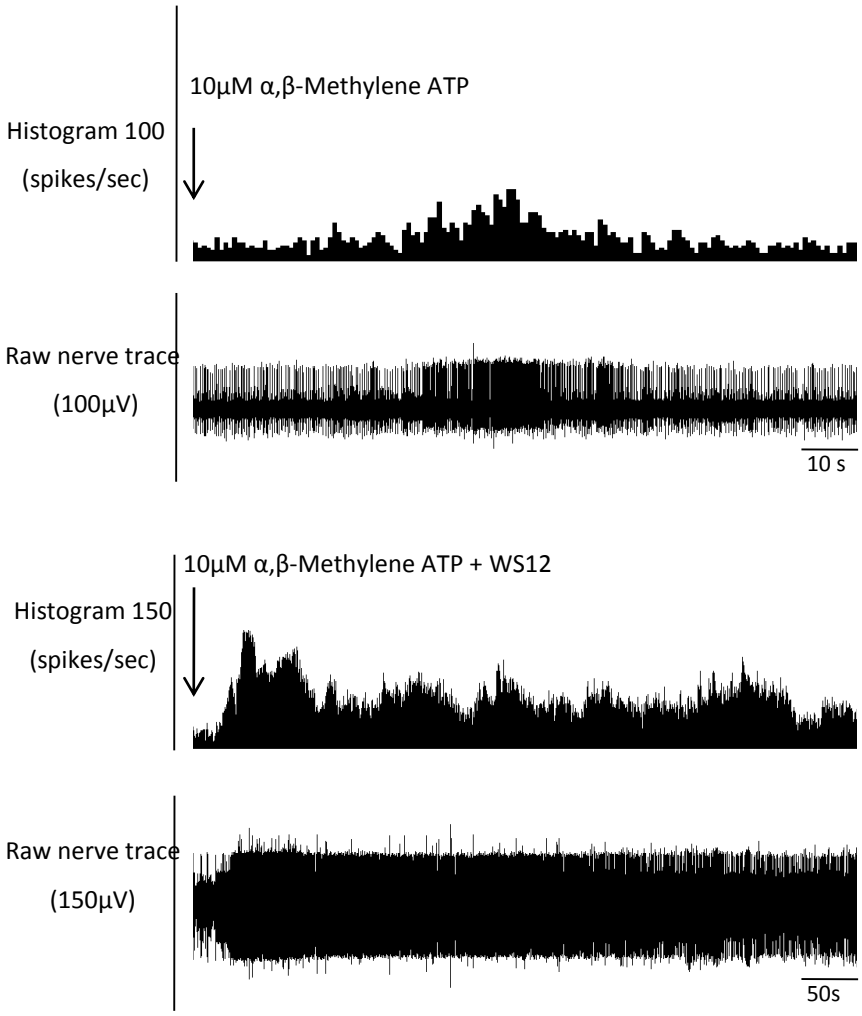


Figure 4.11: examples of raw nerve trace to the application of αβmethylene ATP (10µM) and αβmethylene ATP (10µM) with WS-12. A; The intraluminal application of αβmethyleneATP induced excitation of bladder sensory firing, followed by rapid desensitisation. B; 10µM αβmethylene ATP with WS-12 significantly increased duration and frequency of spikes firing at baseline.

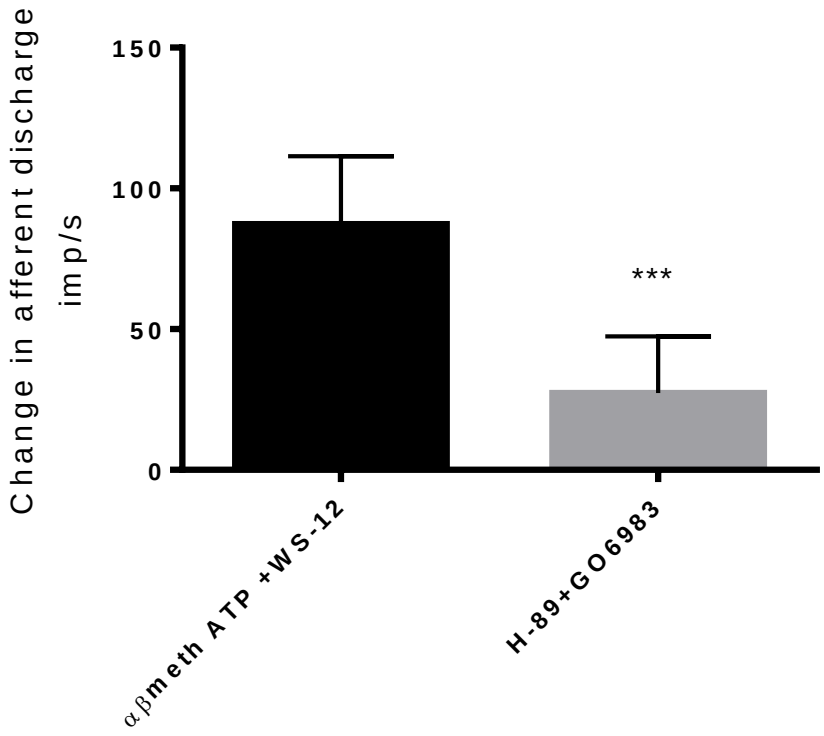


Figure 4.12: Intracellular signalling pathways involved in the interaction between purinergic signalling and TRPM8 activation. The augmentation observed with the combined application of αβmethylene ATP and WS-12 was significantly reduced in the presence of both H-89 and GÖ6983 (***P=0.001, n=6 Student t test).

4.3.6 Effect of WS-12 and Capsaicin on bladder innervating DRGs.

To investigate whether the responses observed in the *in vitro* bladder preparation are a direct effect on the nerve or an indirect effect through the urothelium or a combination of both, DRG's innervating the bladder were cultured and calcium imaging experiments were performed with WS-12 and Capsaicin. Separately the urothelial cells obtained from mouse bladder tissue were cultured and the same experiments were performed to determine if there is any effect on the urothelial cells of these compounds. 100nM capsaicin and 1µM WS-12 induced a significant change in intracellular calcium concentration in DRGs (Figure 4.13). To investigate the interaction between TRPM8 and TRPV1, 1µM WS-12 was administered with 100nM capsaicin. This combination induced no significant changes in the intracellular calcium concentration as expected from the afferent nerve recording data. We also looked at a lower concentration of 1nM capsaicin with WS-12, this combination induced a bigger change ($p=0.001$) in the intracellular calcium concentration (Figure 4.13). 1µM Ws-12, 100nM capsaicin and 10µM AITC had no effect on isolated urothelial cells (Figure 4.14B), hence the combined administration of these drugs were not studied in these cells.

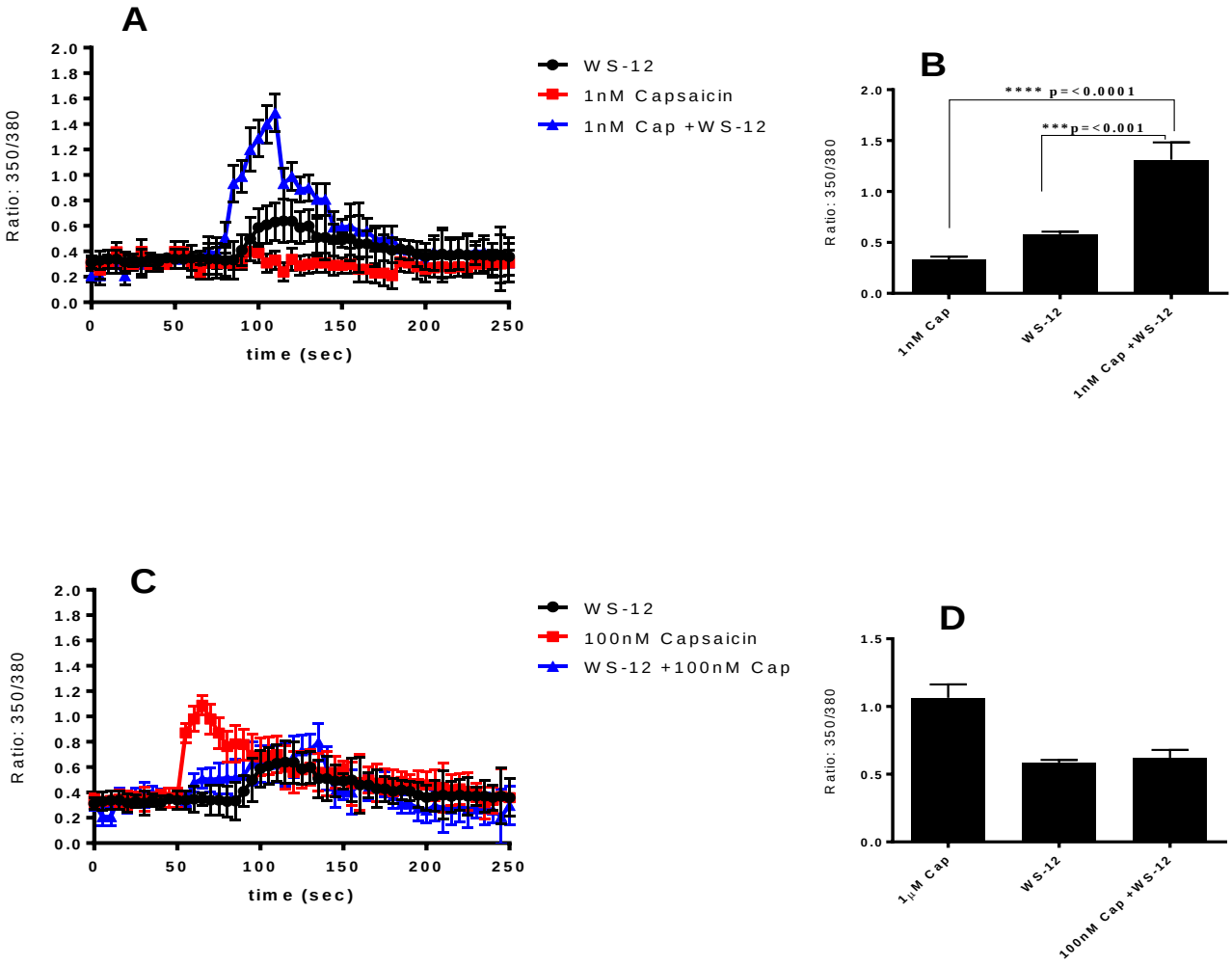


Figure 4.13 Calcium imaging on isolated DRG's. *A*; increased responses to combined application of WS-12 and low concentration of capsaicin (1nM). B; Increased mean response to combined application of WS-12 and 1nM capsaicin (***P<0.001, N=6). C, D; No change in intracellular calcium level with the combined application of 100nM capsaicin and WS-12 (N=6). Values are means ± SEM. All results are analysed with One-Way ANOVA followed by bonferroni test for comparison.

