

**Cholinergic signalling pathways in the superficial
layers of the human bladder; comparing health,
detrusor overactivity and the effect of Botulinum
Toxin Type A**

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Signed Declaration

I, Soumendra Datta confirm that the work presented in this thesis is of my own.
Where information has been derived from other sources, I confirm that this has
been indicated in the thesis.

Soumendra N Datta

Abstract

Introduction

Muscarinic receptors have been identified both in the suburothelium and urothelium of the human bladder. It has been proposed that increased release of Acetylcholine (ACh) from the urothelial and suburothelial nerves may act on suburothelial and detrusor muscarinic receptors, resulting in detrusor overactivity (DO). Neuropeptide Y (NPY) is a cholinergic co-transmitter. Botulinum neurotoxin type A (BoNT/A) is known to act by blocking the release of ACh. We compared expression of M1, M2, M3 receptors, NPY and SNAP-25 in patients with DO, controls and following successful treatment with BoNT/A.

Methods

Flexible cystoscopy bladder biopsies were obtained from 36 patients with DO at baseline, four and sixteen weeks after successful BoNT/A treatment, together with 9 asymptomatic controls. Specimens were immunostained using specific antibodies to the above mentioned antigens. Immunoreactivity (IR) were quantified with image analysis.

Results

Reduced levels of M1 IR were noted in DO patients compared to controls. Following BoNT/A, there were increases and 'normalisation' of M1 IR with similar changes in the urothelium. Significant similar post-BoNT/A increases were seen in M2 IR. Decreased M3 IR was observed at baseline DO compared to controls, with significant increases only in the urothelium following BoNT/A.

SNAP-25 IR showed no changes. NPY IR increased in DO, with a decreasing trend following BoNT/A. Inverse IR correlations were found with frequency and urgency.

Conclusions

Reduced levels of suburothelial muscarinic receptors in DO are in accordance with previous RT-PCR findings, showing reduced mRNA levels in overactive bladders. NPY IR is increased and may illustrate upregulated cholinergic transmission with DO, similar to ATP. SNAP-25 IR demonstrates the presence of BoNT/A sensitive neurones within the suburothelium. Post-BoNT/A changes in suburothelial muscarinic receptors and NPY appear compensatory to the reduced release of ACh, supporting a neuroplastic effect of BoNT/A on bladder afferent pathways as part of its mechanism of action.

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Abbreviations

ABC	Avidin-Biotin Complex
Ach	Acetylcholine
ATP	Adenosine 5'-triphosphate
β3-AR	Beta 3 adrenoreceptor
BOO	Bladder Outflow Obstruction
BoNT/A	Botulinum Neuro-Toxin type A
Botox®	Botulinum neurotoxin type A as marketed by Allergan
BR	Bradykinin Receptor
c-AMP	Cyclic adenosine monophosphate
c-GMP	Cyclic guanosine monophosphate
CGRP	Calcitonin Gene Related Peptide
CISC	Clean Intermittent Self Catheterisation
CTA	Clinical Trials Authorisation
Cx	Connexin
DAB	Diaminobenzidine
DO	Detrusor Overactivity
DSD	Detrusor Sphincter Dyssynergia
Dysport®	Botulinum neurotoxin type A as marketed by Ipsen
EDRF	Endothelin Dependent Relaxing Factor
EUS	External Urethral Sphincter
FDV	First Desire to Void
GCP	Good Clinical Practice

GRK	G protein-coupled Receptor Kinase
HA	Human Albumin
HPF	High Power Field
ICS	International Continence Society
IDO	Idiopathic Detrusor Overactivity
IHC	Immunohistochemistry
IMS	Industrial Methylated Spirit
IPG	Implantable Pulse Generator
IPSS	International Prostate Symptom Score
IR	Immuno-reactivity
KO	Knock Out
LUT	Lower Urinary Tract
MCC	Maximum Cystometric Capacity
MHRA	Medicines and Healthcare products Regulatory Agency
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NA	Noradrenaline
NDO	Neurogenic Detrusor Overactivity
NGF	Nerve Growth Factor
NHNN	National Hospital for Neurology and Neurosurgery
NMDA	N-methyl-D-aspartic acid
NICE	National Institute for Health and Clinical Excellence
NO	Nitric Oxide

NPY	Neuropeptide Y
OAB	Over-Active Bladder syndrome
OCT	Organic Cation Transporters
PAG	Para-Aqueductal Gray
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDET	Detrusor pressure
PIL	Patient Information Leaflet
PKC	Protein Kinase C
PMC	Pontine Micturition Centre
PMN	Parasympathetic Motor Nucleus
PTNS	Posterior Tibial Nerve Stimulation
Qmax	Maximum voiding flow rate
QoL	Quality of Life
RCT	Randomised Controlled Trials
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RTX	Resiniferatoxin
SCI	Spinal Cord Injury
SEM	Standard Error of the Mean
SNAP-25	Synaptosome-Associated Protein of 25000 Daltons
SNS	Sacral Nerve Stimulation
SP	Substance P

SNARE	Soluble N-ethylmaleimide-sensitive fusion Attachment protein Receptor
SV2	Synaptic Vesicle protein 2
TRP	Transient Receptor Potential cation channel
TRPV ₁	Transient Receptor Potential; subfamily V; member 1
Trk-A	Tyrosine Kinase A receptor
TTX	Tetrodotoxin
UCL	University College London
UDIF	Urothelially Derived Inhibitory Factor
UUI	Urgency Urinary Incontinence
VIP	Vasoactive Intestinal Polypeptide

I - Introduction

Chapter 1 - Neural control of the bladder in health and disease

Structure of the lower urinary tract

The role of the lower urinary tract (LUT) is to store and voluntarily periodically release urine. For what seems to be a simple function, it is a structure with remarkably complicated control exerted by the brain, through the spinal cord and peripheral ganglia. The dependence on central nervous control distinguishes it from other visceral organ systems such as the gastrointestinal tract and cardiovascular system, that can maintain a level of function even after extrinsic neural input has been eliminated.

The functional units of the LUT are the bladder and its outlet, which consists of the bladder neck, urethra and striated muscles of the urethral sphincter. The bladder-urethral complex has two exclusive modes; storage and elimination of urine produced from the kidneys. During storage, the bladder fills with urine by remaining compliant and staying at low pressure. For the duration of elimination the outlet resistance is reduced by relaxation at the external urethral sphincter, while the bladder increases its luminal pressure by contracting the surrounding

detrusor in a coordinated manner, to expel the urine down the lowered resistance urethral channel.

Micturition requires the integration of autonomic and somatic efferent mechanisms to co-ordinate the activity of the bladder with that of the urethral striated sphincter. Due to this complexity, the LUT is sensitive to injury or disease affecting the nervous system.

Structure and function of the bladder

Structure

The bladder is a hollow, muscular distensible organ which in the healthy human can hold between 400 – 650 ml of urine. The wall consists of three layers (from inside to outside): urothelium and a suburothelial layer, detrusor muscle and the fibrous adventitia.

The outer most serosa and adventitia is comprised of nerves, blood vessels and soft elastic connective tissue for supporting the bladder wall. The detrusor muscle layer consists of an inner and outer longitudinal layer of smooth muscle with a middle circular layer. The trigone of the bladder is a smooth triangular region between the ureteric orifices and the internal urethral orifice derived from the caudal end of the mesonephric ducts. The trigone has extensive

suburothelial nerve plexuses, considerably more than other parts of the bladder (Gosling and Dixon, 1974).

Urothelium

The urothelium is the epithelial lining of the urinary tract and is composed of basal, intermediate and apical layers of large hexagonal cells (diameter of 25-250µm) known as umbrella cells (Lewis, 2000) (Figure 1). These cells are interconnected by tight junctions and are covered on the apical surface by crystalline proteins called uroplakins. The barrier function of the urothelium is dependent on these tight junctions, uroplakins and lipids, which prevent the movement of ions and solutes as well as reduce the permeability of cells to small molecules such as water, urea and proteins (Acharya et al., 2004). A polysaccharide glycosaminoglycan layer covers the apical layer and is thought to prevent bacterial adherence and defend against infection.

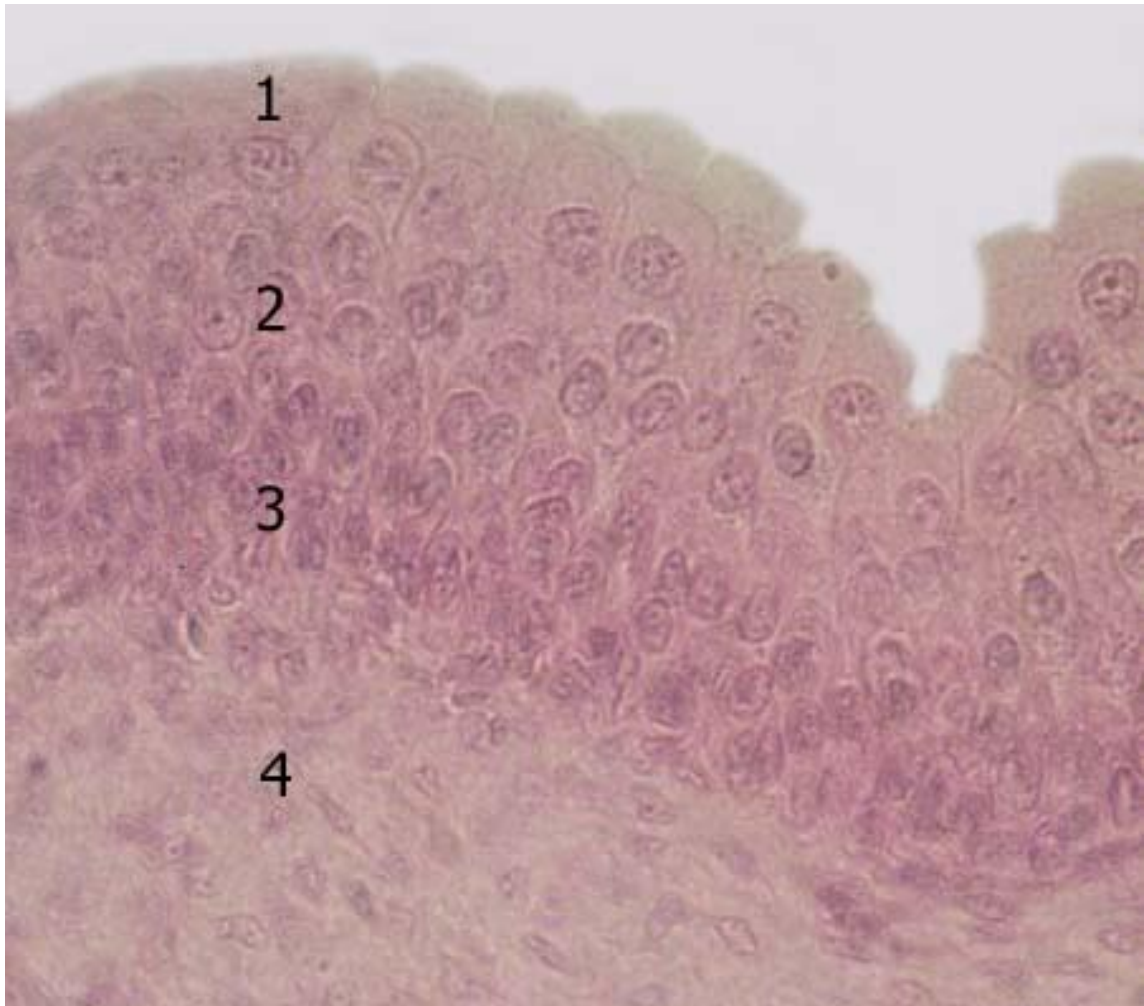


Figure 1: Superficial layer of human urinary bladder stained with haematoxylin and eosin (H+E). (1) Diameter of superficial umbrella cells depends on degree of bladder stretch. (2) Intermediate cells are ~20 μm in diameter (3) Basal urothelial cells are germinal in nature and are ~5-10 μm in diameter. (4) Suburothelium. (Reproduced with permission from Prof A Gunin, Pathologist).

Suburothelium

The suburothelium comprises of loose fibroelastic connective tissue containing axons with their varicosities, fibroblasts, blood capillaries and smooth muscle

cells. The smooth muscle cells have been reported to form a distinct layer termed, by analogy with the structure in the intestine, the 'muscularis mucosae' although it was noted to be discontinuous and less well defined than in the gut. This description has been adopted by urological texts (Wiseman et al., 2003).

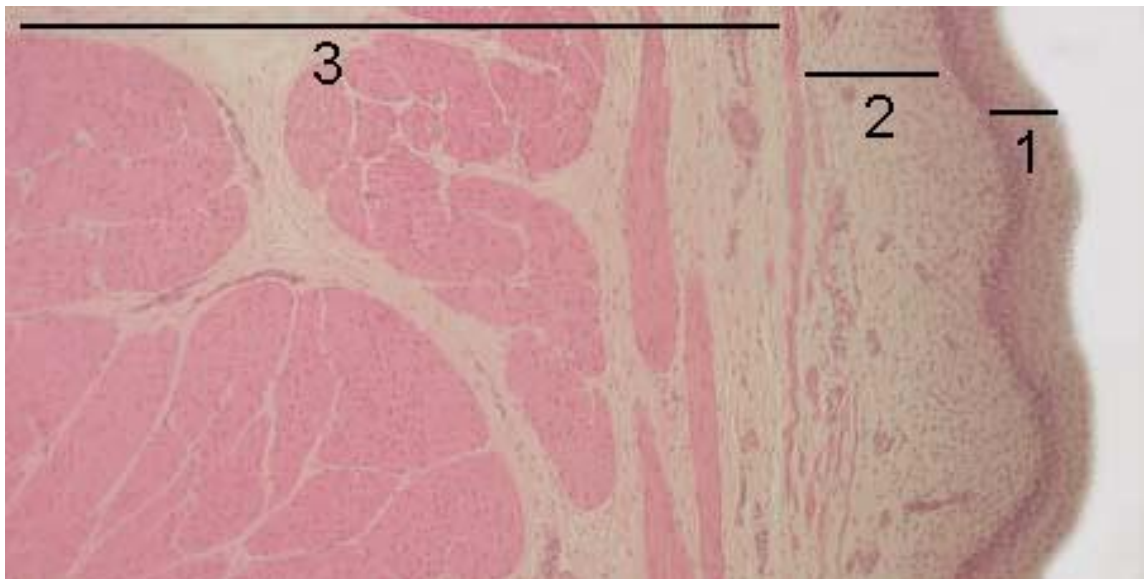


Figure 2: Full H+E cross-section of bladder wall showing the constituent layers (Reproduced with permission from Prof A Gunin, Pathologist). (1) Urothelium, (2) Suburothelium, (3) Muscle Layer.

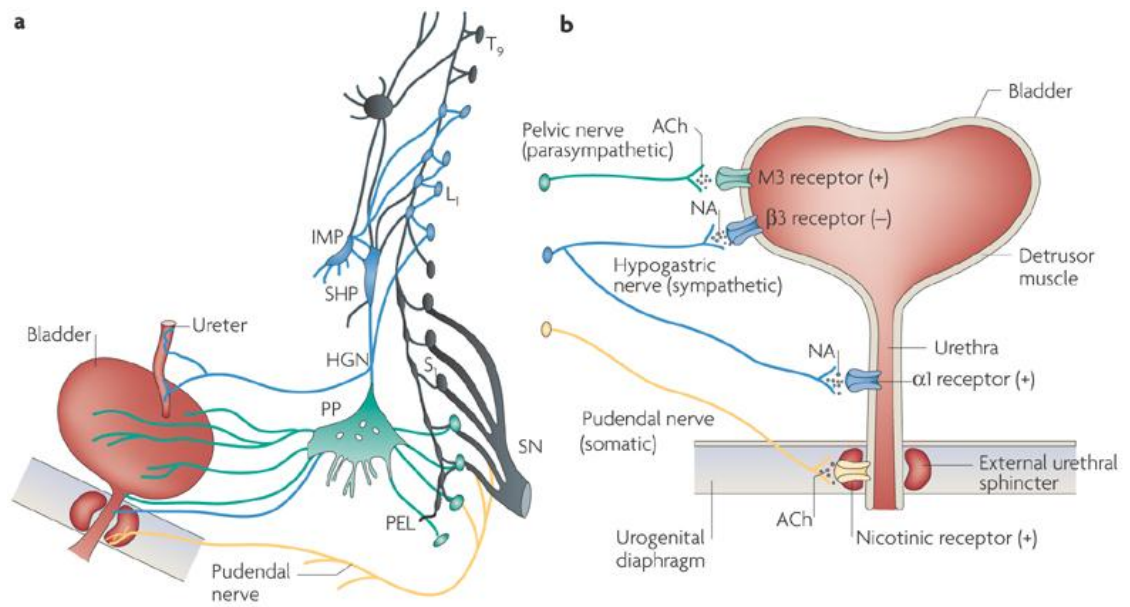
Immunohistochemistry using pan neuronal markers (PGP 9.5, S-100) has shown the presence of a significant number of neurons in this layer, with the density of these fibres changing with disease (Dasgupta et al., 1997; Brady et al., 2004b). Further structural studies have shown these to be afferent neurones presumptively conveying sensory stimuli from the bladder lumen (Dixon and Gilpin, 1987; Gabella and Davis, 1998).

This layer also contains cells with microscopic characteristics of myofibroblasts (Sui et al., 2002; Wiseman et al., 2003). These cells lie in rows below the urothelial layer, with their cell bodies parallel to this layer and have close apposition to unmyelinated nerves, suggesting functional connectivity (Wiseman et al., 2003). It is hypothesised that these cells form part of the sensory response mechanism to bladder filling.

Innervation of the bladder

Efferent innervation of the bladder

Precise coordination and voluntary control of the LUT is achieved by three sets of peripheral nerves: sacral parasympathetic nerves (pelvic nerves), thoracolumbar sympathetic nerves (hypogastric nerves and sympathetic chain) and sacral somatic nerves (pudendal nerves) (Figure 3).



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Figure 3: a | Efferent pathways of the female lower urinary tract (reproduced from The neural control of micturition; Fowler, Griffiths & de Groat; Nature Reviews Neuroscience 9, 453-466 (June 2008). Sympathetic fibres (shown in blue); parasympathetic preganglionic fibres (shown in green); somatic motor nerves (shown in yellow); b | Efferent pathways and neurotransmitter mechanisms that regulate the lower urinary tract.

Parasympathetic outflow

The sacral parasympathetic outflow provides the major excitatory input to the urinary bladder. Preganglionic nicotinic cholinergic fibres originate from the intermediolateral region of the sacral spinal cord at the S2-4 spinal segments, then travel in the pelvic nerve to ganglia in the pelvic plexus and the bladder wall where they synapse (Morgan et al., 1993) (Figure 3). Short post-ganglionic fibres excite detrusor bladder smooth muscle via the release of cholinergic (acetylcholine) and purinergic (ATP) neurotransmitters acting on P2X receptors (Andersson and Arner, 2004) (Ralevic and Burnstock, 1998). Muscarinic

receptors are extensively involved in the control of the bladder as will be discussed later. Postganglionic fibres also contain Vasoactive Intestinal Polypeptide (VIP) and Neuropeptide Y (NPY); these are thought to be co-transmitters released with acetylcholine and ATP to modulate the efferent signal (Keast and De Groat, 1992).

Cholinergic Transmission

The release of acetylcholine is the major excitatory mechanism of the human bladder. Contraction is mediated by acetylcholine acting on muscarinic receptors present on detrusor myocytes and nerve terminals (Chess-Williams, 2002; Andersson and Arner, 2004; Birder, 2005). There are five different muscarinic receptors each encoded by distinct genes (Caulfield and Birdsall, 1998). The receptors have seven transmembrane regions and are G-protein coupled, although the subtypes and second messenger pathways differ. $M_1/M_3/M_5$ are coupled to G proteins $G_{q/11}$ and activate phospholipase C to upregulate inositol triphosphate production, whereas M_2 and M_4 are coupled to G protein $G_{i/o}$ and inhibit the activity of adenylyl cyclase (Andersson and Yoshida, 2003).

mRNA for all muscarinic receptors has been detected in the human bladder; the M_2 and M_3 receptor subtypes being the most frequent in human detrusor muscle (Sigala et al., 2002), whereas all five receptors are present in the urothelium (Kullmann et al., 2008b). M_1 , M_2 and M_3 subtypes have been

detected using immunohistochemistry in urothelium, interstitial cells, nerve fibres and detrusor smooth muscle (Mukerji et al., 2006; Bschiepfer et al., 2007; Zarghooni et al., 2007).

Muscarinic transmission in detrusor smooth muscle

M₃ receptors are thought to be the most important for mediating detrusor contraction. Evidence for this comes from studies using M₃ knockout mice. Bladder strips from these mice had only 5% of the maximal contractile response compared to wild type mice (Matsui et al., 2000). These mice presumably relied on their remaining purinergic pathway for efficient voiding (Igawa et al., 2004). Even in obstructed rat bladder, the M₃ subtype played the predominant role in mediating detrusor contraction (Krichevsky et al., 1999).

M₃ receptor activation is thought to cause detrusor contraction through phosphoinositide hydrolysis by activating protein kinase C (Andersson et al., 1991; Jezior et al., 2001). Studies have shown that contraction is dependent on Ca²⁺ ion entry via nifedipine sensitive channels as well as activation of Rho kinase, which is thought to increase the sensitivity of the contractile machinery to Ca²⁺ ions (Schneider et al., 2004) (Figure 4).

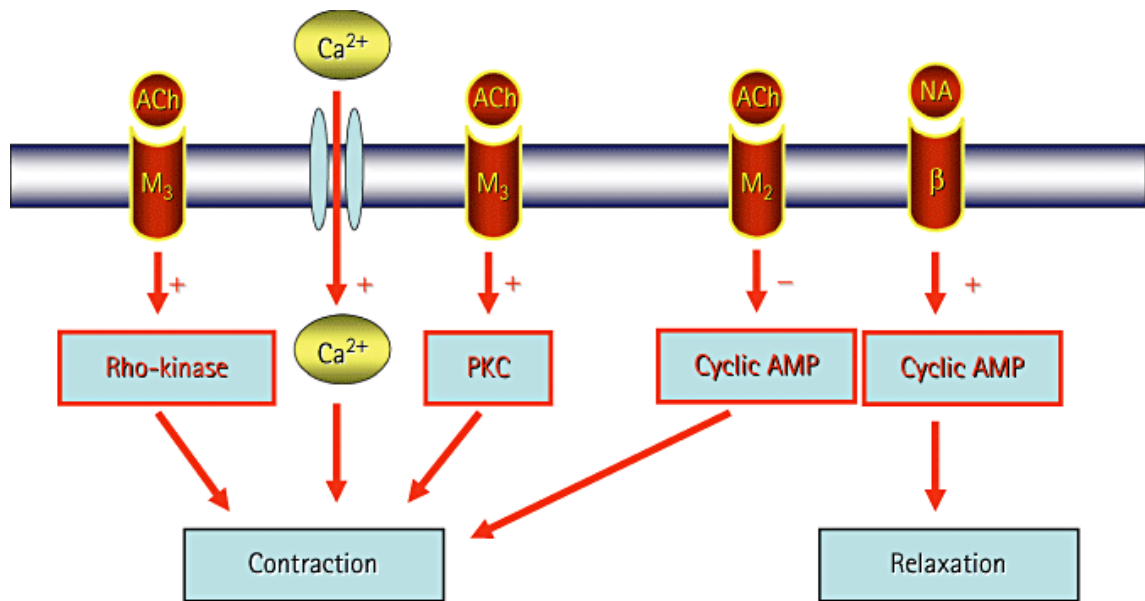


Figure 4: Muscarinic receptor mediated contraction. Stimulation of M_3 receptors by ACh causes contraction of detrusor muscle via activation of protein kinase C and rho-kinase, resulting in an influx of Ca^{2+} ions. An indirect action of ACh is to inhibit the production of cAMP and reverse the relaxation caused by NA on the β -adrenoceptor, promoting contraction. Reproduced from Abrams & Andersson. Muscarinic receptor antagonists for overactive bladder. *BJU International* 100 (5), 987-1006: 2007

M_2 receptor activation can contract the bladder indirectly by inhibition of adenylate cyclase and reversing sympathetically (i.e. beta-adrenoceptor) mediated relaxation, (Hegde et al., 1997). Knockout mice studies have shown that M_2 receptors may indirectly mediate bladder contractions by enhancing the contractile response to M_3 receptor activation and that minor M_2 receptor mediated contractions might occur (Ehlert et al., 2005).

