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THE ROLE OF THE UROTHELIAL MUCOSA IN BLADDER MECHANOSENSATION

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A thesis submitted in partial fulfilment of the requirements of Sheffield Hallam University for the degree of Doctor of Philosophy by Research.

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ABSTRACT

This thesis investigates the role of the urothelium in bladder mechanosensation with focus on the cholinergic signalling pathway. The urothelium can be seen as a first responder to physiological changes in the bladder. It releases a host of mediators including ACh, ATP and NO that play a coordinated role in the stimulation of signalling cascades, which then lead to the onset of afferent nerve activity and detrusor muscle contraction, and finally trigger the micturition reflex. However, the underlying signalling pathways remain elusive. Understanding the signalling pathways involved in the mechanosensation of the urinary bladder will improve the understanding of bladder function, both in health and pathology and ultimately lead to novel treatment options available to patients of lower urinary tract conditions such as overactive bladder syndrome.

Mediator release experiments in the whole, isolated murine bladder depicted that ACh is released in a mechanosensitive manner from the urothelium. Distension of as little as 5 mm Hg stimulated a significantly greater release of ACh compared to resting level. However implementation of rising pressure levels showed that the amount of ACh decreased in a negative correlation to the applied pressure. A stretch dependent choline uptake appears to be a convincing explanation for these results. Spontaneous contractions were also measured in the whole, isolated murine bladder and showed a positive correlation to increasing distension levels. Blocking different components of ACh release and choline uptake in the urothelium showed that interfering at any point in the cholinergic pathway does trigger the same response in the muscle, leading to an extremely contracted detrusor muscle, even at low pressure levels. It has been hypothesized in previous studies, that the balance of inhibitory and excitatory mediators released from the urothelium modulates afferent nerve activity and therefore bladder contractility. This balance might be altered when blocking components of the cholinergic pathway. In the present thesis it was also shown, that application of BoNT/A, a novel treatment option for lower urinary tract symptoms, alters this balance by decreasing the excitatory mediator ATP and by increasing the inhibitory mediator NO. How exactly these mediators are balanced and which imbalances occur in the onset of lower urinary tract symptoms will be the topic of future research.

New insights on the physiological changes occurring in the naturally aged murine bladder have been shown in this thesis. A significantly higher sensitivity of the aged detrusor in the contractile response to purinergic and muscarinic stimulation was observed. Furthermore, urothelial release of ATP was increased while release of ACh was decreased in the aged bladder. Urothelial NO release was not affected by age and Substance P could not be shown to be released by the urothelium of adult or aged murine bladders. Moreover an increased purinergic receptor sensitivity of aged urothelial cells was shown, which is probably facilitated via the purinergic $P2X_3$ receptor. Further characterisations of the studied pathways are now required to fully validate the data, most suitably in human tissue, as it is not clear if the same pathways are affected by age in the human bladder.

Spontaneous contractions and carbachol-induced contractions were measured in male and female porcine bladder strips isolated from the dome, body and trigone region. The amplitude and frequency of these contractions were analysed. After cholinergic stimulation the amplitude of denuded tissue strips was higher compared to the intact counterparts in all bladder regions and in both genders. The existence of an urothelial derived inhibitory factor had been proposed before and would explain these results. Functional differences were also observed between the three bladder regions, particularly the female trigone seems to act differently compared to the bladder dome and body. Gender differences were not observed when comparing spontaneous activity of male and female bladder strips. However, after cholinergic stimulation, female tissue strips from the trigone region showed significantly higher amplitude and frequency in the contractile response. Gender differences and regional disparities should be considered when comparing the findings of detrusor contractility studies. In further experiments it should be examined if mediator release and receptor distribution of the urothelium differs between the different regions of the bladder and between the genders.

In summary it can be stated that the urothelium plays an important role in signalling processes and in detrusor contractility of the porcine and murine urinary bladder. Further research is required to fully understand the signalling pathways in the different bladder cells and their interactions.

DECLARATION

This thesis is submitted to Sheffield Hallam University in partial fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

This research represents my own original work towards this research degree and contains no material which has been previously submitted for a degree at this university or any other institution.

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PUBLICATIONS

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Collins VM, Daly DM, **Liaskos M**, McKay NG, Sellers D, Chapple C & Grundy D (2013) OnabotulinumtoxinA significantly attenuates bladder afferent nerve firing and inhibits ATP release from the urothelium. BJU International, 112(7), 1018-1026.

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ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine 5'-triphosphate
BoNT/A	Botulinum toxin-A
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
CarAT	Carnitine Acetyltransferase
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
ChAT	Choline Acetyltransferase
ChT	High-affinity Choline Transporter
CNS	Central Nervous System
CO_2	Carbon Dioxide
DAG	Diacylglycerol
DO	Detrusor Overactivity
EL	Extraluminal
eNOS	Endothelial Nitric Oxide Synthase
GABA	γ-Aminobutyric Acid
GAG	Glycosaminoglycan
$G_{i/o}$	G-Protein subtype
$G_{q/11}$	G-Protein subtype
GTP	Guanosine Triphosphate
H_2O_2	Hydrogen Peroxide
HRP	Horseradish Peroxidase
ICC	Interstitial Cells of Cajal
ICLC	Interstitial Cajal-like Cells
ICS	International Continence Society
ICs	Interstitial Cells
IgG	Immunoglobulin G
IL	Intraluminal
iNOS	Inducible Nitric Oxide Synthase
IP_3	Inositol Trisphosphate
K^+	Potassium
LUT	Lower Urinary Tract
LUTS	Lower Urinary Tract Symptoms
mAChR	Muscarinic ACh Receptor

MLA	Methyllycaconitine
mRNA	Messenger Ribonucleic Acid
nAChR	Nicotinic ACh Receptor
Na^+	Sodium
NA	Noradrenaline
NDO	Neurogenic Detrusor Overactivity
NGF	Nerve Growth Factor
$NK_{1/2/3}$	Neurokinin 1/2/3 Receptor
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
O_2	Oxygen
OAB	Overactive Bladder
OCT	Organic Cation Transporter
P2X	Purinergic Receptor Subtypes
P2Y	Purinergic Receptor Subtypes
PKA	Protein Kinase A
PLC	Phospholipase C
PP_i	Pyrophosphate
RFU	Relative Fluorescence Units
RLU	Relative Light Units
SEM	Standard Error of the Mean
SNAP-25	Synaptosomal-associated Protein 25
SNARE	Soluble N-ethylmaleimide-sensitive-factor Attachment Protein Receptor
SV2	Synaptic Vesicle Protein 2
TRP	Transient Receptor Potential Channels
TTX	Tetrodotoxin
UDP	Uridine Diphosphate
UDIF	Urothelium-derived Inhibitory Factor
UI	Urge Incontinence
UP	Uroplakin
UT	Urinary Tract
UTP	Uridine Triphosphate
UUT	Upper Urinary Tract
VAChT	Vesicular Acetylcholine Transporter

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1. INTRODUCTION

1.1 ANATOMY AND PHYSIOLOGY OF THE URINARY BLADDER

The urinary tract (UT) can be divided into the upper urinary tract (UUT) which consists of the kidneys and ureters and the lower urinary tract (LUT) which is defined by the bladder and the urethra. Under normal conditions over 0.5 ml of urine per kg of body weight is continuously produced per hour by both kidneys. The urine drains from the kidneys via the ureters through ureteral openings into the bladder (Chapple 2011). The bladder itself is a hollow, muscular organ. The bladder wall consists of the urothelium, the lamina propria with muscularis mucosae and interstitial cells (ICs), the detrusor muscle and connective tissue (Figure 1).

The UT has the function to produce, transport, store and expel urine as well as to homeostatically regulate the blood plasma volume and solute concentration. The fundamental role of the bladder is to collect and store the urine produced by the kidneys at low pressure in order to expel it at an appropriate time and place. Urinary continence is maintained by coordination of the detrusor smooth muscle contraction and the closure pressure of the urethral sphincter, which act as two opposing forces (Martini and Nath 2009, Chapple 2011).

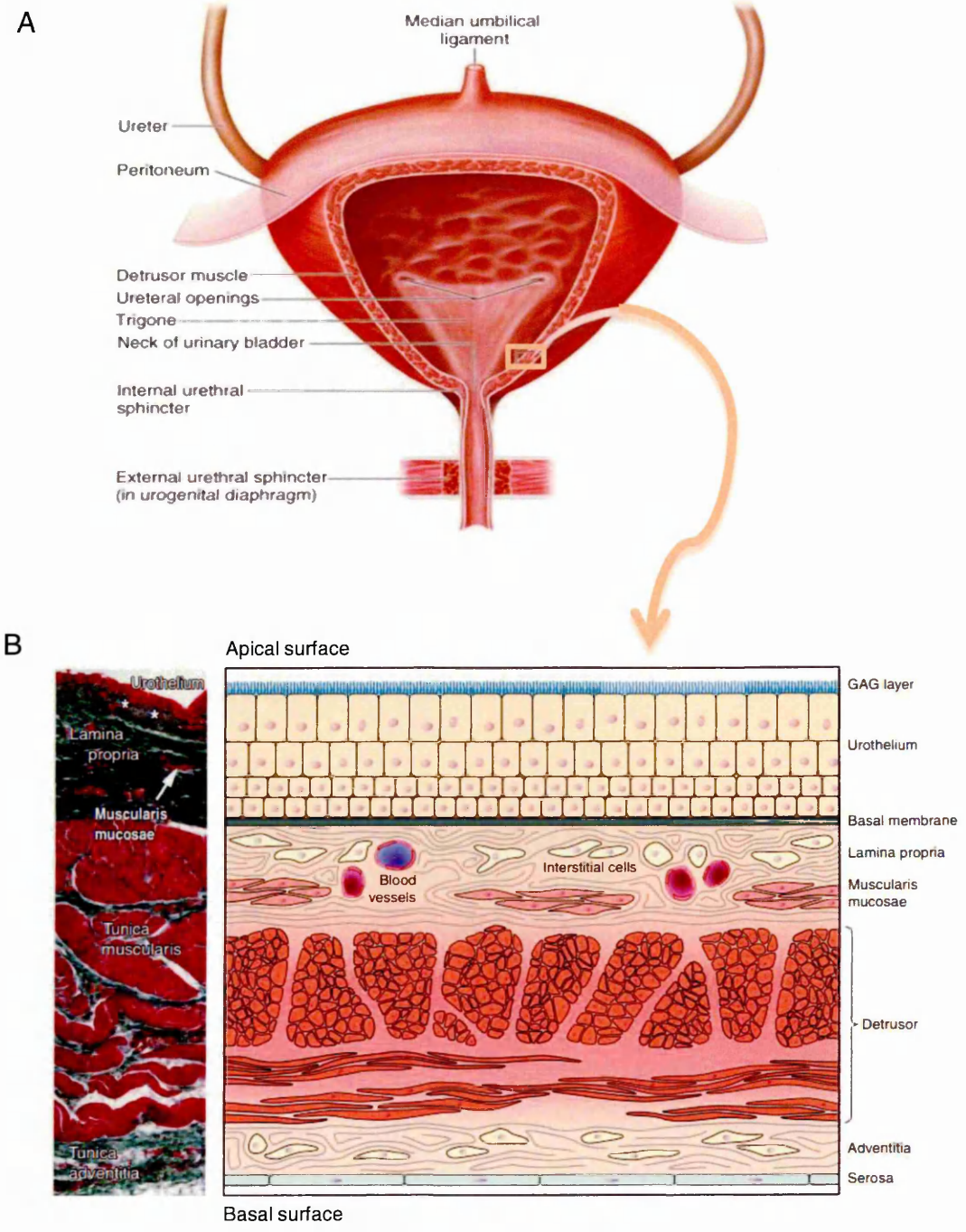


Figure 1 **Schematic of the lower urinary tract and the bladder wall. (A) Anatomy of the human lower urinary tract and (B) cross section of the bladder wall (adapted from McKinley and O'Loughlin 2006 and Birder and Andersson 2013).**

(GAG – Glycosaminoglycan)

1.1.1 DETRUSOR MUSCLE

The detrusor is a smooth muscle which consists of three muscle layers. The cells of the outer and inner layer occur in a longitudinal orientation while the middle layer occurs to have a circular orientation. The individual muscle cell is long, spindle-shaped with a central nucleus and represents a typical smooth muscle cell. Bundles of muscle cells vary in size in the human detrusor and are surrounded by connective tissue rich in collagen. The micturition cycle provides a challenge for the detrusor muscle components, as they have to relax and elongate during the filling stage, while generating force and shortening in a synchronised and fast way during micturition. The processes underlying this mechanical adaptation are not fully understood. Nervous synchronisation of the functional units of smooth muscle cells generates a voiding contraction of the whole bladder. This action is regulated by a cellular signalling network through altering the relations between intracellular Ca^{2+}, myosin phosphorylation and contraction. Furthermore, small numbers of non-smooth muscle cells might have an important function in coordinating contractions, sensing volume, releasing local factors and providing smooth muscle precursors. The most important receptors involved in human detrusor contraction are muscarinic and purinergic receptors while the main pathway for relaxation processes appears to be activated via adrenoceptors (Andersson and Arner 2004).

1.1.2 LAMINA PROPRIA WITH INTERSTITIAL CELLS

The lamina propria is an extracellular matrix positioned between the urothelium and the detrusor muscle (Figure 1 B). It contains numerous types of cells including fibroblasts, adipocytes, interstitial cells (ICs) and sensory nerve endings as well as a vascular network, lymphatic channels, elastic fibres and smooth muscle fascicles forming the muscularis mucosae. The lamina propria plays a role in determining bladder compliance and enabling adaptation to volume changes as well as communicating signals like mechanosensation or nociception to the central nervous system. Furthermore, its components possibly assist in the transmission of information from the urothelium to other components of the bladder wall and contribute to the activation of the detrusor (Andersson and McCloskey 2014).

In the lamina propria of several species, including humans spindle-shaped bladder cells have been discovered. No consensus on the nomenclature has been agreed on yet and these cells are referred to as interstitial cells (ICs), interstitial Cajal-like cells (ICLC), myofibroblasts or telocytes. Cytological ICs have characteristics of myofibroblasts and can be found in close proximity to nerves. ICs have both an afferent as well as efferent nerve supply (Wiseman *et al.*, 2003). In this work these cells will be exclusively referred to as interstitial cells (ICs).

Interstitial cells of Cajal in the gastrointestinal tract - referred to as ICC - are well studied and it has been shown that these cells are electrically coupled to smooth muscle cells. As part of this unit ICC act as pacemaker cells, provide propagation pathways for slow waves and are involved in the transduction of inputs from motor neurons as well as the transduction of mechanosensitivity (Sanders 2014). The physiological role of bladder ICs has not been clearly defined yet. Currently it is suggested that ICs play a role in sensory processing, in the signalling pathways within the layers of the bladder wall and in the modulation of bladder smooth muscle activity. Furthermore, a link has been proposed between the physiological function of ICs and detrusor activity as several studies report an alteration of IC distribution in numerous bladder dysfunctions (McCloskey 2013).

1.1.3 UROTHELIUM

1.1.3.1 ANATOMY

The bladder urothelium - also called uroepithelium or urothelial mucosa - is the innermost layer of the ureters, the bladder and the upper urethra (Figure 1 B, Khandelwal *et al.,* 2009). At cellular level it is composed of three cell layers with morphologically distinct cell types; the basal cells, attached to the lamina propria, an intermediate cell layer and the apical layer of umbrella cells (Figure 2 A, Lewis 2000).

Umbrella Cells

Umbrella cells - also called superficial cells - are hexagonal, polarised cells forming a single layer on the apical side of the urothelium (Figure 2 B). Their diameter varies in size from 25 to 250 μm depending on the filling state of the bladder (Truschel *et al.,* 2002). One distinct feature of these cells is the scalloped appearance of their apical membrane which consists of plaques and hinge regions. The plaque regions are crystalline in nature and comprise of a family of transmembrane proteins called uroplakins (UPIa, UPIb, UPII, UPIIIa and UPIIIb). Due to the crystalline plaque regions, all other nonplaque proteins (e. g. receptors and channels) may be localised in the hinge areas (Khandelwal *et al.,* 2009). The unusual membrane composition is similar to myelin, rich in cholesterol, phosphatidylcholine, phosphatidylethanolamine and cerebroside (Hicks *et al.,* 1974).

Umbrella cells are filled with cytoplasmic vesicles, which are associated with cytokeratin filaments (Figure 2 B). These vesicles accumulate under the apical surface. During bladder filling they traffic to and fuse with the cell membrane to increase the apical surface area (Truschel *et al.,* 2002). This stretch-induced endo- and exocytosis could furthermore be essential in the modulation of mediator release as well as in the regulation of receptor and ion channel expression on the urothelium (Birder and de Groat 2007). In addition, the umbrella cells secrete glycosaminoglycans (GAGs), which cover the apical surface (Figure 1 B) and are thought to play a role in the nonspecific defence mechanisms against infections (Hurst *et al.,* 2007).

Intermediate Cells

Intermediate cells are about 10 to 15 µm in diameter and are connected by desmosomes and by gap junctions. They form one to several cell layers beneath the umbrella cells dependent on the species. In rodents the intermediate cells form one to two layers, while in humans up to five cell layers have been observed. It was suggested that the layers of intermediate cells slide past one another during the filling stage as fewer cell layers were observed in the distended bladder compared to the empty bladder. Intermediate cells just beneath the umbrella cells are partially differentiated, express UPs and contain vesicles. This layer of intermediate cells rapidly differentiates into umbrella cells, when the apical cell layer is disrupted (Khandelwal *et al.*, 2009).

Basal Cells

Basal cells have a similar morphology to intermediate cells. They are attached to the lamina propria in a single cell layer (Figure 1 B). They are the smallest out of the three urothelial cell types with about 10 µm diameter and they are believed to fuse to form intermediate cells (Khandelwal *et al.*, 2009). Basal cells are seen as the precursors for other cell types in the urothelium and exhibit a low turnover rate of three to six months in adult mice under normal conditions (Jost 1989).

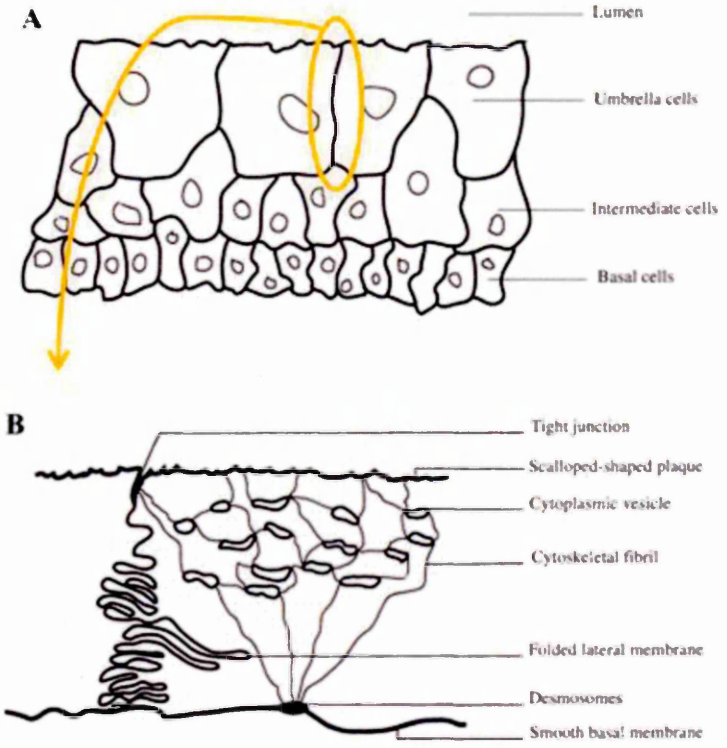


Figure 2 **Schematic of the urothelium (adapted from Lewis 2000). (A) The urothelium consist of three distinct cell layers, the basal cells, the intermediate cells and the umbrella cells. (B) Umbrella cells are filled with cytoplasmic vesicles which accumulate under the apical surface and which are connected via cytoskeletal fibril.**

1.1.3.2 PHYSIOLOGY

The function of the urothelium has for a long time exclusively been seen as a passive barrier with low urea and water permeability. Lewis (2000) summarised only fifteen years ago, that the healthy urothelium possesses four properties to store urine while maintaining a similar urine composition over time, which was regarded as the main function of the urothelium. The first of the four properties is the minimum epithelial surface area compared to urine volume, which prevents substances from moving between urine and blood. The second property is the especially low passive permeability of the umbrella cells due to the membrane composition and the connecting tight junctions, which prevents ions and solutes from passively moving across the epithelium and between cells. Thirdly, it was suggested that the urothelium has a regulated active transport system. For example, active sodium absorption opposes the passive movement of sodium from blood to urine. The fourth stated property is the inertness of the bladder. Substances that are found in the urine or blood do not alter the permeability properties of the apical membrane or tight junctions in healthy conditions (Lewis 2000).

In the last two decades the view about the functional role of the urothelium has changed dramatically and it is now seen to have important mechanosensory functions beyond being simply a barrier with low urea and water permeability. As an active contributor to bladder function the urothelium has a sensory role in transmitting information to underlying nervous and muscular tissue as well as ICs and neighbouring urothelial cells by sensing and communicating changes in the extracellular environment. The urothelium might therefore play an important role in the regulation of the micturition cycle by releasing a number of mediators in response to chemical, mechanical and physical stimuli. Various receptors expressed on urothelial cells could trigger intracellular responses to the stimuli. Soluble factors found in the urine and chemical mediators/ peptides/ transmitters that are released from nerves, inflammatory cells and blood vessels might act on these receptors. Mechanical stimuli could include bladder pressure, tension in the urothelium, torsion, geometrical tension or movement of visceral organs as well as indirect mechanical stimuli such as changes in the cytoskeleton, coactivation of urothelial channel proteins and changes in cell surface molecules such as integrins (Birder and Andersson 2013).

Mechanical stimuli occur when the bladder fills and empties. During this process the urothelium has to accommodate immense changes in stretch to maintain its barrier function. Several mechanisms are in place to achieve this. First of all the surface of the mucosa unfolds at tissue level while at cellular level the membrane of umbrella cells expands following exocytosis mechanisms. After voiding this mechanism is thought to be reversed, the additional membrane is taken up into vesicles by endocytosis and the membrane refolds. Endo- and exocytosis mechanism are also thought to play a crucial role in regulating the expression of UPs, lipids, receptor/ channel proteins and mediators at the apical surface of umbrella cells (Truschel *et al.,* 2002, Khandelwal *et al.,* 2009).

Birder *et al.* (1998) gave the first indirect indication that the urothelium releases mediators and expresses ion channels by demonstrating increased nitric oxide (NO) release in the rat urothelium after treatment with capsaicin, an agonist for the TRPV1 receptor, which is part of a transient receptor potential family of ion channels. Shortly afterwards the expression of vanilloid receptors in the urothelium was demonstrated and a sensory role for the urothelium suggested (Birder *et al.,* 2001). This was enhanced by the finding that vanilloid receptor knock-out mice showed altered urinary bladder function (Birder *et al.,* 2002a). In addition, unmyelated nerves in human urothelial and immediate suburothelial tissue layers were shown by Wiseman *et al.,* (2002), further indicating a sensory role of the urothelium. In the following years the expression of diverse receptors was demonstrated in urothelial cells and functional studies were carried out to verify the suggested sensory role of the urothelium. Overall there are three main reasons - reviewed by Birder (2011) - that provide evidence for the suggestion that the urothelium has sensory properties. First, afferent and efferent bladder nerves are localised close to or within the urothelium. Second, the urothelium expresses various receptors and ion channels, which respond to thermal, mechanical and chemical stimuli. The third line of evidence is the ability of the urothelium to release various mediators itself.

1.2 UROTHELIAL SIGNALLING

It is known today, that the urothelium has a sensory role in bladder function. To accomplish this sensory role the urothelium releases different transmitters and expresses various receptors. Urothelial cells can be activated by either autocrine or paracrine (release from nearby nerves, ICs or muscle cells) mechanisms. Figure 3 illustrates a theoretical model for the possible interactions between urothelial cells, bladder afferent and efferent nerves, smooth muscle cells and ICs. Up to date knowledge on the cholinergic and purinergic signalling pathways as well as NO and Substance P signalling in the urothelium will be summarised in the following.

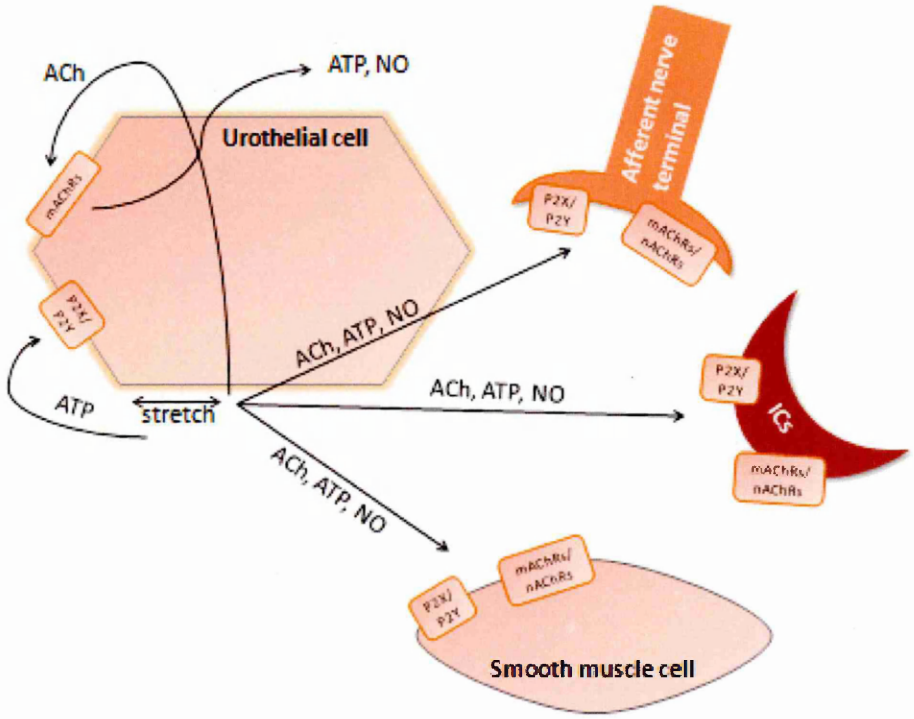


Figure 3 **Possible interactions of mediators and bladder cells. The mediators ACh, ATP and NO are playing a crucial role in signalling the filling state of the bladder by acting on cholinergic, purinergic and NO receptors of urothelial cells, ICs, smooth muscle cells and afferent nerve terminals. Different signalling cascades are activated these cells, leading to the release of mediators, second messengers or the onset of action potentials.**

1.2.1 THE CHOLINERGIC SYSTEM IN THE UROTHELIUM

The cholinergic system consists of the neurotransmitter acetylcholine (ACh) and its transporters, receptors, as well as its synthesising and metabolising enzymes. ACh was the first neurotransmitter that was identified in 1926 by Otto Loewi and it plays a crucial role in the parasympathetic as well as sympathetic nerve system. Its omnipresence in the human body was soon discovered, but research only started in the 1990s to also focus on the non-neuronal cholinergic system (Beckmann and Lips 2013).

Yoshida *et al.* (2006) showed that the human bladder exhibits a non-neuronal cholinergic system by measuring ACh release after treatment with tetrodotoxin (TTX). TTX is a neural toxin which inhibits the firing of action potentials in nerves by blocking the passage of sodium ions into the nerve cells. Tetrodotoxin-insensitive and therefore non-neuronal ACh release could be seen after stretching human bladder strips with and without urothelium. The findings showed no difference in neuronal ACh release when comparing strips with and without urothelium. However the non-neuronal ACh release from strips with urothelium was significantly higher compared to urothelium-denuded strips (Yoshida *et al.,* 2006). This suggests that the urothelium either releases non-nerve-evoked ACh or releases a factor that triggers ACh release from underlying tissues. The first suggestion has now been confirmed, as it has been shown that isolated urothelial cells of different species release non-neuronal ACh (Hanna-Mitchell *et al.,* 2007, Lips *et al.,* 2007, McLatchie *et al.,* 2014). ACh plays a crucial role in the regulation of bladder mechanosensation and is involved in regulation processes in the detrusor muscle, afferent and efferent nerves as well as in the urothelium (see 2.1).

1.2.1.1 MUSCARINIC RECEPTORS (MACHRS)

Muscarinic receptors (mAChRs) are all G-protein coupled and consist of the five subtypes M_1-M_5 (Hulme *et al.,* 1990). G-protein coupled receptors consist of seven transmembrane helices, each containing an extracellular N-terminus and an intracellular C-terminus. Upon stimulation by an agonist the receptor undergoes a conformational change that activates a signal transduction pathway. Depending on the subtype of mAChR this transduction involves either the phophatidylinositol pathway or the cAMP pathway. The muscarinic receptor subtypes M_1, M_3 and M_5 have been shown to typically couple via the α-subunit of $G_{q/11}$, which activates phospholipase C (PLC). PLC then mediates phosphoinositide hydrolysis and Ca^{2+} mobilization. M_2 and M_4 however couple via $G_{i/o}$ α-subunits and inhibit adenylyl cyclase activity, which consequently reduces cAMP levels (Caulfield and Birdsall 1998, Rang *et al.,* 2007). Table 1 summarizes the preferred coupling mechanisms and suggested functional roles of the mAChR subtypes in human cells.

All subtypes are expressed in the human urothelium with density distribution as following, $M_2 >> M_3 = M_5 > M_4 = M_1$. Immunohistochemistry results show expression of the M_1 receptor only in the basal cells of the urothelium whereas expression of M_2 and M_5 receptors was increased in the umbrella cells and decreased in the underlying urothelial cell layers (Bschleipfer *et al.,* 2007, Mansfield 2005, Tyagi *et al.,* 2006). All five mAChR subtypes are also expressed in the mouse urothelium and again a cell-type specific distribution exists within the cell layers. Basal cells exhibit M_1, M_3, M_4 and M_5, intermediate cells exhibit M_3, M_4 and M_5 and umbrella cells exhibit M_2, M_3, M_4 and M_5 (Zarghooni *et al.,* 2007).

The M_2 receptor quantitatively predominates in the urothelium, however the M_3 receptor has been shown to be the major subtype contributing to direct contractions of the detrusor muscle under normal conditions. This was demonstrated in female pigs (Sellers *et al.,* 2000), male and female mice (Choppin and Eglen 2001, Choppin 2002) as well as humans (Chess-Williams *et al.,* 2001).

	M_1	M_2	M_3	M_4	M_5
G-Protein	q/11	i/o	q/11	i/o	q/11
2nd Messenger	↑IP_3, DAG	↓cAMP	↑IP_3, DAG	↓cAMP	↑IP_3, DAG
Functional Response	Excitation ↓ K^+ conductance CNS excitation gastric secretion	Inhibition ↓ Ca^{2+} conductance ↑K^+ conductance neural inhibition central muscarinic effects (for example tremor, hypothermia)	Excitation ↑$[Ca^{2+}]_i$ Smooth muscle contraction Gastric, salivary secretion Vasodilatation Decrease neuro-transmitter release (presynaptic)	Inhibition ↓ Ca^{2+} conductance	not known

Table 1 **Muscarinic receptor subtypes. G-protein coupling, transduction pathways and functional responses in the human body (Caulfield and Birdsall 1998, Rang *et al.*, 2007).**

(DAG - Diacylglycerol, IP_3 - Inositol Triphosphate, cAMP – cyclic Adenosine Monophosphat)

1.2.1.2 NICOTINIC RECEPTORS (NACHRS)

Nicotinic receptors (nAChRs) are transmitter-gated ion channels consisting of 17 different subunits (α1-α10, β1-β4, ε, δ and γ) which form heteromeric or homomeric pentamers (Figure 4 B and C). Homomeric pentamers are the simplest nAChRs and are formed by only one type of subunit while heteromeric receptors are composed of two or more subunit types. Neurons exhibit homomeric pentamers such as the α7 and α9 nicotinic receptor, or heteromeric channels consisting of various subunits (Figure 4 A). In particular α7 nicotinic receptors are widely distributed throughout the nervous system and contribute to multiple physiological functions. Muscles exhibit heteromeric receptors with characteristic electrophysiological and pharmacological properties (Figure 4 A). Stimulation of nAChRs generally results in a conformation change that allows modulation of the flow of ions (Na^+, K^+ and Ca^{2+}) across the cell membrane. The electrogenic activity of these ions depolarises the cell membrane and increases neuronal excitability. Furthermore, Ca^{2+} entry through some nAChRs has effects on an array of intracellular signalling cascades (Hurst *et al.,* 2013, Papke 2014).

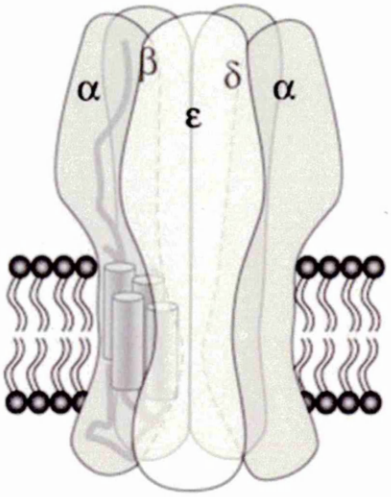
Expression of the subunits α7, α9, α10 was demonstrated in the human urothelium with a density range of α7 >> α10 > α9 (Bschleipfer *et al.,* 2007), whereas in the murine urothelium the subunits α2, α4, α5, α6, α7, α9 and α10 were found (Zarghooni *et al.,* 2007). Basal cells of the murine urothelium were found to exhibit intense immunolabelling for the α5 subtype, while the α7 subtype could be observed throughout all cell layers of the murine urothelium. For the subunits α9 and α10 distinct immunolabelling could be seen on the apical membrane of murine umbrella cells (Zarghooni *et al.,* 2007). In rat urothelial cells mRNA of the α3, α5, α7, β3, and β4 subunits could be identified (Beckel *et al.,* 2006).

A

Heteromeric receptors			
Muscle type			
Ligand binding dimers			Structural
α1δ	α1γ (embryonic)	α1ε (adult)	β1
Neuronal type			
Ligand-binding alphas		Ligand-binding betas	Structural
α2 α3 α4 α6		β2 β4	α5 β3
Homomeric receptors			
α7 (α8)[a] α9 (α10)[b]			

[a] α8 found only in chick.
[b] α10 may form heteromeric receptors with α9.

B



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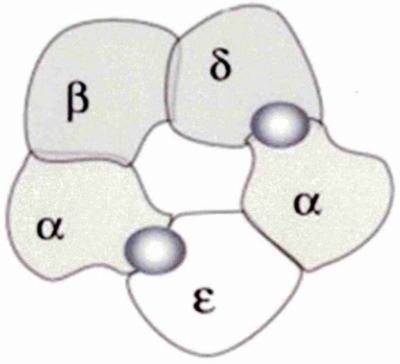


Figure 4 **Nicotinic receptor subtypes (adapted from Hurst *et al.*, 2013 and Papke 2014). (A) nAChR subunits with the division into heteromeric and homomeric subunits as well as division into muscle and neuronal subunits. (B) Schematic representation of a heteromeric nAChR in the cell membrane showing furthermore the muscle subunits and binding sites. (C) Top view of the same heteromeric muscle nAChR with the ACh binding sites at the interface of αε and αδ.**

Numerous important functional roles have been ascribed to the nAChRs especially to the homomeric α7 nicotinic receptor which has been shown to have unique physiological and pharmacological properties, including a high permeability to calcium and rapid desensitization. Beside its important role in neurons this nicotinic receptor subtype has also been demonstrated to have a functionally important role in non-neuronal cells and responds not only to ACh but also to its breakdown product choline (Hurst *et al.,* 2013). The physiological function of nAChRs in non-neuronal tissue has only gained attention recently and includes cell proliferation and differentiation processes, cell motility, modulation of endothelial permeability and release of chemokines/ cytokines (Beckel and Birder 2012). The urothelium is one of these non-neuronal tissues that express nAChRs.

Functional studies performed by Beckel *et al.* (2006) showed an increased intracellular Ca^{2+} concentration after stimulating nAChR of cultured rat urothelial cells with nicotine. This confirms that the expressed subunits form functional receptors in these cells. Furthermore, *in vivo* bladder voiding reflexes in rats were significantly reduced after stimulation of urothelial α7 nicotinic receptors with choline. In a more recent study Beckel and Birder (2012) further examined these findings. The stimulation of α7 nicotinic receptors in rat urothelial cells decreased ATP release and increased intracellular Ca^{2+} through intracellular stores, whereas stimulation of α3 nicotinic receptors increased ATP release and increased intracellular Ca^{2+} through extracellular influx. Furthermore activation of α7 nicotinic receptors inhibited the response to subsequent activation of α3 nicotinic receptors by preventing the increase in intracellular Ca^{2+} (Beckel and Birder 2012) showing the complex interactions between urothelial signalling pathways.

1.2.2 THE PURINERGIC SYSTEM IN THE UROTHELIUM

The purinergic system consists among others of adenosine 5'-triphosphate (ATP) and its transporters, receptors as well as its synthesising and metabolising enzymes. ATP is a molecule which belongs to the purine family. It is made of a nitrogenous base (adenine), a sugar (ribose) and a chain of three ionised groups (phosphates) which are bound to the ribose.

Intracellular ATP is the universal source of readily available chemical energy for living cells (Bodin and Burnstock 2001).The source of extracellular ATP has for a long time been believed to be damaged or dying cells. However in 1972, evidence emerged showing that ATP can mediate nonadrenergic, noncholinergic neurotransmission in nerves supplying the gut and the urinary bladder (Burnstock *et al.,* 1972, Burnstock 1972). It is now clear that extracellular ATP and its metabolites modulate biological processes through activation of purinergic receptors in an autocrine or paracrine fashion in numerous tissues. These processes include platelet aggregation, vascular tone, peripheral and central neurotransmission, cardiac function and muscle contraction (Gordon 1986).

Several physiological studies have been carried out to understand the role of the purinergic system in the bladder, including experiments with knock-out mice showing significantly altered bladder function in animals that lack purinergic ATP receptors. ATP could also be shown to be important for the initiation of the micturition cycle (Cockayne *et al.,* 2000). Furthermore, intraluminally administered ATP has been revealed to induce urinary bladder hyperactivity and stimulate the micturition reflex in awake, freely moving rats (Pandita and Andersson 2002).

ATP is released from many cell types in response to mechanical stimuli such as shear stress, stretch and osmotic swelling (Bodin and Burnstock 2001, Boudreault and Grygorczyk 2004). A large body of evidence exists today showing that also urothelial cells release ATP in response to physiological changes in several species (Ferguson *et al.,* 1997, Birder *et al.,* 2003, Kumar *et al.,* 2004, Lewis and Lewis 2006, Cheng *et al.,* 2011, McLatchie *et al.,* 2014). The released ATP has the potential to activate ionotropic and metabotropic P2 purinergic receptors in the underlying tissues or in the urothelium itself.

1.2.2.1 IONOTROPIC P2X RECEPTORS

Purinergic receptors can be divided into two types, P1 receptors for adenosine and P2 receptors for ATP/ADP. The P2 receptors can be further distinguished into two major families. An ionotropic P2X family of ligand-gated ion channel receptors and a metabotropic P2Y family of G protein-coupled receptors. The ionotropic $P2X_{1-7}$ receptor family are ligand-gated non-selective cation channels with two transmembrane regions. Currently seven P2X subunits are recognised. Any three out of these seven subunits form heteromeric as well as homomeric trimer ion pores which are ATP gated (Burnstock 2007).

The first study showing purinergic receptor expression in the urothelium was conducted by Elneil *et al.* (2001) demonstrating immunoreactivity of the $P2X_3$ receptor protein but not of the $P2X_1$ receptor in both rat and human urothelium. This was the first revelation of a non-neuronal localisation of the $P2X_3$ receptor. As the $P2X_3$ receptor could not be found on sensory nerves in close proximity to the urothelium, it was proposed that the receptor would not have a direct role in the mediation of sensory nerve responses to ATP in the bladder. Instead the receptor could be involved in signal transduction pathways in the urothelial sensory web. It has recently been confirmed that immunoreactivity for the $P2X_3$ receptor could be observed throughout all cell layers of the human urothelium with the umbrella cells labelled the strongest (Sterle *et al.,* 2014).

Contradictory studies exist however regarding the mRNA expression of the purinergic P2X receptors in the human urothelium. Shabir *et al.* (2013) stated that the human urothelium predominantly expresses mRNA for the $P2X_4$ receptor subtype while $P2X_{1-3}$ and $P2X_{5-7}$ were not expressed. Another study by Sterle *et al.* (2014) revealed $P2X_3$ and $P2X_5$ receptor protein expression in the human urothelium whereas Tempest *et al.* (2004) showed that $P2X_2$ and $P2X_3$ receptor protein was expressed in the human urothelium. Translational regulations, feedback mechanisms and mRNA stability could account for these differing results and further studies are needed to confirm the presence of the diverse P2X receptors in the urothelium.

1.2.2.2 METABOTROPIC P2Y RECEPTOR

The metabotropic P2Y receptor family consists of G protein-coupled receptors with seven transmembrane regions. Currently eight P2Y receptor subtypes are recognised, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$. In response to nucleotide activation, P2Y receptors affect intracellular signalling cascades by either activating PLC which leads to the release of intracellular Ca^{2+} or by affecting adenylyl cyclase which results in the alteration of intracellular cAMP levels (Burnstock 2007). As the urothelium expresses ecto-ATPase enzymes (Stella *et al.,* 2010), it is likely that the released ATP is hydrolysed to ADP, AMP and adenosine which then act on the P2Y receptors. It has been shown that twelve nucleotides other than ATP can be detected in human urine samples. Quantification with HPLC showed that the levels of ADP, UTP, UDP and GTP were more than 10 times higher than the level of ATP (Contreras-Sanz *et al.,* 2012). It is however still unknown what role the different nucleotides play in P2Y receptor signalling.

Shabir *et al.* (2013) showed that the human urothelium predominantly expresses mRNA for the $P2Y_1$ and $P2Y_2$ receptor subtype while $P2Y_6$ expression could not be detected and $P2Y_{11}$ detection was marginal. However, in the porcine urothelium expression of $P2Y_6$ could be identified (Bahadory *et al.,* 2013). $P2Y_2$ and $P2Y_4$ receptor expressions could be shown in the rat urothelium (Chopra *et al.,* 2008).

In a recent study the complexity of purinergic receptor signalling was shown by examining the effect of P2 receptor agonists on ATP release from a human urothelial cell line (Mansfield and Hughes 2014). It was demonstrated that the P2Y agonist ADP stimulated ATP release to a level that was 40-fold higher than hypotonic stimulated ATP release. Furthermore, UTP, another P2Y agonist, stimulated ATP release, however to a lesser extent. On the other side, P2X agonists did not seem to stimulate ATP release from the cells. This work is in line with another publication demonstrating that the P2Y agonist UTP stimulated ATP release in guinea pig and human bladder urothelium strips while a P2X agonist had no effect (Sui *et al.,* 2006). It is however not clear which subtype is responsible for these effects, as a lack of subtype selective agonists for individual P2Y receptors makes it difficult to identify the involved subtypes.

Investigations on the physiological function of P2Y receptors in the urothelium are only beginning to attract interest. Recently, it has been demonstrated that activation of the P2Y receptors results in increased spontaneous activity of the rat and human bladder mucosa (Fry *et al.,* 2012).

1.2.3 NITRIC OXIDE (NO)

Another transmitter that is proposed to play a role in the signalling processes of the urothelium is nitric oxide (NO). NO is a free radical with a half-life of under 6 seconds. In mammals including humans NO is an important biological messenger in signal transduction processes. NO is formed by the conversion of amino acid arginine into citrulline (Figure 5). This complex reaction is catalysed by nitric oxide synthase (NOS). NOS consist of three isoforms, inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS), with the latter two being dependent on Ca^{2+} for NO formation. iNOS is only activated upon certain signalling mechanisms, such as inflammatory processes. eNOS and nNOS are constitutively expressed, however at varying quantities depending on the stimuli (Ignarro 2000).

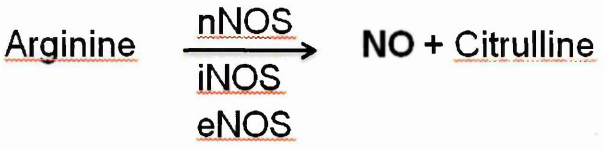


Figure 5 Synthesis of NO. NO is formed by conversion of the amino acid arginine to citrulline in an enzymatic reactions catalysed by either neuronal, inducible or endothelial NO synthase.

NO has been shown to be one of the non-noradrenergic, non-cholinergic transmitter that influence bladder function. It was postulated that NO plays a role in bladder mechanosensation promoting relaxation of the detrusor by modulating parasympathetic signalling and spontaneous activity. The effects of NO donors and inhibitors were tested on pre-contracted human detrusor strips. NO donors, as well as inhibitors, modulated carbachol-evoked contractions however the response was exceptionally complex and was either relaxant, contractile or biphasic (Moon 2002). This implies that several pathways, possibly in different cell types of the urothelium, the ICs and the muscles were affected and influenced by each other.

Formation of NO possibly occurs via iNOS and eNOS in urothelial cells (Birder *et al.,* 1998, Birder *et al.,* 2002b) with iNOS being the predominant isoform for NO synthesis in cell damage and inflammatory response (Persson *et al.,* 1999, Jezernik *et al.,* 2003, Giglio *et al.,* 2005).

1.2.4 SUBSTANCE P

Substance P is a neuropeptide belonging to the tachykinin family. It binds to tachykinin receptors, neurokinin 1 (NK_1), neurokinin 2 (NK_2) and neurokinin 3 (NK_3). These tachykinin receptors are all G-protein coupled transmembrane proteins. Substance P plays an important role in pain perception. This sensory function is thought to be transmitted by NK_1 delivering tissue damage information to the CNS (Satake and Kawanda 2006).

It has been shown that stimulation of tachykinin receptors produces contractions in the human and porcine bladder detrusor, which is predominantly mediated via NK_2 receptor (Bushfield *et al.,* 1995, Templeman *et al.,* 2003, Sadananda *et al.,* 2008). There is no evidence to date that Substance P is released by urothelial cells. However, tachykinin receptor expression as well as Substance P immunoreactivity and mRNA expression has been shown in the urothelium and therefore suggests an involvement of Substance P in urothelial signalling processes. Heng *et al.* (2011) have demonstrated Substance P and NK_1 immunoreactivity as well as mRNA expression in the urothelium of rats. In the urothelium of female pig bladders expression of NK_2 could be shown, however NK_1 was not detected (Bahadory 2013).

It is not clear if Substance P is playing a role in the urothelial communication of mechanosensation in the bladder. Urothelial release of Substance P was therefore investigated in this study.

1.2.5 INTERACTION OF ACH, ATP AND NO

Several studies showing the interaction of ACh, NO and ATP signalling pathways support the hypothesis that mediators released by the urothelium act as part of a sensory network and influence each other. Urothelial NO release has been proposed to be mediated by activation of mAChR leading to attenuation of detrusor contractility (Andersson *et al.,* 2008). Muscarinic agonists also stimulated the release of ATP in cultured urothelial rat cells and it was suggested that altered bladder reflexes after the stimulation of muscarinic receptors are dependent on ATP and NO release (Kullmann *et al.,* 2008). Low levels of muscarinic stimulation had an inhibitory effect on voiding functions in the anesthetized rat with NO being involved in the signal transduction, whereas a higher level of muscarinic stimulation exhibited higher bladder activity with ATP transducing the signal (Kullmann *et al.,* 2008). This link was supported by Lagou *et al.* (2006), showing that NO interacts with interstitial cells and modulates the pattern of muscarinic induced contractile activity. Another study by Hanna-Mitchell *et al.* (2007) showed a significant increase in radiolabelled ACh release of murine urothelial cells upon mechanical stimuli by cellular swelling as well as upon stimulation with ATP. In addition, an inhibitory effect on stretch-evoked urothelial ATP release by anticholinergic agents was shown by Young *et al.* (2012). All these studies strongly suggest a link between the cholinergic system, the purinergic system and NO in the urothelium.

There is also compelling evidence that in bladder dysfunctions the signalling system in the urothelium is disrupted. An alteration in the signalling system of ACh, ATP and NO has been shown in the pathologic bladder (Pandita *et al.,* 2000, Birder 2003, Tempest *et al.,* 2004, Kumar *et al.,* 2010, Munoz *et al.,* 2011). In urothelial cells of cats with feline interstitial cystitis, up-regulation of ATP release could be shown (Birder *et al.,* 2003). This was one of the first links of altered purinergic signalling in bladder pathology. Sun and Chai (2006) proved this finding by showing that human urothelial cells from interstitial cystitis patients release several fold more ATP compared to control samples. The same group had already shown before, that ATP levels were significantly increased in the urine of patients with interstitial cystitis compared to controls. Furthermore, stretch activated ATP release was elevated in these patients in urodynamic tests (Sun *et al.,* 2001). A significant rise in ATP release was seen in human urothelial tissue strips

from bladders of patients with detrusor overactivity compared to controls after applying mechanical stretch and electric field stimulation (Kumar *et al.,* 2010).

In the following years a body of literature has demonstrated altered purinergic signalling in bladder dysfunctions such as interstitial cystitis, overactive bladder, bladder outlet obstruction and other voiding dysfunctions. These alterations were seen throughout the tissues involved in bladder sensation, including the urothelium, detrusor smooth muscle, peripheral nerves and spinal cord (as reviewed by Sun and Chai 2010). Whether this alteration in the purinergic signalling pathway is a primary or secondary effect is however still unknown.

Tempest *et al.* (2004) showed that $P2X_2$ and $P2X_3$ receptor protein was expressed in the human urothelium. The expression of both receptor proteins was greater in urothelium obtained from patients with interstitial cystitis. As $P2X_3$ receptors are already regarded as pain receptors on nerves, the upregulation of this receptor in the interstitial cystitis urothelium could contribute to the pain that these patients experience. Further studies exist that reveal an alteration of the purinergic P2 receptor expression in pathological bladders, suggesting a role of these receptors in bladder dysfunction (Birder *et al.,* 2004). Also, levels of NO as well as NOS expressions are increased in patients with bladder disorders (Logadottier *et al.,* 2004, Birder *et al.,* 2005, Hosseini *et al.,* 2004).

These pathways and their interactions seem to be highly complex and influenced by various stimuli. The mechanosensitive effect on ATP, ACh and NO release from the urothelium will be studied in this thesis to further examine the complex urothelial cross talk.

1.3 MICTURITION CYCLE AND BLADDER INNERVATION

The micturition cycle in the LUT consists of two phases: storage and periodical elimination of urine. The micturition cycle is controlled by complex neural pathways in brain, spinal cord and peripheral ganglia. The LUT depends on CNS control and its pattern of activity consists of only two modes of operation - storage and elimination. Micturition depends on learned behaviour that develops during the maturation of the nervous system and is under voluntary control, whereas other visceral functions, such as the cardiovascular system are regulated involuntarily (Fowler 2008).

The muscles of the LUT are innervated by three sets of peripheral nerves, the pelvic parasympathetic, the hypogastric sympathetic and the pudendal somatic nerves. These all contain both afferent and efferent axons. The pelvic parasympathetic nerves release acetylcholine (ACh) and adenosine triphosphate (ATP), which act on the bladder smooth muscle via stimulation of muscarinic (M_3) and purinergic ($P2X_1$) receptors, leading to bladder contraction. At the same time the urethral smooth muscle is relaxed, mediated by nitric oxide (NO). The hypogastric sympathetic nerve pathway is mediated by noradrenaline (NA) which acts on β_3-adrenoceptors, relaxing the bladder muscle and on α_1-adrenoceptors, contracting the urethral smooth muscle to maintain continence during bladder filling. The third peripheral nerves are the pudendal somatic nerves. They release ACh, which acts on nicotinic receptors resulting in the contraction of the external urethral sphincter (Figure 6, Yoshimura *et al.,* 2014).

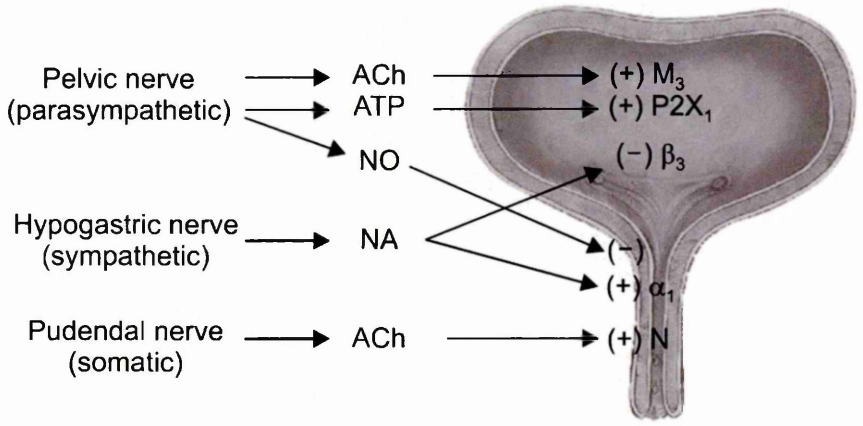


Figure 6 **Sensory nerve innervation and mediators in the muscles of the lower urinary tract (Yoshimura *et al.*, 2014). Pelvic nerves release ACh and ATP, which stimulate M_3 and $P2X_1$ receptors on the bladder smooth muscle respectively, leading to bladder contraction. In addition NO is released by pelvic nerves and relaxes the urethral smooth muscle. Hypogastric nerves release NA which relaxes the bladder muscle via β_3-adrenoceptors and contracts the urethra via α_1-adrenoceptors. Pudendal nerves release ACh which acts on nicotinic receptors, causing the contraction of the external urethral sphincter.**

((+) and (-) indicate neural stimulation and inhibition respectively, ACh - Acetylcholine, ATP – Adenosine Triphosphate, NO – Nitric Oxide, NA – Noradrenaline, M_3 – muscarinic receptor subtype, $P2X_1$ – purinergic receptor subtype, β_3 - adrenergic receptor subtype, α_1 - adrenergic receptor subtype)

Storage Phase

During the storage phase, when the bladder is filling with urine, the detrusor muscle is relaxed, intraluminal pressure is kept at a constant low level and the parasympathetic pathway is quiescent during this phase. The viscoelasticity of the detrusor muscle allows the bladder wall to stretch and therefore adjust to increasing volumes. Stretch-sensitive mechanoreceptors send afferent signals to the spinal cord, from where pudendal somatic efferents subsequently stimulate external urethral sphincter contraction in order to maintain continence. At the same time bladder distension also activates sympathetic nerves that, in return, stimulate contraction of the internal urethral sphincter. Predominantly, the reflex pathways during urine storage are thought to be integrated in the spinal cord. Interesting however is the fact that patients with lesions interrupting the brain stem pathway have impaired voluntary control of micturition, while animals with supraspinal transection of the spinal cord seem to have normal functioning bladder reflexes. It can therefore be assumed that in humans supraspinal input is required to maintain a stable urethral resistance even if the storage reflex seems to be established within the spinal cord (Fowler 2008, Yoshimura *et al.,* 2014).

Elimination Phase

During the elimination phase, sensory awareness of a full bladder leads to voluntary micturition, which depends on input from the micturition centre in the brain. Sensory information is integrated in the micturition centre. Inhibitory input is generated here and transmitted to the sympathetic and the somatic pathways while stimulatory input is transmitted to the parasympathetic pathway. This leads to the relaxation of the external and internal sphincters. The process is followed by the contraction of the detrusor muscle, which leads to an increase in bladder pressure and finally to the release of urine (Fowler 2008, Yoshimura *et al.,* 2014).

All of these bladder innervating nerves can be found in close proximity to the urothelium, as stated previously. Therefore, it is likely that urothelial mediators play a role in the stimulation of afferent nerves, which then excite sensory pathways in the CNS and modulate bladder sensation as well as detrusor contractions.

1.4 SPONTANEOUS ACTIVITY AND ITS PHYSIOLOGICAL SIGNIFICANCE

Smooth muscles are considered to be either phasic or tonic depending on the speed of activation and contraction. Tonic muscles can maintain tension for a long time with low energy expenditure and react with slow and continuous contractions. They are usually activated via membrane receptors for various agonists. Phasic muscles show rapid contraction as well as relaxation and are normally activated by action potentials that occur spontaneously or by neural activity. Depending on the mode of stimulation some muscles can show phasic as well as tonic activity (Kao and Carsten 1997), which seems to be the case in the detrusor muscle.

Smooth muscles in the bladder as well as in the urethra exhibit spontaneous contractile activity – also called autonomous activity or non voiding activity - during the filling phase of the micturition cycle. In the urethra this activity is of tonic nature and contributes to the closure pressure which is essential to maintain continence against the forces of gravity. The detrusor muscle shows atypical activity for a smooth muscle. The contractions rise and fall from a low resting tone and travel over the whole bladder, as relatively isolated contractions in parts of the bladder wall. This localisation of so called micromotions is thought to be the result of poor electrical coupling between the myocytes of the detrusor under normal conditions (Brading 2006).

1.4.1 PHYSIOLOGICAL FUNCTION

The occurrence of spontaneous activity in the bladder during the filling phase was first discovered in 1882 in humans and dogs (Mosso and Pellacani 1882). This study was then confirmed ten years later in monkeys, cats and dogs. It was furthermore shown that spontaneous activity could not be abolished by spinal transection or complete peripheral denervation of the bladder and it was concluded that this contractile activity must be an intrinsic property of the bladder wall (Sherrington 1892). By now, spontaneous contractions have been demonstrated in the whole bladder of the guinea pig (Drake *et al.,* 2003), mouse (Gillespie 2004), rat (Streng *et al.,* 2006) and pig (Dittrich *et al.,* 2007). The discussion of the origin and physiological role of spontaneous contractions

is still ongoing today. Also the factors that regulate the magnitude and frequency of contractions are not fully understood to date.

It has been proposed that spontaneous contractions allow the individual muscle bundle to adjust their length when the bladder is filling (Brading 2006). This is supported by numerous studies showing a link between bladder volume and change in the activity pattern of spontaneous contractions during the micturition cycle. Furthermore, it has been demonstrated that spontaneous activity is accompanied by electrical changes in the detrusor. Spontaneous activity in the bladder could therefore be linked to the generation of afferent discharge from mechanoreceptors in the bladder wall and as a result contributing to sensations communicating bladder volume/ filling state (Streng *et al.*, 2006).

The detrusor is not only stimulated by mechanical stimuli, also mediators released from the urothelium are thought to initiate signalling cascades - as described above - that propagate rapidly throughout the detrusor muscle, and generate spontaneous activity (Hashitani *et al.*, 2001). The urothelium may not only release excitatory but also inhibitory mediators as studies on an unidentified urothelium-derived inhibitory factor (UDIF) suggest (Hawthorn *et al.*, 2000).

1.4.2 SPONTANEOUS ACTIVITY IN AGE AND DISEASE

It has been observed that the pattern of spontaneous activity is dependent on age. Spontaneous activity was shown to be enhanced in mucosal strips from juvenile pig bladders compared to strips from adult pig bladders in a recent study by Vahabi *et al.* (2013). The bladder of neonatal animals is capable of generating comparable prominent spontaneous activity, while bladders of mature animals - when voiding is driven by neural mechanisms - exhibit lower activity. In the aged bladder this development appears to reverse and spontaneous activity reemerges to a high level (Szigeti *et al.*, 2005). It has been proposed that these differences are accounted for by either an alteration in the properties of ICs or an age-dependent change of urothelial signalling (Vahabi *et al.*, 2013).

It is also assumed that a change in the pattern of spontaneous contraction plays an essential role in bladder pathology. In human muscle strips from overactive bladders

spontaneous activity developed more often and at greater amplitude, frequency and basal tension compared to normal bladders (Kinder and Mundy 1987). This is in line with a study on bladder strips from patients with idiopathic detrusor instability, showing altered spontaneous contractile activity as well as an increased electrical coupling of the muscle cells compared to control tissue (Mills *et al.,* 2000).

1.5 BLADDER PATHOLOGY – OVERACTIVE BLADDER (OAB)

Overactive bladder (OAB) syndrome is characterised by lower urinary tract symptoms of unidentified aetiology. The largest population-based surveys assessing the prevalence of OAB among men and women were conducted by Milsom *et al.* (2001) and Irwin *et al.* (2006). OAB appears to affect around 12-17% of the European and American population with rising prevalence in the older population.

Apart from the socioeconomic burden, OAB can have a substantial physical, psychological and economic impact on the patient's quality of life. The disorder often affects work productivity as well as all aspects of social activity. This can lead to low self-esteem, embarrassment and isolation which are reasons why patients are predisposed to depression. Nocturia can furthermore disturb the patient's sleep pattern and result in chronic fatigue (Sacco *et al.*, 2010, Ouslander 2004).

1.5.1 DEFINITION

According to the International Continence Society (ICS) (Abrams *et al.*, 2003) OAB is defined as a symptom complex involving

- urinary urgency, with or without urgency incontinence,
- usually frequency (voiding eight or more times in 24h),
- nocturia (awakening two or more times at night to void).

Moreover other local or metabolic factors that would account for the symptoms, for example bacterial infections, need to be absent. OAB dry is referred to when urinary urge incontinence is absent while OAB wet is referred to when urge incontinence is present.

OAB is a clinical and subjectively-reported diagnosis. It needs to be distinguished from detrusor overactivity (DO), which diagnosis is made on urodynamic assessments. These assessments include a series of tests to measure the pressure-flow relationship of the bladder and the urethra in order to define the functional status of the LUT. The two conditions, OAB and DO, have common characteristics. However only 64% of patients diagnosed with OAB have DO as demonstrated by urodynamic measures (Hashim and Abrams 2006).

1.5.2 CAUSE

The understanding of the pathophysiology of OAB is limited as it is a symptom-based diagnosis and normal micturition regulation is complex, involving both peripheral and central nervous system factors.

According to current knowledge, OAB appears to be an aberration in the voiding reflex, which leads to an atypical increase in pressure during the filling stage and results in involuntary detrusor contractions (Tyagi 2011). The hypotheses to explain this pathophysiology include:

- *A neurogenic hypothesis* stating that damage to inhibitory pathways in the central nervous system or sensitisation of the peripheral afferent terminals in the bladder expose primitive voiding reflexes that trigger bladder overactivity through a generalised, nerve-mediated excitation of the detrusor muscle (de Groat 1997).

- *A myogenic hypothesis* proposing that changes in the properties, structure and innervations of detrusor myocytes lead to increased excitability, resulting in spontaneous rises in pressure, that occur in the overactive bladder. Furthermore, an increased ability of electrical activity to spread between cells could add to the onset of coordinated contraction of the whole detrusor (Brading 1997).

- *A peripheral autonomy hypothesis* suggesting an excessive expression of peripheral autonomous activity, which disturbs the balance of excitation and inhibition in the detrusor (Drake *et al.,* 2001).

- *An urothelial hypothesis* declaring that alterations to the sensory system of the urothelium may lead to the onset of OAB (Birder *et al.,* 2012).

A contribution of more than one of the above stated mechanisms is likely to be involved in the manifestation of OAB. The sensory system in the bladder involves myogenic, neurogenic and urothelial components which are all linked in normal bladder function. Therefore, an aberration in one might influence all other components (Figure 7, Tyagi 2011). It can be stated that the cause of OAB is still not fully understood, but that it is very likely a multifactorial pathogenesis and that the underlying pathophysiological

mechanisms might even differ between patient groups. This might explain the success of medication in some patients whereas other patients do not respond to the same medication.