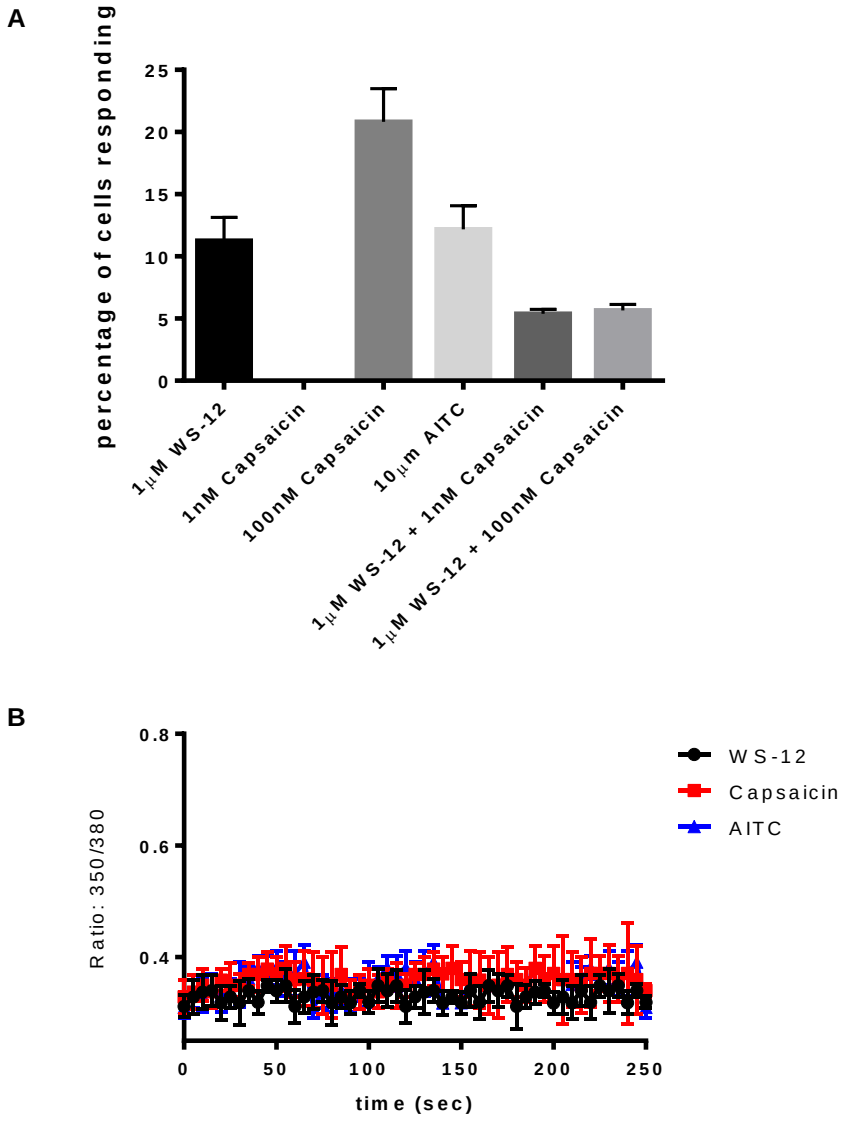


Figure 4.14. A; Percentage of cells responding to the various pharmacological treatments. B; No significant changes in intracellular calcium were observed after application of ws-12, AITC and capsaicin on isolated urothelial cells, (N=6). Values are means ± SEM. All results are analysed with two-Way ANOVA followed by bonferroni test for comparison.

4.3.7 Summary

- This chapter gives an initial insight into the interaction between different receptors, especially TRPM8 and TRPV1.
- The intraluminal infusion of 1, 10 and 30μM capsaicin had no effect on baseline firing, whereas 100μM capsaicin significantly increased baseline afferent response.
- The combined application of 10μM Ws-12 and 1μM capsaicin resulted in a significant increase in baseline firing, whereas the co-administration of 10μM WS-12 and 100μM capsaicin had no effect on baseline firing.
- The afferent responses observed with the co-application of WS-12 and capsaicin were absent in TRPV1 KO mice and were also inhibited in the presence of 10μM PF-05105679 (TRPM8 channel blocker). This suggests a TRPM8 and TRPV1 dependent pathway.
- Similarly, the effect of AITC and α, β-methylene ATP were also significantly greater in the presence of WS-12.
- The exaggerated afferent responses were inhibited in the presence of H-89 and G0-6983 (PKA and PKC inhibitors).
- Studying these questions could provide useful information for the mechanism underlying bladder hypersensitivity.

CHAPTER 5

ROLE OF OESTROGEN RECEPTOR BETA ON BLADDER AFFERENT FIRING

5.2 Experimental protocol

Voiding behaviour assessment

Oestrogen receptor beta male and female knockout mice and their wild type littermates were provided by Pfizer. The mice were singly housed and maintained under standard laboratory conditions. This study was conducted with 34 mice housed under 12:12 hour reversed dark: light cycle with food and water offered *ad libitum*. A total of 8 wild type females and males and 10 KO females and 8 KO males were used in this study. There were no overt differences in feeding behaviour and body weight between WT and KO groups. In each experiment 2 animals were selected from each of these groups and randomised for testing. These mice were assessed for voiding behaviour as described in chapter 2.

Afferent nerve recording were conducted on the mice as described in detail in chapter 2

Effect of 17β estradiol was assessed by cumulative application of 300nM, 1µM, 3µM, 10µM, 30µM, 100µM, 300µM, and 1mM. The parameter analysed were sensory responses during distension and at baseline as well as changes in bladder compliance.

5.3 Results

5.3.1 Comparing of voiding behaviour between ERβ$^{-/-}$ and ERβ$^{+/+}$ mice

Male and female knockout and wildtype mice were assessed for the total volume voided, average volume per void and frequency of voiding. No significant difference was observed between in any of these parameters (P>0.05). No significant difference (P>0.05) was also observed after water loading (20ml/kg) the mice. The voiding parameters between the mice were obtained after the mice were acclimatized for 3 days (3hrs per day).

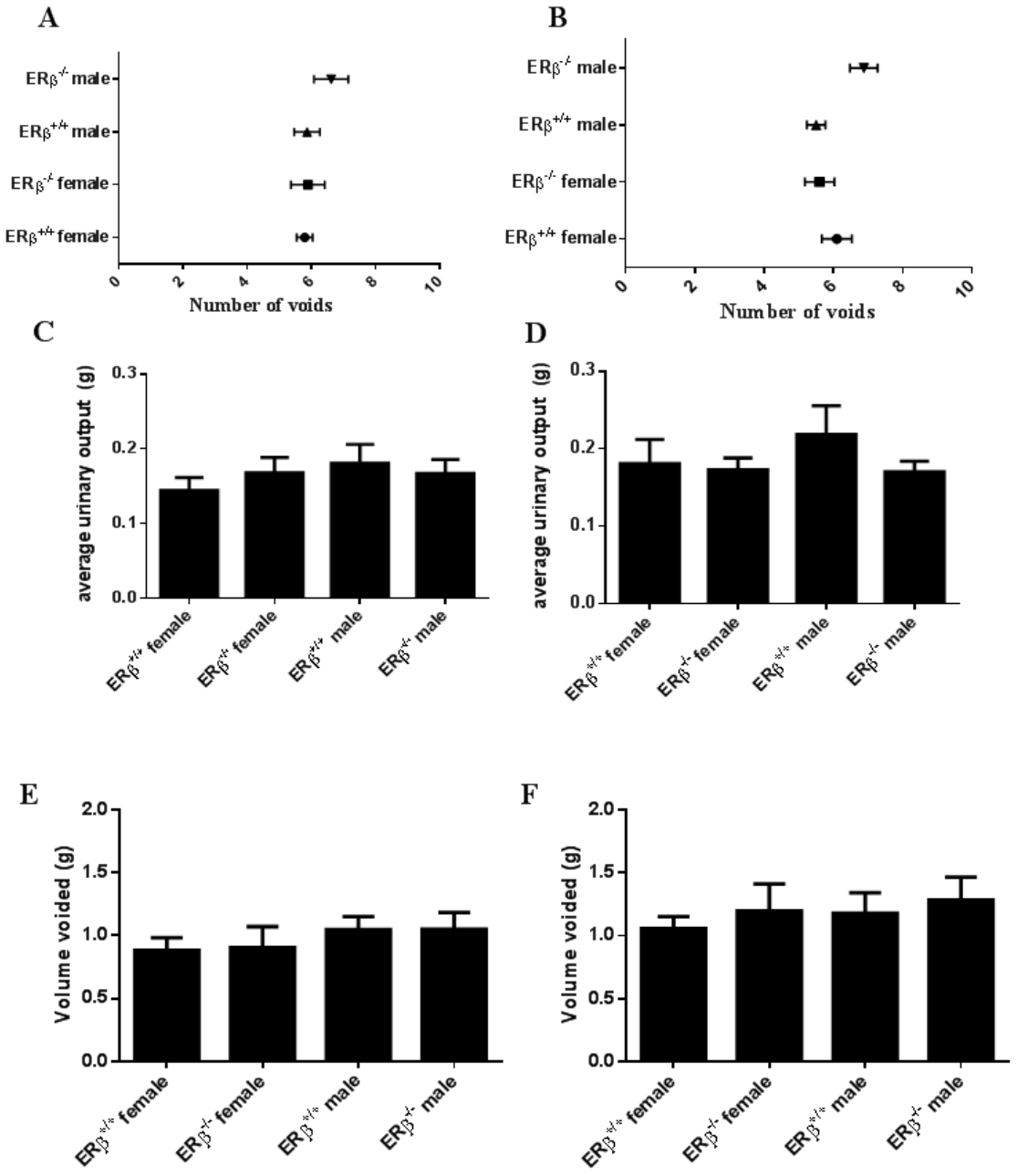


Figure 5.1 voiding behaviour of the ERβ-/- male and female mice and their wild type littermates. No changes were observed in the A + B; the number of voids C+D; average urinary output per void and E+F; the total volume voided over a 6 hour period. Data are expressed as means ± S.E.M. Statistical analysis was carried out using a either a one-way ANOVA followed by a Bonferroni post-test (Significance was set at $P< 0.05$).