In the healthy bladder, M_2 receptors seem to have a lesser contribution to contraction compared to M_3 receptors but this may change with disease states. In the denervated rat bladders, M_2 receptors or a combination of M_2 and M_3

receptors contribute to the contractile response (Braverman et al., 1998). In obstructed hypertrophied rat bladders there was an increase in total muscarinic receptors and M_2 receptor density, with a decrease in M_3 receptor density (Braverman and Ruggieri, 2003). This has not been seen in human detrusor studies. Pontari et al. examined whether there was shift from M_3 to M_2 receptor mediated contraction in patients with NDO following traumatic cervical injuries. Bladder strips from those patients were compared to bladders from organ donors and showed that contractions in NDO patients could partly be mediated by M_2 receptors (Pontari et al., 2004). This was further studied by Stevens et al. in subjects with NDO or IDO undergoing clam cystoplasty compared to patients undergoing cystectomy for bladder cancer using subtype selective antagonists. They showed that although the contraction was enhanced in DO, there was no change in the receptor subtype (M_3) contributing to the contraction in the disease state (Stevens et al., 2007).

Presynaptic nerve terminals

To activate post-junctional muscarinic receptors, ACh controls its own release by muscarinic auto-receptors which are located prejunctionally on cholinergic nerve terminals within the bladder. The inhibitory pathway allows for a muscarinic feedback mechanism to reduce the release of ACh from the cholinergic terminals. The inhibitory prejunctional receptors have been classified in rabbit as M_2 and in humans as M_4 (Inadome et al., 1998; D'Agostino et al., 2000; Takeuchi et al., 2008), whereas the M_1 subtype seems to facilitate

cholinergic transmission in rat and rabbit bladders (Somogyi and de Groat, 1992; Inadome et al., 1998). This same facilitatory mechanism has been shown in human bladder and is mediated by influx of extracellular Ca^{2+} into the parasympathetic nerve terminals via L- and N-type Ca^{2+} channels (Somogyi and de Groat, 1999). Prejunctional M_1 -muscarinic receptors have been identified on cholinergic nerve terminals innervating the bladders of several species, where they enhance transmitter release (Somogyi and de Groat, 1992; Somogyi et al., 1994) and potentiate bladder contractions to electrical field stimulation, (Somogyi and de Groat, 1999) promoting efficient voiding.

In chronic spinal cord transected rats, this facilitation exhibits plasticity and has been found to be up-regulated and primarily mediated by M_3 receptors (Somogyi et al., 2003). Conversely, inhibitory M_2 and M_4 prejunctional receptors may function to promote urinary storage in pathological states such as bladder denervation or spinal cord injury (Chapple, 2000).

Functional role of muscarinic receptors

Pharmacological studies

Molecular studies have classified muscarinic receptors into five subtypes (M_1 – M_5) (Caulfield and Birdsall, 1998). Radioligand studies have shown the bladder of most species including man are principally M_2 and M_3 receptors, with the former predominating (Hegde and Eglén, 1999). These early studies were not sensitive enough to detect receptors in the urothelium and suburothelium, and the potential importance of this superficial layer was not recognised.

Until recently, the functional role of muscarinic receptors has been interpreted from agonist and antagonist studies which had preferential activity for a particular subtype e.g. pirenzepine, darifenacin and 4-DAMP. These studies suggested that M_3 receptors primarily mediate the direct contractile response to agonists in the bladder (Hegde et al., 1997; Chess-Williams, 2002). The initial evidence was that M_3 receptors mediated contraction via activation of phospholipase C, however further studies have shown that contraction is dependent on entry of extracellular calcium through L-type calcium channels and activation of Rho kinase (Schneider et al., 2004). Pharmacological studies have not demonstrated any role of M_2 receptors in mediating contractile responses, and although their role in disease states (NDO) may become more important (Pontari et al., 2004), this has been challenged (Stevens et al., 2007).

Urothelium derived inhibitory factor

The urothelium was traditionally viewed as a passive membrane barrier between the urinary tract and urine itself. There is increasing evidence that the suburothelium and urothelium express a range of receptors that participate in sensory function by releasing neurotransmitters in response to distention and receptor activation (Fry et al., 2004; Abrams et al., 2006; Apostolidis et al., 2006; de Groat, 2006).

There are a number of factors released by the urothelium which decrease detrusor muscle tone. A diffusible "urothelial derived inhibitory factor" (UDIF) released by muscarinic or histamine receptor stimulation, has been shown to inhibit detrusor contraction in a pig by about 35% (Hawthorn et al., 2000; Templeman et al., 2002; Murakami et al., 2007). Muscarinic agonists stimulate the release of this inhibitory factor from the human urothelium; the factor is distinct from nitric oxide and it persists in the presence of beta-adrenoceptor blockade or cyclooxygenase inhibition (Chaiyaprasithi et al., 2003). The release of UDF is also reduced during drug induced inflammation (Inci et al., 2003).

Isoprenaline, a β -adrenoreceptor agonist, causes detrusor muscle relaxation but does not appear to act via NO. Isoprenaline may act directly by relaxing the detrusor muscle or by stimulating the release of this UDIF, as it is more potent at inhibiting carbachol contractions in the presence of the urothelium (Murakami et al., 2007). Further experiments have shown that urothelial beta-

adrenoceptors induce the release of a UDIF, which inhibits the beta-adrenoceptor agonist-induced relaxation of human detrusor smooth muscle and that this inhibitory mechanism does not involve NO (Otsuka et al., 2008).

Gene Knock Out studies

M₃ receptor knockout (KO) mice had their responses to carbachol (muscarinic agonist) attenuated by 95% (Matsui et al., 2000). Although the M₃ KO mice had larger voiding intervals and bladder capacity, they voided to completion and showed no functional impairment, suggesting possible compensation by alternative muscarinic receptors or other non-cholinergic neurotransmitter systems (Hegde, 2006). M₂ and M₃ KO mice showed complete loss of contractile responses, implying that M₂ receptors mediated a small direct contractile response and provided the compensatory response seen in the M₃ KO mice (Matsui et al., 2002). Only male mice showed urinary bladder distension, but all animals were still viable.

Whereas pharmacological studies were limited in their ability to discern whether the prejunctional inhibitory auto-receptors were M₂ or M₄ subtypes, this was clarified by M₄ KO animals, showing M₄ receptor subtypes provided an inhibitory negative feedback mechanism on ACh release (Zhou et al., 2002). The facilitatory role of M₁ receptors is yet to be confirmed with KO studies.

Sympathetic pathways

These originate from the T11 – L2 segments of the cord and run in inferior mesenteric ganglia or pass through the paravertebral chain to enter the bladder via the hypogastric and pelvic nerves respectively. Their main function is thought to be the relaxation of the detrusor muscle via β_3 adrenoceptors and excitation of the urethral sphincter complex via release of norepinephrine on α_1 adrenoceptors (Andersson and Arner, 2004). Sympathetic inputs also modulate cholinergic nicotinic neurotransmission in the pelvic ganglia.

Somatic efferent pathways

Cells in the circumscribed region of lateral ventral horn in the S2-S4 segment of the sacral cord (Onuf's nucleus) (Onufrowicz, 1899) give rise to the motor innervation of the urethral striated sphincter. Motor nerves pass in the pudendal nerve and innervate the neuromuscular junctions of the sphincter muscle.

Coordination of activity by the CNS

The process of voiding is initiated by the sensation of bladder fullness and indeed the overall motivation of the behaviour is to attain relief from this increasingly intense sensation of bladder filling,

The prefrontal cortex is postulated to be involved in the decision of whether or not to micturate (Fowler, 2006). Once the decision to void is made by the

prefrontal cortex, there is activation of the insula, hypothalamus and par-aqueductal gray (PAG), resulting in activation of the pontine micturition centre (PMC) (Blok et al., 1997; Blok et al., 1998; Fowler et al., 2008). There is spinal transmission of activity to sacral segments of the cord, with initial inhibition of external urethral sphincter motor activity, followed by activation of bladder parasympathetic efferents. A combination of excitatory and inhibitory neurotransmitter release results in coordinated voiding (Figure 5). Afferent activity feedback to the PAG and PMC during a sustained void allows complete voiding to occur with subsequent cessation of activity.

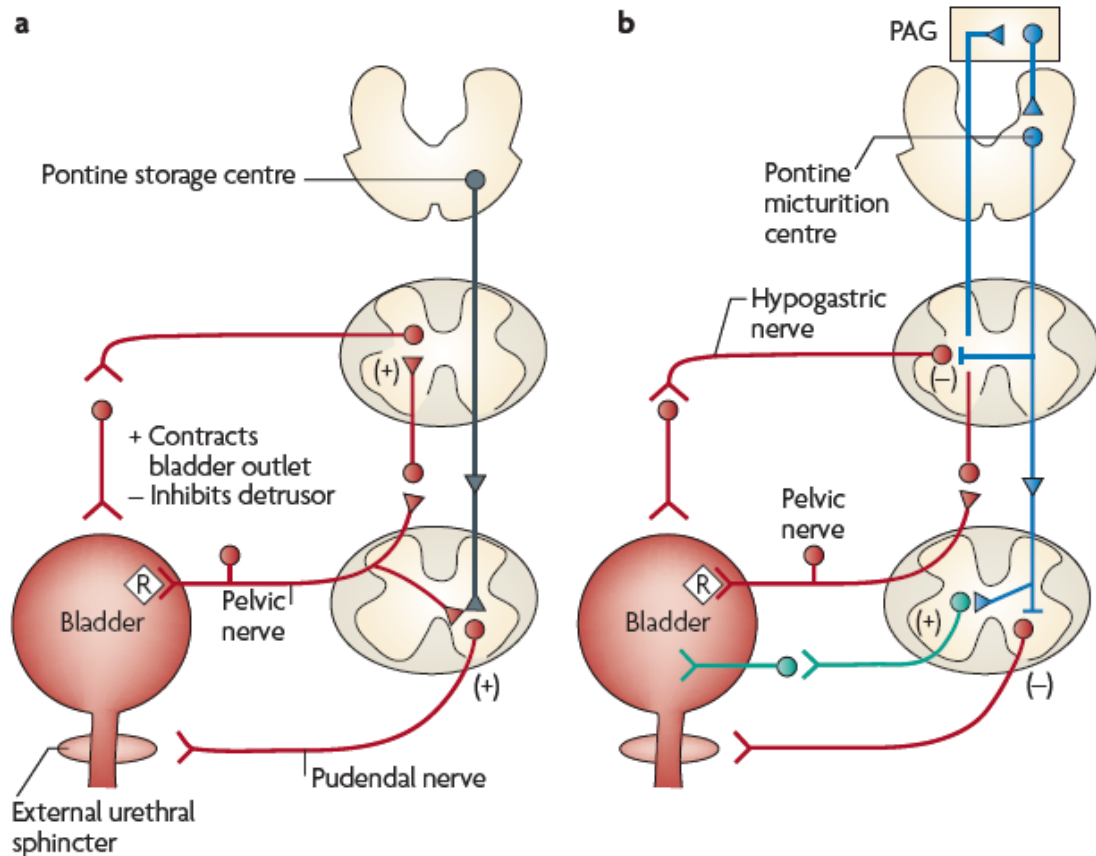


Figure 5: Neural circuits that control continence and micturition. a | Urine storage reflexes. During the storage of urine, distention of the bladder produces low-level vesical afferent firing. This in turn stimulates the sympathetic outflow in the hypogastric nerve to the bladder outlet (the bladder base and the urethra) and the pudendal outflow to the external urethral sphincter. These responses occur by spinal reflex pathways and represent guarding reflexes, which promote continence. Sympathetic firing also inhibits contraction of the detrusor muscle and modulates neurotransmission in bladder ganglia. A region in the rostral pons (the pontine storage centre) might increase striated urethral sphincter activity. b | Voiding reflexes. During the elimination of urine, intense bladder-afferent firing in the pelvic nerve activates spinobulbospinal reflex pathways (shown in blue) that pass through the pontine micturition centre. This stimulates the parasympathetic outflow to the bladder and to the urethral smooth muscle (shown in green) and inhibits the sympathetic and pudendal outflow to the urethral outlet (shown in red). Ascending afferent input from the spinal cord might pass through relay neurons in the peri-aqueductal grey (PAG) before reaching the pontine micturition centre. Note that these diagrams do not address the generation of conscious bladder sensations, nor the mechanisms that underlie the switch from storage to voiding, both of which presumably involve cerebral circuits above the PAG. R represents receptors on afferent nerve terminals. Reproduced from (Fowler et al., 2008).

Normal Bladder Function

One of the aspects of human behavioural development is the ability to plan to void at a socially acceptable time and place. This involves being aware of one's environment and the availability and accessibility of a toilet, together with attentiveness to sensory signals indicating bladder fullness. It is critical that the brain can be given sufficient time and warning prior to the requirement to void. The vast majority (99%) of our normal lives we are in storage mode (Fowler, 2006).

Urodynamic studies in healthy controls performed 5 years apart have shown that the first sensation of bladder fullness occurs approximately at 40% of capacity (Wyndaele and De Wachter, 2002). The first desire to void (FDV) has been defined by the International Continence Society (ICS) as “the feeling, during cystometry, that would lead the patient to pass urine at the next convenient moment, but voiding can be delayed if necessary”, and occurs at 60% of capacity (Abrams et al., 2002; Wyndaele and De Wachter, 2002). At 90% capacity, patients report a strong desire to void which the ICS define as “a persistent desire to void without the fear of leakage”. Psychological mental state can alter the perception of bladder fullness similar to pain (Valet et al., 2004); anxiety can increase the level of desire to void whereas distractions reduce it.

Afferent pathways

Sensations of bladder fullness are conveyed to the spinal cord in the pelvic and hypogastric nerves (Janig and Morrison, 1986). Input from the bladder neck and urethra are conveyed in the pudendal and hypogastric nerves (Morisson et al., 2005). The most important components of the sensory inputs are the small myelinated (A δ) and unmyelinated (C) fibres which convey impulses from tension and volume receptors as well as nociceptors in the bladder to neurones in the spinal cord (de Groat, 1997). The afferent nerves are located in the smooth muscle, as well as the serosal and suburothelial layers of the bladder (Gosling and Dixon, 1974).

A δ fibres

A δ fibres are mechano-sensitive, respond to passive distention and active contraction and convey information regarding bladder filling (Janig and Morrison, 1986). Afferent pathways from the bladder project into Lissauer's tract and then synapse onto interneurons. These neurones send long projections transmitting sensory input into supraspinal centres like the PAG and gracile nucleus (Blok et al., 1995). The PAG relays information during filling to the PMC, maintaining an excitatory effect on Onuf's nucleus. This in turn maintains sphincter contraction and inhibits the Parasympathetic Motor Nucleus (PMN) inhibiting bladder contraction. When the bladder is sufficiently full and volitional control is relaxed, the PAG signals the PMC to initiate the micturition reflex by inhibiting Onuf's nucleus and activating the PMN. This integrated system

receives higher control from the hypothalamus, cingulate gyrus and forebrain to ensure social and emotional control over initiation or inhibition of the micturition reflex, by influencing the activity of the PAG or PMC itself (Fowler et al., 2008).

C-fibres

Based on their sensitivity to capsaicin, it is thought that 70% of the afferent nerves from the bladder are C-fibres (Yoshimura and de Groat, 1999). C-fibres are thought to be normally quiescent and only become activated during inflammation, irritation or cooling (Fall et al., 1990), therefore being insensitive to bladder filling under physiological conditions. C-fibre afferents are sensitive to noxious stimuli like the vanilloids capsaicin and resiniferatoxin (RTX) as well as tachykinins, NO, ATP, temperature and pH (Dasgupta et al., 2000; Rong et al., 2002; Cheng and de Groat, 2004). These substances modulate afferent activity excitability and thus alter sensory transduction. Both A δ and C-fibres have cell bodies located in the dorsal root ganglia at S2-S4 and T11-T12 spinal segments. These axons synapse with interneurons which project to higher brain centres.

ATP is thought to be a modulator of afferent nerve excitability, as immunohistochemical studies have revealed that P2X and P2Y receptors are expressed in the bladder afferent nerves and urothelial cells (Lee et al., 2000). There is now considerable evidence suggesting that extracellular ATP modulates afferent nerve excitability by activating P2X receptors (Burnstock,

2000; Yu and de Groat, 2008). The purinergic receptor P2Y₆ is thought to be present on suburothelial myofibroblasts in the suburothelium. These cells respond to ATP by generating an intracellular Ca²⁺ transient (Sui et al., 2006).

Bladder sensory nerves and innervation of the urothelium

Both humans and animals have a dense nexus of sensory nerves in the suburothelial layer of the urinary bladder (Gosling and Dixon, 1974; Gabella and Davis, 1998). Terminal fibres of these nerves project through the suburothelium, occasionally reaching the urothelium (Birder et al., 2001; Wiseman et al., 2002). The plexus is thickest in the neck of the bladder and in the initial portion of the urethra, becoming progressively less dense and sparse towards the dome (Gabella and Davis, 1998).

Numerous studies have shown both afferent and autonomic efferent nerves are located in close proximity to the urothelium (Birder et al., 2001; Dickson et al., 2006). Peptide and TRPV-1 immunoreactive fibres are located in a plexus underneath the urothelium and these extend into the urothelium (Avelino et al., 2002). Cholinergic and adrenergic fibres have also been detected here.

The urothelium is no longer regarded as a passive barrier; it has substantial sensory and signalling properties (Birder et al., 1998) and engages in reciprocal chemical communication with the adjacent nerves in the suburothelium (Birder et al., 2001; de Groat, 2006; Birder and de Groat, 2007). Studies over several

years indicate that urothelial cells display a number of properties similar to those of sensory neurons such as nociceptors and mechanoreceptors, and that both types of cells use diverse signal-transduction mechanisms to detect physiological stimuli (Birder and de Groat, 2007).

Several substances have been shown to be released from urothelial cells following physical and chemical stimulation: ATP, NO, substance P, acetylcholine, adenosine, UDIF, cytokines and prostanoids (Birder and de Groat, 2007).

These substances contribute to autocrine and paracrine mechanism of signal transmission. Autocrine signalling is where a cell releases a neurotransmitter that binds to receptors on the same cell type, leading to changes within that cell e.g. release of ACh from the urothelium acting on the urothelium itself. This is in contrast to paracrine signalling, in which the target is near the signal releasing cell e.g. release of ACh from the urothelium acting on underlying nerve fibres and cells in the suburothelium. The signalling molecules involved degrade very quickly, are taken up quickly or can travel only short distances, therefore limiting their effect to the local surrounding environment.

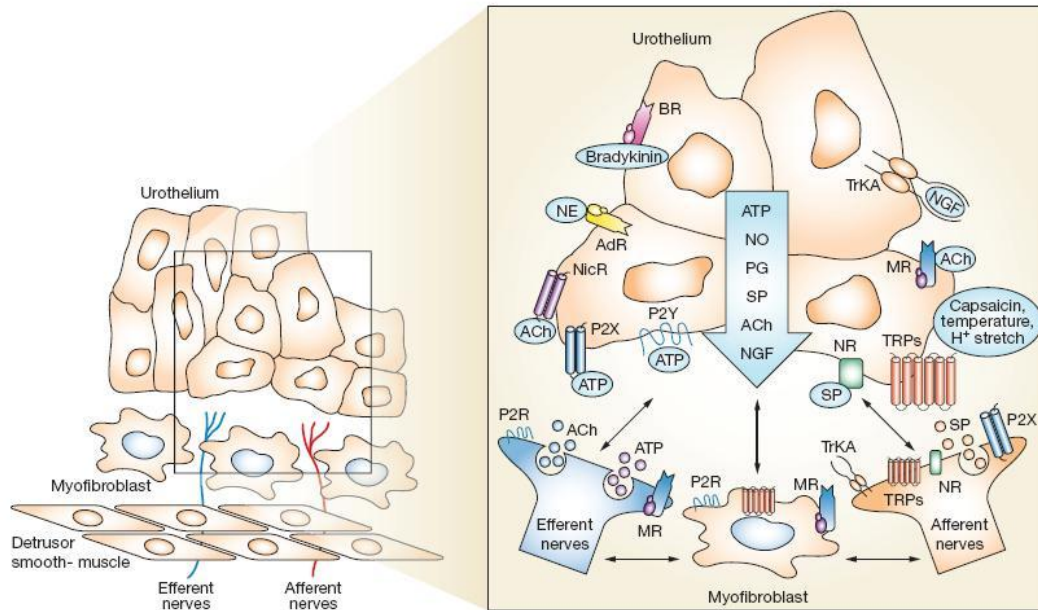


Figure 6: Hypothetical model depicting possible interactions between bladder afferent and efferent nerves, urothelial cells, smooth muscle and myofibroblast. Urothelial cells can be activated by either autocrine or paracrine mechanisms. Stimulations of urothelial cells can release mediators that activate bladder nerves. Reproduced from *Mechanisms of disease: involvement of the urothelium in bladder dysfunction*. Nat Clin Pract Urol. Jan;4(1):46-54. Review (Birder and de Groat, 2007).

Sensory molecules, receptors and ion channels of the urothelium

There is expression of a multitude of receptors (nicotinic, noradrenergic α and β , muscarinic, tachykinin, purines P2X and P2Y, adrenergic, bradykinin, neurotrophin Trk-A and p75, vanilloid) responding to released neurotransmitter (Figure 6). The urothelium has a close physical association with afferent nerves which can relay sensory information back to the spinal cord. (Beckel et al., 2006; Birder and de Groat, 2007). Further examples of identified neuronal 'sensory molecules' include mechanosensitive Na^+ channels and a number of TRP channels (TRPV1, TRPV2, TRPV4 and TRPM8) (Ferguson et al., 1997;

Chopra et al., 2005; Beckel et al., 2006). There is evidence that urothelium expresses the full complement of muscarinic receptors (M_1 - M_5) (Chess-Williams, 2002; Abrams et al., 2006; Bschleipfer et al., 2007).

ATP

The role of urothelial released ATP as part of the afferent system was first identified when it was found to be released with bladder distension and was increased in experimental NDO (Ferguson et al., 1997). P2X and P2Y purinergic receptor subtypes are expressed in the urothelium, nerves and myofibroblasts, suggesting an important role in cell communication. ATP causes an increase in intracellular Ca^{2+} release in those cells (Wu et al., 2004). Intravesical administration of ATP increases firing of afferent terminals and enhances the firing as a result of bladder distension (de Groat, 2006). Its intravesical administration in rats has been found to induce bladder overactivity and this effect was blocked by P2X antagonists (Nishiguchi et al., 2005). P2X₃ receptors have been identified in the urothelium in humans (Elneil et al., 2001), suggesting an autocrine role for urothelial released ATP.

Bladder efferent activity from P2X₃ knockout mice was reduced compared to normal mice during bladder distension, strongly suggesting a sensory role for ATP released from the urothelium acting at this receptor (Vlaskovska et al., 2001). It is thought that 90% of bladder afferents are excited by ATP, activating

P2X_{2/3} receptors (Zhong et al., 2003), and that its main role is in autocrine and paracrine signalling from the urothelium.