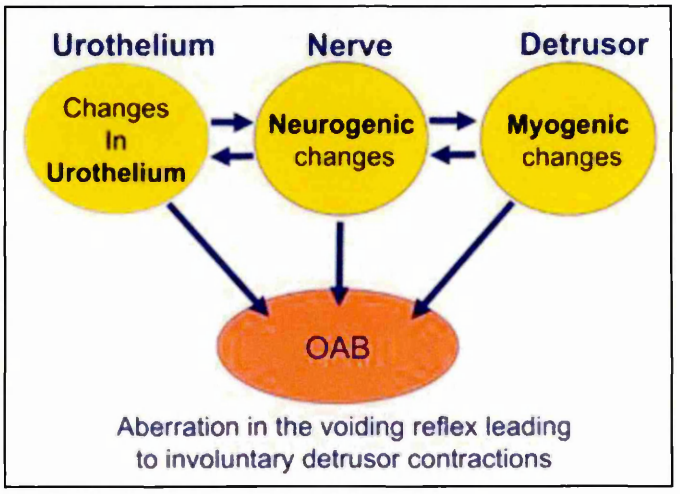


Figure 7 **Potential mechanism and interaction underlying OAB pathophysiology (Tyagi 2011). Changes in the urothelium, the nerves and the detrusor can influence each other and lead to the manifestation of OAB.**

(OAB – Overactive Bladder)

1.5.3 PREVALENCE

OAB has been shown to be a common disorder that seriously affects the quality of life of about 16.6% of the European population over 40 years of age (Milsom *et al.,* 2001). For this study the data from 16 776 individuals, over 40 years of age, from six European countries - France, Germany, Italy, Spain, Sweden and the United Kingdom – were collected. It has also been shown in this study that the prevalence of OAB in both men and women increases dramatically with age (Figure 8). A more recent study and the largest population-based survey assessing the prevalence of LUT symptoms to date estimated the prevalence of OAB among men and women over 18 years of age at 11.8% of the total population. This finding is based on a survey undertaken with 19 165 individuals in five countries - Canada, Germany, Italy, Sweden, and the United Kingdom - using the 2002 ICS definition of OAB (Irwin *et al.,* 2006). The higher prevalence in the study by Milsom *et al.* (2001) is likely to be accounted for by the older age group and possibly the use of an older definition of OAB.

Due to the demographic change with an ageing population and the high prevalence of OAB in age, a growing number of people will be affected by OAB in the near future. Irwin *et al.* (2011) made estimations based on the above mentioned study along with gender- and age predictions worldwide and came to the result that by 2018 an estimated 2.3 billion individuals will be affected by at least one LUT symptom (18.4% increase 2008-2018), with 546 million suffering from OAB (20.1% increase 2008-2018).

Overall no gender difference in the occurrence of OAB has been reported in the above mentioned studies. Irwin *et al.* (2006) states that 12.8% of women and 10.8% of men reported OAB symptoms. However, while nearly half of the women who reported OAB were affected by UI, only a quarter of the men experienced UI. Furthermore, a gender difference could be seen in both studies when comparing different age groups. Women have a higher prevalence of OAB before the age of 60 years compared to men, while men have a higher prevalence of OAB after the age of 60 compared to women (Milsom *et al.,* 2001, Irwin *et al.,* 2011).

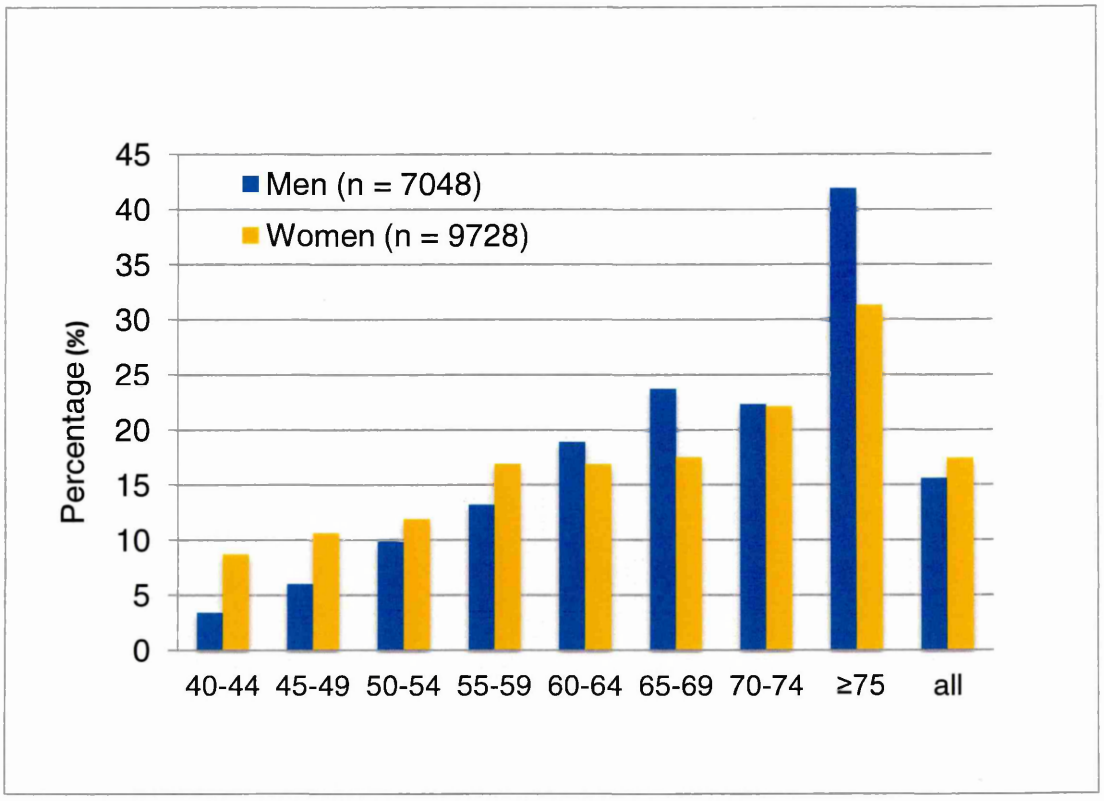


Figure 8 **Prevalence of OAB (Graph constructed from data obtained from Milsom *et al.*, 2001). Prevalence of OAB in %, grouped according to age and gender in a population ≥40 in a random sample of 16 776 subjects from six European countries.**

1.5.4 CURRENT THERAPEUTIC MEASURES

At present there is no cure for OAB but treatment options aiming to provide symptomatic relief are available. First line measures are conservative treatments including bladder training, restriction of fluid intake, prevention of caffeinated, acidic and carbonated drinks (Henderson and Drake 2010).

Antimuscarinic Drugs

If the patient does not respond to these behavioural methods pharmacological treatment will be advised. Antimuscarinic drugs which competitively inhibit muscarinic receptors are still the mainstay therapy for OAB and aim to improve the symptoms of urgency, frequency and urgency incontinence. However, it is not fully understood if antimuscarinics act on the detrusor muscle, on the urothelium or on sensory nerves. Several antimuscarinics are on the market with inadequate evidence to promote one drug over another (Henderson and Drake 2010). Adverse effects with antimuscarinic agents are relatively common, including dry mouth, pruritus, digestive disorders and effects on the CNS. Furthermore the marketed dose of some agents lack efficacy and patient's therapy persistence is impaired due to these two factors (Sacco *et al.,* 2010). It is not expected that future developments on anticholinergic agents will have a beneficial impact on the efficacy or safety of these drugs. Therefore, the development of alternative treatment options has attracted increasing interest (Mangera and Chapple 2011).

Botulinum Neurotoxin A (BoNT/A)

For patients resistant to or not compliant with antimuscarinics, injection of Botulinum neurotoxin A (BoNT/A) into the bladder wall has become a promising, alternative treatment option in the clinical practice over the last years. Seven serotypes of Botulinum neurotoxin are produced by Clostridium Botulinum, which are gram-positive, anaerobic bacteria. Botulinum neurotoxin type A (BoNT/A) has the longest duration of action and is most commonly used. Two toxins with different formulations and different dosage requirements are on the market known as onabotulinumtoxin A

which is available as Botox® (Allergan) and abobotulinumtoxin A which is available as Dysport® (Ipsen) (Dolly and Lawrence 2014).

A large systematic review for the use of Botox in the treatment of LUT disorders has shown a significant improvement in all NDO and OAB outcomes compared with placebo in a large number of randomised control trials (Mangera *et al.,* 2014). Several neurological side effects such as dry mouth and muscle weakness as well as urological complications like hematuria, urinary retention and urinary tract infection can occur after the treatment with BoNT/A for OABS. However these side effects were transient, mild and did not require treatment (Bauer *et al.,* 2011). BoNT/A has now received regulatory approval for clinical use for NDO and OAB patients.

Further Treatment Options

Several other emerging treatment options are under investigation, aiming to reduce afferent nerve activity, muscle contractility or neuromuscular transmission. Special focus lies on transient receptor potential (TRP) channels, phosphodiesterases and β_3-adrenoceptor of the detrusor muscle (Henderson and Drake 2010). An oral β_3-adrenoceptor agonist has now passed phase III clinical trials and is available as mirabegron. Other promising treatment options for bladder dysfunction are tissue engineering and stem cell transplantation in order to obtain tissue regeneration (Kim *et al.,* 2014) or device-based interventions such as sacral or tibial nerve stimulation.

In severe cases, surgical interventions can be offered to treat OAB. They are however not widely available as a consequence of relatively high costs and stringent selection criteria. Reconstructive surgery is also a major undertaking and can have inadequate outcomes and side effects (Henderson and Drake 2010).

1.6 ANIMAL MODELS

All experiments for this thesis have been carried out *in vitro*. While *in vivo* experiments make it possible to study the physiological or pathophysiological properties of a living being in conditions closest to real life, it is difficult to simplify *in vivo* models in order to answer specific scientific questions. In an *in vitro* experiment it is easier to control parameters that might affect the outcomes of the study and the great variability that living organisms exhibit can be reduced. Furthermore, animal welfare issues make it essential to use anaesthesia in most *in vivo* experiments which again might affect the outcomes of the study. In an *in vivo* bladder experiment efferent nerve pathways would for example still be intact, however the anaesthesia of the animal might inhibit afferent pathways or affect central control centres. Throughout the *in vitro* experiments carried out for this thesis, it was attempted to establish conditions close to the normal physiological surroundings of the tissue. The tissue was for example provided with adequate oxygen and nutrient supply and kept at body temperature.

Murine and porcine tissue has been used in this work. The porcine bladder has been recognised to be an appropriate model for the human bladder in various studies regarding size, bladder capacity, micturition pattern, electrical properties and even purinergic signalling pathways (Hashitani and Brading 2003, Templeman *et al.,* 2003, Kumar *et al.,* 2004, Sadananda et al 2008). The pig also showed comparable urodynamic characteristics to humans (Crowe and Burnstock 1989). Bladders from male and female pigs of approximately six months of age were used to investigate the gender difference of spontaneous activity in this thesis. Castration of male piglets is common practice to reduce the level of steroid hormones in the pig carcasses which are associated with unpleasant odours in the meat (Brooks and Pearson 1986). This practice might however change the physiological behaviour of the tissue. For the experiments in this thesis, it was made sure that the male pigs were not castrated. It was however not possible to standardise the time point of the female oestrous cycle. This was taken into consideration when interpreting the results.

The pig has the advantage to be comparable to the human bladder regarding various factors stated above. However, the housing conditions and the environmental factors cannot be standardised when obtaining the tissue from an abattoir. Experiments with male mice bladders were therefore carried out to investigate specific signalling

pathways. The mouse model was also used to investigate age-related changes in bladder physiology as the ageing time in mice is comparably short.

The experiments in this thesis were carried out on single bladder cells, on bladder strips and on the whole bladder organ. All these models have both advantages and disadvantages. Experiments on isolated cells make it possible to study specific signalling pathways because isolated components can be studied without the complex interactions of the surrounding tissue. Studies with urothelial cells in culture have however shown that receptor expression can be lost when its function is no longer needed. It has also been shown that cultured cells do not necessarily mimic the actions exerted by intact tissues (Bahadory *et al.,* 2013). Furthermore, the whole spectrum of interactions between urothelial cells, muscle cells and ICs only function in the intact organ. Isolated whole murine bladders set up in a micro organ bath under physiological conditions were therefore used in this thesis for the examination of mediator release. Murine bladders were favoured in this experiment, as the size of the porcine bladder presented difficulties. Tissue strip experiments with both murine and porcine bladder strips were carried out for pharmacological investigation of the receptor agonists and antagonists. Isolated organ and tissue preparations easily allow changing parameters such as the oxygen availability, the perfusate or the drug administration, while having the complexity and functionality of different cell types. The organ and tissue strip bath experiments can therefore be seen as a bridge between cell culture experiments and *in vivo* experiments. Tissue bath experiments can for example be used to quantify the physiological effect of molecular test results.

As cellular responses and receptor expressions are often species-specific, extrapolating findings obtained from animal experiments to humans is often problematic and has to be done with precaution. For example, as stated above, differing subtypes of muscarinic and nicotinic receptors are expressed in the human bladder compared to the mice bladder and they are also in different distributions. However, it is essential to use animal models as human tissue is not readily available for experimental investigations. The current knowledge about the bladder has been mainly obtained from animal experiments and extrapolating animal findings to the human situation is a necessary procedure. *In vitro* methods are also vital to optimise a specific experimental method that can then be repeated directly with human tissue once it becomes available.

1.7 AIMS AND OBJECTIVES

The overall aim of this thesis is to further investigate the mechanisms and signalling pathways involved in the mechanosensation of the bladder - with focus on the urothelium. Various lines of evidence, as previously stated, suggest that non-neuronal mediator play a key role in the mechanosensation of the urinary bladder and that the urothelium is the source of this mechanically stimulated mediator release. It was tested if the mediators ACh and ATP are released in a mechanosensitive manner. Special attention is given to the cholinergic pathway, as antimuscarinic treatment is the mainstay therapy for OAB - however lacking efficiency. Therefore, it is important to improve our understanding of the non-neuronal signalling pathways in the bladder to be able to find new pharmacological targets for bladder dysfunctions.

As the demographic change leads to an ageing population and as the prevalence of OAB is increasing with age, it is also essential to understand the changes occurring in bladder function throughout life. Therefore, it was studied in the isolated whole murine bladder if urothelial mediator release undergoes age-related changes. Further investigations were carried out on the purinergic signalling pathway of isolated urothelial cells from aged and control mice. Furthermore, age-related changes in the mechanosensitivity of the detrusor muscle were examined. Another factor, influencing the occurrence of OAB in the different age groups, is the gender. Hence, variations in cholinergic stimulated activity in female and male pig detrusor strips were studied.

Due to a number of different methods used throughout this thesis, the methods will be explained prior to each chapter.

This work was conducted as part of a Marie Curie Initial Training Network and experiments were carried out alongside the work of other Marie Curie Fellows. A number of experimental protocols have therefore been adjusted to the protocols of previous experiments to ensure that the obtained data can be compared and integrated with the whole program.

Aims

- Investigation of intra- and extraluminal mediator release (ACh, ATP, NO and Substance P) in the isolated, whole murine bladder under varying conditions:
    1. Different rates of intraluminal pressure.
    2. Application of BoNT/A.
    3. Application of fesoterodine.
    4. Effects of ageing.
    5. Effects of blocking specific components of the cholinergic signalling pathway.

- Investigation of spontaneous contractions in the isolated whole mouse bladder.

- Investigation of age-related changes in the mouse bladder regarding detrusor mechanosensitivity and signalling pathways.

- Investigation of regional disparities and gender related differences in the cholinergic pathway of the porcine bladder in presence/ absence of the urothelium.

2. MEDIATORS INVOLVED IN THE MECHANOSENSATION OF THE ISOLATED MURINE BLADDER

2.1 INTRODUCTION

The study of mechanosensation and mechanotransduction, the ability and the mechanism of cells to recognise, analyse and respond to changing mechanical stimuli, is still in its early stages regarding the bladder urothelium. It has been proposed however, that the urothelium plays an essential role in sensing the filling state of the bladder by converting changes of stretch (Yu *et al.,* 2009, Tanaka *et al.,* 2011) and pressure (Ferguson *et al.,* 1997, Wang *et al.,* 2003, Olsen *et al.,* 2011) into biochemical signals.

As explained in Chapter 1, the urothelium is an active contributor to bladder function and responds to mechanical stimuli by releasing an array of mediators that then act on neighbouring urothelial cells, sensory nerve terminals, ICs or underlying muscle cells to allow the perception of bladder fullness (Apodaca *et al.,* 2007, Birder and de Groat 2007, Birder 2011). In response to distension the urothelium has been found to release several mediators including ACh (Yoshida *et al.,* 2004, Yoshida *et al.,* 2006), ATP (Ferguson *et al.,* 1997, Birder 2003) and NO (Birder *et al.,* 1998, Moon 2002, Munoz 2011).

ACh

ACh is a ubiquitous neurotransmitter in the human body and plays a crucial role in the regulation of bladder mechanosensation and bladder contraction. This mediator is involved in the regulation processes in the detrusor muscle, in afferent and efferent nerves as well as in the urothelium. In addition to the neuronal cholinergic system, it has been shown that the bladder exhibits a non-neuronal cholinergic system. Tetrodotoxin-resistant and therefore non-neuronal ACh is released in the human bladder which mainly originates from the urothelium (Yoshida *et al.,* 2004, Yoshida *et al.,* 2006).

Hanna-Mitchell *et al.* (2007) showed a significant increase in radiolabelled ACh release from murine urothelial cells after mechanical stimuli by cellular swelling and after stimulation with ATP. Furthermore the molecular components involved in the cholinergic pathway were studied in urothelial cells using RT-PCR. The expression of the high-affinity choline transporter (ChT) as well as the enzymes necessary for ACh synthesis could be shown in murine urothelial cells. The latter enzymes are choline acetyltransferase (ChAT), which are also found in the nervous system, and carnitine acetyltransferase (CarAT), which are also found in muscle cells. It has also been proposed by Hanna-Mitchell *et al.* (2007), that ACh is unlikely to be stored and released via vesicles in urothelial cells, as the vesicular acetylcholine transporter (VAChT) is not expressed in the urothelium. VAChT is necessary to transfer ACh into synaptic vesicles in cholinergic neurons. Moreover, ACh release was not blocked in experiments with brefeldin - a substance that blocks the formation of transport vesicles. It could instead be demonstrated that organic cation transporter 3 (OCT 3) is expressed in urothelial cells. This is a subtype of the polyspecific organic cation transporters (OCTs) and it was hypothesised that this transporter is most likely responsible for the transfer of ACh across the plasma membranes in the urothelium (Hanna-Mitchell *et al.,* 2007). Interestingly OCTs work in a bidirectional manner depending on membrane potential and substrate concentration (Koepsell *et al.,* 2003). Therefore these transporters may not only mediate ACh release but also ACh uptake into urothelial cells.

The findings with murine urothelial cells were consistent with studies by Lips *et al.* (2007) using human urothelial cells. The group showed expression of CarAT, OCT1 and OCT3, whereas VACht was not detected at mRNA or at protein level. Furthermore the investigators did not find ChAT expression (Lips *et al.,* 2007). As ChAT had already been found in murine (Hanna-Mitchell *et al.,* 2007) as well as in human urothelial cells (Yoshida *et al.,* 2006), several examinations were carried out by Lips *et al.* (2007) and the authors proposed that the positive immunolabelling that had already been obtained with ChAT antiserum, might have been caused by CarAT, due to a structural similarity of ChAT and CarAT. The authors were able to reproduce the positive findings for ChAT with the antiserum that had been used before. This indicates that ChAT does not exist in the urothelium and that CarAT is therefore the major ACh synthesizing enzyme in human urothelial cells with choline being the rate limiting substance.

Choline uptake is dependent on the distribution of ChT in the cell membrane. ChT is a 13 transmembrane domain protein that appears to be phosphorylated as well as glycosylated. The physiological mechanism of this transporter is not yet fully understood. It is however known, that the presence of ChT protein at the cell surface is limited by rapid endocytosis in clathrin-coated pits. Surprisingly, only a small fraction of the transporters are found to be associated with the cell surface, the predominant quantity of ChT is located in intracellular vesicular structures of endocytotic origin (Ribeiro *et al.,* 2006). However, the regulatory signals controlling relocation of ChT via decrease or increase in endocytosis and exocytosis respectively are unknown.

A recent publication by McLatchie *et al.* (2014) suggests that the cystic fibrosis transmembrane conductance regulator (CFTR) plays a role in the release of ACh and ATP in urothelial cells of guinea pigs. Blocking OCT 1-3 with quinine had no significant effect on the release of neither ACh, nor ATP whereas an inhibitor of CFTR significantly reduced ACh as well as ATP release. These results suggest that OCTs might not be involved in the release mechanism of ACh/ATP, whereas CFTR could play an important role. In addition, immunohistochemistry experiments demonstrated the expression of the CFTR protein within the urothelium. Studies on human urothelial cells need to be conducted to ensure that these results are not species specific. CFTR is a member of the ATP binding cassette (ABC) transporter gene family. It regulates the movement of chloride ions through the cell membranes and opens or closes when ATP binds to its cytoplasmic domain after being phosphorylated by protein kinase A (PKA) (Gadsby *et al.,* 2006). Recently, evidence emerged that changes in membrane tension (stretch or pressure change) can open the CFTR channel independently of phosphorylation and ATP-binding mechanisms (Zhang *et al.,* 2010).

Based on the previously stated literature, Figure 9 illustrates the possible non-neuronal cholinergic pathway in bladder urothelial cells that is regulated by mechanosensation. ACh is most likely not released in an exocytotic way, but instead ACh release into the lumen could be enabled via OCT3 or CFTR following mechanical or chemical stimuli. ACh is then broken down by acetylcholine esterase (AChE) in to choline and acetic acid. Subsequently, choline is taken up into urothelial cells by ChT. In the cell, choline is resynthesized to ACh while reacting with Acetyl Coenzyme A. This reaction is normally catalysed by ChAT, but in urothelial cells the reaction is more likely to be

catalysed by CarAT. However, it is also possible that intact ACh is taken up into urothelial cells by the bidirectional OCTs. ACh acts in an autocrine or paracrine fashion to signal the filling state of the bladder to neighbouring urothelial cells, as well as underlying sensory nerves, ICs and muscle cells by acting on cholinergic receptors in these tissues.

This chapter tests elements of this hypothesised model in the whole, isolated murine bladder.

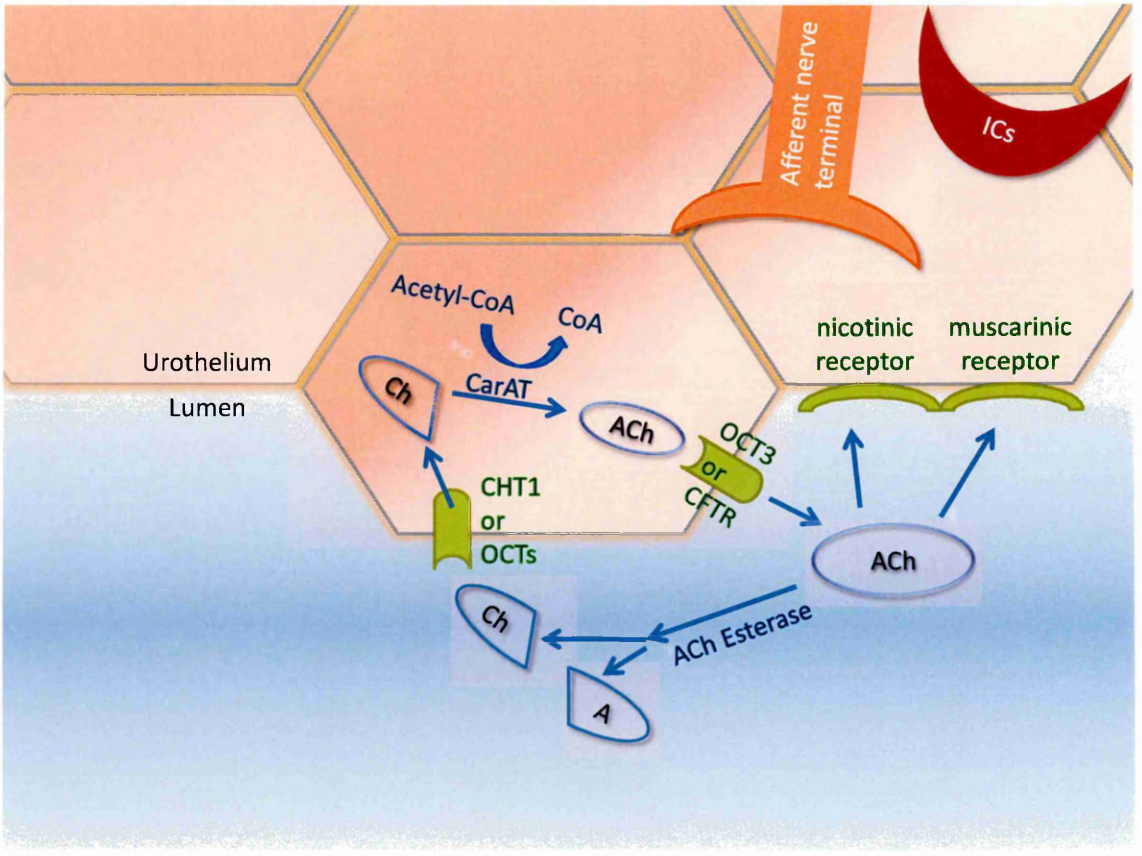


Figure 9 **Hypothetical model showing the cholinergic pathway in urothelial cells. ACh is released either via OCTs or CFTR into the bladder lumen and acts on cholinergic receptors found on neighbouring urothelial cells as well as on nerve endings and ICs in close proximity to the urothelium. Choline, the breakdown product of ACh is taken up into urothelial cells by ChT or bidirectional OCTs. In the cell, choline is resynthesized to ACh while reacting with Acetyl Coenzyme A. This reaction is possibly catalysed by CarAT.**

(ACh – Acetylcholine, OCT – Organic Cation Transporter, CFTR - Cystic Fibrosis Transmembrane conductance Regulator, ICs - Interstitial Cells, ChT – Choline Transporter 1, CarAT - Carnitine Acetyltransferase)

ATP

Another mediator contributing to the contractile responses of the urinary bladder and playing a role in urothelial signalling is ATP. Non-neuronal ATP release in the urothelium was first shown by Ferguson *et al.* (1997). It has been demonstrated in this study that ATP was released from the urothelial side of rabbit urinary tissue strips mounted in an Ussing chamber. This is a scientific device consisting of two chambers with the tissue mounted in between, allowing the separation of the apical and basal side of the tissue. The release was three times higher on the apical side compared to the release on the basal side, suggesting an important physiological function of ATP in the urothelium. Furthermore, organ bath experiments with intact, denuded and urothelial tissue strips following electrical stimulation, showed that ATP release from the isolated urothelial strips was of the same magnitude as the ATP release from intact bladder strips while the denuded smooth muscle strip showed very little ATP release. This suggested that the main source of ATP, released from the bladder strips, is the urothelium. Additionally, TTX had no effect on electrically induced ATP release from the urothelium showing its non-neuronal nature (Ferguson *et al.,* 1997).

In the following years urothelial ATP release could be shown in different species. Feline urothelial cells released ATP in response to hypotonic swelling – which is supposed to have common characteristics with mechanical stretch or distension. It was proposed, that this swelling-mediated ATP release is due to both Ca^{2+} influx and Ca^{2+} release from intracellular stores (Birder *et al.,* 2003). Also rabbit urothelium set up in an Ussing chamber showed bidirectional ATP release from both the apical and basal side of the urothelium (Lewis and Lewis 2006). In porcine and human bladders, augmented ATP release could be shown after mechanical and electrical stimulation. The main source of ATP release was again stated to be the urothelium from predominantly non-neuronal sources (Kumar *et al.,* 2004). Furthermore, ATP release was demonstrated in porcine cell culture of urothelial cells, ICs and muscle cells. It could be shown that baseline ATP was higher in urothelial cell and ICs culture compared to muscle cell culture. ATP release was significantly stimulated by stretch in all three cell cultures whereas only urothelial cells responded to low pH with release of ATP (Cheng *et al.,* 2011).

The presented findings propose that the purinergic signalling pathway is embedded in a complicated sensory web in the urothelium and that various stimuli result in an altered release of ATP from the urothelium. The released ATP has the potential to activate ionotropic and metabotropic P2 purinergic receptors in the underlying tissues or in the urothelium itself. It is widely known that afferent nerves express purinergic receptors and ICs have been shown to express purinergic P2Y (Wu *et al.,* 2004) as well as P2X receptors (Liu *et al.,* 2009). Furthermore, purinergic P2 receptors are expressed in the urothelium to receive the urothelial, extracellular ATP signal in an autocrine fashion or act to ATP released from other neighbouring cells. Mansfield and Hughes (2014) recently studied the complex interaction of ATP release, breakdown and P2 receptor signalling. The breakdown products of ATP, such as ADP or AMP also have an effect on the purinergic receptors located on the urothelium (see Figure 10).

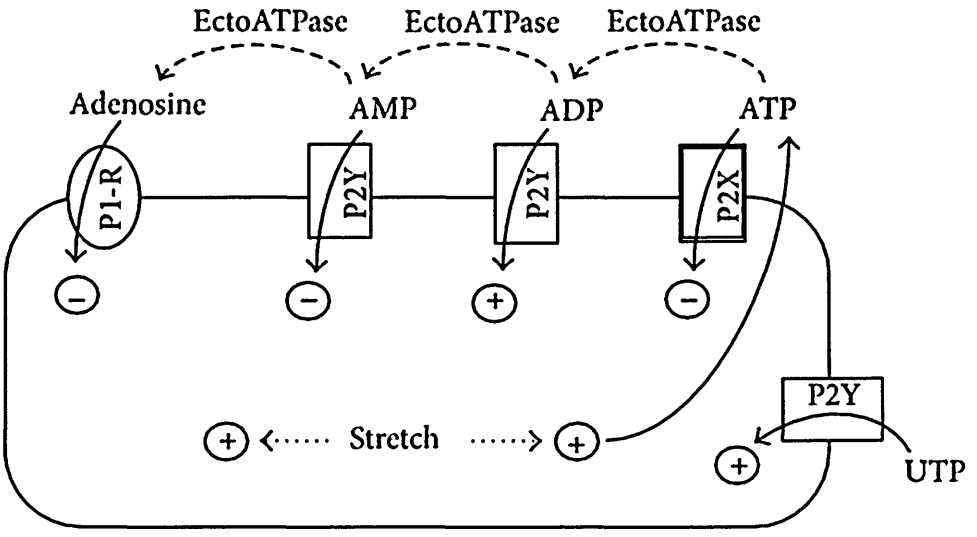


Figure 10 **Schematic representation of the proposed effect of nucleotides and stretch on urothelial ATP release mediated through purinergic receptors (Mansfield and Hughes 2014). Stretch results in ATP release from the urothelium, where it binds to P2X receptors and signals negative feedback. Furthermore membrane-bound ectoATPases metabolise ATP to ADP. ADP then acts on P2Y receptors stimulating further ATP release from the urothelial cell via activation of intracellular Ca^{2+} transients. Further breakdown of ADP to AMP and adenosine in turn inhibits ATP release by acting on P2Y and P1 adenosine receptors. ATP release is also stimulated by UTP acting on P2Y receptors.**

(ATP – Adenosine Triphosphate, P1-R – adenosine receptor, P2X/P2Y – purinergic receptors, ADP – Adenosine Diphosphate, AMP – Adenosine Monophosphate, UTP – Uridine Triphosphate)

NO

A further mediator that seems to play a role in the urothelial signalling network is NO. It was shown that adrenergic stimulation of rat bladder strips released NO, which source was mainly the urothelium as adrenergic evoked release of NO was reduced by 85% after removal of the urothelium (Birder *et al.,* 1998). Expression of endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) but not neuronal NO synthase (nNOS) have been identified in rat urothelial cells (Birder *et al.,* 2002b). eNOS has also been identified in the urothelium of rats using immunohistochemistry and it was furthermore shown that a NO synthase inhibitor increased the contractile effect of a cholinergic agonist (Giglio *et al.,* 2005). This suggests a link between cholinergic and NO signalling in the urothelium. Giglio *et al.* (2005) also showed that eNOS was upregulated in rats with cyclophosphamide induced cystitis compared to controls (Giglio *et al.,* 2005). This is an indicator that NO synthase in the urothelium plays a role in pathogenesis of urinary bladder disorders.

Pharmacological Therapy for OAB

The main pharmacological therapy for OAB is the oral administration of antimuscarinic drugs such as fesoterodine. Fesoterodine binds to muscarinic receptors in the bladder urothelium and detrusor muscle in a competitive and reversible manner (Yoshida *et al.,* 2013) and aims to improve the symptoms of urgency, frequency and urgency incontinence. Another promising treatment option is the injection of Botulinum neurotoxin A (BoNT/A) into the bladder wall. The mechanism of action of these two treatment options in the bladder is not fully understood. Therefore this chapter examines the effect of BoNT/A and fesoterodine on the release of the previously discussed mediators in the whole, isolated murine bladder.

BoNT/A was also used to examine the effect of blocking vesicular mediator release in the bladder. The effect of BoNT/A at neuromuscular junctions is well documented. Here it obstructs parasympathetic, presynaptic release of ACh by preventing synaptic vesicle fusion which results in the inhibition of muscle contraction. The underlying mechanism involves several steps, including internalisation of BoNT/A after binding synaptic vesicle protein 2 (SV2) at the membrane and cleavage of synaptosomal-associated

protein 25 (SNAP-25), which is a membrane protein essential for synaptic vesicle fusion (Figure 11, Cruz 2014). The suggestion that ACh is released in a non vesicular way from the urothelium was tested in this chapter with the help of BoNT/A.

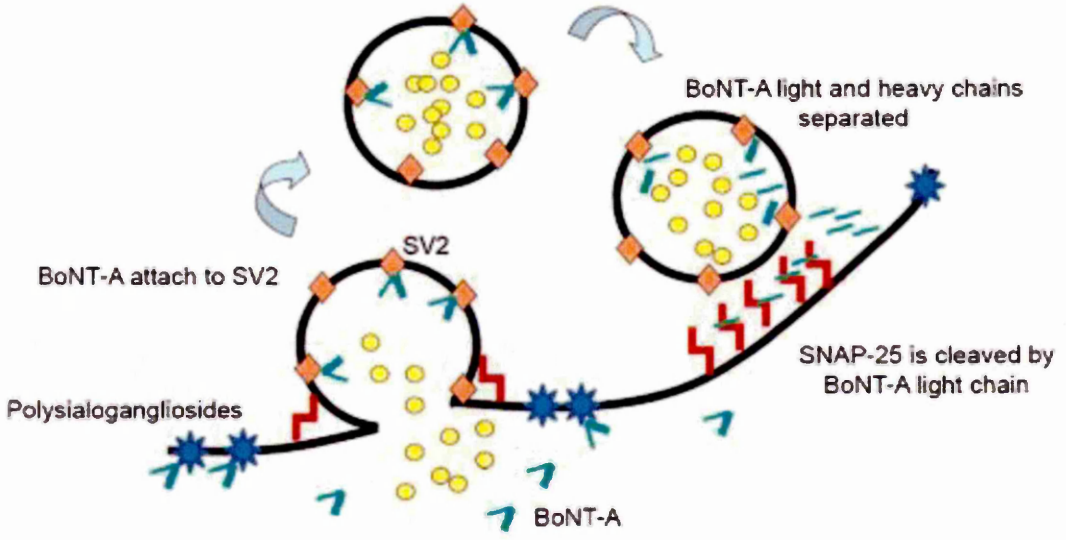


Figure 11 Mechanism of action of BoNT/A in a neuronal ending (Cruz 2014). BoNT/A binds to SV2 at the membrane and gets internalised by endocytosis. After internalisation, the light and heavy chain of the BoNT/A molecule separate and the light chain cleaves membrane protein SNAP-25. As this protein is essential for synaptic vesicle fusion, the release of neurotransmitter is now impaired.

(SV2 – synaptic vesicle protein 2, SNAP-25 – synaptosomal-associated protein 25)

Aims

In order to further gain insight into the role of the different mediators involved in mechanosensation of the urothelium, the amount of ACh, ATP and NO was determined in the whole, isolated murine bladder under different conditions. The bladders were set up in a purpose built micro organ bath under physiological conditions and distended to predetermined pressures at a set perfusion rate. Intra- and extraluminal samples were then taken and the amount of mediators was quantified with assay kits.

Following properties were tested:

- The effect of bladder distension on the amount of ACh in intra- and extraluminal samples.
- The effect of BoNT/A on the amount of ACh, ATP and NO in intra- and extraluminal samples.
- The effect of varying degrees of distension on the amount of ACh and ATP in intra- and extraluminal samples.
- The effect of blocking several components of the cholinergic pathways on the amount of ACh in intra- and extraluminal samples.

The term 'whole isolated bladder' is used throughout this thesis, referring to the fact that experiments were performed *in vitro* using the intact bladder organ compared to experiments with tissue strips.

The data of this chapter are partly published in Collins *et al.* (2013).

2.2 MATERIAL AND METHODS

2.2.1 PRESSURE TRANSDUCER MICRO-ORGAN BATH

2.2.1.1 TISSUE PREPARATION

Male C57/B6 Mice, aged approximately 3-6 months were obtained from Harlan (UK) and housed in the University of Sheffield Field Labs. Handling followed principles of good laboratory practice in compliance with UK laws and regulations. The mice were killed by cervical dislocation; a Home Office approved Schedule One procedure. The whole, isolated bladders were immediately dissected from the animals and placed in Krebs' solution (37 °C, pH ~7.4, oxygenated with 95% O_2 and 5% CO_2, formulation see 2.2.4). The bladders were then catheterised using a dual-lumen cannula (Polythene Tubing, inner diameter 0.28mm, outer diameter 0.38mm) via the urethra under a dissection microscope. The bladder neck, including the ureters, was closed off with surgical suture (size 4-0) to prevent leaks and to hold the catheter in place.

2.2.1.2 MICRO ORGAN BATH SET –UP

A purpose built micro organ bath for isolated whole murine bladders was set up and validated (Figure 12). Catheterised, empty mouse bladders were placed into a micro organ bath filled with 500µl Krebs' solution (pH 7.4) and oxygenated with 95% O_2 and 5% CO_2 at 37°C. The catheter was connected to both an infusion pump filled with buffered saline (0.9% NaCl buffered with NaOH, pH 7.4-7.8) and a pressure transducer (BD DTXPlus™). The transducer signal was amplified and recorded with Spike2 software (Cambridge Electronic Design, UK). An overflow tap was mounted in front of the pressure transducer, allowing the collection of intraluminal samples by emptying the bladder with the help of capillary action. The tube supplying the outflow tap was kept as short as possible in order to collect the maximal amount of intraluminal sample. This set up allowed infusion and withdrawal of medium (Krebs' solution plus possible drugs) at a constant rate and the recording of intraluminal pressure while filling/emptying the bladder accordingly. Intra- and extraluminal samples were collected to examine the amount of ACh, ATP and NO.

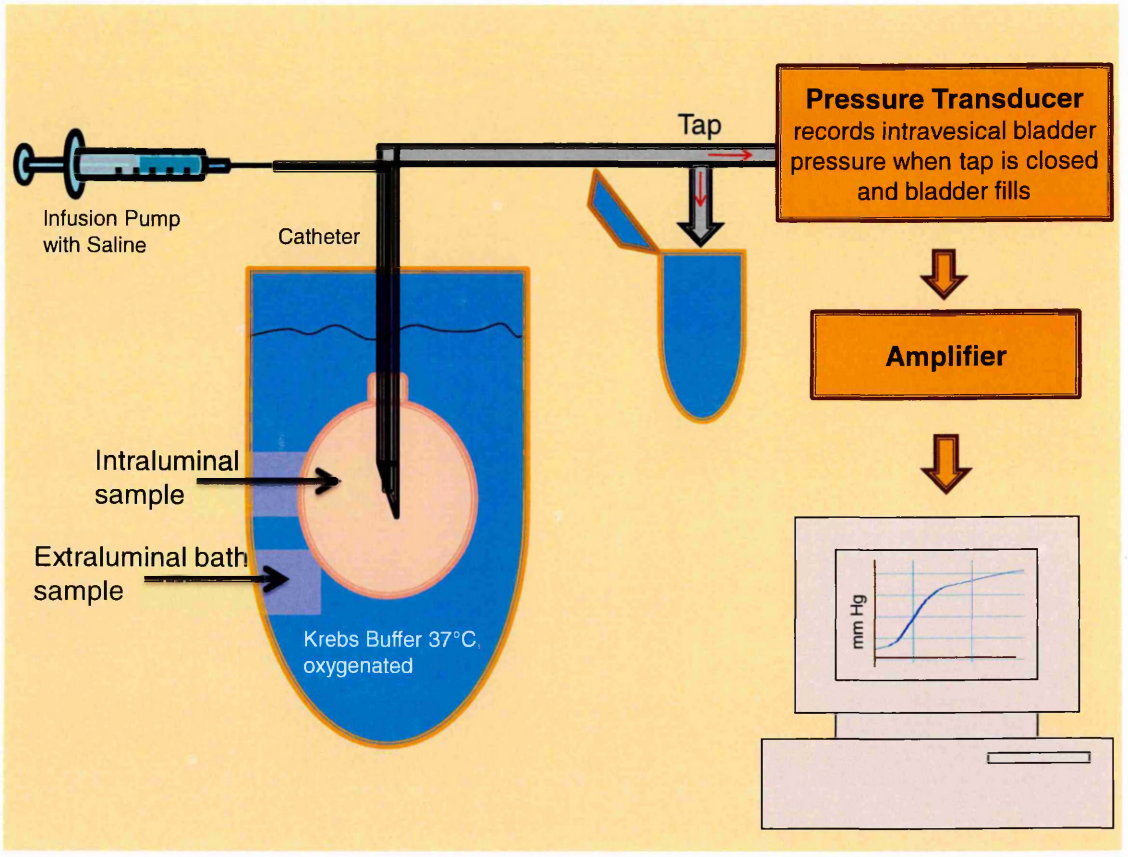


Figure 12 **Micro organ bath set up. A catheterised mouse bladder was mounted in a whole organ tissue bath filled with 500 µl Krebs' solution and oxygenated with 95% O_2 and 5% CO_2 at 37°C. The catheter was connected to both an infusion pump filled with saline and to a pressure transducer with an overflow tap. The pressure signal was amplified and made visible on the computer screen.**

2.2.2 PROTOCOLS

After mounting the bladder in the micro organ bath, a single distension to 30 mm Hg was carried out with buffered saline to ensure that the set-up was functioning. The drained bladder was then left empty for 30 minutes to equilibrate.

Three extra- and intraluminal basal samples were then taken at the beginning of every experiment. Intraluminal basal samples were collected without distending the bladder wall, by constantly perfusing the bladder with buffered saline at 40 µl/min with the overflow tap open and positioned in a collection tube on dry ice (-60°C). Instant collection on dry ice was performed to prevent enzymatic degradation. The collection tube was exchanged every ten minutes. Extraluminal basal samples were also taken every ten minutes by collecting the total bath fluid with a syringe into a collection tube on dry ice. For every extraluminal sample taken, the total bath fluid of 500 µl oxygenated, 37°C Krebs' solution was collected and replaced.

After the basal samples were collected, the overflow tap was closed and distensions were performed with buffered saline while the rise in pressure was monitored. The perfusion rate of saline flowing into the bladder as well as the maximal pressure varied for the different protocols used. These protocols are described below. Intraluminal samples were collected into a collection tube on dry ice via opening the overflow tap, extraluminal samples were taken by collecting the total bath fluid with a syringe into a collection tube on dry ice. The volume of the intraluminal samples varied according to the dissimilarity in bladder size and the compliance of the individual bladder. Samples were stored at -80°C in the freezer until measurements of ACh, ATP and NO were carried out using the relevant assay kits.

In addition spontaneous activity was recorded as small transient rises in intraluminal pressure and the traces were analysed for amplitude and frequency measurements.

The experiments have to be viewed in context with other experiments as part of the Marie-Curie project. In some experiments specific perfusion rates and maximum pressure levels have been used in order to be able to compare the results with those of colleagues.

Protocols

1. Three basal samples were collected at a perfusion rate of 40 µl/min. The mouse bladders were then distended ten times with buffered saline at a perfusion rate of 150 µl/min to an initial pressure of 50 mm Hg before the perfusion was stopped and the outflow tap opened. Intra- and extraluminal samples were collected immediately after the 50 mm Hg pressure threshold was reached and the bladder was distended again after a ten minute interval (Chapter 2.3.1).
2. The effect of BoNT/A on ACh, ATP and NO content in intra- and extraluminal samples was examined. The mouse bladders were distended several times with buffered saline containing BoNT/A (2U/bladder) to a pressure of 40 mm Hg at a perfusion rate of 100 µl/min over a 2 h time period. Intra- and extraluminal samples were collected immediately after the 40 mm Hg pressure threshold had been reached and the bladder was distended again after a ten minute interval. Collections were carried out for over 2 hours. Time-matched vehicle controls with buffered saline were conducted using the same protocol (Chapter 2.3.2).
3. To further examine the effect of pressure on neurotransmitter release (Chapter 2.3.3), collections of intra- and extraluminal samples were carried out after gradually distending the bladder to varying pressure levels. Three basal samples were collected at a perfusion rate of 40 µl/min. Distensions were performed to initial pressure levels of 20, 30, 40, 50 and 60 mm Hg at a perfusion rate of 150 µl/min before the perfusion was stopped and the outflow tap opened. Intra- and extraluminal samples were collected immediately after the pressure threshold was reached and the bladder was distended again after a ten minute interval.
   Moreover distensions to lower distension levels of 5, 10, 15 and 20 mm Hg were carried out at a perfusion rate of 40 µl/min to apply a more physiological stimulus. For each distension pressure, three samples were collected to obtain a sufficient sample size for the biochemical assays. All intra- and extraluminal samples were collected after ten minutes to ensure minimal variations between the samples. Adjusting for time differences of collection time was therefore unnecessary. The bladders were then left empty for five minutes to equilibrate before the next distension was carried out. The order of filling to 5, 10, 15 and 20 mm Hg was randomised in blocks of three distensions at each pressure level

(see Figure 13). A subsequent set of experiments were performed with distensions to 20, 30 and 40 mm Hg in randomised order following the same protocol.

4. The cholinergic pathway was studied further by blocking the reuptake of choline with hemicholinium-3, by blocking OCT1 with decynium and by blocking α7 nicotinic receptors with MLA. The experiment was set up as in the protocol described in Figure 13, however for intraluminal application of the drugs the saline solutions contained 5 nM hemicholinium-3, 1 μM decynium 22 and 10 nM MLA respectively. Krebs' solution was used in the bath. The order of filling to 5, 10, 15 and 20 mm Hg was again randomised in blocks of three distensions at each pressure level (see Figure 13, Chapter 2.3.4).
5. The effect of the antimuscarinic drug fesoterodine on ACh and ATP content in intra- and extraluminal samples was examined. Bladders were distended to 50 mm Hg with bath application of 10 μM fesoterodine at a perfusion rate of 100 μl/min. Intra- and extraluminal samples were collected immediately after the 50 mm Hg pressure threshold was reached and the bladder was distended again after a ten minute interval (Chapter 2.3.4).

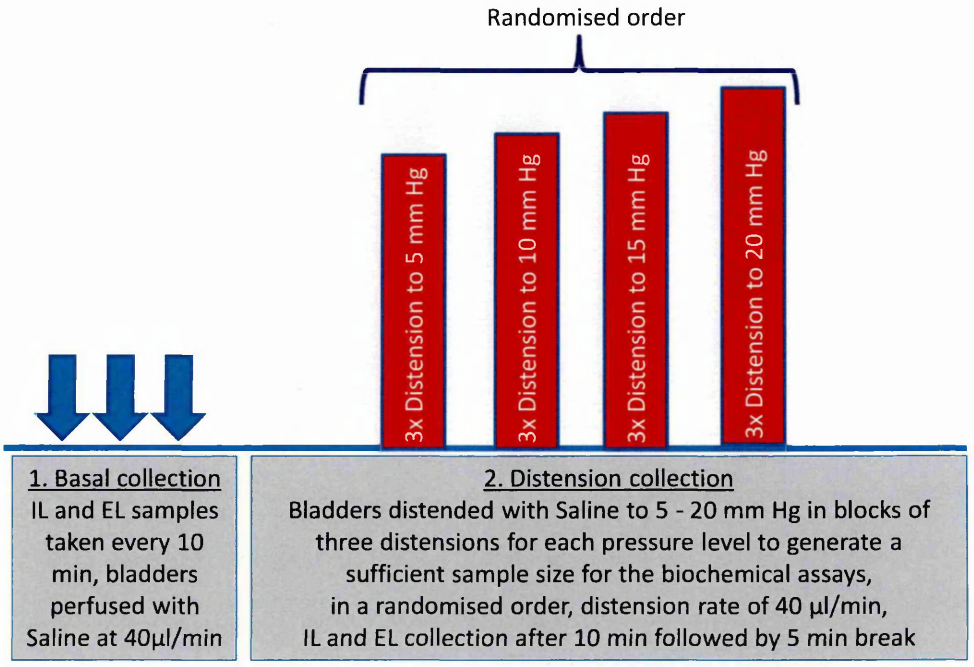


Figure 13 Protocol for intra- and extraluminal sample collection. (1.) Basal collection was performed with buffered saline at a perfusion rate of 40 µl/min. The intraluminal sample drained into the collection tube on dry ice. The tube was exchanged every ten minutes. (2.) Distensions were carried out to initial pressure levels of 5, 10, 15 and 20 mm Hg at 40 µl saline/min. Four blocks of three distensions at the same pressure level were carried out in a randomised order. Intra- and extraluminal samples were collected after ten minutes followed by a five minute equilibration period.

(IL – Intraluminal, EL – Extraluminal)

2.2.3 BIOCHEMICAL ASSAYS

Assays for ACh, ATP and NO were performed as described below according to the manufacturers' protocols. The concentrations of the stated mediators in intra- and extraluminal samples were calculated using standard curves which were plotted using known concentrations of the mediator. The data points were then extrapolated from the curves.

2.2.3.1 ACETYLCHOLINE (ACH)

Concentrations of ACh in intra- and extraluminal samples from murine bladders were measured using the fluorescence based Amplex®Red Acetylcholine/Acetylcholinesterase Assay Kit (Molecular Probes, Oregon, USA). The amount of released ACh is detected indirectly by measuring hydrogen peroxide (H_2O_2), generated from the oxidation of choline, using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2.

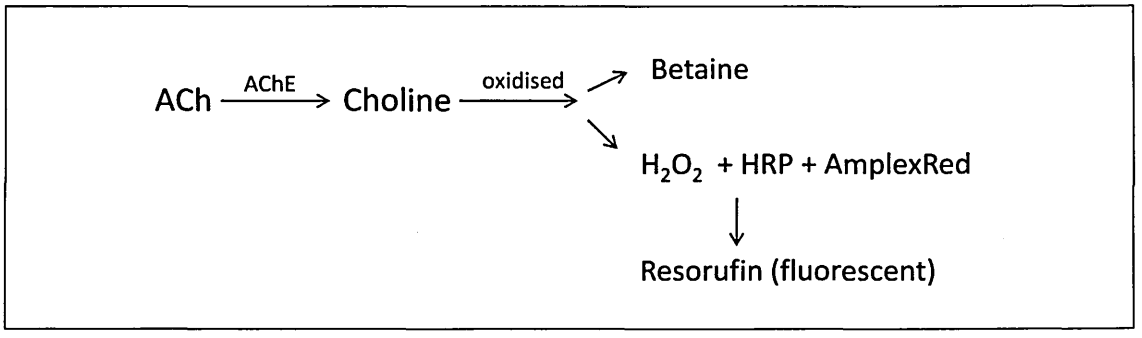


Figure 14 Reactions occurring in the conversion of ACh to the highly fluorescent product resorufin.

The enzyme reactions are shown in a simplified way in Figure 14. In the first step AChE converts acetylcholine to choline. Choline is subsequently oxidised by choline oxidase to betaine and H_2O_2. H_2O_2 then reacts with Amplex Red, in the presence of horseradish peroxidase (HRP), in a 1:1 stoichiometry to resorufin. This is a highly fluorescent product with an absorption and fluorescence emission maxima of approximately 571 nm and 585 nm respectively. Therefore little interference from autofluorescence in biological samples was expected. The range of detection covers 0.3

μM to 100 μM acetylcholine. The assay kit indirectly detects ACh by measuring the breakdown product choline. Therefore, not only the amount of ACh in the samples, but also any ACh that has been broken down during the time of sample collection is measured. Figure 15 shows an example of an ACh standard curve. For each assay, a new standard curve was conducted and each well plate contained two wells with the vehicle, which was either Krebs' solution or saline. The mean of the measurement of these wells was subtracted from the results prior to analyses. Fluorescence was read using a FLUOstar OPTIMA Plate reader (BMG, Germany) with 544 nm excitation and 590 nm emission wavelengths.

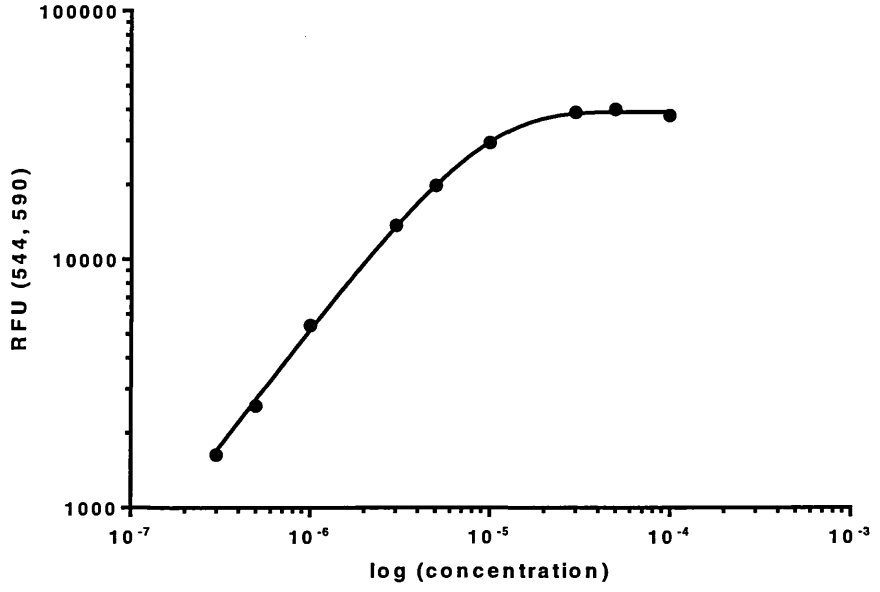


Figure 15 Typical standard curve of Relative Fluorescence Units (RFU) to known concentrations of ACh (300 nM-100 μM). Fluorescence was read using 544 nm excitation and 590 nm emission wavelengths.

2.2.3.2 ADENOSINE-TRIPHOSPHATE (ATP)

Concentrations of ATP in intra- and extraluminal samples from murine bladders were measured using the luminescence based Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma, UK). This kit provides a method for quantitative determination of ATP in the range $2x10^{-12}$ to $8x10^{-5}$ moles/litre.

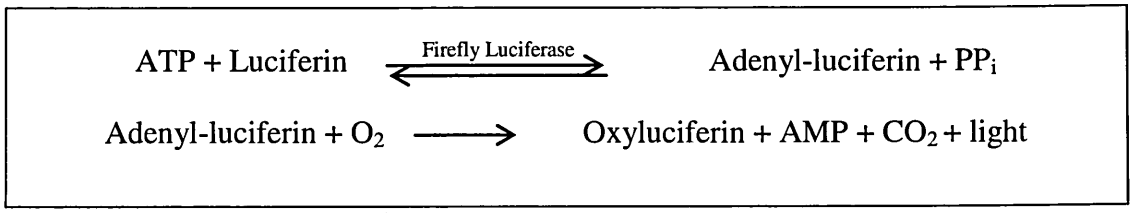


Figure 16 Reaction of ATP and Luciferin leading to the emission of light.

The assay is based on the ability of ATP to produce light in a reaction catalysed by Firefly Luciferase. ATP reacts with Luciferin forming adenyl-luciferin and pyrophosphate (PP_i). In a second reaction step the oxidised adenyl-luciferin reacts with oxygen and light is released (Figure 16). The amount of ATP present in the analysed sample is directly proportional to the released light and can be detected with a luminometer. An example of a typical standard curve is shown in Figure 17. For each assay a new standard curve was conducted and each well plate contained two wells with the vehicle, which was either Krebs' solution or saline. The mean of the measurement of these wells was subtracted from the results prior to analyses. Luminescence was read using a FLUOstar OPTIMA Plate reader (BMG, Germany).

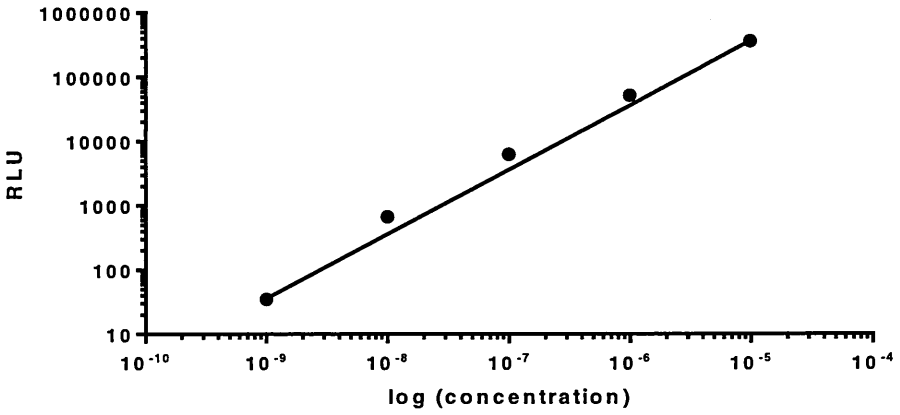


Figure 17 Typical ATP standard curve of Relative Light Units (RLU) to known concentrations of ATP (1 nM – 10 µM).

2.2.3.3 NITRIC OXIDE (NO)

Concentrations of NO in intra- and extraluminal samples from murine bladders were measured using the Nitric Oxide Fluorometric Assay Kit (BioVision). The assay kit provides a measurement of total nitrite (NO_2^-)/ nitrate (NO_3^-) concentration. NO converts rapidly into NO_2^- and NO_3^-. Consequently, the kit uses the total concentration of nitrite/nitrate as a quantitative measure of NO present in the samples. Initially, all nitrate is converted to nitrite, catalysed by nitrate reductase. Subsequently, nitrite reacts with the fluorescent probe DAN (2, 3-diaminonaphthalene) (Figure 18). The amount of emerging fluorescence is proportional to the total NO in the sample.

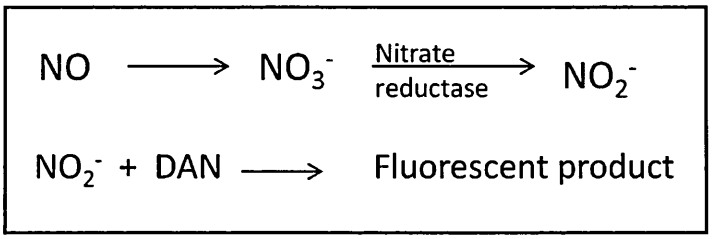


Figure 18 Two-step reaction of NO leading to the emission of fluorescence. In the first step, nitrate is converted to nitrite by nitrate reductase. In the second step, total nitrite reacts with the fluorescent probe DAN (2, 3- diaminonaphthalene) in to a fluorescent product. The fluorescent intensity is proportional to the total amount of NO in the analysed sample.

Figure 19 shows a NO standard curve. For each assay a new standard curve was conducted and each well plate contained two wells with the vehicle, either Krebs' solution or saline. The mean of the measurement of these wells was subtracted from the results prior to analyses. Fluorescence was read using a FLUOstar OPTIMA Plate reader (BMG, Germany) with 340 nm excitation and 460 nm emission wavelengths.

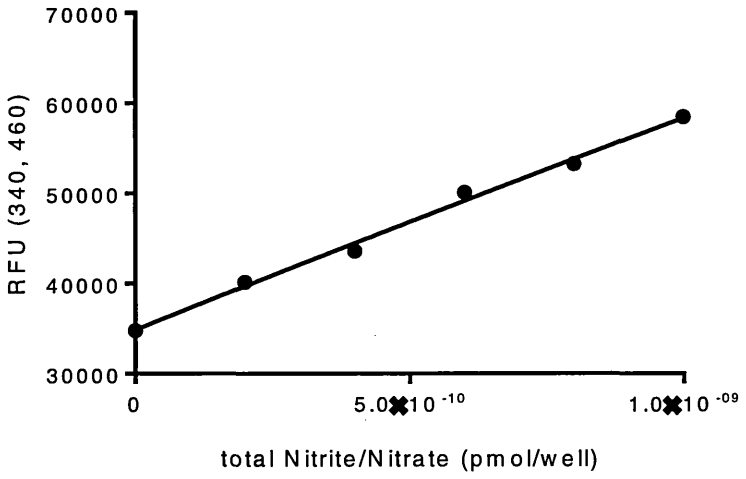


Figure 19 Standard curve of Relative Fluorescence Units (RFU) to known concentrations of NO (0-1nM). Fluorescence was measured using 340 nm excitation and 460 nm emission wavelengths.

2.2.4 DRUGS AND SOLUTIONS

Krebs' solutions consisted of NaCl 118.4 mM, Glucose 11.7 mM, $NaHCO_2$ 24.9 mM, KCl 4.7 mM, $MgSO_4$ 1.15 mM, KH_2PO_4 1.15 mM and $CaCl_2$ 1.9 mM. Chemicals were obtained from VWR (Leicestershire, UK).

The assay kits were obtained from the companies previously stated. Hemicholinium-3 was acquired from Sigma (Poole, UK), decynium 22 and MLA from Tocris Bioscience (Bristol, UK) and fesoterodine from Pfizer (Surrey, UK). Stock solutions and dilutions were prepared in Krebs' solution or saline where indicated.

For BoNT/A experiments OnaBotA (Allergan, USA) was reconstituted using sterile saline to a stock concentration of 100 U/ml. The solution was aliquoted and stored at -20°C for up to two months.

2.2.5 DATA ANALYSIS

Data are presented as mean ±SEM with n being the number of bladders used. Statistical analysis was carried out using paired or unpaired Student's T test, unpaired Student's T test with Welch's correction of %change, 1-way ANOVA with Tukey's post-test or 1-way ANOVA with Dunnett's multiple comparison post-test where indicated. Statistical significance was considered whenever reaching a 95% confidence interval with p values of ≤ 0.05 considered as significant. All statistics were calculated using Prism 6 (GraphPad Prism, San Diego, USA).

Results were corrected for bladder volume and expressed as nM/µl and pM/µl respectively. This was carried out to compensate for variations in bladder size and therefore variations in urothelial surface area releasing mediators. Whenever the time frame of collection did vary between the bladders in the same experiment, the results were also adjusted for filling time and expressed as pmole/min.

Spontaneous activity

The fluctuation of pressure in the bladder was observed as a proxy for spontaneous muscle activity. Spontaneous activity indicated by small transient rises in pressure was

measured and analysed using a script custom designed by Cambridge Electronic Design. Maximum peak to peak amplitude (max-min) of contractions was calculated over ten minutes. For frequency analyses the number of contractions per minute was calculated. A contraction event was defined whenever the peak exceeded the amplitude of 0.5 mm Hg. It was manually confirmed that no noise was recorded.

Three distensions to each pressure level were performed to collect sufficient solution to carry out the assays as previously stated. For the analyses of spontaneous activity the trace of the 2^{nd} distension has been used. Furthermore, the entire time frame from starting the distension to opening the tap for collection (10 minutes) has been used to analyse amplitude and frequency of activity.

Bladder Compliance

Bladder compliance is defined as the relationship between the change in bladder volume and the change in detrusor pressure ($\Delta V/\Delta P$). The detrusor pressure is calculated by subtracting the abdominal pressure from the intraluminal pressure, measured with a bladder catheter. It gives an indication on how the different mechanisms in the bladder wall react to stretching (Abrams *et al.,* 2003, Wyndaele *et al.,* 2011). Taking into consideration that no abdominal pressure exists in the experimental set up used in this study, the vesical pressure was simply used as detrusor pressure measured via pressure transducer.