5.3.2 Afferent nerve firing between ERβ$^{-/-}$ and ERβ$^{+/+}$ mice

After measuring the voiding behaviour of the mice, the mice were sacrificed to analyse the sensory function between the knockout mice and their wildtype littermates. There was no significant difference in the baseline firing and the sensory response to distension between ERβ wt and ERβ ko in both the males and female mice. There was a significant difference in the bladder compliance between the male ERβ wt (n=6) and ERβ ko (n=7) ($P<0.05$). There was no significant difference in compliance between the female ERβ wt (n=6) and ERβ ko (n=6) mice ($P<0.05$).

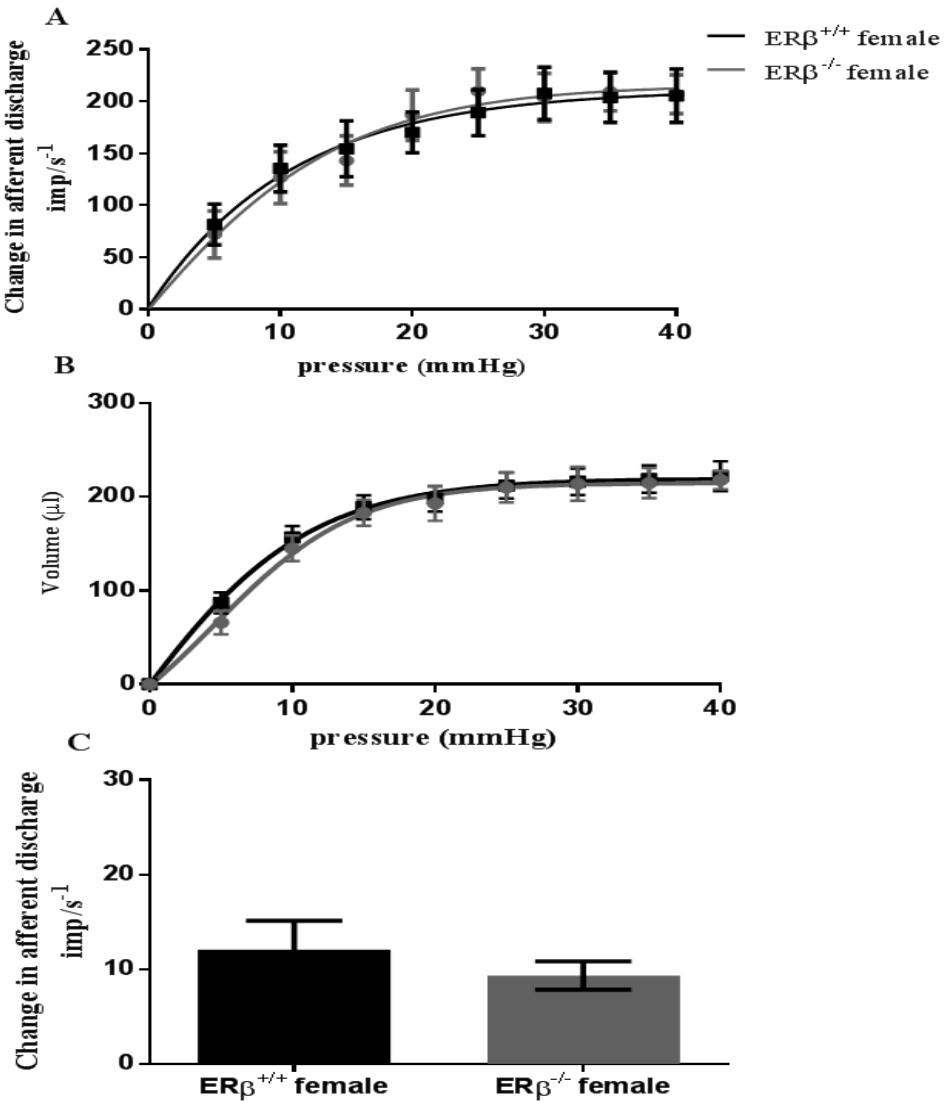


Figure 5.2 Comparison of afferent firing between ERβ$^{-/-}$ female mice and their wild type littermates. No changes were observed in the A; sensory firing in response to distension B; Bladder compliance and C; baseline firing. Data are expressed as means ± S.E.M. Statistical analysis was carried out using either two way ANOVA and one-way ANOVA followed by a Bonferroni post-test (Significance was set at $P< 0.05$).

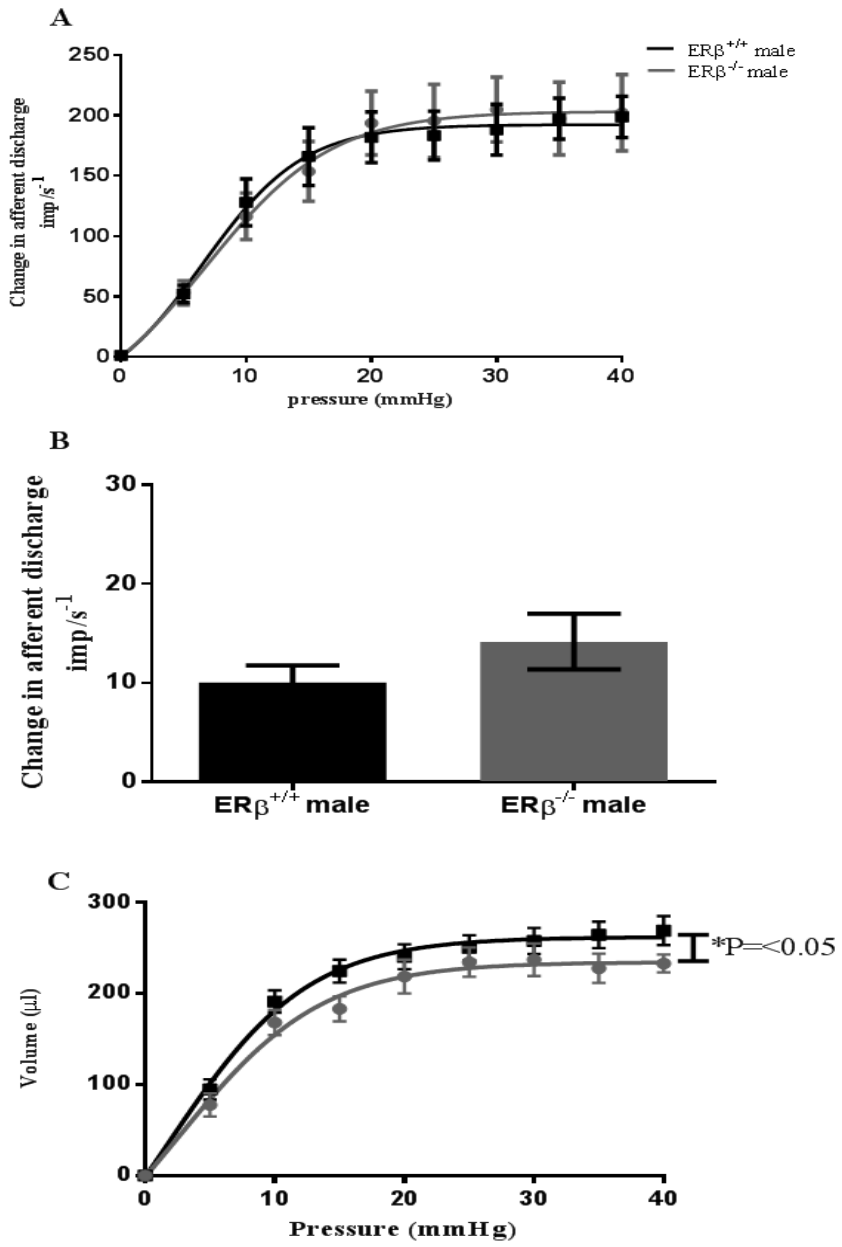


Figure 5.3: Comparison of afferent firing between ERβ$^{-/-}$ male mice and their wild type littermates. No changes were observed in the A; sensory firing in response to distension and B; Baseline firing. The bladder compliance C: was significantly reduced in the KO mice (P<0.05 two way ANOVA followed by a Bonferroni post-test) Data are expressed as means ± S.E.M. was set at P< 0.05).

5.3.3 The effect of 17β estradiol on bladder sensory firing and compliance.

To investigate the effect of oestrogen directly on bladder sensation and compliance, 17β estradiol was applied cumulatively at various concentrations (300nM, 1μM, 3μM, 10μM, 30μM, 100μM, 300μM, and 1mM). 17β estradiol had no effect on the afferent firing to distension at any of the above-mentioned concentrations (Figure 5.5A and 5.5B). However, the baseline firing was significantly increased at a concentration of 300μM in all $ER\beta^{-/-}$ mice and their wild type littermates (Figure 5.6). In the female mice estradiol had a significant effect even at lower concentrations (30μM and 100μM $ER\beta^{+/+}$ and 10μM and 100μM $ER\beta^{-/-}$**)** (Figure 5.6A +B). Significant increases were observed in the bladder compliance at a concentration of 300μM in all the mice (Figure 5.7).