Capsaicin and TRPV₁

Another important group of urothelial sensory molecules is the transient receptor potential cation channel family (TRPV). TRPV1 is thought to have a role in nociception as well as normal bladder function. The heat and pain induced by chilli peppers is caused by stimulation of TRPV1 receptors (Caterina et al., 1997). This ion channel receptor protein is activated by moderate heat, protons and lipid metabolites such as anandamide. TRPV1 is expressed on urinary bladder c-fibres and on urothelial cells (Birder et al., 2001; Lazzeri et al., 2004). TRPV1 is thought to amplify the response to various stimuli especially in the irritated bladder. However, in TRPV1 knock-out mice it was observed that bladder function was altered and there were reductions in ATP release (Birder et al., 2002) indicating that TRPV1 participates in normal bladder function and is part of the mechanically evoked urothelial purinergic signalling mechanism.

Basal cell layer TRPV1 immunoreactivity has been shown to be significantly increased in NDO compared with control bladders. Following successful treatment with RTX, basal cell layer and total urothelial TRPV1 immunoreactivity decreased significantly (Apostolidis et al., 2005a). Decreases in the basal cell layer TRPV1 immunoreactivity after RTX were comparable to the parallel decreases in suburothelial TRPV1 nerve fibres (Brady et al., 2004b).

Studies have found that symptoms of sensory urgency are associated with increased TRPV1 expression in the trigonal mucosa (Liu et al., 2007).

The TRPV4 cation channel has been found to mediate stretch-evoked Ca^{2+} influx and ATP release in primary urothelial cell cultures, suggesting this is a sensor molecule in detecting bladder distension (Mochizuki et al., 2009). Further evidence that TRPV4 plays a critical role in urothelium-mediated transduction comes from TRPV4-deficient mice, which exhibit a lower frequency of voiding contractions as well as a higher frequency of non-voiding contractions (Gevaert et al., 2007). Stretch evoked ATP release has been shown to be decreased in isolated whole bladders from TRPV4-deficient mice. The role of TRPV4 in normal human physiology and pathophysiology is yet to be determined, but if found to be critical, TRPV4 antagonists could potentially modulate the sensory transduction process and open up a novel class of drug.

Suburothelium

Together with the urothelium, the suburothelium plays a substantial role in sensory function. Transmission electron microscopy has revealed a complex sensory nexus of vesicle packed naked axons. The exact contents of these dense core and clear vesicles are yet to be elucidated (Wiseman et al., 2003).

Immunohistochemical techniques have demonstrated unmyelinated nerves staining for CGRP, substance P, VIP, acetylcholine and ATP. TRPV1 and P2X_3

immunoreactive fibres have been identified within the suburothelium and detrusor layers (Yiangou et al., 2001).

Suburothelial myofibroblasts

There is a layer of cells beneath the urothelium in the lamina propria that have microscopic characteristics of myofibroblasts (Wiseman et al., 2003). These cells have been described “with the cytological characteristics of both fibroblasts and smooth muscle cells, that included bundles of fine cytoplasmic filaments, dense bodies, linear arrays of subsurface vacuoles, and the presence of an interrupted basal lamina” with close apposition to suburothelial unmyelinated nerve fibres (Wiseman et al., 2003) .

There are also cells in the detrusor layer with similar appearances termed “interstitial cells”, but it is not known whether they are of the same type as the suburothelial myofibroblasts. They have been found to be innervated by fibres that increase cellular cGMP and may also release NO (Smet et al., 1996). The detrusor interstitial cells lack the fibronexus and abundant rough ER of the archetypal myofibroblast and thus can be differentiated from myofibroblasts by using transmission electron microscopy (Drake et al., 2006).

The suburothelial myofibroblasts characteristically lie in rows with their cell bodies and projections parallel to the base of the urothelium. They display cell to matrix connections and intracellular stress fibres (Drake et al., 2006) and

respond to exogenous ATP by generating intracellular Ca^{2+} transients (Wu et al., 2004). Immunolabeling has been possible with vimentin and connexin 43 (Cx43) antibodies, showing that they form a syncytium through extensive Cx43 gap junction coupling (Fry et al., 2007).

Physiologically suburothelial myofibroblasts cells show spontaneous intracellular Ca^{2+} and membrane potential transients. ATP is thought to initiate these membrane potentials via the P2Y_6 receptor subtype, but these can be generated by reduction of extracellular pH and generation of nitric oxide (Fry et al., 2007). Signals initiated by one group of cells can be transmitted considerable distances in the suburothelial layer. It is hypothesised that the close relationship between nerves and myofibroblasts allows for variable amplification of an afferent signal in response to ATP release. Suburothelial myofibroblasts respond to several physiological and artificial interventions that are known to influence bladder activity either directly or through activation of sensory mechanisms. It is proposed that these cells could modulate bladder responses by being a link between urothelial signalling during bladder filling and afferent fibre stimulation (Sui et al., 2008).

In the rat model of NDO, there was marked upregulation of suburothelial gap junctions Cx26 and Cx43 (urothelial and suburothelial respectively) in neonatal and spinal cord transected animals. Administration of a gap junction blocker was shown to reduce spontaneous bladder contractions (Ikeda et al., 2007).

Immunofluorescence studies have also shown an increased number of gap junction protein Cx43 on myofibroblasts in human NDO and IDO, which is not altered by successful BoNT/A treatment (Roosen et al., 2008).

Non-neuronal cholinergic system in the urothelium and suburothelium

Muscarinic receptors have been detected in the urothelium and suburothelium but their functional importance has not been defined as yet (Mansfield et al., 2005; Mukerji et al., 2006; Mansfield et al., 2007).

It has been suggested that non-neuronal ACh synthesised in cells other than parasympathetic neurons may act in an autocrine or paracrine manner (Hanna-Mitchell et al., 2007). The traditional view of ACh acting solely as a neurotransmitter had to be revised based on the findings published both early and late in the last century, demonstrating the non-neuronal cholinergic system. Cholinergic communication is highly conserved from the beginning of life and is found in primitive uni- and multicellular organisms. (Horiuchi et al., 2003). Evidence for ACh synthesis is not only provided by positive anti-ChAT immunoreactivity, but ChAT enzyme activity and/or ACh content have also been determined in the majority of epithelial cells e.g. vagina, urothelium, endothelium, cornea and placenta (Wessler and Kirkpatrick, 2008). Knowledge of the release mechanism of ACh from the various epithelial cells is scarce. Organic Cation Transporters (OCT) are believed to be involved in mediating the release of non-neuronal ACh. In numerous organ systems, non-neuronal ACh

has been found to modify signal transduction via nicotinic and muscarinic receptors (Wessler and Kirkpatrick, 2008).

Immunohistochemical staining of human bladder tissue with antibody against choline acetyltransferase has shown the presence of a cholinergic system in the urothelium / suburothelium. Tetrodotoxin (TTX) has been used to block neuronal release of ACh, with experiments demonstrating that a small amount of ACh was released despite the presence of TTX, implying a non-neuronal system. Stripping the urothelium off detrusor specimens has shown that the urothelium contributed significantly to overall ACh release by the bladder (Yoshida et al., 2004), and is likely to be the source of the non-neuronal ACh. Release of non-neuronal (TTX resistant) ACh has been shown to be evoked by stretch and to increase with age (Yoshida et al., 2008).

There is evidence to suggest that urothelial ACh is not stored and released via vesicles, as Vesicular Acetylcholine Transporter (VACHT) was not found in urothelial cells and brefeldin, a chemical that blocks vesicle formation, did not block the release of ACh from urothelial cells (Hanna-Mitchell et al., 2007). Non-neuronal urothelial ACh release occurred in the storage phase of the voiding cycle and from this work, proposals were made that anti-muscarinic drugs might be acting at the urothelial/suburothelial level (Yoshida et al., 2004). In-vivo studies on mice have suggested that muscarinic receptor agonists and acetylcholine have an inhibitory role in bladder afferent activity, and that this

effect is not mediated by M₃ receptors (Daly et al., 2010). It has been suggested that the alterations of non-neuronal cholinergic system may contribute to the pathophysiology of human bladder.

In a study looking at muscarinic receptors in human bladder, Mukerji et al. showed that there was M₂ and M₃ receptor immunoreactivity in the urothelium, nerve fibres and detrusor layers. “Myofibroblast like staining” was stated by authors to be observed in the suburothelial and detrusor layers when stained for M₂, M₃ and vimentin. There is currently controversy with regard to pathological changes of receptors with detrusor overactivity. Mukerji et al. showed significant increase in suburothelial myofibroblast like M₂ and M₃ staining in patients with Idiopathic Detrusor Overactivity (IDO) compared to control subjects, whereas Mansfield et al. showed a decrease in urothelial and suburothelial M₃ mRNA expression in IDO patients compared to controls (Mukerji et al., 2006; Mansfield et al., 2007). Immunocytochemical evidence from guinea pigs has shown the presence of M₃ receptors located on interstitial cells in the suburothelium (Gillespie et al., 2003). It has been proposed that suburothelial muscarinic receptors predominantly detect the non-neuronal acetylcholine release from the urothelium.

Immunohistochemical studies have demonstrated all five muscarinic receptors being present on human urothelium, with M₂ and M₃ subtypes being the most

common (Bschleipfer et al., 2007). M₁ IR was found to be highly localised to basal cells in the urothelium.

It is clear that the functional role of muscarinic receptors in the urothelium and suburothelium is highly complex and not well understood; further work is required to clarify their roles in normal and pathological bladder function. It is controversial as to whether anti-muscarinic agents in the treatment of DO and OAB function at the level of the muscle or on the sensory mechanism involving the complex of urothelium, suburothelium and myofibroblasts.

Neuropeptide Y (NPY)

Peptides such as ATP and NPY have been implicated as co-transmitters in cholinergic pathways as well as the sympathetic nervous system. Neuropeptide Y is a 36 amino acid peptide and is the most abundant peptide neurotransmitter in the normal human bladder, with a rich distribution in the detrusor muscle as well as in the suburothelium (Crowe et al., 1991; Dixon et al., 2000). In the human body NPY coexists with noradrenaline (NA) in neurones in the brainstem (Everitt et al., 1984), peripheral sympathetic ganglia on blood vessels (Ekblad et al., 1984) and mouse vas (Stjarne et al., 1986). The presence of NPY in parasympathetic cholinergic neurones has been demonstrated in the otic and internal carotid ganglia (Suzuki et al., 1990), major pelvic ganglia in the rat (Keast, 1991) and ganglia of the guinea pig and human urinary bladder (James and Burnstock, 1988; Dixon et al., 2000).

95% of all cholinergic neurones of the human detrusor muscle were found to also contain neuropeptide Y (Dixon et al., 2000). In peripheral neurones, NPY appears to have several functions, including a direct post-junctional excitatory action as well as pre and post junctional modulatory effects. In the vas deferens, NPY acts pre-junctionally to inhibit NA release (Lundberg and Stjarne, 1984; Ellis and Burnstock, 1990) and post-junctionally to enhance the action of NA (Ellis and Burnstock, 1990), but does not directly induce smooth muscle contraction.

NPY has an inhibitory regulatory role on the release of cholinergic and adrenergic neurotransmitters and induces a reversible reduction of the contractile response to field stimulation of the urinary bladder (Lundberg et al., 1984; Tran et al., 1994). NPY also reduces the cholinergic component of electrically induced contraction of rat detrusor strips (Zoubek et al., 1993). The inhibition is due to its prejunctional action possibly by Y₂ subtype receptors to inhibit ACh release (Wahlestedt et al., 1990; Tran et al., 1994).

Changes showing nerve degeneration and regeneration have been described in bladder detrusor biopsies from NDO patients (Haferkamp et al., 2003a). Lower densities of cholinergic fibres have also been described in NDO patients with meningocele, indicating partial denervation of the smooth muscle (German et al., 1995). Drake et al. found that there was a reduction of NPY

containing nerves corresponding to partial nerve degeneration in NDO patients with heterogenous spinal cord injury (Drake et al., 2000). Haferkamp et al. showed a reduced frequency of NPY containing nerves in the detrusor of NDO patients, without a corresponding decrease in PGP 9.5 immunoreactive nerve fibres. They proposed that this reduced the inhibitory action of NPY on detrusor overactivity contractions (Haferkamp et al., 2006). The role of NPY in the suburothelium is unknown and changes in human DO are yet to be elucidated. It is postulated that NPY containing nerves in the suburothelium may be upregulated in response to enhanced cholinergic pathway in DO.

Bladder dysfunction - Overactive Bladder and Detrusor Overactivity

Current causation theories

The precise cause of OAB and DO has not been elucidated so far. There are animal experimental models of DO but not OAB, due to the symptomatic nature of the diagnosis. Most animal work is careful scientific observation between control animals and those with induced DO, and postulating their importance in the mechanisms that underlie DO. Three main theories have been proposed regarding the cause of DO.

It is likely that the true cause of DO and OAB may be different in different situations or individuals, and may involve one or more of the hypotheses and each hypothesis is not mutually exclusive. There are some overlaps between the each of these models.

Myogenic Theory

This theory is based on the findings of altered properties of detrusor myocytes which are thought to be responsible for the involuntary detrusor contraction (Brading, 1997). Brading's theory is based on partial denervation setting in motion changes in detrusor physiological properties leading to supersensitivity to agonists, increased spontaneous excitability and increased electrical coupling between cells. Due to a lower threshold of excitability, a smaller local stimulus

may initiate a myogenic contraction of the entire bladder giving rise to DO. The greater excitability of smooth muscle cells are thought to come about due to increases in gap junctions and changes in cellular ultrastructure with the formation of protrusion junctions and ultra-close abutments, facilitating propagation of any electrical activity (Elbadawi et al., 1993; Haferkamp et al., 2003b). The local electrical stimulus can be initiated by stretch detected by afferent fibres or by normal firing in efferent pre-ganglionic fibres. Brading postulates that these changes come about as a response to a reduction in the normal excitatory input to the bladder, and do not preclude alteration in neural pathways of micturition.

Neurogenic Theory

This theory supported by de Groat suggests that changes in peripheral and central neural pathways can lead to detrusor overactivity (de Groat, 1997). It is based on 1) a reduction in peripheral or central inhibition, 2) an enhancement of excitatory transmission in the micturition reflex pathway, 3) increased afferent input and 4) emergence of bladder reflexes that are resistant to central inhibition. The above may not be present in all cases and different causes may be present in different groups of patients.

Disruption of suprapontine circuits by anterior cerebral lesions or degeneration of dopaminergic neurones in Parkinson's disease removes inhibitory control on the PMC. Studies looking in rat models of cerebral infarction suggest

suprapontine lesions alter signalling to the PMC, giving rise to DO (Fowler, 1999; Yokoyama et al., 2002; Yokoyama et al., 2003).

Spinal cord injury above lumbosacral segments prevents voluntary and supraspinal control of voiding, initially leading to a hypotonic bladder followed by development of autonomic micturition and NDO mediated by spinal reflex pathways (de Groat and Yoshimura, 2006). Voiding can be complicated by detrusor sphincter dyssynergia (DSD), which is caused by simultaneous contraction of the bladder and the urethral sphincter due to lack of central coordination, resulting in post-micturition residual urine volumes.

When these pathways are disconnected by spinal cord demyelinating plaques (MS) or injury, there emerge segmental reflexes which cause detrusor contractions in response to bladder distension. Animal experiments have shown activation of normally quiescent c-fibres following spinal cord injury, which become mechano-sensitive responding to bladder filling and stretch (de Groat and Yoshimura, 2006).

Between 52-97% of patients with multiple sclerosis (MS) exhibit bladder symptoms and signs associated with spinal cord involvement (Litwiller et al., 1999). Symptoms of frequency, urgency and nocturia are reported by both sexes, with incontinence reported more by women (Koldewijn et al., 1995). As described above, direct pathways project from the PMC to the sacral segments

of the cord (S2-S4), determining parasympathetic outflow to the detrusor as well as activity to the external urethral sphincter.

Normally there is central inhibition of parasympathetic innervation to the detrusor, but in the absence of this, detrusor contractions are caused by sacral afferent activity causing synaptic activity in the sacral segment of the cord. This can give rise to the common urinary complaints of MS patients. As spinal cord involvement of MS increases, so does the worsening of bladder dysfunction (Betts et al., 1993), but it is rare to see upper urinary tract involvement (Koldewijn et al., 1995).

However following traumatic spinal cord injury, most patients lose the reciprocal relationship between bladder and urethral sphincter function, resulting in detrusor sphincter dyssynergia (DSD). During bladder emptying, the urethral sphincter simultaneously contracts, causing bladder outflow obstruction. Up to half of patients with untreated DSD have high intravesical pressures, which in the long term may cause upper tract dilatation, reflux, progressive renal damage and failure (Hackler, 1977).

There is accumulating recent evidence that aberrant afferent activity plays an important role in the pathogenesis of OAB and DO.

In the urothelium, studies have shown interstitial cystitis results in augmented ATP release which can cause painful sensation by exciting purinergic P2X receptors on sensory fibres (Sun et al., 2001; Birder et al., 2003). Increased basal urothelial levels of TRPV1 have been shown in human NDO (Apostolidis et al., 2005a).

Various studies support the presence of suburothelial innervation expressing the capsaicin receptor TRPV1 (Brady et al., 2004b), purinergic receptor P2X₃ (Brady et al., 2004a), and the sensory neuropeptides substance P (SP) and CGRP (Smet et al., 1997) in the pathology of human DO. Patients with NDO and IDO were found to have increased TRPV1 and P2X₃ suburothelial innervation compared to controls (Apostolidis et al., 2005b). Following BoNT/A treatment both receptors populations normalised to control levels. Intravesical instillations of the c-fibre toxin RTX were found to delay or suppress involuntary detrusor contractions in patients with IDO (Silva et al., 2002) and decrease TRPV1 and P2X₃ immunoreactive fibres in NDO responders (Brady et al., 2004a; Brady et al., 2004b). Women with IDO were found to have increased density of suburothelial SP and CGRP immunoreactive fibres compared to controls (Smet et al., 1997). Men with obstructive DO had an increased incidence of a positive ice water test, which is related to c-fibre afferent evoked bladder reflex (Chai et al., 1998).

Autonomous bladder theory

This theory suggests that the organisation of muscle regions in the bladder is modular, with circumscribed areas of muscle supplied by individual intramural bladder ganglia or by a node of interstitial cells, which collectively are termed the "myovesical plexus" (Drake et al., 2001). There is synchronisation between modules either through nerves, interstitial cells or direct communication via muscle cells. The theory suggests that during bladder filling there is autonomous activity with non-micturition contractions and phasic sensory discharges. This activity can shift in pathological conditions to more excitatory inputs with reduction of inhibitory inputs, leading to inappropriate augmentation of autonomous activity and to DO. Any factor enhancing communication between modules will predispose to detrusor overactivity. It is similar to the myogenic model but proposes that all structural and functional alterations associated with both increased filling sensation (urgency and frequency) and DO are summated and integrated peripherally, contributing to the overall level of excitation (Hashim and Abrams, 2007b).

Functional MRI studies have suggested that inadequate activation of the frontal cortex may result in "poor bladder control", suggesting a central cause in the absence of overt neurological lesion (Griffiths et al., 2005).

In summary, the pathophysiology in OAB and DO is controversial with various proposed theories. There are multiple disease processes with associated DO

and a large cohort of patients with idiopathic DO, but no single unifying pathophysiological mechanism to explain causation.

Urotheliogenic theory

This is based on changes occurring in the urothelium and suburothelium causing an increase the sensitivity in the suburothelial network of nerves and myofibroblasts resulting in enhanced spontaneous detrusor activity. There are substantial signalling and sensory properties in the superficial layers of the bladder which have been noted to undergo upregulation in experimental detrusor overactivity. ATP and acetylcholine appear to be important neurotransmitters in sensory transduction. The important known constituents of the sensory pathway in the are documented on pages 36-49.

Chapter 2 - Detrusor Overactivity

Detrusor Overactivity (DO) is defined by the ICS as “a urodynamic observation characterised by involuntary detrusor contractions during the filling phase which may be spontaneous or provoked” (Abrams et al., 2002). The Over-Active Bladder (OAB) syndrome is a symptom complex characterised by urgency, with or without urgency incontinence, usually accompanied by frequency and nocturia. Urinary urgency is a pathological sensation although defined as a “sudden compelling desire to void that is difficult to deter”, although there is much debate about the definition and translation of the word; especially defining the difference between the meaning of “strong urge” and “urgency”. OAB is based on clinical diagnosis while DO is based on involuntary detrusor contraction with reproduction of symptoms during the filling phase of urodynamics. The populations studied in the continence literature are quite varied, with conclusions applied to both OAB and DO populations. DO is further subdivided according to associated disease; when there is concurrent neurological disease, the condition is termed Neurogenic Detrusor Overactivity (NDO), and where the cause is unknown or undiagnosed it is labelled Idiopathic Detrusor Overactivity (IDO).

Overactive Bladder Syndrome

Population based estimates suggest that OAB affects 12-17% of adults in Europe and the United States (Milsom et al., 2001; Stewart et al., 2003; Irwin et al., 2006b), with significant negative effect on health related quality of life

(Coyne et al., 2004). Not all patients with OAB have DO and neither will all patients with DO have OAB, leading some authors to state that the bladder is not always a reliable witness (Hashim and Abrams, 2006). It was not until a few years ago that the prevalence of OAB was identified. Previous studies had looked at urinary incontinence but it was not until the standardisation of the definition of OAB that a large study be performed. In a review of 36 general population studies from 17 countries, the prevalence of urinary incontinence ranged from 5 to 69%, with most studies reporting a prevalence of any urinary incontinence in the range of 25–45% (Hunikaar et al., 2005). The recent EPIC study (Figure 7) of 19165 adult participants is the largest multinational (Canada, Germany, Italy, Sweden, and the United Kingdom) population based cross-sectional survey and gives a good estimate of the prevalence of lower urinary tract symptoms using current ICS definitions (Irwin et al., 2006b). The prevalence of OAB was 11.8% (10.8% in men and 12.8% in women) and increased with age. Before the age of 60, it was more common in women than in men. In those with OAB, incontinence was present in 49.2% of women and 28.7% of men. Disappointingly, there are virtually no population based studies of the prevalence of incontinence in developing countries (Milsom, 2009).

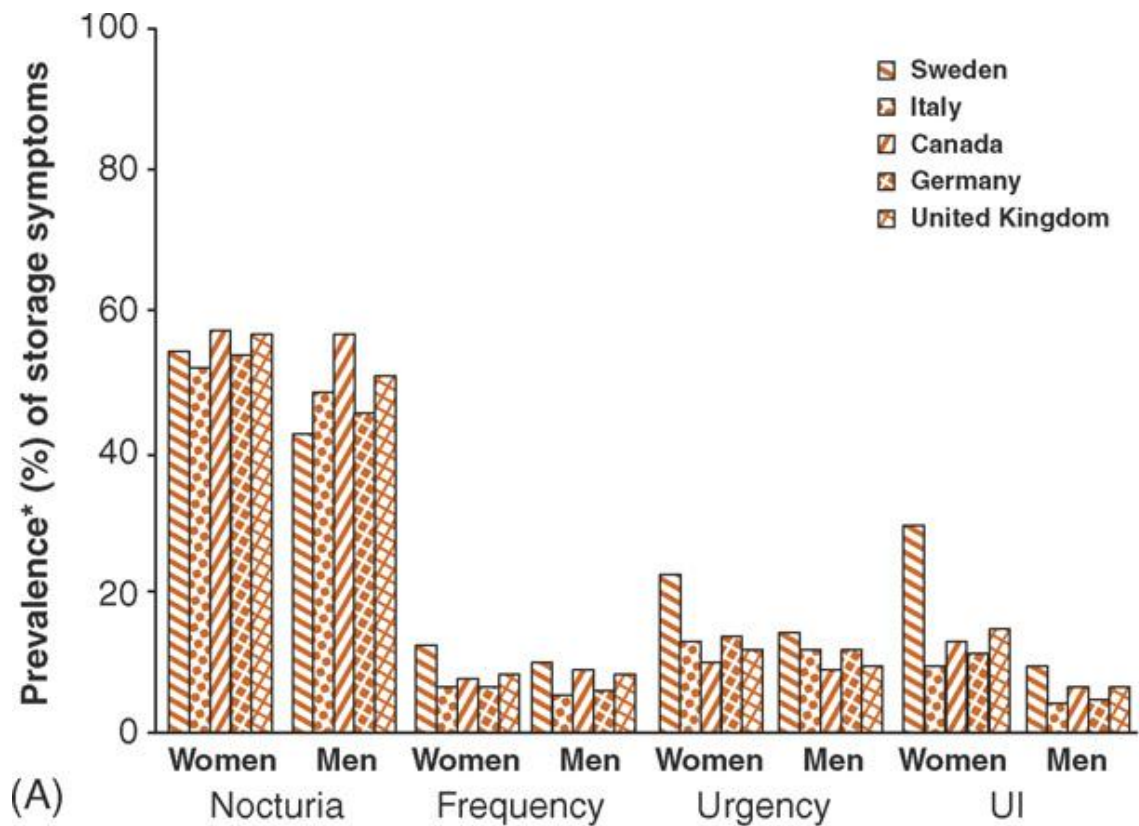


Figure 7: Prevalence of storage lower urinary tract symptoms by country and gender. UI = Urinary Incontinence. Reproduced from (Irwin et al., 2006b).