2.3 RESULTS

2.3.1 STANDARD CURVES WITH DATA POINTS

Whole, isolated murine bladders were set up in a micro organ bath as previously described. Intra- and extraluminal samples were obtained and were analysed for their ACh, ATP and NO content. Typical standard curves of these three mediators are shown in the following.

ACh

A typical ACh standard curve with the interpolated data points is shown in Figure 20.

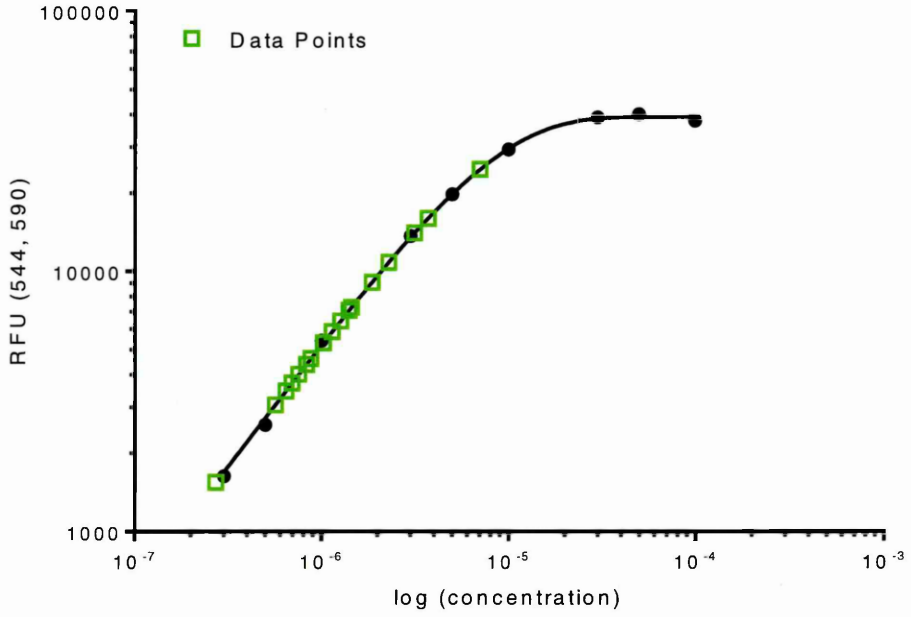


Figure 20 Standard curve of Relative Fluorescence Units (RFU) to known concentrations of ACh (300 nM-100 µM) with interpolated data points shown in green. Fluorescence was read using 544 nm excitation and 590 nm emission wavelengths.

ATP

A typical ATP standard curve with interpolated data points is shown in Figure 21.

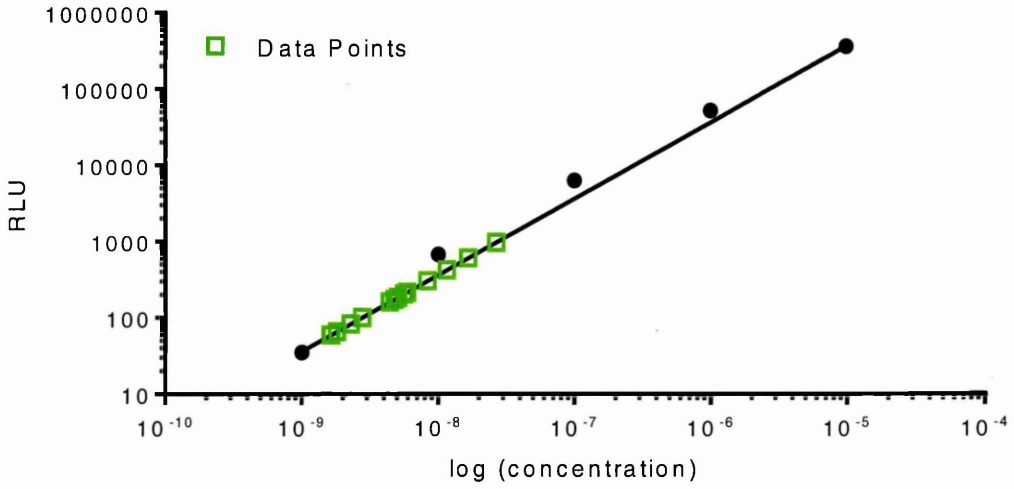


Figure 21 Standard curve of Relative Light Unit (RLU) to known concentration of ATP (1 nM – 10 µM). Interpolated data points are shown in green.

NO

A typical NO standard curve with interpolated data points is shown in Figure 22. The first point on the standard curve (lowest standard) is zero. Small amounts of NO could be detected in the intraluminal samples and all data points were positioned in between the first three standards.

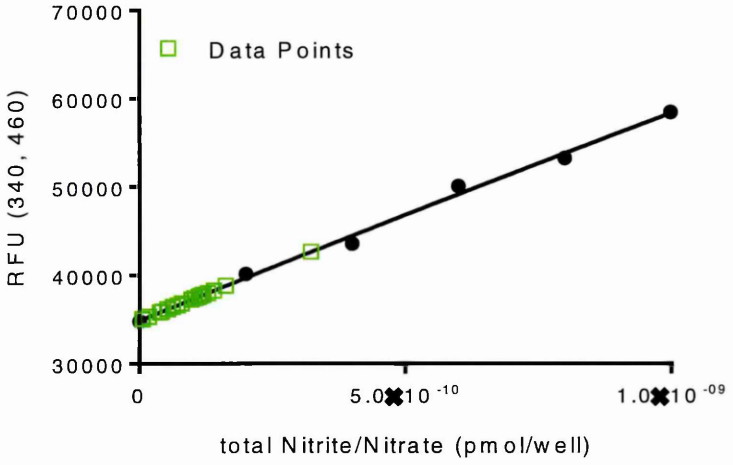


Figure 22 Standard curve of Relative Fluorescence Units (RFU) to known concentrations of NO (0-1nM) with interpolated data points shown in green. Fluorescence was read using 340 nm excitation and 460 nm emission wavelengths.

2.3.2 EFFECT OF DISTENSION ON ACH CONTENT IN INTRA- AND EXTRALUMINAL SAMPLES OF THE ISOLATED MURINE BLADDER

The amount of ACh was analysed in intra- and extraluminal samples of the whole, isolated mouse bladder. Basal sample collection was carried out at a perfusion rate of 40 µl/min, followed by ten successive distensions to 50 mm Hg at a perfusion rate of 150 µl/min as described in 2.2.2 (protocol 1).

The data are shown in pmole/min to compensate for the differences in collection time between basal samples (collection after 10 min) and distension samples (collection was carried out directly after distension, see protocol).

Intraluminal ACh

At basal level the amount of intraluminal ACh was measured at 11.46 ±6.06 pmole/min (n=5). After distension to 50 mm Hg a significant increase to 90.39 ±7.68 pmole/min was observed (paired Student's T test, n=5, p=0.003, Figure 23 A). The mean of ten distensions to 50 mm Hg was used for this analysis.

Extraluminal ACh

At basal level the amount of extraluminal ACh was measured at 158.48 ±21.29 pmole/min (n=5). After distension to 50 mm Hg a significant increase to 472.75 ±51.74 pmole/min was observed (paired Student's T test, n=5, p=0.012, Figure 23 B). The mean of ten distensions to 50 mm Hg was used for this analysis.

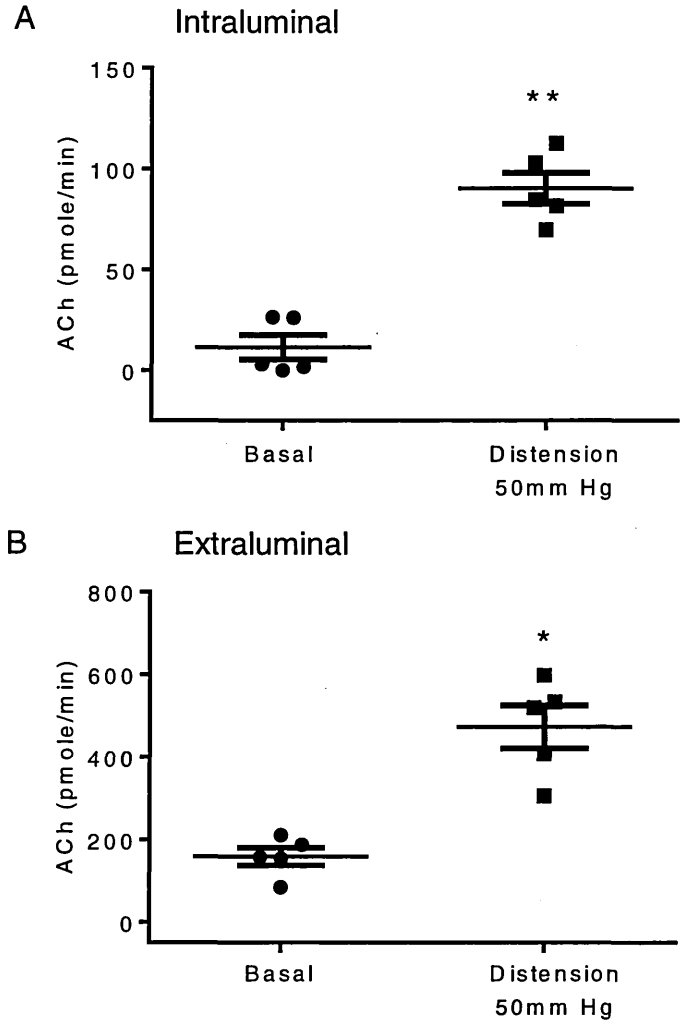


Figure 23 ACh in intra- and extraluminal samples at basal level and after distension to 50 mm Hg (shown as single data points with mean ±SEM). (A) Intraluminal ACh during distensions to 50 mm Hg was significantly higher than basal release (paired Student's T test, n=5, ** indicates $p \leq 0.01$). (B) Extraluminal ACh content was also significantly increased from basal release to release after distension to 50 mm Hg (paired Student's T test, n=5, * indicates $p \leq 0.05$).

ACh release during ten successive distensions to 50 mm Hg

Ten successive distensions to 50 mm Hg were carried out and the amount of ACh measured in intra- and extraluminal samples after each of the ten distensions. A stable amount of ACh was released in intra- and extraluminal samples over time. There was no significant difference in the amount of detected ACh between the ten distensions, neither in intra- nor extraluminal samples. Intraluminally an average of 90.39 (±16.88) pmol/min ACh and extraluminally an average of 472.75 (±51.74) pmol/min ACh was measured (1-way ANOVA with Tukey's post-test, n=5, Figure 24).

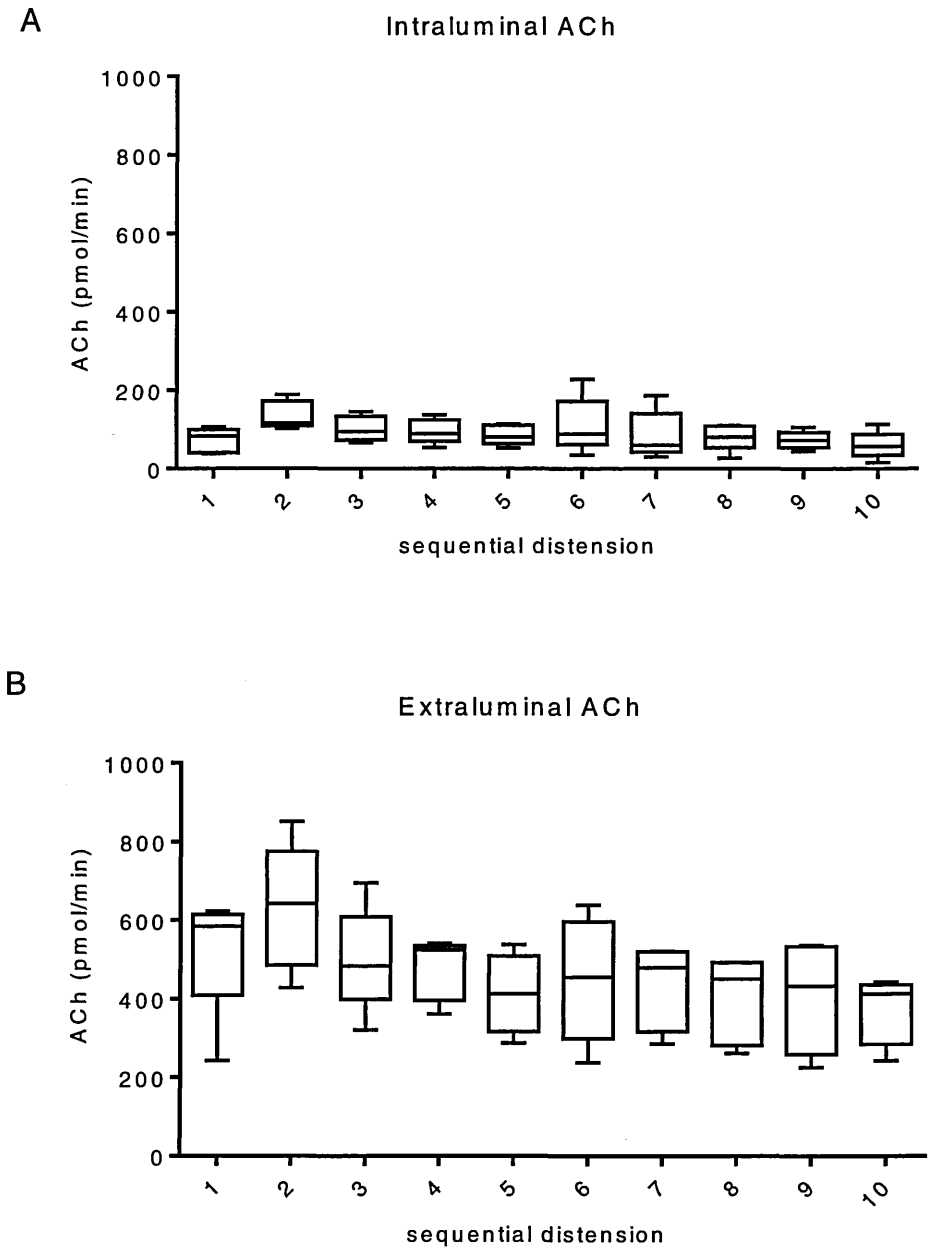


Figure 24 **ACh in intra- and extraluminal samples during ten successive distensions to 50 mm Hg (box = 25th and 75th percentiles with median, bars = min and max values). No significant difference observed between sequential distensions over time in (A) intra- and (B) extraluminal samples (1-way ANOVA with Tukey's post-test, n=5).**

2.3.3 EFFECT OF BOTULINUM TOXIN ON MEDIATORS IN INTRA- AND EXTRALUMINAL SAMPLES OF THE ISOLATED MURINE BLADDER

The amount of ACh, ATP and NO was analysed in intra- and extraluminal samples of the whole, isolated mouse bladder after treatment with BoNT/A (2U per bladder) and compared to time matched controls. Successive distensions to 40 mm Hg were carried out at a perfusion rate of 100 µl/min over 2 h as described in 2.2.2 (protocol 2).

The data are shown in pmole/min to compensate for the differences in collection time between basal samples (collection after 10 min) and distension samples (collection was carried out directly after distension, see protocol).

Intraluminal ACh

At the time point of 1 h post BoNT/A application ACh was measured at 57.67 ±9.34 pmol/min in the control group and 96.61 ±19.78 pmol/min in the BoNT/A treated group. No significant difference was observed between the two groups (unpaired Student's T test, n=15, Figure 25 A). At the time point of 2 h post BoNT/A application ACh was measured at 69.23 ±18.11 pmol/min in the control group and at 84.58 ±12.32 pmol/min in the BoNT/A treated group. Again no significant difference was observed between the two groups (unpaired Student's T test, n=15, Figure 25 A).

Before application of BoNT/A (0 h) the measured amount of ACh after distension to 40 mm Hg was 103.50 (±23.95) pmol/min in the control group and 339.60 (±89.76) pmol/min in the BoNT/A group. A significant difference was observed between the two groups (n=15, p=0.017, unpaired Student's T test, Figure 25 A). The results were therefore analysed as proportional change (%Δ release BoNT/A vs control). But again no significant effect was observed between samples of BoNT/A treated bladders and time-matched vehicle controls at 1 h and 2 h post BoNT/A application (unpaired Student's T test with Welch's correction of %change, n=15, Figure 25 B).

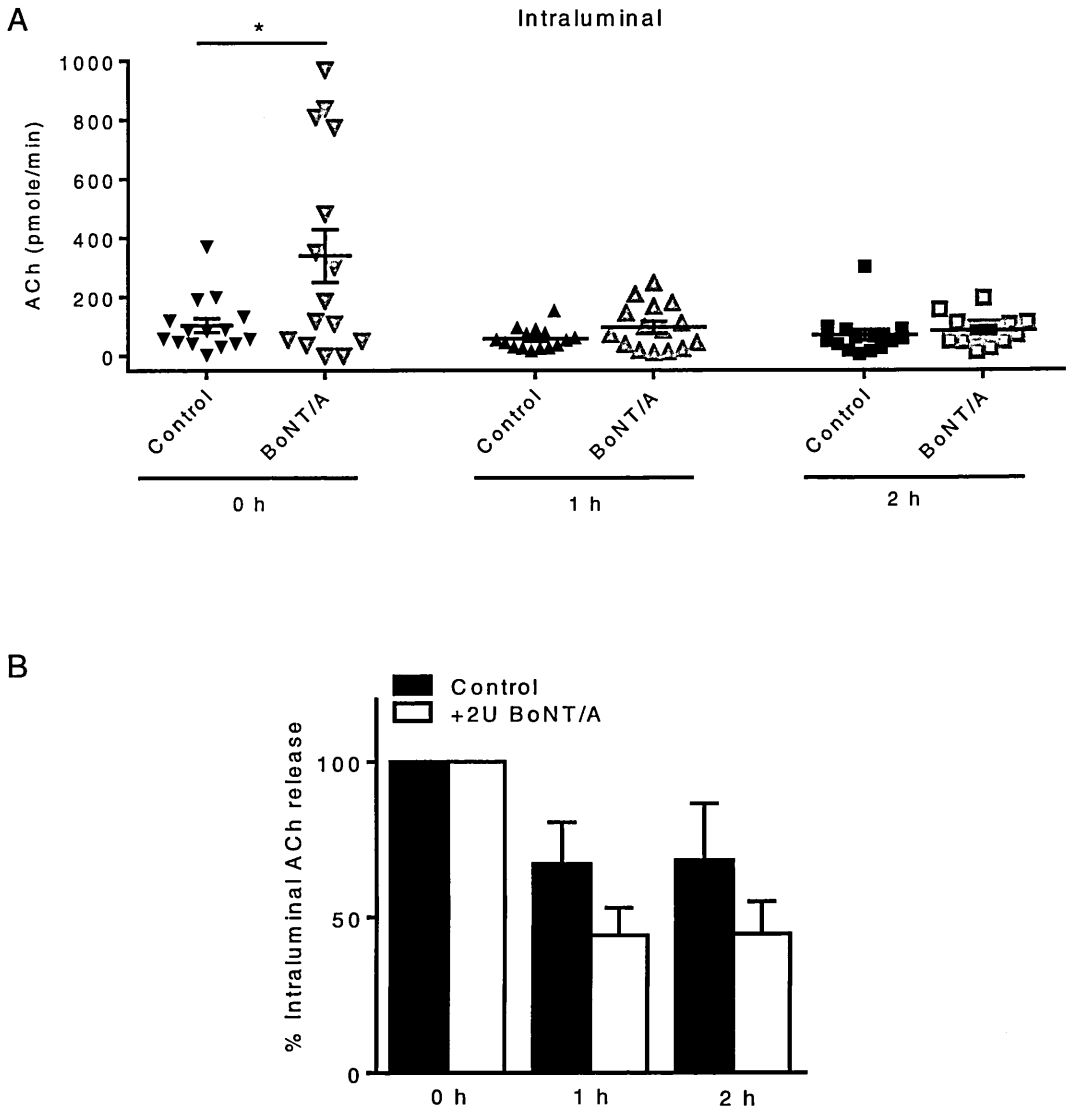


Figure 25 **Intraluminal ACh before application of BoNT/A (0 h) and at 1 h and 2 h post application compared to time matched controls (mean ±SEM). (A) No significant difference observed between control and BoNT/A treated bladders at 1 h and 2 h post application, however before application of BoNT/A the ACh level in the BoNT/A group was significantly higher compared to control group (unpaired Student's T test, n=15, * indicates $p \leq 0.05$). (B) Proportional change of ACh release (%Δ release BoNT/A vs control). No significant effect was observed between samples of BoNT/A treated bladders and time-matched vehicle controls at 1 h and 2 h post BoNT/A treatment (unpaired Student's T test with Welch's correction of %change, n=15).**

Extraluminal ACh

At the time point of 1 h post BoNT/A application extraluminal ACh was measured at 141.41 (±35.46) pmol/min in the control group and 210.25 (±34.49) pmol/min in the BoNT/A treated group. No significant difference was observed between the two groups (unpaired Student's T test, n=15-16, Figure 26 A). At the time point of 2 h post BoNT/A application ACh was measured at 111.10 (±31.08) pmol/min in the control group and at 174.95 (±35.96) pmol/min in the BoNT/A treated group. Again no significant difference was observed between the two groups (unpaired Student's T test, n=15-16, Figure 26 A).

Before application of BoNT/A (0 h) the measured amount of ACh after distension to 40 mm Hg was 173.82 (±27.55) pmol/min in the control group and 287.31 (±40.10) pmol/min in the BoNT/A group. A significant difference was observed between the two groups (n=15-16, p=0.025, unpaired Student's T test, Figure 26 A). The results were therefore analysed as proportional change (%Δ release BoNT/A vs control). But again no significant effect was observed between samples of BoNT/A treated bladders and time-matched vehicle controls at 1 h and 2 h post BoNT/A application (unpaired Student's T test with Welch's correction of %change, n=15-16, Figure 26 B).

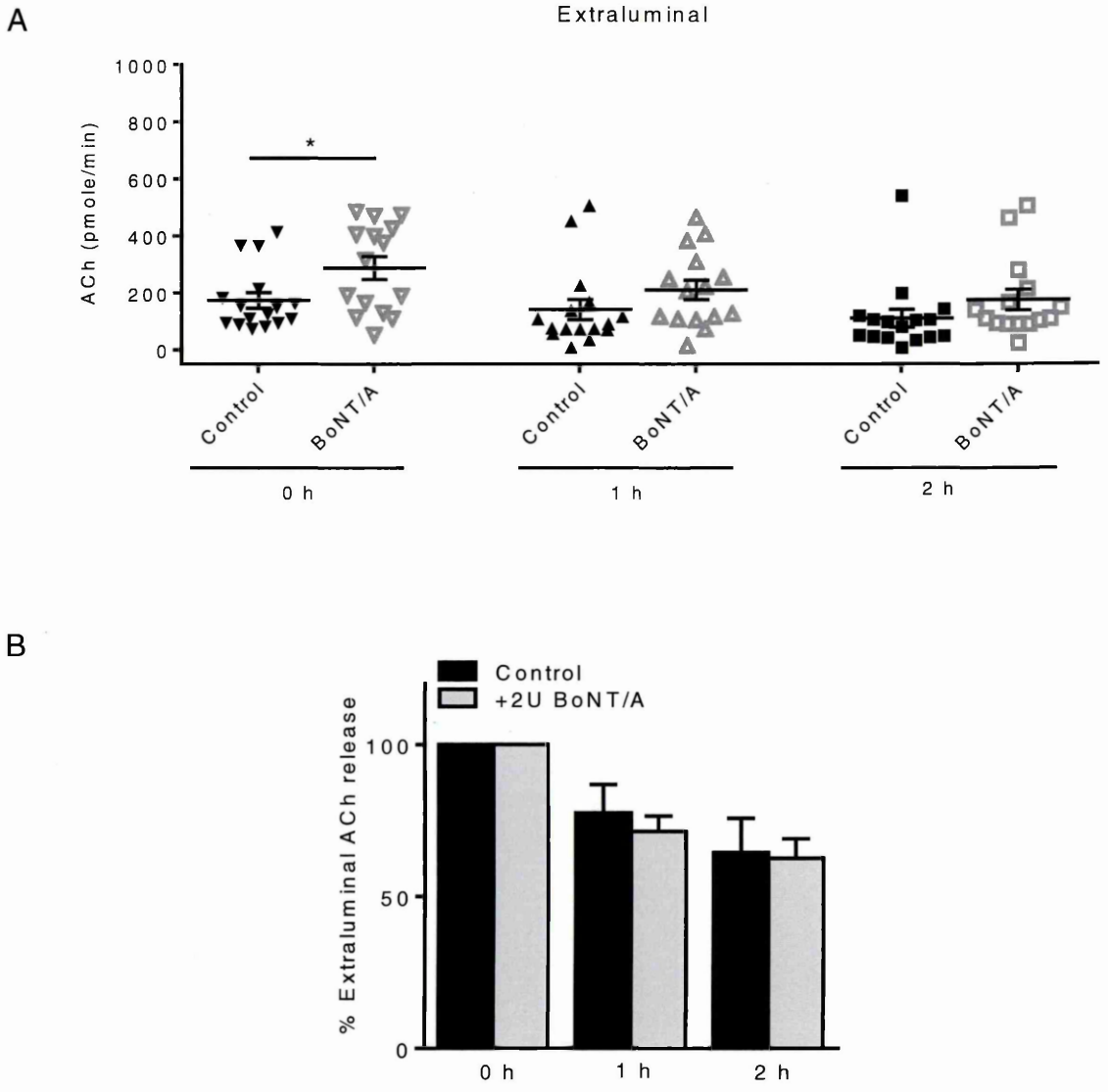


Figure 26 **Extraluminal ACh before application of BoNT/A and at 1 h and 2 h post application compared to time matched controls (mean ±SEM). (A) No significant difference observed between control and BoNT/A treated bladders at 1 h and 2 h post application, however before application of BoNT/A the ACh level in the BoNT/A group was significantly higher compared to control group (unpaired Student's T test, n=15-16, * indicates $p \leq 0.05$). (B) Proportional change of ACh release (%Δ release BoNT/A vs control). No significant effect was observed between samples of BoNT/A treated bladders and time-matched vehicle controls at 1 h and 2 h post BoNT/A treatment (unpaired Student's T test with Welch's correction of %change, n=15-16).**

Intraluminal ATP

The measurement of intraluminal ATP before application of BoNT/A (0 h) was 7.94 (±1.96) pmol/min in the control group and 14.23 (±3.17) pmol/min in the BoNT/A treated group. No significant difference was measured between the two groups (unpaired Student's T test, n=9-13, Figure 27 A).

At the time point of 1 h post BoNT/A application ATP was measured at 6.08 (±1.57) pmol/min in the control group and 7.73 (±2.24) pmol/min in the BoNT/A treated group. No significant difference was observed between the two groups (unpaired Student's T test, n=9-13, Figure 27 A). At the time point of 2 h post BoNT/A application ATP was measured at 8.17 (±2.33) pmol/min in the control group and at 6.35 (±1.93) pmol/min in the BoNT/A treated group. Again no significant difference was observed between the two groups (unpaired Student's T test, n=9-13, Figure 27 A).

The results were also analysed as proportional change (%Δ release BoNT/A vs control). Intraluminal ATP was significantly reduced two hours post BoNT/A application by >30% from 99.94 (±11.70)% to 64.66 (±15.81)% (unpaired Student's T test with Welch's correction of %change, n=9-13, p=0.043, Figure 27 B).

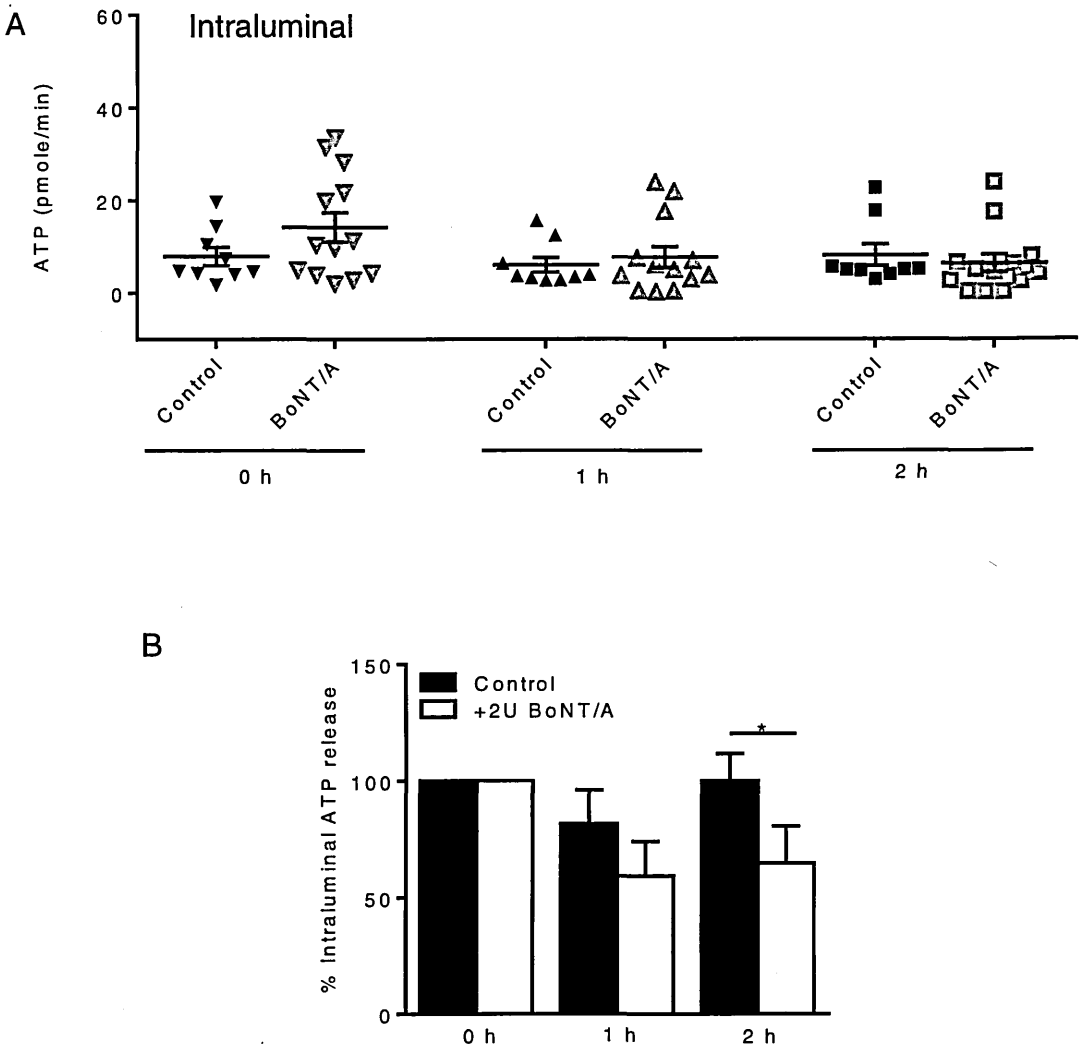


Figure 27 **Intraluminal ATP before application of BoNT/A and at 1 h and 2 h post application compared to time matched controls (mean ±SEM). (A) No significant difference could be observed between control and BoNT/A treated intraluminal samples at basal, 1 h and 2 h post application (unpaired Student's T test, n=9-13). (B) Proportional change of ATP release (%Δ release BoNT/A vs control). No significant effect was observed between samples of BoNT/A treated bladders and time-matched vehicle controls at 1 h post BoNT/A treatment. However at 2 h post BoNT/A treatment the amount of measured ATP was significantly lower in the BoNT/A treated group compared to the time-matched vehicle controls (%Δ release, unpaired Student's T test with Welch's correction of %change, n=9-13, * indicates $p \leq 0.05$).**

Extraluminal ATP

At the time point of 1 h post BoNT/A application extraluminal ATP was measured at 13.06 (±3.61) pmol/min in the control group and 5.75 (±0.37) pmol/min in the BoNT/A treated group. The amount of ATP was significantly lower in the BoNT/A treated group compared to the control (unpaired Student's T test, n=9-11, p=0.038, Figure 28 A). At the time point of 2 h post BoNT/A application ACh was measured at 18.97 (±4.28) pmol/min in the control group and at 6.26 (±0.89) pmol/min in the BoNT/A treated group. Again the amount of ATP was significantly lower in the BoNT/A treated group compared to the control (unpaired Student's T test, n=9-11, p=0.005, Figure 28 A).

Before application of BoNT/A (0 h) the measured amount of ATP after distension to 40 mm Hg was 15.39 (±2.72) pmol/min in the control group and 5.95 (±1.24) pmol/min in the BoNT/A treated group. A significant difference was observed between the two groups (unpaired Student's T test, n=9-11, p=0.003, Figure 28 A). The results were therefore analysed as proportional change (%Δ release BoNT/A vs control). No significant difference was observed between samples of BoNT/A treated bladders and time-matched vehicle controls at 1 h and 2 h post BoNT/A application (unpaired Student's T test with Welch's correction of %change, n=9-13, Figure 28 B).

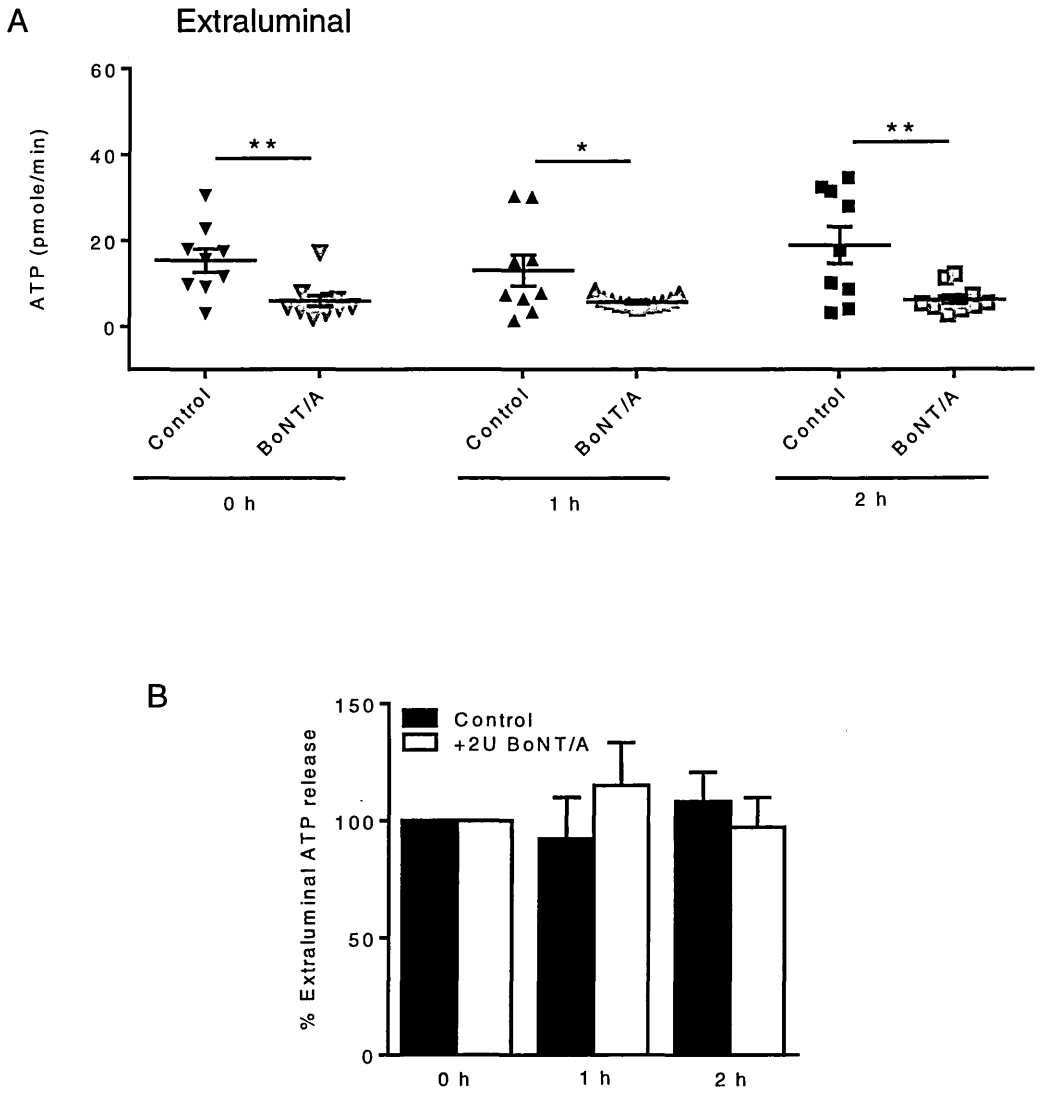


Figure 28 **Extraluminal ATP before application of BoNT/A and at 1 h and 2 h post application compared to time matched controls (mean ±SEM). (A) Before application of BoNT/A the ATP level in the BoNT/A group was significantly lower compared to the time matched controls (n=9-11). The amount of ATP was significantly lower in the BoNT/A treated group compared to controls 1 h post BoNT/A application (n=9-11) and 2 h post BoNT/A application (unpaired Student's T test, n=9-11, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$). (B) Proportional change of ATP release (%Δ release BoNT/A vs control). No significant effect was observed between samples of BoNT/A treated bladders and time-matched vehicle controls at 1 h and 2 h post BoNT/A treatment (unpaired Student's T test with Welch's correction of %change, n=9-13).**

Intraluminal NO

Intraluminal samples from BoNT/A treated bladders and time-matched vehicle control bladders were also analysed for NO. Samples for the NO measurement were taken 1 h after BoNT/A application. NO was found in all eight intraluminal samples from BoNT/A treated bladders and in five out of eight time-matched control samples. The amount of intraluminal NO in control samples was 47.61 (±18.34) pmole/min. In BoNT/A treated samples the amount of NO was significantly greater at 239.70 (±64.81) pmole/min (unpaired Student's T Test, n=8, p=0.013, Figure 29).

Due to restricted assay and sample size, NO was only measured in intraluminal samples 1 h after BoNT/A treatment.

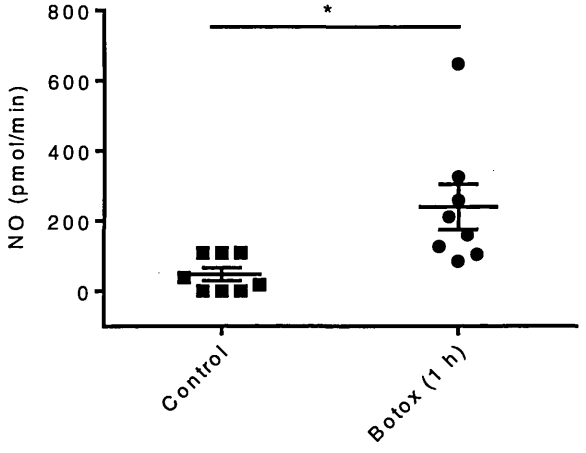


Figure 29 Intraluminally measured NO 1 h post application of BoNT/A compared to time matched controls (mean ±SEM). The amount of NO was significantly greater in the BoNT/A treated group compared to controls (unpaired Student's T test, n=8, * indicates p≤0.05).

2.3.4 EFFECT OF VARYING GRADES OF DISTENSION ON ACH AND ATP IN INTRA- AND EXTRALUMINAL SAMPLES OF THE ISOLATED MURINE BLADDER

Effect of pressure changes from 20 – 60 mm Hg on ACh release

Distensions were performed to initial pressure levels of 20, 30, 40, 50 and 60 mm Hg at a filling rate of 150 µl/min as described in Chapter 2.2.2 (protocol 3). ACh was identified in both intra- and extraluminal samples at all pressure levels. The amount of ACh was not significantly different between the varying grades of distension, neither intra- nor extraluminally (1-way ANOVA with Tukey's post-test, n=5 and n=4 respectively, Figure 30).

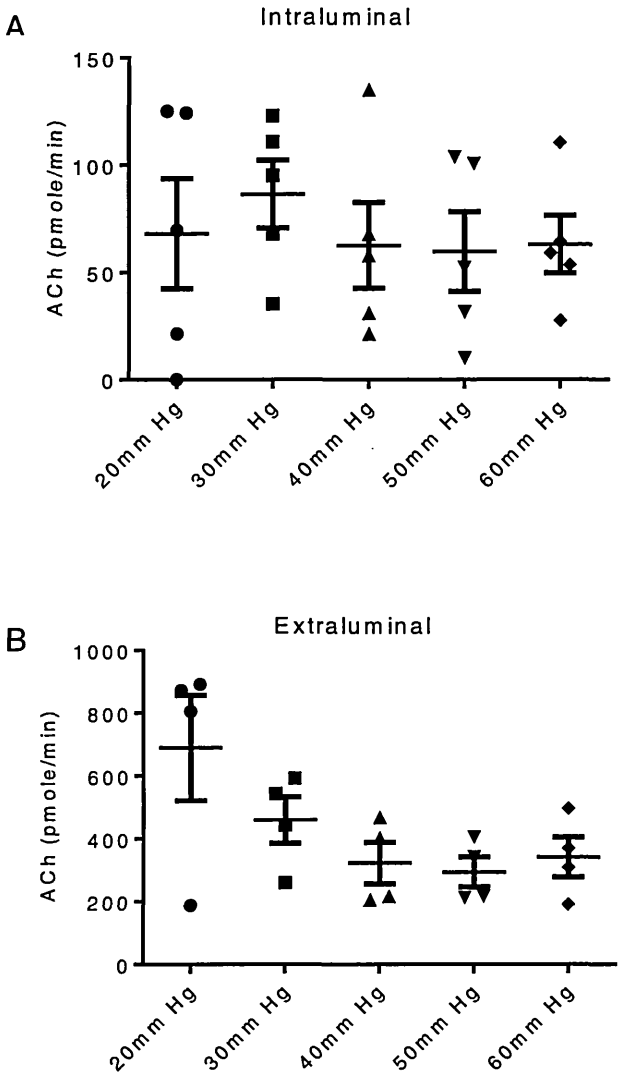


Figure 30 **Intra- and extraluminally measured ACh after graded distensions (at 150 µl/min) from 20 to 60 mm Hg (mean ±SEM). No significant difference observed after application of varying grades of distension, neither intra- nor extraluminal (1-way ANOVA with Tukey's post-test, n=5 and n=4 respectively).**

Effect of pressure changes from 5 – 20 mm Hg on ACh release

Further experiments were carried out to observe the effect of lower pressure levels of 5-20 mm Hg on the intra- and extraluminal release of ACh. Mouse bladders were perfused at 40 µl/min to pressure levels of 5, 10, 15 and 20 mm Hg in a randomised order as described in Chapter 2.2.2 (protocol 3). This protocol allowed a more physiological set-up as the pressure levels and the perfusion rate are set at a lower level. Furthermore the bladders were kept at isovolumetric conditions for ten minutes at each pressure level. Collection of all samples was performed after these 10 minutes followed by five min equilibration. This allowed the comparison of all samples in nM/µl without having to adjust for any differences in collection time. Data were analysed in nM/µl compared to pmole/min in earlier experiments. In addition this set up allowed monitoring spontaneous contractions at the different distension levels over the ten minute time period.

At basal level the amount of intraluminal ACh was measured at 3.95 (±0.65) nM/µl. Basal measurement was significantly lower compared to distension to 5 mm Hg ($p \leq 0.0001$) and to 10 mm Hg ($p \leq 0.05$, n=5/4, 1-way ANOVA with Dunnett's multiple comparison post-test, Figure 31 A).

The amount of extraluminally measured ACh was 6.47 (±1.08) nM/µl at basal level. No significant difference could be seen in extraluminal samples when comparing the amount of ACh at basal level to distension levels at 5, 10, 15 and 20 mm Hg (1-way ANOVA with Dunnett's multiple comparison post-test, n=4-5 Figure 31 B).

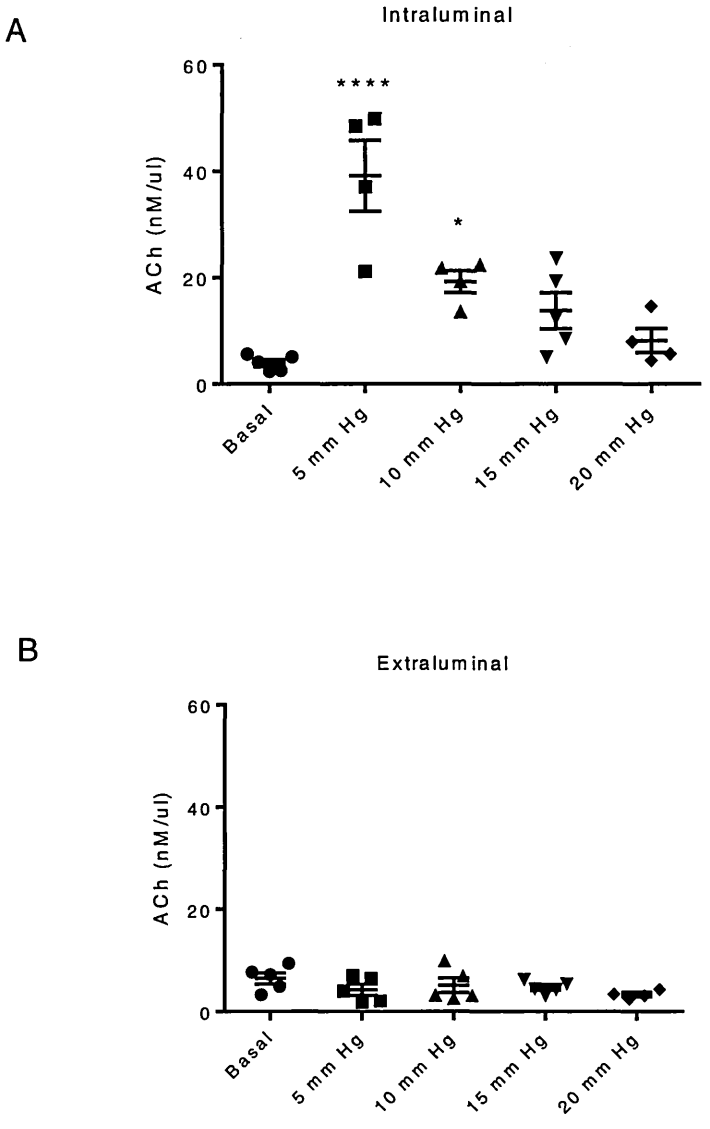


Figure 31 **Intra- and extraluminally identified ACh during basal collection and during distension to 5, 10, 15 and 20 mm Hg (mean ±SEM). (A) Compared to basal measurement (3.95 ±0.65 nM/µl, n=5), a significant increase of ACh could be observed at 5 mm Hg (39.16 ±6.64 nM/µl, n=4) and 10 mm Hg (19.26 ±2.01 nM/µl, n=4) (1-way ANOVA with Dunnett's multiple comparison post-test, * indicates $p \leq 0.05$, **** indicates $p \leq 0.0001$). (B) In extraluminal samples no significant difference could be observed when comparing distension measurements to basal measurements (1-way ANOVA with Dunnett's multiple comparison post-test, n=4-5).**

Effect of pressure changes from 5 – 40 mm Hg on ACh release

ACh measurements have also been carried out at higher distension levels of 20, 30 and 40 mm Hg following the same protocol with a physiological perfusion rate of 40 µl/min. The data have been pooled as %change of the basal measurement with the data already obtained for distension levels to 5, 10, 15 and 20 mm Hg shown in Figure 31.

Increasing the distension levels from 5, 10, 15, 20, 30 to 40 mm Hg in a randomised order showed a significant decrease of intraluminal ACh (1-way ANOVA, n=3-7, $p \leq 0.0001$, Figure 32 A). In extraluminal samples the amount of ACh did not differ significantly between the different distension levels (1-way ANOVA, n=3/5/7, Figure 32 B).

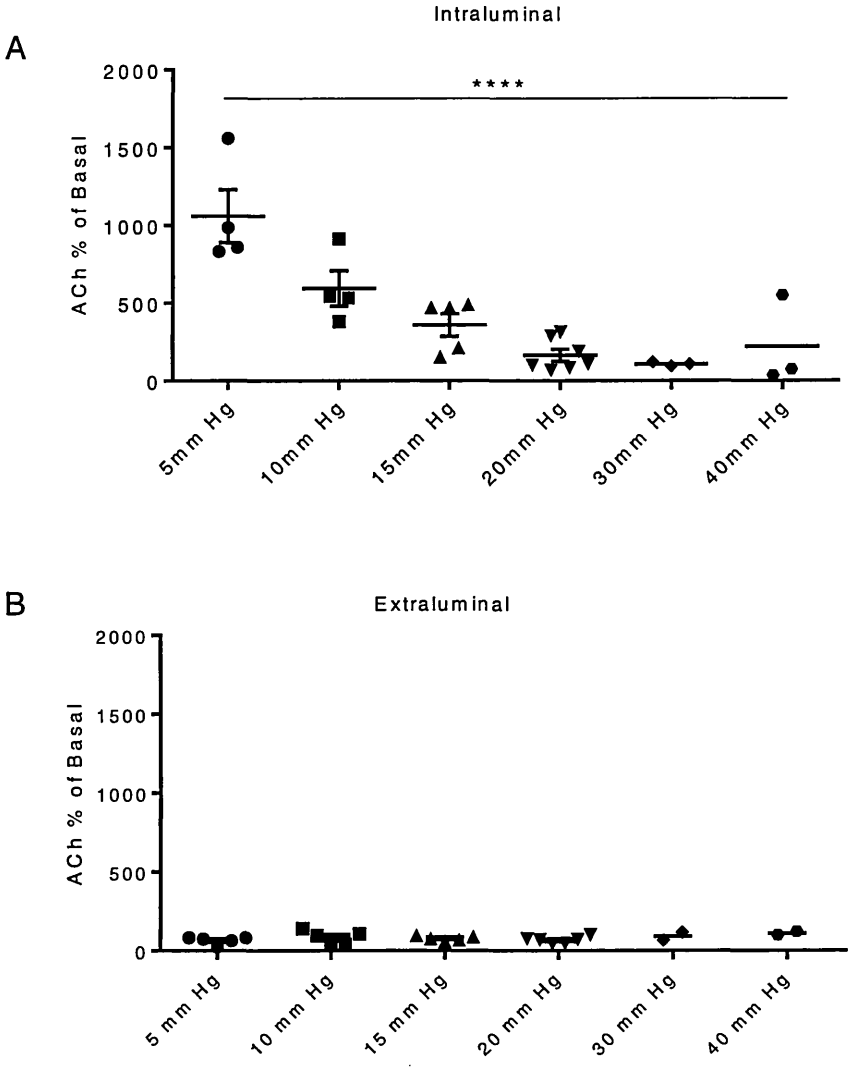


Figure 32 Amount of ACh as % of basal measurement in intra- an extraluminal samples after distensions to 5, 10, 15, 20, 30 and 40 mm Hg (mean ±SEM). (A) A significant decrease of ACh could be observed from 5 mm Hg to 40 mm Hg (1-way ANOVA, n=3-7, $p \leq 0.0001$, ** indicates $p \leq 0.0001$). (B) In extraluminal samples no significant difference could be observed between the different distension levels (1-way ANOVA, n=2-6).**

Effect of pressure changes from 5 – 20 mm Hg on ATP release

ATP levels were measured in intra- and extraluminal samples following the same protocol. Basal measurement of intraluminal ATP was measured at 10.71 (±1.22) pM/µl. The amount of ACh in basal samples was not significantly different compared to the amount in the samples after distension to 5, 10, 15, or 20 mm Hg (1-way ANOVA with Dunnett's multiple comparison post-test, n=4-5, Figure 33 A).

Basal measurement of extraluminal ATP was measured at 12.36 (±6.13) pM/µl. No significant difference could be seen in extraluminal ATP when comparing basal level to distension levels at 5, 10, 15, or 20 mm Hg (1-way ANOVA with Dunnett's multiple comparison post-test, n=4-5, Figure 33 B).

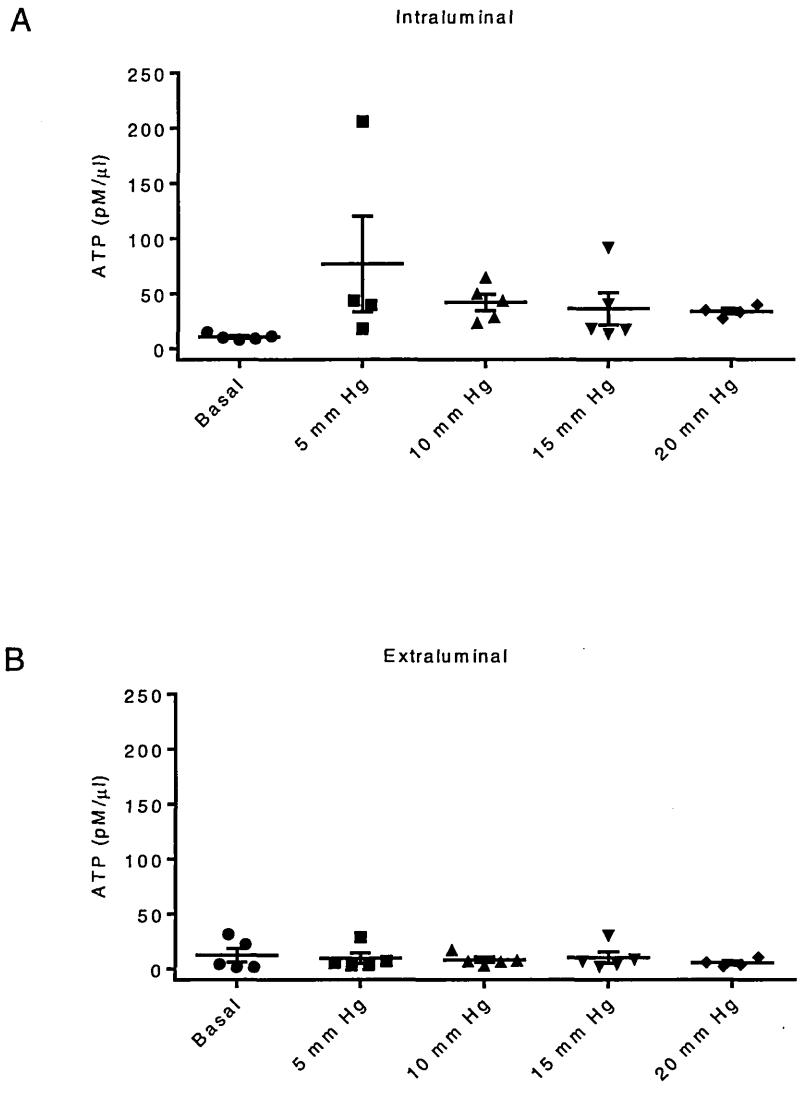


Figure 33 **Intra- and extraluminally measured ATP during basal collection and after distension to 5, 10, 15 and 20 mm Hg (mean ±SEM). (A) Intraluminally measured ATP was not significantly different between basal level and distensions to 5, 10, 15 and 20 mm Hg (1-way ANOVA with Dunnett's multiple comparison post-test, n=4-5). (B) In extraluminal samples no significant difference could be observed when comparing the measurements after distension to basal measurement (1-way ANOVA with Dunnett's multiple comparison post-test, n=4-5).**

Effect of pressure changes from 5 – 40 mm Hg on ATP release

ATP measurements have also been carried out at higher distension levels of 20, 30 and 40 mm Hg following the same protocol with a physiological perfusion rate of 40 µl/min. The data have been pooled as %change of the basal measurement with the data already obtained for distension levels to 5, 10, 15 and 20 mm Hg shown in Figure 33.

Intraluminal ATP did not change significantly when increasing the distension levels from 5, 10, 15, 20, 30 and to 40 mm Hg in a randomised order (1-way ANOVA, n=3-7, Figure 34 A). In extraluminal samples however, increasing distension levels from 5, 10, 15, 20, 30 to 40 mm Hg showed a significant increase of intraluminal ATP (1-way ANOVA, n=3-7, p=0.0063, Figure 34 B).

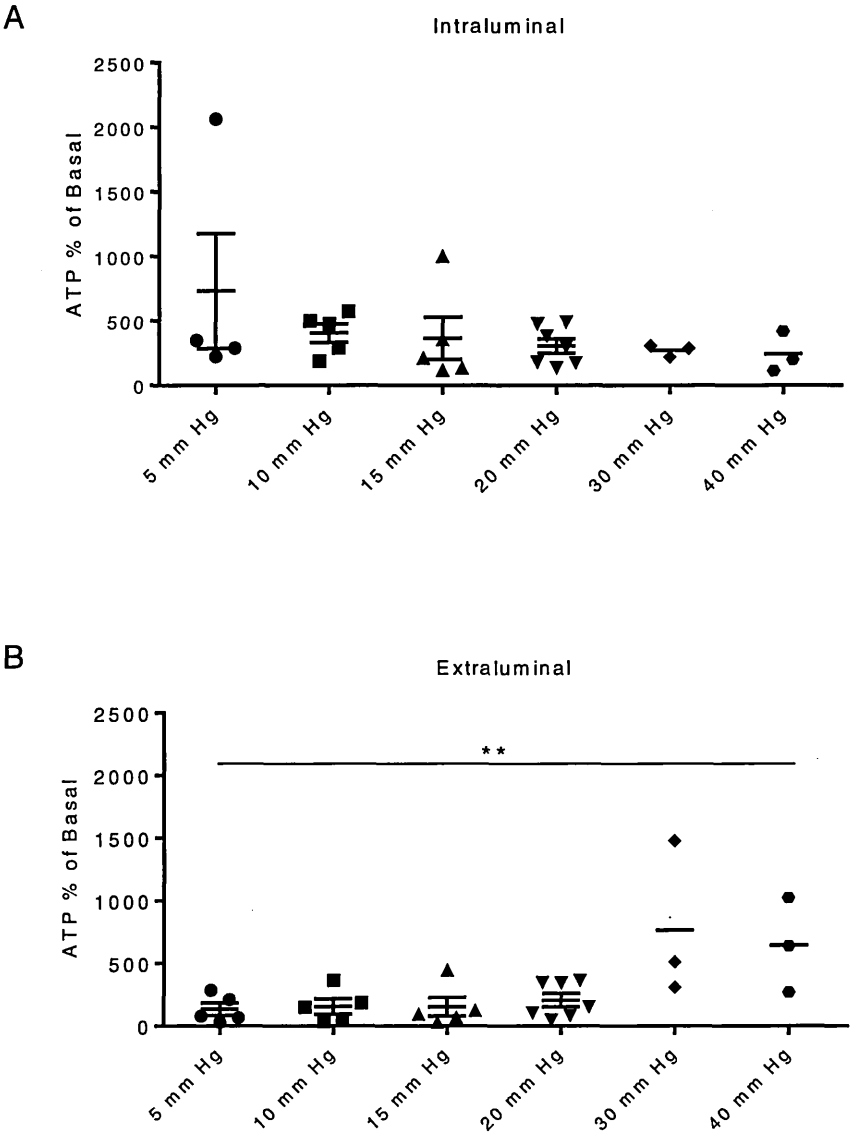


Figure 34 Amount of ATP as % of basal measurement in intra- an extraluminal samples after distension to 5, 10, 15, 20, 30 and 40 mm Hg (mean ±SEM). (A) No significant change of ATP could be observed from 5 mm Hg to 40 mm Hg (1-way ANOVA, n=3-7). (B) In extraluminal samples a significant increase could be observed from 5 to 40 mm Hg (1-way ANOVA, n=2-6, ** indicates $p \leq 0.01$).

Spontaneous activity

Spontaneous activity was measured as small transient rises in pressure. Figure 35 shows an example of traces obtained at different pressure levels. One out of five bladders showed no activity.

A significant rise in the mean amplitude was observed when comparing spontaneous activity at distension levels from 5, 10, 15 to 20 mm Hg (5mm Hg: 3.50 ±0.45 mm Hg, n=4; 10mm Hg: 6.74 ±0.13 mm Hg, n=4; 15mm Hg: 11.25 ±1.30 mm Hg, n=4; 20mm Hg: 18.26 ±1.43 mm Hg; n=3, 1-way ANOVA, $p \leq 0.0001$, Figure 36 A). Conversely no difference could be seen when comparing the frequency of contractions for the different levels of distension (1-way ANOVA, n=3-4, Figure 36 B).

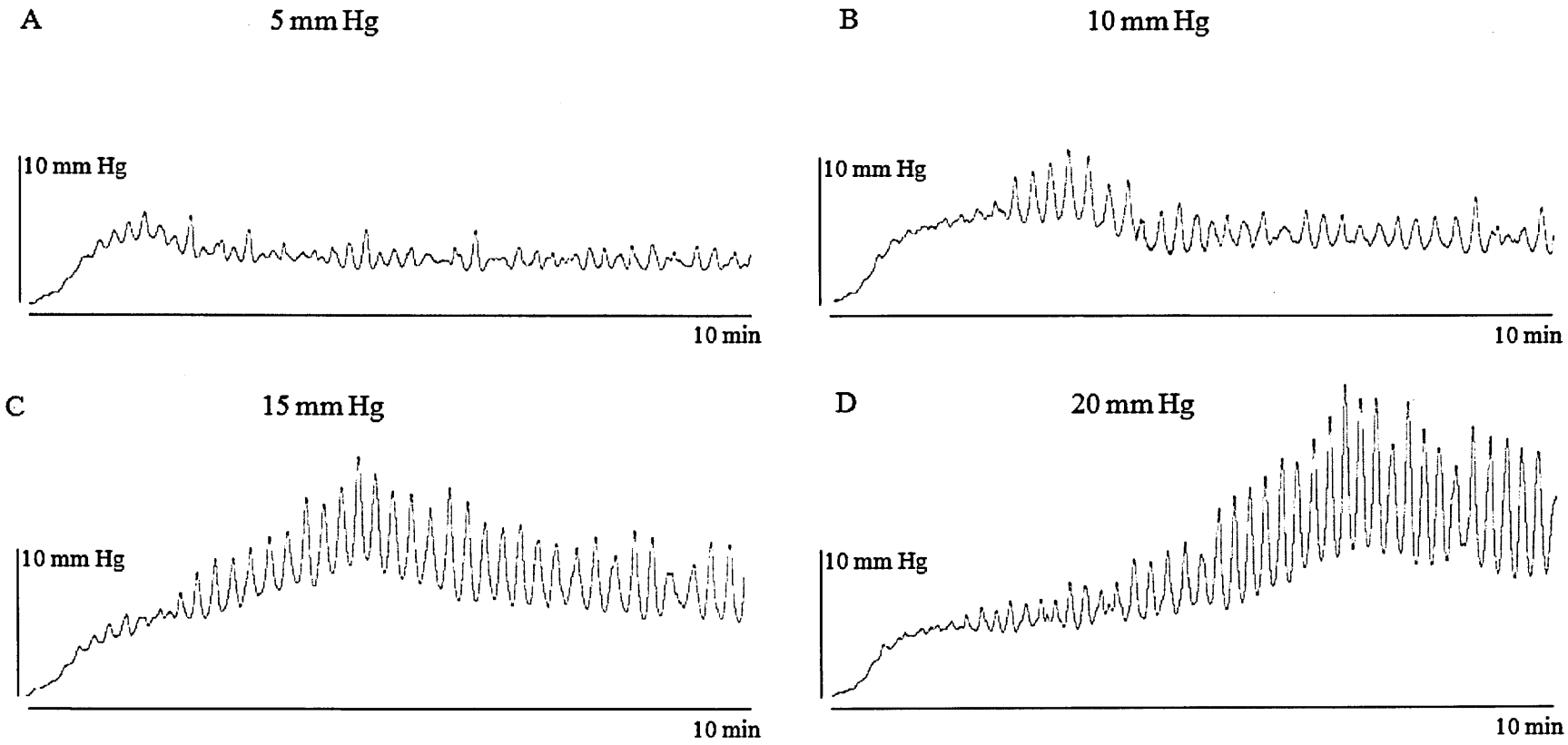


Figure 35 Traces of pressure induced spontaneous activity at distensions to 5mm Hg (A), 10mm Hg (B), 15mm Hg (C) and 20mm Hg (D) over a time frame of 10min.