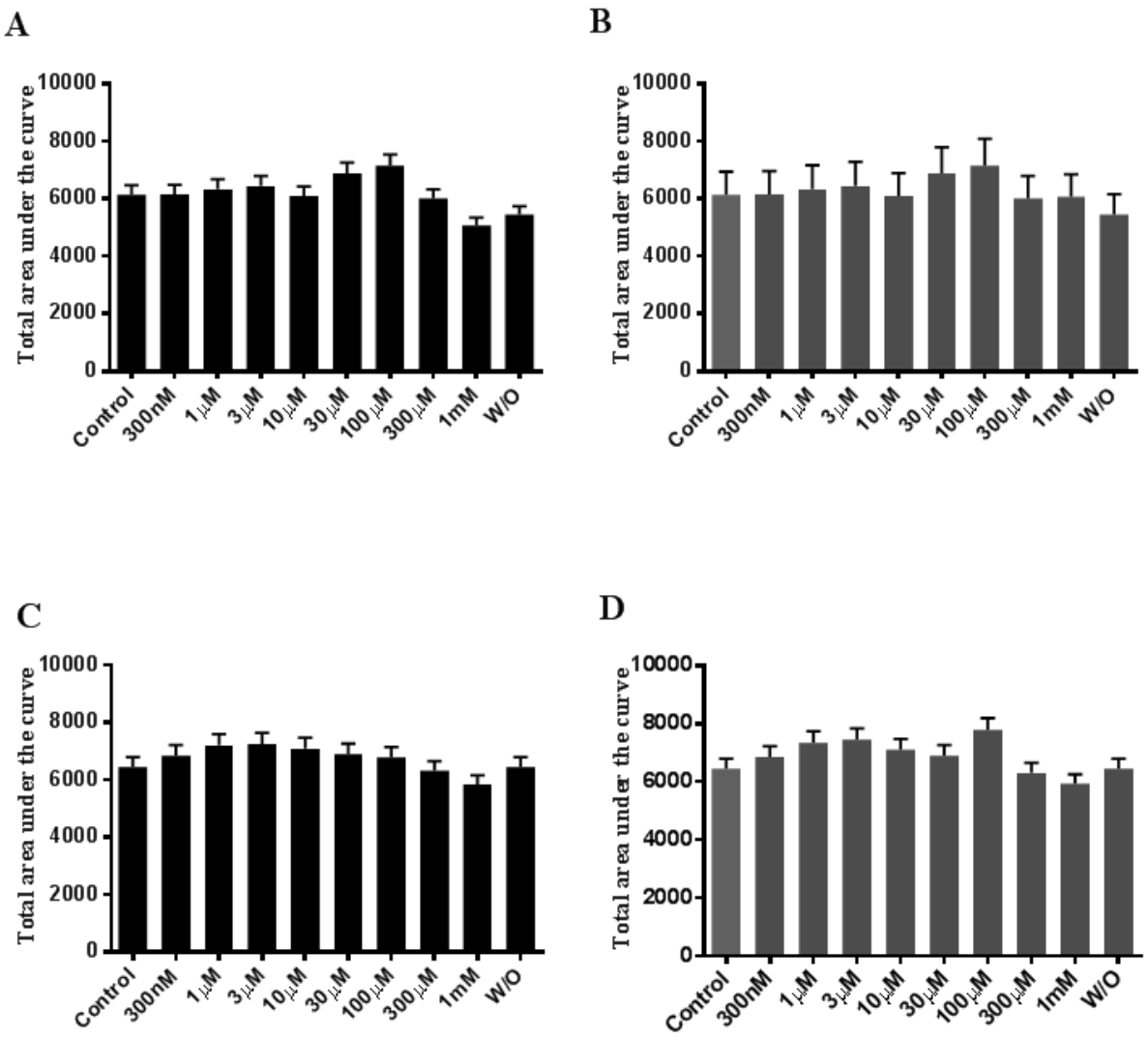


Figure 5.4 effect of 17estradiol on afferent response to distension between ERβ$^{-/-}$ ERβ$^{+/+}$ mice. No changes were observed in the afferent firing with 17estradiol in A; female ERβ$^{+/+}$ B, female ERβ$^{-/-}$ C, male ERβ$^{+/+}$ and D, male ERβ$^{-/-}$ **.** (P>0.05 one way ANOVA followed by a Bonferroni post-test) Data are expressed as means ± S.E.M.

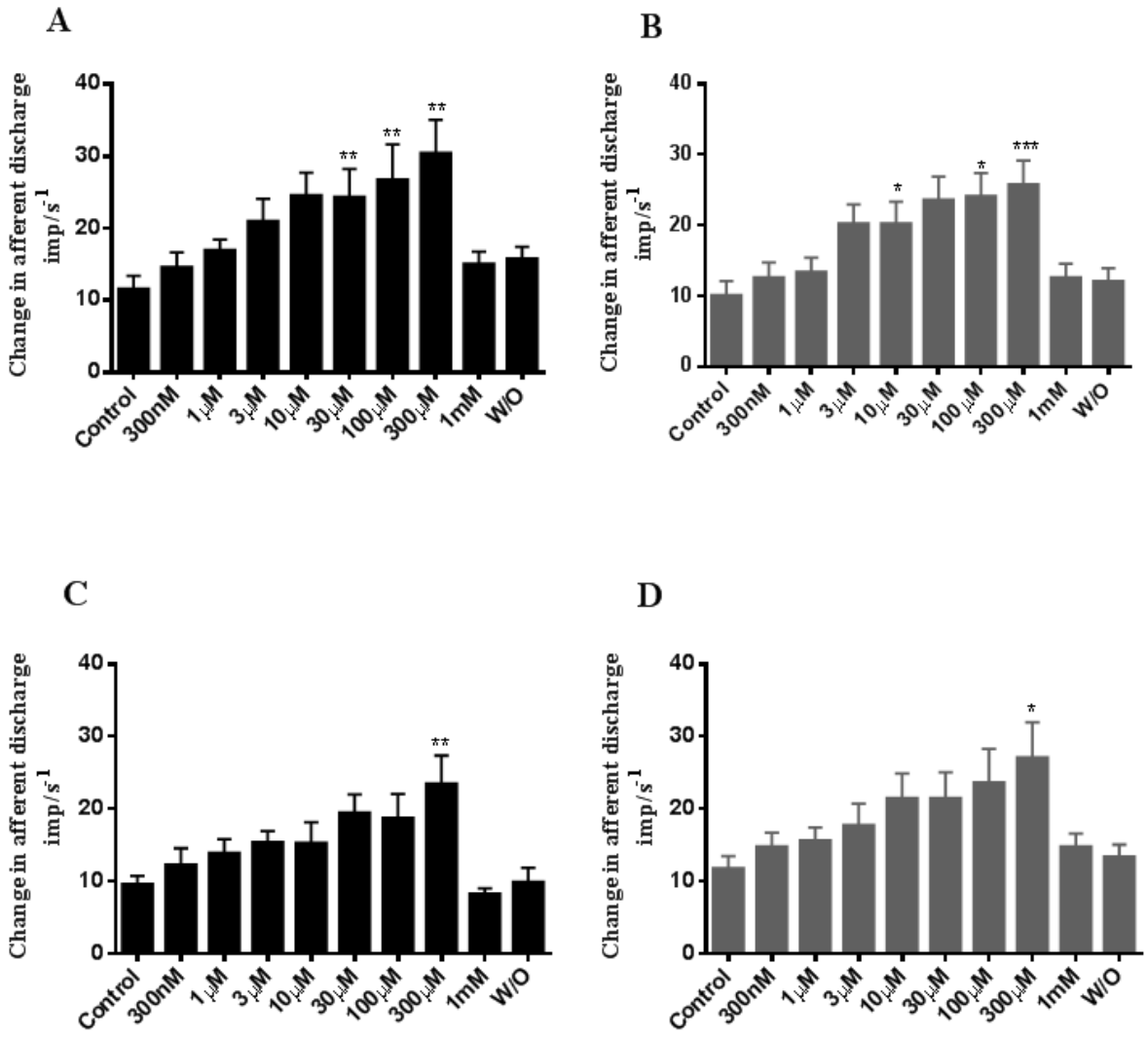


Figure 5.5 effect of 17β estradiol on the baseline firing between ERβ$^{-/-}$ ERβ$^{+/+}$ mice. 17β estradiol significantly increased baseline firing in A; female ERβ$^{+/+}$ (**P<0.01) B, female ERβ$^{-/-}$ (*P<0.05, ***P<0.001) C, male ERβ$^{+/+}$ (**P<0.01) and D, male ERβ$^{-/-}$ (*P0.05) one way ANOVA followed by a Bonferroni post-test) Data are expressed as means ± S.E.M.