Impact of incontinence

OAB is a condition that causes a significant impact on all aspects of the quality of life (QoL) of an individual, including social, psychological, occupational, domestic, physical and sexual aspects (Brown et al., 2000a). One European cross-sectional population-based survey of 11 521 individuals, with 1272 sufferers, showed 21% of OAB sufferers worried about interruptions to meetings and work because of frequent visits to the toilet, with 3% of sufferers needing to change jobs or becoming unemployed due to bladder control problems (Irwin et al., 2006a). 32% of sufferers felt depressed as a direct result of their symptoms,

and reported that these bladder symptoms were a source of great concern and made them feel uncomfortable in social situations. The associated incontinence made sufferers make decisions about voluntary or early retirement (27% men vs. 4% women). The additive effects of urgency combined with the fear of incontinence made sufferers shy away from participating in activities far from home and in situations involving people they know well, as well as those they do not know well. Despite the debilitating effects of OAB symptoms, most individuals with OAB reported that their symptoms were something they had to learn to live with (74.3%). Just under half (43%) felt that their symptoms were not worth discussing with their doctor. These symptoms had a significant effect on the emotional well-being as well as the productivity of sufferers at home and at work. A study looking at health-related quality of life among adults with symptoms of overactive bladder showed that OAB had greater impact on QoL than diabetes (Figure 8) (Abrams et al., 2000).

Elderly patients with OAB have an increased risk of urinary tract infections and visit the toilet more often, doubling the chance of becoming injured in a fall. Weekly or more frequent urgency incontinence was associated independently with an increased risk of falls and fractures in older women (Brown et al., 2000b).

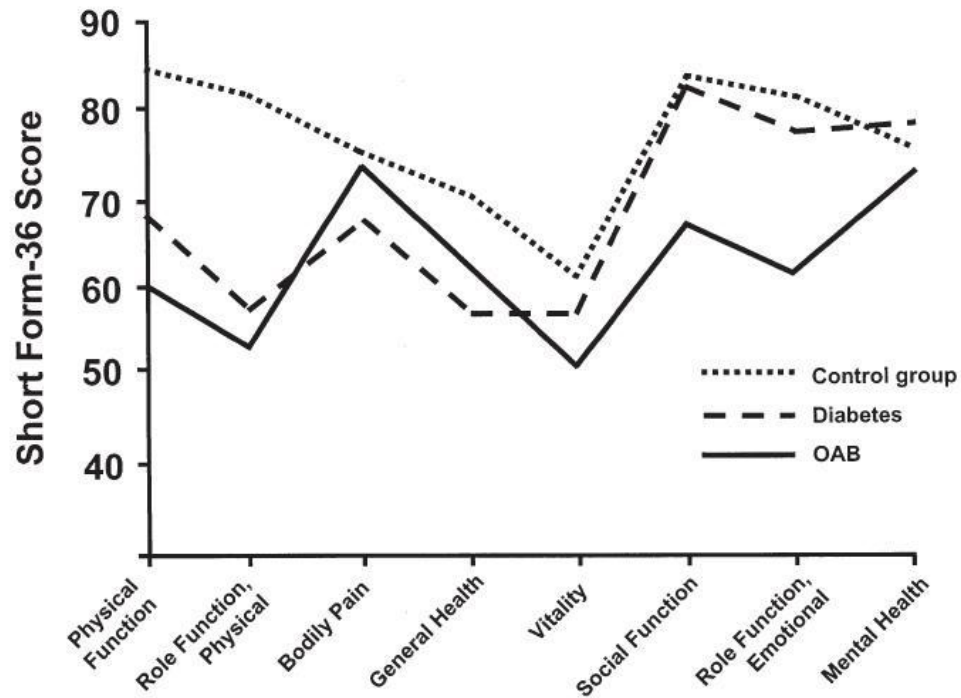


Figure 8: Comparison of Quality-of-Life Scores in Patients with OAB, Diabetes, and Normal Subjects. Source: Abrams P, Wein AJ. *The Overactive Bladder: A Widespread But Treatable Condition* Stockholm, Sweden: Erik Sparre Medical AB; 1998

Nocturia can interfere with sleeping and rest, resulting in reduced energy, chronic fatigue and greater impairment of work and activity (Kobelt et al., 2003). In the elderly, this can lead to falls as alertness is impaired on waking and lighting is often at lower levels.

Rates of depression and anxiety are increased in OAB with incontinence (Irwin et al., 2006a). OAB without incontinence causes anxiety, fear of incontinence, a sense of depression and hopelessness, all of which are worse for those with incontinence (Nicolson et al., 2007).

Cost consequence of OAB

There is considerable economic burden to society from this disease. Direct costs include those of routine care, diagnosis, treatment and consequence costs. There are further indirect costs incurred from lost wages to patients and lost productivity as a result of morbidity and mortality. Intangible costs reflect the suffering and decreased QoL associated with the condition (Hu and Wagner, 2000). An estimate of the economic costs of OAB in the United States in the year 2000 was \$12.6 billion (Hu et al., 2003). Three quarters of this were spent in the community (female and male \$7.37 and \$1.79 billion respectively). This ranked fifth for cost after arthritis, pneumonia and osteoporosis (Hashim and Abrams, 2007b).

In Europe, the direct cost of OAB in 2000 was estimated to be €4.2 billion affecting 20.2 million people over 40 across five European countries. Average annual expenditure was estimated to be between €269 to €706 per patient, with the major cost being for containment (Reeves et al., 2006). OAB incurs a substantial direct cost that is anticipated to increase in the future in line with aging populations.

Clinical assessment of OAB

OAB is a clinical diagnosis and is based on a history of frequency, urgency and nocturia with or without incontinence. The 3rd International Consultation on Incontinence 2004 defines the basic assessment necessary in the evaluation of OAB (Abrams et al., 2005). This includes a full history, a focused physical examination and appropriate investigations. The investigations should include a urinalysis ranging from dipstick to urine microscopy and culture to detect blood, glycosuria and occult infection. In addition, measurement of a post void residual using ultrasound or single "in and out" catheterisation, a frequency volume chart and QoL questionnaire are all recommended. Objective measurement of variables using uroflowmetry such as maximum flow rate and volume of residual urine after voiding gives useful information on micturition. Urinary tract ultrasound is also highly recommended if an anatomical cause is suspected. Urodynamic testing is recommended prior to invasive treatments, after treatment failure or in "complicated incontinence". The aim of the urodynamic evaluation is to assess bladder sensation, detect DO, assess urethral competence during filling, determine detrusor function during voiding, assess the outlet during voiding and to measure the residual urine. Visual inspection of the urethra and bladder by cysto-urethroscopy is important in patients with persistent dysuria or haematuria, as it can show mucosal abnormalities such as bladder tumours and strictures.

The frequency / volume chart should be kept for a minimum of 3 days in order to quantify the number of frequency episodes, the fluid input and output, estimate the functional capacity of the bladder and exclude polyuria (Dmochowski et al., 2005).

A disease specific OAB QoL questionnaire (e.g. ICIQ-OAB QOL) captures the severity of LUT symptoms and establishes a baseline from which treatments can be assessed. The National Institute for Health and Clinical Excellence (NICE) guidelines for the management of urinary incontinence in women suggest that incontinence should be categorised according to the patients' symptoms and treatment directed towards the predominant symptom (NICE, 2006). The guidance further states that 3-day bladder diary assessment should be used. Urodynamics is not recommended routinely prior to conservative management.

Chapter 3 - Treatment of OAB / DO

Treatments are based on clinical diagnosis with exclusion of other disease processes i.e. urinary tract infection, bladder tumour, calculi or diabetes mellitus.

Conservative management

When symptoms are mild, simple lifestyle interventions such as moderation of fluid intake to 1-1.5 litres a day and reduction of alcohol and caffeine can make a difference (Switinhbank et al., 2005), especially in the elderly. Patients are advised to avoid fluids four hours before bedtime, try reducing weight and cease smoking (Hannestad et al., 2003).

Physiotherapy

Behavioural therapy for DO was first reported in the 1960s advocating “voiding by the clock” (Jeffcoate and Francis, 1966). It is aimed at training the patient to re-learn cortical inhibition of detrusor contractions, thereby increasing voided volumes and the time interval between voids (Burgio, 2004). It requires high levels of motivation and encouragement. A systematic review in 2000 showed weak evidence that “bladder drill” (delaying micturition by progressively increasing intervals until a target of one void every 4 hours is met) is more effective than no treatment, and weak evidence that bladder drill is better than drug (oxybutynin IR, flavoxate and imipramine) treatment (Berghmans et al., 2000). Bladder training is usually supplemented by pelvic floor exercises.

Patients are taught to tighten the pelvic floor when they sense an involuntary contraction. There is evidence that this can potentiate the guarding reflex, inhibiting detrusor contraction.

NICE recommends offering patients bladder training for a minimum of 6 weeks as first line treatment of OAB (NICE, 2006). Further well designed trials are required to determine the effect of bladder retraining and pelvic floor exercises, as current studies in this area remain very heterogenous in type of therapy and populations studied.

Antimuscarinic agents

It is generally accepted that activation of muscarinic receptors on the detrusor is one of the mechanisms driving abnormal detrusor overactivity in the diseased bladder (de Groat, 1997). Regardless of the precise locus of action of antimuscarinic drugs (i.e. afferent or efferent), their widespread use for the treatment of OAB over the past three decades underscores the critical role of muscarinic receptors in the pathophysiology of this disorder.

Antimuscarinics include oxybutynin, tolterodine, solifenacin, trospium chloride, propiverine and fesoterodine (table 1).

Table 1: Current UK licensed antimuscarinic medications

Generic Name	Trade Name ®	Dose (mg)	Frequency	Receptor subtype selectivity	Elimination half life (hours)
Tolterodine tartrate	Detrusitol	2	BD	Non-selective	2.4
Tolterodine tartrate XL	Detrusitol XL	4	OD	Non-selective	8.4
Trospium chloride	Regurin	20	BD	Non-selective	20
Trospium chloride XL	Regurin XL	60	OD	Non-selective	38.5
Oxybutynin chloride		2.5 - 5	BD-QDS	Non-selective	2.3
Oxybutynin chloride XL	Lyrinel XL	5 - 30	OD	Non-selective	13.2
Oxybutynin Transdermal	Kentera	3.9 / 24 hours	Twice weekly	Non-selective	8
Propiverine hydrochloride	Detrunorm	15	OD-QDS	Non-selective	4.1
Darifenacin	Emselex	7.5 - 15	OD	Selective M3 receptor antagonist	3.1
Solifenacin	Vesicare	5 - 10	OD	Selective M2/M3 antagonist	40 – 68
Fesoterodine	Toviaz	4-8	OD	Non-selective	6 - 9

Antimuscarinic mechanisms of action

This class of drugs blocks muscarinic receptors with variable selectivity. Until a few years ago, the received wisdom was that antimuscarinic drugs act by blocking the detrusor muscarinic receptors which are stimulated by the release of acetylcholine (ACh) from the parasympathetic, cholinergic innervation. However this traditional view was challenged when, based on the observation that they act mainly during the storage phase decreasing symptoms of urgency and increasing bladder capacity, it was proposed their action was predominantly on afferents (Andersson, 2003, 2004).

There is good experimental evidence that antimuscarinics act on the storage phase by decreasing afferent nerve (A δ and c fibre) activity (De Laet et al., 2006; Iijima et al., 2007; Daly et al., 2010). The antimuscarinic drugs are all competitive antagonists, and during micturition where there is a large release of ACh the effect of the antimuscarinic drug would be overcome by endogenous agonist. If the sole effect of antimuscarinics were to be on the efferent pathway, we should clinically observe decrease in voiding efficiency. Although large doses of antimuscarinics can cause urinary retention, in the dose ranges used for OAB / DO, there is little evidence for increased residual volumes, (Finney et al., 2006).

Yokoyama et al. administered intravenous and intravesical tolterodine to rats with DO induced by cerebral artery occlusion, with and without RTX (an

analogue of capsaicin which induces c-fibre afferent de-sensitisation) (Yokoyama et al., 2005). Tolterodine increased bladder capacity at lower doses in untreated rats, but was ineffective in those treated with RTX. The authors suggest that the afferent nerves (A δ and C-fibres) are fundamental to antimuscarinic mode of action.

Systemic oxybutynin (De Laet et al., 2006) and darifenacin (Iijima et al., 2007) have been shown to reduce bladder afferent activity mediated by both A δ and c-fibres in pelvic nerve studies in rats. The inhibitory effect of darifenacin was more pronounced on c-fibre as compared to oxybutynin, which affected both types of fibres. Both these studies suggest that antimuscarinics directly or indirectly influence bladder sensory nerves, inhibiting the afferent part of the micturition reflex. Conversely, muscarinic agonists have also been shown to significantly inhibit the afferent response to bladder distension in wild type mice (Daly et al., 2010).

In healthy women, tolterodine has been shown to increase the sensory threshold to electrical stimulation of the bladder, which the authors here speculated could be due to tolterodine's effect on the suburothelium (Boy et al., 2007).

Muscarinic receptors are found on bladder urothelial and suburothelial cells (Mukerji et al., 2006; Birder and de Groat, 2007). There is release of ACh in

response to stretch from the urothelium and suburothelium (Yoshida et al., 2006; Yoshida et al., 2008). A significant increase in suburothelial cell M2 and M3 receptor immunoreactivity has been found in human IDO compared to controls (Mukerji et al., 2006). It has been postulated that it is a combination of ACh and Adenosine 5'-triphosphate (ATP) released during the storage phase has an excitatory effect on nerves in the suburothelium and the detrusor and which give rise to bladder afferent activity (Andersson et al., 2009). These experimental observations all point to the conclusion that the urothelium and suburothelium muscarinic receptors may be the key therapeutic target of antimuscarinic drugs.

Clinical efficacy

All the above antimuscarinics (Table 1) have evidence to support their efficacy in the treatment of DO and OAB. Large scale, randomised, placebo controlled studies have shown significant reductions in urinary frequency, urgency episodes, and Urgency Urinary Incontinence (UUI) episodes (Herbison et al., 2003; Chapple et al., 2005).

The clinical relevance of the efficacy of antimuscarinic drugs was questioned by the Herbison's systematic review in 2003, with the conclusion that although they produce statistically significant improvements over placebo, the benefits are of limited clinical significance (Herbison et al., 2003). Larger, more recent meta-analyses have refuted this view by including currently used drugs, showing that

this class of drugs are of clinically significant benefit (Chapple et al., 2005; Chapple et al., 2008a; Novara et al., 2008).

Chapple's 2005 systematic review attempted to address the criticisms of the Herbison review by looking at quality of life data in included trials and attempted to 'split' different drugs to assess any variation between the different drugs (Chapple et al., 2005). The review conclusions suggested that antimuscarinics were efficacious, safe and well tolerated, with marked improvements in quality of life. There were more trials included in this study with active comparator studies also allowed. The study criticised the quality of reporting in the studies, minimal data on older patients and limited quality of life data. Oxybutynin Immediate Release (IR) was found to be least tolerable and associated consistently with greater adverse events. The systematic review found that that population studied was very restrictive and not reflective of daily clinical practice. Few trials looked at older patients (>65 years) and at patients with NDO.

An update in 2008 looked at 73 blinded, randomised placebo and active controlled trials of oral and transdermal antimuscarinics to treat OAB (from over 12,000 references) (Chapple et al., 2008a). Antimuscarinics were found to be more effective than placebo, with good tolerability. The mean change with anticholinergic medication was between 0.4 to 1.1 incontinence episodes per day. There were no statistically significant differences for efficacy among

treatments in meta-analyses of active controlled trials. All drugs were well tolerated but oxybutynin IR was associated with the highest risk of trial withdrawal. Tolterodine 2mg IR and Oxybutynin Transdermal System were associated with adverse events similar to placebo, the other agents (Fesoterodine, Solifenacin, Propiverine IR and Extended Release [ER], Oxybutynin IR and ER, Trospium IR) had an adverse event profile greater than that of placebo. Dry mouth was the most common adverse event, others being constipation, pruritus and headache. The newer drugs allowed dose flexibility and titration of medication depending on efficacy and tolerability. This study again highlighted the limited evidence available in elderly patients with significant co-morbidity. There was very limited data on CNS adverse events, such as memory impairment. The conclusions were that this group of drugs was efficacious, safe and well tolerated, with improved Health Related Quality of Life (Chapple et al., 2008a).

Novara et al (2008) reviewed 50 Randomised Controlled Trials (RCT) and three pooled analyses to evaluate the efficacy and safety of antimuscarinics. They concluded that the trials had good methodological quality showing efficacy of antimuscarinics, that extended release were preferable to immediate release agents and the transdermal route of administration did not confer a significant advantage over the oral route due to local site reactions (Novara et al., 2008). Few studies did long-term follow-up of patients with patient reported outcomes. The authors pointed out that the majority of studies were designed to reflect the

needs of the company for registration rather than address the question relevant for clinical practice, which is fair criticism of industry funded trials. However, the finance required to sponsor such large expensive large-scale trials is beyond the scope of independent researchers. Further independent comparative clinical studies are required.

Onset of action

The onset of action of antimuscarinics can be quite variable. Tolterodine ER in an open labelled study reduced micturitions, urgency and UUI episodes as early as on day 5 of treatment (Sussman et al., 2007). Darifenacin significantly reduced similar parameters in patients with OAB at 2 weeks of treatment (Haab et al., 2004), whereas patients taking trospium showed improvements in UUI and urgency episodes as early as on days 1 to 5 (Landis et al., 2004).

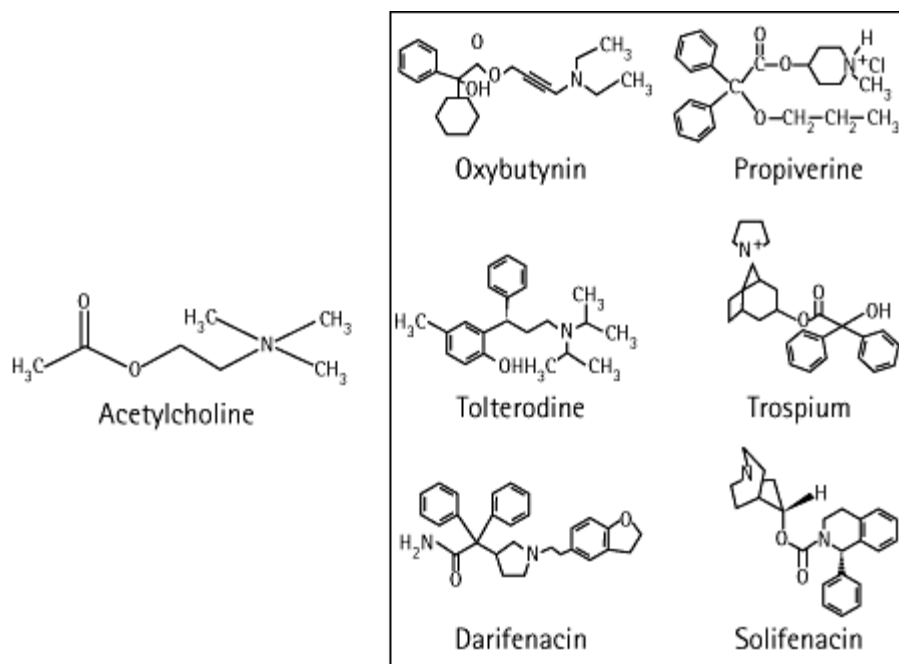


Figure 9: Antimuscarinic agents. Note the structural differences between tertiary (all except trospium) and quaternary (trospium) amines. Reproduced from Abrams P & Andersson KE Muscarinic receptor antagonists for overactive bladder. *BJU International* 100 (5), 987-1006.

Pharmacokinetics

Antimuscarinic drugs (Figure 9) are divided into tertiary and quaternary amines and they impart the pharmacokinetics of the molecule. Tertiary amines have no ionic charge, are lipophilic and more readily absorbed through the gastrointestinal tract, as well as cross the blood brain barrier easily. In contrast, positively charged quaternary amines i.e. trospium chloride are hydrophilic and do not easily pass into the brain (Pak et al., 2003), but still produce peripheral antimuscarinic effects. Tertiary amines undergo significant cytochrome P450 metabolism, whereas trospium has little enzyme metabolism, with the vast

majority excreted in the urine unchanged compared to oxybutynin or tolterodine; reducing the potential for drug interaction.

Drug delivery

The vast majority of antimuscarinic drugs are oral agents, however they have been used intravesically and transdermally. Extended release formulations have been licensed and prolong the duration of release of active drug, allowing once daily administration. Currently only oxybutynin is licensed as a transdermal sustained delivery patch, which allows for more stable serum concentrations and avoids first pass hepatic metabolism (Bang et al., 2003).

Tolerability and safety

Antimuscarinic agents block acetylcholine binding at many subtypes of muscarinic receptor not just in the bladder but in other organ systems, leading to adverse effects. Blockade of M₃ receptors in the salivary gland, lower bowel and ciliary smooth muscle are consistent with the mechanism of action of antimuscarinics and give rise to the most widely reported side effects of dry mouth, constipation and blurred vision. Transdermal formulation of oxybutynin avoids first pass metabolism and the formation of the active metabolite N-desethyloxybutynin, which has greater affinity for parotid gland muscarinic receptors, resulting in a much reduced rate (94% vs. 34%) of dry mouth in RCTs comparing immediate release oxybutynin with the transdermal formulation (Davila et al., 2001).

Elderly patients account for a large number of OAB patients; they are more susceptible to cognitive effects and constipation, both of which can further exacerbate urinary symptoms.

Although most studies state low withdrawal rates from adverse effects, in clinical practice, patients do complain of the side-effects. Although these may be tolerated for short periods similar to the follow-up reported in trials, in the long term many patients discontinue their treatments. Antimuscarinics are not optimum agents for OAB and DO given their limited efficacy and non-selectivity, but they are currently the first line drugs of choice, due to limited alternative oral agents.

Alternate classes of drugs

Alternative pharmaceutical classes of drugs used to treat OAB symptoms with placebo controlled trial evidence include desmopressin and duloxetine (Hashim and Abrams, 2007a; Steers et al., 2007). Nicorandil, a potassium channel opener together with a nitric oxide donor has been tried in animal models, with results showing suppression of DO in neurogenic and myogenic models (Kamiyama et al., 2008).