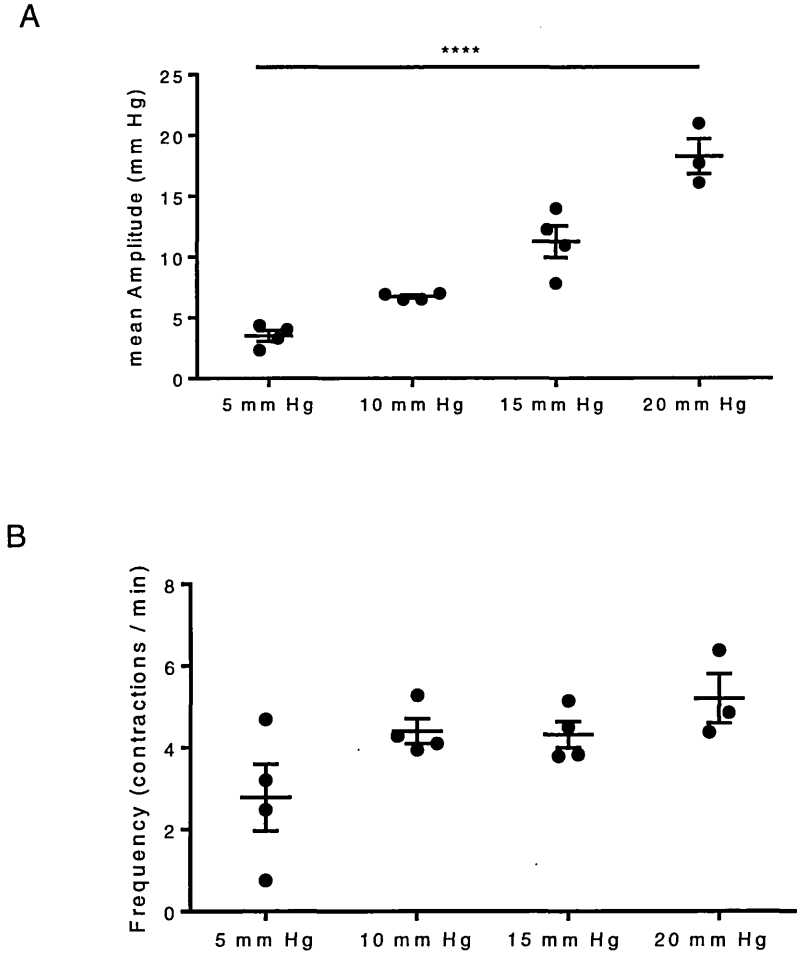


Figure 36 Amplitude and frequency of spontaneous activity in the whole isolated murine bladder (mean ±SEM). (A) Mean Amplitude was significantly increased with rising pressure (applied in randomised order) from 5, 10, 15 to 20 mm Hg (1-way ANOVA, n=3-4, ** indicates $p \leq 0.0001$). (B) However frequency of spontaneous activity did not significantly change from 5 to 20 mm Hg (1-way ANOVA, n=3-4).**

2.3.5 EFFECT OF HEMICHOLINIUM-3, DECYNIUM 22, MLA AND FESOTERODINE ON ACH RELEASE AND COMPLIANCE

Experiments were carried out to explore the cholinergic pathway in the urothelium further. Hemicholinium-3 was used to block CHT1 to prevent the reuptake of choline, decynium 22 was used to inhibit OCT3 and Methyllycaconitine (MLA) was used to block α7 nicotinic receptor.

Intraluminal ACh was analysed and compared to control measurements after intravesicular application of either 5 nM hemicholinium-3, 1 μM decynium 22 or 10 nM MLA respectively at basal level and at 5, 10, 15 and 20 mm Hg distension following the protocol described in 2.2.2. After application of the drugs, the bladders contracted to such an extent that it was not always possible to collect a sufficient sample size to run the ACh assay. Consequently the n numbers are highly varied.

At basal level a significantly lower amount of intraluminal ACh was detected after application of all three drugs compared to control measurement (control: 3.95 ±0.65nM/μl, n=5; hemicholinium-3: 0.13 ±0.05nM/μl, n=6, $p \leq 0.0001$; decynium 22: 0.80 ±0.54 nM/μl, n=5, $p \leq 0.001$; MLA: 0.49 ±0.14 nM/μl, n=6, $p \leq 0.0001$; 1-way ANOVA with Dunnett's multiple comparisons test, Figure 37).

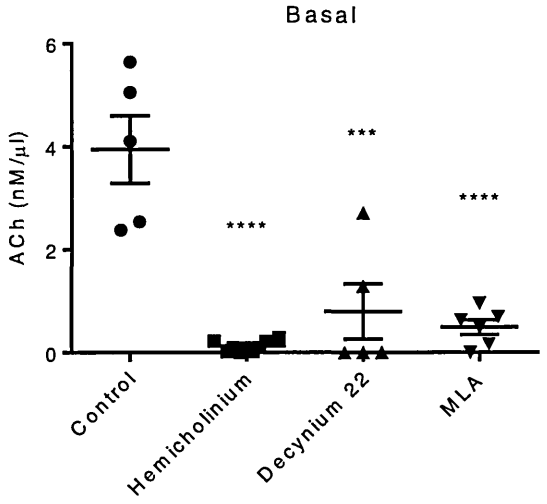


Figure 37 Intraluminal amount of ACh in basal samples of control and after application of hemicholinium-3, decynium 22 and MLA (mean ±SEM). At basal level a significant decrease of intraluminal ACh was seen after application of hemicholinium-3, decynium 22 and MLA compared to control (1-way ANOVA with Dunnett's multiple comparisons test, n=5-6, * indicates p≤0.001, **** indicates p≤0.0001).**

A similar pattern could be shown after distending the bladders to 5 mm Hg. Application of either hemicholinium-3 or MLA significantly reduced the level of ACh in the intraluminal samples compared to control measurement (control: 39.16 ±6.64 nM/µl, n=4; hemicholinium-3: 0.26 ±0.26 nM/µl, n=5, p=0.0003; MLA: 4.12 nM/µl, n=2; 1-way ANOVA with Dunnett's multiple comparisons test, Figure 38 A). The sample volume available after application of decynium 22 has not been sufficient to carry out the ACh assay as the bladder was highly contracted.

At distension to 10, 15 and 20 mm Hg no significant difference could be seen in the amount of intraluminal ACh after application of the drugs compared to control measurement (1-way ANOVA with Dunnett's multiple comparisons test, Figure 38 B, C, D).

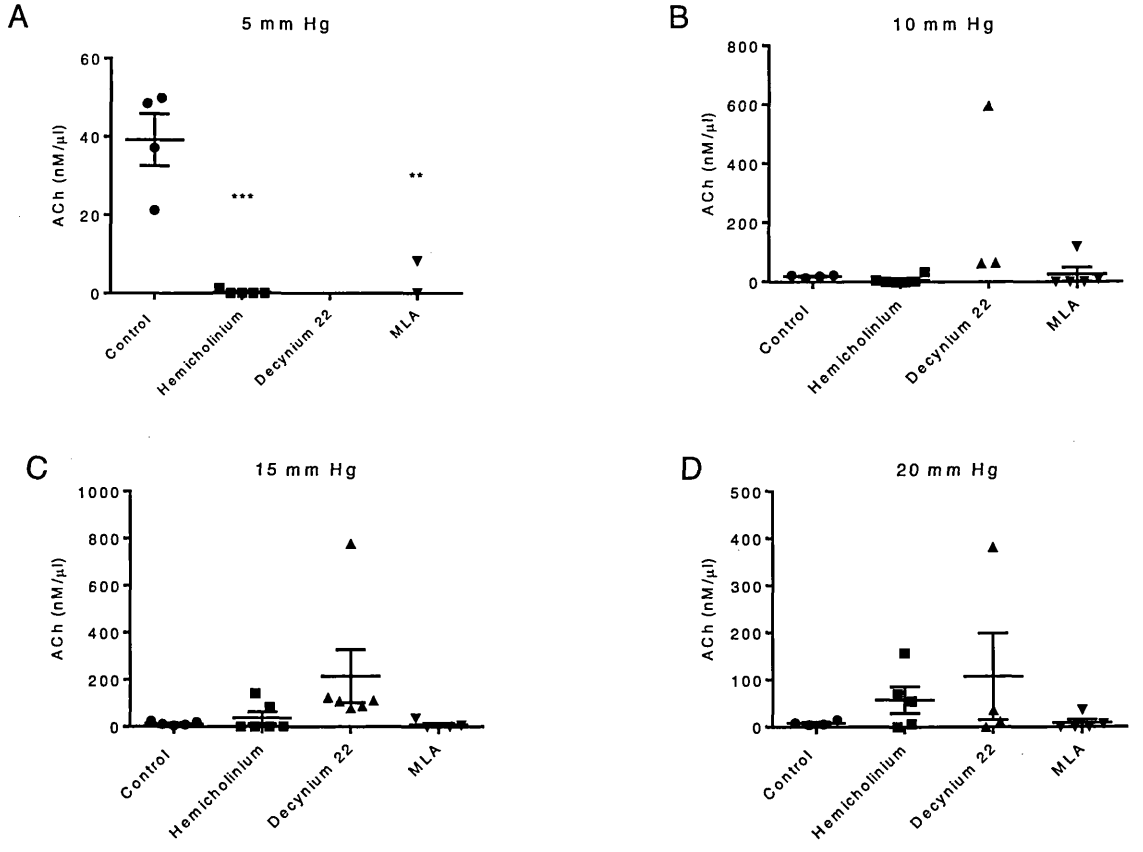


Figure 38 **Intraluminal ACh after application of hemicholinium-3, decynium 22 and MLA compared to control measurements (mean ±SEM). (A) A significant decrease of intraluminal ACh was seen at distension to 5 mm Hg after application of hemicholinium-3 and MLA compared to control (1-way ANOVA with Dunnett's multiple comparisons test, n=2-5, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$). (B, C, D) No significant change could be seen after distension to 10, 15 and 20 mm Hg compared to control measurements (1-way ANOVA with Dunnett's multiple comparisons test).**

Compliance

Compliance was analysed for all data recorded (n=5-6). A significant decrease in compliance (ΔV/ΔP) was found after application of hemicholinium-3, decynium 22 and MLA at all distension levels compared to control measurement (1-way ANOVA with Dunnett's multiple comparisons test). Results are shown in Table 2 and in Figure 39.

	Control	Hemicholinium-3	Decynium 22	MLA
5 mm Hg	14.77 ±4.30 n=5	3.36 ±1.19 n=6 p≤0.01	1.63 ±0.23 n=6 p≤0.001	1.53 ±0.30 n=6 p≤0.001
10 mm Hg	11.07 ±1.35 n=5	3.99 ±1.45 n=6 p≤0.001	0.97 ±0.23 n=4 p≤0.0001	1.12 ±0.14 n=6 p≤0.0001
15 mm Hg	11.92 ±1.68 n=5	3.10 ±0.89 n=6 p≤0.0001	1.37 ±0.29 n=6 p≤0.0001	1.04 ±0.16 n=6 p≤0.0001
20 mm Hg	11.83 ±1.46 n=4	3.51 ±0.96 n=6 p≤0.0001	0.81 ±0.13 n=6 p≤0.0001	1.37 ±0.30 n=6 p≤0.0001

Table 2 Compliance (ΔV/ΔP) at different distension levels after application of hemicholinium-3, decynium 22 and MLA compared to control measurements (mean ±SEM). After application of all three drugs, a significant decrease in compliance could be seen at all distension levels.

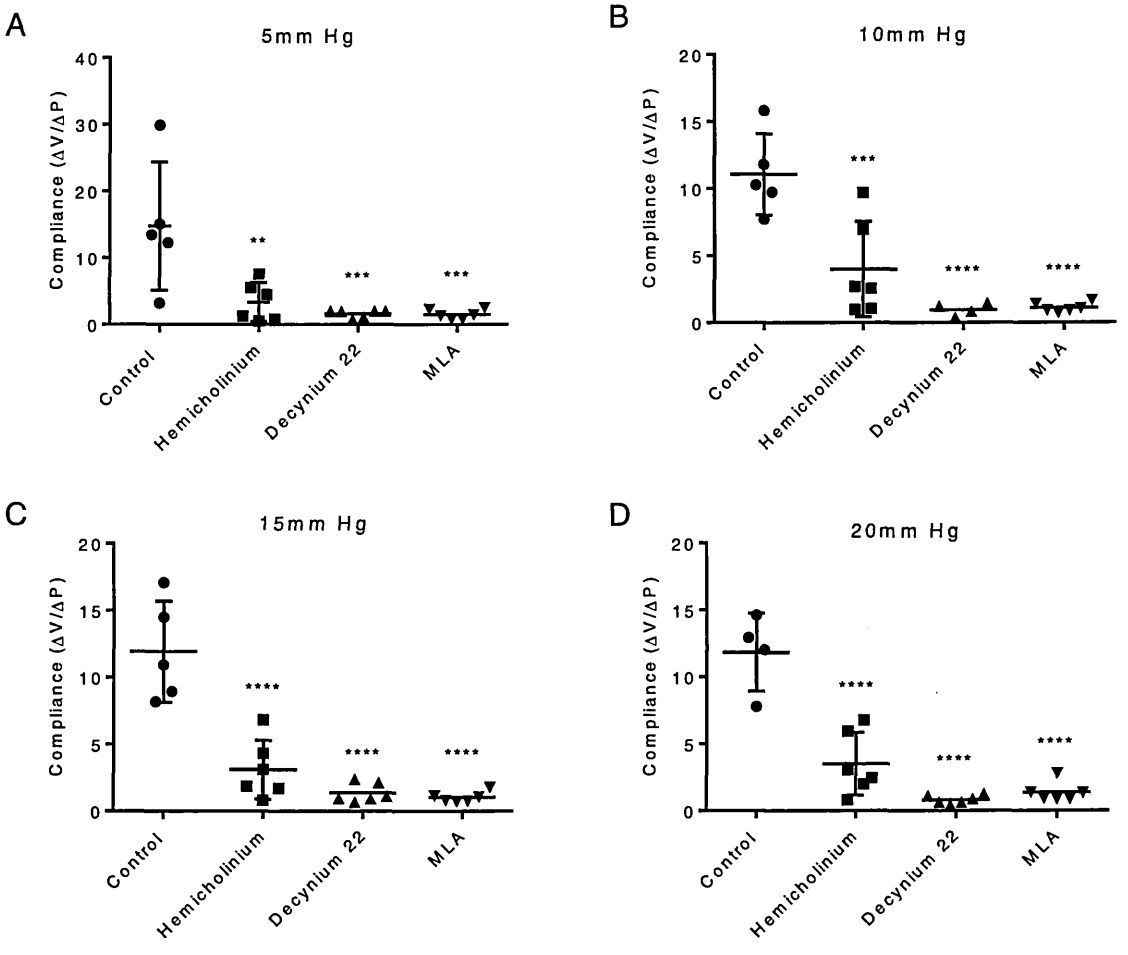


Figure 39 Change in compliance (ΔV/ΔP) after application of hemicholinium-3, decynium 22 and MLA at distension to 5, 10, 15 and 20 mm Hg compared to controls (mean ±SEM). At all four distension levels compliance was reduced after application of the drugs compared to control (1-way ANOVA with Dunnett's multiple comparisons test, n=4-6, ** indicates $p \leq 0.01$, * indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$).**

Fesoterodine

Distensions of the bladder at 100 µl/min to 50 mm Hg at ten minute intervals was carried out with the application of 10 µM fesoterodine as described in Chapter 2.2.2 (protocol 5). The protocol was chosen to be able to compare the results with other experiments using nerve stimulation that were carried out at the same time by colleagues. Samples were taken before and 30 minutes after application of fesoterodine. The amount of ACh and ATP was measured in intra- and extraluminal samples. Furthermore the bladder compliance ($\Delta V/\Delta P$) was analysed.

The content of ACh in intraluminal samples did not differ significantly between the control samples (84.28 ±17.83 pmol/min) and the fesoterodine samples (75.08 ±13.15 pmol/min, paired Student's T test, n=6, Figure 40 A). No significant difference could be seen in extraluminal samples between the control group (114.30 ±37.01 pmol/min) and the fesoterodine group (83.97 ±26.46 pmol/min, paired Student's T test, n=6, Figure 40 B).

The content of intraluminal ATP did not differ significantly between the control samples (0.69 ±0.04 pmol/min) and the fesoterodine samples (0.65 ±0.07 pmol/min, paired Student's T test, n=6, Figure 41 A). No significant difference could be seen in extraluminal samples between the control group (0.41 ±0.07 pmol/min) and the fesoterodine group (0.42 ±0.05 pmol/min, paired Student's T test, n=6, Figure 41 B).

In addition bladder compliance was analysed before and 10, 20 and 30 minutes post application of fesoterodine. No difference could be observed (1-way ANOVA with Tukey's post-test, n=6, Figure 42).

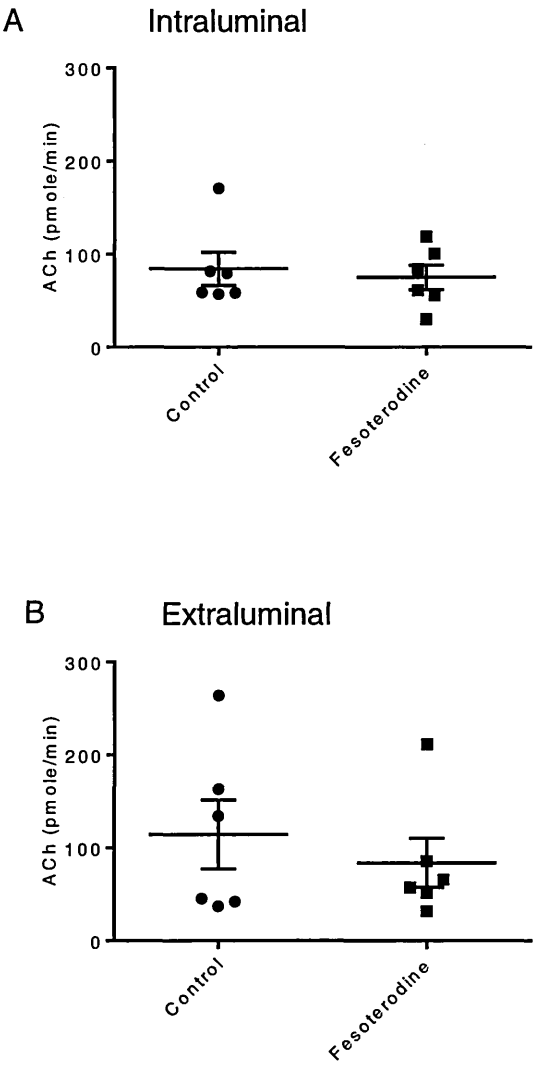


Figure 40 ACh in intra- and extraluminal samples before and after application of 10 µM fesoterodine (mean ±SEM). (A and B) Fesoterodine did not affect the amount of detected ACh in intra- and extraluminal samples (paired Student's T test, n=6).

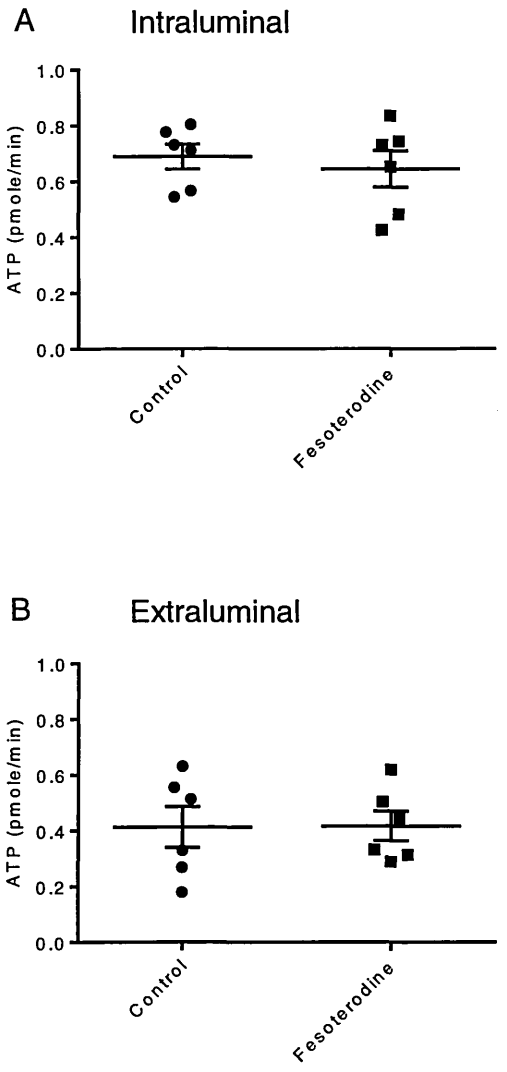


Figure 41 ATP in intra- and extraluminal samples before and after application of 10 µM fesoterodine (mean ±SEM). (A and B) Fesoterodine did not affect the amount of detected ATP in intra- and extraluminal samples (paired Student's T test, n=6).

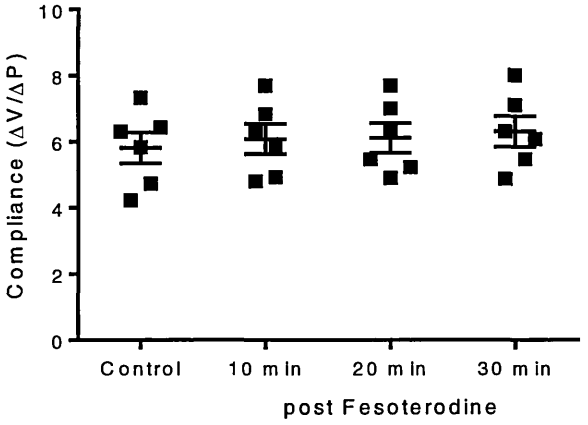


Figure 42 Compliance (ΔV/ΔP) before and at 10, 20 and 30 minutes after application of fesoterodine (mean ±SEM). No significant difference observed (1-way ANOVA with Tukey's post-test, n=6).

2.4 DISCUSSION

This chapter further examined the role of different mediators in the mechanosensation of the whole, isolated murine bladder under varying conditions. The purpose-built and optimised micro organ bath successfully provided samples of intraluminal and extraluminal fluids that were analysed for ACh, ATP and NO. The measurements are showing a steady-state situation of release, break-down and uptake of the quantified mediators.

2.4.1 DISTENSION CAUSED A SIGNIFICANT INCREASE IN THE AMOUNT OF ACH IN INTRA- AND EXTRALUMINAL SAMPLES

The first experiment described in this chapter was performed in order to verify if reproducible data can be obtained with the used set-up and if distension of the whole, isolated bladder has an effect on the release of ACh.

The described method of intra- as well as extraluminal sample collection on dry ice using a purpose build micro organ bath has been shown to generate reproducible data. Ten successive distensions up to 50mm Hg showed a stable response of intra- and extraluminal ACh release to distension. These results suggest that the tissue function is retained over the given 2 h time period.

Basal sample collection was performed by consistently perfusing the bladder with buffered saline. Even though the bladder is scarcely distended at this point, urothelial cells might be slightly stimulated by the flow of saline passing slowly through the bladder.

The distension to 50 mm Hg evoked a significant increase of ACh in intra- and extraluminal samples compared to basal measurement (Figure 23). These results are consistent with publications by Yoshida *et al.* (2006), showing stretch-evoked, non-neuronal ACh release from human bladder strips and Hanna-Mitchell *et al.* (2007) showing a significant increase in radiolabelled ACh release from murine urothelial cells after mechanical stimulation by cellular swelling.

Data are presented in pmole/min in Figure 23. The samples were taken directly after the predetermined pressure had been reached. The time point for reaching this pressure varied, due to natural variation in bladder size and compliance. Consequently it was important to adjust for time differences as the amount of released mediators might be dependent on the period of distension.

Basal ACh in intraluminal samples was found to be around 14 times lower than basal ACh in extraluminal samples (IL: 11 pmole/min to EL: 158 pmole/min, Figure 23). However, after distending to 50 mm Hg, intraluminal ACh was only around 5 times lower than extraluminal ACh (IL: 90 pmole/min, EL: 473 pmole/min, Figure 23). The rise in intraluminal ACh after distension was unequally higher compared to extraluminal ACh and suggests that the urothelium plays an important role in the mechanotransduction of distensions. The cell populations found in the urothelium and the detrusor muscle seemed to respond differently to the onset of stretch and pressure change.

Numerous studies have shown the release of mediators by the urothelium (Ferguson *et al.*, 1997, Birder *et al.*, 1998, Moon 2002, Birder 2002, Yoshida *et al.*, 2004, Yoshida *et al.*, 2006, Munoz 2011). However, it is difficult to compare the quantity of urothelial released neurotransmitter between different studies, as no uniform unit exists for transmitter release. Transmitter release is expressed per gram of tissue weight, per minute, per µl or as percentage increase from control or from basal release. Furthermore various animal models and experimental set-ups have been used in the different studies.

2.4.2 BOTULINUM TOXIN HAS NO EFFECT ON ACH, BUT DECREASES ATP AND INCREASES NO IN INTRALUMINAL SAMPLES

ACh

It is assumed that the source of intraluminal ACh is the urothelium, however it could also derive from ICs or nerve endings that can be found in close proximity to the urothelium. There is no known study demonstrating the release of ACh by ICs and it has been shown that ICs are rarely seen in close proximity to cholinergic nerves in the mouse bladder

(Lagou *et al.,* 2006). This indicates that ICs are not likely to be involved in the cholinergic transmission. To determine if intraluminally detected ACh is released by nerve endings, samples were taken after intraluminal application of 2 units/bladder BoNT/A. This toxin blocks the vesicular release of ACh from neurons. As previously stated, evidence exists that urothelial cells do not release ACh in a vesicular way in murine and human urothelium (Hanna-Mitchell *et al.,* 2007, Lips *et al.,* 2007). Urothelial ACh release should therefore not be influenced by BoNT/A.

TTX is generally used to block ACh release from neurons in scientific experiments. However it has been shown that spontaneous TTX-resistant release of ACh from autonomic nerves occurred in guinea pig and rat bladder strips (Zagorodnyuk 2009). Therefore, BoNT/A application was preferably used to block vesicular release of ACh from neurons in this study.

The amount of ACh in intraluminal samples did not change significantly after application of BoNT/A at time points of 1 h and 2 h post application compared to time matched controls. These findings confirm the hypothesis that intraluminally detected ACh is not released by neurons, but most likely by the urothelium. Supporting evidence comes from McLatchie *et al.* (2014) who showed that BoNT/A had no effect on non-neuronal ACh release from guinea pig urothelial cells. The data in this thesis also validate the suggestion that ACh is not stored and released via vesicles in urothelial cells as already proposed by Hanna-Mitchell *et al.* (2007) and Lips *et al.* (2007).

Extraluminally detected ACh was also not influenced by BoNT/A treatment. BoNT/A was instilled intraluminally and therefore an effect on extraluminal ACh was not expected.

ATP

To further investigate the mechanism of action of BoNT/A in the bladder urothelium, the effect of BoNT/A on ATP release was measured. In contrast to ACh, ATP has been demonstrated to be released in vesicles from urothelial cells (Birder *et al.,* 2003, Wang *et al.,* 2005, Hanna-Mitchell *et al.,* 2007). It was therefore expected that release was blocked by application of BoNT/A.

There was no difference between control samples and samples treated with BoNT/A 1 h post drug application. However 2 h post BoNT/A treatment intraluminally detected ATP was decreased in the treated bladders compared to control bladders. This is in line with a study by Smith *et al.* (2008) showing that BoNT/A treatment significantly reduced evoked ATP release in bladders of spinal cord injured rats.

The release mechanism of ATP in the urothelium is still questionable. Besides the studies showing vesicular release of ATP in the urothelium (Birder *et al.,* 2003, Wang *et al.,* 2005, Hanna-Mitchell *et al.,* 2007), evidence exists that ATP is also released via pannexin channels in the urothelium (Negoro *et al.,* 2014, Timoteo *et al.,* 2014). Additionally, Sui *et al.* (2014) showed that there are two further mechanisms of ATP release in urothelial cells, uridine triphosphate (UTP)-mediated calcium-dependent ATP release and carbachol-mediated calcium-independent ATP release. These further ATP release mechanisms might explain the remaining amount of ATP found in the intraluminal samples after BoNT/A treatment in the above experiments.

A body of literature suggests that various mediators in the urothelium influence each other (Lagou *et al.,* 2006, Hanna-Mitchell *et al.,* 2007, Kullmann *et al.,* 2008, Young *et al.,* 2012, see 1.2.5). The change in ATP 2 h post BoNT/A treatment could therefore also be the secondary effect of BoNT/A changing the release of other vesicularly released mediators such as serotonin or noradrenaline.

BoNT/A had no effect on extraluminal ATP in the above experiment. As BoNT/A was instilled intraluminally, an effect on extraluminal ATP was not expected.

NO

To further investigate the mechanism of action of BoNT/A in the bladder urothelium, the effect of BoNT/A on NO release was measured. NO is a gaseous substance and it is assumed that it therefore cannot be stored in or be released via vesicles. This would mean that neither storage nor release of NO is vesicular in the urothelium and that NO should not be affected by BoNT/A treatment.

In the present study, small amounts of NO could be detected in intraluminal control samples after distension. After treatment with BoNT/A the amount of NO in the intraluminal samples increased significantly by approximately five fold upon the time-matched control measurement. The source of NO could be ICs in the suburothelial cell layers or urothelial cells. BoNT/A is unlikely to have acted directly on the release of NO. Instead, the release of other mediators might have changed due to the inhibition of vesicular release and resulted in a signalling cascade that eventually caused the increase in NO.

Injection of BoNT/A into the bladder wall has become a promising treatment option for NDO and OAB in recent years (see 1.5.4). It was believed that BoNT/A blocks ACh release at the neuromuscular junctions and therefore inhibits detrusor muscle contraction. However evidence exist that BoNT/A instead acts on the afferent and efferent system (Ikeda 2012). The mechanism of BoNT/A was further investigated by colleagues and it could be shown that BoNT/A in clinically effective doses attenuates afferent nerves involved in micturition and pain sensation. Intraluminal perfusion of BoNT/A during distensions resulted in a graded inhibition of firing, while no effect could be seen on bladder compliance suggesting that the effect is not secondary to altered muscle tone and compliance. It can therefore be suggested that BoNT/A acts on the sensory transduction in the bladder with little direct effect on the detrusor neuromuscular junctions (Collins *et al.,* 2013).

It has been hypothesized in previous studies, that the balance of inhibitory and excitatory mediators released from the urothelium modulates afferent activity (Smith *et al.,* 2008). It was shown in this present study, that application of BoNT/A alters this balance, decreasing the excitatory mediator ATP and increasing the inhibitory mediator NO. Regarding the

results from colleagues published in Collins *et al.* (2013), it can be hypothesised that this change altered neuronal function and caused decreased mechanosensation.

BoNT/A is injected into the bladder wall of patients as it has been shown to have greater efficacy than intraluminal application (Smith *et al.,* 2008). Therefore it is possible that the drug did not work in the same way as in clinically applied situations. Further mediator release studies should be conducted with BoNT/A injections into the wall of the whole mouse bladder, ideally in the living animal.

2.4.3 ACH RELEASE IN PROPORTION TO THE EXTENT OF DISTENSION

To test the hypothesis that the amount of released ACh is proportional to the extent of distension, the isolated bladders were distended to varying pressure levels and the amount of ACh analysed in intra- and extraluminal samples.

Graded distensions from 20 to 60 mm Hg at a perfusion rate of 150 µl/min did not show a significant change in intra- or extraluminal ACh release in this study. Contradicting results by Yoshida *et al.* (2006) show, that release of non-nerve evoked ACh increases linear when applying stretch from 0 to 40 mN to human bladder strips. The difference might be due to the models used, bladder strips versus whole, isolated bladder. It could also be due to species specific differences in mouse and human bladder.

The pressure levels and perfusion rates used in this study have been used to examine the mechanosensitive effect on ACh release in the bladder at very high mechanical stimulation. They are however supraphysiological for the mouse bladder. The values were chosen to compare the findings to work by colleagues of the Marie Curie project. The protocol is well established in the laboratory to conduct afferent recordings, as using these high pressure levels ensures that all nerves are excited. The protocol was therefore amended to a more physiological set up.

The voiding contraction in the mature mouse sets in at around 30 cm of water, which can be compared to 22 mm Hg (Smith *et al.,* 2012). Therefore, lower pressure levels of 5, 10, 15 and 20 mm Hg have also been investigated. Moreover the used perfusion rate of 150 µl/min

is supraphysiological for the mouse, as the mean inter-void interval in the mature mouse is approximately 200 s, and the mean void volume 0.1 ml (Smith *et al.,* 2012). This would compare to a perfusion rate of 30 µl/min. Experiments would take relatively long at this perfusion rate and the tissue might deteriorate, therefore the perfusion rate was set slightly higher for these experiment, at 40 µl/ min.

The time point of sample collection might also be important, as earlier distensions to different pressure levels could influence the transmitter release. Therefore the distensions to different pressure levels were randomised (see protocol 2.2.2, Figure 13). The bladders were also left distended at the predetermined pressure for exactly ten minutes before collecting the intra- and extraluminal samples to eliminate the necessary adjustment for time. Leaving the bladder distended at the predetermined pressure for a set period of time before collecting the samples will also provide a result closer to the steady state situation occurring in the physiologically filling bladder.

The obtained results show that with a perfusion rate of 40 µl/ min, intraluminal ACh is released at about 3 nM/ µl at resting level (basal). After applying a pressure change of only 5 mm Hg, the release of ACh significantly rose to around 40 nM/ µl, more than tenfold higher than at basal level (Figure 31). It can therefore be assumed that ACh release is very sensitively tuned and a slight change in pressure already activates the release mechanism. These results are consistent with a recent publication suggesting that urothelial release of ACh has a small dynamic range and might follow an 'all or nothing' rule, communicating the occurrence of stretch rather than its magnitude, as minimal mechanical stimuli of urothelial guinea pig cells achieved maximum ACh release (McLatchie *et al.,* 2014).

It was expected to see a positive correlation between rising pressure and the amount of intraluminally detected ACh, as ACh is thought to be released in a mechanosensitive manner (Yoshida *et al.,* 2006). Surprisingly however, a significant decrease could be seen in the amount of ACh released intraluminally with rising pressure from 5-40 mm Hg (Figure 32). Considering that the bladder has already gone through all the lower distension levels when for example reaching 20 mm Hg, it needs to be considered that a higher choline uptake accounts for the findings. The ACh assay kit used in the experiment detects the total amount of choline - the breakdown product of ACh - as a sum of released ACh and

metabolised ACh in the samples. Therefore, it can be ruled out that an activation of cholinesterase activity with increasing pressure alone is responsible for the drop in detected ACh. If this is the case, it must also be linked to choline uptake by urothelial cells or nerve terminals. All the samples in this study were taken for analyses after a ten minute period. A time dependent or ACh provoked choline uptake can therefore be ruled out. A stretch dependent choline uptake appears to be a convincing explanation for the decline of detected ACh with increasing pressure levels.

Interesting is the fact, that the amount of ACh at 20, 30 and 40 mm Hg returns to basal level while the voiding contraction in the mouse bladder sets in at around 20 mm Hg (Smith *et al.,* 2012). This, combined with the fact that ACh is released during the storage phase at low pressure stretch, leads to the suggestion that ACh release is high during storage phase, acting as an inhibitory neurotransmitter on afferent nerves to suppress sensory responses and therefore bladder contractions. This suppression might be in place until a certain pressure level is reached, where ACh release drops under a threshold and afferent signals are leading to the onset of voiding contractions – around 20 mm Hg in the mature mouse. This hypothesis is underlined by a study by Daly *et al.* (2010) showing that stimulation of muscarinic receptor pathways in murine bladders inhibited the afferent response to bladder distension.

The previous mentioned study by McLatchie *et al.* (2014) showed that ACh release in urothelial cell culture is not linked to OCT transport mechanisms but instead is dependent on CFTR. CFTR is activated by pressure levels of only 5 mm Hg in cellular human airway epithelial cells as well as in mouse intestinal tissue (Zhang *et al.,* 2010). Interestingly, in the present study the highest release of ACh is found at this pressure level of 5 mm Hg (Figure 31 and Figure 32).

The opposing results of Yoshida *et al.* (2006) showing an increase of ACh release by three times after stretching human bladder strips to 40 mN could be due to the fact that a strip model was used and that tetrodotoxin was applied to prevent neuronal ACh release. As all sensory neurons are suppressed with tetrodotoxin, the whole signalling mechanism will be inactivated. It can also however be a species specific disparity, as animals differ considerably in their bladder signalling pathways from humans. The shown data in this

thesis are also a preliminary investigation with a low number of observations and further investigations need to be conducted to confirm these findings.

Noteworthy is the fact, that at basal level the amount of identified ACh in extraluminal samples is similar to the amount intraluminal samples. Extraluminally ACh release is not influenced by a change in pressure in the present study. It could be hypothesised that this basal release is the background ACh release that is always present in the bladder.

Distending the bladder to 50 mm Hg at a high flow rate of 150 µl/ min did show a significant increase in extraluminal ACh compared to basal measurement, while distending the bladder at the lower flow rate of 40 µl/ min did not significantly change extraluminal ACh to basal measurement (Figure 23 and Figure 32). It can be hypothesised that the detrusor muscle does not release ACh in a mechanosensitive way under normal physiological condition but might do so as a stress response when pressure and filling rate are higher than physiologically normal. Under supraphysiological pressure, various stretch induced ion channels and membrane transporters will be activated and might account for the results shown. Sensory nerves are also highly activated at the level of distension to 50 mm Hg and might add to the increase of ACh in extraluminal samples.

In the present study, spontaneous contractions occurred in the bladder during the filling phase and were measured as transient rises in intraluminal pressure. In was shown in this study that the amplitude of spontaneous contractions increased with rising pressure from 5-20 mm Hg. This was negatively correlated to the increasing ACh release seen with increasing pressure. This is a further indication that ACh might act as an inhibitory mediator. As previously stated this could be due to an inhibition of afferent response to bladder distension mediated by the stimulation of muscarinic receptor (Daly *et al.,* 2010).

Interesting is the observation, that in contrast to amplitude, frequency of contractions did not change with a change in pressure. This contrasts a publication showing a rise in the frequency of contractions when applying increasing stretch on bladder tissue strips (Lagou 2004). However, whole, isolated bladders were filled to a preset pressure level in the above described experiment, which might have different effects than stretch of an isolated tissue strip.

In consideration of the above findings, the following hypothesis of the cholinergic pathway in the urothelium was formulated. ACh is released intraluminally whenever small changes in pressure are perceived by the sensory urothelium mediated through OCT3. However also CFTR could be responsible for the ACh release, opening its pore at pressure changes of only 5 mm Hg. ACh might then act as an inhibitory neurotransmitter on muscarinic receptors, suppressing afferent responses to distension and therefore keeping the amplitude of spontaneous contractions low. With rising pressure less ACh is released by urothelial cells. At the same time AChesterase and CHT1 are activated, leading to metabolisation of ACh and the reuptake of choline into the urothelial cells. Whenever the amount of freely available ACh drops under a certain threshold, afferent nerves are activated and lead to the onset of voiding contraction, which is around 20 mm Hg in the mature mouse. ATP release might be triggered by ACh release as shown in the study by McLatchie *et al.* (2014). This is a simplified hypothesis and there is most likely a more complex signalling mechanism taking place in the mechanosensing of the urothelium and the whole bladder respectively. To test parts of this hypothesis, the cholinergic pathway in the mouse bladder was studied further by blocking different components of ACh release and choline reuptake (2.4.5).

2.4.4 ATP RELEASE IN PROPORTION TO THE EXTENT OF DISTENSION

To further test if also the amount of released ATP is proportional to the extent of distension, the isolated bladders were distended to varying pressure levels and the amount of ATP analysed in intra- and extraluminal samples.

The bladders were distended at 40 µl/ min to pressure levels of 5, 10, 15 and 20 mm Hg. The amount of intraluminal ATP after distension to 10 and 20 mm Hg was significantly higher compared to basal level (Figure 33). However no significant difference in ATP could be seen between the different distension levels (Figure 34). Extraluminal ATP was identified at the same level as intraluminal ATP (approximately 10 pM/ µl) at basal level and did not alter after distension from 5-20 mm Hg.

There is convincing evidence, that urothelial cells release ATP in response to stretch (Ferguson *et al.,* 1997, Cheng *et al.,* 2011, Dunning-Davis *et al.,* 2013).

However this is the first study known to measure ATP in the steady state situation of a filled isolated bladder. The reason that ATP could not been shown to be released in a mechanosensitive manner might be due to the short half-life of ATP in the intact bladder. The concentration of ATP is regulated by ATP release and its breakdown by ectonucleotidases. Ectonucleotidases exist in the bladder wall (Lewis and Lewis 2006) and ATP released into the lumen could therefore be broken down before the samples were taken. The breakdown product adenosine could then modulate sensory afferent function as well as the contraction of the detrusor cells (Yu *et al.,* 2009). Further investigations should be done looking at intraluminal adenosine and its change with raising distension levels.

McLatchie *et al.* (2014) demonstrated a release of ACh five times higher than ATP after mechanically stimulating urothelial guinea pig cells in culture. In the above experiments, release of ACh was 400 times higher than ATP release at basal level and 1200 times higher at distension to 5mm Hg. The differences might be due to the dissimilar experimental set-ups and different animal models.

2.4.5 EFFECT OF BLOCKING DIFFERENT COMPONENTS OF THE CHOLINERGIC PATHWAY IN THE MOUSE BLADDER

The cholinergic pathway in the urothelium is not fully understood, as it differs from the well-studied cholinergic pathway found in neurons as previously discussed. Different components of the cholinergic pathway of the urothelium were blocked by intraluminally applying hemicholinium, decynium 22, MLA and fesoterodine. The bladder was then distended to different pressure levels and the change in intraluminal ACh levels was measured. This study was designed to give further insight in to the cholinergic pathway of the urothelium.

Figure 43 illustrates the drugs used and the expected site of action. Hemicholinium was used to block ChT to prevent choline - the breakdown product of ACh - being taken up into the urothelial cells. Decynium 22 was used to block OCTs, to prevent ACh release. MLA was used to block α7 nicotinic receptor and fesoterodine was used to block muscarinic receptors.

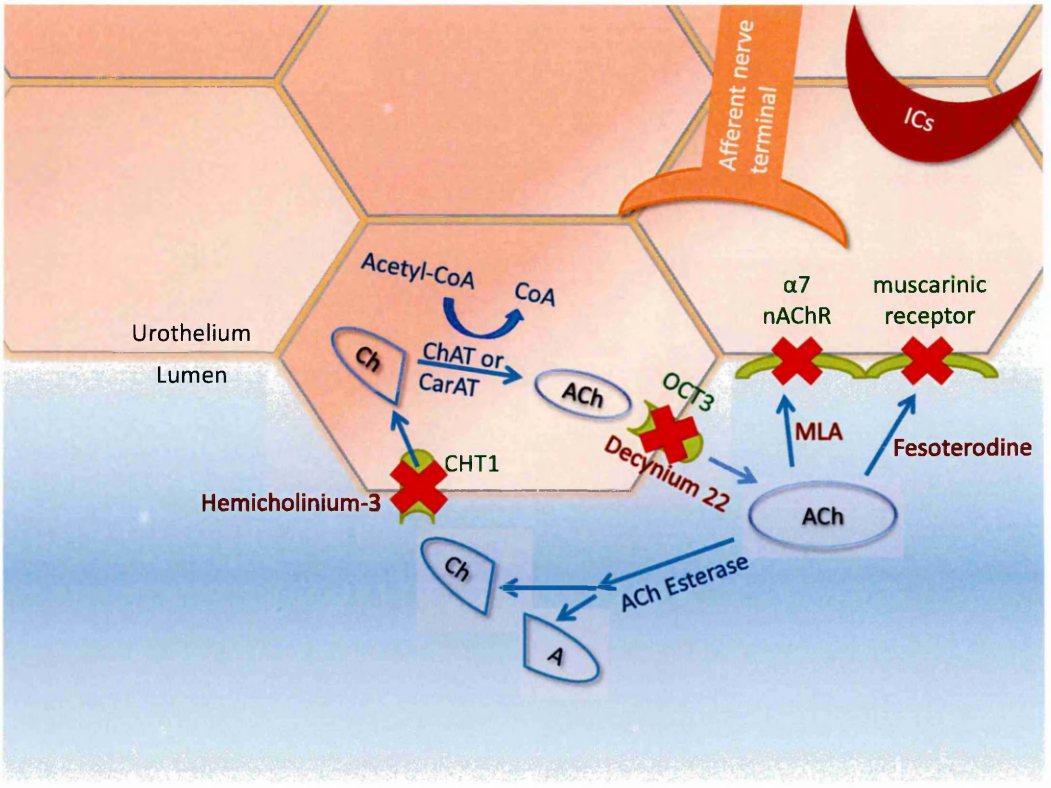


Figure 43 **Hypothetical model showing the cholinergic pathway in urothelial cells and the drugs used to block certain components (red crosses). ACh is released either via OCTs (blocked with decynium 22) or CFTR into the bladder lumen and acts on the cholinergic receptors found on neighbouring urothelial cells as well as on nerve endings and ICs in close proximity to the urothelium (muscarinic receptors blocked with fesoterodine, α7 nAChR blocked with MLA). Choline, the breakdown product of ACh gets taken up into urothelial cells by ChT (blocked with hemicholinium-3) or by bidirectional OCTs. In the cell, choline is resynthesized in to ACh while reacting with Acetyl Coenzyme A. This reaction is possibly catalysed by CarAT.**

(ACh – Acetylcholine, OCT – Organic Cation Transporter, CFTR - Cystic Fibrosis Transmembrane conductance Regulator, ICs - Interstitial Cells, ChT – Choline Transporter 1, CarAT - Carnitine Acetyltransferase)

Hemicholinium-3

Hemicholinium-3 was applied to assess the contribution of choline reuptake to the decrease of intraluminally released ACh, when applying increasing pressure.

The amount of ACh was reduced after application of hemicholinium-3 in comparison to control measurements at basal level and after distension to 5 mm Hg. Hemicholinium-3 prevents the reuptake of choline by blocking the ChT, which could lead to an increase in the amount of choline acting on nicotinic receptor in the urothelium and in response lead to a decrease in ACh release. Over time, it might however cause a depletion of ACh stores in urothelial cells, as choline is the rate limiting step for ACh production. This might explain the reduction of ACh after application of hemicholinium-3 compared to control. Another possible explanation would be an existing feedback mechanism, whereupon ACh only gets released by urothelial cells when the ChT is activated.

Also OCTs are expressed in urothelial cells (Hanna-Mitchell *et al.,* 2007) and have been shown to work in a bidirectional manner depending on membrane potential and substrate concentration (Koepsell *et al.,* 2003). This could be another mechanism of ACh uptake into urothelial cells and explain the low choline concentration at basal level.

No significant change in ACh release could be seen at distension levels of 10, 15 and 20 mm Hg between control and hemicholinium-3 treated samples. This might be due to the increased stretch of the urothelium that results in different accommodation mechanisms. The surface of the urothelium unfolds at tissue level and the membrane of umbrella cells unfolds at cell level through exocytosis. Possibly this also includes the exocytosis of ChT as endo- and exocytosis mechanism are thought to play a crucial role in regulating the expression of lipids, receptor/ channel proteins and mediators at the apical surface of umbrella cells (Truschel *et al.,* 2002, Khandelwal *et al.,* 2009). Furthermore ChT proteins are stored in vesicle membranes to allow the rapid increase of cell surface ChT levels (Black and Rylett 2012).

Hemicholinium-3 was used at a concentration of 5 nM. Alexander *et al.* (2011) suggested an IC_{50} of 10 nM. However, at concentrations of 1 mM and 10 nM the tissue contracted to a

degree that made it impossible to carry out the experiment. Therefore a concentration of 5 nM hemicholinium-3 was used to perform the experiment.

Decynium 22

Decynium 22 was used at a concentration of 1 μM to selectively block OCT3. This transporter was proposed to be responsible for the transfer of ACh across the urothelial plasma membranes (Hanna-Mitchell *et al.,* 2007). K_i values for inhibition of OCT1 are ten times higher than for OCT3, therefore selective targeting of OCT3 can be expected at the used concentration (Alexander *et al.,* 2011).

ACh levels in intraluminal samples were significantly reduced at basal level after application of decynium 22. This underlines the hypothesis that OCT3 transporter are involved in the release mechanism of ACh in urothelial cells. After distension to 5 mm Hg the bladders treated with decynium 22 were contracted to an extent that made it impossible to collect intraluminal samples and no measurements are available. After distension to 10, 15 and 20 mm Hg no significant difference could be observed between the samples from treated bladders and the samples from control bladders. It was recently proposed by (McLatchie *et al.,* 2014) that the CFTR transporter plays a role in the release of ACh in urothelial cells as previously stated. CFTR changes it conformation with increasing pressure and might be responsible for the ACh release after application of higher pressure levels while OCT3 transporter might be responsible for ACh release at basal level.

However, the decynium 22 also blocks monoamine transporter. Therefore, transporter for monoamine neurotransmitter such as serotonin, dopamine, norepinephrine and epinephrine might also be affected by decynium 22. As these could also play a role in bladder signalling pathways the results prevent conclusive interpretation.

Methyllycaconitine (MLA)

MLA is a cholinergic antagonist acting on nicotinic but not muscarinic sites (Benn and Jacyno 1983). The drug was used at a concentration of 10 nM, as it has been shown that the IC50 to block α7 is 0.64 nM (Briggs and McKenna 1996) and the IC50 to block α3 is 3.8 μM (Bryant *et al.,* 2002). Therefore, selective blocking of α7 nicotinic receptors at the concentration used should be possible. α7 nicotinic receptors were blocked in this experiment as they have been shown to be expressed most intensely in human and murine urothelial cells (Bschleipfer *et al.,* 2007, Zarghooni *et al.,* 2007) and have important physiological function in bladder voiding reflexes (Beckel *et al.,* 2006).

ACh levels in intraluminal samples were significantly reduced at basal level after application of MLA suggesting that α7 nicotinic receptors are involved in the urothelial signalling system. It has been shown by Beckel and Birder (2012) that stimulation of α7 nicotinic receptors in rat urothelial cells decreased ATP release. This could be a secondary effect to a decrease in ACh release or vice versa.

After distension to 10, 15 and 20 mm Hg no significant difference could be observed between the samples from treated bladders and the samples from control bladders. This might be due to a desensitization of α7 nicotinic receptors during the time frame of the tissue being exposed to MLA. Friis *et al.* (2009) as well as Giniatullin *et al.* (2005) have reported a rapid desensitization of α7 nicotinic receptors.

The results of these experiments are challenging to interpret as neuronal ACh release has not been suppressed in the experimental set up. The drugs were applied intraluminally only; however neurons are ending in close proximity to the urothelium and might have been affected by the drugs, resulting in a change of neuronal transmitter release, which could then influence bladder signalling pathways. It would therefore be necessary to assess if a suppression of neuronal activity would make a difference to the above findings. However, this will also affect other bladder signalling pathways and spontaneous contractions. Therefore, isolated models such as cell culture experiments, ideally with human cells, will be most suitable to answer specific questions about the urothelial cholinergic pathway.

Compliance

Bladder compliance was also measured after application of hemicholinium-3, decynium 22 and MLA. All three drugs had the same reducing effect on bladder compliance (Figure 39). This suggests that interfering at any point in the cholinergic pathway does trigger the same response in the muscle, leading to an extremely contracted detrusor muscle, even at very low pressure levels. The fact that the muscle contracted already at the low pressure levels, made it difficult to retain a big enough sample size to run the assays. Therefore numbers of observation were low.

Under healthy conditions bladder pressure should only change slightly during the filling phase, however certain voiding dysfunctions are associated with low bladder compliance. Bladder outlet obstruction is one of these dysfunctions and it was hypothesised that a build up of detrusor mass and collagen leads to the decline in bladder compliance (Mauroy 1997). Detrusor overactivity (DO) during filling has also been linked to low bladder compliance which was suggested to be the result of a reduction in detrusor nerve density and connective tissue (Madersbacher *et al.,* 1999). The results from this study however suggest that bladder dysfunctions with low compliance measurements might also be due to changes in the urothelial cholinergic pathway. This is an interesting point for future research.

Fesoterodine

Fesoterodine was applied to the detrusor muscle to investigate the effect of blocking muscarinic receptor on ACh and ATP release. Fesoterodine is a commonly used antimuscarinic drug to treat OAB syndrome. It was assumed that antimuscarinics antagonise muscarinic receptors on the detrusor muscle and consequently inhibit muscle contraction, which are normally induced by the action of ACh released from efferent nerve fibres. This theory has however been questioned as antimuscarinics have little effect on voiding contractions at the clinically recommended doses. Instead antimuscarinics reduce bladder tone during storage and increase bladder capacity (Andersson and Yoshida 2003,

Finney *et al.,* 2006). The exact working mechanism of antimuscarinics is not clear to date. It was therefore examined how bath applied fesoterodine effects the intra- and extraluminal release of ACh and ATP during distension to 50 mm Hg.

In the present study, bath application of fesoterodine to the intact mouse bladder had no significant influence on intra- or extraluminal ACh and ATP release. Compliance was also not significantly different between the bladders treated with fesoterodine and the controls.

Fesoterodine is a pro-drug that is rapidly hydrolysed by non-specific plasma esterases to 5-hydroxymethyl tolterodine, its primary active metabolite (Simon and Malhotra 2009). Further studies are needed to show that the used amount of fesoterodine is converted to tolterodine in the experimental system used. Additional studies should also be carried out with other well characterized antimuscarinic drugs.

Antimuscarinics block muscarinic receptors in a competitive and reversible manner (Yoshida *et al.,* 2013). The samples in this study were taken 30 minutes after application of fesoterodine which might have been enough time for the bladder to regenerate a steady state situation of ACh release.

It would also be interesting to study if intraluminally applied fesoterodine has an influence on ACh and ATP as it is not confirmed that antimuscarinics act on the muscle alone. The urothelium could also be the side of action for these drugs and a signalling cascade might start in the urothelium, finally affecting the detrusor muscle. The pressure and perfusion rate applied was supraphysiological for the mouse bladder and should be adjusted in future experiments. The high pressure levels were chosen to compare the data to the results from colleagues doing afferent nerve recordings with application of fesoterodine. These experiments have found that fesoterodine significantly inhibited afferent responses to bladder distension without an effect on bladder compliance (Daly, unpublished). It was also observed that fesoterodine significantly increased the amplitude of spontaneous contractions measured as small rises in pressure. The working mechanism of antimuscarinic drugs requires further examination.

3. AGE-RELATED CHANGES IN THE MECHANOSENSATION OF THE ISOLATED MURINE BLADDER

3.1 INTRODUCTION

The prevalence of LUT dysfunctions including OAB considerably increases with age. As previously cited, about 15% of the western population are affected by OAB. The prevalence rises sharply in the aged population from about 4% of men affected at the age of 40 to over 40% of men affected at the age of 75 (Milsom *et al.,* 2001, Irwin *et al.,* 2006). Taking into consideration the demographic change of an ageing population with the high prevalence of OAB in the elderly, an estimated 2.3 billion individuals worldwide over 20 years of age are expected to be affected by at least one LUT symptom with 546 million suffering from OAB by 2018 population (Irwin *et al.,* 2011).

The high prevalence of LUT symptoms in the elderly population is most likely due to age-related changes in the urinary tract. These include considerable changes of detrusor morphology, bladder innervation and bladder metabolism. The high prevalence of comorbidity and polypharmacy with medication side effects in the aged also increases the risk of developing LUT symptoms. Changes in the LUT also occur following diseases in the CNS or the vascular system. For example, the onset of metabolic syndrome - which is defined as abdominal obesity associated with hyperinsulinemia, insulin resistance and two additional cardiovascular risk factors - has been shown to be associated with LUT syndromes (Madersbacher *et al.,* 1998, Dubeau 2006, Cruz and Desgrandchamps 2010).

It is important to understand the physiological changes occurring in the ageing bladder to successfully treat OAB. The major difficulty of conducting ageing studies is to find an appropriate model of ageing. Human studies are not easily carried out due to the relatively long lifespan of humans, however a variety of animal models are available for scientific experiments. Extrapolating the data from these models into humans has to be done with

caution as pathways that are relevant in ageing in a certain model may be irrelevant in humans due to species specific differences.

Ageing Models

The range of models available is diverse. Due to the short life cycle and the ability of genetic manipulation, the following organisms are the main models used for ageing studies: human cells, unicellular organisms such as the yeast Saccharomyces cerevisiae, the nematode worm Caenorhabditis elegans, the fruitfly Drosophila melanogaster and the mouse. However the use of these models in ageing studies has been discussed controversially as it is not easy to decide on the exact age in some of these models and it is often hard to distinguish between accidental and age-related death when researching effects on the lifespan of an organism. The laboratory environment has also an impact on the study outcomes, genetic divergences between strains occur over time and a reduced lifespan has been observed due to inbreeding (Partridge and Gems 2007). Apart from the mentioned physiologically aged animal models, ageing induced animal models exist. Examples of these are mice with specific mutations in the nuclear or mitochondrial genomes or a cellular ageing model where DNA damage is experimentally induced by hydroxyurea treatment (Dong *et al.,* 2014). How comparable these induced ageing models are to the physiologically aged organisms is not clear to date and more research is needed before conclusions can be drawn.

Physiologically aged mice were used in the present study. The mouse is a suitable animal model as it is very similar to humans in anatomy, physiology and genetics. About 99% of the encoded gene sequences are similar between humans and mice. Mice also experience many of the common human diseases. Further advantages of the mouse model are the availability of mutants and the possibility of genetic manipulation. The short lifespan of around two to three years also makes the mouse a cost-effective and efficient tool for ageing and life span studies in the physiologically aged animal (Waterston *et al.,* 2002, Nguyen and Xu 2008).

In the present study the bladders of physiologically aged C57 mice have been used. The representative life phase equivalencies of C57 mice compared to humans are shown in Figure 44 using a survival curve based on a cohort of 300 mice (Flurkey *et al.,* 2007). The bladders used in this study were obtained from mice aged 3 months (referred to as adult mice throughout this thesis) and from mice aged 24 months (referred to as aged mice throughout this thesis). This would compare to a human age of about 20 and 69 years respectively.

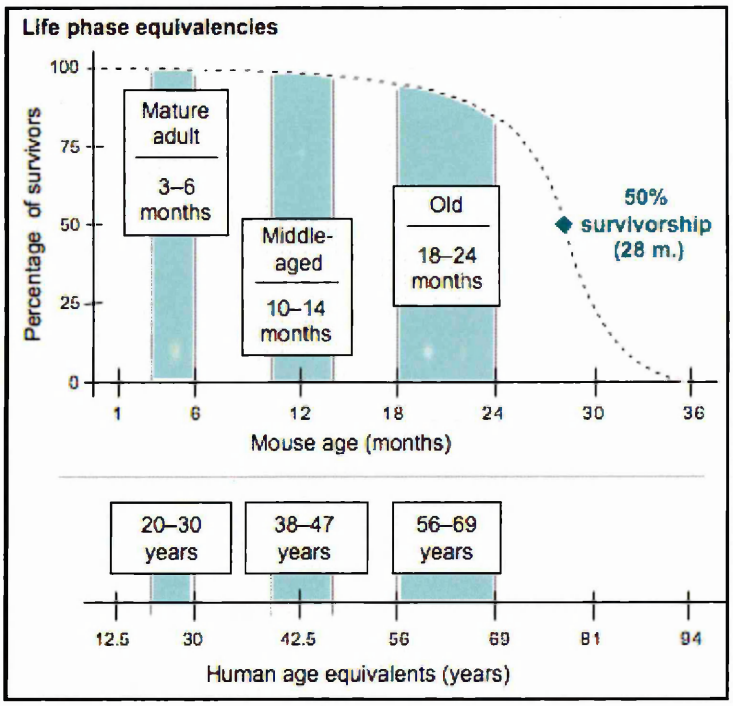


Figure 44 Representative life phase equivalencies of C57 mice compared to humans (Flurkey *et al.,* 2007).

Functional and structural changes in the ageing bladder

Several urodynamic studies have demonstrated a number of functional alterations in the aged bladder including reduced bladder capacity, decreased urinary flow rate, incomplete bladder voiding and a reduced voiding volume (Elbadawi *et al.,* 1998, Madersbacher *et al.,* 1998, Pagala *et al.,* 2001). Structural changes have also been shown in the aged bladder, including increased collagen content, decreased innervation and alterations in cell junctions (Warburton and Santer 1994, Hotta *et al.,* 1995, Elbadawi 1995).

However, relatively few studies exist that investigate the underlying mechanisms of these functional and structural changes occurring in the bladder during ageing. The first published study examining age-related changes in signalling pathways was conducted by Kolta *et al.* (1984). The investigators showed that the contractile response to ACh was increased by 63% in bladder strips of 29 month old rats compared to the bladders of 7 month old rats. It was also shown in this study that the increased sensitivity was due to an increase in the number of muscarinic receptors. Yoshida *et al.* (2004, 2006) reported a negative correlation between age and non-neuronal ACh release in human urinary bladder strips whereas a positive correlation between age and ATP release was shown. It was also shown that the cholinergic component of contractile responses decreased with age, while the purinergic component of contractile responses increased with age.

Colleagues have reported an age-related change in the voiding pattern and an increase in the number of voiding events in the mouse (Daly *et al.,* 2014). Bladders from adult animals (3-4 months old) and from aged animals (24 months old) were used for the investigations. Awake mice were housed separately for 4 h in cages that were lined with filter paper with free access to food and water. Aged and adult mice both tended to urinate in one corner of the cage and no significant difference could be seen in the total area of urine voided. However the filter papers from aged mice showed significantly more urine spots compared to the filter papers from the control group (Figure 45). Additionally, significantly more small urine spots (<0.2 cm^2) could be seen on the filter papers from aged mice and the spots were more scattered. Osmolality was not significantly altered between adult and aged mice. This suggests that the results are not affected by alterations in renal function or by an

increase in urine production. The results propose that aged mice void smaller volumes of urine in a single voiding event while the number of voiding events increases.

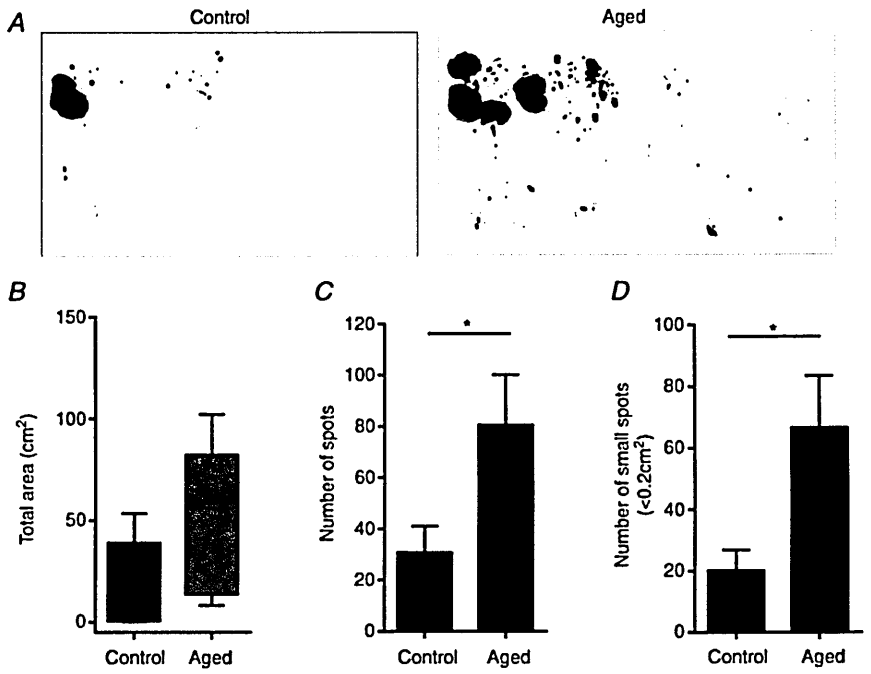


Figure 45 Voiding pattern and number of voiding events in the aged and control mice (Daly *et al.*, 2014). (A) Representative, digitised traces showing the urine spot patterns obtained from a control and an aged mouse over a 4 h period. (B) Total urine spot area was not significantly different between aged and control mice (Student's T test, n=6). (C) The number of urine spots detected was greater in aged mice than in controls (* indicates $p \leq 0.05$). (D) The number of small urine spots (<0.2 cm^2) was also significantly greater in aged mice than in controls (* indicates $p \leq 0.05$).