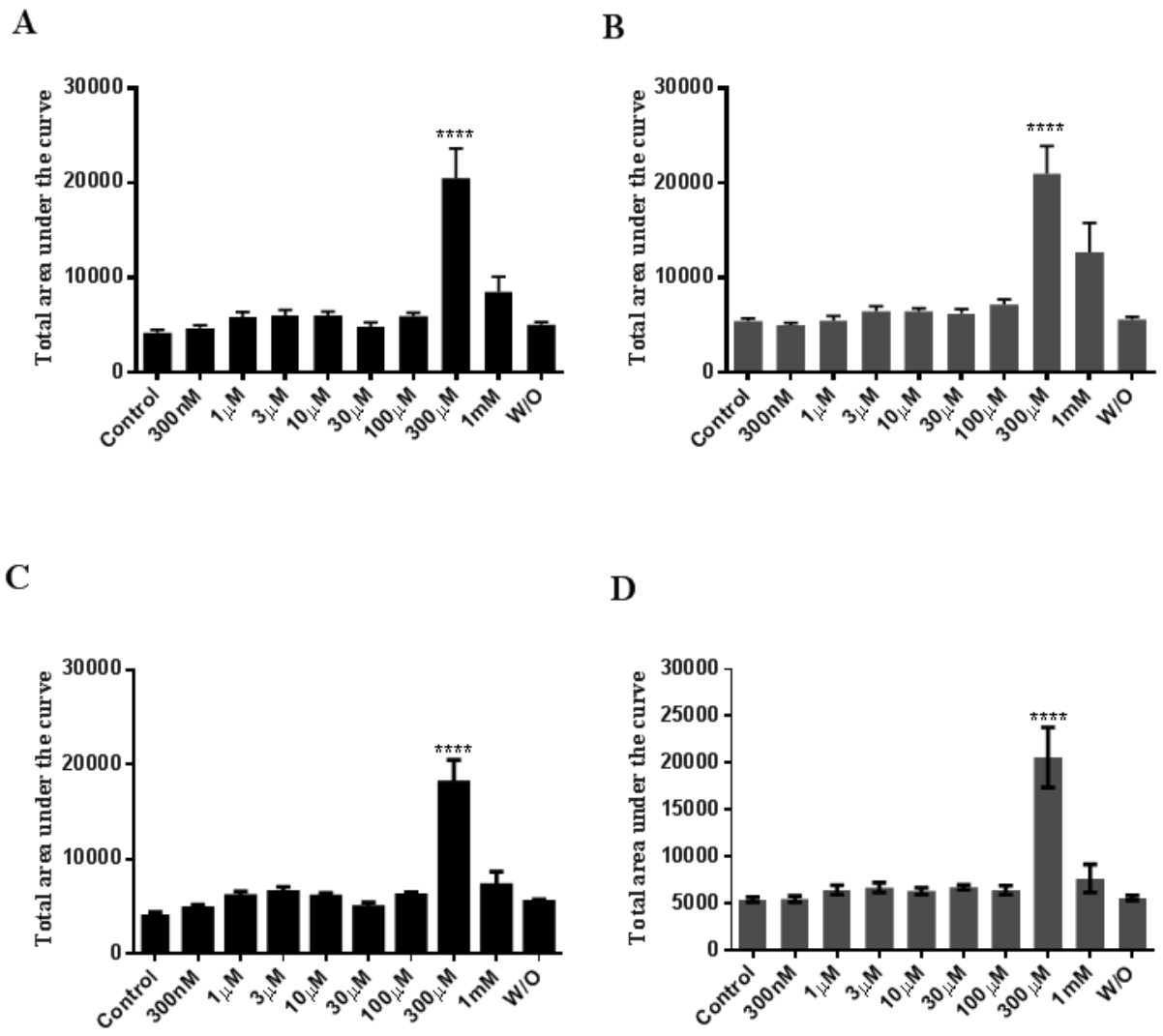


Figure 5.6 effect of 17β estradiol on bladder compliance between ERβ$^{-/-}$ ERβ$^{+/+}$ mice. 17β estradiol significantly increased compliance at a concentration of 300M in A; female ERβ$^{+/+}$, B: female ERβ$^{-/-}$, C: male ERβ$^{+/+}$ and D: male ERβ$^{-/-}$ (****P<0.0001) one way ANOVA followed by a Bonferroni post-test) Data are expressed as means ± S.E.M.

Table 5.1 General information regarding the mice used in this study

Animal identification	Body weight (g)	Strain	sex	Age	Delivery date
1	34.2	KO	F	N/A	28/04/2010
2	42.5	KO	F	N/A	28/04/2010
3	46	KO	F	N/A	28/04/2010
4	39.9	KO	F	N/A	28/04/2010
5	41.3	KO	F	N/A	28/04/2010
6	40.8	KO	F	N/A	28/04/2010
7	33.2	KO	F	N/A	28/04/2010
8	49	KO	F	N/A	28/04/2010
9	41.9	KO	F	N/A	28/04/2010
10	38.3	KO	F	N/A	28/04/2010
11	35.5	WT	F	N/A	28/04/2010
12	44.4	WT	F	N/A	28/04/2010
13	40.8	WT	F	N/A	28/04/2010
14	43.1	WT	F	N/A	28/04/2010
15	33.7	WT	F	N/A	28/04/2010
16	45.7	WT	F	N/A	28/04/2010
17	49.3	WT	F	N/A	28/04/2010
18	53.3	WT	F	N/A	28/04/2010
19	52.5	WT	M	N/A	28/04/2010
20	39.4	WT	M	N/A	28/04/2010
21	31.7	WT	M	N/A	28/04/2010
22	40.1	WT	M	N/A	28/04/2010
23	44.8	WT	M	N/A	28/04/2010
24	42.9	WT	M	N/A	28/04/2010
25	49.4	WT	M	N/A	28/04/2010
26	51.6	WT	M	N/A	28/04/2010
27	51.9	KO	M	N/A	28/04/2010
28	51.6	KO	M	N/A	28/04/2010
29	42.8	KO	M	N/A	28/04/2010
30	48.2	KO	M	N/A	28/04/2010
31	48.7	KO	M	N/A	28/04/2010
32	41.1	KO	M	N/A	28/04/2010
33	46.8	KO	M	N/A	28/04/2010
34	43.6	KO	M	N/A	28/04/2010

5.4 Summary

- This Study investigated the role of oestrogen receptor β in bladder afferent signalling. The study used *in vivo* metabolic cage experiments and *in vitro* afferent nerve recording to measure the voiding parameter between male and female ER-β ko and WT mice as well as look at the changes in afferent nerve activity between the different groups.
- No significant difference were observed in the voiding parameters between the male $er\beta^{+/+}$ and $er\beta^{-/-}$ mice and the female $er\beta^{+/+}$ and $er\beta^{-/-}$ mice.
- No changes were also observed in the afferent response between the male $er\beta^{+/+}$ and $er\beta^{-/-}$ mice and the female $er\beta^{+/+}$ and $er\beta^{-/-}$ mice.
- However, the bladder compliance was significantly reduced in the male KO mice compared to the WT mice.
- No changes were observed in the bladder compliance between the female WT and KO mice.
- 300μM estradiol significantly increased the bladder compliance and the baseline afferent response in the male and female WT and KO mice.
- This study indicates a potential role of oestrogen in bladder sensation; in particular there are significant changes in bladder compliance. However, further studies are required to classify oestrogen receptor beta knockout mice as a model of interstitial cystitis.

CHAPTER 6
DISCUSSION

Historically, the main focus in the field of bladder research was given to the mechanism underlying efferent function and detrusor contraction. In the last decade the importance of afferent signalling in bladder function has been realised. Mechanosensitivity is the ability of the nerve to detect mechanical changes. This is vital for the on-off circuit of micturition. The afferent nerve innervating the bladder send information to the CNS, which allows voiding reflexes to be generated via the efferent input to the bladder (Birder., 2013). Although the exact mechanisms involved in the control of micturition reflex are still unclear, a lot of focus has been given to the urothelium in relying information to the underlying afferent nerves. However, to regulate the mechanosensitivity there must be a system that coordinates the excitability of the bladder afferents.

As previously discussed, TRP channels may govern the mechanosensitivity of these afferents. Over-expression of these channels has been observed in various pathological states in the bladder (Lashinger *et al.,* 2008, Tsukimi *et al.,* 2005 and Mukerji *et al.,* 2006). Therefore, investigating the functional significance of TRP channels in normal bladder physiology may help to understand the molecular mechanism associated with bladder dysfunction. However, the study of TRP channels has proven to be challenging, because many compounds seem to interact with multiple TP channels (Zheng *et al.,* 2013).

This thesis focuses on investigating these channels in bladder mechano-sensitivity; In particular the role of TRPM8, TRPV1 and TRPA1 has been studies. A lot of importance has been given to the interaction between these receptors, since these channels may act in a cascade, and changes in one receptor may influence changes in another receptor, hence changing the excitability of afferent and then finally leading to increased/decreased sensation.

TRPM8 channel and bladder function

Studies conducted by *Stein et al* and *Mukerji et al,* initiated an interest in the function of TRPM8 in the bladder, especially in the pathophysiology of the bladder-cooling reflex and as potential target for future drug treatments for DO/OAB. *Stein et al*, reported the activation of TRPM8 by menthol and cool temperatures (8C to 28C) and proposed TRPM8 as a contributor to the diagnostic ice water test (Stein *et al.,* 2004). *Mukerji et al,* reported a correlation between the expression of TRPM8 in nerve fibres and severity of OAB (i.e. there is an increase with increasing severity). Additionally, they suggested the involvement of TRPM8 in the symptomatology and pathophysiology of these disorders. The most interesting finding was provided in 2006, where exaggerated pain score were reported in PBS patients with the intravesical instillation of ice water but not by other patients (Mukerji *et al.,* 2006a and Mukerji *et al.,* 2006b). Similarly, Lashinger *et al* (Lashinger *et al.,* 2008) with the aid of AMTB (TRPM8 antagonist), has reported the contribution of TRPM8 in the micturition reflex and nociceptive signalling in rats, further suggesting a need for the investigation of TRPM8 in bladder sensory signalling.

However, despite the large body of work, the functional significance of TRPM8 in normal bladder function remains unidentified. There is also no conclusive evidence to date for the receptor mechanism involved in the bladder-cooling reflex and hence, the ice water test. The main focus of this thesis was to investigate the role of TRPM8 in bladder afferent signalling.

Comparison in bladder sensory firing between WT and TRPM8 KO mice

This is the first study to compare bladder afferent response between $TRPM8^{+/+}$ and $TRPM8^{-/-}$ mice. The current study compares the afferent response during bladder distension and at rest. By

monitoring effects on bladder compliance enables any effects secondary changes in muscle tone to be determined. However, no differences between TRPM8$^{+/+}$ and TRPM8$^{-/-}$ mice were found in any of the above-mentioned parameter. This may not be too surprising since the thermo-sensitive properties of TRPM8 would be functionally silent in the bladder under normal physiological condition. They might only become recruited or sensitised under pathological conditions. This would be consistent with the negative cold saline test except in patients with painful bladder syndrome. Conversely, there could be compensatory mechanisms, such as the up-regulation of other TRP channels, which may account for the lack of differences observed between WT and KO mice. However, no molecular or protein analysis studies were conducted to confirm this.

Effect of menthol on bladder afferent firing

Studying the short-term effects of agonist/antagonist application provides a way of determining the functional role of TRPM8 in modulating bladder afferent sensitivity, avoiding long-term compensatory mechanism that may occur in knockout animals. In this respect menthol has been used extensively to gain understanding. In the present study, administration of menthol had both inhibitory and excitatory effects on bladder afferent sensitivity. The excitatory effects, observed at low concentrations, appear to be TRPM8 dependent since they are absent in the TRPM8 KO. However, the inhibitory effect would appear to be independent of TRPM8 since this persisted in the KO animals. Thus, specificity of menthol to TRPM8 is concentration dependent; 150μM menthol results in an increase in afferent firing in the WT, but is absent in the KO mice, suggesting a TRPM8 dependent pathway. However, at higher concentrations (2mM) an inhibitory response is observed, that is TRPM8 independent. This study therefore provides insight into the direct effect of menthol on bladder afferents, as well as the non-specific nature of the compound. The effect of

menthol on sensory afferents has not been studied before; however, the effect of menthol on detrusor muscle contraction and micturition reflex has been studied in several studies. *Nomoto et al* used cystometry to study the effect of infusing 1-3mM menthol into the bladder of conscious female rat and observed an enhanced micturition reflex. In contrast, menthol was also shown to inhibit carbachol-induced contraction of detrusor smooth muscle (Nomoto *et al*., 2008). However, in the study conducted by *Nomoto et al,* they provided no evidence that inhibition was mediated via a TRPM8 dependent pathway. In a separate study, 100μM menthol has been shown to reduce basal tone and amplitude of spontaneous bladder contractions. In detrusor muscle carbachol induced contractions were inhibited by menthol. These studies show that menthol can influence detrusor muscle contraction. The influence on detrusor muscle may consequently affect nerve activity. However, this effect of menthol is possibly due to the blocking of calcium channels (as acknowledged by the authors), rather than through TRPM8 pathway (Paduraru *et al.*, 2011). Since the responses to menthol have been investigated in the absence of a knockout or an antagonist, the evidence to conclude that the effect is TRPM8 dependent is lacking.