Another drug class showing promise are the beta 3 adrenoceptor agonists which promote bladder relaxation. It has been shown in dog models that these

agonists cause detrusor relaxation as well as increases in the micturition reflex threshold (Hicks et al., 2007).

In-vitro studies have shown that non-selective β -adrenoreceptor agonists like isoprenaline have a pronounced inhibitory effect on human bladder, causing increases in bladder capacity (Anderson, 1993). This has not been followed up as a therapeutic option because of probable cardiac side effects. Subtypes of the β -adrenoreceptor (β -AR) have been identified both in the detrusor and in the urothelium (Michel and Vrydag, 2006; Otsuka et al., 2008), with evidence that these are functionally active (Leon et al., 2008). RT-PCR experiments have identified β 3-AR as being predominantly expressed in human detrusor (Michel and Vrydag, 2006). It is thought that β -AR causes relaxation of the detrusor by activation of adenylyl cyclase together with formation of cAMP (Frazier et al., 2008). Animal in-vivo studies have shown that β 3-AR agonists increase bladder capacity without affecting voiding parameters (Kaidoh et al., 2002; Takeda et al., 2002).

Two current β 3-AR agonists are being evaluated in human studies as treatment in DO: solabegron (GW427353) and mirabegron (YM178) (Drake, 2008). Reporting in abstract form by Chapple et al. showed primary efficacy by significant reduction in mean micturition frequency with mirabegron compared to placebo (Chapple et al., 2008b). Secondary variables included significant improvements in mean volume per micturition, mean number of incontinence episodes, nocturia episodes and urgency incontinence episodes. This study

demonstrates the proof of principle of β -AR agonists being useful agents in symptoms of OAB and paves the way for further large randomised controlled trials.

Recently Cizolirtine citrate (cizolirtine), an inhibitor of CGRP and Substance P release has been shown to be efficacious compared to placebo in a phase 2 proof of concept study (Zat'ura et al., 2009).

Sacral Nerve Stimulation (SNS)

Sacral nerve stimulation or neuromodulation is recommended by NICE for women who have DO refractory to conservative and medical therapy (NICE, 2006). It involves the stimulation of the S3 nerve root by an Implantable Pulse Generator (IPG) (Interstim® and Interstim II®, Medtronic Inc, Minnesota, Minneapolis, USA), and has been shown to reduce episodes of frequency, urgency and urgency incontinence. It is an expensive treatment costing £7,000 for the implant alone and requires expert assessment and management, especially where there are complications. The exact mechanism of action is unknown but theories postulate that both afferent and efferent mechanisms may be involved.

In long term case series studies, symptom improvement was observed in about 50% of patients with frequency and urgency (Table 2). Patients with incontinence have similar reported efficacy, however a lot of studies report

improvements rather than measureable objective indicators. The original Interstim® IPG lasted approximately 7 years, needing to be replaced with a new unit, which normally sits in a subcutaneous pocket either in the buttock or the abdomen. The new Interstim II® IPG system is smaller, with fewer connectors but also with a reduced battery life.

In long term series, over half the women implanted suffered a complication such as infection, box site pain, lead migration or leg pain, with most remedied with medication or surgery (Datta et al., 2008). There is hope that the new two stage implantation will improve the efficacy, reduce the number of complications and successfully treat more patients.

Posterior Tibial Nerve Stimulation (PTNS)

PTNS is second line therapy for OAB syndrome that is approved by NICE. It is administered by placement of a fine gauge needle adjacent to the tibial nerve just above the ankle. This is connected to a low voltage stimulator. The mechanism of action is unknown but is thought to be by neuromodulation of the sacral nerve plexus. Treatment involves 12 sessions lasting 30 minutes each usually performed weekly. Further sessions are required to maintain efficacy. Two randomised clinical trials compared PTNS to placebo or anticholinergics have demonstrated moderate/marked improvement in 55% and 80% of patients respectively with significant improvements in quality of life (Peters et al., 2009; Peters et al., 2010). There are no major safety issue with treatment.

Table 2 : Published reports of efficacy of Sacral Neuromodulation in Urgency Incontinence (UI) and Urgency Frequency (U/F).

Study	Total		Patients with UI		Patients with U/F		Follow-up
		Cured	>50% improvement	Improved	>50% improvement	Improved	
US Patient Registry (Pettit et al., 2002)	81		27/43		10/19		
(Amundsen and Webster, 2002)	12	2/12	12/12				7.8 months
(Bosch and Groen, 2000)	45		27/45	9/45			47.1 months
(Siegel et al., 2000)	112		21/41	19/41	16/29	5/29	24-36 months
(Schmidt et al., 1999)	34	16/34	10/34	26/34			6 months
(Hassouna et al., 2000)	25				14/25		24 months
(Elhilali et al., 2005)				1/6		10/22	77.4 months
(van Voskuilen et al., 2006)	149			89/107			70 months

Surgery

Clam augmentation cystoplasty

The principle of bladder augmentation is to increase bladder capacity and compliance and to decrease overactivity. It is performed by isolating a detubularised segment of intestine segment (most commonly ileum) and suturing it to the top of a bi-valved bladder. It is a procedure indicated in patients with low capacity bladder who are refractory to all therapies, and whose quality of life is severely hampered. It results in improvements in symptoms and bladder capacity plus reductions in maximum detrusor pressures in spinal cord injured patients (Khastgir et al., 2003). However, it fails to resolve OAB symptoms in approximately 8% of patients with NDO and 40% of patients with IDO (Greenwell et al., 2001). The complications of the procedure are that patients often have to self-catheterise, have problems with mucus retention, stones and a 5% long-term risk of adenocarcinoma if large bowel is used. If ileum is used for augmentation, there are further risks of hyperchloraemic acidosis, vitamin B₁₂ and folate malabsorption. The most dreaded complication is spontaneous perforation, which carries a mortality of about 25%.

Urinary diversion

In patients with severe detrusor overactivity who cannot or will not manage clean intermittent catheterisation, an option is to perform a urinary diversion. Urine then drains into an ileal conduit, which is attached to the abdominal wall

as a stoma. The urine then collects into an appliance bag. If a cystectomy is not simultaneously performed, there are long-term risks of bladder cancer, pyocystis and spasticity.

More complex continent diversions such as Mitrofanoff and Koch pouches can be reconstructed if patients are willing to catheterise an opening in the abdominal wall. There is a high revision rate for continent catheterisable vesicostomy, mainly for stomal stenosis or difficulty in catheterisation (Van der Aa et al., 2009). The risk of complications and the irreversibility necessitate that surgery remains the option of last resort in the management of detrusor overactivity.

Botulinum toxin in detrusor overactivity

Rationale for use of BoNT/A in OAB and DO

There are significant limitations of antimuscarinic medication and surgical procedures with their high incidence of side effects. Intermediate treatments that fill the gap between oral medication and bladder surgery have failed to produce robust clinical results. Intravesical instilled neurotoxins, namely capsaicin and RTX, remain unlicensed 15 years after their introduction, with conflicting results of efficacy in randomised placebo controlled trials (Silva et al., 2005; Rios et al., 2007). Sacral neuromodulation is an invasive and costly procedure involving significant complications and limited only for IDO patients. A serious alternative treatment was lacking until the turn of the last century.

Since ACh is the main neurotransmitter involved in detrusor contraction, it was thought that suppressing its release in DO with BoNT/A, a toxin known to inhibit Ach release, would be of clinical benefit.

Botulinum neurotoxin type A (BoNT/A)

The botulinum toxins are among the most potent toxins known, and were first identified in 1897 as the product of the gram positive anaerobe *Clostridium botulinum*, the causative agent for botulism food poisoning (Schantz and Johnson, 1997). Although all serotypes block transmission at the neuromuscular junction, thereby reducing muscle activity to varying degrees, BoNT/A has the

most prolonged effect and has been studied extensively, especially in striated muscle.

BoNT/A has been approved for use in various conditions of excess skeletal muscle contractility (e.g. cervical dystonia, blepharospasm and limb spasticity) and oversecretion of sweat (hyperhidrosis). Its efficacy in disorders such as oesophageal spasm and DO have prompted some observers to note that its effect in smooth muscle is similar to skeletal muscle, but with a longer duration of action. (Storr et al., 2001).

Molecular basis of BoNT activity

Seven immunologically distinct serotypes of this neurotoxin exist (designated A to G). BoNT/A is synthesised as a single-chain polypeptide of 150 kDa molecular mass, consisting of 1285 amino acids (Lacy et al., 1998). The toxin associates with additional non-toxic proteins to form larger molecules of between 300 – 900 kDa. When the single chain is cleaved by an endogenous clostridial protease to a 100 kDa heavy chain and a 50 kDa active light chain, maximum biological activity is gained (Simpson, 2004). The two chains are still linked by disulphide and non-covalent interactions (Kozaki et al., 1989).

BoNT/A is folded into three functional domains that directly affect the toxin's action on the target cell. The domains work together via three stages: (i) binding and internalisation, (ii) translocation and (iii) exocytosis inhibition (Figure 10).

The c-terminal of the heavy chain binds to ecto-acceptors on the pre-synaptic cholinergic nerve terminal and is taken up into neurones by endocytosis (Dolly et al., 1984; Black and Dolly, 1986). BoNT/A enters neurons by binding to the synaptic vesicle protein SV2, the expression of which is enhanced by synaptic activity (Dong et al., 2006).

Once internalised and present within a vesicle, BoNT/A undergoes a pH-dependent conformational change which causes disassociation of the light and heavy chains (Li and Singh, 2000). The heavy chain forms a pore in the vesicle by inducing a conformational change, allowing the light chain to enter the cytoplasm and function as a zinc dependent protease, targeted specifically towards SNARE (Soluble N-ethylmaleimide-sensitive fusion Attachment protein Receptor) proteins which mediate fusion of cellular transport vesicles with cell membranes (Schiavo et al., 1992b; Schiavo et al., 1992a).

The light chains of the different serotypes act to disable the vesicle docking system of exocytosis by cleaving different SNARE components. BoNT/A cleaves SNAP-25 by removing 9 amino acids from the C-terminus, whereas BoNT/E cleaves 26 amino acids (Dolly, 2003). The cleavage by BoNT/A is highly specific as it recognises a highly conserved, nine-residue binding motif that occurs with the SNAP-25 protein (Washbourne et al., 1997). The other toxin serotypes cleave syntaxin and synaptobrevin at various other locations.

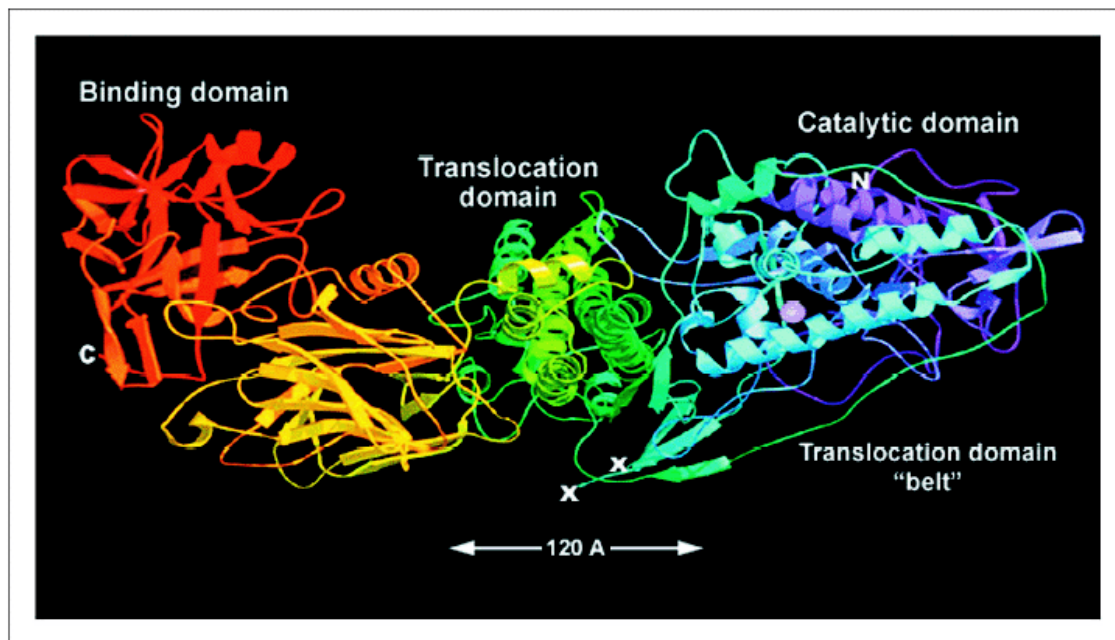


Figure 10: Three-dimensional structure of botulinum neurotoxin type A (BoNT/A). The binding domain regions in the C-terminal of the heavy chain, are indicated in red and yellow. The translocation domain in the N-terminal of the heavy chain (green) contains 2 parallel helices, whereas the toxic moiety (blue) resides in the light chain which possesses the protease with zinc (lilac dot) bound at the active site. Reproduced from Dolly (Dolly, 2003).

BoNT/A has the greatest stability of proteolytic activity when compared to other subtypes of toxin, with activity for over 31 days in cerebellar granular neurones. BoNT/C1 showed activity for 25 days, BoNT/B for 10 days and BoNT E for 0.8 days (Foran et al., 2003). The cleavage product of SNAP-25 is SNAP-25_A, which is also antagonistic and inhibitory to the function of unproteolysed SNAP-25 (O'Sullivan et al., 1999). These properties make BoNT/A ideally suited to clinical use for long-term inhibition of neurotransmitter release (Dolly and Aoki, 2006). Recovery of cholinergic neurotransmission in striated muscle is

dependent on the removal of the BoNT/A light chain protease and restoration of intact SNARE proteins (Foran et al., 2003).



Figure 11: Inhibition of acetylcholine exocytosis by BoNT/A. (A/B = Docking of vesicle to synaptic cleft; C = Membrane fusion and Ach released. D= BoNT/A binds to SV2 and enters by endocytosis; E= Light Chain released into cytosol by conformational change in heavy chain; F= Light Chain of BoNT/A cleaves SNAP-25 preventing assembly of SNARE complex and further blocking Ach release. Reproduced from *Nat Clin Pract Urol.* (2008) Drug Insight: biological effects of botulinum toxin A in the lower urinary tract (Chancellor et al., 2008).

Action on striated versus smooth muscle

In striated muscle, BoNT/A temporarily inactivates neurotransmission and acts as a biochemical neuromodulator. It blocks the signalling between α and γ motor neurones and extrafusal muscle fibres and spindles (Rosales et al., 1996). Cells show temporary signs of denervation with central mitochondria dispersed to the periphery within a few weeks of injection (Borodic and Ferrante, 1992). The physiological changes include reduction in the resting membrane potential as well as elimination of extra-junctional acetylcholinesterase activity (Thesleff et al., 1990). There is compensatory sprouting and growth of extra-junctional motor nerve terminals which are rich in dense core vesicles (de Paiva et al., 1999). Once there is return of vesicle turnover to the original parent terminals, there is elimination of the superfluous sprouts.

Comparatively in detrusor smooth muscle there is no evidence of nerve sprouting in detrusor muscle following BoNT/A (Haferkamp et al., 2004). BoNT/A injections do not appear to be producing significant inflammatory changes, fibrosis, or dysplastic changes in human bladder urothelium/suburothelium after a single injection (Apostolidis et al., 2008). NDO patients treated with BoNT/A had less bladder wall fibrosis than those not treated (Comperat et al., 2006). There was no evidence of apoptosis following the 1st injection of BoNT/A in human detrusor (Kessler et al., 2009).

Clinical data from intradetrusor BoNT/A

The first publication of the use of intra-detrusor BoNT/A injection was in 1999 (Stohrer et al., 1999) and was followed by several open labelled studies confirming impressive clinical results in patients with spinal cord injury and NDO. These papers all described improvements in clinically measurable urodynamic parameters such as bladder capacity, volume at first contraction, decreases in detrusor pressure during filling, as well as patient reported parameters such as frequency and urgency incontinence (Schurch et al., 2000; Popat et al., 2005; Schurch et al., 2005). In 200 spinal NDO patients, continence was restored in 73%, with results lasting up to 9 months following a single BoNT/A injection (Reitz et al., 2004). A further American study of 42 patients with OAB of various aetiologies (NDO, IDO, sensory urgency and interstitial cystitis) had return to continence in 81% of the study population with sustained results to 6 months (Smith et al., 2005).

Preparations

The most common serotype of BoNT is BoNT/A which was licensed under the brand name of Botox® (Allergan Inc., Irvine, CA, USA). Another commercially available form of BoNT/A is Dysport® (Ipsen Ltd., Slough, Berkshire, UK), which has different associated proteins. Both products have different dosage, efficacy and safety profiles and should not be considered as bioequivalent (Table 3) (Wenzel et al., 2007). There is inevitable commercial rivalry about the bioequivalence of the two preparations, however the majority of the clinical trials of the bladder have used the Allergan Botox® preparation.

The recently released BoNT/B products Myobloc® and Neurobloc® (Solstice Neurosciences Inc, San Francisco, CA) are not clinically equivalent with BoNT/A products, as they have very different product characteristics and have been noted to have systemic antimuscarinic adverse effects (Dressler and Eleopra, 2006; Ghei et al., 2006)

Table 3 : Comparison of Botox and Dysport characteristics

	Dysport®	Botox®
Complex molecular weight (kDa)	500–900	900
Units/package	500	100
Neurotoxin protein content (ng/vial)	4.35	c. 5
Formulation	Lyophilized	Vacuum dried
pH	c. 7.0	c. 7.0
Excipient	HA with lactose	HA with 0.9% NaCl
Target	SNAP-25	SNAP-25
Approval	1989 (US)	1991 (EU)

Table modified from (Wenzel et al., 2007) with data from (Pickett et al., 2007)

Randomised controlled trials (RCTs)

In a placebo controlled randomised double blind trial studying two different BoNT/A doses (200U and 300U BOTOX®) versus placebo, Schurch et al demonstrated significant improvements in incontinence episodes, frequency and urodynamic variables only in the toxin-treated groups. The placebo-treated group failed to demonstrate improvements in any of the study outcomes up to 24 weeks post-treatment. Results were highly significant despite the small numbers of patients recruited (19 patients in each toxin group, 21 patients in the placebo group) (Schurch et al., 2005).

In IDO patients, there have been comparable urodynamic and symptomatic improvements to those found in NDO patients (Kessler et al., 2005; Popat et al., 2005). The largest IDO series of 100 patients showed low dose (100U Botox®) BoNT/A restored continence in 86% of patients, with normalisation of frequency within 1-2 weeks and a mean duration of response of 6 months (Schmid et al., 2006).

A single centre, randomised double-blind, placebo controlled trial in 16 patients with IDO and 18 placebo controls demonstrated 200 U (Botox®) improved frequency, urgency and urgency incontinence episodes at 4 and 12 weeks after BoNT/A injection (Sahai et al., 2007). Urodynamic investigations demonstrated an increase in mean final cystometric capacity of 145ml compared to placebo,

with improvements sustained out to six months. Six patients were required to perform CISC due to high residual volumes. Significant improvements in QoL outcome measures were also observed in this study following BoNT/A.

A head to head prospective RCT of BoNT/A and intravesical RTX in spinal NDO patients showed superior efficacy of BoNT/A in clinical and urodynamic study endpoints at 6, 12 and 18 month follow-up (Giannantoni et al., 2004).

Botulinum neurotoxin type B (BoNT/B) has also showed efficacy in a randomised, double blind, placebo controlled crossover trial in a mixed population of 3 NDO and 17 IDO patients. BoNT/B improved bladder capacity, frequency, incontinence and QoL but its effect lasted about 6 weeks, much less than the duration of BoNT/A (Ghei et al., 2005).

Onset and duration of effect

Patients report improvements soon after the treatment. A study looking at daily changes occurring in the first week after bladder BoNT/A injections in a group of 29 patients with DO showed decline in urgency, frequency and nocturia at day 2, followed by a reduction in incontinence episodes at day 3 (Kalsi et al., 2007). This was confirmed in a larger study of 100 patients with a mean time to loss of urgency of 5 days, sustained at 4 weeks in 82% of patients (Schmid et al., 2006).

The largest studies of Botox® in 200 patients showed a mean duration of action of between 9 and 11 months, with repeat injections showing equally sustained beneficial effect (Reitz et al., 2004; Grosse et al., 2005). A cohort study showed similar results, in that the mean time to symptom return was 10.4 months (Popat et al., 2005). Differences in dose, injection technique, formulation of BoNT/A, volume of diluent and number of injection sites may play a role in the variability of effect in different patients.

Evidence for an effect of BoNT/A on afferent pathways

In the bladder, the action of BoNT/A was initially thought to be via inhibition of presynaptic vesicular Ach release through the cleavage of synaptosomal-associated protein of 25kDa (SNAP-25), inducing detrusor muscle relaxation (Schurch et al., 2000). However, BoNT/A also has an inhibitory effect on SNARE dependent expression and vesicular release of other neuropeptides, neurotransmitters and receptors mediating sensory neurotransmission. BoNT/A has been shown to induce long lasting inhibition of SP following cleavage of SNAP-25 (Welch et al., 2000) as well as release of glutamate in a model of inflammatory pain (Cui et al., 2004). Other studies have shown BoNT/A inhibition of exocytosis of TRPV₁ receptors to the plasma membrane (Morenilla-Palao et al., 2004),

Additional studies in a rat pain model have demonstrated inhibition of CGRP release from superficial layers in the bladder, together with an increase in the interval between bladder contractions (Chuang et al., 2004).

BoNT/A effect on afferent bladder pathways

In rat models of NDO, BoNT/A significantly reduced the abnormal distension evoked urothelial release of ATP (Khera et al., 2004) and the contractions evoked by application of ATP or capsaicin to rat bladders in-vivo (Atiemo et al., 2005).

Biopsies taken from patients with IDO and NDO before and after BoNT/A showed a progressive decrease with normalisation in sub-urothelial P2X₃ and TRPV₁ receptor immunoreactivity indicating that BoNT/A affected sensory afferents in this layer. Changes in P2X₃ receptors coincided with clinical improvements in patient reported episodes of urgency (Apostolidis et al., 2005b).

Further supporting evidence comes from a study which looked at homogenised bladder tissue levels of Nerve Growth Factor (NGF) in patients with NDO; these were reported to be significantly reduced up to 3 months after treatment with BoNT/A, suggesting that BoNT/A can modulate the production or release of NGF from the bladder (Giannantoni et al., 2006). A follow-on study looked at urinary levels of NGF and found that it was increased in patients with NDO and

IDO, however following successful treatment with BoNT/A these decreased significantly towards control levels. The authors concluded that NGF was a sensitive biomarker for DO and could be used to evaluate successful treatment after BoNT/A (Liu et al., 2008) and antimuscarinic therapy (Liu et al., 2009).

BoNT/A effect on efferent bladder pathways

Although BoNT/A is proposed to exert its therapeutic effect on patient symptoms via an effect on the afferent pathway, it does also elicit changes in the efferent pathway of bladder contraction. Electrical field stimulation experiments designed to mimic parasympathetic nerve overactivity in DO have demonstrated decreases in the neuronal release of Ach in bladder strips treated with BoNT/A compared to sham treated animals (Smith et al., 2003).

Clinical studies have also demonstrated post-BoNT/A decreases in detrusor pressures in both filling and voiding phases, as well as increases in post-void residual volumes (Popat et al., 2005; Schurch et al., 2005), suggesting an inhibitory effect on the motor nerves supplying the detrusor muscle.

Chapter 4 - Aim and Objectives of thesis

The use of intra-detrusor BoNT/A has been revolutionary in the treatment of patients who are refractory to anti-cholinergic therapy. It is repeatable and the treatment appears to be safe and durable in the short to medium term. However, several key areas remain unexplained, including the pathophysiology of DO and the mechanism of action of BoNT/A, especially in reducing urgency, frequency and nocturia.