It was also shown, that spontaneous afferent nerve activity was significantly higher in aged mice compared to control mice, while bladder compliance was not affected by age (Daly *et al.,* 2014). The reason for this altered sensory nerve transduction in the aged animals could be due to changes in the (1) nerves itself, in the (2) muscle or in the (3) urothelium as distinct subpopulations of afferent nerves innervate the muscle and the urothelium and are able to detect changes in the bladder wall environment (Zagorodnyuk *et al.,* 2007).

(1) As earlier studies have shown that the general pattern of neural innervation is conserved during ageing (Nakayama *et al.,* 1998, Mohammed and Santer 2002, Aizawa *et al.,* 2011), changes in the morphology and function of the sensory nerves are unlikely to contribute to the increased nerve activity.

(2) A change in the contractile abilities of the detrusor muscle or a change in detrusor tone could account for the increased nerve activity as afferent fibres lie within the muscle layer (Zagorodnyuk *et al.,* 2007, Xu and Gebhart 2008). In this work organ bath studies were performed with denuded detrusor strips of adult and aged mice to evaluate how the contractile response of the bladder detrusor changes during ageing. The main types of stimulus in the detrusor tissue are of purinergic and cholinergic origin (Ford *et al.,* 2006). Therefore the non-selective purinergic receptor agonist ATP and the non-selective muscarinic agonist bethanechol were bath applied at different concentrations to determine the age dependent effect on the contractile response. Another reason for the increased nerve activity could be the higher susceptibility to depolarisation in aged cells. Contractions of aged and adult mouse detrusor strips were therefore recorded after exposure of the tissue strips to KCl enriched Krebs' solution to examine the effect of depolarisation.

(3) The third possible reason for the increased afferent nerve activity in the aged mice bladder is a change in urothelial function. The urothelium is now known to release a host of excitatory and inhibitory mediators which could act on the afferent terminals to modulate neural excitability or act in an autocrine/paracrine manner on urothelial cells to set off downstream signalling cascades. Calcium imaging experiments were therefore carried out on murine urothelial cells to assess if purinergic or cholinergic receptor signalling is altered as a result of ageing. Results from related studies in the laboratory showed a significantly increased cell signalling in response to the non-selective purinergic receptor agonist ATP

(100 μM) in cells from aged bladders compared to cells from adult bladders. The number of urothelial cells responding to ATP as well as the magnitude of response was significantly increased in the cells from aged mice compared to cells of adult mice (Daly *et al.,* 2014, Figure 46). Further studies showed that the response to the non-selective muscarinic receptor agonist bethanechol (100 μM) did not significantly differ between the two groups (Daly *et al.,* 2014, Figure 46). These results indicate that one of the purinergic receptors is responsible for the increased signalling seen in the aged mice following ATP administration. To reveal this purinergic receptor, calcium imaging experiments were carried out in the present study with the selective $P2X_1$ and $P2X_3$ agonist αβ-Methylene ATP and the $P2X_1$ agonist βγ-Methylene ATP.

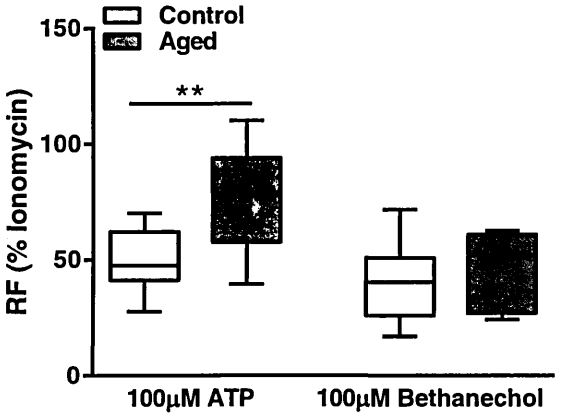


Figure 46 Fluorescent response of urothelial cells to ATP and bethanechol (Daly *et al.,* 2014). Responses to ATP stimulation (100 μM) were significantly higher in urothelial cells from aged mice compared to urothelial cells from adult mice (indicates $p \leq 0.01$) whereas the response to bethanechol was not significantly different between the two groups.**

Further experiments were performed to investigate if urothelial release of ACh, ATP, NO and Substance P changes in ageing. A change in urothelial signalling might account for the increased sensory nerve activity in the aged mice bladders. The mediators ACh, ATP and NO play an important part in bladder signalling pathways as excitatory and inhibitory mediators (see 1.2). Release of Substance P from urothelial cells has not been shown to date, however tachykinin receptor expression as well as Substance P immunoreactivity and mRNA expression has been shown in the urothelium and therefore suggests an involvement of Substance P in urothelial signalling processes (Heng *et al.,* 2011, Bahadory 2013). Substance P has furthermore been shown to play a role in inflammation processes (O'Connor *et al.,* 2004) which play an important role in ageing.

In this study the mediators ACh, ATP, NO and Substance P were therefore quantified in intra- and extraluminal samples after distension of the whole isolated bladder of the adult and the aged mouse.

Aims

The demographic forecast clearly shows that understanding age-related changes in bladder physiology and signalling mechanisms is crucial to further develop treatment options for LUT symptoms in an increasingly ageing population. The aim of this study was to identify age-related changes in signalling mechanisms and detrusor contractility in the naturally aged murine bladder.

- The contractility of denuded detrusor strips from adult and aged mice was measured after application of muscarinic and purinergic agonists.
- Intra- and extraluminal samples of the whole, isolated bladder from adult and aged mice were analysed for various mediators.
- Spontaneous contractions of the whole, isolated bladders from adult and aged mice were recorded and analysed.
- Investigations into the purinergic signalling pathways in urothelial cells were carried out using calcium imaging technique.

The results could also give an insight in to the mechanisms involved in general pathophysiological bladder conditions unrelated to ageing.

The data of this chapter are published in Daly *et al.* (2014).

3.2 MATERIAL AND METHODS

3.2.1 RECORDING OF DETRUSOR MUSCLE CONTRACTIONS IN DENUDED TISSUE STRIPS

Tissue Preparation and Organ Bath Set-up

Male C57/B6 mice, aged 3-4 months (adult mice), and male C57/B6 mice, aged 24 months (aged mice), were obtained from Harlan, UK and Charles River, UK respectively. The animals were housed in the Field Labs at the University of Sheffield. Handling followed the principles of good laboratory practice in compliance with UK laws and regulations. The mice were killed by cervical dislocation, a Home Office approved Schedule One procedure. The whole bladders were immediately isolated from the animal and placed in oxygenated Krebs' solution at room temperature (chemical composition described in 2.2.4).

Following dissection, the bladders were trimmed of excess connective tissue and were carefully cut open along a vertical line from the urethral opening. The bladder sheet was pinned down with the urothelium upper most and the mucosa was then dissected from the detrusor muscle in one sheet under a dissecting microscope (Figure 47). The detrusor strip without the urothelium will be referred to as denuded in the subsequent text. The denuded detrusor was longitudinally cut in half and the two tissue strips mounted in an organ bath. Hence two denuded strips were prepared from each bladder. In the rare case that one of the two tissue strips got damaged during the mounting process, only one detrusor strip was used for that specific bladder.

The tissue strips were mounted in a 10 ml organ bath. One end of the tissue strip was attached to the tissue holder; the other end was connected to a UF1 force transducer (Pioden Controls Ltd., UK) using surgical thread. The force transducer registered the tension of the tissue strip and sent an electrical signal to an amplifier. After amplification the signal was recorded using a PowerLab data acquisition system and Chart software (ADInstrument, Colorado Springs, USA). The resting tension was adjusted to 1 g for 30 minutes. The force transducer organ bath set-up is shown in Figure 48. The tissue was maintained at 37°C in Krebs' solution and aerated with 95% O_2 and 5% CO_2 at all times.

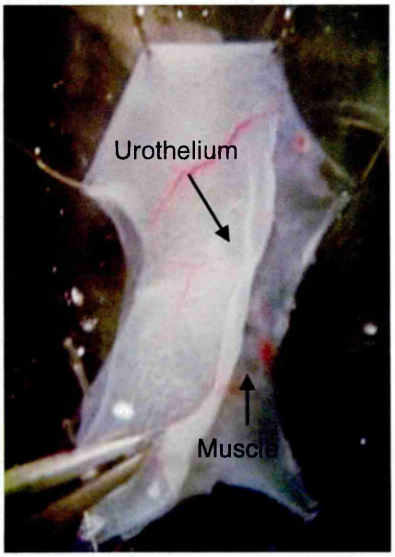


Figure 47 Dissection of the murine bladder. The urothelium was separated from the muscle by blunt dissection under the microscope.

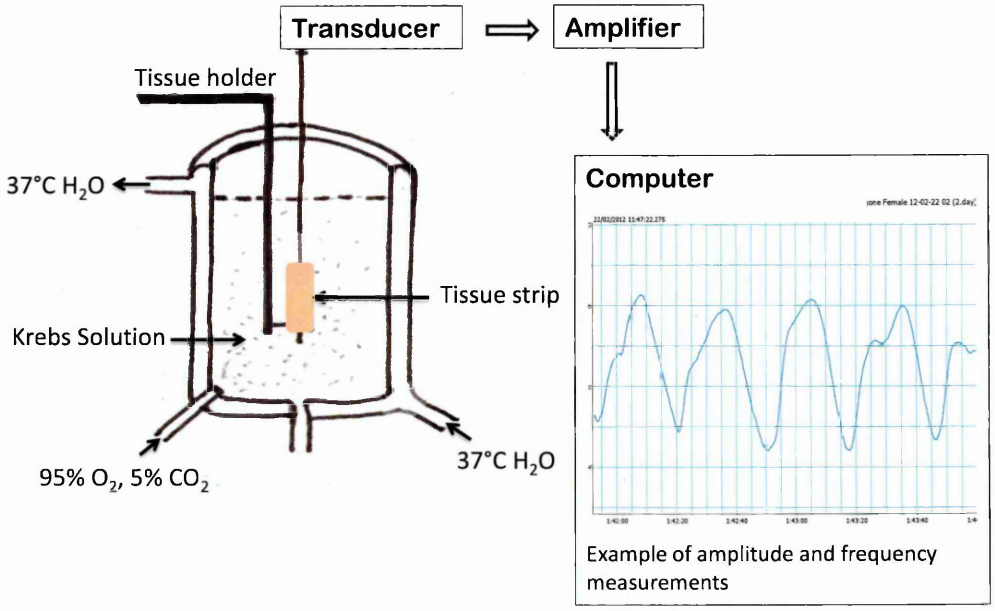


Figure 48 Organ bath set-up. Detrusor tissue strips were mounted in a double walled glass chamber, which was aerated with 95% O_2 and 5% CO_2 and kept at 37°C to maintain physiological conditions. The tissue was hooked up at the bottom of the chamber via the tissue holder and at the top via suture connecting the tissue strip to a UF1 force transducer. Contractions were registered by a transducer, amplified and made visible on the computer screen in order to analyse amplitude and frequency measurements.

Protocol

During the equilibration time of 30 minutes the Krebs' solution was replaced every 10 minutes and the resting tension was adjusted to 1 g. The last two minutes of this 30-minute period were used as a baseline measurement for amplitude and frequency of contractile activity. ATP concentrations of 10 μM, 100 μM and 1 mM were subsequently bath applied for five minutes each with a 20 minute washout period between concentrations. Subsequent cumulative concentrations of bethanechol were applied at concentrations of 1, 10 and 100 μM. The higher concentration of bethanechol was applied whenever the peak of contraction plateaued. After a 30 min washout period, KCl was added to final bath concentrations of 25 and 65 mM for five minutes each with a 30 min washout period between concentrations. Contractile activity was recorded and analysed for amplitude and frequency.

Data Analysis

Amplitude and frequency of spontaneous activity was analysed over a two-minute period post drug application. Maximum peak to peak amplitude (max-min) of contractions was calculated over the described period. For frequency analysis a threshold of 30% of the mean amplitude was calculated to define single contraction events as peaks above this threshold line. A peak is counted as one contraction event, when the relaxing phase does not return below the 30% threshold level before contracting again (Figure 49). This method was proposed by Imai *et al.* (2001) and has been previously used in the laboratory by colleagues (Vahabi *et al.*, 2011, Nyamwaro 2012). Furthermore, a contraction curve was only counted if the shape and size was not affected by noise spikes.

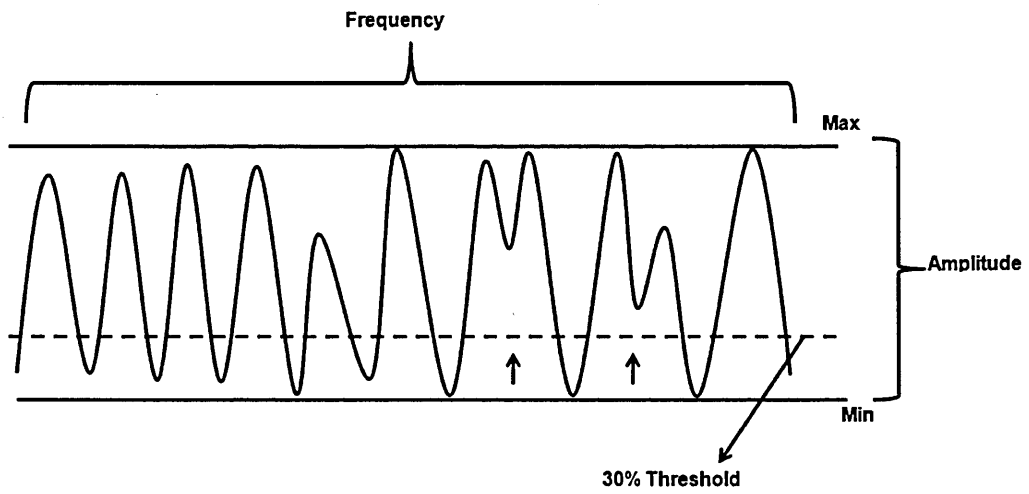


Figure 49 **Analyses of frequency and amplitude of contractions in murine bladder strips. Amplitude was measured as peak amplitude (max-min). The 30% threshold of the peak amplitude was calculated and any contraction exceeding this threshold was calculated to the frequency measurement. Superimposing contractions that did not return below threshold line before contracting again were only counted as single contractile event (illustrated by arrows) (adapted from Imai *et al.*, 2001).**

Amplitude data were normalised for wet tissue weight to account for the variations in tissue size and are shown as absolute grams of tension per mg of tissue weight. Frequency was expressed as number of contractions per minute.

Data were expressed as mean (±SEM) with N being the number of mice used, whereas n shows the number of tissue strips. Unpaired Student's T test was performed for comparison of the two groups, adult and aged. 2-way ANOVA with Bonferroni's post-test was used to analyse the response to cumulative application of Bethanechol. Statistical significance was considered whenever reaching a 95% confidence interval with p values ≤ 0.05 considered as significant. All statistics were calculated using Prism 5 (GraphPad Prism, San Diego, USA).

Drugs and Solutions

ATP disodium salt hydrate and bethanechol chloride (carbamyl-β-methylcholine chloride) were obtained from Sigma (Poole, UK). KCl was obtained from VWR (Leicestershire, UK). Stock solutions were freshly prepared in Krebs' solution.

3.2.2 SAMPLE COLLECTION FOR BIOCHEMICAL ASSAYS AND RECORDING OF SPONTANEOUS CONTRACTIONS IN THE WHOLE, ISOLATED MURINE BLADDER

Micro Organ Bath Set-up

The set-up was described in detail in 2.2.1.2, Figure 12. Whole and intact mouse bladders of adult and aged mice were removed immediately after the animals were humanely killed by cervical dislocation and kept in 37 °C Krebs' solution oxygenated with 95% O_2 and 5% CO_2. The bladders were then catheterised using a dual-lumen cannula (Polythene Tubing, inner diameter 0.28 mm and outer diameter 0.38 mm) via the urethra and sealed off with surgical thread. After mounting the bladders into the micro organ bath filled with 500 µl oxygenated Krebs' solution at 37 °C, the bladders were allowed to equilibrate for 30 minutes.

The catheter was connected to an infusion pump on one side and a pressure transducer (BD DTXPlus™) on the other side. With a set perfusion rate the bladder was filled via the infusion pump with buffered saline (0.9%, pH 7.4 - 7.8) while the rise in pressure was registered by the transducer, amplified and monitored on a computer screen. After maintaining isovolumetric conditions for 10 minutes, an overflow tap was opened to completely empty the bladder and the intraluminal samples were collected on dry ice. At the same time the extraluminal bath samples of 500 µl were also collected on dry ice and the bath was then refilled. The bladders were distended to an intraluminal pressure of 50 mm Hg.

After ten distensions to 50 mm Hg the bladder was left distended at 10 and 20 mm Hg respectively for 30 minutes to record spontaneous activity as small transient rises in intraluminal pressure using Spike 2 software (Cambridge Electronic Design, UK).

Intra- and extraluminal samples were analysed for ACh, ATP and NO following the techniques described in Chapter 2.2.3. Substance P was measured as described in the following.

Substance P

Concentrations of Substance P in intraluminal samples from murine bladders were measured using the EIA Assay (Cayman Chemical). The kit provides an enzyme-linked immunosorbent assay (ELISA) for quantitative measurement of Substance P at a range of 3.9 to 500 pg/ml.

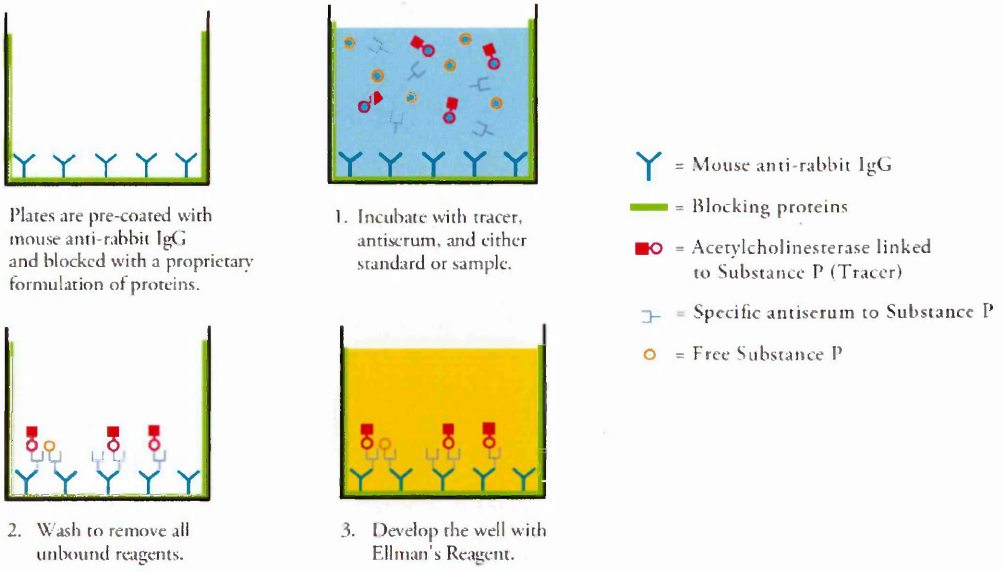


Figure 50 Schematic of the reaction steps occurring in the Substance P EIA assay (Cayman Chemical Company 2011). (1.) Well plates, which are pre-coated with mouse anti-rabbit IgG, were incubated with AChE Tracer, rabbit antiserum with Substance P specific binding sites and either the sample or standard. (2.) A wash was performed to remove all unbound reagents. (3.) The well plate was then developed with Ellman's Reagent which consisted of the AChE substrate. The yellow coloured product of this enzymatic reaction quantifies the binding of the Substance P Tracer, which is inversely proportional to the amount of free Substance P present in the well during the incubation.

The assay consists of a well plate coated with mouse anti-rabbit IgG antibodies to which rabbit antiserum with Substance P specific binding sites was added. The assay is based on the competitive binding action between Substance P and a Substance P AChE conjugate (Tracer) for a limited amount of Substance P-specific rabbit antiserum binding sites. The binding of the Tracer was quantified after an enzymatic reaction that occurred by adding Ellman's Reagent consisting of the AChE substrate (Figure 50). The coloured product of this reaction absorbs strongly at 412 nm and is proportional to the amount of Substance P Tracer bound to the well. This in turn is inversely proportional to the amount of free Substance P present in the well during incubation. An example of the standard curve is shown in Figure 51.

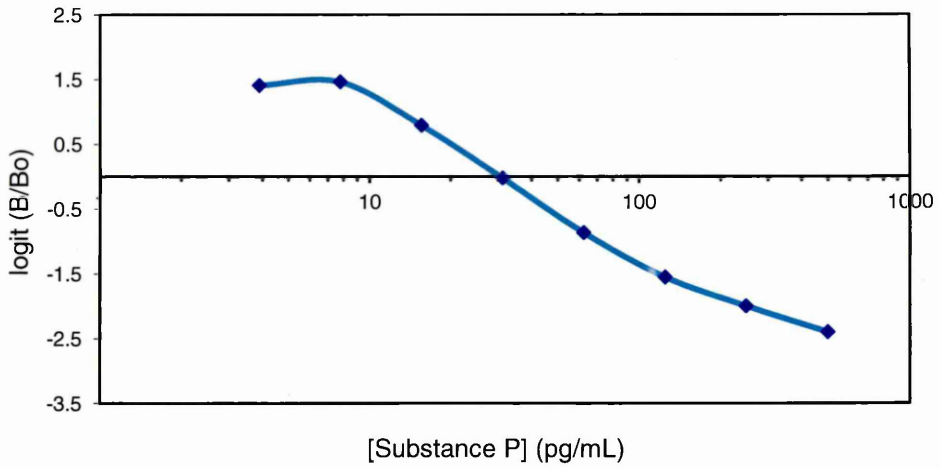


Figure 51 Standard curve of spectrophotometer readings for the detection of Substance P (3.9-500 pg/ml) using the Substance P EIA Assay kit. Absorbance was measured with a microplate reader at a wavelength of 405 nm. Logit (B/B_0) describes the inverse of the sigmoidal function of standard bound B/maximum bound B_0.

Protocol

After the bladders from adult and aged animals equilibrated in oxygenated Krebs' solution for 30 minutes, basal sample collection was carried out. Three intra- and extraluminal samples were taken every ten minutes while perfusing the bladder intraluminally with buffered Saline at 30 µl/ min.

Ten distensions to 50 mm Hg at 100 µl/ min were then performed with buffered saline. Every distension was kept at isovolumetric conditions for 10 minutes, where after intra- and extraluminal samples were collected on dry ice. The organ bath was then refilled again with 500 µl Krebs' solution and the bladders were distended again to 50 mm Hg with saline.

Following the ten distensions to 50 mm Hg pressure, the bladders were distended to 10 mm Hg at 100 µl/ min perfusion rate and left for 30 minutes at isovolumetric conditions in order to record amplitude and frequency of spontaneous activity. The bladders were then filled to 20 mm Hg at 100 µl/ min perfusion rate and again left for 30 minutes at isovolumetric conditions to record spontaneous activity.

Data Analyses

Data are presented as mean ±SEM with n being the number of mice/bladders used. Statistical analysis was carried out using unpaired Student's T test. Statistical significance was considered whenever reaching a 95% confidence interval with p values of ≤ 0.05 considered as significant. All statistics were calculated using Prism 6 (GraphPad Prism, San Diego, USA).

Results were corrected for bladder volume to compensate for variations in bladder size and therefore variations in urothelial surface area releasing mediators. As sample collection was carried out as soon as reaching a 50 mm Hg threshold, the time of collection varied between the bladders in the same experiment. The results were therefore also adjusted for filling time and expressed as pmole/min.

Spontaneous Activity

Spontaneous activity indicated by small transient rises in pressure was analysed over a five minute period at the end of a 30 minute equilibration period at either 10 or 20 mm Hg. Frequency and amplitude were analysed using a script that was custom designed by Cambridge Electronic Design as described in Chapter 2.2.5. Maximum peak to peak amplitude (min-max) of contractions was calculated over the described period of five minutes. For frequency analyses the number of contractions over this five minute period was analysed and expressed as frequency per minute. A contraction event was defined whenever the peak exceeded the amplitude of 0.5 mm Hg. It was manually confirmed that no noise peaks were included in the analyses.

3.2.3 CA^{2+} IMAGING

Cell Culture

Isolation of urothelial cells from the murine bladder was carried out according to previous studies (Birder *et al.,* 2002, Everaerts *et al.,* 2010) and optimised for highest cell yield. Bladders from adult and aged mice were dissected following cervical dislocation of the animals. The urethra and excess connective tissue were trimmed off, the bladder cut open along a vertical line from the urethral opening and the bladder sheet pinned down flat with the urothelial side up in a Sylgard-coated dish.

The bladder sheet was washed twice with modified eagle medium (MEM) containing 1% antibiotic-antimycotic media 100x (PSF) and 0.7% 1M HEPES buffer. The bladder sheet was then incubated with MEM containing 2.5 mg/ml dispase for 2 hours at room temperature under the tissue culture hood. The MEM solution was discarded and urothelial cells were collected by gentle scraping with a scalpel blade under a dissecting microscope. The removed cells were dissociated in 0.025% Trypsin EDTA for ten minutes at 37°C by repeat pipetting with a Pasteur pipette every few minutes until the cells were not clustered any longer. The dissociation was stopped by adding MEM media containing 12.5% fetal bovine serum (FBS) to the solution. After centrifuging the solution at 1500 rpm for 5 minutes at room temperature, the pellet was resuspended in keratinocyte serum-free media (KSFM) and further centrifuged at 1500 rpm for 10 minutes. After discarding the media, the pellet was resuspended in fresh KSFM. The amount of KSFM was calculated in order to have 30 µl cell solution per coverslip used. The cells were plated on glass coverslips (13 mm) that were coated with collagen IV (1:20 dilution of collagen solution in PBS). The coverslips were placed in a 24-well plate and incubated for 1.5 hours at 37°C in order to facilitate attachment of the cells to the collagen. Thereafter the wells were filled with 1 ml of KSFM and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2.

Ca^{2+} Imaging Technique

Calcium Imaging Technique uses calcium indicators, which are fluorescent molecules that can respond to the binding of Ca^{2+} by changing their fluorescent properties. Ca^{2+} is a universal 2nd messenger involved in the regulation of cellular processes. Changes in intracellular Ca^{2+} concentration were measured using fura-2-acetoxymethyl ester (fura2-AM), which is a fluorescent dye that distributes inside the cells and binds to free intracellular Ca^{2+}. Its fluorescence properties are activated upon binding to Ca^{2+} making it possible to record the calcium status of the cells. Intracellular signalling transduction can be made visible with this method, as a change in the amount of intracellular Ca^{2+} concentration leads to a change in the fluorescence signal.

Following over night incubation, the urothelial cells grown on coverslips were loaded with 2 μM fura2-AM for 30 minutes at 37°C in a darkened room. The coverslips were placed into a perfusion chamber and mounted on the imaging rig which consists of an epifluorescent microscope with excitation and emission filter, a gravity perfusion system and a camera linked to a computer where the Ca^{2+} signal is made visible on the screen. The whole set-up was placed on a vibration isolation table. All calcium imaging experiments were performed at room temperature.

The cells were continually perfused with HEPES buffer (10 mM Hepes, 135 mM NaCl, 5 mM KCl, 10 mM Glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, adjusted to pH 7.4 with NaOH) at a rate of 1.5 ml/min for 30 minutes in order to remove any fura2-AM that has not been taken up into the cells. Cells were then stimulated for 3 minutes with HEPES containing either αβ-Methylene ATP or βγ-Methylene ATP. The changes in intracellular calcium $[Ca^{2+}]_i$ were monitored in real-time as change of fluorescence. The drugs were washed out with HEPES buffer and subsequently stimulated with 5μM Ionomycin, which is a calcium ionophore working as positive control.

Analysis

The camera was adjusted to a single view field in the middle of the coverslip and individual, fura2-AM loaded cells were marked as regions of interest (Figure 52). Furthermore, a blank spot was marked to perceive a background light emission reading and correction for this background signal was carried out. Only cells that responded to the positive control Ionomycin were included in the analysis.

The change in intracellular calcium concentration $[Ca^{2+}]_i$ was measured as the ratio between the fluorescence signal emitted at 350 nm and at 380 nm during a 0.1 s exposure of light. The 350 nm signal did not change over time while the 380 nm signal decreased as $[Ca^{2+}]_i$ increased. The results were calculated as ratio of the fluorescence at both excitation wavelengths (relative fluorescence - RF) after correction for the individual background light emission.

Data are presented as mean ±SEM. Statistical analysis was carried out using unpaired Student's T test, and significance was confirmed at $p \leq 0.05$. N represents number of mice while n represents number of cells. The results were expressed as a percentage of responding cells and as a percentage of Ionomycin fluorescence response.

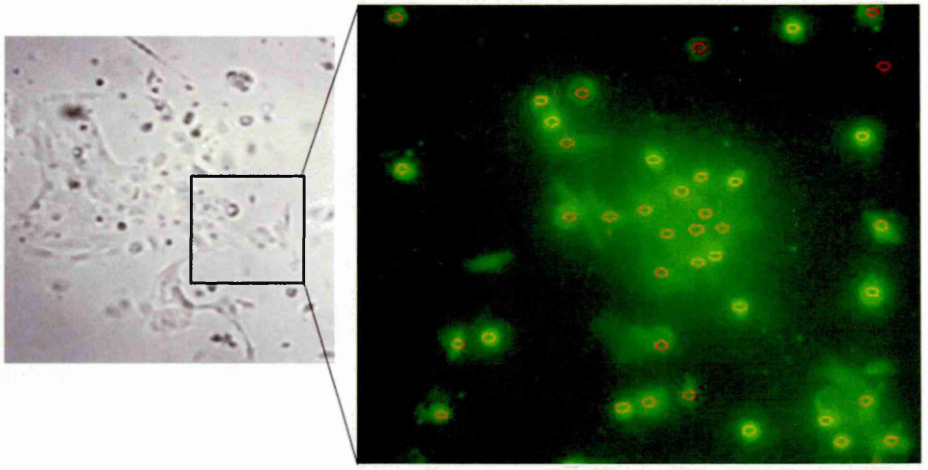


Figure 52 Example of a single view field of urothelial cells loaded with the calcium-sensitive dye fura2-AM. Individual urothelial cells were marked (red circles) to allow recordings of the intracellular Ca^{2+} signal.

Drugs and Solutions

MEM, dispase, PSF, Trypsin EDTA, FBS, Keratinocyte media, fura-2-acetoxymethyl ester (fura2-AM) were obtained from Invitrogen (California, USA). Collagen type IV, Ionomycin, HEPES buffer salt, αβ-Methylene ATP and βγ-Methylene ATP were obtained from Sigma Aldrich (Poole, UK).

3.3 RESULTS

3.3.1 EFFECT OF AGEING ON THE CONTRACTILE RESPONSE TO CHOLINERGIC AND PURINERGIC STIMULATION OF THE MURINE DETRUSOR

Tissue weight and baseline measurements of contractions in adult and aged detrusor strips

The wet tissue weight was measured to normalise the obtained results for tissue size. No significant difference in the weight of the detrusor strips of adult (10.2 ±0.6 mg) and aged (8.6 ±0.7 mg) mice could be detected. This suggests that the muscle mass does not differ between the two groups (Student's T test, N=6, n=10, Figure 53 A).

Peak amplitude and frequency of the contractile response was analysed for denuded detrusor strips of adult and aged mice. Baseline measurements over a time frame of two minutes before drugs were added did not show a significant difference in peak amplitude between adult (0.015 ±0.001 g/mg tissue) and aged (0.020 ±0.002 g/mg tissue) tissue strips of the mouse detrusor (Student's T test, N=6, n=10, Figure 53 B). Furthermore, no significant difference could be observed comparing frequency of adult (7.40 ±1.59 contractions per minute) and aged (10.05 ±2.65 contractions per minute) detrusor strips (Student's T test, N=6, n=10, Figure 53 C).

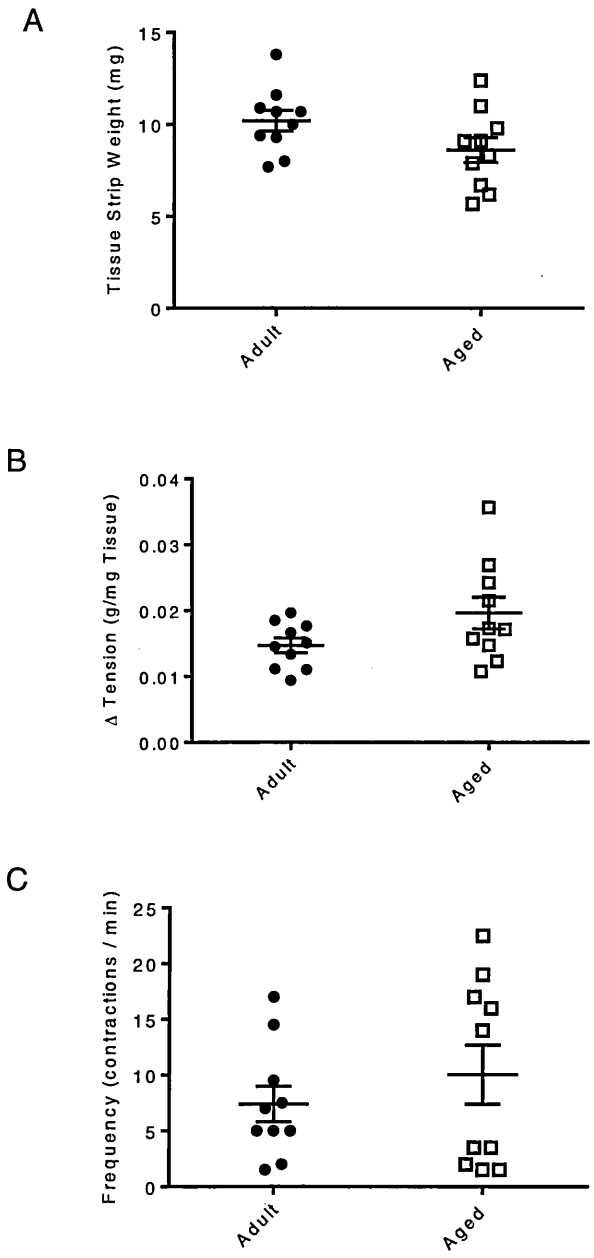


Figure 53 **Wet tissue weight and baseline measurements of amplitude and frequency of spontaneous contraction (mean ±SEM). (A) Tissue weight did not significantly differ between adult and aged detrusor strips (unpaired Student's T test, N=6, n=10). (B) No significant difference could be observed in the amplitude (change in tension) of baseline measurements between adult and aged detrusor strips (unpaired Student's T test, N=6, n=10). (C) No significant difference could be observed in the frequency (contractions/min) of baseline measurements between adult and aged detrusor strips (unpaired Student's T test, N=6, n=10).**

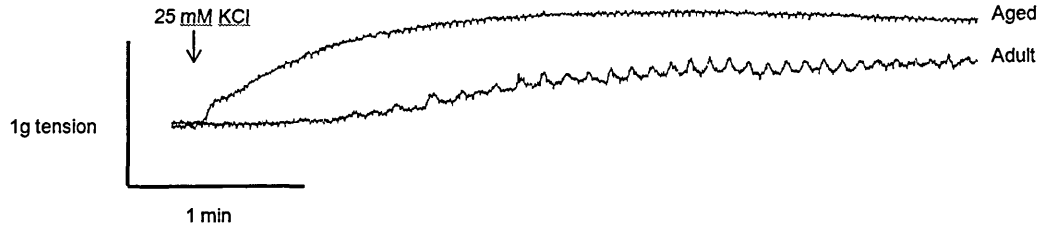
Contractile response to depolarisation with KCl

Sample traces of the contractile response to depolarisation with 25 and 65 mM KCl in aged and adult mice detrusor strips are shown in Figure 54 A and B.

Peak amplitude after application of KCl was significantly greater in aged mice tissue strips compared to adult mice tissue strips at 25 mM (0.04 ±0.008 to 0.09 ±0.013 g/mg tissue; Student's T test; N=6, n=10; p=0.005) as well as at 65 mM (0.11 ±0.018 to 0.19 ±0.020 g/mg tissue; Student's T test; N=6, n=10; p=0.009) (Figure 55 A).

Frequency of contractile response was not significantly different between adult and aged mice tissue strips at 25 mM KCl (1.64 ±0.68 to 0.91 ±0.40 contractions/min; Student's T test, N=6, n=10). In the aged tissue strips no contractions were counted after application of 65 mM KCl, as the contractions did not return below the 30% threshold, which was necessary to define a new contraction event with the used definition for analyses of frequency (see 3.2.1.) (0.23 ±0.16 to 0.00 ±0.00 contractions/min; N=6, n=10; Figure 55 B).

A



B

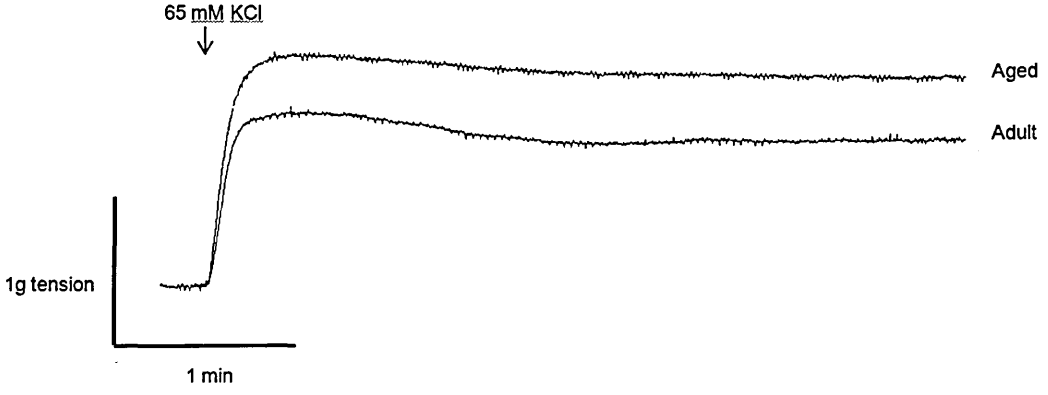


Figure 54 Sample traces of the contractile response to 25 mM KCl (A) and 65 mM KCl (B) in adult and aged tissue strips of the murine detrusor (not normalised for weight).

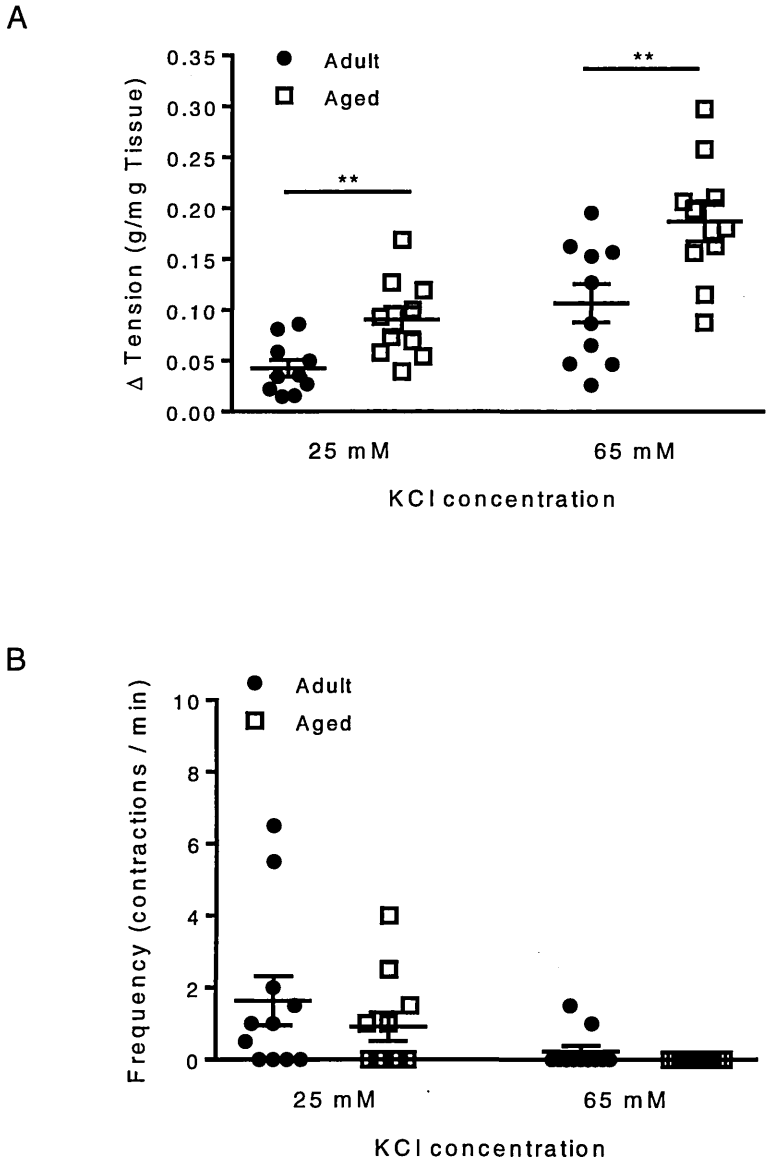


Figure 55 **Contractile response of aged and adult mouse detrusor strips to depolarising solution of KCl (mean ±SEM). (A) In aged muscle strips the amplitude of the contractile responses to 25 mM as well as to 65 mM KCl were significantly greater than in adult muscle strips (unpaired Student's T test, N=6, n=10, ** indicates $p \leq 0.01$). (B) No significant difference could be observed in the frequency of contractions between adult and aged muscle strips (unpaired Student's T test, N=6, n=10).**

Contractile response to ATP

ATP was bath applied at concentrations of 10 µM, 100 µM and 1 mM. The amplitude of the contractile response to ATP was significantly greater in muscle strips from aged mice compared to muscle strips from adult mice at concentrations of 10 µM (p=0.0034) and 1 mM (p=0.0095), whereas application of 100 µM showed no significant difference (unpaired Student's T test, N=6, n=10; Table 3, Figure 56 A).

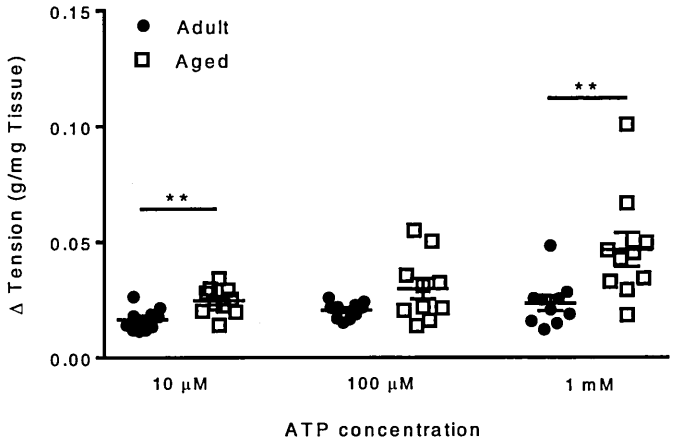
The frequency of the contractile response to ATP did not show a significant difference between tissue strips of adult and aged mice at any of the applied concentrations (unpaired Student's T test, N=6, n=10; Table 3, Figure 56 B).

	Adult	Aged	Significance
Amplitude (g/mg tension)			
10 µM ATP	0.017 ±0.002	0.025 ±0.002	p=0.003
100 µM ATP	0.021 ±0.001	0.030 ±0.004	n.s.
1 mM ATP	0.024 ±0.003	0.047 ±0.007	p=0.010
Frequency (contractions/min)			
10 µM ATP	9.600 ±2.326	8.050 ±2.130	n.s.
100 µM ATP	10.300 ±2.470	7.550 ±1.669	n.s.
1 mM ATP	10.850 ±1.780	9.300 ±2.459	n.s.

Table 3 Contractile response to ATP and bethanechol in denuded detrusor strips of adult and aged murine bladders (mean ±SEM; N=6, n=10).

(N = number of mice, n = number of tissue strips, n.s. – not significant)

A



B

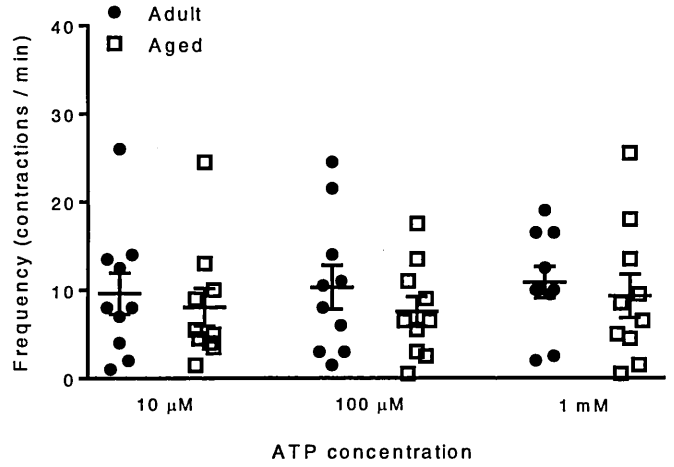


Figure 56 Contractile response in denuded detrusor strips of adult and aged mice after application of ATP (mean ±SEM). (A) Peak amplitude of contractions significantly differed after application of 10 µM and 1 mM ATP between tissue strips of adult and aged mice whereas no significant difference could be seen after application of 100 µM ATP (unpaired Student's T test, N=6, n=10; ** indicates $p \leq 0.01$). (B) Frequency of contractions did not significantly differ between adult and aged tissue strips at any concentration used (unpaired Student's T test, N=6, n=10).

Contractile response to Bethanechol

Bethanechol was cumulatively bath applied at concentrations of 1, 10 and 100 μM and therefore presented as a logarithmic graph (Figure 57). Peak amplitude of the contractile response to bethanechol was significantly greater in muscle strips from aged mice compared to muscle strips from adult mice (2-way ANOVA with Bonferroni's post-test, N=6, n=10; $p \leq 0.0001$; Table 4, Figure 57 A). Frequency of the contractile response to bethanechol did not show a significant difference between tissue strips from adult and aged mice (2-way ANOVA with Bonferroni's post-test, N=6, n=10; Table 4, Figure 57 B).

	Adult	Aged	Significance
Amplitude (g/mg tension)			
1 μM bethanechol	0.037 ±0.005	0.052 ±0.005	$p \leq 0.0001$
10 μM bethanechol	0.119 ±0.021	0.175 ±0.017	
100 μM bethanechol	0.169 ±0.027	0.257 ±0.027	
Frequency (contractions/min)			
1 μM bethanechol	3.550 ±1.237	4.400 ±1.108	n.s
10 μM bethanechol	0.750 ±0.485	0.150 ±0.107	
100 μM bethanechol	1.000 ±0.489	0.250 ±0.250	

Table 4 Contractile response to bethanechol in denuded detrusor strips of adult and aged murine bladders (mean ±SEM; N=6, n=10).

(N = number of mice, n = number of tissue strips, n.s. – not significant)

A

B

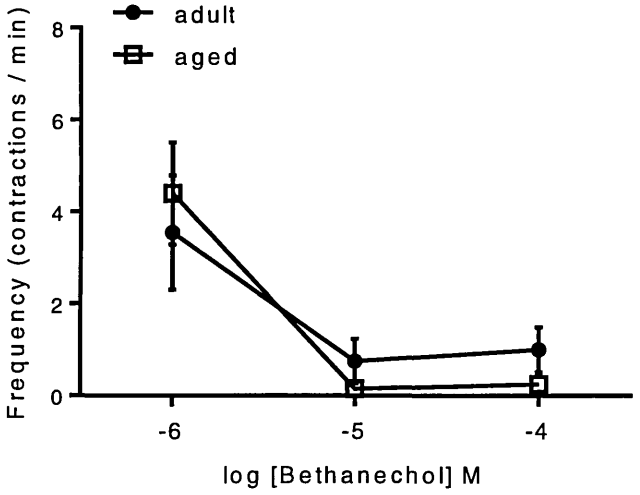


Figure 57 Contractile response in denuded detrusor strips of adult and aged mice after application of 1, 10 and 100 µM bethanechol. (A) Cumulative application of bethanechol did reveal a significant higher peak amplitude in tissue strips of aged mice compared to tissue strips of adult mice (2-way ANOVA with Bonferroni's post-test, N=6, n=10; ** indicates p≤0.01, ** indicates p≤0.0001). (B) Frequency of contractions did not significantly differ between adult and aged tissue strips (2-way ANOVA with Bonferroni's post-test, N=6, n=10).**

3.3.2 EFFECT OF AGEING ON MEDIATORS RELEASED FROM THE INTACT, ISOLATED MURINE BLADDER

Intra- and extraluminal samples of the intact, isolated murine bladder were screened for ACh, ATP, NO and Substance P. Bladder volume was calculated as filling rate (100 µl/min) multiplied by the time it took for the bladder to reach 50 mm Hg. The volume did not significantly differ between adult (283 ±14 µl, n=6) and aged (361 ±33 µl, n=8) mice (unpaired Student's T test, Figure 58). Therefore, compliance (ΔV/ΔP) is also not significantly different between adult and aged mice as the same pressure was applied to all bladders.

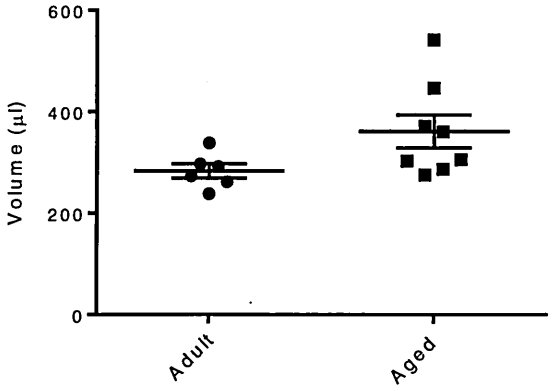


Figure 58 Bladder volume of aged and adult murine bladders after distension to 50 mm Hg (mean ±SEM). Bladder volume did not significantly differ between adult and aged mice bladders (n=6 and n=8 respectively).

ACh, ATP, NO and Substance P in Intraluminal Samples

ACh, ATP and NO could be detected in all tested intraluminal samples, however Substance P was not detected in any of the analysed intraluminal samples.

ACh was detected at 67 (±16) pmole/min in intraluminal samples of the adult mice. In intraluminal samples of the aged mice the amount of detected ACh was significantly lower at 17 (±8) pmole/min (unpaired Student's T test, n=6 and 7 respectively, p=0.011, Figure 59 A).

ATP was detected at 0.42 (±0.15) pmole/min in intraluminal samples of the adult mice. In intraluminal samples of the aged mice the amount of detected ATP was significantly greater at 0.94 ±0.12 pmole/min (unpaired Student's T test, n=6, p=0.023, Figure 59 B).

In intraluminal samples of adult mice bladders NO was identified at 117 (±21) pmole/min. This did not significantly differ to the amount of NO identified in intraluminal samples of aged mice bladders at 123 (±22) pmole/min (unpaired Student's T test, n=6 and 8 respectively, Figure 59 C).

Substance P could neither be detected in intraluminal samples from control bladders nor in intraluminal samples from aged bladders (n=9 and 8 respectively). The standard curve (3.9-500 pg/ml, Figure 51) showed a linear line from 8-150 pg/ml and was in accordance to the manufacturer's protocol. All the used standard samples included in the kit were identified accurately; however Substance P could not be detected in any of the tested intraluminal samples of the adult and aged mice bladder.

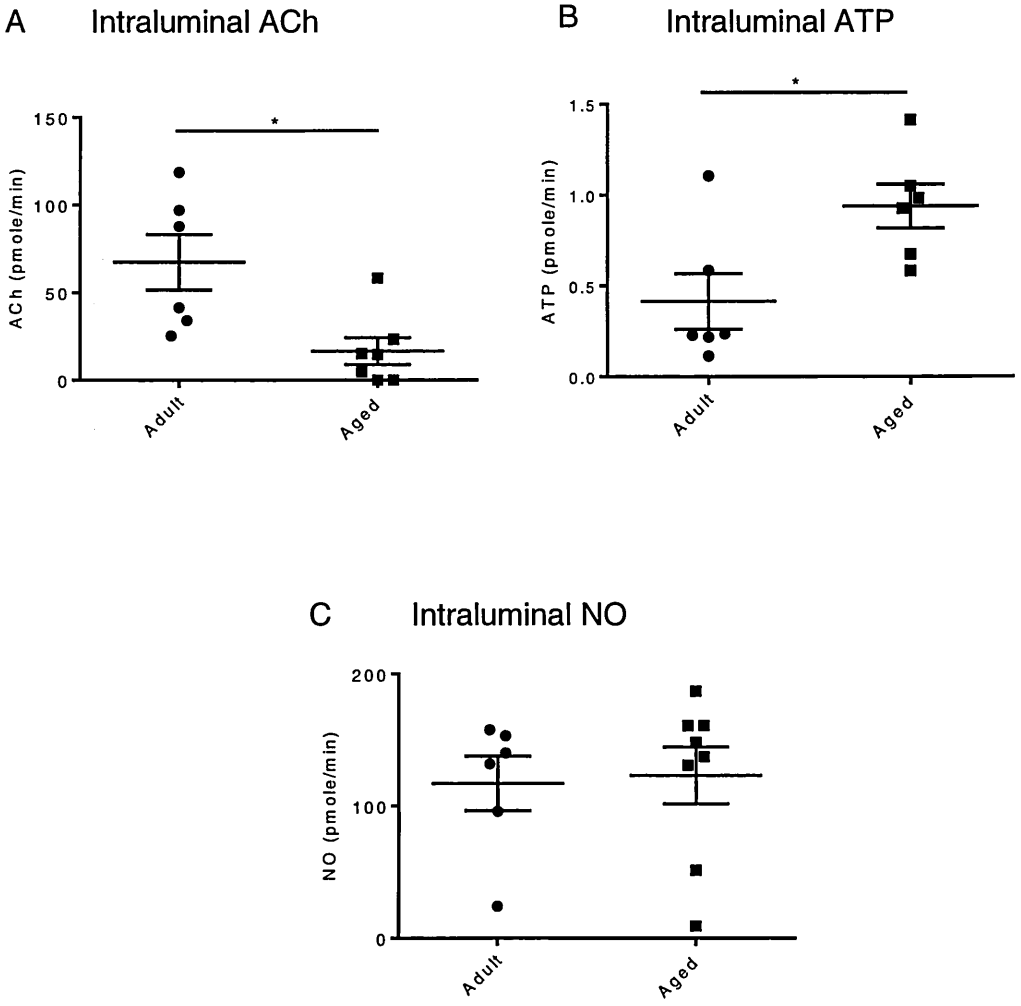


Figure 59 **ACh, ATP and NO in intraluminal samples of adult and aged murine bladders after distension to 50 mm Hg (mean ±SEM). (A) ACh levels were significantly lower in the intraluminal samples of aged bladders compared to the samples of adult bladders, whereas (B) intraluminally detected ATP was significantly increased in the aged bladder compared to adult bladder (unpaired Student's T test, n=6 and n=7 respectively, * indicates $p \leq 0.05$). (C) Intraluminally detected NO did not significantly vary between adult and aged mice bladders (unpaired Student's T test, n=6 and n=8 respectively).**

ACh and ATP in Extraluminal Samples

Extraluminal samples were analysed for ACh and ATP. Due to restricted sample volume and assay size, extraluminal samples were not examined for NO and Substance P.

ACh was detected at 54 (±28) pmole/min in extraluminal samples of the adult mice. In extraluminal samples of the aged mice the amount of detected ACh was significantly greater at 182 (±36) pmole/min (unpaired Student's T test, n=6 and 7 respectively, p=0.020, Figure 60 A).

ATP was detected at 1.40 (±0.33) pmole/min in extraluminal samples of the adult mice. This was not significantly different to the amount of ATP found in intraluminal samples of the aged mice at 1.75 (±0.13) pmole/min (unpaired Student's T test, n=6 and 7 respectively, Figure 60 B).

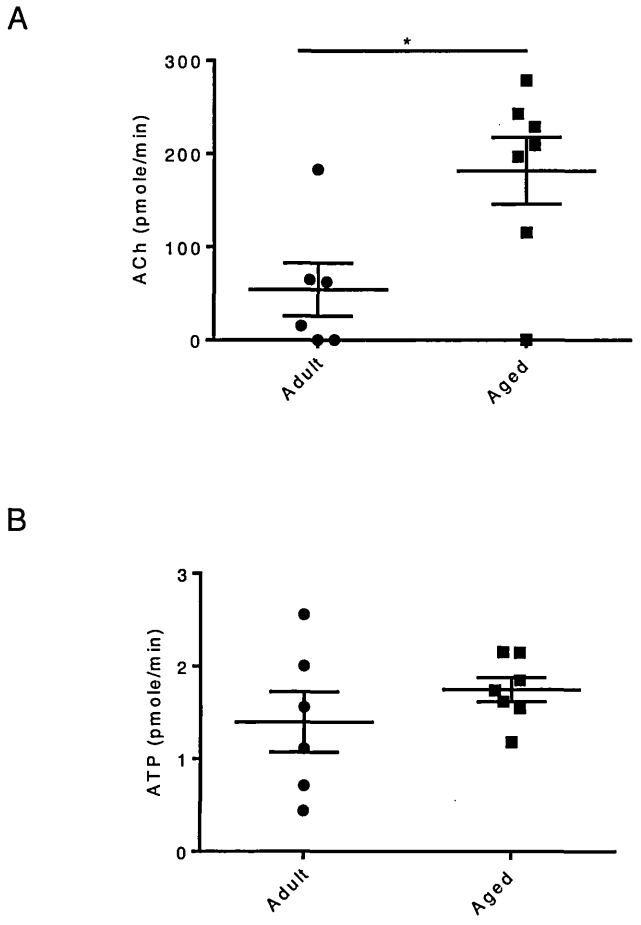


Figure 60 **ACh and ATP in extraluminal samples of adult and aged murine bladders after distension to 50 mm Hg (mean ±SEM). (A) ACh levels were significantly higher in the extraluminal samples of aged bladders compared to the samples of adult bladders (unpaired Student's T test, n=6 and n=7 respectively, * indicates $p \leq 0.05$). (B) No significant difference could be seen when comparing ATP levels in extraluminal samples of adult and aged bladders (unpaired Student's T test, n=6 and n=7 respectively).**

3.3.3 EFFECT OF AGEING ON SPONTANEOUS CONTRACTIONS IN THE WHOLE, ISOLATED MURINE BLADDER

Spontaneous contractions were observed as small transient rises in intraluminal pressure in the whole isolated murine bladder. Bladders of adult and aged mice were distended to 10 and 20 mm Hg while the contractions were recorded. An example of a typical trace can be found in Figure 61 A. Amplitude and frequency measurements were analysed using a custom designed script.

After distension to 10 mm Hg, spontaneous contractions were seen in 6 out of 8 aged mice bladders (75%) and in 5 out of 6 adult mice bladders (83.33%). After distension to 20 mm Hg, spontaneous contractions could be observed in all aged mice bladders (100%) and again in 5 out of 6 adult mice bladders (83.33%) (Figure 61 B).

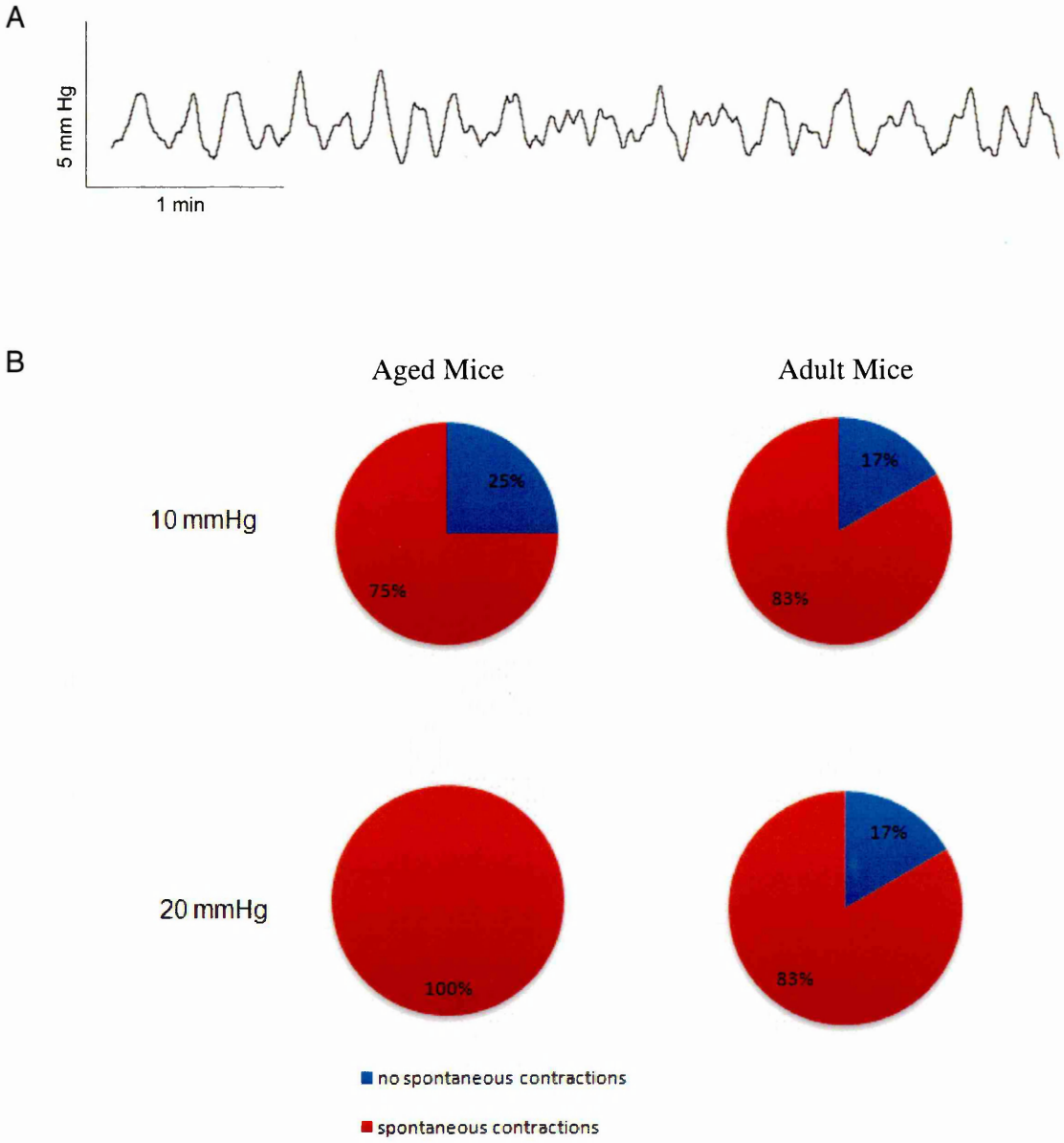


Figure 61 **Sample trace and occurrence of spontaneous contractions in aged and adult mice bladders. (A) Typical sample trace of spontaneous contractions occurring in the mouse bladder after distension. (B) Percentage of spontaneous contractions occurring in isolated, whole bladders of aged (n=8) and adult (n=6) mice after distension to 10 and 20 mm Hg.**

The amplitude of spontaneous contractions was measured at 2.14 (±0.73) mm Hg in adult murine bladders and at 2.56 (±0.62) mm Hg in aged murine bladders after distension to 10 mm Hg. After distension to 20 mm Hg the amplitude of spontaneous contractions was measured at 2.48 (±0.91) in adult murine bladders and at 2.90 (±0.72) mm Hg in aged murine bladders. No significant difference was observed between adult and aged bladders, neither at distension to 10 mm Hg not at distension to 20 mm Hg (unpaired Student's T test, adult n=6, aged n=8, Figure 62 A).