According to our study, responses to menthol may be also due to pathways independent of TRPM8, as shown by the knockout studies in this thesis. Although, it needs to be acknowledged that there are species difference between the studies. Moreover we didn't study detrusor muscle contraction directly. However, in our experimental setup no changes were observed on the bladder compliance in our current study.

Effect of WS-12 on bladder afferent firing

The lack of specificity of menthol necessitated use of more selective compounds. In this study the effect of WS-12 on bladder afferent firing was found to be TRPM8-specific over a range of concentrations since responses were absent in the TRPM8 KO. Low concentrations of WS-12

(10μM) had a profound effect on sensory firing in comparison to menthol. Both intra- and extraluminal infusion of 10μM WS-12 resulted in an increase in sensory firing to distension, which was TRPM8 dependent. We found Ws-12 to be acting through the activation of TRPM8. Similarly, Beck *et al*, investigated the effect of WS compounds on TRPM8 channels and WS-12 was found to have an EC50 of 30nM, which is 2000 times more potent than that of menthol and 20 than that of icilin. The authors concluded that WS-12 is probably the highest-affinity TRPM8 agonist available (Beck *et al.,* 2007). *Bodding et al*, also concluded that WS-12 is most potent than menthol for TRPM8 activation. They also examined a range of other TRP channels which were not activated by WS-12 at μM concentrations. WS-12's efficacy was also shown to be similar to icilin (Bodding *et al.,* 2007 and Sherkheli *et al*., 2010). Other TRP receptors, including TRPV1, TRPV2, TRPV3, TRPV4 and TRPA1 have been shown not to be activated at concentration up to 1mM (Ma *et al*., 2008).

The excitatory effect of WS-12 on bladder afferent signalling was observed only during distension and had no effect on baseline firing. In contrast, menthol had a dramatic effect on baseline firing that was absent in the TRPM8 KO. This suggests that the activation of TRPM8 through WS-12 requires an additional mechanical force.

Afferent response to the intraluminal infusion of cold saline into the bladder

A role of TRPM8 in the bladder-cooling reflex has been postulated in various studies. *Mukerji et al*, observed exaggerated pain sensation in PBS patients to the infusion of ice water in comparison filling with saline at room temperature (Mukerji *et al.,* 2006). Additionally, they showed an

increase in TRPM8 expression in the bladder of PBS patients, consistent with a role for TRPM8 in its pathogenesis. However, the receptor mechanism underlying this exaggerated pain perception of cold in PBS patients is unclear. Experiments to identify the effect of cold on bladder afferent signaling were therefore conducted to better understand the basis of symptom generation in the ice water test.

Intraluminal infusion of cold saline into the bladder of TRPM8 $^{+/+}$ results in an increase in the afferent response to distension. This augmentation of the distension response was absent in the KO. Interestingly, this increase in afferent response was only observed during bladder distension and absent at rest. Similar to WS-12, cold saline may not be sufficient to activate TRPM8 under baseline conditions, and requires the additional mechanical stress for activation. In this respect distension with ice cold water is necessary to evoke pain in PBS patients. However, unlike menthol and WS-12 the effect of cold saline was only observed during the first distension and was not reproducible in the consequent distensions. Only with alternating infusion of cold and warm saline could repeatable responses to cold be evoked while warm saline (38°C) alone had no effect on bladder afferent firing in either the WT or TRPM8 KO mice. A possible reason for this observation could be that the duration between two distensions wasn't sufficient to bring the local tissue temperature back to 36°C and so the TRPM8 receptor remained desensitized.

Another reason could be, with the infusion of cold, TRPM8 under goes a significant conformational change, changing the opening probability of the channel. Upon opening, the influx of calcium consequently activates PLC, and results in PIP2 depletion, hence inactivating or desensitising the channel. The resensitisation of the channel requires PKC dependent pathway. The continued exposure of cold may cause the channel to desensitize, as well as slowing down the

activity of the PKC dependent restoration of channel activity. Hence infusion of warm (allows the stimulus to be removed) saline is required to restore this process, before the second application of cold saline.

Nevertheless, the current study suggests that TRPM8 is involved in cold perception within the bladder and may possibly account for the painful response observed during the ice water test.

Effect of PF-05105679 on the bladder afferent firing

Several TRPM8 antagonists have been developed (Table 3.2). However, the lack of specificity with activity at other TRP receptors has always presented difficulties when studying the functional role of TRPM8. PF-05105679 is a compound manufactured by Pfizer**.** The current study aimed to investigate the effect of this drug on bladder afferent signalling. Infusion of 10µM PF-05105679 had no effect on sensory firing or bladder compliance. This observation supports the previous conclusion, that TRPM8 may not have a significant role in normal bladder filling, as seen by the TRPM8-/- study. However, pre-incubation of PF-05105679 reversed the potentiating induced by 1µm WS-12, hence appears to be an antagonist for the TRPM8 receptor in the model studied. However, this observation doesn't confirm that this antagonist wouldn't be acting on other channels as well.

Effect of WS-12, cold and menthol on DRGs

To study whether WS-12 is acting directly on afferent nerves terminals on the urothelium or both we repeated the same experiments in cultured mouse DRG's. The effect of WS-12 was also studied on isolated mouse DRGs. The DRGs were obtained from section T-13 to L-2 and L6-S2 since these regions have previously been shown by retrograde labelling to innervate the bladder

(Yoshimura *et al.,* 1999). This increases the probability of bladder specific response being obtained. However, even so, only a small percentage of DRG neurons project to the bladder and the responses obtained from these experiments may be a general feature of sensory neurons rather than specific to the bladder innervation. Sensitivity to WS-12 was only reliably observed when the experiment was carried out at 35°C (close to body temperature). When experiments were conducted at room temperature, which is normal in cell culture studies, responses presented considerable variation in magnitude and time course of response. The variation might be due to the fact that TRPM8 might already be activated at the varying temperature of the room. The increase in calcium influx, due to the opening of the channel is already observed in the baseline response. Hence an additional activator (WS-12) will not have an additional effect. The percentage of cells responding to WS-12 were small, which is again consistent with previous studies (Peier *et al.,*2002 and Story *et al.,* 2003). The low percentage of cells may not have a significant impact on nerve sensation, but as reported by *mukerji et al.,* there is an increase in TRPM8 expression with increasing severity of disease in PBS. This change may have a significant input in sensation and contribute the pain felt during the ice water test in PBS patients (Mukerji *et al*., 2006).

Interaction of TRPM8 and TRPV1

This is the first study to show sensitization of TRPV1 to capasicin through TRPM8 in bladder afferents. The mechanism underlying sensitization of TRPV1 has gained a lot of interest in recent years, since this phenomenon has been shown to play an important role in the development of hyperalgesia. Reduced thermal hyperalgesia has been reported in$TRPV1^{-/-}$ mice during inflammation (Caterina *et al.,* 2000 and Davis *et al.,* 2000). Moreover, reduced mechanical

hyperalgesia has been observed in studies using TRPV1 antagonists (Lee *et al.*, 2005, Pomonis *et al.,* 2003 and Walker *et al.,* 2003). Hence, the involvement of TRPM8 in sensitization of TRPV1 makes it a potential target for novel analgesic.

Daly et al, have also observed similar response to 100 µM capsaicin (Daly *et al.,* 2007). Interestingly, within the jejunum 1µM capsaicin has been shown to induce a period of intense discharge (Rong *et al.,* 2004). The difference in concentration might be attributed to the urothelium, presenting an impermeable barrier. Alternatively, there might be a different activation threshold of TRPV1 through capsaicin between the bladder and gut. The response to 100µM capsaicin was comparable to previous studies (Daly *et al.,* 2007 and Rong *et al.,* 2004). A transient response, brief excitation followed by desensitisation of the afferents. Desensitisation is characterised by a significant reduction in baseline firing and reduced afferent response to distension. Reproducible responses to capsaicin were also observed, but after 1 hour of washout. $TRPV1^{-/-}$ mice exhibited neither excitatory nor desensitisation effects to 100µM capsaicin, confirming specificity to TRPV1.

Administration of WS-12 together with capsaicin resulted in a significantly augmented response to 1µM, 3µM, 10µM and 30µM capsaicin. The magnitude of response together with the duration of response was significantly increased. Hence the activation of TRPM8 resulted in sensitization of TRPV1 to capsaicin. Moreover, a loss of desensitization was also observed. Paradoxically, the response to 100µM capsaicin was completely inhibited in the presence of WS-12 (there was no alteration of baseline firing). Phosphorylation of TRPV1 might shift of the concentration response curve towards left, so an increased sensitisation is seen with 1µM.

The increase with the co-application of WS12 and 1µM capsaicin and the decrease with WS-12

and 100µM capsaicin may be acting through two different mechanisms. As previously mentioned, the urothelium is a strong barrier, which is why we need 100µM capsaicin on its own to see an effect, as only a small amount of that might reach the nerve terminal to initiate a response. If that is the case than the inhibitory effect with 100µM capsaicin and WS-12 is primarily occurring at the nerve terminal. Whereas, the increase seen with 1µM capsaicin and WS-12 might be primarily due to the urothelium instead, since this concentration is too low to cross the barrier and have an effect on nerve terminal (1µM capsaicin on its own did not change afferent nerve firing). Hence both of the opposing effects seen with the different concentration of capsaicin might be mediated through two different mechanisms.

TRPV1$^{-/-}$ mice exhibited neither excitatory nor desensitisation effects to the infusion of 1µM capsaicin in the presence WS-12, confirming specificity to TRPV1. The augmented response was also reduced in the presence of PF-05105679, suggesting involvement of TRPM8; although its needs to be noted that the response was not completely inhibited.