Both muscarinic receptors and NPY are involved in afferent neurotransmission in the bladder. Little is known regarding the expression of superficial muscarinic receptors in those with DO as compared to control subjects. We aimed to examine a possible role of urothelial and suburothelial muscarinic receptors in human NDO/IDO and whether the action of BoNT/A is partly exerted through an effect on those receptors.

NPY is a known co-transmitter in neurones containing ACh and is thought to inhibit the contractile response of the detrusor. NPY has been shown to change with disease states. We aimed to examine the possible role of NPY in human NDO/IDO and whether the action of BoNT/A affects NPY density.

BoNT/A has been speculated to act on afferents in the superficial bladder layer via its action on SNAP-25. We aimed to examine if SNAP-25 is expressed in human urothelium or suburothelium.

The aim of this thesis was to look at differences in neurotransmitter and neurotransmitter receptor expression in patients with DO before and after treatment. The project required the collection of biopsies, acquisition of skills necessary to perform immunohistochemistry and development of new protocols to successfully stain for the different receptors and peptides. The analysis of the slides required skills in computer quantification, database construction and statistical testing.

Hypotheses

1. We hypothesised that there was involvement of the afferent cholinergic muscarinic system in the development of detrusor overactivity. We proposed to test this hypothesis by comparing muscarinic receptors and neuropeptide Y density within the urothelium and suburothelium in patients with DO, as compared to control subjects.
2. BoNT/A has a highly specific action on SNAP-25. We hypothesised that SNAP-25 was present in the superficial layers of human bladder and this was unaltered with disease. We proposed to test if there were differences in SNAP-25 immunoreactivity between controls, patients with DO and in those treated with BoNT/A.

3. Our final hypothesis was based around the mechanism of action of botulinum toxin. There is debate as to how the toxin acts to produce amelioration of symptoms. We hypothesised that its marked effect on sensory symptoms was based on its action at the afferent level, by modulating receptor and neurotransmitter expression. We aimed to test this by using immunohistochemistry to compare the density of muscarinic receptors and the neurotransmitter NPY before and after application of BoNT/A.

II – Methods

Chapter 5 - Clinical Methods

Ethical and Regulatory Approval

The immunohistochemistry component of this study was part of a larger, ethically approved, prospective, single centre phase II open labelled study looking into the clinical effects of BoNT/A in patients with detrusor overactivity (Popat et al., 2005). All of the investigations performed as part of this thesis followed the approval of the study by the local ethics committee (Joint Ethics Committee of UCLH / NHNN Trust / Institute of Neurology and Ealing Hospital). The permission of this committee is detailed in Appendix A. Clinical Trial Authorisation (CTA) for the use of Botox® was sought from the Medicines and Healthcare products Regulatory Agency (MHRA) and its predecessor Medicines Control Agency (Exceptions from License); the document is detailed in Appendix B. University College London Hospitals Trust Research and Development Approval was also received and is listed in Appendix C. The study was approved prior to the 2004 EU clinical trial legislation and was updated to meet the requirements subsequently.

Informed Consent

Patients were recruited to this study from referrals to the Department of Uro-neurology at the National Hospital for Neurology and Neurosurgery and the

Department of Urology, Ealing Hospital. All BoNT/A treated subjects in the study had a history of urgency, frequency and / or urgency incontinence for more than one year and were refractory to oral antimuscarinic treatment (at least two different formulations), due to either lack of efficacy or intolerable adverse effects. Subjects were provided with a patient information leaflet (PIL) (Appendix D) and invited to participate by signing a trial specific consent form (Appendix E).

Control tissue was obtained from asymptomatic subjects (screened for the absence of OAB symptoms) at Ealing Hospital NHS Trust undergoing flexible cystoscopy for haematuria. Subjects were also provided with a control biopsy specific PIL (Appendix D) and consent form (Appendix E). This study was carried out according to principles set out in the Declaration of Helsinki and EU Good Clinical Practice (GCP). All health professionals including the subjects' General Practitioner were kept fully informed.

DO Patient Group

Patients with a history of urgency, frequency and/or urgency incontinence (as defined by the International Continence Society) for more than a year who could not be managed by oral medication with or without use of clean intermittent self-catheterization (CISC), or who had intolerable adverse events from therapeutic doses of anticholinergic therapy were considered for this study. There were a

total of 36 patients (13 men and 23 women) with 17 having IDO and 19 having NDO.

They underwent a full history, examination, with urine dipstick collection of 3 day voiding diaries and urodynamic investigations before a diagnosis of DO was made. There were two subgroups: IDO and NDO patients. The causes of NDO were multiple sclerosis (15), spastic paraparesis, spina bifida, transverse melitis, and spinal cord injury. Mean age was 45.7 years (range 19-63 years).

Inclusion criteria required patients to be willing to perform CISC, in case this was required following treatment and the ability to attend afterwards for study tests. Exclusion criteria included; women who were pregnant or breast feeding or planning pregnancy, patients on anticoagulant therapy, patients with neuromuscular transmission disorders and those taking drugs that might interfere with neuromuscular transmission (e.g. aminoglycosides).

Urodynamic methods

Subjects were assessed with standard subtraction cystometry using a filling rate of 30 ml per minute, and those with urodynamic evidence of phasic or terminal DO were considered suitable to enter the study. Filling was stopped when the patient could no longer delay micturition or upon terminal DO; the volume was then recorded as the maximum cystometric capacity (MCC). Maximum detrusor pressure during filling ($P_{det_{max}}$) was also recorded as the maximum pressure

during phasic or terminal DO. Patients with reduced bladder compliance (increased detrusor pressure at low cystometric capacity) in the absence of obvious DO were excluded from the study.

Intra-detrusor Injection of Botox®

Prior to treatment, urine was checked with dipstick for signs of infection and if present, a urine specimen was sent for culture, the patient was treated with empirical antibiotics and the procedure was postponed.

The skin was prepared with antiseptic solution and 1% lignocaine gel was applied to the urethra for lubrication and local anaesthesia. A flexible cystoscope (CYF-5, Olympus Keymed, UK) was inserted into the bladder under direct vision and the bladder wall examined. Only if the bladder wall was considered healthy did the clinician proceed further. All procedures were carried out under the supervision of a consultant urologist, Mr Prokar Dasgupta. If there was discomfort with the procedure, 20ml of lignocaine gel was injected per urethra into the bladder, and the procedure recommenced after 20 minutes.

The Botox® (Allergan Pharmaceuticals, Ireland) was reconstituted and diluted in saline solution prior to injection. Patients were injected by using a disposable 27 gauge flexible needle enclosed in a sterile reusable sheath (NM-101C-0427 set includes 1 x reusable sheath and 6 sterile needles, Olympus Keymed, UK) via a

flexible cystoscope with a 2.2 mm working channel (Harper et al., 2003; Sahai et al., 2006).The toxin was injected in a grid like pattern avoiding the trigone.

Neurogenic patients were treated with 300 units and idiopathic patients received 200 units of Botox® injected at 30 and 20 sites respectively. Prophylactic oral trimethoprim antibiotic were administered prior to injection with two further doses given for the patient to take at home.

Chapter 6 - Laboratory methods

Tissue Retrieval

Baseline and follow-up biopsies were taken by flexible cystoscope using the Olympus 21SX biopsy forceps (Olympus, Tokyo, Japan) from a consistent area 2cm above and lateral to the ureteric orifices, which collected approximately 1mm³ of tissue per biopsy. Follow-up biopsies were obtained during check flexible cystoscopy at 4 and 16 weeks after each treatment session. Identical methodology was used for control subjects.

Three or four biopsies were taken from each subject prior to injection of BoNT/A. One specimen was immediately fixed in Zamboni's solution (buffered picric acid-formaldehyde (Stefanini et al., 1967), while the other two were snap frozen in liquid nitrogen. After two hours, the biopsy in Zamboni's solution was transferred to sucrose based preservative solution (15% sucrose and 0.01% sodium azide as cytoprotectant and preservative) and preserved in 4°C. These biopsies were used for IHC purposes and for histological examination (Apostolidis et al., 2008). The 4th biopsy was fixed in 3% glutaraldehyde or 3% glutaraldehyde + 0.1% PFA solutions and was then used for electron microscopy examination as part of a different project.

Slide mounting

All samples obtained for IHC purposes were embedded in optimum cutting temperature medium (Tissue-Tek®, Sakura USA) and kept frozen at -60°C, until cut with a histology cryostat microtome to 10-12 µm sections and collected on 3-aminopropyltriethoxy-silane (APES) coated Superfrost® slides (VWR, USA). Three serially cut sections from each biopsy were placed on each slide to capture as much of the same level of the biopsy in each receptor staining. Investigators were blinded to the identity of the samples (IDO, NDO, Control, Pre or Post BoNT/A) by assigning a unique code at tissue collection. The study was unblinded following analysis of all slides at the end of the study.

Bladder Immunohistochemistry

This protocol was devised at the Institute of Neurology, Queen Square based on modifying available protocols using antibody staining with diamino-benzidine (DAB) chromogen immuno-localisation from the Hammersmith Hospital.

IHC was extremely labour intensive and required a method development period of 18 months and a budget of approximately £7000 in optimising the appropriate staining protocol in human tissue and confirming specific immunoreactivity in other species. Several experimental trial runs were required to optimise the methodology. The problems encountered were antibody specific staining in human tissue which required testing of several different commercial antibodies (up to 5 different manufacturers) with accurate titration of antibody and using

prefixed as opposed to snap frozen tissue. The other difficulty was in the reduction of background non-specific staining which required a complex series of washes after each stage, reduction of time spent in DAB developer and changes in the blocking serum. Specificity of staining was checked with known animal tissue staining and western blots using the same antibody. The final slides were checked with Dr Alex Freeman, Consultant Pathologist at University College Hospital and presented at departmental meetings.

Each experiment required 3 days of preparation with cutting, preparing tissue, solutions and slides prior to IHC process which required 2 days. Analysis of results took a further week with photographing and quantifying the stained tissue. The method development, experimental and analysis phase took a total of 3 years to complete.

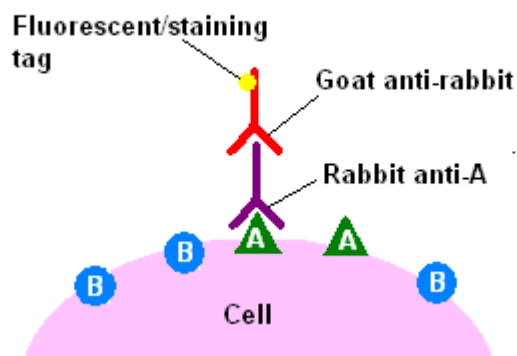


Figure 12: The indirect method of immunohistochemical staining uses one antibody against the antigen being probed for, and a second, labelled antibody against the first. (Diagram created by Viki Male in Wikipedia, 14th September 2005)

An indirect method of immunohistochemical detection of antigens in tissue was used throughout (Figure 12).

This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The secondary antibodies were all biotinylated, exploiting the very strong affinity avidin has for biotin. Once the avidin-biotin complexes had formed, the cut sections were then reacted with 3,3'-Diaminobenzidine (DAB) to produce a brown stain wherever primary and secondary antibodies were attached. This reaction was further enhanced using nickel, producing a deep blue/black stain.

Antibody specificity

Negative controls with no antibody applied were included in each run. Mouse cerebellum and heart specimens were also immunostained for M₁, M₂ and M₃ receptors as positive controls. Since cognates for pre-absorption tests were commercially unavailable and antibody sequences were protected, we performed Western blots on fresh tissue homogenates from mouse cerebellum and heart using additional immunoprecipitation. Immunoblots revealed appropriate major and minor bands for muscarinic receptor antigen.

IHC Protocol

This protocol was used successfully for staining of M₁, M₂, M₃ receptors, SNAP-25 and Neuropeptide Y. All similar groups of slides were stained together; in batches of between 50 and 80 slides. The critical differences in technique for the different antigens stained were the primary antibody used and time required for final developing.

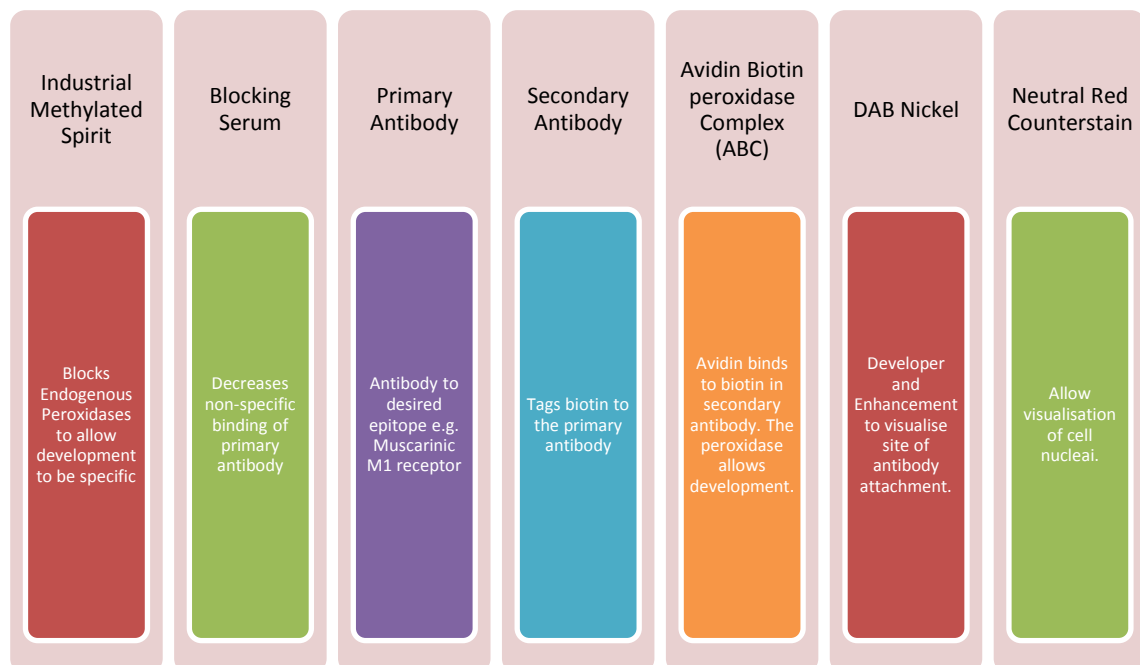
M₂ receptor staining was only successful with snap frozen tissue, and this was fixed in 4% Paraformaldehyde solution for 30 minutes, followed by 3 washings in Phosphate Buffered Saline (PBS, Oxoid®, Dulbecco 'A' Tablets U.K.) for 5 minutes. Prefixed tissue (Zamboni's fixed specimens) was used in M₁, M₃, NPY and SNAP-25 staining and did not require the initial paraformaldehyde step. Sections were then transferred to 0.3% hydrogen peroxide in Industrial Methylated Spirit (IMS, 90% Ethanol and 10% Methanol) for 30 minutes to inhibit endogenous peroxidases. Following three further washes with PBS as described above, blocking serum was applied for 30 minutes. This was followed by another series of PBS washes and the application of the primary antibody which was incubated overnight at room temperature.

Table 4 : Blocking sera, primary and secondary antibodies used in IHC protocol.

Primary Antibody	Manufacturer / Antibody	Blocking Serum	Immunogen	Secondary antibody
Rabbit polyclonal to muscarinic Acetylcholine Receptor 1 1:1000 concentration	Abcam ab3480	1/30 Normal Goat Serum with 1% BSA	Fusion protein containing amino acids 269-320 from human m1 AChR	Biotinylated Anti-Rabbit 5 µl/ml
Rabbit polyclonal to muscarinic Acetylcholine Receptor 2 1:500 concentration	Affinity Bioreagents PA1-23537	1/30 Normal Goat Serum with 1% BSA	3rd cytoplasmic domain of human Muscarinic Acetylcholine Receptor M2	Biotinylated Anti-Rabbit 5 µl/ml
Rabbit polyclonal to Muscarinic Acetylcholine Receptor 3 1:500 Concentration	Abcam ab13063	1/30 Normal Goat Serum with 1% BSA	2nd cytoplasmic loop of human synthetic peptide	Biotinylated Anti-Rabbit 5 µl/ml
Mouse monoclonal to SNAP-25 1:2000 concentration	Abcam ab24737	1/30 Normal Horse Serum with 1% BSA	Full length protein	Biotinylated Anti-Mouse 10 µl/ml
Rabbit polyclonal to Neuropeptide Y 1:5000 concentration	Abcam ab10980	1/30 Normal Goat Serum with 1% BSA	Full length Neuropeptide Y	Biotinylated Anti-Rabbit 5 µl/ml

On the following day after washing with PBS, slides were incubated in corresponding secondary antibodies (Vector Laboratories, USA) and made up in blocking serum for 45 minutes. This was followed by amplification and development of the signal using the avidin-biotin complex (ABC) method together with DAB (Shu et al., 1988). The slides were then left in ABC mixture (5µl/ml solution A, 5µl / ml solution B in 1% BSA and PBS, VECTASTAIN® Elite ABC reagent, Vectorlabs, USA) for 30 minutes, followed by further washes with PBS (as above). A proportion of the avidin is also bound to biotinylated peroxidase and to biotinylated secondary antibody; this is crucial to the final step of development where the peroxidase is developed by DAB-nickel.

Figure 13: Summary of bladder immunohistochemistry protocol



One trough (sufficient for 25 slides) of developing solution was produced by adding 40ml of 1 M Sodium Acetate (acidified with glacial acetic acid to pH 6.0) solution diluted in 360 ml distilled water. To this solution, 10g of Nickel ammonium sulphate powder was added. Further to this, 400mg of D-glucose was added with 80mg of Ammonium Chloride. At the final stage, 100 mg DAB was added together with 4mg of glucose oxidase to catalyse the reaction. The DAB-nickel solution in the presence of peroxidase from the ABC converts to a blue/black stain which delineates the area where the primary antibody has bound. The amount of time spent in the developing solution was highly variable depending on concentration and type of antibody used. Development was stopped when an adequate signal to noise ratio was achieved. This was decided by comparing the amount of cellular or nerve fibre IR to background IR.

Table 5: Time spent in DAB-nickel developer by primary antibody

Antibody	Time in DAB-nickel developer.
M1	5 minutes
M2	5 minutes
M3	8 minutes
SNAP-25	4 minutes
NPY	5 minutes

Following development, slides were washed in distilled water and placed in increasing concentrations of alcohol (50%, 70%, 90% and 100%) and finally xylene to achieve dehydration. The slides were incubated in a counter-stain of neutral red (0.1%, 0.4g in 400ml with 2 drops of glacial acetic acid) for 20 seconds and washed with distilled water (x2 for 5 minutes) to label nuclei and allowing identification of the urothelium and sub-urothelium. Slides were then air dried and cover-slipped with Depex® (Gurr, England) slide mounting media, prior to quantification.

Positive and negative controls

Specificity of the immunostaining was confirmed by the use of both negative and positive controls. In each run, two random sections were selected where instead of primary Ab, only the blocking serum was added, in order to serve as negative controls. For external positive controls mouse cerebellum and heart tissue were used, the former are abundant in M1 and M3 receptors and the latter in M2 receptors. In addition, human detrusor muscle containing biopsies from cystectomy specimens were used as internal positive controls.

Image capture

Slides were analysed on a light microscope (Axioskop 40, Zeiss Germany) with images captured by a digital imaging system (Cell D, Olympus UK) based on calibrated 5 megapixel images stored at 2576 x 1932 pixels. Between 3 and 20 digital images taken at x20 high power magnification field were taken per

subject slide. The coding on the slides allowed complete blinding for the microscope operator as to the diagnosis of the subject and the time-point at which the biopsy was taken.

Computer Quantification

All slides were analysed by using Cell D image quantification software (Olympus, Tokyo, Japan), which allowed threshold to be set around the DAB-nickel blue-black chromogen. A filter was then set, capturing the blue-black elements in the HPF. Holes or tears in the tissue were excluded either by cropping the image or the computer detecting areas corresponding to artefacts and removing this from the total image area. Areas detected as artefact or analysable tissues were checked by the author. The software allowed analysis of every image taken and determined the stained percentage per HPF. This same filter was then applied to all the sections in that staining run. Each image was checked for “real” staining and any artefacts were excluded. The quantification results were exported to a spread sheet (Excel 2007, Microsoft Seattle, WA USA) where all the results from each individual subject were then combined. A mean percentage and standard error of the mean (SEM) were then calculated. This was transferred to a database (Access 2007, Microsoft Seattle, WA USA) where the results could be unblinded and analysed for differences between groups.

Quality control

Semi quantification

During analysis of the M₁ receptor slides, a blinded visual grading system of scoring (0-3) was adopted according to the numbers of suburothelial nerve fibres displaying M₁ receptor staining. This went up in increments of 0.5, each increment being equivalent to up to 5 additional immunoreactive fibres per section. These results were then compared to results obtained by computer quantification and showed good correlation and validity. This method had been successfully used in earlier studies, as a semi-qualitative measure (Apostolidis et al., 2005a; Apostolidis et al., 2005b).

Blinded repeatability analysis

To ensure computer quantification was accurate we devised a repeatability test using three different subjects' bladder biopsies. Four slides containing serial sections from each subject were used to determine the mean staining density. These slides were blinded from the investigator and randomised. These 12 slides were stained for SNAP-25 and computer quantified. Once the analysis of staining density was complete, the slides were unblinded and each individual slide correlated to the population mean (combination of three slides) for the subject. The calculated correlation co-efficient was 0.756, indicating validity of the computer quantification process.

Statistical Methodology

For clinical urodynamic results we used one way repeated measure ANOVA with Bonferroni's multiple comparison test correction.

We applied the same statistical plan consistently to our IHC M₁, M₂, M₃, NPY, SNAP-25 and urothelial M₁ / M₃ analyses. Two tests were used for the bulk of all analyses. All datasets were skewed and did not normalise using logarithmic transformations, consequently nonparametric tests were used.

Comparing control and baseline DO patients, we used a non-parametric test (Mann Whitney) to statistically show if groups were different.

To evaluate for a treatment effect of BoNT/A, we used a non parametric analysis of variance test ("Kruskal-Wallis test"). This prevents bias from repeat testing. Only when this was significant did we perform a post-test (Dunn's multiple comparison test) for comparison between the three groups (baseline, 4 weeks, 16 weeks).

Statistical advice and planning for this project was in conjunction with Dr Aviva Petrie, Head of Biostatistics Unit and Senior Lecturer at UCL. Specialist statistical analytical software was used (GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

III - RESULTS

From the 36 DO patients involved in the study, only a proportion of biopsies were available for staining and analysis for each antigen (Table 6). There were a total of 9 subjects control available for control. Complete biopsies sets were not available for analysis for the following reasons; patient discomfort, infection, loss of tissue on slide, inadequate fixation, tearing of biopsy, folding of specimen on slide and small size inadequate for analysis. To account for incomplete sets, unpaired analyses were performed. The M3 group in particular had few paired biopsies. No duplication of biopsies occurred within each studied antigen group.

Table 6: Total number of patients in each part of the study and the number of biopsies available for analysis at each time point.

Studied antigen	Control Biopsies	Total number of DO Patients	Baseline DO Biopsies	Post – BoNT/A 4 week DO Biopsies	Post – BoNT/A 16 week DO Biopsies
M1	6	28	24	18	16
M2	7	36	19	15	17
M3	5	34	15	11	9
SNAP-25	8	32	27	21	17
NPY	9	31	21	16	14

Chapter 7 - Clinical Urodynamic results

Of the 36 patients, 35 patients had evaluable traces at all stages of the study. One patient had infection prior to follow-up cystometry and did not have a full set of results. There were 13 men and 23 women, with 17 having IDO and 19 having NDO.

Table 7 summaries the cystometry, bladder diary and QOL results from the patients at baseline, 4 weeks and 16 weeks after intradetrusor BoNT/A. All patients in this cohort achieved significant improvements.