The frequency of spontaneous contractions was measured at 4.77 (±0.47) contractions/min in adult murine bladders and at 4.50 (±0.33) contractions/min in aged murine bladders after distension to 10 mm Hg. After distension to 20 mm Hg the frequency of spontaneous contractions was measured at 4.43 (±0.39) contractions/min in adult murine bladders and at 4.30 (±0.36) contractions/min in aged murine bladders. No significant difference was observed between adult and aged bladders, neither at distension to 10 mm Hg not at distension to 20 mm Hg (unpaired Student's T test, adult n=6, aged n=8, Figure 62 B).

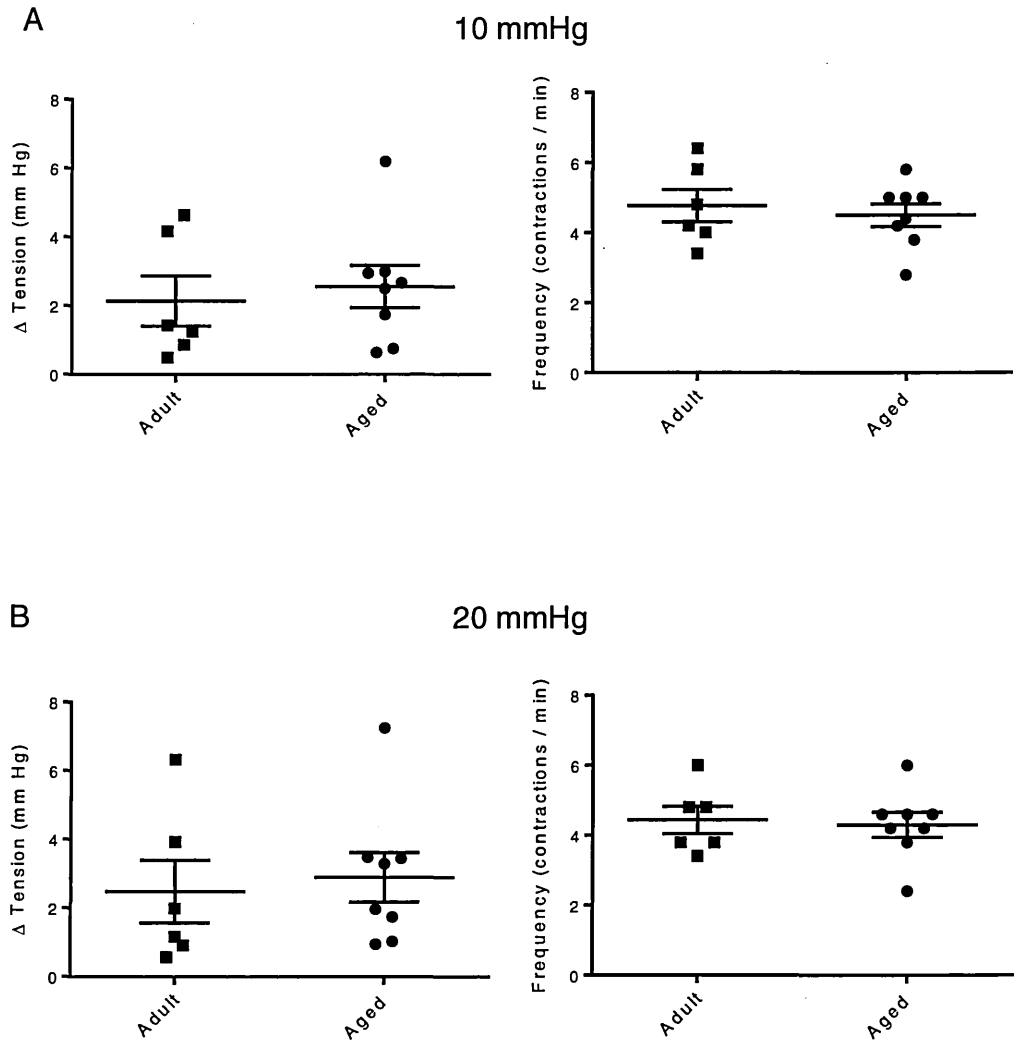


Figure 62 Amplitude and frequency of contractions in the whole, isolated bladder of aged and adult mice (mean ±SEM). No significant difference could be detected in the frequency and amplitude between aged and adult mice bladders at distension to 10 (A) and 20 mm Hg (B) (unpaired Student's T test, adult n=6, aged n=8).

3.3.4 EFFECT OF AGEING ON CA^{2+} RELEASE IN PURINERGIC SIGNALLING PATHWAYS IN UROTHELIAL CELLS

Calcium imaging experiments on isolated urothelial cells were carried out to investigate the effect of ageing on specific purinergic signalling pathways in these cells. Figure 63 shows a typical trace of the change in fluorescence in urothelial cells in response to ATP and ionomycin.

Two purinergic agonists were used and concentrations calculated using suggestions by Alexander *et al.* (2011). The agonist of $P2X_1$ and $P2X_3$ receptor, αβMeth-ATP was used at a working concentration of 30 μM. The agonist of $P2X_1$ receptor, βγMeth-ATP was used at a working concentration of 100 μM.

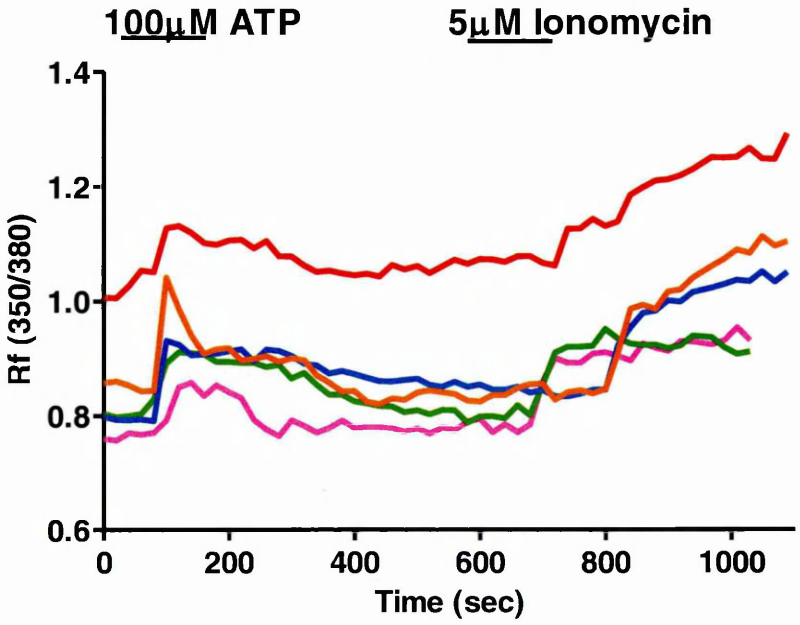


Figure 63 Sample traces of intracellular calcium response in five urothelial cells to ATP (100 μM) and to the ionophore ionomycin (5 μM) expressed as relative fluorescence (RF 350/380 nm).

Significantly more urothelial cells from aged bladders (N=5, n=218) responded to $P2X_1$ and $P2X_3$ receptor agonist αβMeth-ATP compared to adult cells (N=5, n=175, unpaired Student's T test, $p \leq 0.01$,). The magnitude of response to αβMeth-ATP between cells from adult (33.4 ±3.9% Ionomycin) and aged mice (42.6 ±7.0% Ionomycin) was not significantly increased (unpaired Student's T test, Figure 64).

The proportion of responding cells to the $P2X_1$ receptor agonist βγMeth-ATP did in contrast not change between cells from adult (N=4, n=189) and aged bladders (N=5, n=239, unpaired Student's T test). Also the magnitude of response of βγMeth-ATP did not show a difference between cells from adult (44.9 ±3.5% Ionomycin) and aged (36.1 ±6.4% Ionomycin) mice bladders (unpaired Student's T test, Figure 64).

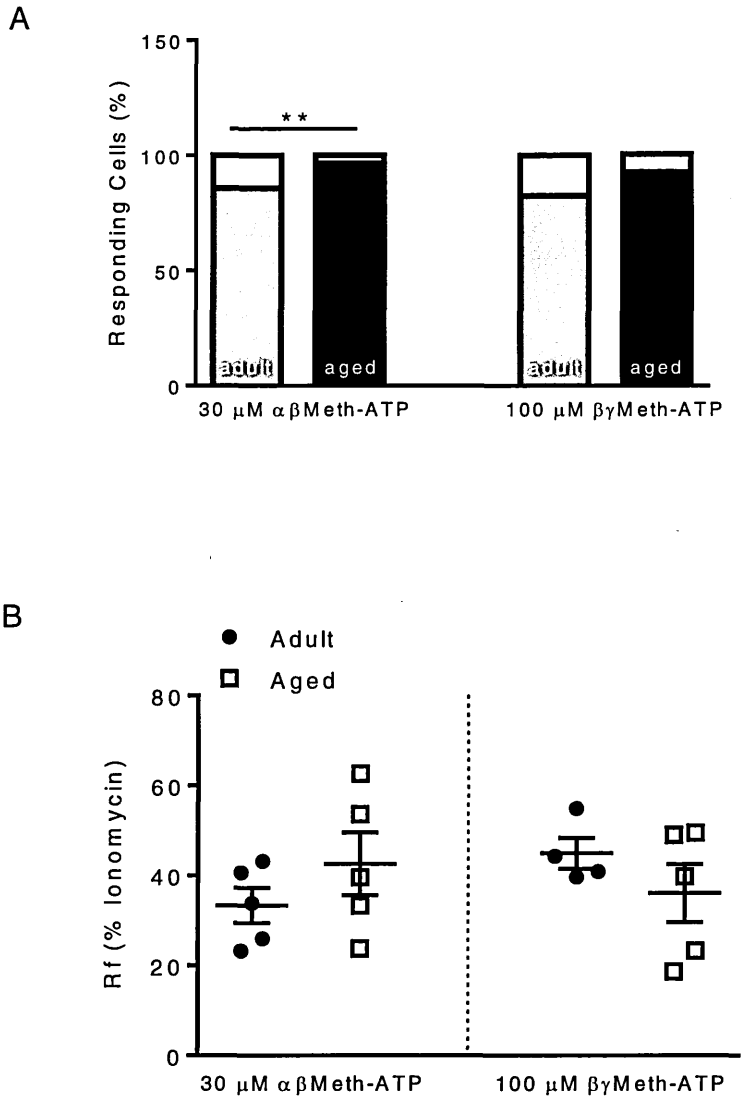


Figure 64 **Percentage of responding cells and magnitude of response to the selective $P2X_{1/3}$ receptor agonist αβMeth-ATP and the selective $P2X_1$ receptor agonist βγMeth-ATP. (A) The responding cells are presented in % of the total amount of cells observed. In aged mice the number of urothelial cells responding to αβMeth-ATP (30 μM) was significantly greater compared to cells from adult mice (unpaired Student's T test, adult: N=5, n=175, aged: N=5, n=218, ** indicates $p \leq 0.01$). The number of cells responding to βγMeth-ATP (100 μM) does however not change between the two groups (unpaired Student's T test, adult: N=4, n=189, aged: N=5, n=239). (B) The magnitude of response was not significantly different between the two groups after application of αβMeth-ATP or βγMeth-ATP (mean ±SEM, unpaired Student's T test).**

(N = number of mice, n = number of cells, RF - ratio fluorescence)

3.4 DISCUSSION

The aim of this chapter was to investigate age-related changes in urothelial signalling and in detrusor function. In aged mice altered ATP and ACh bioavailability was measured as transmitter release into the bladder lumen. These changes were concurrent with increased detrusor contractility in the aged bladders after stimulation of purinergic and muscarinic receptor. It was shown in Ca^{2+} imaging experiments that these changes possibly occur due to changes in $P2X_3$ receptor sensitivity.

3.4.1 AGEING ENHANCED AGONIST EVOKED DETRUSOR CONTRACTIONS

Spontaneous contractions (baseline measurement without agonist added) were measured in the whole, isolated bladder of adult and aged animals as transient rises in intraluminal pressure. In addition, spontaneous contractions were measured in denuded bladder strips of adult and aged mice. The detrusor was used in isolation to clearly assign the effects to the bladder muscle without having to consider the influence of the urothelium. The urothelium was therefore carefully removed from the detrusor and used for Ca^{2+} imaging experiments.

The whole, isolated bladders of adult and aged mice did not reveal a significant difference in amplitude and frequency of spontaneous contractions after distension to 10 and 20 mm Hg. Moreover, amplitude and frequency of spontaneous contractions in the denuded detrusor strips did not significantly differ between the two groups. These results suggests that the contractility of the bladder is not changing during ageing, neither in the denuded detrusor muscle strips nor in the intact, *in vitro* bladder organ after distension.

A recent urodynamic study by Smith *et al.* (2012) showed no significant alterations in nonvoiding contractions with increasing age in the female mice under *in vivo* conditions. Another urodynamic study on men and women over 40 years of age demonstrated no significant change for maximum detrusor pressure and detrusor pressure at peak flow rate with increasing age (Madersbacher *et al.*, 1998). It can therefore be assumed that detrusor contraction strength does not decline in the ageing bladder.

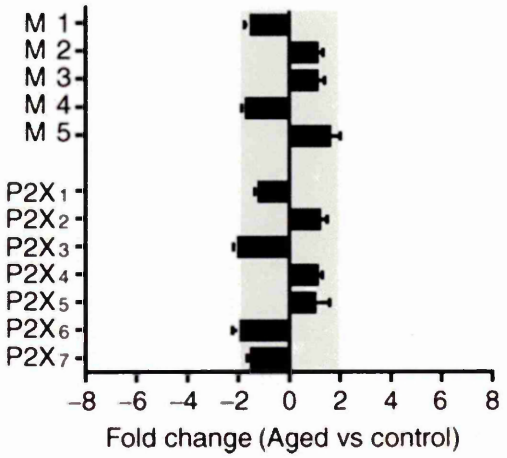
A difference between bladders from adult and aged mice could however be seen with the occurrence of spontaneous activity at specific pressure levels. While 83% of adult bladders showed spontaneous contractions at 10 as well as 20 mm Hg, only 75% of aged bladders showed spontaneous contractions after distension to 10 mm Hg and all aged bladders showed spontaneous contractions after distension to 20 mm Hg. Results of voiding pattern analyses demonstrated that aged mice show an increase of voiding events, while voiding smaller volumes of urine compared to adult mice (Daly *et al.,* 2014). Considering that the voiding contractions set in around 22 mm Hg in the adult mouse (Smith *et al.,* 2012), it could be proposed that the voiding threshold lowers with advancing age. The underlying mechanism of this change in voiding pattern in the aged mice could be of morphological origin. The detrusor contraction strength has however been shown to be similar in the two age groups. Therefore changes in the signalling pathways in the aged mice bladder were studied.

The amplitude of ATP and bethanechol-induced contractions were indeed significantly increased in the denuded detrusor strips from aged mice compared to adult mice while the frequency of contractions was not significantly different. This suggests a higher sensitivity of the aged detrusor tissue to the used purinergic and muscarinic agonist, which results in an increased strength of contraction but has no effect on the number of contractions. Muscarinic receptor density has been reported to be increased in the bladder base of old rats (Kolta *et al.,* 1984) and rabbits (Latifpour *et al.,* 1990) compared to adult animals. The higher sensitivity of the aged bladder tissue to the applied agonists could therefore be due to a higher density of receptors in the aged mouse.

Studies by colleagues have however shown that mRNA expression for muscarinic and purinergic receptors in the mouse detrusor are not influenced by age (Daly *et al.,* 2014). Expression of mRNA was identified for all five muscarinic receptor genes, M1 to M5, with M2 and M3 exhibiting the greatest expression, and all seven of the P2X receptor genes, $P2X_1$ to $P2X_7$, with $P2X_1$ exhibiting the greatest expression. The results were expressed as relative expression to the housekeeping gene GAPDH and the fold change compared to the expression in adult bladder tissue was calculated. No significant difference in gene expression was detected between adult and aged detrusor samples (Figure 65 A). The

increased overall contractile ability of the aged mouse detrusor could nevertheless be due to an increase in receptor expression in the aged mouse detrusor, as various processes are in place to regulate mRNA translation into protein. These could be translational regulations, protein degradation regulation, or post-transcriptional regulations such as splicing. Further experiments are needed to reveal whether the receptor protein expression changes in the mouse detrusor with advancing age. Furthermore the data need to be confirmed in human tissue. An age-related decrease of $P2X_1$ mRNA expression was detected in detrusor biopsy specimen from men aged 30-86 years (Chua *et al.,* 2007). It could therefore be possible that various regulation processes are affected by ageing in the human bladder but not the murine bladder.

A Detrusor



B Urothelium

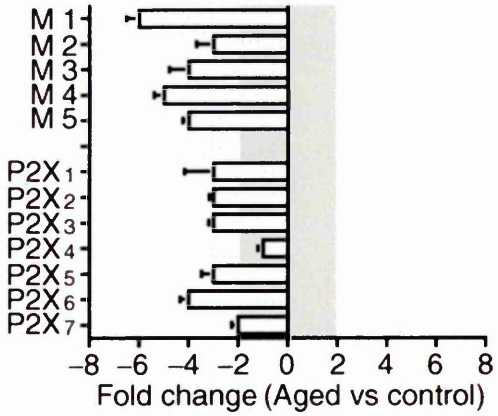


Figure 65 Fold change in gene expression in detrusor and urothelium of aged mouse relative to samples of the adult mouse (Daly *et al.*, 2014). (A) No significant difference was observed regarding the fold change of gene expression of aged detrusor samples relative to adult detrusor samples (adult n=9 and aged n=6). (B) A significant reduction in all muscarinic and all P2X receptor genes, except of $P2X_4$, was observed in the gene expression of aged urothelium samples relative to adult urothelium samples (adult n=8 and aged n=6).

The contractile response to chemical depolarisation with KCl was also significantly increased in the detrusor strips of aged animals compared to strips of adult animals. Possible explanations for this greater contractile response to depolarisation are changes in the ion permeability of the cell membranes, faster travelling action potentials, or a decline in the activation threshold. The results are in line with a study by Longhurst *et al.* (1992) showing an increase of the contractile response to 60 mM KCl in 24 month old male and female rats compared to 3 month old male and female rats.

The tested agonists, ATP and bethanechol, as well as the depolarisation with KCl, induced a greater contractility in the aged detrusor strips compared with the adult detrusor strips, while spontaneous contractions in tissue strips and in the whole organ were not altered. Furthermore, the expression of muscarinic and purinergic receptor expression in the detrusor was not significantly different between adult and aged bladders. Therefore the functional changes seen in the voiding pattern of the aged mice could be due to changes in urothelial signalling function. It could indeed be demonstrated in the lab that gene and protein expression in the urothelium are altered in the aged bladder compared to the adult bladder (Daly *et al.,* 2014). PCR analyses of mRNA expression found a significant reduction of all muscarinic and all P2X receptor genes, apart from $P2X_4$, in the aged urothelium compared to the adult urothelium (Figure 65 B). The reason for this down regulation could be an increased release of urothelial mediators. To further investigate whether the role of the urothelium in signalling processes changes with advancing age, experiments were carried out to determine release of ACh, ATP, NO and Substance P in intra- and extraluminal samples of the whole, isolated bladder of adult and aged mice.

3.4.2 AGEING IS ASSOCIATED WITH AN INCREASE IN ATP AND DECREASE IN ACH IN INTRALUMINAL BLADDER SAMPLES

While the bladder volume of adult and aged animals was comparable, ACh detected in intraluminal samples was significantly reduced, whereas intraluminal ATP was significantly increased in aged bladders compared to the adult bladder. The obtained results are in line with studies by Yoshida *et al.* (2001, 2004), showing that increasing age is associated in a linear manner with a decrease in ACh release and an increase in ATP release from human bladder strips (Figure 66). This indicates that the alterations occurring in the urothelial signalling pathways during age in the murine bladder can be compared to the human bladder.

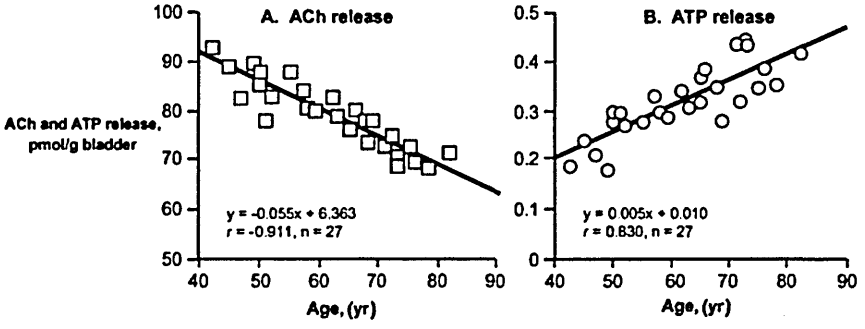


Figure 66 Correlation between age and ACh/ATP release from human bladder strips (Yoshida 2004). (A) Negative correlation could be seen between age and ACh release. (B) Positive correlation could be seen between age and ATP release.

Further mediators that were analysed were NO and Substance P. Nerves containing NO synthase have been shown to innervate the urothelium in 3 month old as well as 24 month old male rats with a slightly reduced nerve density in the aged rat urothelium (Mohammed and Santer 2001). The amount of NO found in intraluminal samples of the adult and the aged mouse bladder in this study was however extremely similar. It can be assumed that NO does not play an important role in the physiological changes occurring in the aged bladder.

Substance P containing nerve fibres have been detected in both the rat and human bladder and have been reported to be increased in the urothelium (but not the detrusor) of IC patients (Pang *et al.,* 1995). Substance P was analysed in intraluminal samples of the mouse bladder to examine age-related changes of this mediator. Substance P could however not be detected in any of the samples of adult or aged mice. Studies exist on different species that indicate a role of Substance P in urothelial signalling. Tachykinin receptors are expressed in cat urothelial cells (Birder *et al.,* 2010) and in porcine urothelial cells (Bahadory 2013). Furthermore Substance P had been demonstrated in urothelial cell lysates of cats and an increase in Substance P could be shown in cats with feline cystitis (Birder *et al.,* 2010). The role of Substance P in signalling processes in the urothelium might therefore be species specific or dependent on certain circumstances such as the onset of a specific disease.

In the present study, the concentration of mediators in samples of the whole, isolated bladder was measured. Taking the knowledge of urothelial signalling and the results from Chapter 2 into consideration, it can be assumed that the urothelium is the main source of intraluminal mediators. It is however possible that other cell types, such as nerve endings or ICs, are releasing the mediators detected in the examined samples. It is also possible that the breakdown and reuptake of mediators has changed in the aged bladder and not the assumed release of mediators. Therefore further calcium imaging experiments were carried out with isolated urothelial cells, to further investigate altered signalling pathways.

Ageing is Associated with an Increase in ACh in Extraluminal Bladder Samples

ACh levels were significantly higher in the extraluminal samples of aged bladders compared to the samples of adult bladders, while no significant difference could be seen when comparing ATP levels. It is not clear which cell types release the mediators but it is likely to be the detrusor muscle cells and nerve cells. It can therefore be suggested that ACh mediated cholinergic components of detrusor signalling become more important with age. This is in line with the obtained results, showing a significant increase in the contractile response of aged detrusor strips after muscarinic stimulation. Due to restricted assay and sample size, NO and Substance P were not measured in extraluminal samples.

3.4.3 AGEING ALTERED THE PURINERGIC SIGNALLING TRANSDUCTION IN UROTHELIAL CELLS

Intracellular signalling transduction can be made visible with calcium imaging technique as fluorescence properties get activated upon binding to Ca^{2+} making it possible to record the calcium status of the cells.

Calcium imaging experiments carried out by colleagues (Daly *et al.*, 2014) showed that ageing had no effect on urothelial response to the muscarinic agonist bethanechol. However the mobilisation of Ca^{2+} in urothelial cells from aged mice was increased by the purinergic agonist ATP. Therefore the effect of agonists on specific purinergic receptor subtypes was studied as part of this thesis. The selective $P2X_1$ and $P2X_3$ receptor agonist αβMeth-ATP produced a greater signal in urothelial cells from aged bladders, while no significant difference could be detected after applying the $P2X_1$ selective agonist βγMeth-ATP. Consequently it was assumed that the altered purinergic signalling is likely to be mediated via the $P2X_3$ receptor.

Colleagues verified the presented calcium imaging data by showing that the $P2X_3$ receptor expression is significantly higher in the aged urothelium compared to adult urothelium using Western blot analyses (Daly *et al.*, 2014). In contrary mRNA expression was significantly decreased in the urothelium of aged mice compared to adult mice, suggesting

that regulation processes from mRNA translation into protein are altered in the aged mouse urothelium.

The shown data provide interesting insights of physiological changes occurring in the bladder of the naturally aged mouse, suggesting a higher activity and sensitivity of the aged bladder. The results revealed an increase of ATP and decrease of ACh in intraluminal samples, as well as increased purinergic receptor sensitivity. This is possibly facilitated via the purinergic $P2X_3$ receptor. Further characterisations of the studied pathways are now required to fully validate the data, most suitably in human tissue, as it is not clear if the same pathways are affected by age in the human bladder.

4. THE ROLE OF THE UROTHELIUM AND REGIONAL DISPARITIES IN THE CONTRACTILITY OF TISSUE STRIPS FROM THE PORCINE BLADDER

4.1 INTRODUCTION

The physiological relevance of spontaneous activity in the bladder detrusor and the factors that regulate the magnitude and frequency of these contractions are not fully understood to date. It is assumed that these contractions communicate information on the bladder filling state. In the present study spontaneous activity and carbachol-induced activity from intact and denuded bladder strips of the porcine bladder were examined. The data were analysed for differences between intact and denuded tissue strips individually for the three bladder regions. Furthermore, regional disparities between dome, body and trigone were studied and a possible gender difference of the contractile activity was examined.

Figure 67 shows the three regions of the bladder, the dome, the body and the trigone.

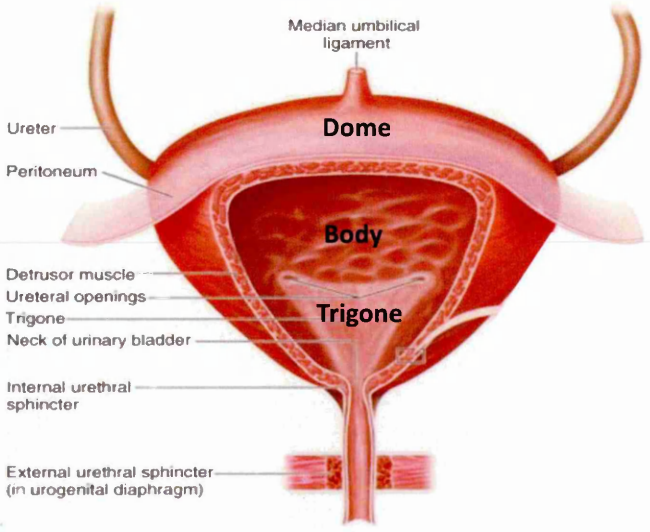


Figure 67 The three regions of the urinary bladder, dome, body and trigone (adapted from McKinley and O'Loughlin 2006).

Role of the Urothelium in Bladder Contractions

As previously outlined, the urothelium is not just a barrier with low water and urea permeability which has the ability to accommodate stretch, it also has a sensory role in transmitting information to underlying nervous and muscular tissue as well as ICs and neighbouring urothelial cells by sensing and communicating changes in the extracellular environment. The urothelium may therefore play an important role in processing bladder mechanosensation and in modulating the contractile activity of the bladder detrusor.

The urothelium has indeed been shown to inhibit contractile activity induced by various mediators. For example, Substance P responses in the guinea pig detrusor were reduced by the presence of the urothelium (Maggi *et al.,* 1987). The effect was first thought to be due to mechanical obstruction by the urothelium, which blocked the free access of the mediator to the underlying muscle. However, it could later be shown, that the inhibitory effect of the urothelium to the Substance P response is due to the high peptidase activity in the urothelium which breaks down the peptide Substance P (Saban *et al.,* 1992). Other mechanisms must however be in place, as the urothelium also inhibits responses to muscarinic agonists, purinergic agonists, potassium, or electric field stimulation as shown by Levin *et al.* (1995). Removal of the urothelium from pig bladder strips of the dome region furthermore resulted in postponed development of spontaneous activity compared to intact tissue strips (Akino *et al.,* 2008). This also indicated a role for the urothelium in the development and regulation of contractile activity of the detrusor.

Hawthorn *et al.* (2000) was the first to show, that bladder contractions of a denuded pig bladder strip were inhibited in the presence of a second intact bladder strip. There was no inhibition if the second strip was also denuded. It was suggested, that the urothelium releases a diffusible inhibitory factor, the urothelial-derived inhibitory factor (UDIF), as the inhibition could transfuse from one strip to another. Potassium stimulated contractions were not influenced by the presence of the urothelium and it was therefore suggested, that the inhibition is receptor mediated, possibly by muscarinic and/ or nicotinic receptors. Conversely Levin *et al.* (1995) stated that potassium stimulated contractions were inhibited by the urothelium in feline detrusor. This might be due to a species specific difference. It is

still controversial, whether the urothelium can inhibit potassium stimulated contractions and therefore if the inhibitory effect is receptor mediated.

Pharmacological investigation by Hawthorn *et al.* (2000) demonstrated that the UDIF is unlikely to be NO, adenosine, a cyclo-oxygenase product, a catecholamine or GABA, which represent the majority of well-established detrusor inhibitory pathways. Its identity has not been determined so far, and the inhibitory effect of the urothelium might be mediated by various mediators activating signalling cascades acting on different receptors of the detrusor.

This urothelium-derived inhibition also exists in the human bladder (Chaiyaprasithi *et al.,* 2003). It is furthermore reduced in diabetic rats, a model for detrusor overactivity (Kosan *et al.,* 2005). Therefore the reduction of UDIF could be one factor in the symptom complex overactive bladder.

For the mentioned study by Hawthorn *et al.* (2000) bladder strips from the dome region were used. Another study looking at strips from the trigone region also found that contractile responses to carbachol as well as to histamine were reduced in the presence of the urothelium (Templeman *et al.* 2002).

It is often not quoted in studies from which part of the bladder tissue strips were taken and different experiments on the function of the urothelium might not be comparable when taken from different bladder parts. In the present study not only the effect of the urothelium, but also the regional differences in the development of spontaneous and carbachol-induced contractility of the porcine bladder strips were therefore analysed.

Regional Disparities of Bladder Function

Limited information can be found on regional disparities in the development of spontaneous contractions. Sibley (1984) showed that 20% of 139 intact strips from the human dome developed spontaneous contractions while 71% of 7 strips from the human trigone showed spontaneous activity. Akino *et al.* (2008) studied the effect of urothelial removal on spontaneous activity in the dome and trigone of female pigs. Tissue strips from

the dome showed a delayed development of spontaneous contractions after removal of the urothelium compared to intact tissue strips. However in the strips from the trigone region spontaneous activity developed quickly, irrespective of the presence or absence of the urothelium. This suggests that the urothelium has little effect on the generation of spontaneous contractions in the trigone, while the urothelium has an influence on the development of spontaneous contraction in the dome, possibly through the release of mediators (Akino *et al.,* 2008). Another study by Sanchez Freire *et al.* (2011) looking at bladder biopsies revealed structural differences between the bladder dome and trigone. The investigators revealed that the dome contained higher smooth muscle content in comparison with the trigone, which implies a strongly developed network of myofibroblasts and muscularis mucosa. Furthermore, higher levels of mRNA expression for $P2X_1$, M2 and M3 could be found in the dome in comparison with the trigone indicating that cholinergic receptors might be up-regulated in the dome. mRNA expression for cellular adhesion and tight junction proteins were however up-regulated in the trigone compared to the dome (Sanchez Freire *et al.,* 2011). Regional differences could also be seen between dome, body and trigone by immunohistological studies looking at the cell distribution of neuronal NO synthase and cGMP in the guinea pig urothelium (Gillespie *et al.,* 2004). These studies demonstrate that structural and physiological differences between the different bladder regions exist. The present study addressed the question, whether frequency and amplitude of spontaneous and carbachol-induced contractions differ between the bladder dome, body and trigone.

Influence of Gender on Bladder Function

Sexual dimorphisms occur not only in the appearance of males and females of the same species, but also in multiple physiological functions in the body and in the prevalence of many diseases. Several studies have revealed that gender is a key biological variable that should be considered in all basic physiological and biological research (Gesensway 2001, Legato 2003, Arnold *et al.,* 2009).

At the age of 40-50 years lower urinary tract symptoms start to develop in both genders (Milsom *et al.,* 2001) and urodynamic finding reveal differences between men and women. In this decade, the male maximum flow rate lowers to about 60% of the female maximum flow rate. Corresponding residual urine and detrusor pressure in men are significantly higher than in females (Madersbacher *et al.,* 1998). The largest population-based study by Irwin *et al.* (2006) showed that the prevalence for storage lower urinary tract symptoms is greater in women than men while voiding and postmicturition symptoms occur more often in men than women. Regarding OAB, overall about 12% of the population are affected with an age dependent increase (see 1.5.3). However, women suffer more from OAB with urge incontinence (wet OAB) while men suffer more from OAB without urge incontinence (dry OAB) (Irwin *et al.,* 2006). Gender differences also exist in the incidence of detrusor overactivity, which has been reported significantly more often in males compared to females. Detrusor overactivity might not be the major underlying cause for female OAB while this is possible for the male OAB (Sekido *et al.,* 2006, Al-Ghazo *et al.,* 2011).

Urinary incontinence occurs generally twice as often in females than in males. The natural risk factors for incontinence in women are pregnancy and vaginal child birth which lead to persistent overstraining of the connective tissue and bladder descent (Findik *et al.,* 2012). Further risk factors for incontinence in women are diabetes mellitus, obesity as well as a genetic risk factor (Stothers and Friedman 2011). The main risk factor for incontinence in adult men is prostate surgery. In addition male urinary incontinence is linked to prostate cancer and prostatic hyperplasia (Stothers 2004).

While the mentioned studies show the clinical existence of gender differences in bladder function, limited data are published about the possible underlying mechanisms. This study investigates functional gender differences in the contractility of tissue strips from male and female porcine bladders, in order to reveal new insights into gender dependent differences in bladder physiology. This might be important to evaluate the requirement of gender-specific treatment for bladder disorders like OAB.

Aims

The aim of this chapter was to further characterise spontaneous and carbachol-induced contractions in the porcine bladder. Therefore the contractility of intact and denuded bladder strips was examined regarding the effect of the urothelium, regional differences between dome, body and trigone and possible gender differences.

Spontaneous activity as well as carbachol-induced contractions of intact and denuded strips of the three bladder regions dome, body and trigone from male and female porcine bladders was recorded. The data were analysed

- for differences in the development of spontaneous activity as well as carbachol-induced contractions between intact and denuded bladder strips.

- for regional disparities in the development of spontaneous activity as well as carbachol-induced contractions between bladder dome, body and trigone.

- for possible gender differences in the development of spontaneous activity as well as carbachol-induced contractions.

4.2 METHODS

4.2.1 CONTRACTION RECORDINGS OF INTACT AND DENUDED TISSUE STRIPS FROM THE PORCINE BLADDER

Tissue Preparation

Whole urinary bladders from male and female 'Large White Yorkshire pigs' and 'British White Landrace pigs' – aged 6-7 month – were collected from a local abattoir and placed in cold Krebs' solution within 30 minutes of the animal's death. Upon arrival in the laboratory, after about 40 minutes travel time, the tissue was processed immediately, stored in Krebs' solution at a temperature of 5°C and used within 24 hours. Male and female bladders could be distinguished easily by the differing anatomy of the urinary bladders (Figure 68). Male bladders were larger in size. Moreover the seminal vesicles and prostate glands could be located on male bladders.

The connective tissue and excessive fat was removed from the bladders. The bladder was then dissected as shown in Figure 69. The dome was located as the top third of the bladder and separated from the rest of the organ. The bladder was then cut open on the ventral side in a longitudinal direction and the bladder sheet spread out. After separating the bladder body and trigone, the tissue was stored in Krebs' solution in the fridge at 5°C.

Immediately before the tissue was set up in the organ bath, paired tissue strips of approximately 1cm length x 0.3cm width were cut in longitudinal direction from the dome, body and trigone sheets. The urothelium was carefully removed from one strip of each pair along the natural plane of dissection with scissors, while the second strip was left intact. This dissection method has been previously described by Sellers *et al.,* (2000), Templeman *et al.* (2002) and Akino *et al.* (2008). It is important to mention that through the blunt dissection several suburothelial cells might have been removed as well. In the following it will be referred to 'intact' and 'denuded' strips, to describe strips of tissue with the urothelium present and absent respectively.

All waste tissue was disposed off according to the university guidelines regarding animal tissue handling.

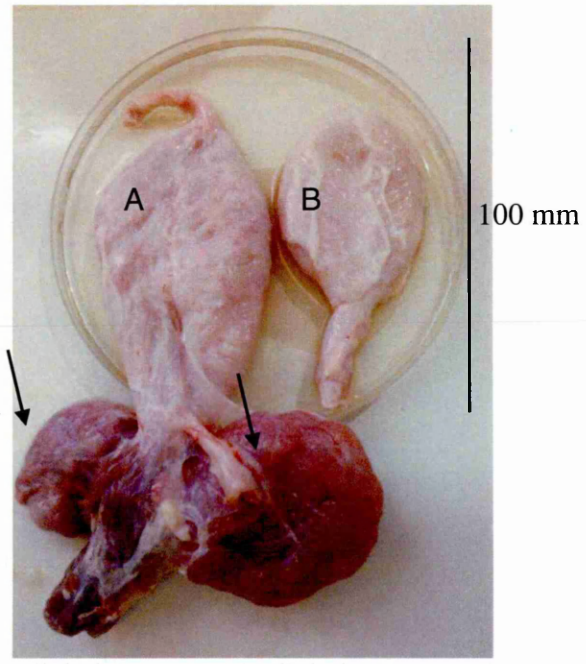


Figure 68 Male and female porcine bladders. (A) At the bladder neck of male bladders, the seminal vesicles (arrow) are very prominent. Furthermore the prostate gland could be located. (B) Female bladders were smaller in size.

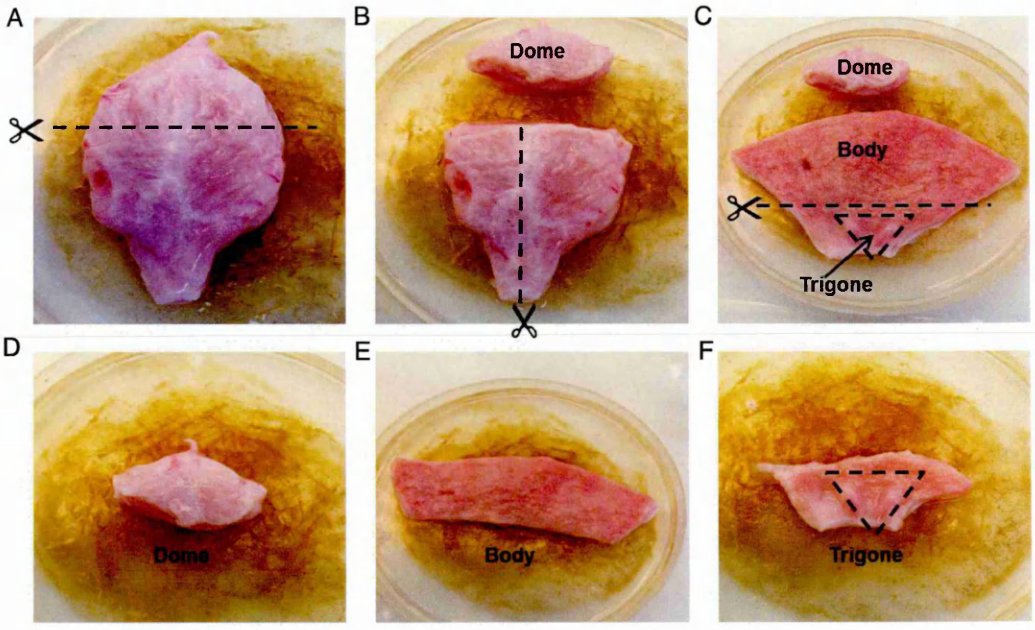


Figure 69 **Dissection of the porcine bladder to separate dome, body and trigone. (A) Separation of the dome. (B) Longitudinal cut through the ventral side of the bladder. (C) Body region is separated from the trigone. Trigone region is well defined by the urethral openings. (D, E, F) Separated dome, body and trigone region.**

Organ Bath set up

Pairs of intact and denuded strips from dome, body and trigone were mounted in 15 ml organ baths filled with Krebs' solution (pH 7.4). Physiological conditions were maintained by keeping the bathing solution constantly at 37°C and aerated with 95% O_2 and 5% CO_2. The force transducer organ bath set up is shown in Figure 70. The tissue was attached to a tissue holder on the bottom, while surgical suture (size 4-0) connected the top side of the strip to a UF1 force transducer (Pioden Controls Ltd., UK). The transducer registered isometric contractions by measuring the tension developed by the tissue and sent an electrical signal to an amplifier (ADInstrument, Colorado Springs, USA). After amplification, the signal was recorded using a PowerLab data acquisition system and Chart software (both ADInstrument, Colorado Springs, USA).

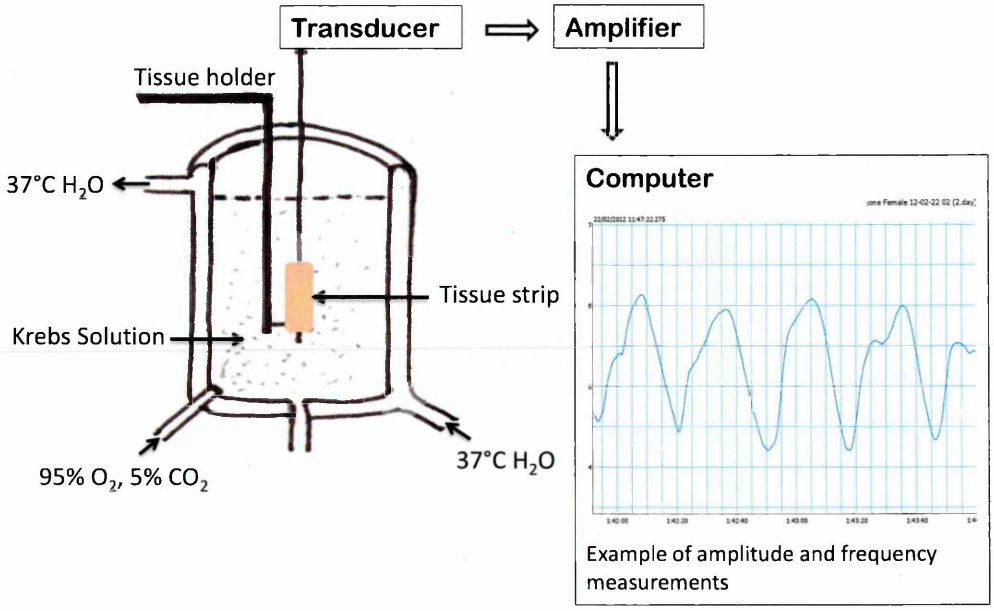


Figure 70 Organ bath set up. Isolated tissue strips were mounted in a double walled glass chamber, which is aerated with 95% O_2 and 5% CO_2 and kept at 37°C to maintain physiological conditions. The tissue was attached to a tissue holder, while suture was connecting the tissue strip to a UF1 force transducer at the top. Contractions were registered by a transducer, amplified and made visible on the computer screen.

4.2.2 PROTOCOL

After 60 minutes equilibration time during which the Krebs' solution was exchanged every 15 minutes and the resting tension maintained at 1 g, baseline measurements of spontaneous contractions were taken. Cumulative concentrations of carbamylcholine chloride (carbachol) (0.01, 0.03, 0.05, 0.07, 0.1, 0.2, 0.3, 0.4 and 0.5 μM) were then applied directly to the Krebs' solution in the organ bath. The strips were incubated with each concentration of carbachol for 10 minutes before the next concentration was added to the Krebs' solution.

4.2.3 DATA ANALYSIS

The last five minutes of an equilibration period of 60 minutes was used for the analyses of basal measurements. Contractions occurring without any stimulation of the tissue strip are referred to as spontaneous activity in this work, while the contractions after stimulation with carbachol are referred to as carbachol-induced contractions. The effect of carbachol on contractility of the tissue was analysed over the last 5 minutes of each concentration of carbachol when a stable response was established.

Maximum peak to peak amplitude of contractions was calculated over the described period of 5 minutes. For frequency analyses a threshold of 30% of the mean amplitude was calculated to define single contraction events as peaks above this threshold line. A peak is counted as one contraction event when the relaxing phase does not return below the 30% threshold level before contracting again (Figure 71). This method was proposed by Imai *et al.* (2001) and has previously been used in the laboratory (Vahabi *et al.*, 2011, Nyamwaro 2012). Furthermore a contraction curve was only counted if its shape and size was not affected by noise spikes. Amplitude data were normalised for wet tissue weight to account for variations in tissue size and are shown as absolute grams of tension per g of tissue weight. Frequency was expressed as number of contractions per minute.

Data were expressed as mean (±SEM) with n being the number of tissue strips used. Unpaired Student's T test was performed for comparison of two groups, for example

comparison of basal contractions in male versus female tissue strips. Statistical comparison of basal contractions in the three bladder regions was performed using 1-way ANOVA with Tukey's post-test. 2-way ANOVA with Tukey's post-test was used to compare carbachol-induced contractions in the three bladder regions. For the comparison of gender differences after stimulation of the tissue with carbachol, 2-way ANOVA with Bonferroni's post-test was used.

Statistical significance was considered whenever reaching a 95% confidence interval with p values ≤ 0.05 considered as significant. All statistics were calculated using Prism 5 (GraphPad Prism, San Diego, USA). Experiments for basal measurements were performed on n=16 tissue strips for dome, body and trigone. This was performed for both male and female bladders. Experiments for carbachol-induced measurements were performed on n=8 tissue strips for dome, body as well as trigone. This was also performed for both male and female bladders. Each pair of intact and denuded strips was dissected from a separate bladder.

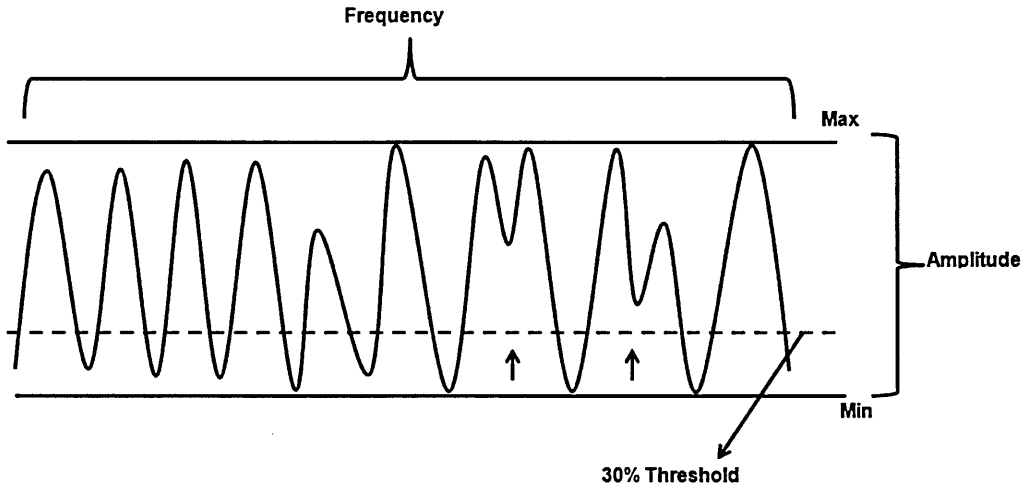


Figure 71 **Analyses of frequency and amplitude of contractions in porcine bladder strips. Amplitude was measured as peak amplitude (max-min). The 30% threshold of the peak amplitude was calculated and any contraction exceeding this threshold was calculated to the frequency measurement. Superimposing contractions that did not return below threshold line before contracting again were only counted as single contractile event (illustrated by arrows) (adapted from Imai *et al.*, 2001).**

4.2.4 DRUGS AND SOLUTIONS

Chemical composition of Krebs' solution as described in 2.2.4. Carbachol chloride ($C_6H_{15}ClN_2O_2$) was obtained from Sigma (Poole, UK). Stock solutions were freshly prepared in distilled water. All dilutions were made in Krebs' solution.

4.3 RESULTS

4.3.1 OCCURRENCE OF SPONTANEOUS ACTIVITY IN INTACT AND DENUDED TISSUE STRIPS OF BODY, DOME AND TRIGONE FROM THE MALE AND FEMALE PORCINE BLADDER

Spontaneous activity was measured in denuded and intact tissue strips from the dome, body and trigone region of female and male porcine bladders. Examples of experimental traces recorded of female and male strips from all three regions are shown in Figure 72 and Figure 73.

Contractile activity without any stimulation is referred to as spontaneous activity in this work in contrast to carbachol-induced contractile activity as previously mentioned.

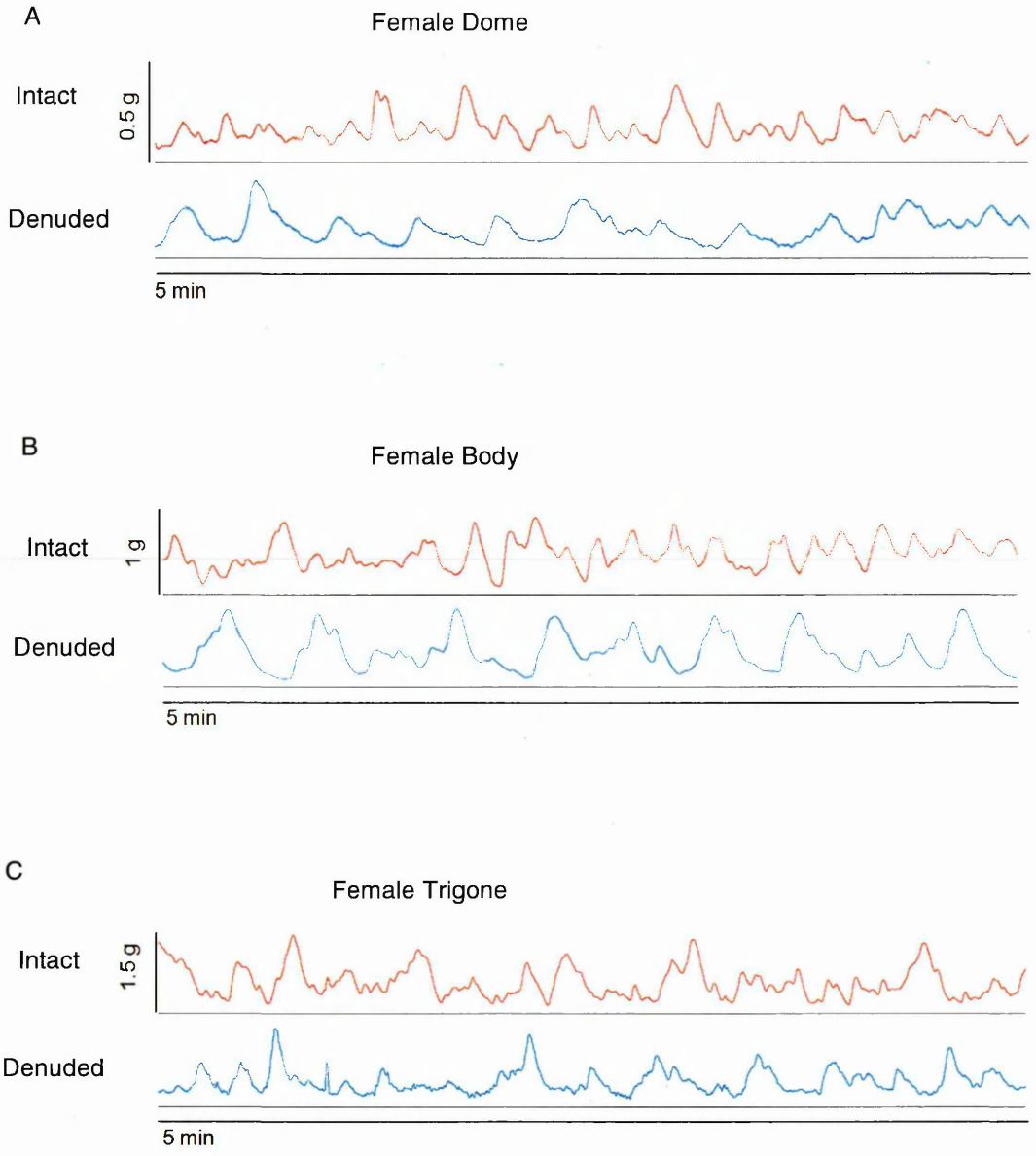


Figure 72 Representative traces showing the spontaneous contractions of intact and denuded tissue strips from the dome, body and trigone region of the female porcine bladder. The differences in scale are due to the fact that the traces are not adjusted for tissue weight yet.

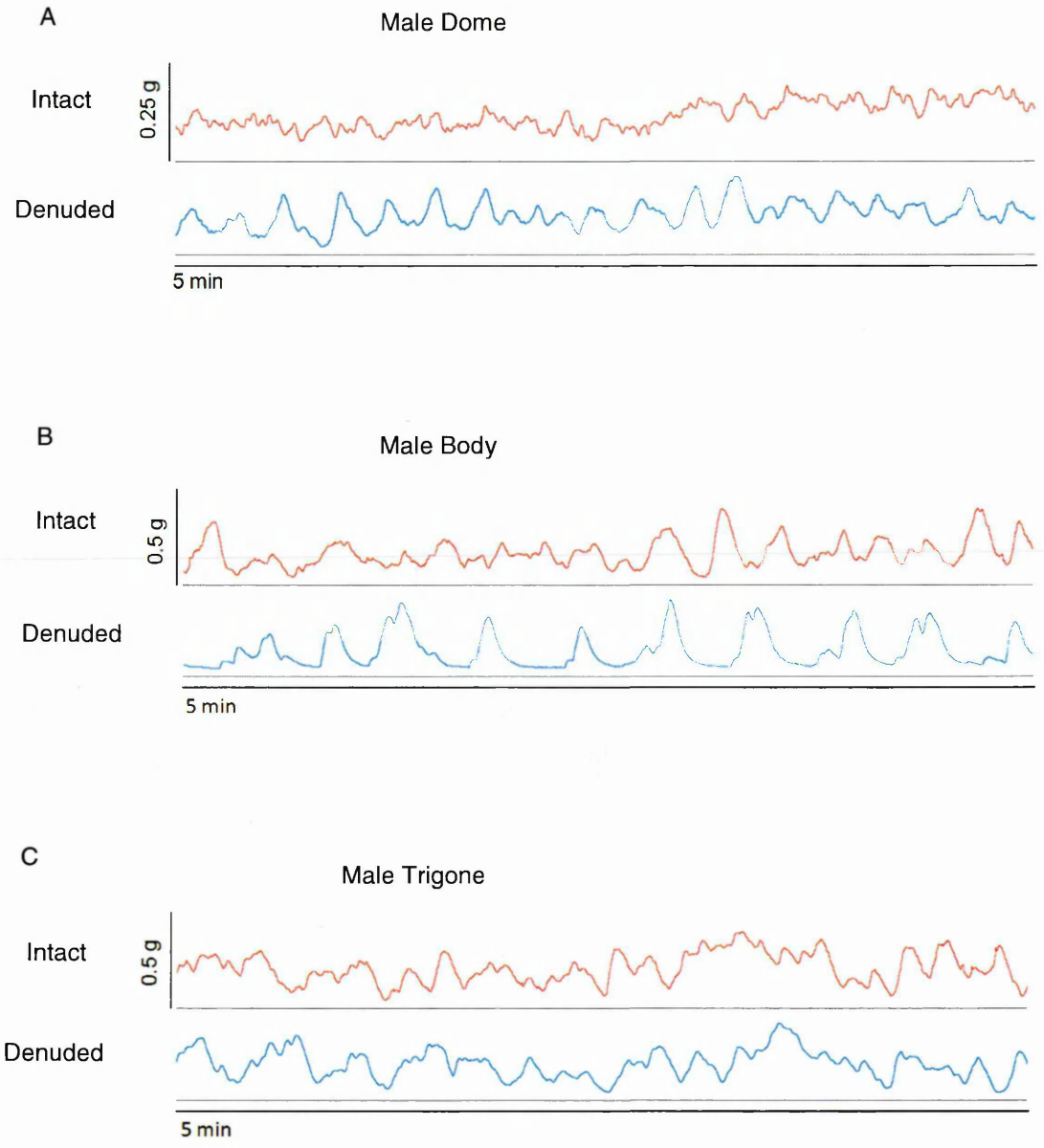


Figure 73 Representative traces showing the spontaneous contractions of intact and denuded tissue strips from the dome, body and trigone region of the male porcine bladder. The differences in scale are due to the fact that the traces are not adjusted for tissue weight yet.

Not all examined bladder strips developed spontaneous activity. The percentage of intact and denuded bladder strips developing spontaneous activity after 55 minutes equilibration time is shown in Figure 74 A and B. Each bar represents the data of n=16 tissue strips.

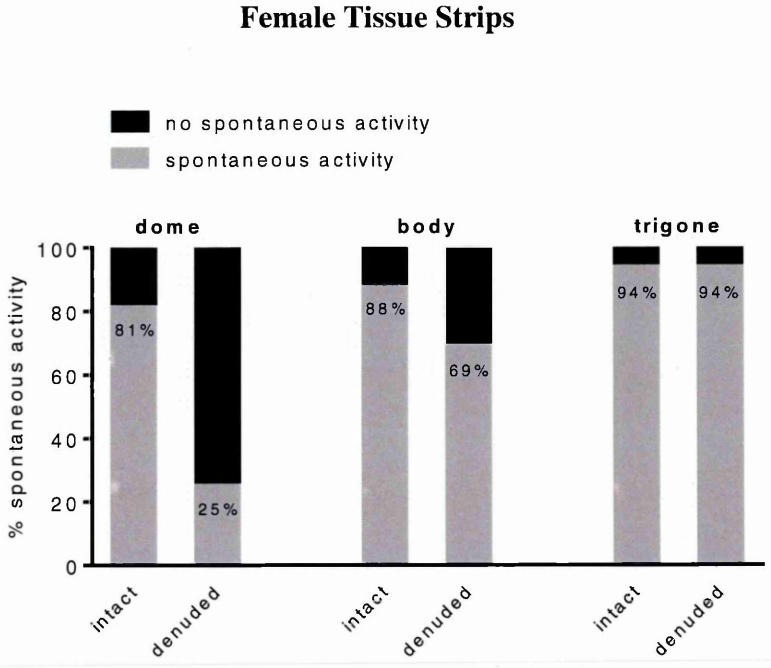
The most prevalent difference between intact and denuded tissue strips could be seen in the dome region from male as well as female bladders. Only 25% of female denuded bladder strips developed spontaneous activity compared to 81% of the intact counterpart and only 19% of male denuded bladder strips developed spontaneous activity compared to 94% of the intact counterpart. 88% of intact body strips from the female bladder developed spontaneous activity compared to 69% of denuded tissue strips. Male bladder strips developed spontaneous activity in 81% of the intact strips compared to 38% of denuded strips. Tissue strips from the female trigone were unaffected by the removal of the urothelium, 94% of both intact and denuded tissue strips developed spontaneous activity. However more tissue strips of the male trigone developed spontaneous activity after removal of the urothelium (69% to 88%). These data show that the removal of the urothelium had an impact on the development of spontaneous activity in tissue strips of all regions from both genders apart from female trigone strips.

The percentage of intact and denuded tissue strips developing spontaneous activity also varies between the regions. The data already suggest a difference in the contractile behaviour between the three bladder regions.

A prominent gender difference could be seen in the intact trigone with 25% fewer male strips showing contractile activity compared to female strips. In the denuded tissue strips the most prevalent gender difference was observed in the body with 31% fewer male strips showing contractile activity compared to female strips.

In the following, only the bladder strips that were developing contractile activity were included in the analyses of spontaneous activity.

A



B

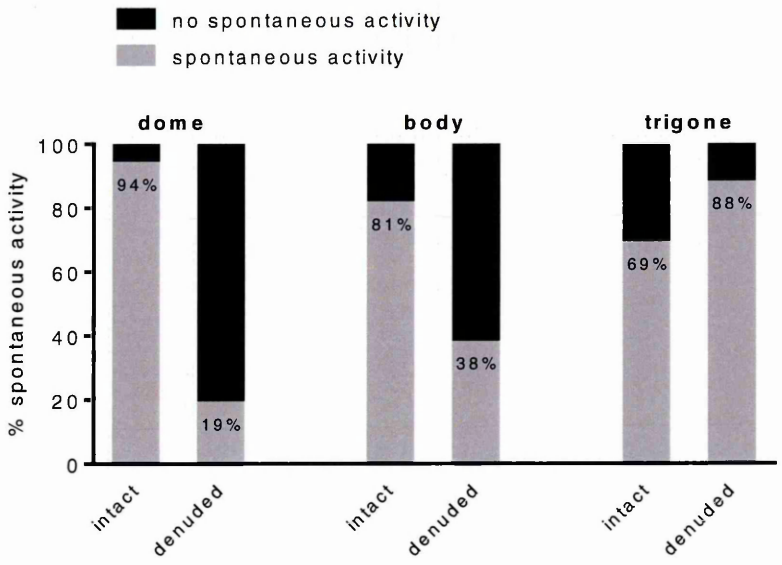


Figure 74 Development of spontaneous activity in percentage of total number of tissue strips. (A) Occurrence of spontaneous activity (%) in female tissue strips of the three bladder regions from intact and denuded porcine bladders (n=16). (B) Occurrence of spontaneous activity (%) in male tissue strips of the three bladder regions from intact and denuded porcine bladders (n=16 tissue strips per bar).

4.3.2 CONTRACTILE ACTIVITY OF BLADDER STRIPS FROM THE FEMALE PORCINE BLADDER

4.3.2.1 AMPLITUDE AND FREQUENCY OF SPONTANEOUS ACTIVITY IN INTACT AND DENUDED TISSUE STRIPS FROM THE FEMALE PORCINE BLADDER

Role of the Urothelium

Spontaneous activity was measured in intact and denuded tissue strips from the dome, body and trigone region of female porcine bladders and peak amplitude and frequency analysed. Examples of the experimental traces of intact and denuded strips from all three regions of the female bladder have previously been shown in Figure 73.

The removal of the urothelium caused a significant increase in the amplitude of spontaneous contraction in the strips from the trigone region from 5.12 (±0.86) to 9.31 (±1.41) g/ g tissue ($p=0.017$). No significant difference in the amplitude of spontaneous contractions could be seen between the intact and denuded tissue strips of the dome and body region (unpaired Student's T test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 75).

The removal of the urothelium caused a significant decrease in the frequency of spontaneous contraction in the strips of the dome region from 3.59 (±0.46) to 0.45 (±0.05) g/ g tissue ($p=0.002$) and in the strips of the body region from 3.23 (±0.29) to 1.42 (±0.30) g/ g tissue ($p=0.0003$), while no significant difference could be observed in the trigone (unpaired Student's T test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 76).