Various intracellular signalling pathways have been reported to regulate the sensitisation of TRPV1; hence these signalling pathways may also be involved in the interaction between TRPM8 and TRPV1. In particular, the phosphorylation of TRPV1 by protein kinases has been reported to influence TRPV1 sensitisation. For example, PKC has been reported to phosphorylate various residues on rat TRPV1 (Bhave *et al.,* 2003 and Numazaki *et al.,* 2002). On the other hand, PKA has also been shown to phosphorylate TRPV1 (Bhave *et al.,* 2002 and Mohapatra *et al.,* 2003). These protein kinases may increase the opening probability of TRPV1, resulting in channel sensitization (Vellani *et al.,* 2001). Moreover, endogenous also modulates activation of TRPV1 (Chuang *et al.,* 2001 and Prescott *et al.,* 2003). Removal of PIP_2 by PLC application increases the current initiated by capsaicin, heat and protons on membrane patches (Chuang *et al.,* 2001).

Therefore, we examined whether, PLC, PKC and PKA are involved in the intracellular mechanisms involved in the interaction between TRPM8 and TRPV1. LPC (U-73122) inhibitor did not affect the interaction between WS-12 and Capsaicin. However, PKA (H-89, 1µM) and PKC (GÖ6983, 1µM) inhibitor both reduced the augmentation significant, especially when combined together. Suggesting that the augmentation observed involves both PKC and PKA. This observation, suggests that the sensitisation seen by TRPM8 and TRPV1 might be due to phosphorylation of these channels. The concentration of H-89 used is higher than its IC50 value (135nm). The reason to use this concentration was based on similar studies that have used concentrations ranging from1µM-10µM (Armstrong *et al.,* 1995, Frazier *et al.,* 2005 and Hristov *et al.,* 2008). Nevertheless, it should be noted that H-89 can be non-specific and interact with other protein kinases at that concentration.

The current study is restricted by the unavailability of specific phospho-antibodies for TRPM8 and TRPV1 that might be able to detect phosphorylation. One of the obstacles faced by researchers in the field of TRP channel is the non-specificity of TRP antibodies. Moreover, the majority of drugs seem to interact with more than one TRP channel. One of the options we considered to study this mechanism further was to use mass spectrometry. Mass spectrometry is based on measuring the mass-to-charge ratio of charged molecules. In the case of the current study, phosphorylation of TRPM8 and TRPV1 would be investigated by measuring the change in the mass of peptide sequences of the ion channels in isolated bladder DRGs. In this case alteration of the original mass by the addition of phosphate groups to the daughter ions of TRPM8 or TRPV1 would be expected. Unfortunately, due to time constraints this part of the study was not completed, but would be an exciting area for further research.

Throughout the study there is an assumption that the activation leads to the sensitisation of TRPV1. There is high probability that the augmentation observed is through the sensitisation of TRPM8 or both TRPM8 and TRPV1. Forskolin and PMA were used to see whether the same augmentation could be reproduced by intraluminally administering either WS-12 or capsaicin with these compounds. This might allow us to assess whether the augmentation observed is through he sensitisation of TRPM8 or TRPV1 or both.

Foskolin is used to increase the levels of cyclic AMP, which activates cAMP dependent pathways, such as PKA. On the other hand PMA, which is structurally analogues to diacylglycerol, directly activated PKC (Hurley *et al.*, 1997 and McEwan *et al.*, 2007). Strangely, the application of these compounds with WS-12 or capsaicin did not result in any significant changes. The reason behind the lack of response seen with the combination of the drugs (forskolin and PMA) and WS-12 could be because PKA and PKC activation has been shown to inhibit TRPM8 (Premkumar *et al.*, 2005 and Abe *et al.*, 2006). However, the reasoning behind the lack of response seen with TRPV1 is unclear, because the phosphorylation of TRPV1 through PKA and PKC is well recognized (Huang *et al.*, 2006 and Palazzo *et al.*, 2012). There is a possibility that the isoform of PKC involved in TRPV1 phosphorylation is different to the one that PMA is activating. However, studies have suggested that the isoform involved in TRPV1 phosphorylation are α and ε, and PMA seems to activate these isoforms. Alternatively, the EC-50 of PMA is in the range of 10-100ng/ml), however the concentration of PMA used in the nerve recording experiments is 10μM. this concentration might be too high and instead may result in the down-regulation of the PKC pathway, hence leading to the inhibition of TRPV1.

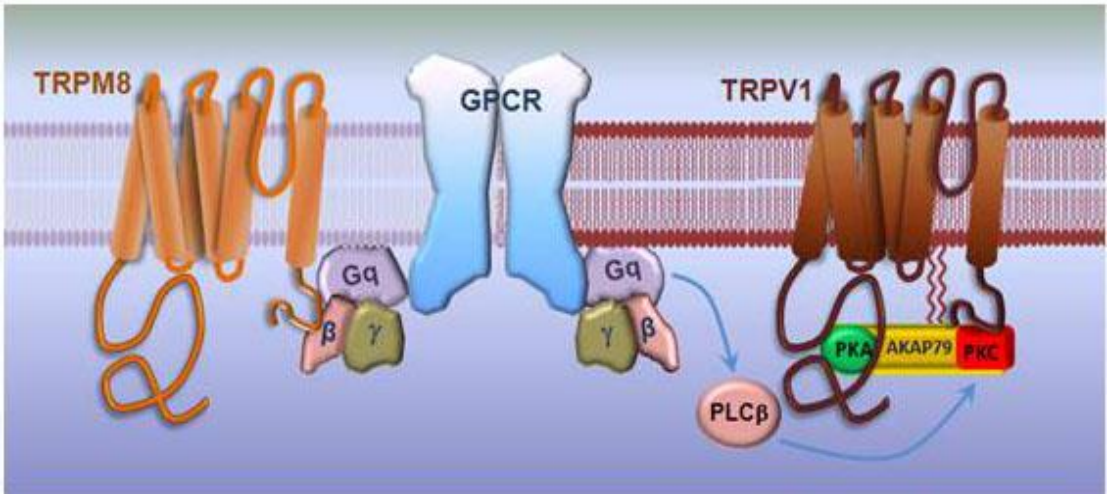
The idea that TPM8 can influence the gating of TRPV1, suggests a vital role of TRPM8 in bladder dysfunction. Previously, *Harrington et al* (2011), reported that a subpopulation of splanchnic afferents responding to icilin (TRPM8 agonist), also responded directly to capsaicin (3 μmol/L), and icilin reduced the direct chemosensory response to capsaicin. Within the gut, 3μM capsaicin is sufficient to significantly increase afferent firing (Rong *et al.*, 2004). Similarly, our data shows that WS-12 can inhibit the afferent response to a 100μM capsaicin (significantly increases afferent firing in the bladder). Surprisingly, our study also reports the significant augmentation of afferent signalling with the administration of WS-12 with lower concentration of capsaicin. This dual effect on afferent signalling via the interaction of TRPM8 and TRPV1 is a novel finding, which requires further exploration in order to elucidate the exact mechanism underlying this interaction.

If the interaction between TRPM8 and TRPV1 result in phosphorylation of the channel, what are the pathways that might be involved?

There could be direct interaction between TRPM8 and TRPV1. Previously, mice studies have refuted the co-expression of TRPM8 and TRPV1 (Peier *et al.,* 2002 and Story *et al.,* 2003). Conversely, the upregulation of both the receptor have been reported in pathological states, this upregulation may increase/ induce the co-expression of TRPM8 and TRPV1, hence causing a direct interaction between the channels.

On the other hand indirect interaction between TRPM8 and TRPV1 through, for example Gq-coupled GPCR is also suggested (Figure 6.1) (Zhang *et al.*, 2012). TRPM8 and TRPV1 have been suggested to modulate each other responses through the activation of Gq-coupled GPCR. The activation of GPCR might result in inhibition of TRPM8 through a protein complex formed by Gq and TRPM8 (Zhang *et al.,* 2012). Activated Gq directly inhibits TRPM8 channel. However,

TRPV1 sensitization through Gq-coupled GPCR might involve phosphorylation-dependent mechanism through PKA and PKC. The activation of TRPV1 inturn inhibits TRPM8 as both of the channels seem to act in the opposite direction. Similarly inflammatory mediators such as bradykinin and histamine have been shown to inhibit TRPM8 in sensory nerves through G-protein subunit Gq. whereas; the same mediators have resulted in the sensitisation of TRPV1 through phosphorylation. Interestingly, a recent study has also reported an interaction between TRPM8 and Gq (Klasen *et al.*, 2012), however in this study the activation of TRPM8 leads to the downstream activation of the Gq pathway. These reports suggest that both Gq and the gating of TRPM8 can modulate each other activity. The second scenario may be involved in the current study, where the activation of TRPM8 could activate Gq, which would then inhibit TRPM8. Additionally, the activated Gq, could also lead to the sensitization of TRPV1.



(Zhang *et al.*, 2012)

Figure 6.1: indirect interaction between TRPM8 and TRPV1

An important step in the study was to investigate whether there is interaction between

Effect of capsaicin and WS-12 isolated mouse DRGs (T13-L2 and L6-S2)

To investigate the interaction between WS-12 and capsaicin on DRG's, we established reproducible responses to 1μM WS-12 and 100nM capsaicin. The individual application of these drugs significantly increased calcium influx into the cells. 1nM capsaicin was also administered, but didn't have a significant effect on the calcium levels. The application of 1μM WS-12 with 1nM capsaicin significantly altered the calcium level when compared to individual application of these drugs. These results are comparable to the afferent nerve recording data observed in the *in vitro* preparation, suggesting a similar conclusion. However, because these cells didn't show an inhibitory response to 100nM capsaicin and WS-12, the idea that the inhibitory effect seen in the nerve preparation is happening at the nerve terminals seems unlikely.

Studying the interaction further in normal bladder function and also assessing the changes that occur in dysfunction may provide more information on the functional significance of these receptors in maintaining normal bladder sensation. It may also lead to the identification of new targets for therapy for patients with bladder hypersensitivity.

Effect of AITC, CA and hydrogen sulphide on bladder afferent

TRPA1 is a TRP channel activated by noxious cold. TRPA1 has been located in sensory nerves innervating the rodent bladder (Nagata *et al.,* 2005 and Streng *et al.,* 2008) and in the urothelium of both human and rat (Du *et al.,* 2007 and Streng *et al*., 2008). Upregulation of this channel has been shown in the mucosa of patients with bladder outlet obstruction (Du *et al.,* 2008). It has been

previously shown that the activation of TRPA1 trough AITC may result in contraction of the rat bladder (Andrade *et al*., 2006)

Administration of AITC and CA did not alter afferent nerve firing or bladder compliance. This observation contradicts previous findings by *Minagawa et al.* they reported significant increases in bladder afferent firing and detrusor over-activity (Minagawa *et al.,* 2013). Contrary to the above-mentioned study, all the TRPA1 agonists in our study were applied in the absence of protamine sulphate. Minagawa *et al.,* used protamine sulphate in their study to disrupt the urothelial barrier, however protamine sulphate may have other nonspecific effect that may contribute the results observed, any results obtained after the administration of this compound may be secondary to tissue injury observed with administration the of protamine sulphate and hence, difficult to interpret. The urothelium might present a strong barrier, or it could be that the activation of TRPA1 requires tissue injury.