Highly significant mean improvements were noted in urodynamic parameters and symptoms assessed by bladder diaries in both the NDO and IDO patients and at both follow-ups. Symptomatically and objectively there were considerable improvements in patient symptoms of frequency, urgency and urgency incontinence both at 4 and 16 weeks after BoNT/A injection. Quality of life improved greatly in both patient groups. One way repeated measure ANOVA with Bonferroni's Multiple Comparison Test Correction was used to determine significance. Efficacy was maintained when patients on antimuscarinic medication were analysed as a separate group.

Table 7: Summary of urodynamic and clinical results from the patients who provided bladder tissue for the purposes of this study.

		Pre	4/52	16/52	P Value Pre vs. 4/52	P Value Pre vs. 16/52
MCC (ml)	IDO	225.6 ± 27.8	363.9 ± 41.8	304.9 ± 36.5	0.0008	<0.05
	NDO	216.2 ± 33.3	533.8 ± 39.0	425.4 ± 37.1	< 0.0001	< 0.0001
Pdet (cmH20)	IDO	66.5 ± 11.5	36.1 ± 7.7	49.6 ± 7.8	0.0001	0.0001
	NDO	65.8 ± 8.0	28.8 ± 4.4	26.5 ± 3.7	< 0.0001	< 0.0001
Frequency	IDO	13.4 ± 1.2	7.1 ± 1.2	7.5 ± 0.8	< 0.0001	< 0.0001
	NDO	12.3 ± 1.1	5.3 ± 1.1	6.2 ± 0.4	< 0.0001	< 0.0001
Leak	IDO	5.4± 1.0	0.3 ± 0.1	0.6 ± 0.3	< 0.0001	< 0.0001
	NDO	4.0 ± 0.7	0.3 ± 0.7	0.9 ± 0.2	< 0.0001	< 0.0001
Urgency	IDO	11.1 ± 1.7	4.3 ± 0.9	5.1 ± 1.2	0.0002	<0.01
	NDO	7.6 ± 1.0	1.3 ± 0.3	1.4 ± 0.3	< 0.0001	< 0.0001
QOL	IDO	116.0 ± 7.8	91.5 ± 7.8	84.8 ± 7.2	<0.05	<0.0026
	NDO	121.4 ± 6.4	77.9 ± 5.1	84.1 ± 5.1	< 0.0001	< 0.0001

MCC: maximum cystometric capacity, Pdet: maximum detrusor pressure during filling, Urgency: number of micturition episodes per 24 hours associated with urgency, Leak: number of urgency incontinence episodes per 24 hours, Frequency: total number of micturition episodes per 24 hours, QoL: quality of life scores assessed with the ICS-BPH questionnaire.

Chapter 8 - Muscarinic Receptor 1

A total of 28 patients' and 6 control subjects' biopsies were stained with M₁ receptor antibody. There were six controls (mean age 58.3 years, range 30.0 – 75.4 years), 40 NDO biopsies (mean age 47.3 years, range 29.2 – 66.3 years) and 18 IDO biopsies (mean age 45.9 years, range 27.3 – 60.7 years). Over 581 HPF images were analysed. Mean percentage of M₁ staining per x20 High Power Field (HPF) values were taken from each slide. Staining patterns showed the presence of M₁ receptors in the urothelium and sub-urothelium (Figure 14 & Figure 15) with a higher power image (x40) in Figure 16.

A positive control slide showed positive cellular M₁ immunoreactivity in mouse cerebellum (Figure 17).

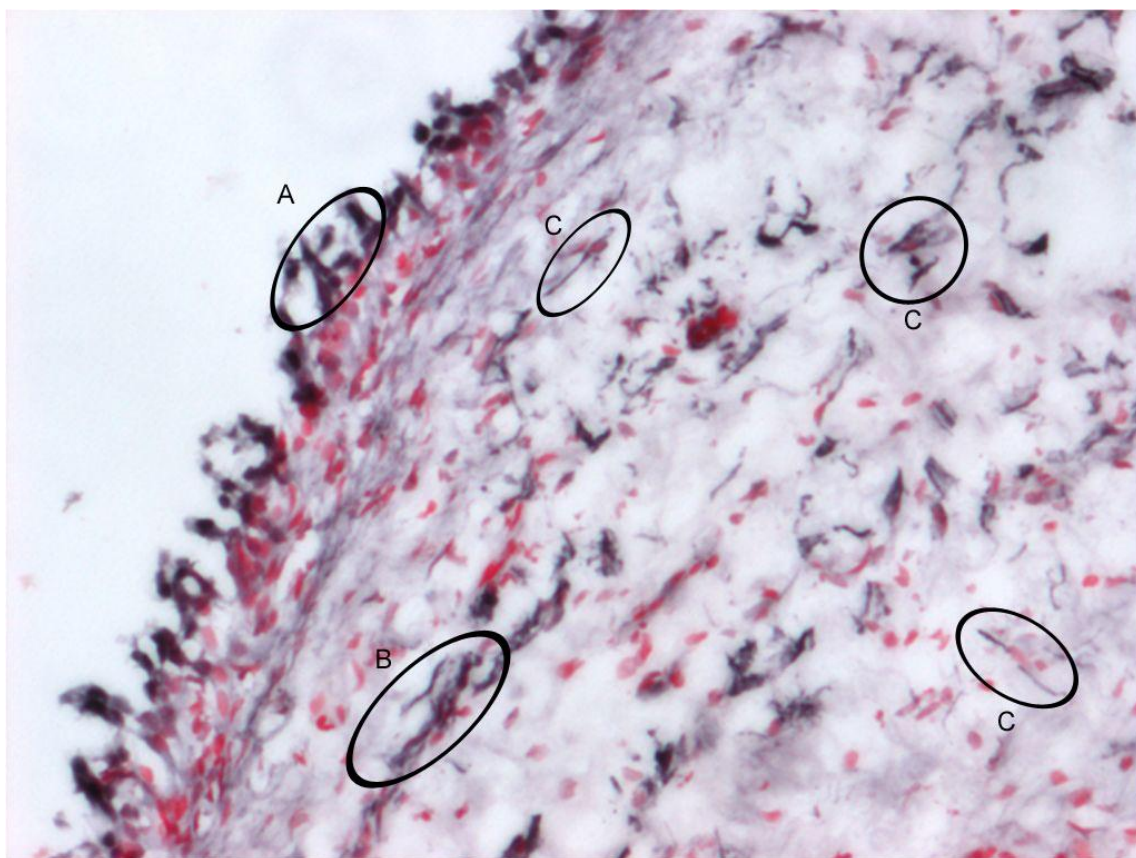


Figure 14: M1 immunostained slide showing apical urothelial immunostaining (A), staining of fibre like structures (B) and cellular staining in the form of either cytoplasmic immunostaining or that of bipolar cellular processes (C).

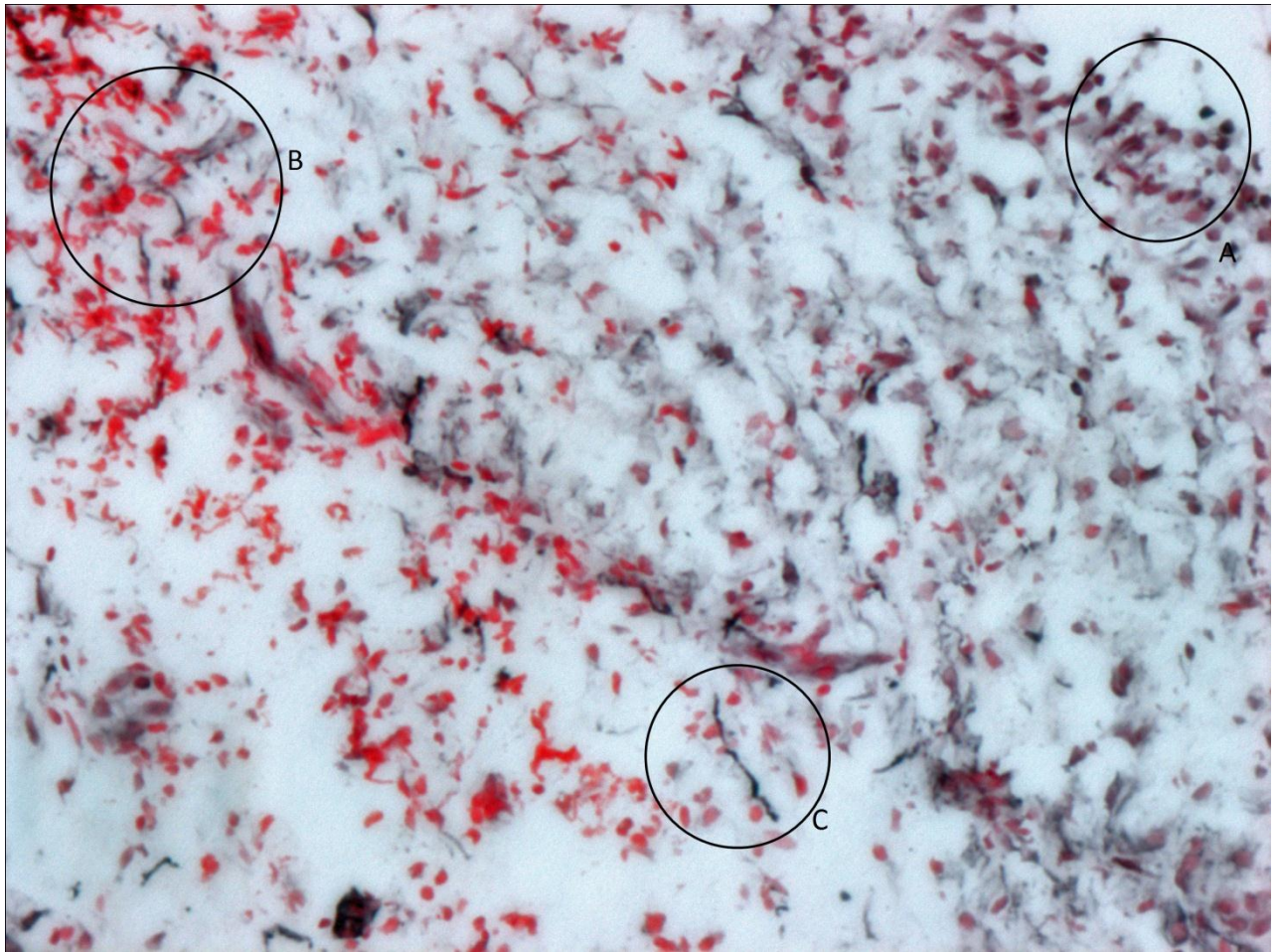


Figure 15: M1 Immunostaining in the suburothelial layer. The urothelium which also shows staining (A) is present at the edge. Multiple fibre like structures (B) and bipolar cellular processes (C) are also seen.

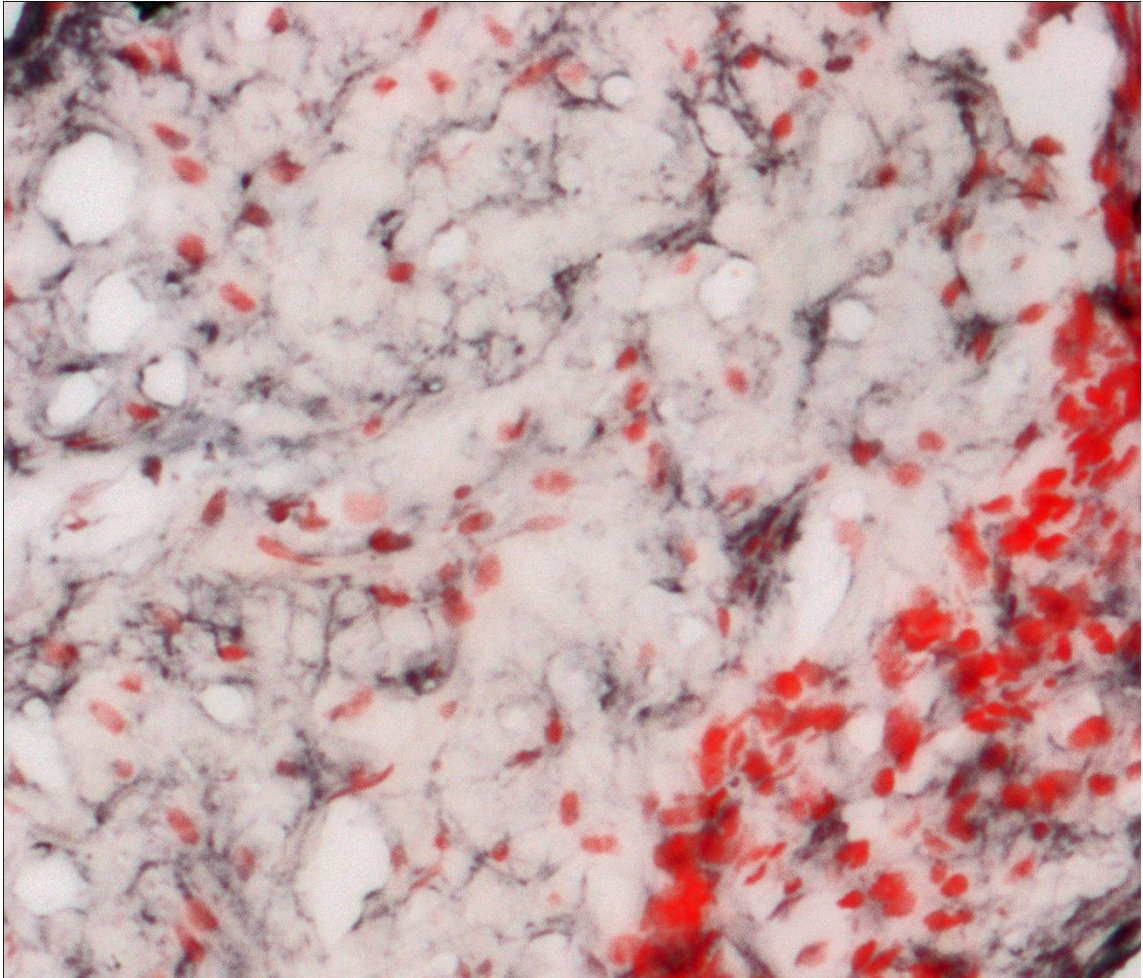


Figure 16: M1 Suburothelium; Higher power view (x40) of fine fibre like projections in the suburothelium.

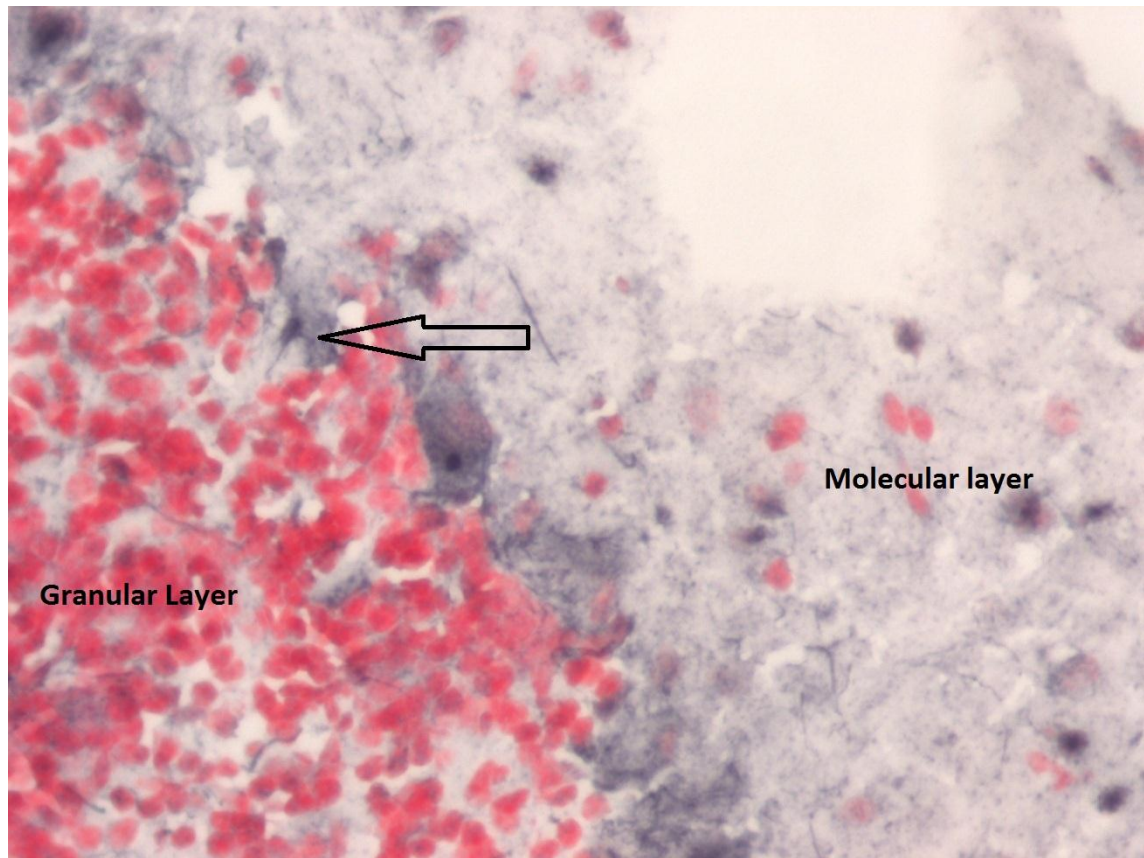


Figure 17: M₁ receptor cellular immunoreactivity present on Purkinje cells (arrow) and in the molecular layer of the mouse cerebellum (Positive Control)

There was observed presence of M₁ immunoreactivity in the apical layer of the urothelium (Figure 14). No M₁ labelled nerves were noted to be present within the urothelium. In the sub-urothelial layer there was abundant immunostaining of fibre like structures (Figure 15). There was also evidence of immunostaining on cells with bipolar processes. Negative controls showed no staining (Figure 18).

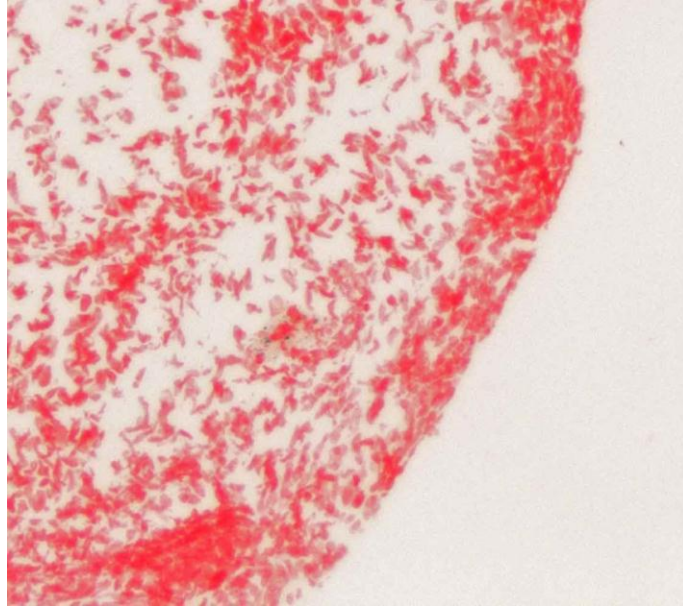


Figure 18: Negative control slide in M₁ receptor staining protocol

The results and statistical analysis on the suburothelial M₁ receptors are shown below. There was a significant mean reduction (Figure 19) in M₁ suburothelial IR (immunoreactivity) from 1.71% in the control group to 0.63% in the IDO subjects and 0.88% in the NDO subjects ($p=0.014$ and 0.036 respectively).

Following BoNT/A, there was a significant increase (Figure 20) in M₁ suburothelial staining from 0.794% to 1.61% (4 weeks) and 1.73% (16 weeks) ($p=0.036$ and 0.019 respectively), with a return to control value levels.

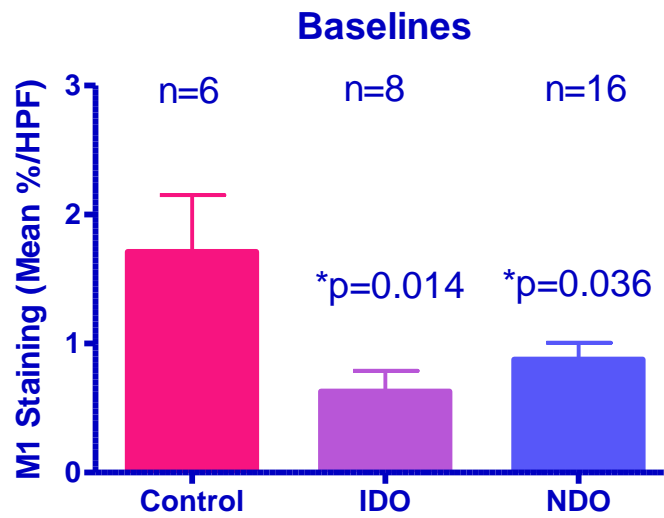


Figure 19: Statistically significant difference in suburothelial M1 immunostaining between Controls and Baseline IDO and NDO subjects

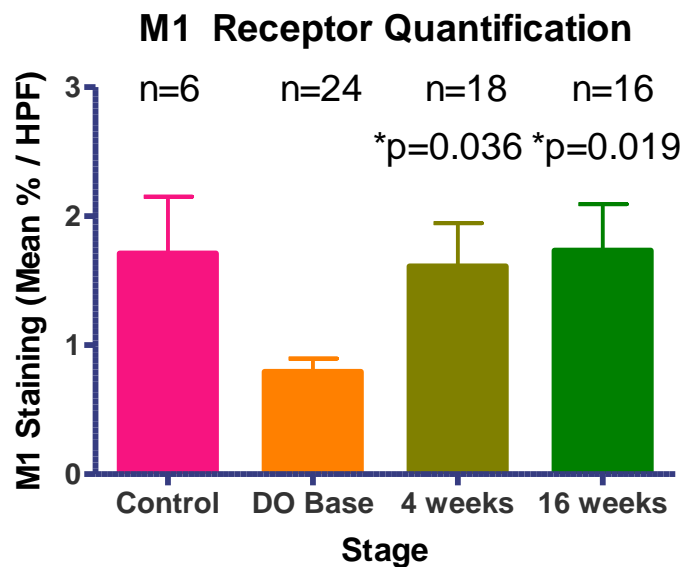


Figure 20: Normalisation of M1 suburothelial immunostaining following application of BoNT/A at 4 and 16 weeks

In the urothelium, there were fewer biopsies for analysis (controls=6, baseline DO=14, 4 weeks=13, 16 weeks=9), but there was a similar marked decrease (Figure 21) in M₁ IR in patients with DO at baseline, when compared to controls (3.2% vs. 19.5%). M1 receptor IR statistically increased 16 weeks after BoNT/A injection to 8.2% (significance not noted at 4 weeks).