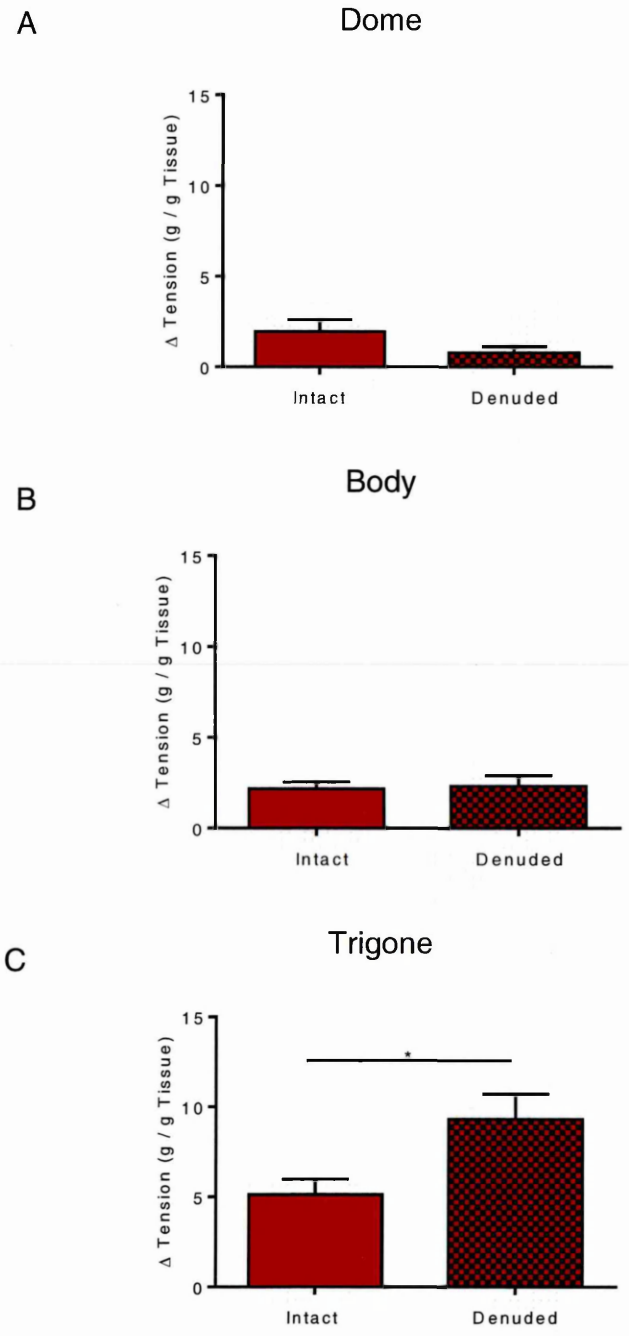


Figure 75 Effect of the urothelium on amplitude of spontaneous contractions in female bladder strips of the three regions (mean ±SEM). (A and B) Amplitude is not significantly different between intact and denuded tissue strips of the dome and body. (C) Amplitude is significantly greater in denuded strips of the trigone compared to the intact counterpart (unpaired Student's T test, n=16 minus strips without contractile activity, * indicates $p \leq 0.05$).

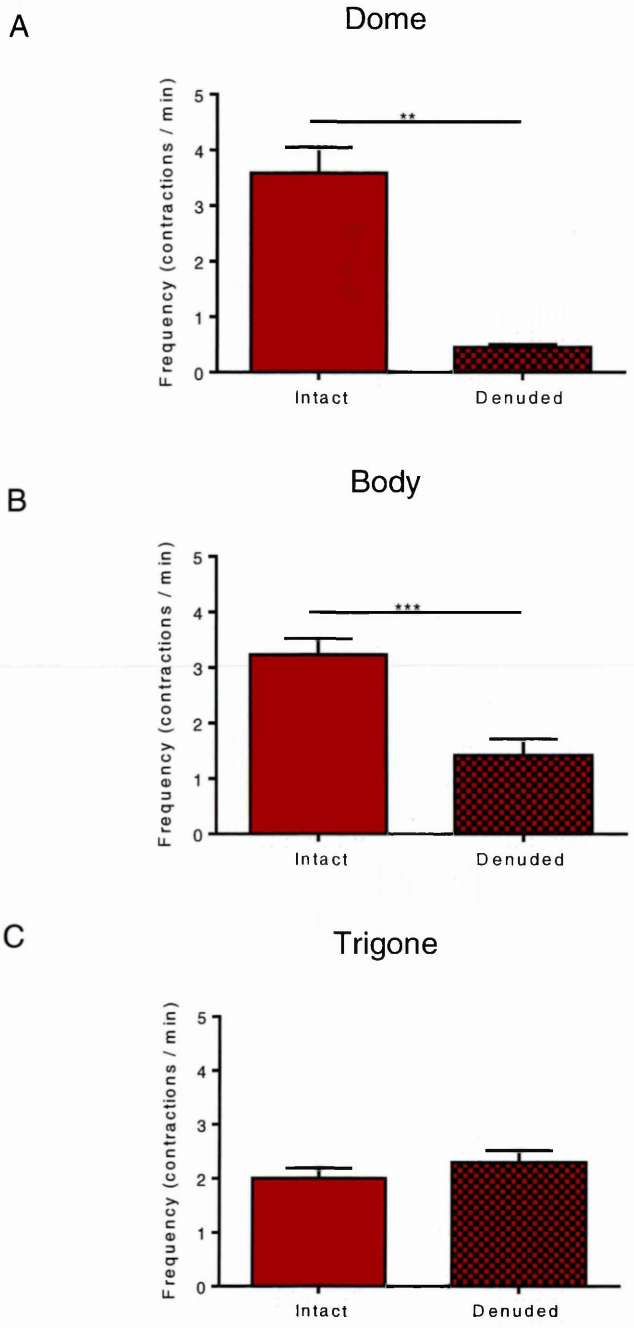


Figure 76 Effect of the urothelium on frequency of spontaneous contractions in female bladder strips of the three regions (mean ±SEM). (A) Frequency is significantly lower in denuded strips of the dome compared to the intact counterpart. (B) Frequency is also significantly lower in denuded strips of the body compared to the intact counterpart. (C) No differences observed in the trigone (unpaired Student's T test, n=16 minus strips without contractile activity, ** indicates $p \leq 0.01$, * indicates $p \leq 0.001$).**

Regional Differences

Regional differences were analysed between tissue strips from the dome, body and trigone region of the female bladder.

In intact tissue strips the amplitude of spontaneous contractions was significantly greater in tissue strips from the trigone region (5.13 ±0.86 g/ g tissue) compared to tissue strips from the body region (2.17 ±0.37 g/ g tissue, $p \leq 0.01$) and from the dome region (1.95 ±0.65 g/ g tissue, $p \leq 0.01$) (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 77 A).

In denuded tissue strips the amplitude of spontaneous contractions was also significantly greater in tissue strips from the trigone region (9.31 ±1.41 g/ g tissue) compared to tissue strips from body region (2.32 ±0.59 g/ g tissue, $p \leq 0.001$) and dome region (0.79 ±0.35 g/ g tissue, $p \leq 0.01$) (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 77 B).

In intact tissue strips the frequency of spontaneous activity was significantly decreased in strips from the trigone region (2.00 ±0.19 g/ g tissue) compared to tissue strips from the dome region (3.59 ±0.46 g/ g tissue, $p \leq 0.01$) and the body region (3.23 ±0.29 g/ g tissue, $p \leq 0.05$) (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 78 A).

In denuded tissue strips the frequency of spontaneous activity was significantly decreased in strips from the trigone region (2.29 ±0.22 g/ g tissue) compared to tissue strips form the dome region (0.45 ±0.05 g/ g tissue, $p \leq 0.01$) and the body region (1.42 ±0.30 g/ g tissue, $p \leq 0.05$) (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 78 B).

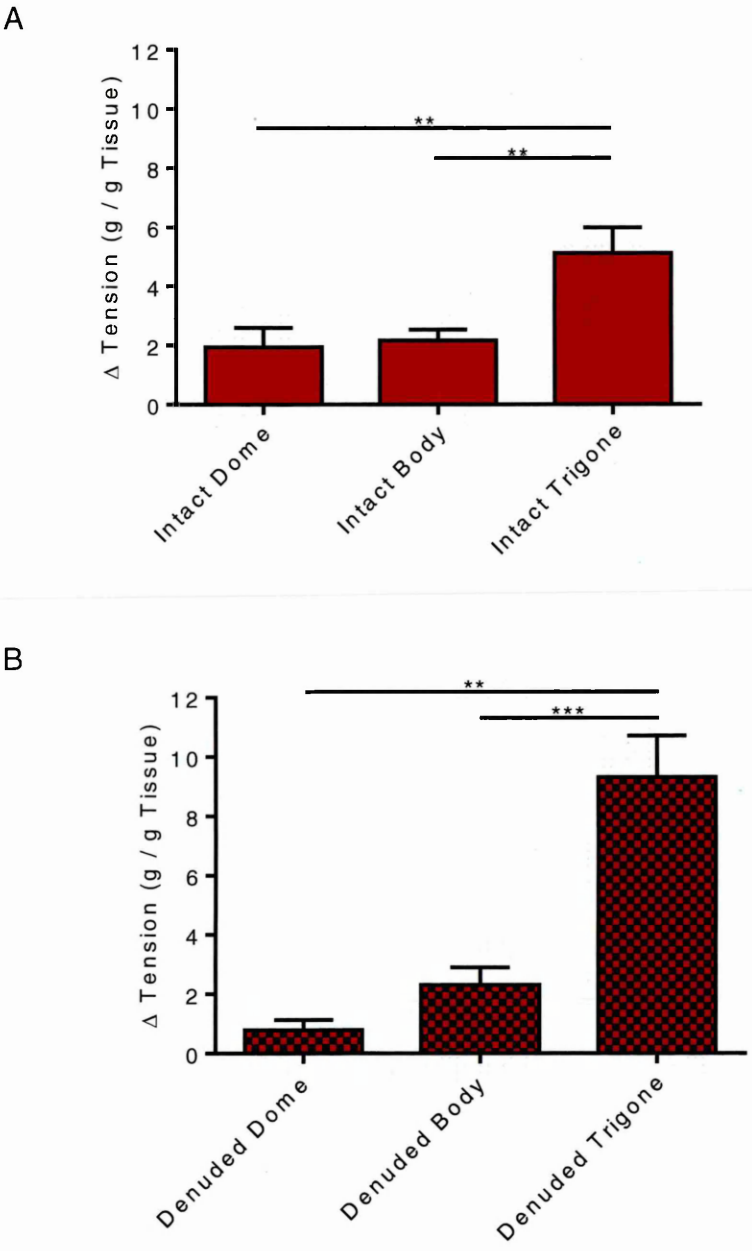


Figure 77 Regional disparities in amplitude of spontaneous contractions in intact and denuded female bladder strips (mean ±SEM). (A and B) Amplitude of the contractile activity in intact and denuded tissue strips from the trigone is significantly elevated compared to activity in dome and body strips. (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity, ** indicates p≤0.01, * indicates p≤0.001).**

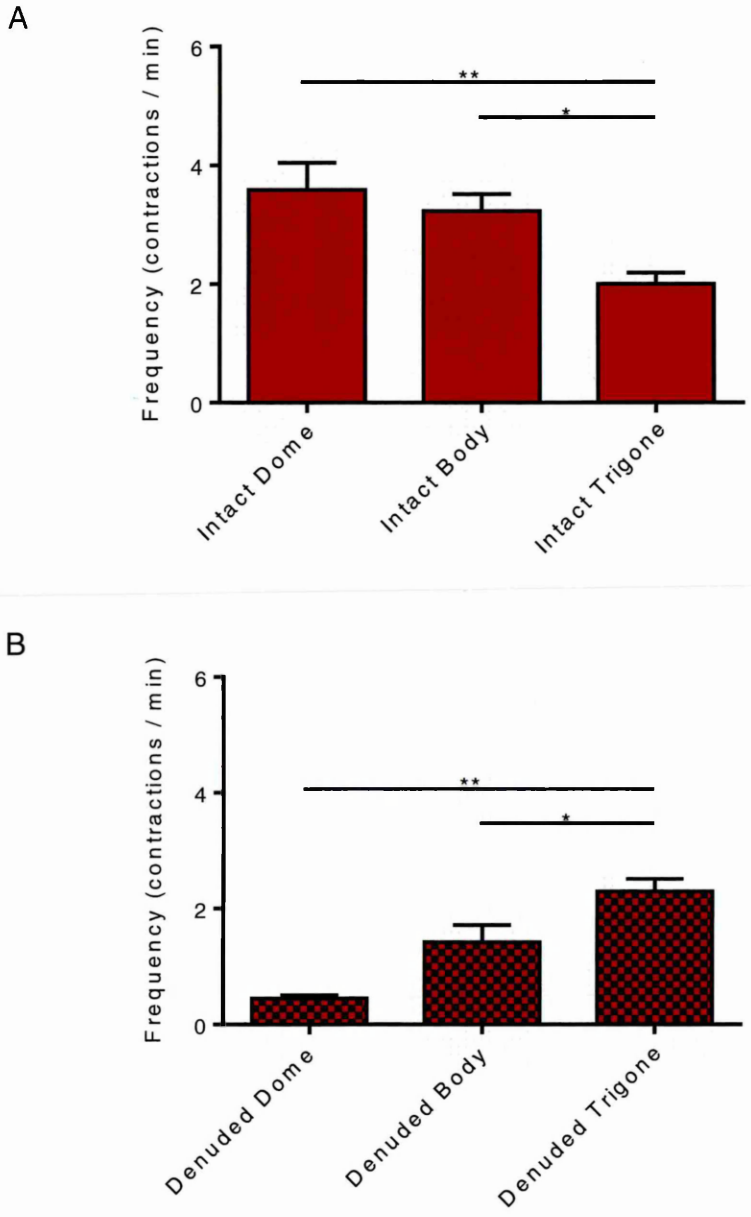


Figure 78 Regional disparities in frequency of spontaneous contractions in intact and denuded female bladder strips (mean ±SEM). (A) Frequency of the contractile activity in intact tissue strips is significantly decreased in trigone strips compared to the activity of dome and body strips. (B) Frequency of denuded tissue strips is significantly increased in trigone strips compared to activity in dome and body strips (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$).

4.3.2.2 AMPLITUDE AND FREQUENCY OF CARBACHOL-INDUCED CONTRACTIONS IN INTACT AND DENUDED TISSUE STRIPS FROM THE FEMALE PORCINE BLADDER

Amplitude and frequency of carbachol-induced contractions were analysed in intact and denuded tissue strips of the female porcine bladder.

Amplitude

The amplitude of carbachol-induced contractions was significantly greater in denuded tissue strips of the dome at concentrations of 0.2, 0.3, 0.4 and 0.5 μM carbachol compared to the intact counterparts ($p \leq 0.05$). Removal of the urothelium did not result in a significant difference in the amplitude between intact and denuded tissue strips from the body and trigone region, however the amplitude was higher in the denuded tissue strips of both regions at all concentrations (2-way ANOVA with Bonferroni's post-test, n=8, Figure 79).

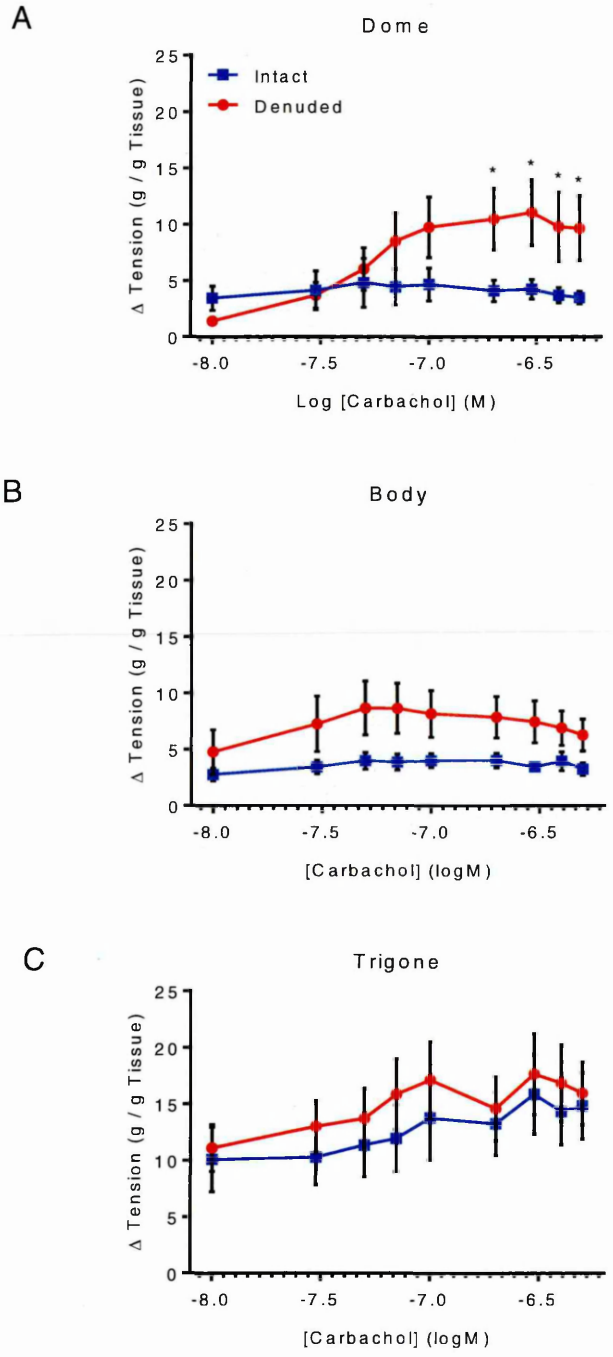


Figure 79 Effect of the urothelium on amplitude of carbachol-induced contractions in female bladder strips from dome, body and trigone (mean ±SEM). (A) Amplitude was significantly greater in denuded tissue strips of the dome at concentrations of 0.2, 0.3, 0.4 and 0.5 µM carbachol. (B and C) No significant differences could be observed in the amplitude between intact and denuded tissue strips of body and trigone (2-way ANOVA with Bonferroni's post-test, n=8, * indicates $p \leq 0.05$).

Frequency

The frequency of carbachol-induced contractions was significantly greater in intact tissue strips of the dome at concentrations of 0.01 μM carbachol compared to the denuded counterparts ($p \leq 0.05$). In tissue strips of the body region, frequency was significantly greater in intact tissue strips at concentrations of 0.01 μM ($p \leq 0.001$) and 0.03 μM ($p \leq 0.01$) carbachol. Removal of the urothelium did not make a significant difference regarding the frequency of carbachol-induced contractions in tissue strips of the trigone, however frequency was higher in the denuded trigone strips at all carbachol concentrations (2-way ANOVA with Bonferroni's post-test, n=8, Figure 80).

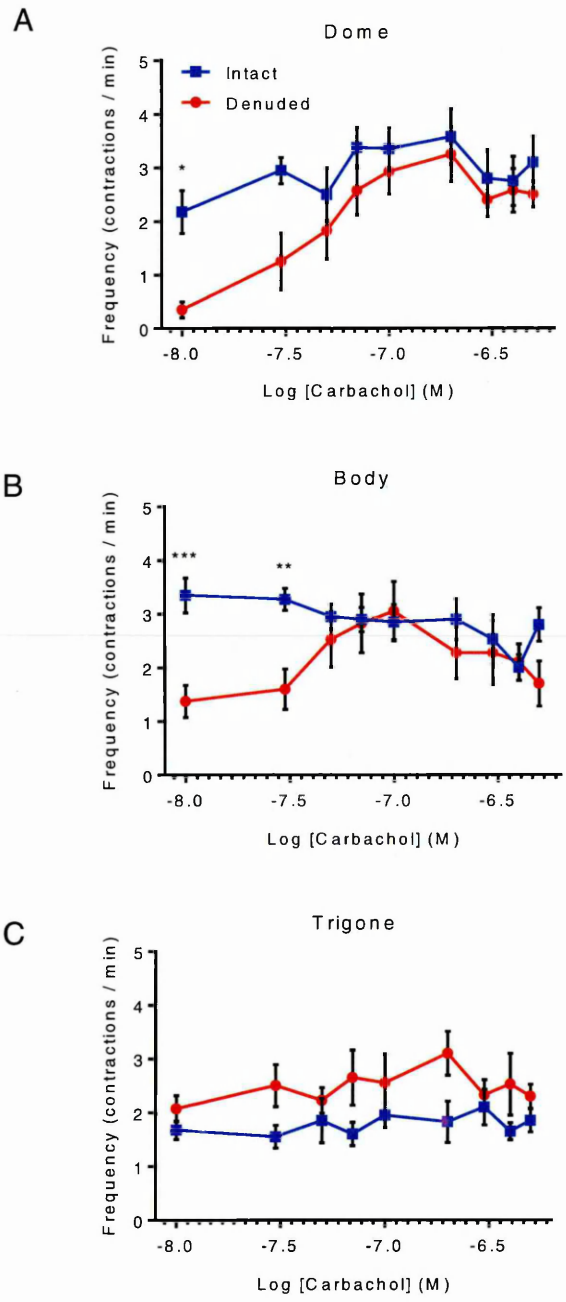


Figure 80 Effect of the urothelium on frequency of carbachol-induced contractions in female bladder strips from dome, body and trigone (mean ±SEM). (A) Frequency was significantly greater in intact tissue strips of the dome at concentrations of 0.01 µM carbachol. (B) Frequency was significantly greater in intact tissue strips of the body at concentrations of 0.01 and 0.03 µM carbachol. (C) No significant differences could be observed in the frequency between intact and denuded tissue strips of the trigone (2-way ANOVA with Bonferroni's post-test, n=8, * indicates $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

4.3.2.3 REGIONAL DIFFERENCES IN AMPLITUDE AND FREQUENCY OF CARBACHOL-INDUCED CONTRACTIONS IN INTACT AND DENUDED TISSUE STRIPS FROM THE FEMALE PORCINE BLADDER

Regional differences between the dome, body and trigone region in amplitude and frequency of carbachol-induced contractions were analysed in intact as well as denuded bladder strips of the female porcine bladder.

Regional Differences in the Amplitude of Carbachol-induced Contractions

At all concentrations of carbachol tested, the amplitude of contractions in the intact trigone strips was significantly higher compared to intact strips of the body and to intact strips of the dome. This was also the case after removal of the urothelium, the denuded trigone strips showed a significantly greater amplitude than the denuded body and dome strips.

There were no significant differences in the amplitude between intact dome and intact body strips at any used concentration of carbachol.

Significant differences were seen between denuded dome and denuded body strips at carbachol concentrations of 0.01, 0.03, 0.3 and 0.5 μM (2-way ANOVA with Tukey's post-test, n=8, Figure 81, Table 5 and Table 6).

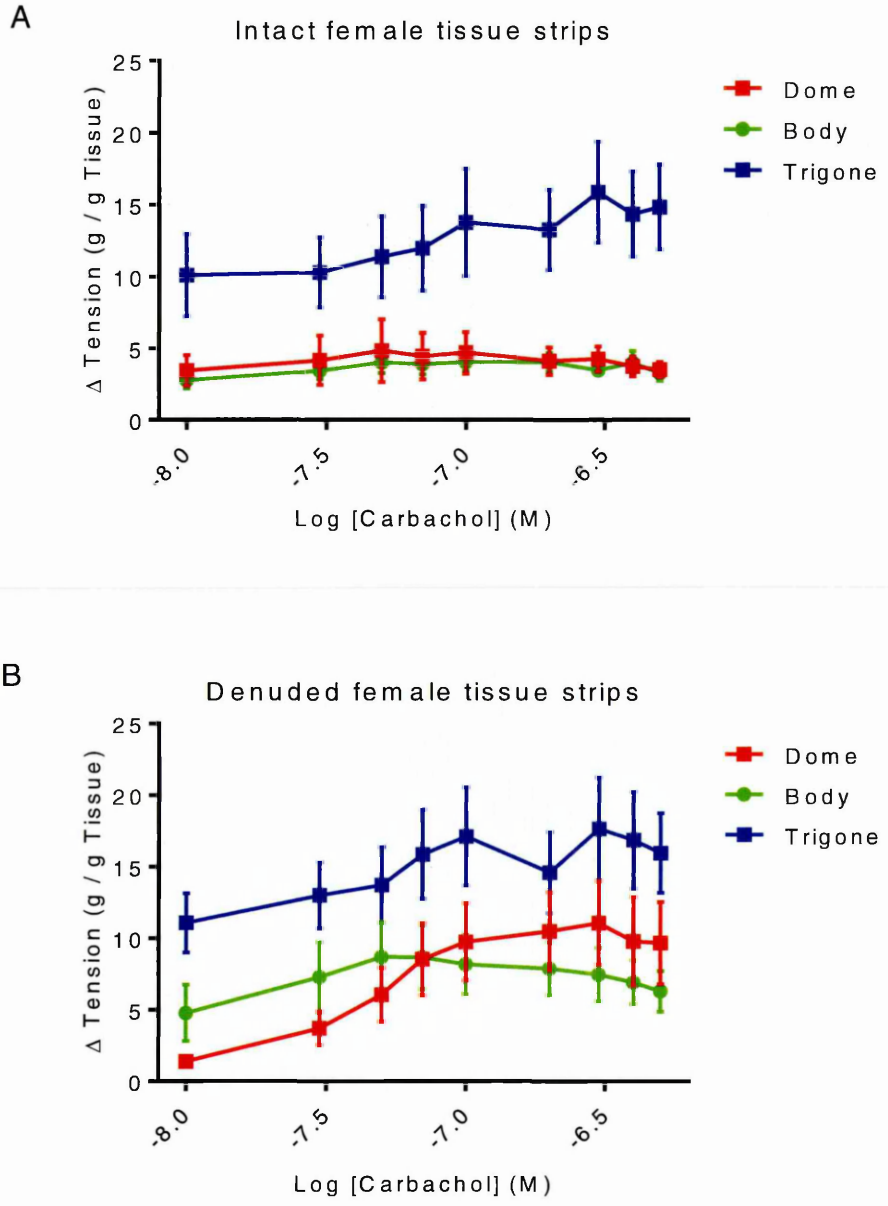


Figure 81 **Regional differences in amplitude of carbachol-induced (0.01-0.5 µM) contractions in intact and denuded female bladder strips (mean ±SEM). (A) Amplitude in intact tissue strips of the dome, body and trigone from the female porcine bladder. (B) Amplitude in denuded tissue strips of the dome, body and trigone from the female porcine bladder (2-way ANOVA with Tukey's post-test, n=8, numbers and significance see Table 5 and Table 6).**

Carbachol conc. (μM)	Amplitude Intact Tissue Strips	Amplitude 1 vs. (g/g tissue)	Amplitude 2 (g/g tissue)	Significance
0.01	Dome vs. Body	3.43	2.76	ns
	Dome vs. Trigone	3.43	10.07	****
	Body vs. Trigone	2.76	10.07	****
0.03	Dome vs. Body	4.14	3.42	ns
	Dome vs. Trigone	4.14	10.27	****
	Body vs. Trigone	3.42	10.27	****
0.05	Dome vs. Body	4.81	3.99	ns
	Dome vs. Trigone	4.81	11.35	****
	Body vs. Trigone	3.99	11.35	****
0.07	Dome vs. Body	4.43	3.87	ns
	Dome vs. Trigone	4.43	11.94	****
	Body vs. Trigone	3.87	11.94	****
0.1	Dome vs. Body	4.67	4.00	ns
	Dome vs. Trigone	4.67	13.75	****
	Body vs. Trigone	4.00	13.75	****
0.2	Dome vs. Body	4.09	4.01	ns
	Dome vs. Trigone	4.09	13.23	****
	Body vs. Trigone	4.01	13.23	****
0.3	Dome vs. Body	4.24	3.45	ns
	Dome vs. Trigone	4.24	15.85	****
	Body vs. Trigone	3.45	15.85	****
0.4	Dome vs. Body	3.72	3.96	ns
	Dome vs. Trigone	3.72	14.33	****
	Body vs. Trigone	3.96	14.33	****
0.5	Dome vs. Body	3.494	3.244	ns
	Dome vs. Trigone	3.494	14.82	****
	Body vs. Trigone	3.244	14.82	****

Table 5 **Regional differences in the amplitude of carbachol-induced contractions in intact tissue strips of dome, body and trigone from the female porcine bladder. The mean of amplitude measurements (g/g tissue) and the significance of difference is shown (2-way ANOVA with Tukey's post-test, n=8, **** indicates $p \leq 0.0001$).**

Carbachol conc. (μM)	Amplitude Denuded Tissue Strips	Amplitude 1 vs. (g/g tissue)	Amplitude 2 (g/g tissue)	Significance
0.01	Dome vs. Body	1.38	4.76	*
	Dome vs. Trigone	1.38	11.07	****
	Body vs. Trigone	4.76	11.07	****
0.03	Dome vs. Body	3.69	7.26	*
	Dome vs. Trigone	3.69	12.99	****
	Body vs. Trigone	7.26	12.99	***
0.05	Dome vs. Body	6.04	8.68	ns
	Dome vs. Trigone	6.04	13.70	****
	Body vs. Trigone	8.68	13.70	**
0.07	Dome vs. Body	8.50	8.65	ns
	Dome vs. Trigone	8.50	15.85	****
	Body vs. Trigone	8.65	15.85	****
0.1	Dome vs. Body	9.74	8.15	ns
	Dome vs. Trigone	9.74	17.10	****
	Body vs. Trigone	8.15	17.10	****
0.2	Dome vs. Body	10.46	7.86	ns
	Dome vs. Trigone	10.46	14.57	*
	Body vs. Trigone	7.86	14.57	****
0.3	Dome vs. Body	11.04	7.45	*
	Dome vs. Trigone	11.04	17.64	****
	Body vs. Trigone	7.45	17.64	****
0.4	Dome vs. Body	9.78	6.90	ns
	Dome vs. Trigone	9.78	16.84	****
	Body vs. Trigone	6.90	16.84	****
0.5	Dome vs. Body	9.65	6.28	*
	Dome vs. Trigone	9.65	15.94	****
	Body vs. Trigone	6.28	15.94	****

Table 6 **Regional differences in the amplitude of carbachol-induced contractions in denuded tissue strips of dome, body and trigone from the female porcine bladder. The mean of amplitude measurements (g/g tissue) and the significance of difference is shown (2-way ANOVA with Tukey's post-test, n=8, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$).**

Regional Differences in the Frequency of Carbachol-induced Contractions

Intact tissue strips of the trigone showed less contractions at all used concentrations of carbachol compared to intact strips of the body and the dome. Intact strips of the body showed significantly greater frequency at concentrations of 0.01, 0.03 and 0.05 μM carbachol compared to trigone and dome strips. At higher concentrations of 0.07 to 0.5 μM carbachol the intact strips of the dome showed significantly more contractions compared to the body and trigone (2-way ANOVA with Tukey's post-test, n=8, Figure 82 and Table 7).

After removal of the urothelium, denuded strips of the trigone showed significantly greater frequency at concentrations of 0.01 and 0.03 μM carbachol compared to denuded dome strips. At concentrations of 0.05 to 0.5 μM carbachol no significant difference could be observed between the three regions. Increasing stimulation with carbachol seemed to make the frequency responses of the denuded tissue strips more similar (2-way ANOVA with Tukey's post-test, n=8, Figure 82 and Table 8).

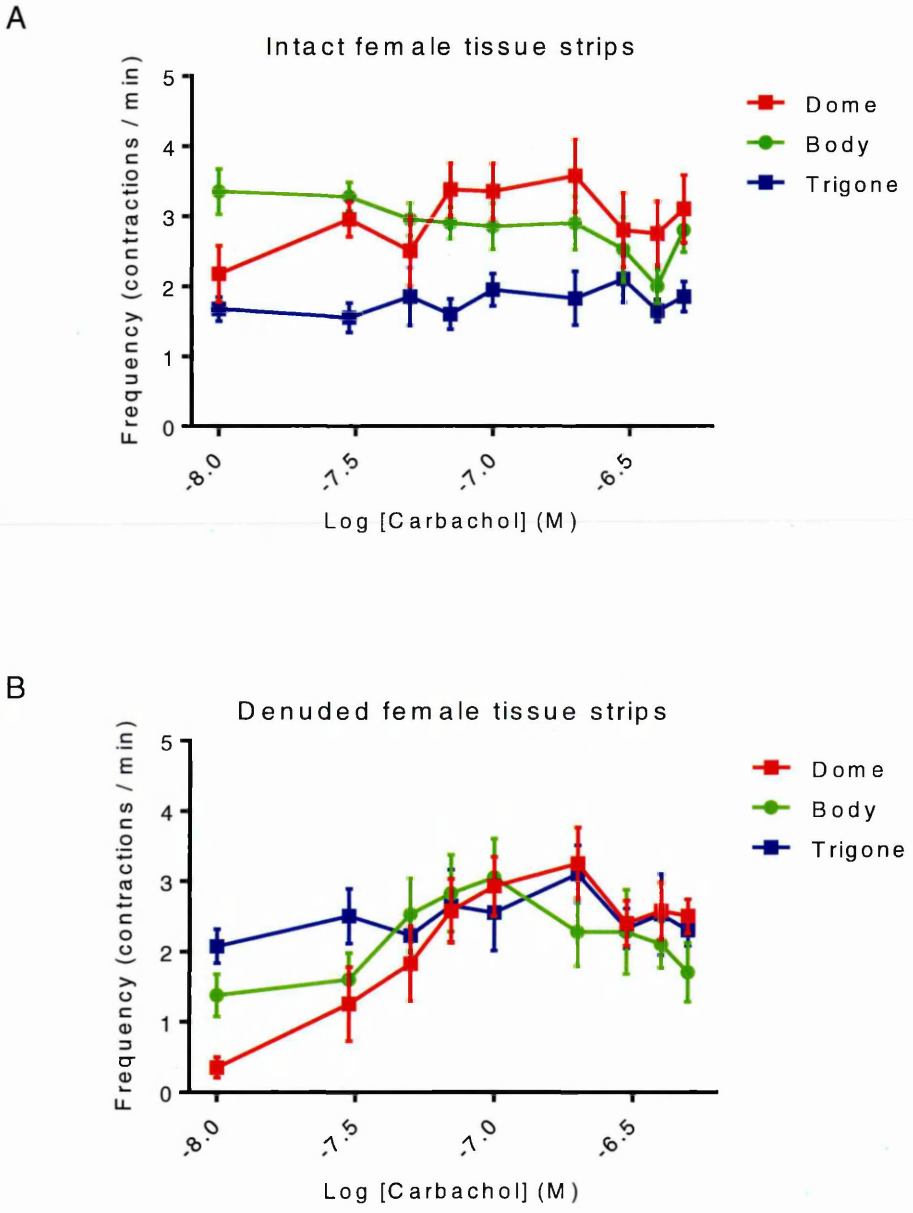


Figure 82 Regional differences in frequency of carbachol-induced (0.01-0.5 μM) contractions in intact and denuded female bladder strips (mean ±SEM). (A) Contractions per min in intact tissue strips of the dome, body and trigone from the female porcine bladder. (B) Contractions per min in denuded tissue strips of the dome, body and trigone from the female porcine bladder (2-way ANOVA with Tukey's post-test, n=8, results see Table 7 and Table 8).

Carbachol conc. (μM)	Frequency Intact Tissue Strips	Frequency 1 vs. (contractions/min)	Frequency 2 (contractions/min)	Significance
0.01	Dome vs. Body	2.18	3.35	*
	Dome vs. Trigone	2.18	1.68	ns
	Body vs. Trigone	3.35	1.68	***
0.03	Dome vs. Body	2.95	3.28	ns
	Dome vs. Trigone	2.95	1.55	**
	Body vs. Trigone	3.28	1.55	***
0.05	Dome vs. Body	2.50	2.95	ns
	Dome vs. Trigone	2.50	1.85	ns
	Body vs. Trigone	2.95	1.85	*
0.07	Dome vs. Body	3.38	2.90	ns
	Dome vs. Trigone	3.38	1.60	****
	Body vs. Trigone	2.90	1.60	**
0.1	Dome vs. Body	3.35	2.85	ns
	Dome vs. Trigone	3.35	1.95	**
	Body vs. Trigone	2.85	1.95	ns
0.2	Dome vs. Body	3.58	2.90	ns
	Dome vs. Trigone	3.58	1.83	***
	Body vs. Trigone	2.90	1.83	*
0.3	Dome vs. Body	2.80	2.53	ns
	Dome vs. Trigone	2.80	2.10	ns
	Body vs. Trigone	2.53	2.10	ns
0.4	Dome vs. Body	2.75	2.00	ns
	Dome vs. Trigone	2.75	1.65	*
	Body vs. Trigone	2.00	1.65	ns
0.5	Dome vs. Body	3.10	2.80	ns
	Dome vs. Trigone	3.10	1.85	**
	Body vs. Trigone	2.80	1.85	ns

Table 7 **Regional differences in the frequency of carbachol-induced contractions in intact tissue strips of dome, body and trigone from the female porcine bladder. The mean of frequency measurements (contractions/ min) and the significance of difference is shown (2-way ANOVA with Tukey's post-test, n=8, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$).**

Carbachol conc. (μM)	Frequency Denuded Tissue Strips	Frequency 1 vs. (contractions/min)	Frequency 2 (contractions/min)	Significance
0.01	Dome vs. Body	0.35	1.38	ns
	Dome vs. Trigone	0.35	2.08	***
	Body vs. Trigone	1.38	2.08	ns
0.03	Dome vs. Body	1.25	1.60	ns
	Dome vs. Trigone	1.25	2.50	*
	Body vs. Trigone	1.60	2.50	ns
0.05	Dome vs. Body	1.83	2.53	ns
	Dome vs. Trigone	1.83	2.23	ns
	Body vs. Trigone	2.53	2.23	ns
0.07	Dome vs. Body	2.58	2.83	ns
	Dome vs. Trigone	2.58	2.65	ns
	Body vs. Trigone	2.83	2.65	ns
0.1	Dome vs. Body	2.93	3.05	ns
	Dome vs. Trigone	2.93	2.55	ns
	Body vs. Trigone	3.05	2.55	ns
0.2	Dome vs. Body	3.25	2.28	ns
	Dome vs. Trigone	3.25	3.10	ns
	Body vs. Trigone	2.28	3.10	ns
0.3	Dome vs. Body	2.40	2.28	ns
	Dome vs. Trigone	2.40	2.33	ns
	Body vs. Trigone	2.28	2.33	ns
0.4	Dome vs. Body	2.58	2.10	ns
	Dome vs. Trigone	2.58	2.53	ns
	Body vs. Trigone	2.10	2.53	ns
0.5	Dome vs. Body	2.50	1.70	ns
	Dome vs. Trigone	2.50	2.30	ns
	Body vs. Trigone	1.70	2.30	ns

Table 8 **Regional differences in the frequency of carbachol-induced contractions in denuded tissue strips of dome, body and trigone from the female porcine bladder. The mean of frequency measurements (contractions/ min) and the significance of difference is shown (2-way ANOVA with Tukey's post-test, n=8, * indicates $p \leq 0.05$, *** indicates $p \leq 0.001$).**

4.3.3 CONTRACTILE ACTIVITY OF BLADDER STRIPS FROM THE MALE PORCINE BLADDER

4.3.3.1 AMPLITUDE AND FREQUENCY OF SPONTANEOUS ACTIVITY IN INTACT AND DENUDED TISSUE STRIPS FROM THE MALE PORCINE BLADDER

Role of the Urothelium

Spontaneous activity was measured in intact and denuded tissue strips from the dome, body and trigone region of male porcine bladders and peak amplitude and frequency analysed. Examples of the experimental traces of intact and denuded strips from all three regions of the male bladder have previously been shown in Figure 73.

The removal of the urothelium caused a significant decrease in the amplitude of spontaneous contraction in the strips from the body region from 1.34 (±0.27) to 0.35 (±0.06) g/ g tissue ($p=0.023$). No significant difference in the amplitude of spontaneous contractions could be seen between the intact and denuded tissue strips of the dome and trigone region (unpaired Student's T test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 83).

The removal of the urothelium caused a significant decrease in the frequency of spontaneous contraction in the strips from the body region from 2.15 (±0.26) to 1.07 (±0.36) g/ g tissue ($p=0.028$). No significant difference in the frequency of spontaneous contractions could be observed between the intact and denuded tissue strips of the dome and trigone region (unpaired Student's T test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 84).

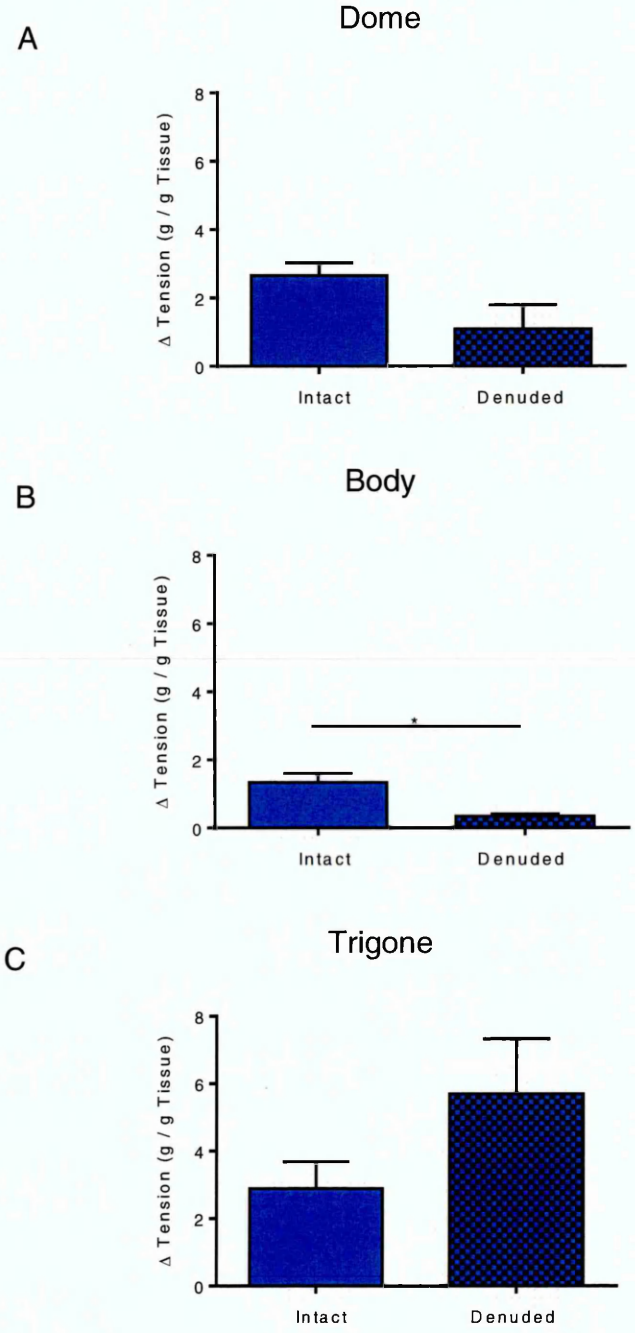


Figure 83 Effect of the urothelium on amplitude of spontaneous contractions in male bladder strips of the three regions (mean ±SEM). (A and C) Amplitude is not significantly different between intact and denuded tissue strips of the dome and trigone. (B) Amplitude is significantly smaller in denuded strips of the body compared to the intact counterpart (unpaired Student's T test, n=16 minus strips without contractile activity, * indicates p≤0.05).

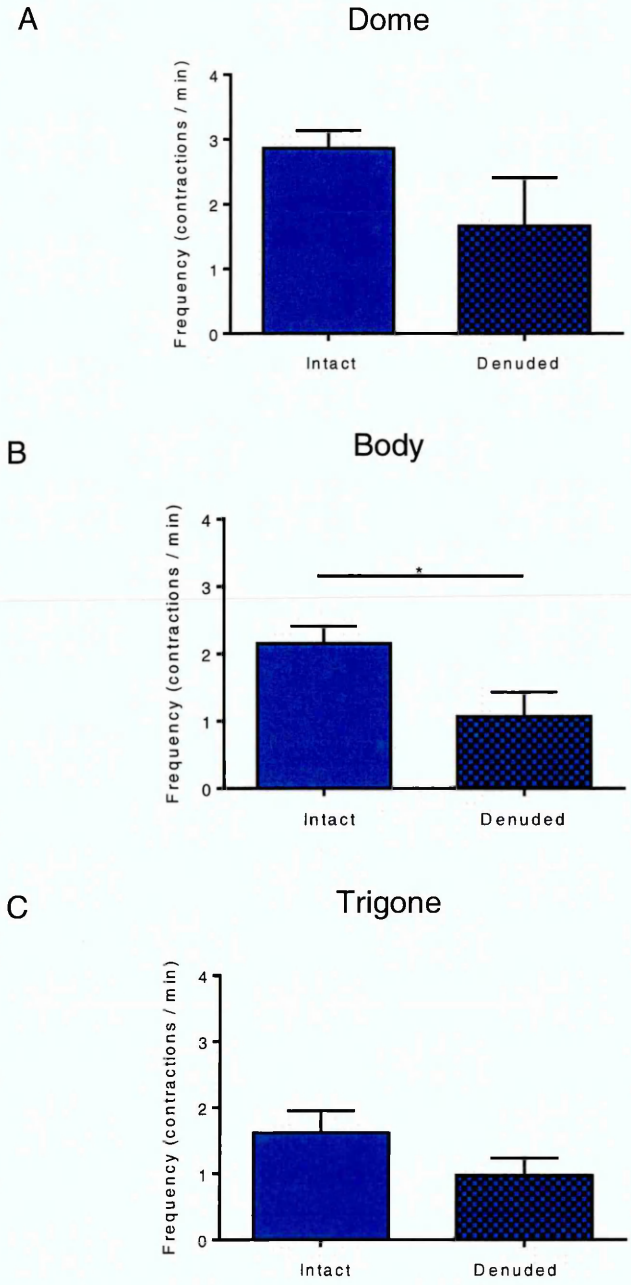


Figure 84 Effect of the urothelium on frequency of spontaneous contractions in male bladder strips of the three regions (mean ±SEM). (A and C) Frequency of contractions is not significantly different between intact and denuded strips of the dome and the trigone. (C) Frequency is significantly lower in denuded strips of the body compared to the intact counterpart (unpaired Student's T test, n=16 minus strips without contractile activity, * indicates $p \leq 0.05$).

Regional Differences

Regional differences were analysed between tissue strips from the dome, body and trigone region of the male bladder.

The amplitude of spontaneous contractions was not significantly different between the three bladder regions, neither in intact nor in denuded tissue strips (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 85 A and B).

The frequency of spontaneous activity was significantly decreased in intact tissue strips from the trigone region (1.62 ±0.33 g/ g tissue) compared to tissue strips from the dome region (2.87 ±0.27 g/ g tissue, $p \leq 0.05$) but no significant difference was seen between trigone and body and between body and dome (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 86 A).

In denuded tissue strips the frequency of spontaneous activity was not significantly different between the three bladder regions (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 86 B).

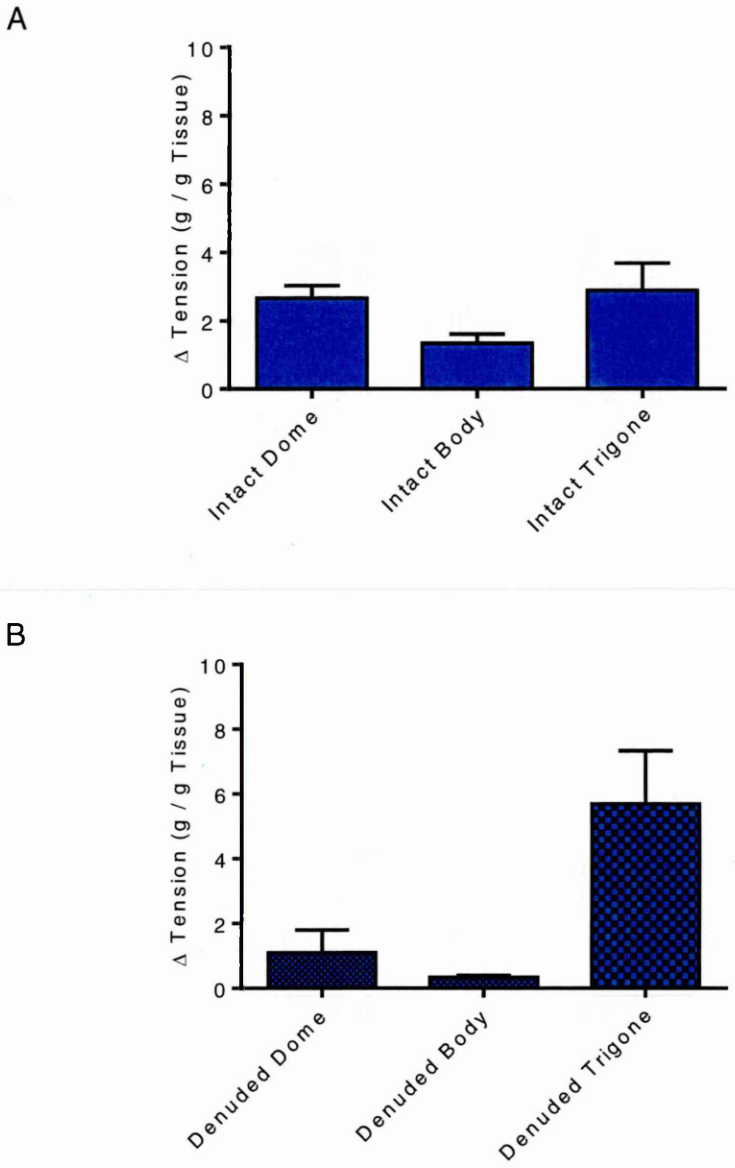


Figure 85 **Regional disparities in amplitude of spontaneous contractions in intact and denuded male bladder strips (mean ±SEM). (A) Amplitude of the spontaneous contractions does not significantly differ between the three regions in the intact tissue strips. (B) Amplitude of the spontaneous contractions does also not significantly differ between the three regions in the denuded tissue strips (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity).**

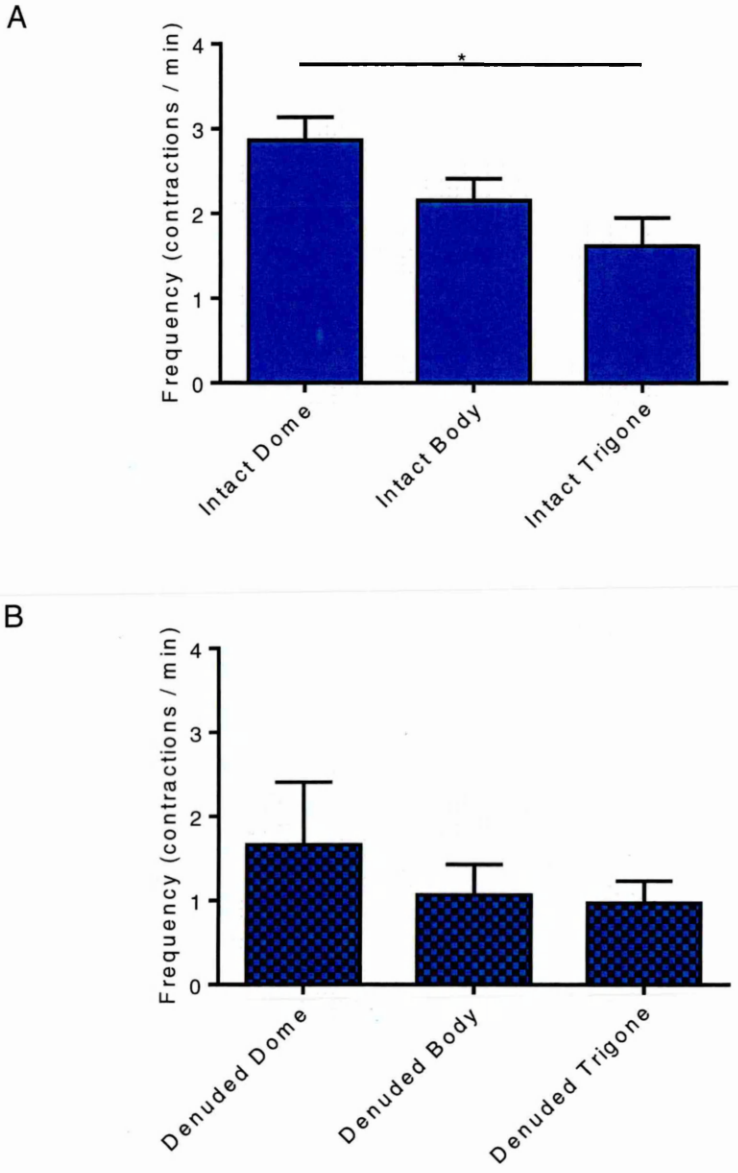


Figure 86 **Regional disparities in frequency of spontaneous contractions in intact and denuded male bladder strips (mean ±SEM). (A) Frequency of the contractile activity in intact tissue strips is significantly decreased in trigone strips compared to the activity of dome strips. (B) No significant regional difference could be seen in the frequency of contractions in denuded tissue (1-way ANOVA with Tukey's post-test, n=16 minus strips without contractile activity (Figure 74), * indicates $p \leq 0.05$).**

4.3.3.2 AMPLITUDE AND FREQUENCY OF CARBACHOL-INDUCED CONTRACTIONS IN INTACT AND DENUDED TISSUE STRIPS FROM THE MALE PORCINE BLADDER

Amplitude and frequency of carbachol-induced contractions were analysed in intact and denuded tissue strips of the male porcine bladder.

Amplitude

The amplitude of carbachol-induced contractions was significantly greater in denuded tissue strips of the dome at concentrations of 0.07 μM ($p \leq 0.001$), 0.1 μM ($p \leq 0.0001$), 0.2 μM ($p \leq 0.001$), 0.3 μM ($p \leq 0.001$), 0.4 μM ($p \leq 0.01$) and 0.5 μM ($p \leq 0.05$) carbachol compared to the intact counterparts. In denuded tissue strips of the body the amplitude of carbachol-induced contractions was significantly greater in denuded tissue strips at concentrations of 0.03, 0.05 and 0.07 μM ($p \leq 0.05$). Removal of the urothelium did not result in a significant difference in the amplitude between intact and denuded tissue strips of the trigone region at any concentrations (2-way ANOVA with Bonferroni's post-test, n=8, Figure 87).

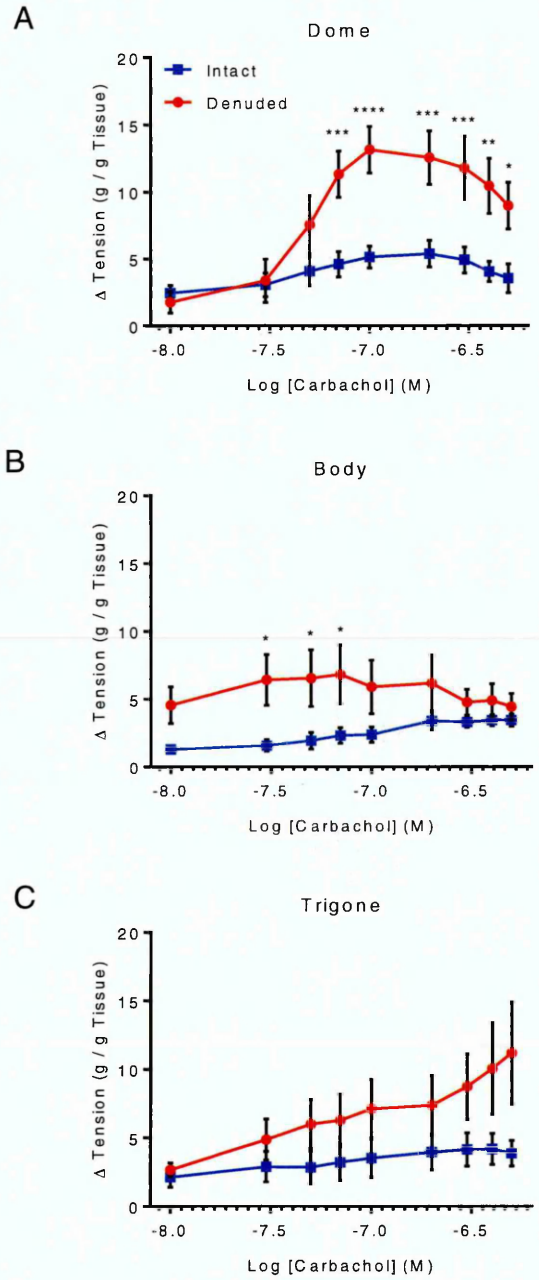


Figure 87 Effect of the urothelium on amplitude of carbachol-induced contractions in male bladder strips from dome, body and trigone (mean ±SEM). (A) Amplitude was significantly greater in denuded tissue strips of the dome at concentrations of 0.07, 0.1, 0.2, 0.3, 0.4 and 0.5 µM carbachol. (B) Amplitude of denuded dome strips was significantly greater at concentrations of 0.03, 0.05 and 0.07 µM carbachol. (C) No significant differences could be observed in the amplitude between intact and denuded tissue strips of the trigone region (2-way ANOVA with Bonferroni's post-test, n=8, * indicates p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001).

Frequency

The frequency of carbachol-induced contractions was significantly greater in intact tissue strips of the dome at concentrations of 0.01 μM ($p \leq 0.01$) and 0.03 μM ($p \leq 0.05$) carbachol compared to the intact counterparts. Removal of the urothelium did not make a significant difference regarding the frequency of carbachol-induced contractions in tissue strips of the dome and the trigone (2-way ANOVA with Bonferroni's post-test, n=8, Figure 88Figure 80).

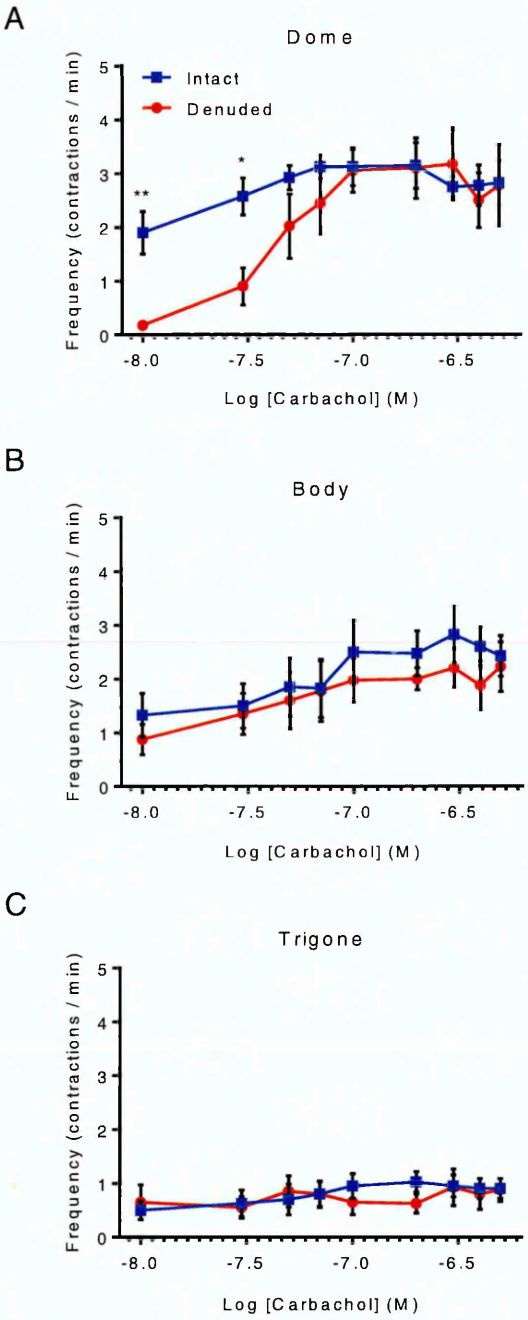


Figure 88 Effect of the urothelium on frequency of carbachol-induced contractions in male bladder strips from dome, body and trigone (mean ±SEM). (A) Frequency was significantly greater in intact tissue strips of the dome at concentrations of 0.01 and 0.03 µM carbachol. (B and C) No significant differences could be observed in the frequency between intact and denuded tissue strips of the body and the trigone (2-way ANOVA with Bonferroni's post-test, n=8, * indicates $p \leq 0.05$, ** $p \leq 0.01$).

4.3.3.3 REGIONAL DIFFERENCES IN AMPLITUDE AND FREQUENCY OF CARBACHOL-INDUCED CONTRACTIONS IN INTACT AND DENUDED TISSUE STRIPS FROM THE MALE PORCINE BLADDER

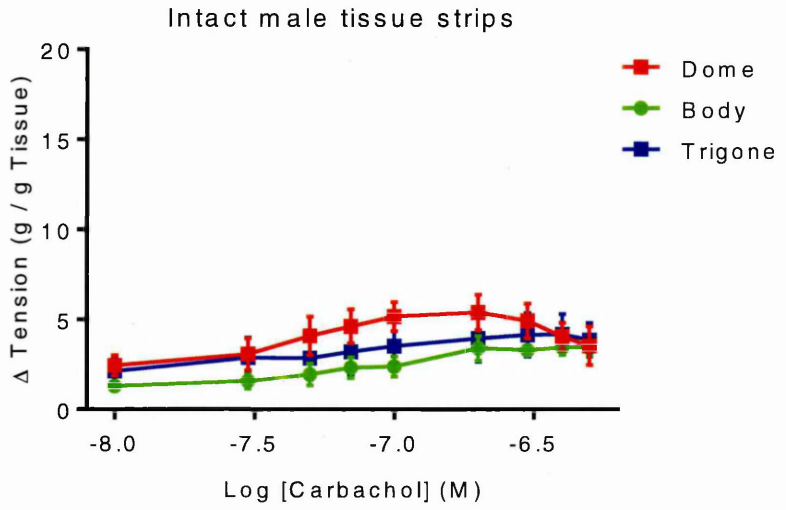
Regional differences between the dome, body and trigone region in amplitude and frequency of carbachol-induced contractions were analysed in intact as well as denuded bladder strips of the male porcine bladder.

Regional Differences in Amplitude of Carbachol-induced Contractions

The amplitude of carbachol-induced contractions in the intact dome strips was significantly greater compared to body strips at concentrations of 0.05, 0.07, 0.1, 0.2 and 0.3 μM carbachol and compared to trigone strips at 0.1 μM carbachol (2-way ANOVA with Tukey's post-test, n=8, Figure 89 A and Table 9).

Denuded tissue strips from the dome region showed a significantly greater amplitude compared to body strips at concentrations of 0.07, 0.1, 0.2, 0.3, 0.4 and 0.5 μM carbachol and compared to trigone strips at concentrations of 0.07, 0.1, 0.2 μM carbachol. Furthermore a significant difference could be observed between trigone and body strips at concentrations of 0.3, 0.4 and 0.5 μM carbachol (2-way ANOVA with Tukey's post-test, n=8, Figure 89 B and Table 10).

A



B

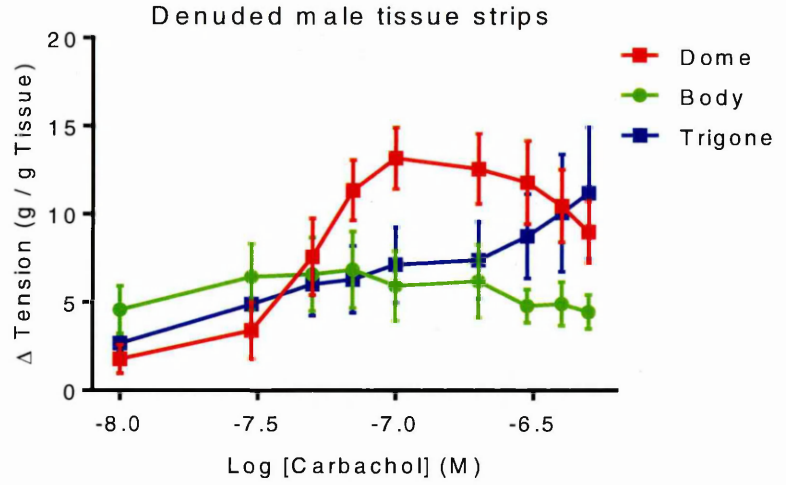


Figure 89 Regional differences in amplitude of carbachol-induced (0.01-0.5 μM) contractions in intact and denuded male bladder strips (mean ±SEM). (A) Amplitude in intact tissue strips of the dome, body and trigone from the male porcine bladder. (B) Amplitude in denuded tissue strips of the dome, body and trigone from the male porcine bladder (2-way ANOVA with Tukey's post-test, n=8, numbers and significance see Table 9 and Table 10).