It was also suggested that, TRPM8 might couple to TRPV1 and TRPA1 to inhibit their downstream chemosensory and mechanosensory responses (Harrington *et al.,* 2011). In our study, we didn't observe any excitatory response; instead we observed inhibition of baseline firing in response to the administration of AITC on its own. However, with the combined administration of AITC with WS-12, this inhibition was prevented and augmentation of sensory signalling was seen. Whether, this effect is direct on the signalling of TRPA1 or indirect remains to be identified. One possibility could be that the augmentation observed might act through or require the activation of TRPV1. Co-expression of both TRPV1 and TRPA1 (Story *et al.,* 2003) has been reported in a subset of sensory neurons innervating the bladder, whereas the co-expression of TRPM8 and TRPA1 has not been shown within bladder afferents. Similar to the interaction between TRPM8 and TRPV1,

we found the involvement of protein kinases, however as previously mentioned further investigation needs to be carried out to confirm the molecular mechanism involved in the interaction.

Conversely, hydrogen sulphide resulted in a significant increase in baseline firing; however, the specificity of hydrogen sulphide to TRPA1 needs to be confirmed. As previously observed with low concentration of capsaicin, the administration of WS-12 together with AITC resulted in a significant increase in baseline firing. Interestingly, the intraluminal application of WS-12 and AITC alone did not have an effect of baseline firing. As observed with capsaicin, both the magnitude of effect and duration of response were significantly increased, and this augmentation of response was inhibited by the administration of PKA and PKC inhibitors.

The next step in this study was to investigate whether the interaction between TRPM8 is limited to TRP channel or extends to other ion channel as well. This would indicate whether the augmentation observed is specific or results from an overall increase in excitability, such that the response to all receptor stimulation is increased.

TRPM8 and purinergic signaling (Effect of α, β methylene ATP on bladder afferent)

The purinergic receptors expressed on sensory nerves are divided into the ionotropic P2X receptors and metabotropic P2Y receptors (Ralevic *et al.*, 1998). α,β-Methylene ATP has been shown to act on P2X receptors. Alteration of purineric signaling has been shown in overactive and painful bladder diseases. Increase in the ATP released from the urothelium has also been reported in patients with interstitial cystitis as well as animal models of cystitis and neurogenic bladder (Smith

et al., 2008, Salas *et al*., 2007, Sun and Chai, 2006 and Birder *et al.,* 2003). P2X antagonists inhibited mechanosensitive bladder afferent discharge in isolated bladders from cyclophosphamide-treated rats (Yu and de Groat, 2008). These and various other data, show the importance of purinergic signaling in bladder function. The influence of TRPM8 activation on this signaling pathway (direct or indirect) indicates the importance of this interaction as a potential target for therapy.

The combined application of αβMethylene ATP and WS-12 also significantly increased the magnitude and duration of response. The protein kinase inhibitors (PKA and PKC) also inhibited this response. Similar to our current observation, the potentiating effects of extracellular ATP have been reported to be reduced by PKC inhibitors (Tominaga *et al*., 2001 and Tominaga *et al*., 2004). However, whether the same mechanism is involved in the interaction between TRPM8 and TRPV1/TRPA1/P2x receptors remains to be confirmed. There is lack of evidence for the phosphorylation of P2X receptors; however the activation of P2X could in turn phosphorylate TRPM8, through PKA and PKC dependent pathways.

From the data it is clear that there is an interaction between TRPM8, TRPV1, TRPA1 and P2X signaling. However, whether the interaction between the studied channels involves the same mechanism is unclear. It could be that the activating of TRPM8 changes the overall gating of the cell membrane, hence affecting the gating properties of the channels expressed on the surface membrane. However, the exact mechanisms involved in this process are still unclear.

The role of oestrogen receptor-β in bladder afferent signaling

One investigation that was briefly carried out in the course of this study was to assess whether oestrogen receptor beta deficient mice can be used as a model for interstitial cystitis. Interstitial cystitis (IC) also known as painful bladder syndrome is a chronic pelvic syndrome with no generally accepted treatment (Dell *et al.,* 2009). This clinical condition is manifested by sensory hypersensitivity of the urinary bladder, leading to exaggerated pain sensation and/or pressure in response to small volume of urine. IC is characterized by the symptoms of pain, frequency, urgency, and nocturia in the absence of bacterial infection or any other identifiable pathology (Butrick, 2003) and diagnosed mainly in women. It has been reported that IC affects more than one million people in the US alone (Clemens *et al.,* 2007).

So far, more than 20 animal models of IC have been reported. Unlike the oestrogen receptor beta-knockout mice, many models reported involve inducing bladder inflammation through chemical compounds. Some models have infiltration of immune cells in the bladder through the systemic instillation of self/foreign antigens, systemic viral infection has also been employed to induce bladder epithelial damage (Bon *et al.,* 2003, Bjorling *et al*., 2007, Birder *et al.,* 2005, Chen *et al.,* 2006, Fraser *et al.,* 2001, Chuang *et al.,* 2003, Guerios *et al.,* 2006, Hauser *et al.,* 2009, Kirimoto *et al.,* 2007, Lavelle *et al*., 2002, Lin *et al.,* 2008, Randich *et al.,* 2009 and Soler *et al*., 2008)

To date there has been no animal model described, with alteration of afferent signalling, that may contribute to pain perception observed in IC patients, and hence the research for drug targets is limited.

Epidemiological studies have long linked oestrogen deficiency with the increased occurrence of lower urinary tract dysfunction, with age, especially in women (Losif and Batra., 1984).

Consequently, various experimental and clinical studies have focused on the role of oestrogen in bladder dysfunction. In the current study, we evaluated the voiding parameters between ERβ and their wild-type littermates in both males and females. The voiding parameters measured in this study were total voided volume, voiding frequency and average urinary output per void. No changes were observed in any of the parameters stated above. Similarly, *Schroder et al* performed control cystometry in conscious $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice and mice that are lack both the receptor subtypes and also found no significant difference in the voiding pattern compared to their wild type litter mates, suggesting, that the knockout of the oestrogen does not affect normal voiding.

This is the first study to compare the sensory responses between $ER\beta^{+/+}$ and $ER\beta^{-/-}$ mice. However, similar to the voiding pattern, no changes were also observed in the afferent firing between the wild type and the KO mice (both male and female). The lack of differences observed in our study may be attributed to the age of the mice, since mice were above 8 month old, at which they reach reproductive insufficiency, which is equivalent to postmenopausal in women. However, there are various reports that indicate a role of oestrogen in regulation of nociception and pelvic pain (Vasudevan *et al*., 2008 and Lonze *et al.,* 2002), for example the identification of oestrogen receptor in the primary afferent neurons in the bladder. Moreover, expression of oestrogen receptors has also been found in the majority of lumbro-sacral bladder sensory neurons. Hence, we investigated the direct application of 17estradiol on sensory nerves. A significant increase was observed in baseline firing, suggesting that ERs are functionally expressed in the terminals of bladder afferents and can influence sensation. Whether, this phenomenon is the case in ERβ knockout models remains unclear.

Conversely, there was a significant reduction of bladder compliance in the male KO mice compared to their wild type litter mates. Surprisingly, this finding was absent in the female mice. This result suggests that oestrogen can influence detrusor muscle.

The concentration response relationship of 17β- estradiol was also investigated on bladder sensory function as well as bladder compliance in these mice. 17β- estradiol is the most potent oestrogen in the circulatory system that acts both on ERα and ERβ. The present results show that 17β-estradiol induced a concentration dependent increase in compliance and baseline firing. It is possible that the increase in sensory function observed may be secondary to the bladder compliance. In line with this finding, a reduction in bladder compliance in ovariectomised rats has also been reported (Aizawa *et al.,* 2011). Oestrogen is produced by ovaries, hence removing ovaries have shown to reduce the level of oestrogen in the circulatory system, leading to a reduction in bladder compliance.

The exact mechanism of how oestrogen effects bladder compliance is unknown. The expression of both ERs have been found on the detrusor muscle. It has been suggested that oestrogen can direct influence (non-genomic) the function of the detrusor muscle through the modulation of muscarinic receptors (Batra *et al.*, 1989) and by inhibition intracellular calcium influx into muscle cells (Elliott *et al.,* 1992). Similarly, it has been reported that estradiol attenuates both the amplitude frequency of spontaneous contractions of the detrusor muscle (Shenfield *et al.,* 1998)

One major aspect that might have limited the investigation is the age of mice studied. The mice used in these experiments were above 12 months old; Mice and rats are sexually mature by 3 to 6 months of age and at 9 months of age they are equivalent of human perimenopause by 9 months of age (Flurkey *et al.,* 2007 and Mobbs *et al.,* 1984). Mice become reproductive senescence between 9-12 months (Mobbs *et al.,* 1984). So the aged any changes that could have been present

between wild type adult (premenopausal) mice (when oestrogen plays a primary role) and ERβ KO mice were not investigated.

Another factor that makes the study difficult to interpret is the concentration of estradiol used. It has been shown that the concentration of estradiol in female mouse serum is <4pg/ml (Daniel *et al.,* 2011). This is far lower than the concentration used in the current experiment. The high concentration is probably having numerous non-specific effects.

This study indicates a potential role of oestrogen in bladder sensation; in particular there are significant changes in bladder compliance. However, further studies are required to classify oestrogen receptor beta knockout mice as a model of interstitial cystitis.

This study does point out the need for an appropriate model for IC, especially in term of changes in afferent signalling, that may account for the pain seen in IC. Such a model would then also be useful for the investigation of ion channel activation that leads to altered sensory signalling. This is an interesting idea, however, identifying and characterizing such a model has many challenges.

This thesis has many interesting observations that look into the role of TRPM8 in bladder afferent signalling. This investigation further extends to the interaction of TRPM8 with TRPV1, TRPA1 and purinergic signalling. However, the study needs to be taken further in order to understand the role of these interactions in the whole afferent limb of the micturition reflex pathway.

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