Carbachol conc. (μM)	Amplitude Intact Tissue Strips	Amplitude 1 vs. (g/g tissue)	Amplitude 2 (g/g tissue)	Significance
0.01	Dome vs. Body	2.45	1.29	ns
	Dome vs. Trigone	2.45	2.13	ns
	Body vs. Trigone	1.29	2.13	ns
0.03	Dome vs. Body	3.07	1.57	ns
	Dome vs. Trigone	3.07	2.88	ns
	Body vs. Trigone	1.57	2.88	ns
0.05	Dome vs. Body	4.08	1.94	**
	Dome vs. Trigone	4.08	2.85	ns
	Body vs. Trigone	1.94	2.85	ns
0.07	Dome vs. Body	4.61	2.32	**
	Dome vs. Trigone	4.61	3.22	ns
	Body vs. Trigone	2.32	3.22	ns
0.1	Dome vs. Body	5.15	2.38	****
	Dome vs. Trigone	5.15	3.53	*
	Body vs. Trigone	2.38	3.53	ns
0.2	Dome vs. Body	5.39	3.40	**
	Dome vs. Trigone	5.39	3.95	ns
	Body vs. Trigone	3.40	3.95	ns
0.3	Dome vs. Body	4.92	3.30	*
	Dome vs. Trigone	4.92	4.15	ns
	Body vs. Trigone	3.30	4.15	ns
0.4	Dome vs. Body	4.06	3.45	ns
	Dome vs. Trigone	4.06	4.19	ns
	Body vs. Trigone	3.45	4.19	ns
0.5	Dome vs. Body	3.54	3.45	ns
	Dome vs. Trigone	3.54	3.88	ns
	Body vs. Trigone	3.45	3.88	ns

Table 9 **Regional differences in the amplitude of carbachol-induced contractions in intact tissue strips of dome, body and trigone from the male porcine bladder. The mean of amplitude measurements (g/g tissue) and the significance of difference is shown (2-way ANOVA with Tukey's post-test, n=8, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, **** indicates $p \leq 0.0001$).**

Carbachol conc. (μM)	Amplitude Denuded Tissue Strips	Amplitude 1 vs. (g/g tissue)	Amplitude 2 (g/g tissue)	Significance
0.01	Dome vs. Body	1.77	4.57	ns
	Dome vs. Trigone	1.77	2.67	ns
	Body vs. Trigone	4.57	2.67	ns
0.03	Dome vs. Body	3.38	6.42	ns
	Dome vs. Trigone	3.38	4.88	ns
	Body vs. Trigone	6.42	4.88	ns
0.05	Dome vs. Body	7.56	6.56	ns
	Dome vs. Trigone	7.56	6.00	ns
	Body vs. Trigone	6.56	6.00	ns
0.07	Dome vs. Body	11.33	6.83	**
	Dome vs. Trigone	11.33	6.28	**
	Body vs. Trigone	6.83	6.28	ns
0.1	Dome vs. Body	13.15	5.91	****
	Dome vs. Trigone	13.15	7.12	***
	Body vs. Trigone	5.91	7.12	ns
0.2	Dome vs. Body	12.56	6.18	****
	Dome vs. Trigone	12.56	7.38	**
	Body vs. Trigone	6.18	7.38	ns
0.3	Dome vs. Body	11.78	4.77	****
	Dome vs. Trigone	11.78	8.74	ns
	Body vs. Trigone	4.77	8.74	*
0.4	Dome vs. Body	10.45	4.89	***
	Dome vs. Trigone	10.45	10.05	ns
	Body vs. Trigone	4.89	10.05	**
0.5	Dome vs. Body	8.97	4.44	**
	Dome vs. Trigone	8.97	11.18	ns
	Body vs. Trigone	4.44	11.18	****

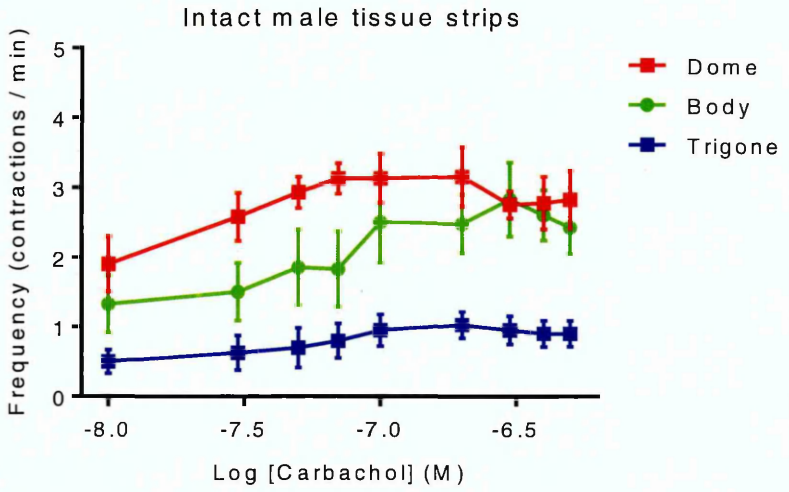
Table 10 **Regional differences in the amplitude of carbachol-induced contractions in denuded tissue strips of dome, body and trigone from the male porcine bladder. The mean of amplitude measurements (g/g tissue) and the significance of difference is shown (2-way ANOVA with Tukey's post-test, n=8, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$).**

Regional Difference in the Frequency of Carbachol-induced Contractions

Intact tissue strips of the trigone showed significantly fewer contractions at all used concentrations of carbachol compared to intact tissue strips of the body and the dome. Intact strips of the dome and body did significantly differ from each other at concentrations of 0.03, 0.05 and 0.07 μM carbachol (2-way ANOVA with Tukey's post-test, n=8, Figure 90 A and Table 11).

After removal of the urothelium, denuded strips of the trigone strips still showed the lowest frequency compared to the denuded strips of the body and the dome, apart from the lowest concentration of carbachol at 0.01 μM. Significant differences were seen between denuded strips of trigone and dome at all concentrations above 0.03 μM carbachol. Furthermore significant differences were observed between denuded strips of the trigone and the body at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 μM carbachol (2-way ANOVA with Tukey's post-test, n=8, Figure 90 B and Table 12).

A



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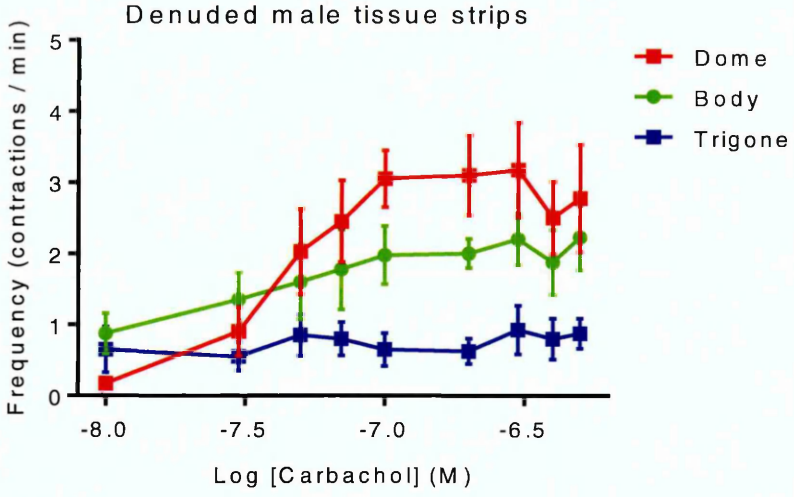

Figure 90 Regional differences in freqeuncy of carbachol-induced (0.01-0.5 µM) contractions in intact and denuded male bladder strips (mean ±SEM). (A) Contractions per min in intact tissue strips of the dome, body and trigone from the male porcine bladder. (B) Contractions per min in denuded tissue strips of the dome, body and trigone from the male porcine bladder (2-way ANOVA with Tukey's post-test, n=8, results see Table 11 and Table 12).

Carbachol conc. (μM)	Frequency Intact Tissue Strips	Frequency 1 vs. (contractions/min)	Frequency 2 (contractions/min)	Significance
0.01	Dome vs. Body	1.90	1.33	ns
	Dome vs. Trigone	1.90	0.50	***
	Body vs. Trigone	1.33	0.50	*
0.03	Dome vs. Body	2.58	1.50	**
	Dome vs. Trigone	2.58	0.63	****
	Body vs. Trigone	1.50	0.63	*
0.05	Dome vs. Body	2.93	1.85	**
	Dome vs. Trigone	2.93	0.70	****
	Body vs. Trigone	1.85	0.70	**
0.07	Dome vs. Body	3.13	1.83	***
	Dome vs. Trigone	3.13	0.80	****
	Body vs. Trigone	1.83	0.80	*
0.1	Dome vs. Body	3.13	2.50	ns
	Dome vs. Trigone	3.13	0.95	****
	Body vs. Trigone	2.50	0.95	****
0.2	Dome vs. Body	3.15	2.48	ns
	Dome vs. Trigone	3.15	1.03	****
	Body vs. Trigone	2.48	1.03	***
0.3	Dome vs. Body	2.75	2.83	ns
	Dome vs. Trigone	2.75	0.95	****
	Body vs. Trigone	2.83	0.95	****
0.4	Dome vs. Body	2.78	2.60	ns
	Dome vs. Trigone	2.78	0.90	****
	Body vs. Trigone	2.60	0.90	****
0.5	Dome vs. Body	2.83	2.43	ns
	Dome vs. Trigone	2.83	0.90	****
	Body vs. Trigone	2.43	0.90	****

Table 11 **Regional differences in the frequency of carbachol-induced contractions in intact tissue strips of dome, body and trigone from the male porcine bladder. The mean of frequency measurements (contractions/ min) and the significance of difference is shown (2-way ANOVA with Tukey's post-test, n=8, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$).**

Carbachol conc. (μM)	Frequency Den. Tissue Strips	Frequency 1 vs. (contractions/min)	Frequency 2 (contractions/min)	Significance
0.01	Dome vs. Body	0.18	0.88	ns
	Dome vs. Trigone	0.18	0.65	ns
	Body vs. Trigone	0.88	0.65	ns
0.03	Dome vs. Body	0.90	1.35	ns
	Dome vs. Trigone	0.90	0.55	ns
	Body vs. Trigone	1.35	0.55	ns
0.05	Dome vs. Body	2.03	1.60	ns
	Dome vs. Trigone	2.03	0.85	*
	Body vs. Trigone	1.60	0.85	ns
0.07	Dome vs. Body	2.45	1.78	ns
	Dome vs. Trigone	2.45	0.80	***
	Body vs. Trigone	1.78	0.80	ns
0.1	Dome vs. Body	3.05	1.98	*
	Dome vs. Trigone	3.05	0.65	****
	Body vs. Trigone	1.98	0.65	**
0.2	Dome vs. Body	3.10	2.00	*
	Dome vs. Trigone	3.10	0.63	****
	Body vs. Trigone	2.00	0.63	**
0.3	Dome vs. Body	3.18	2.20	ns
	Dome vs. Trigone	3.18	0.93	****
	Body vs. Trigone	2.20	0.93	**
0.4	Dome vs. Body	2.50	1.88	ns
	Dome vs. Trigone	2.50	0.80	***
	Body vs. Trigone	1.88	0.80	*
0.5	Dome vs. Body	2.78	2.23	ns
	Dome vs. Trigone	2.78	0.88	****
	Body vs. Trigone	2.23	0.88	**

Table 12 **Regional differences in the frequency of carbachol-induced contractions in denuded tissue strips of dome, body and trigone from the male porcine bladder. The mean of frequency measurements (contractions/ min) and the significance of difference is shown (2-way ANOVA with Tukey's post-test, n=8, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$, **** indicates $p \leq 0.0001$).**

4.3.4 GENDER DIFFERENCES IN THE CONTRACTILE ACTIVITY OF DENUDED AND INTACT BLADDER STRIPS FROM THE DOME, BODY AND TRIGONE REGION

4.3.4.1 GENDER DIFFERENCES IN SPONTANEOUS ACTIVITY OF DENUDED AND INTACT BLADDER STRIPS FROM THE DOME, BODY AND TRIGONE REGION

Following on from the regional disparities, gender differences in the amplitude and frequency of baseline spontaneous activity were analysed in intact and denuded tissue strips of all three regions. The number of tissue strips used for the intact as well as denuded data set of each region was 16 minus the strips that did not show any contractions (see 4.3.1).

Gender differences in intact tissue strips

Amplitude of spontaneous activity was analysed as previously described. No significant gender differences were observed in intact bladder strips from dome, body or trigone region (unpaired Student's T test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 91 A).

Frequency of spontaneous activity was not significantly different between the gender in tissue strips from the dome and trigone region. However, significantly more contractions per minute could be detected in female tissue strips of the intact body (3.23 ±0.29 contractions/ min) compared to the male counterpart (2.15 ±0.26 contractions/ min) ($p=0.011$, unpaired Student's T test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 91 B).

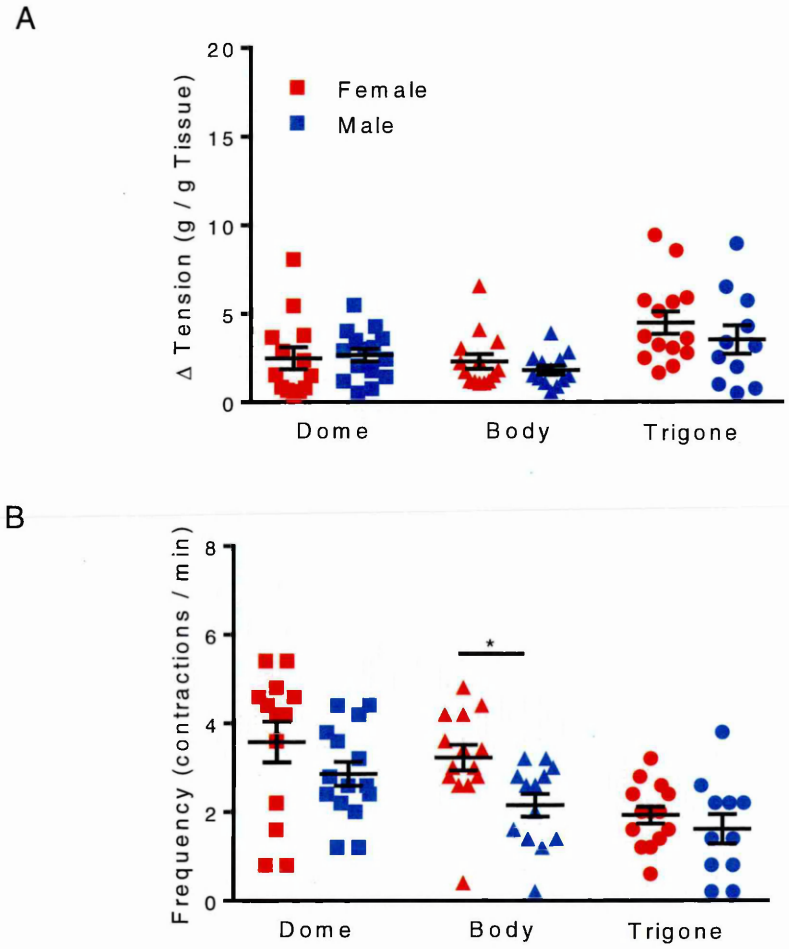

Figure 91 **Gender differences in amplitude and frequency of spontaneous activity in intact tissue strips of the three bladder regions (mean ±SEM). (A) Amplitude did not significantly differ between genders in any of the three regions. (B) Significantly less contractions were observed in the male body compared to female intact tissue strips (unpaired Student's T test, n=16 minus strips without contractile activity, * indicates $p \leq 0.05$).**

Gender differences in denuded tissue strips

Amplitude of spontaneous activity was not significantly different between the genders in tissue strips from the dome, body and trigone (unpaired Student's T test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 92 A).

Frequency of spontaneous activity was not significantly different between the gender in tissue strips from the dome and body region. However, significantly more contractions per minute could be detected in female tissue strips of the denuded trigone (2.29 ±0.22 contractions/ min) compared to the male counterpart (0.97 ±0.26 contractions/ min) ($p=0.043$, unpaired Student's T test, n=16 minus strips without contractile activity as described in 4.3.1, Figure 92 B).

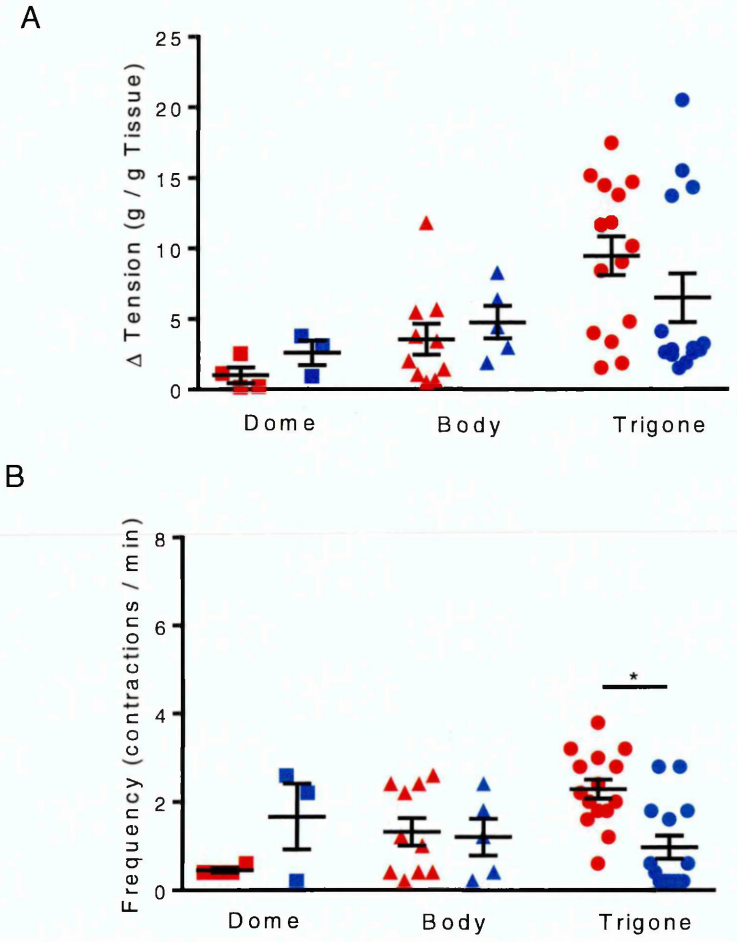

Figure 92 **Gender differences in amplitude and frequency of spontaneous activity in denuded tissue strips of the three bladder regions (mean ±SEM). (A) Amplitude did not significantly differ between genders in any of the three regions. (B) Significantly more contractions were observed in the female trigone compared to the male counterpart (unpaired Student's T test, n=16 minus strips without contractile activity, * indicates $p \leq 0.05$).**

4.3.4.2 GENDER DIFFERENCES IN CARBACHOL-INDUCED CONTRACTILITY OF DENUDED AND INTACT BLADDER STRIPS FROM THE DOME, BODY AND TRIGONE REGION

Amplitude

After cholinergic stimulation all tissue strips developed contractile activity. The amplitude of contractions did not significantly differ between female and male tissue strips of the dome and the body, neither in intact nor in denuded strips (Figure 93 A, B, C and D). However the amplitude of contractions was significantly increased in the intact as well as denuded tissue strips from the female trigone at all concentrations of carbachol used (Figure 93 E and F, 2-way ANOVA with Bonferroni's post-test, n=8).

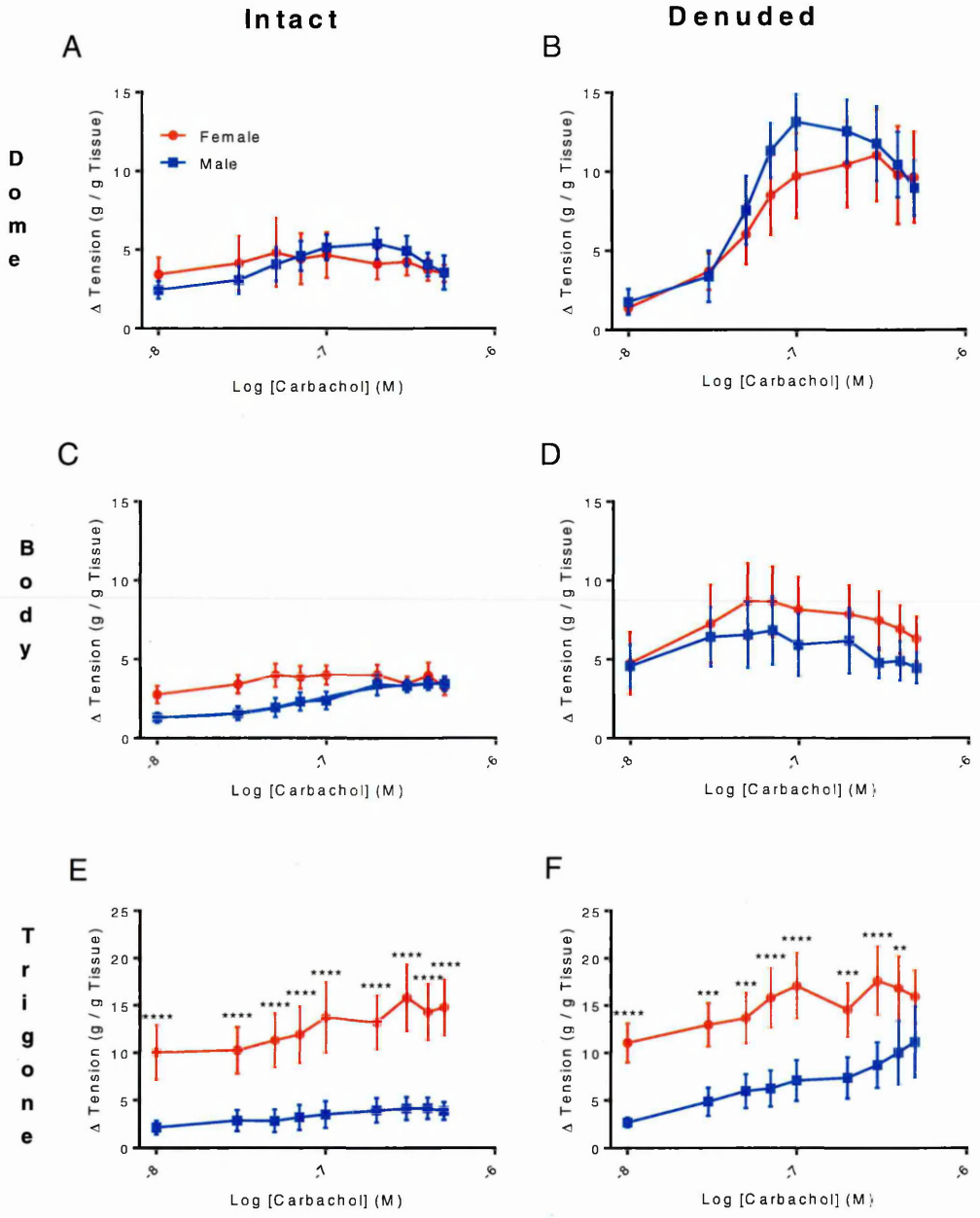

Figure 93 **Gender difference in the amplitude of carbachol-induced contractions (mean ±SEM). (A, B, C, D) No significant difference in the amplitude could be observed between female and male tissue strips of the dome and the body; neither in intact nor in denuded strips. (E, F) Intact as well as denuded tissue strips from the female trigone showed significantly greater amplitude of contractions at all used carbachol concentrations (2-way ANOVA with Bonferroni's post-test, n=8, ** indicates p≤0.01, *** indicates p≤0.001, **** indicates p≤0.0001).**

Frequency

The frequency of contractions did not significantly differ between female and male tissue strips of the dome; neither in intact nor in denuded strips (Figure 94 A and B).

Intact tissue strips of the female body showed significantly greater frequency at 0.01 and 0.05 μM carbachol compared to the male counterpart (Figure 94 C). No significant gender difference could be observed in the denuded body strips (Figure 94 D).

Intact as well as denuded tissue strips of the female trigone showed significantly higher frequency throughout all carbachol concentrations (0.01 to 0.5 μM) compared to the male counterparts (Figure 94 E and F, 2-way ANOVA with Bonferroni's post-test, n=8).

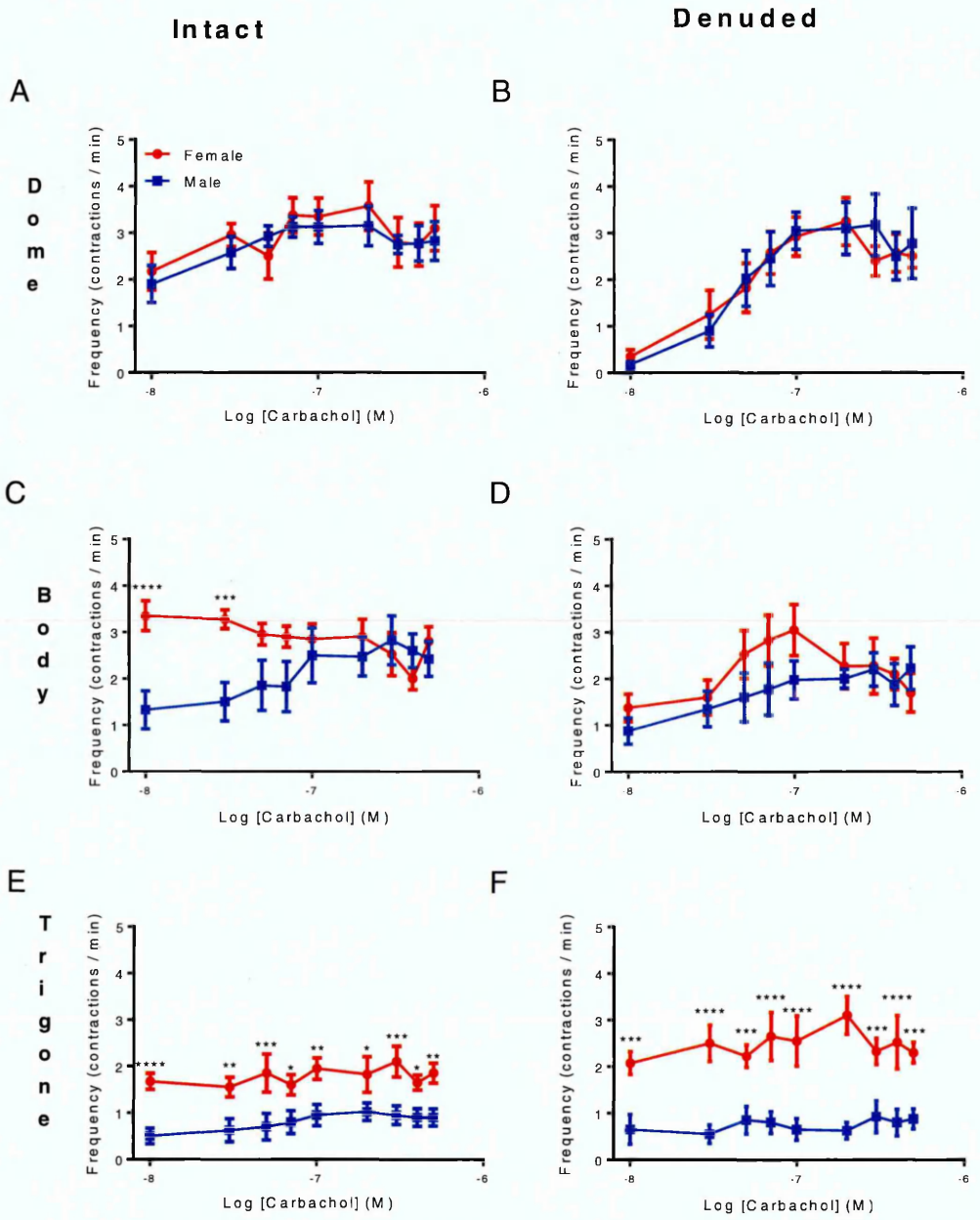

Figure 94 **Gender difference in the frequency of carbachol-induced contractions (mean ±SEM). (A, B) No significant difference could be observed between female and male tissue strips of the dome, neither in intact or in denuded strips. (C, D) In the body the intact tissue strips of the female bladder showed significantly more contractions at 0.01 and 0.05µM carbachol. No significant difference was observed in the denuded tissue strips. (E, F) Intact as well as denuded tissue strips from the female trigone showed significantly more contractions at all carbachol concentrations compared to male tissue strips (2-way ANOVA with Bonferroni's post-test, n=8, * indicates p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).**

4.4 DISCUSSION

The aim of this chapter was to investigate differences in the contractile response of porcine bladder strips regarding the effect of the urothelium, regarding regional differences between dome, body and trigone and regarding possible gender differences.

Occurrence of Spontaneous Activity

Spontaneous contractions have been shown to develop without any stimulation in isolated, whole bladders and in bladder strips from many species (Sibley 1984, Hawthorn *et al.,* 2000, Roosen *et al.,* 2009, Parson *et al.,* 2012). The results in 4.3.1 show that the development of spontaneous activity varied according to region, gender and between intact and denuded tissue strips of the porcine bladder. Not all tissue strips developed spontaneous activity. Fewer strips of the denuded dome and body developed spontaneous contractions compared to their intact counterparts in both genders. Damage of the tissue following the dissection of the urothelium may lead to an impairment of function and therefore the decreased ability to develop spontaneous activity. This assumption is however challenged by the fact that the same percentage of intact and denuded strips from the female trigone developed spontaneous activity (94%) and a higher percentage of denuded strips from the male trigone showed spontaneous activity compared to the intact counterpart (69% vs. 88%).

The results shown in this thesis are in line with a study by Akino *et al.* (2008) showing that female porcine tissue strips from the trigone region developed spontaneous activity irrespective of the presence or absence of the urothelium while tissue strips from the dome area showed a delayed development of spontaneous contractions after removal of the urothelium compared to intact tissue strips. The results suggest that the generation of spontaneous contraction is dependent on the urothelium in the dome and body but not in the trigone.

It can therefore be assumed that depending on the bladder region the urothelium plays a role in the modification of contractility. This might be through the release of specific

mediators such as ACh and ATP acting on neighbouring cells and underlying tissue. Different cellular mechanisms may be initiated that are responsible for the onset of spontaneous contractions in the bladder.

Sibley (1984) underlined this regional disparity by showing that only 19% of 191 muscle strips from the pig bladder dome developed spontaneous contractions while 89% of 19 strips from the trigone showed activity. In the results shown in this thesis, the same trend could be observed for the denuded tissue strips. While only 25% of female and 19% of male dome strips developed spontaneous contractions, 94% of female and 88% of male trigone strips developed spontaneous contractions. A regional disparity in the development of spontaneous contractions seems to exist in both genders and furthermore in different species as it could be shown for pig and human bladder strips (Sibley 1984) and for the guinea-pig (Roosen *et al.,* 2009). Roosen *et al.* (2009) reported that only 38% of 21 intact strips from the male guinea-pig bladder dome developed spontaneous activity while 68% of 28 strips from the trigone showed activity. Sibley (1984) stated that 20% of 139 intact strips from the human dome and 71% of 7 strips from the human trigone developed spontaneous contractions.

Regarding these studies and the results shown in this thesis, it can be stated that a region dependent difference seems to exist in the occurrence of spontaneous activity, which might reflect specific physiological roles of the different bladder regions. This could explain the contradicting results of organ bath strip experiments found in the literature as the outcome of the study highly depends on the bladder region the tissue strips were taken from. It is important that in further studies the exact location of the used specimen is noted.

Amplitude and Frequency of Spontaneous Activity

Amplitude and frequency of spontaneous activity were analysed in the present study. The amplitude of spontaneous activity was significantly greater in denuded strips from female trigone compared to the intact counterpart. However there was no significant difference between the intact and denuded dome and body strips of the female bladder.

While in male trigone strips spontaneous activity was also higher in the denuded strips from the trigone compared to the intact counterpart, this was not significant. Furthermore intact strips of the male body region showed significant greater amplitude than the denuded strips.

Hawthorn *et al.* (2000) showed increased carbachol induced activity of tissue strips after removal of the urothelium and suggested the presence of an urothelium-derived inhibitory factor (UDIF). The presence of an UDIF is supported by the results in the trigone area showing that the spontaneous activity was significantly greater in denuded strips compared to the intact counterpart. However there was no significant difference between the intact and denuded dome and body strips of the female bladder and trigone and dome strips of the male bladder. The proposed UDIF might therefore only be released after stimulation of cholinergic receptor as Hawthorn *et al.* (2000) used carbachol to stimulate activity before comparing the activity of intact and denuded tissue strips.

These results are difficult to compare to literature as limited information are accessible regarding the difference of amplitude of spontaneous contractions between intact and denuded tissue strips in the three different bladder parts. Akino *et al.* (2008) demonstrated however that there are no significant differences in amplitude between intact and denuded strips from the dome and from the trigone region of female pigs. The contradicting results might be due to different tissue preparations or experimental set ups and need to be further investigated.

Regional disparities in the amplitude of spontaneous contractions were found in the female bladder but not in the male bladder. Female trigone strips showed significantly greater amplitude compared to body and dome strips irrespective of the presence or absence of the urothelium. These results are in line with studies by Nyamwaro (2012) showing a higher amplitude in intact as well as denuded tissue strips of the trigone compared to dome and body strips in the female bladder.

Akino *et al.* (2008) studied the effect of time to the onset of spontaneous activity in intact and denuded tissue strips of the dome and trigone region. It was shown, that the tissue strips from the dome demonstrated a delayed development of spontaneous contractions

after removal of the urothelium compared to intact tissue strips with some strips taking more than 70 minutes to develop full activity. Spontaneous activity in tissue strips from the trigone region developed quickly within ten minutes, irrespective of the presence or absence of the urothelium. It might therefore be time dependent when spontaneous contractions occur in the three regions and the different results published might be partly due to the different time points of measurement. It would be interesting to obtain experiments with a longer time scale than the 60 minutes incubation period used in this study as this might not be long enough for spontaneous activity to fully develop in the dome. Less than a quarter of the 16 used dome tissue strips developed spontaneous activity in this thesis and were taken into consideration for the analyses.

Drake *et al.* (2003) demonstrated the spread of bladder contractile activity in the isolated guinea pig bladder via multiple-point motion analysis. It was observed that the autonomous activity was highly complex and comprised of localised micro-contractions in single or multiple discrete regions, waves of activity and micro-stretches. Therefore it would be interesting to compare regional activity in the whole pig organ to observe how contractions in specific regions spread and interact with each other. Regional difference in the onset of contractile activity might reflect specific physiological roles of the three regions.

Variations in the morphology of the bladder wall in the different bladder regions might account for the found regional disparity. Histological studies have revealed a significant regional difference in the thickness of the detrusor muscle in female porcine bladders while the thickness of the urothelium did not significantly change between the three regions. The detrusor was significantly thicker in strips from the body region compared to strips from both trigone and dome (Nyamwaro 2012). It was therefore expected, that the contractile force is greater in tissue strips from the body region. The outcomes of the experiments in this thesis showed however, that intact as well as denuded tissue strips of the female trigone revealed significantly greater amplitude of spontaneous contractions, as well as frequency compared to intact dome and body strips. Therefore it can be hypothesised, that the thickness of the detrusor does not reflect the ability of the tissue strip to contract and that regional differences in the bladder wall rather occur at cellular level than at structural level.

The reason for the demonstrated regional disparity could therefore rather be due to differences in the distribution of receptors and ion channels in the urothelium and the detrusor that leads to differences in signalling transduction and therefore differences in the contractile activity. Published data on the receptor distribution between the three regions in the porcine bladder were not available. It was however observed that muscarinic receptor subtype expression differs between the intact dome, body and trigone in the human bladder (Sigala 2002). A difference in the contractile properties of the three regions might therefore be due to a variation in muscarinic receptor expression. This could lead to a difference in the sensitivity to ACh, the main mediator involved in bladder contractions which is also released by the urothelium. Further studies should be conducted to investigate if these spontaneous contractions are due to ACh release and the activation of muscarinic receptor. This could be performed by using atropine, a non-selective muscarinic antagonist.

Carbachol-induced Contractile Activity

The cholinergic agonist carbachol was used to mimic the effect of ACh on the contractility of male and female porcine bladder strips of the three regions. After cholinergic modulation with carbachol (0.01 – 0.5 μM), the amplitude of denuded tissue strips was greater compared to the intact counterparts in all regions and both genders. This was significant in dome strips of both genders at concentrations of 0.2, 0.3, 0.4 and 0.5 μM carbachol for female dome strips and at concentrations of 0.07, 0.1, 0.2, 0.3, 0.4 and 0.5 μM carbachol for male dome strips.

The urothelium was causing an inhibition of carbachol-induced contractions, which was particularly pronounced in tissue strips of the dome region. The mechanism underlying this inhibition is not clear. As previously mentioned, the release of an urothelium-derived inhibitory factor has been proposed (Hawthorn *et al.,* 2000). This UDIF is probably only released after cholinergic stimulation as it has been shown in this thesis that spontaneous contractions were not influenced by the presence of the urothelium in all bladder regions.

The identity of this UDIF has not been revealed so far, it was however shown that it is unlikely to be NO, adenosine, a cyclo-oxygenase product, a catecholamine or GABA,

which represent the majority of well-established detrusor inhibitory pathways (Hawthorn *et al.,* 2000).

The urothelium has been shown to release various mediators such as ATP (Ferguson *et al.,* 1997), ACh (Yoshida *et al.,* 2006) and NO (Birder 2006). These released mediators can act on neighbouring urothelial cells as a variety of receptors and ion channels are present in the urothelium. The inhibitory effect of the urothelium might therefore be due to a whole array of mediators that are initiating signalling cascades acting on the detrusor muscle to inhibit the cholinergic induced contractions.

Apart from the differences between intact and denuded tissue strips, regional differences were demonstrated in amplitude and frequency of carbachol-induced contractions. In intact as well as denuded tissue strips from the female bladder the amplitude of contractions was significantly higher in the trigone compared to the dome and body. The frequency was however lowest in the intact trigone showing the relation of frequency of amplitude. A contraction higher in amplitude needs longer to develop which means that the frequency decreases. Tissue strips from male bladders showed lowest contraction levels in the body when comparing intact strips and highest in the dome when comparing denuded strips.

These regional disparities might be due to differences in the signalling transduction of released mediators to the underlying detrusor muscle. As previously stated no data were available on the muscarinic receptor distribution between the three regions in the porcine bladder. In intact human bladder strips it could however been shown, that the female trigone expresses low levels of mRNA encoding for M_1, M_3 and M_4 muscarinic receptor subtypes and lacking the expression of M_2 and M_5, while in the female body and dome all five receptor subtypes M_1 - M_5 were expressed. The male trigone however expressed all five receptor subtypes with M_4 being expressed at a low level while the male body and dome also expressed all five receptor subtypes with low expression for M_3 and M_4 (Sigala 2002). Another study demonstrated that the dome contained higher levels of mRNA expression for $P2X_1$, M_2 and M_3 in comparison with the trigone while mRNA expression for cellular adhesion and tight junction proteins were higher in the trigone compared to the dome (Sanchez Freire *et al.,* 2011). These regional differences in the receptor expression of muscarinic and purinergic receptor might explain the differences seen in the carbachol-

induced contractions in the three bladder regions. As activation of urothelial muscarinic receptor leads to the release of other mediators including ATP (Kullmann *et al.,* 2008), cholinergic modulation of bladder contractions will not only stimulate muscarinic and nicotinic but also for example purinergic receptor.

The identification of the functional responses of the muscarinic receptor subtypes have not been very successful so far, due to the lack of selectivity of the available agonists and antagonists (Scarpero and Dmochowski 2003) and the fact that the examined tissues often express all five receptor subtypes. Therefore no conclusion can be drawn on the functional effect these subtypes might have in the different bladder regions. In further experiments the muscarinic receptor distribution in the porcine bladder should be studied to see if a link can be drawn from the regional differences in carbachol-induced contractions to the distribution of muscarinic receptors in the bladder.

Gender Differences of the Contractile Activity

The data were analysed for possible gender differences, as differences in the pattern of spontaneous as well as carbachol-induced contractions were noticed between male and female porcine bladder strips. Furthermore the symptoms of bladder dysfunctions differ between the genders as previously stated in 4.1 (Madersbacher *et al.,* 1998, Irwin *et al.,* 2006, Findik *et al.,* 2012).

Distinct gender differences in the occurrence of spontaneous activity were shown in the intact trigone with 94% of female tissue strips developing activity compared to 69% of male tissue strips. Furthermore in the denuded body with 69% of female tissue strips developing activity compared to 38% of male tissue strips. Amplitude of spontaneous activity was however not significantly different between the genders neither in intact nor in denuded tissue strips. However the frequency of contractions was higher in female tissue strips of the intact body and of the denuded trigone.

Gender differences could also be seen in the trigone after cholinergic stimulation. Female tissue strips showed significantly higher amplitude and frequency in the contractile response. This was the case for both intact and denuded tissue strips.

These results suggest that the female trigone strips develop spontaneous activity faster and at a higher frequency. The female trigone is also more sensitive to carbachol stimulation, showing a greater amplitude and frequency of contractions compared to the male counterpart.

Only few studies have been published looking at the influence of gender on the contractility of the bladder in response to cholinergic stimulation. Longhurst and Levendusky (2000) showed that intact bladder strips from female rats developed a greater contractile response to stimulation with carbachol compared to male bladder strips. This higher sensitivity of female bladder strips to carbachol could be seen at all stages of the oestrous cycle of the rat. Another study showed that carbachol-induced contractions were more potent in female rat detrusor compared to the male counterpart, in both intact and denuded tissue strips of the dome (Liang and Leung 2012). These study outcomes are in line with the above shown results.

However a contradicting study by Fry *et al.* (2011) showed that there was no gender difference in carbachol-induced contractions of human bladder samples. The investigators only used one concentration of 10μM carbachol and it was again not stated from which part of the bladder the biopsies were taken. In the current study various concentrations of carbachol were compared and strips were taken from the three regions dome, body and trigone which might account for the contradictory results.

The underlying cause for the observed gender differences could be a difference in the expression of several receptors. Experiments on the distribution of cholinergic receptors in human urothelial tissue have revealed, that levels of mRNA for the M1, M3, M4 and M5 muscarinic receptor subtypes were not influenced by gender while the level of mRNA for the M2 muscarinic receptor subtype was higher in adult woman compared to man (Arrighi *et al.,* 2008). As the quoted study was carried out on human biopsies, it is not stated from which part of the bladder the tissue was taken. Therefore it is difficult to draw a conclusion as it has already been stated before how regional differences influence the outcome of bladder studies. Another study by Kories *et al.* (2003) showed that number and function of the M2 and M3 muscarinic subtypes did not differ between urinary bladder of male and female rats. Again the part of the bladder from which the strip was taken is not quoted. This

and a difference between species might be the reason for the differing results between the two quoted studies.

The gender difference after cholinergic stimulation could be seen in intact and denuded tissue strips. It will be interesting to examine if cholinergic receptor in the porcine urothelium and in the detrusor show a gender specific distribution.

Apart from a difference in the distribution of cholinergic receptors, sex specific hormones like androgens and estrogens as well as their receptor distribution could be responsible for gender differences in bladder contractility. Estrogen therapy has indeed been shown to be beneficial in easing symptoms of OAB in postmenopausal women and decreased estrogen levels have linked been linked to the increased occurrence of OAB in postmenopausal women (Hanna-Mitchell *et al.,* 2013, Robinson *et al.,* 2014). Furthermore several mechanism of estrogen have been suggested which could influence cell contractility such as the endothelial-dependent release of NO in arteries of rats (Broughton *et al.,* 2010), the activation of protein kinases in guinea-pig neurons (Kelly *et al.,* 1999) and the activation of potassium channels (Rosenfeld and Roy 2012).

Male as well as female pigs of around 6 months of age were used for the experiments of this study. The used breeds have reached sexual maturity at this age and the female pigs have therefore started their oestrous cycle. Female pigs have an oestrous cycle of 21 days rather than a menstrual cycle like humans (Tumbleson and Schook 1996) and it was not known at which point of the cycle the pigs were situated when used in the above experiments. The male pig bladders were taken from pigs that did not undergo castration after birth. Therefore the male as well as the female porcine tissue used for this study will have been influenced by sex specific hormones. Standardising the time point of the oestrous cycle might be important to conduct further experiments. However this is not easily feasible when obtaining tissue from an abattoir. It would also be interesting to study if the efficacy of anticholinergic drugs changes during the menstrual cycle in humans or looking at the distribution of sex specific hormone receptors in the bladder.

Concluding it can be stated that this study revealed a role of the urothelium as well as region and gender dependent differences in spontaneous activity as well as in carbachol-induced contractions in the porcine bladder. Predominantly the trigone region seems to play a distinctive role in the contractile properties of the bladder. The results added new information to the understanding of bladder contractile physiology. The gender and regional disparities of the contractile properties of the bladder should be considered when planning further studies.

5. CONCLUSION

This thesis illustrates a body of work investigating various mechanisms and signalling pathways that are involved in the mechanosensation of the urinary bladder.

Lower urinary tract symptoms such as overactive bladder (OAB) are distressing conditions that affect more and more people worldwide. OAB is defined as 'urgency with or without urge incontinence, usually with frequency and nocturia' (Abrams *et al.,* 2003) and is characterised by increased spontaneous activity of the detrusor smooth muscle during the filling phase (Andersson 2010). It is estimated that about 11.8% of the total population over 18 years of age are affected by OAB (Irwin *et al.,* 2006) with an estimated increase to 546 million individuals by 2020 (Irwin *et al.,* 2011).

The aetiology of OAB is still unknown but one convincing hypothesis states, that alterations to the sensory system of the urothelium play an important role. It has been suggested that a change in mediator release from the bladder urothelium influences afferent nerves, ICs and detrusor muscle excitability and finally leads to the onset of OAB (Birder *et al.,* 2012). Understanding these urothelial signalling pathways that are involved in the mechanosensation of the urinary bladder will improve the understanding of bladder function, both in health and pathology and ultimately lead to more effective treatment options available to the patient. The overall aim of this thesis was to further investigate these mechanisms and signalling pathways involved in the mechanosensation of the bladder urothelium, with focus on the cholinergic system.

Role of the Cholinergic Pathway in Mechanosensation of the Urothelium

Various lines of evidence suggest that non-neuronal mediator release plays a key role in the mechanosensation of the urinary bladder and that the urothelium is the source of this mechanically stimulated mediator release. To analyse mediators in intraluminal samples, a purpose-built micro organ bath for isolated, whole mouse bladders was successfully established and optimised in this thesis. Intra- and extraluminal samples for mediator analyses were obtained under various levels of mechanical stimulation and under the influence of specific drugs.

The main focus of this thesis laid on the cholinergic pathway in the bladder, as antimuscarinic treatment is the mainstay therapy for OAB even though it is often lacking in efficacy. In contrast to the neuronal cholinergic signalling pathways found in other tissues, the urothelium inhibits a non-neuronal cholinergic signalling pathway which could be exclusively targeted by a pharmacological approach and provide novel targets for the therapeutic intervention of bladder disorders.

There is extensive evidence that the urothelium releases ACh (Yoshida 2006, Hanna-Mitchell *et al.,* 2007, McLatchie *et al.,* 2014). It could be shown in this thesis that distension of the isolated, whole mouse bladder evoked a significant increase of ACh in intra- and extraluminal samples compared to basal measurements. Rise in intraluminal ACh after distension was unequally higher than the rise in extraluminal ACh. Furthermore, ACh found in extraluminal samples was not influenced by changes in pressure. This suggests that the detrusor muscle does not release ACh in a mechanosensitive way, while the urothelium seems to communicate the received mechanical signal via ACh.

The source of ACh identified in the intraluminal samples could either be ICs, nerve terminals or the urothelium. Literature showed that ICs are rarely seen in close proximity to cholinergic nerves in the mouse bladder (Lagou *et al.,* 2006). There is furthermore no known study demonstrating the release of ACh by ICs and also the number of ICs is comparably low. Investigations with Botulinum toxin (BoNT/A) in the present thesis furthermore implied, that ACh found in the intraluminal samples was not released by nerve endings but most likely by the urothelium. BoNT/A blocks the vesicular release of ACh

and there is strong evidence, that ACh is not released in a vesicular way in the urothelium, while this is the case for neurons.

Graded distensions from 20 to 60 mm Hg at a perfusion rate of 150 µl/ min did not show a significant change in ACh release, neither intra- nor extraluminally. However after applying a more physiological set-up for the mouse bladder with a perfusion rate of 40 µl/ min and pressure levels from 5 - 20 mm Hg, ACh content in intraluminal samples rose significantly from about 3 nM/ µl at resting level to 40 nM/ µl at 5 mm Hg and dropped down with higher pressure levels until back at basal level at 20, 30 and 40 mm Hg. It can be assumed that ACh release is very sensitively tuned and a slight change in pressure already activates the release mechanism. These results are in line with a recent publication demonstrating that a minimal mechanical stimuli of isolated urothelial guinea pig cells already achieved maximum ACh release, which also suggests that urothelial release of ACh has a small dynamic range (McLatchie *et al.,* 2014). However more experiments need to be conducted to verify these results. The concentration of ACh in intraluminal samples decreased with rising pressure levels. A stretch dependent choline uptake appears to be a convincing explanation for this decline of ACh. Spontaneous contractions were measured as transient rises in intraluminal pressure and occurred in the detrusor muscle during the filling phase. Spontaneous activity showed a positive correlation to rising pressure levels and the amount of ACh decreased with rising pressure. Further studies need to be carried out to investigate if ACh release from the urothelium actually inhibits spontaneous contractions.

The cholinergic pathway in the mouse bladder was studied further by blocking different distinct components of ACh release and choline reuptake. Hemicholinium was used to block ChT to prevent choline - the breakdown product of ACh - being taken up into the urothelial cells. Decynium 22 was used to block OCTs, to prevent ACh release. MLA was used to block α7 nicotinic receptor. All three drugs reduced bladder compliance and the detrusor contracted to a degree that made it difficult to obtain an appropriate sample size to run the ACh assay. Further optimisation is needed to obtain useful results with this approach. It can however be suggested that interfering at any point in the cholinergic

pathway does trigger the same response in the muscle, leading to an extremely contracted detrusor muscle, already at low pressure levels.

Another mediator that has been shown to be released by the urothelium is ATP (Ferguson *et al.,* 1997, Birder *et al.,* 2003, Cheng *et al.,* 2011, McLatchie *et al.,* 2014). In this thesis, ATP was seen to be significantly bigger in intraluminal samples after distension compared to basal measurement. ATP could however not be shown to be released in relation to increasing stretch. This could be due to the short half-life of ATP in the intact bladder. The concentration of ATP is regulated by ATP release and its breakdown by ectonucleotidases. Ectonucleotidases exist in the bladder wall (Lewis and Lewis 2006) and ATP released into the lumen could therefore be broken down relatively quickly. ATP release might be triggered by ACh release as shown in the study by McLatchie *et al.* (2014), but might be metabolised too quickly into adenosine to be detected with the above described experimental set up. In former studies, adenosine, the breakdown product of ATP, has been shown to modulate afferent function and the contraction of the detrusor (Yu *et al.,* 2009). Further investigations should therefore be carried out to investigate if intraluminal adenosine levels change with varying degrees of bladder pressure.

Small amounts of NO (0 – 200 pM) could also be detected in intraluminal samples of the distended, isolated mouse bladder. After treatment with BoNT/A, the amount of NO in the intraluminal samples increased significantly by approximately five times. BoNT/A has probably not acted directly on the release of NO. Instead, the release of other mediators could have changed due to the inhibition of vesicular release and resulted in a signalling cascade that eventually caused the increase in NO.

It has been hypothesized in previous studies, that the balance of inhibitory and excitatory mediator released from the urothelium modulates afferent nerve activity. In the present study, application of BoNT/A alters this balance, by decreasing the excitatory mediator ATP and by increasing the inhibitory mediator NO. It has been shown by colleagues that this change altered neuronal function and caused decreased mechanosensation (Daly *et al.,* 2014). Studying the imbalance of these mediators released by the urothelium in a whole, isolated bladder in various disease states will be an interesting approach for future research.

Impact of Ageing on the Mechanosensation of the Mouse Bladder

It is clearly important to understand the physiological changes that are occurring in the bladder function throughout life, when considering the increasing prevalence of OAB in the aged population. About 20% of the population is affected by OAB at the age of 70 (Milsom *et al.,* 2001) and evidence exists about altered signalling pathways in the bladders of aged animals.

It has been demonstrated that aged mice show a significant increase in natural bladder voiding and an augmented afferent nerve firing during bladder filling compared to adult mice (Daly *et al.,* 2014). This increased mechanosensitivity could be due to age-related changes in morphology and function of the sensory nerves, the detrusor muscle or the urothelium. Several studies have however shown that the general pattern of innervation seemed to be conserved during ageing (Nakayama *et al.,* 1998, Mohammed and Santer 2002, Aizawa *et al.,* 2011) and it is therefore most likely that changes in the detrusor or the urothelium account for the increased mechanosensitivity in the aged animals.

It was demonstrated in this thesis that amplitude and frequency of spontaneous contractions did not significantly differ between denuded detrusor strips of adult and aged mice. Furthermore, no significant difference could be seen in the development of spontaneous activity in the whole, isolated bladder of adult and aged mice. These results are in line with a recent study by Smith *et al.,* (2012), showing no change in nonvoiding contractions with increasing age in the female mice under *in vivo* conditions. Furthermore, no significant changes for maximum detrusor pressure and detrusor pressure at peak flow rate could be seen in an urodynamic study on men and women over 40 years of age (Madersbacher *et al.,* 1998). It can therefore be assumed that detrusor contraction strength does not decline in the ageing bladder.

However, the muscarinic and purinergic agonists, bethanechol and ATP, evoked detrusor contractions that were enhanced in aged murine detrusor strips compared to the adult counterpart. This suggests a higher sensitivity of the aged detrusor tissue to purinergic and muscarinic stimulation. Muscarinic receptor density has been reported to be increased in the bladder base of old rats (Kolta *et al.,* 1984) and rabbits (Latifpour *et al.,* 1990)

compared to adult animals. The higher sensitivity of the aged bladder tissue to the applied agonists could therefore be due to a higher density of receptors in the aged mouse.

Colleagues demonstrated indeed that gene and protein expression in the urothelium are altered in the aged bladder compared to the adult bladder. PCR analyses of mRNA expression in the aged urothelium found however a significant reduction, not increase, of all muscarinic and all P2X receptor genes, apart from $P2X_4$, compared to the adult urothelium (Daly *et al.,* 2014). This down regulation could be due to an increased mediator release from the urothelium. It was therefore investigated in this thesis if urothelial release of ACh and ATP is altered between adult and aged mice. ACh was shown to be significantly decreased and ATP significantly increased in the intraluminal samples of the whole, isolated aged murine bladder compared to samples of the adult bladders. These results are in line with studies by Yoshida *et al.* (2000, 2004) carried out on human bladder strips, showing that increasing age is associated in a linear manner with a decrease in ACh release and an increase in ATP release. Furthermore, no significant change in the amount of NO could be found in intraluminal samples from aged and adult mice in this study.

As far as is known, this is the first study attempting to demonstrate the release of Substance P in the isolated mouse bladder. There are several studies indicating a role of Substance P in urothelial signalling. Tachykinin receptors are expressed in cat urothelial cells (Birder *et al.,* 2010) and in porcine urothelial cells (Bahadory 2013). Furthermore, Substance P had been demonstrated in urothelial cell lysates of cats and an increase of Substance P could be shown in cats with feline cystitis (Birder *et al.,* 2010). In this study, Substance P could not be detected in intraluminal samples under normal conditions or in samples of aged mice. The role of Substance P in urothelial signalling processes might be species specific or dependent on certain circumstances such as the onset of a specific disease.

As stated before, it is assumed that the mediators analysed in intraluminal samples are released by the urothelium. It is however possible that other cell types, such as nerve endings or ICs, are releasing these mediators. It is also possible that the breakdown and reuptake of mediators has changed in the aged bladder and not the release of mediators. These concerns need further investigation.

Calcium imaging experiments were carried out with isolated urothelial cells to further investigate altered signalling pathways. Experiments carried out by colleagues showed that ageing had no effect on the urothelial response to the muscarinic agonist bethanechol. However the mobilisation of Ca^{2+} in urothelial cells from aged mice was increased by the purinergic agonist ATP (Daly *et al.,* 2014). In this thesis it could be shown that the selective $P2X_1$ and $P2X_3$ receptor agonist αβMeth-ATP produced a significantly greater signal in urothelial cells from aged bladders, while no significant difference could be detected after applying the $P2X_1$ selective agonist βγMeth-ATP. Consequently, it was assumed that the altered purinergic signalling response is likely to be mediated via the $P2X_3$ receptor. Colleagues verified this hypothesis by using Western blot analyses to show that the $P2X_3$ receptor expression is significantly higher in the aged urothelium compared to the adult urothelium (Daly *et al.,* 2014). In contrary, mRNA expression was significantly decreased in the urothelium, suggesting that regulation processes from mRNA translation into protein are altered during ageing in the mouse urothelium.

New insights on the physiological changes occurring in the bladder of the naturally aged mouse have been shown in this thesis. A higher sensitivity of the aged bladder in response to purinergic and muscarinic stimulation could be revealed. Furthermore, the aged urothelium showed an increase in ATP and a decrease in ACh release, as well as an increased purinergic receptor sensitivity of urothelial cells, which is probably facilitated via the purinergic $P2X_3$ receptor. Further characterisations of the studied pathways are now required to fully validate the data, most suitably in human tissue, as it is not clear if the same pathways are affected by age in the human bladder.

Spontaneous and Carbachol-induced Activity of Porcine Tissue Strips

To further evaluate the involvement of the cholinergic pathway in bladder contractility, experiments with porcine tissue strips were carried out. Amplitude and frequency of spontaneous and of carbachol-induced contractions were investigated.

Not all tissue strips developed spontaneous activity. The occurrence of spontaneous activity varied according to region, gender and between intact and denuded tissue strips. Fewer strips of the denuded dome and body developed spontaneous contractions compared to their

intact counterparts in both genders. However, the same percentage of intact and denuded tissue strips from the female trigone developed spontaneous activity (94%) while an even higher percentage of denuded tissue strips from the male trigone showed spontaneous activity compared to the intact counterpart. A region dependent difference could also be observed with most contractions occurring in the trigone area. The same trend has been shown before by Sibley (1984) in pig bladder strips as well as human bladder strips of the trigone and dome and by Roosen et al (2009) in guinea-pig bladder dome and trigone.

Amplitude of spontaneous activity of female porcine tissue strips was higher in denuded strips from the trigone compared to the intact counterpart. The finding supports the hypothesis of the existence of an urothelium-derived inhibitory factor as suggested by Hawthorn *et al.* (2000). However, there was no significant difference in the contractility between the intact and denuded strips from the female bladder dome and body, while the denuded strips from the male body area showed significant lower amplitude than the intact counterparts. It would be worth to investigate a longer time scale than the 60 minutes incubation period used in this study, as this might not be long enough for spontaneous activity to fully develop in the dome. It has been shown by Akino *et al.* (2008) that spontaneous activity in the trigone develops relatively quickly, within ten minutes while spontaneous activity in the dome takes sometimes more than 70 minutes to develop. In this study, less than a quarter of the 16 dome tissue strips developed spontaneous activity.

The findings indicate furthermore that the role of the urothelium varies according to the region and that the trigone plays an exceptional role. In further experiments it should be examined if mediator release and receptor distribution of the urothelium differs between the different regions of the bladder. Evidence exists showing that the bladder base is differently innervated compared to the rest of the bladder (Grol *et al.,* 2008), which would also be an interesting point for further examinations.

A regional dependent disparity in the development of spontaneous contractions appears to exist in both genders and furthermore in different species. This could explain the contradicting results of organ bath strip experiments in previous studies, as the outcome of the study is highly dependent on the bladder area that the tissue strip is taken from. It is therefore important that in further studies the exact location of the used specimen is noted.

It was hypothesised that morphological variations in the bladder wall are responsible for the differences in contractility. Histological studies have demonstrated that the detrusor muscle was significantly thicker in strips taken from the body area compared to strips taken from both trigone and dome area (Nyamwaro 2012), it was expected that the contractile force is higher in tissue strips from the body area. However, the thickness of the detrusor does not appear to reflect the contractile ability, as intact tissue strips from the female trigone revealed a significantly higher amplitude and frequency of spontaneous contractions compared to intact dome and body strips. Differences in the contractile ability of the bladder regions might therefore occur at cellular level rather than at structural level. The disparities could for example be due to the distribution of receptors and ion channels in the urothelium and the detrusor throughout the bladder. The cholinergic agonist carbachol was therefore used as a contractile agent, to mimic the effect of ACh on the contractility of male and female porcine bladder strips from the three regions of the bladder.

After application of carbachol (0.01 - 0.5 μM), the amplitude of denuded tissue strips was higher compared to the intact counterparts in all bladder regions and in both genders. The mechanism underlying this inhibition by the urothelium is not clear to date. The identity of the proposed urothelium-derived inhibitory factor is not known to date, it is however unlikely to be NO, adenosine, a cyclo-oxygenase product, a catecholamine or GABA, which represent the majority of the well-established detrusor inhibitory pathways (Hawthorn *et al.,* 2009). The inhibitory effect of the urothelium might not be due to one single mediator, but to a whole array of mediators that are initiating signalling cascades acting on the detrusor muscle to inhibit the cholinergic induced contractions.

Furthermore, the amplitude of carbachol-induced contractions was significantly higher in the trigone compared to the dome and body in intact as well as denuded tissue strips from the female bladder. It was challenging to make out a distinctive pattern regarding regional differences in the contractile response after cholinergic modulation. However, it appears that the trigone plays an important role in the bladder contractile regulation.

Gender differences in spontaneous and carbachol-induced contractility of the bladder tissue were investigated. While the amplitude of spontaneous activity was not significantly different between the genders, gender differences could be seen in the trigone after

cholinergic stimulation. The female tissue strips from the trigone region showed significantly higher amplitude and frequency in the contractile response at all used concentrations from 0.01 - 0.5 µM carbachol. This was the case for both intact and denuded tissue strips. No significant gender difference could be observed in strips from the bladder body and dome after cholinergic stimulation. Several studies are showing a gender difference in the contractile response to stimulation with carbachol in rats. Liang and Leung (2012) showed that the maximal carbachol response was greater in both intact and denuded dome strips from female rat bladders compared to the male counterpart. Another study showed that body strips from the rat bladder were significantly more sensitive to carbachol than the male counterparts (Longhurst and Levendusky 2000). In the present study a significant gender difference could only be seen in the trigone of the mouse bladder, not in the dome or body. This might be a species specific difference. Human bladder samples were analysed by Fry *et al.* (2011) and it was shown that there was no gender difference in carbachol-induced contractions in denuded bladder strips. However the investigators only used one concentration of 10 µM carbachol and it was not stated from which part of the bladder the biopsies were taken. Further studies on carbachol induced contractility in tissue strips of the different parts of human bladder are needed to draw any conclusions.

It should also be examined whether or not the cholinergic receptor in the porcine urothelium and in the detrusor show a gender specific distribution, as contradicting results revealed no gender differences in the distribution of muscarinic receptor subtypes in rats (Kories *et al.,* 2003) and a gender difference for the distribution of M2 muscarinic receptor in humans (Arrighi *et al.,* 2008). As it has not been stated from which part of the bladder the tissue specimen were taken, it is difficult to draw a conclusion.

It may also be important to standardise the time point of the oestrous cycle in future experiments, as sex specific hormones such as oestrogen and their receptor distribution could play a role in the gender differences found in bladder contractility. As the tissue has been obtained from an abattoir, this is not easily feasible. Looking at the distribution of sex specific hormone receptors in the bladder would be another interesting experiment.

Gender differences and regional disparities within the bladder should be considered when comparing the findings of published studies.

To conclude, it can be stated that the results of this thesis have added new information to the understanding of the mechanosensation and the contractile properties of the bladder. The urothelium releases a number of mediators such as ACh, ATP and NO that play a coordinated role in the stimulation of signalling cascades, which then lead to the onset of afferent nerve activity and detrusor muscle contraction, and finally trigger the micturition reflex. How these mediators and signalling pathways are balanced and which imbalances occur in the onset of lower urinary tract symptoms will be the topic of future research.

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