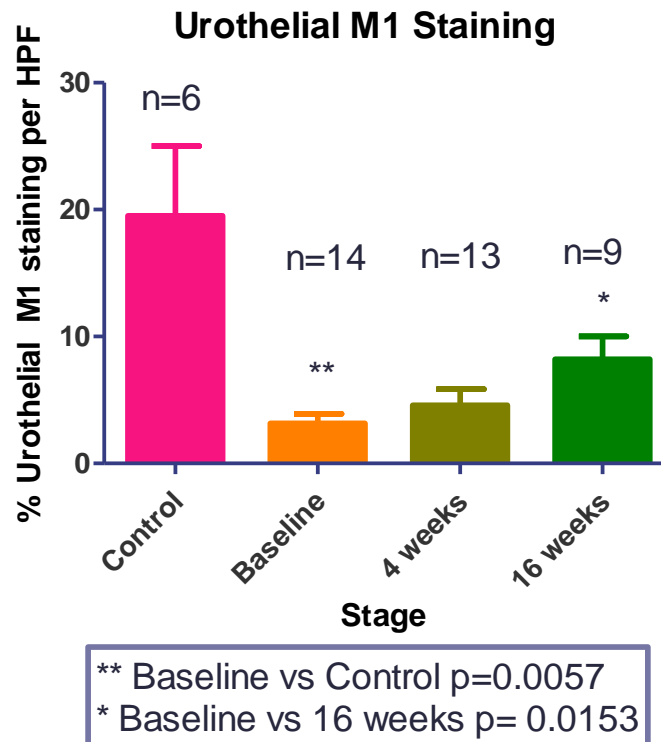


Figure 21: Urothelial M1 receptor staining. Statistical significant difference between Control (Mean 19.5%) and Baseline (3.2%) DO patients. Following BoNT/A, there is a significant increase in urothelial staining at 16 weeks (8.2%)

Chapter 9 - Muscarinic Receptor 2

41 subject slides were stained with antibody for the Muscarinic M₂ receptor (controls =7, IDO =10, NDO = 24). 19 biopsies were obtained from baseline DO patients (Mean 43.0 years, Range 19.6 - 61.6 years), 15 biopsies (Mean 44.0 years, Range 19.6 - 61.6 years) from patients who had 4 week follow-up after BoNT/A injection, and 17 biopsies from patients (Mean 46.1 years, Range 19.6 - 60.5 years) who had 16 week follow-up biopsies.

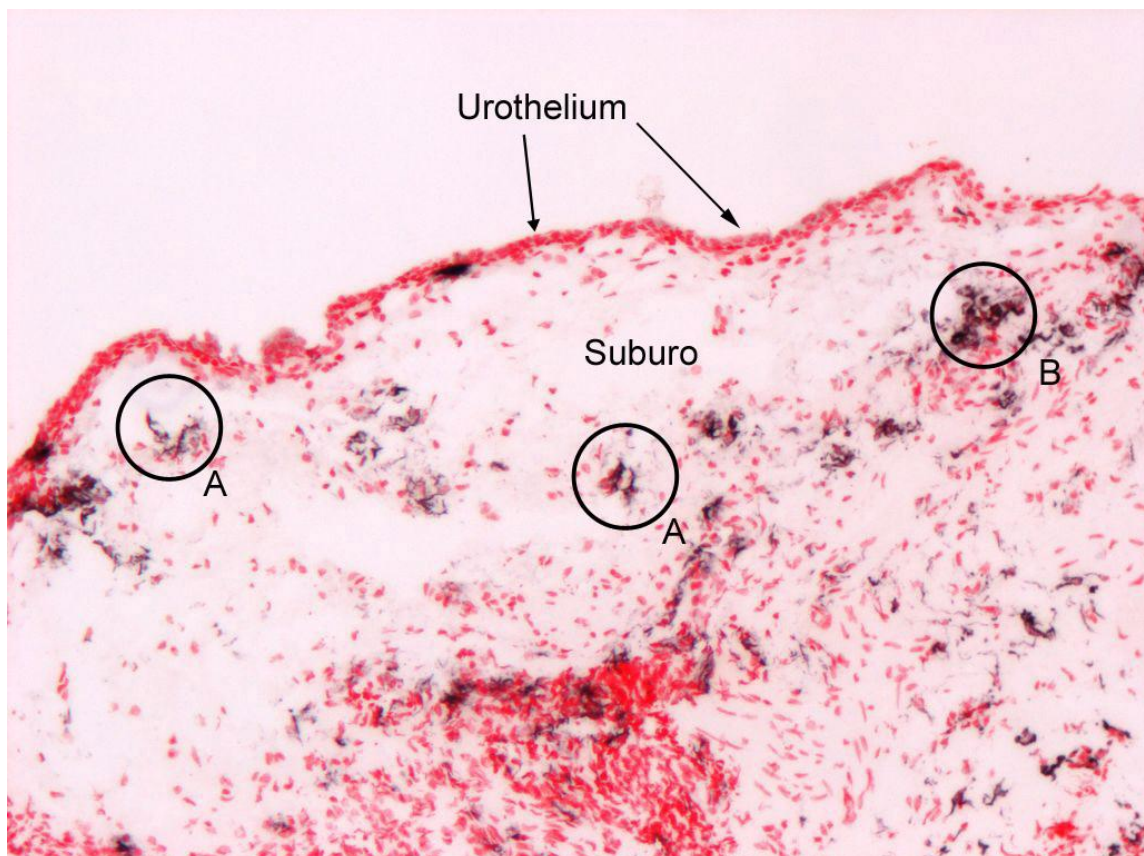


Figure 22: M2 Receptor staining showing fibre like neuronal staining (A) and cellular staining from cytoplasmic immunoreactivity or from cellular processes (B) in the suburothelial layer (x 20 HPF). Clusters of suburothelial M2 staining were noted.

M₂ receptor staining showed no background staining (Figure 22 and Figure 23).

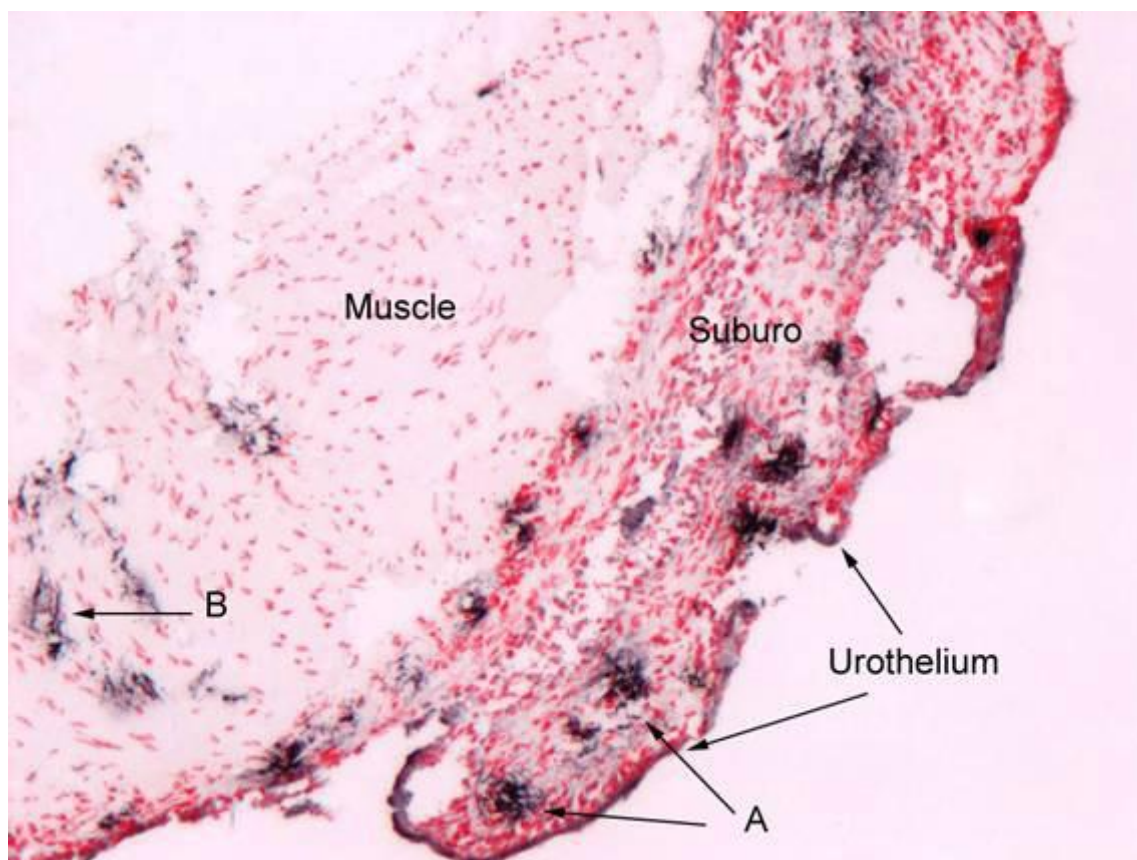


Figure 23: M2 Staining predominantly in sub-urothelial layer. This section has muscle present also. Clusters of M2 fibre like staining (A) present in a layer in the sub-urothelium. Staining of fibres within muscle was also demonstrated (B) (Image Taken at x10 HPF).

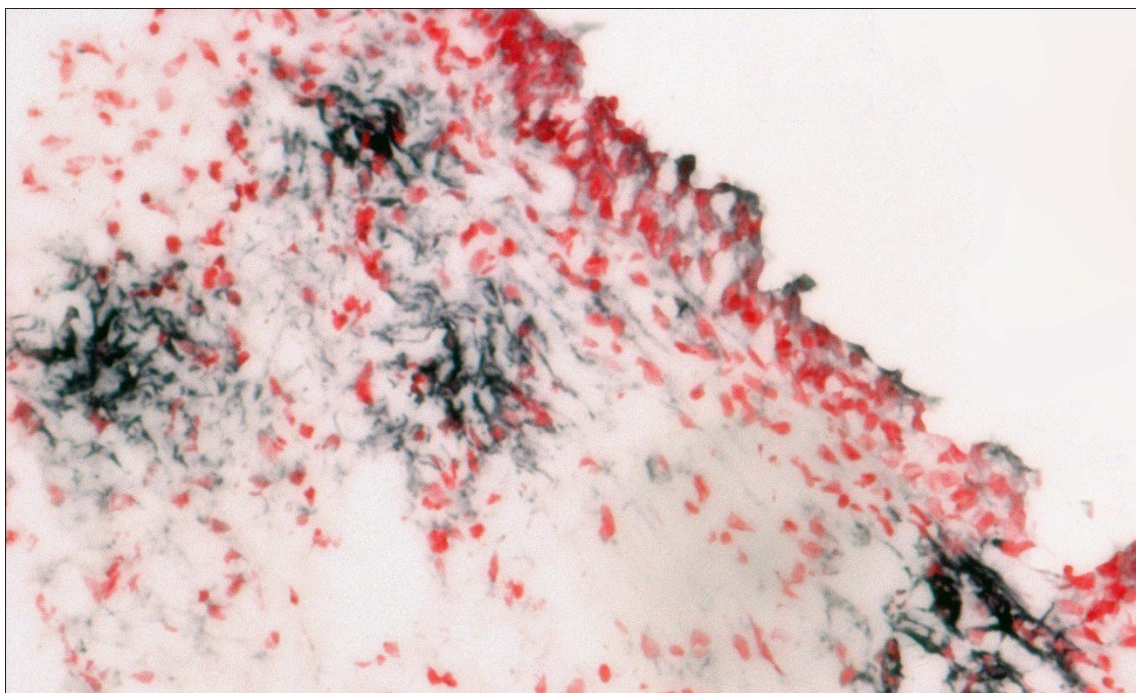


Figure 24: High power view (x20) of cluster of M2 fibres in the suburothelium.

Immunostaining was confined mainly to the sub-urothelial layer with little IR in the urothelium (Figure 23 and Figure 24). IR distribution highlighted fibre like structures as well as cellular staining in discrete clusters in the suburothelium (Figure 24). A few specimens had muscle tissue within the biopsy, and these showed fibre neuronal staining (Figure 23). Negative controls showed no DAB chromogen staining, whereas mouse heart tissue showed positive IR (Figure 25).

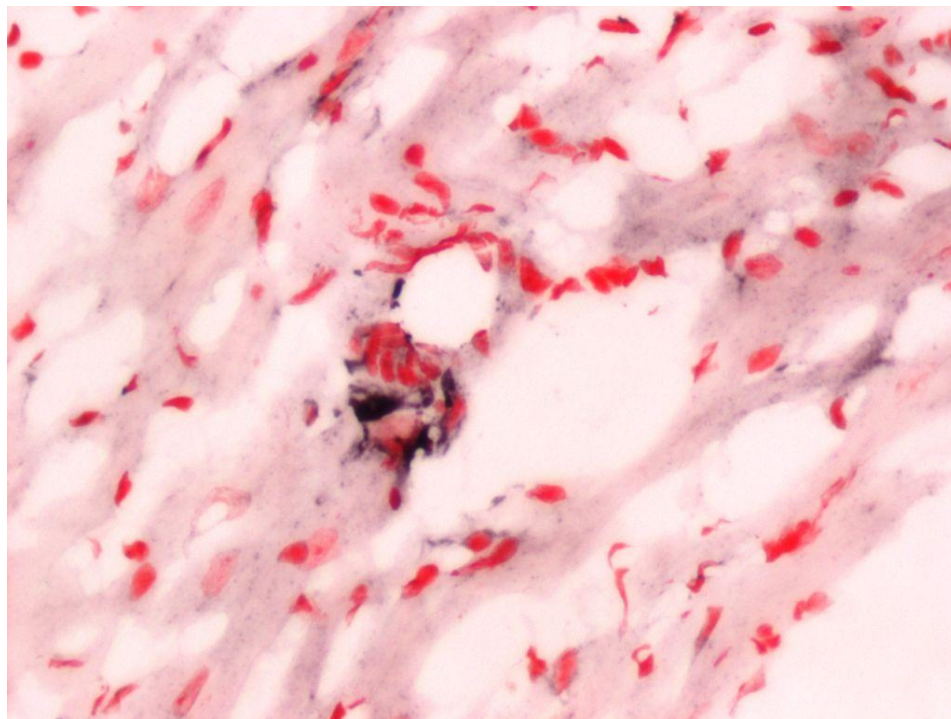


Figure 25: Positive control mouse heart tissue showing M2 immunoreactivity. These receptors have been shown to be present on mammalian heart tissue (Sharma et al., 1997)

A total of 494 HPFs with urothelium and suburothelium were taken from the 59 slides and analysed based on DAB-nickel chromogen. There was no statistical difference found in M₂ immunoreactivity density between controls and patients with IDO or NDO (Figure 26), but there was a decreasing trend.

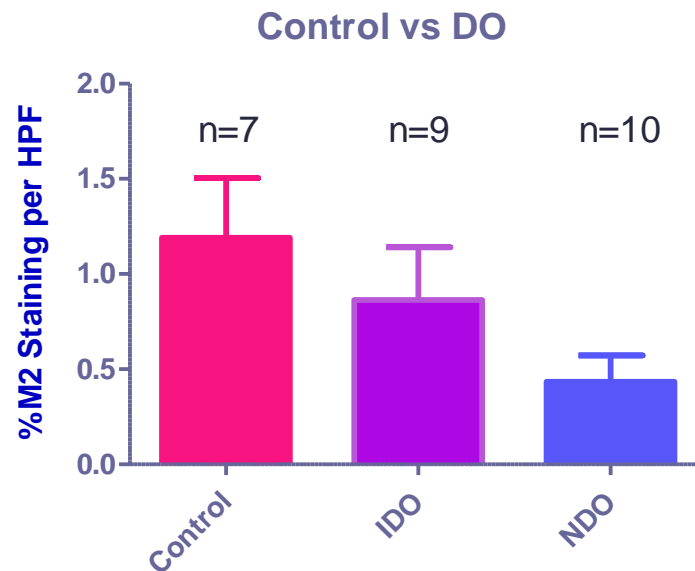


Figure 26: No difference between control and baseline DO M2 immunoreactivity

Following BoNT/A administration, there was a significant increase in mean M₂ receptor suburothelial IR from baseline (0.74%) to 4 weeks (1.62%) and 16 weeks (1.35%) (p=0.013 and 0.018 respectively) (Figure 27).

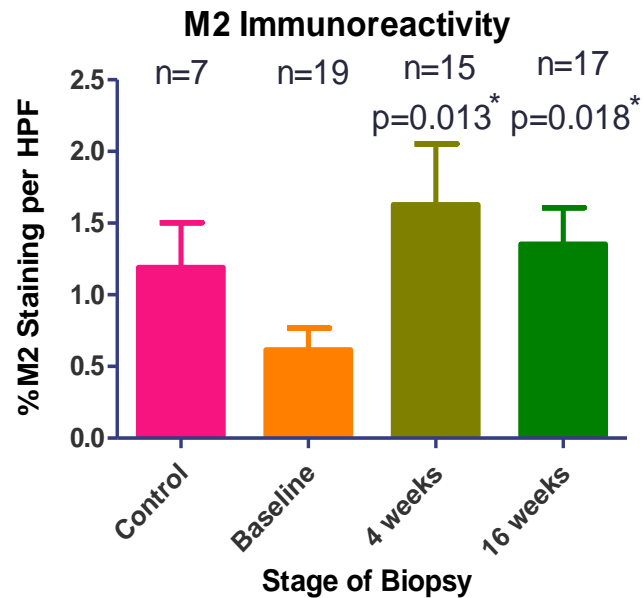


Figure 27: Significant increase in M2 receptor immunoreactivity at 4 and 16 weeks after BoNT/A.

A subgroup analysis showed there was an increase in the NDO group after BoNT/A, but not in the IDO population. This could have been due to smaller number of specimens when performing a subgroup analysis (Figure 28 and Figure 29). The timing of change after BoNT/A was different with NDO cohort responding earlier than the IDO cohort.

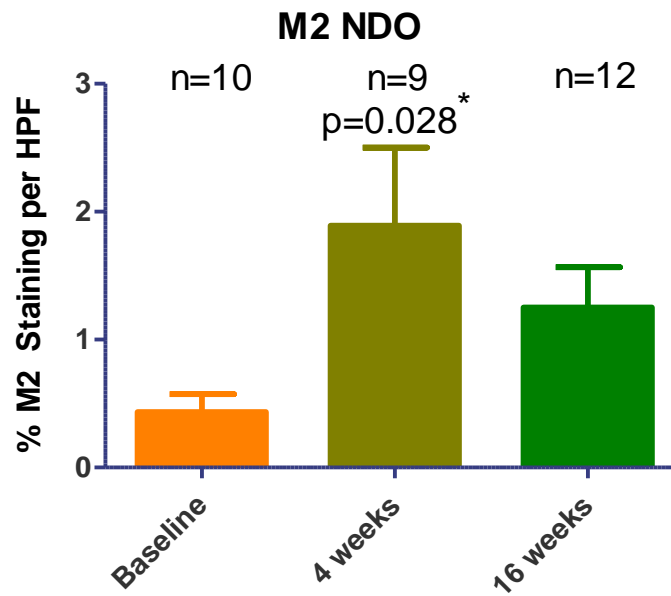


Figure 28: NDO cohort showing an increase in M2 receptor IR after 4 weeks following BoNT/A (p=0.028).

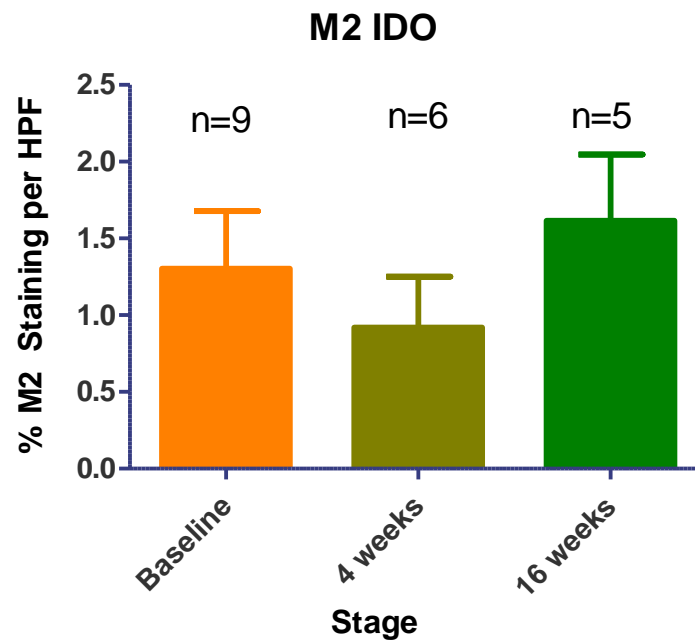


Figure 29: IDO sub-group showing no significant change in M2 IR after BoNT/A

Chapter 10 - Muscarinic Receptor 3

41 patients' biopsies (controls=5, IDO =17, NDO= 19) were stained for suburothelial and urothelial M3 receptors. Of 47 slides, 9 slides were excluded due to poor quality of sections which were unsuitable for analysis. There were five controls (mean 48.9, range 32.4 – 72.0 years), 13 baseline DO biopsies (mean age 46.1, range 29.2 – 61.6 years), eleven 4-week follow-up biopsies (mean age 43.8, range 21.1 – 60.7 years) and nine 16-week follow-up biopsies (mean age 46.5, range 27.3 – 61.6 years).

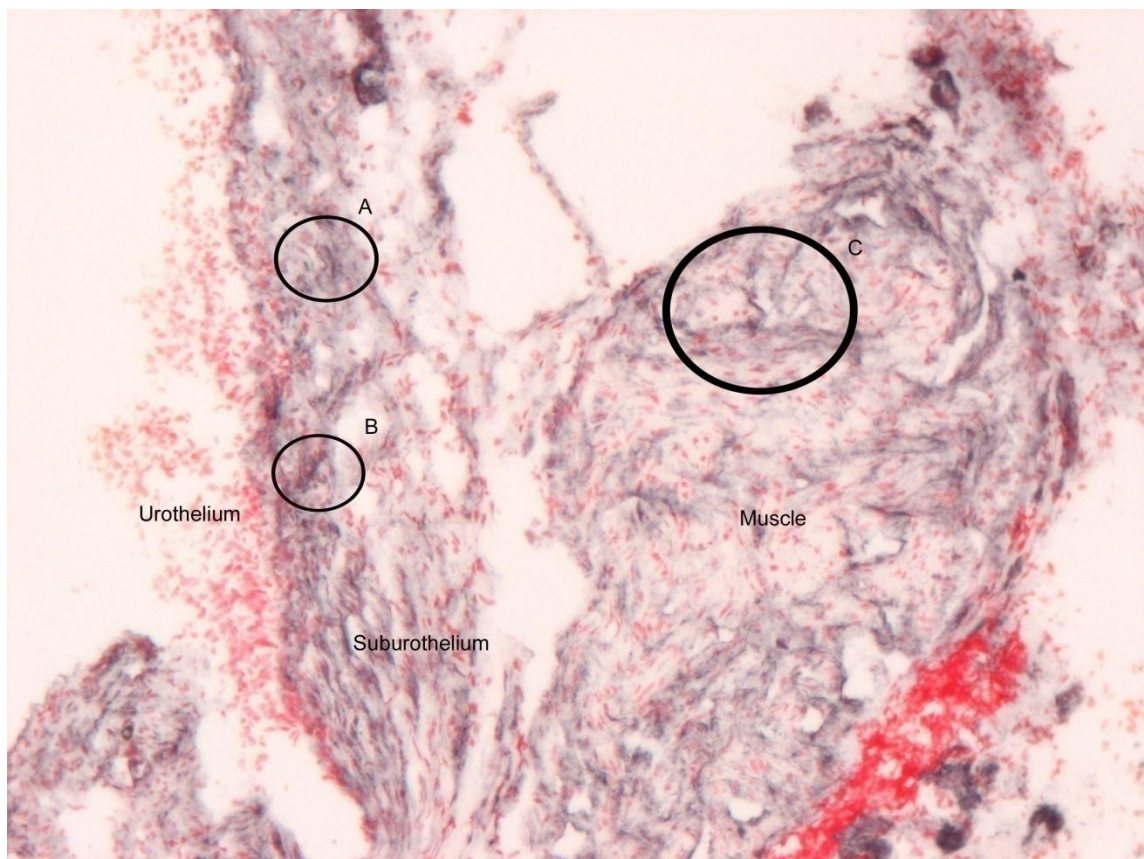


Figure 30: M3 receptor immunostaining in suburothelium showing neuronal staining (A) and cell body staining (B). Neuronal and cell body staining is also noted in the detrusor muscle layer (C) x 10 HPF. The urothelium here is not completely intact.

M₃ receptor immunoreactivity was present in the apical layer of the urothelium, suburothelium, detrusor muscle layer and blood vessel endothelial cells (Figure 30 and Figure 31).

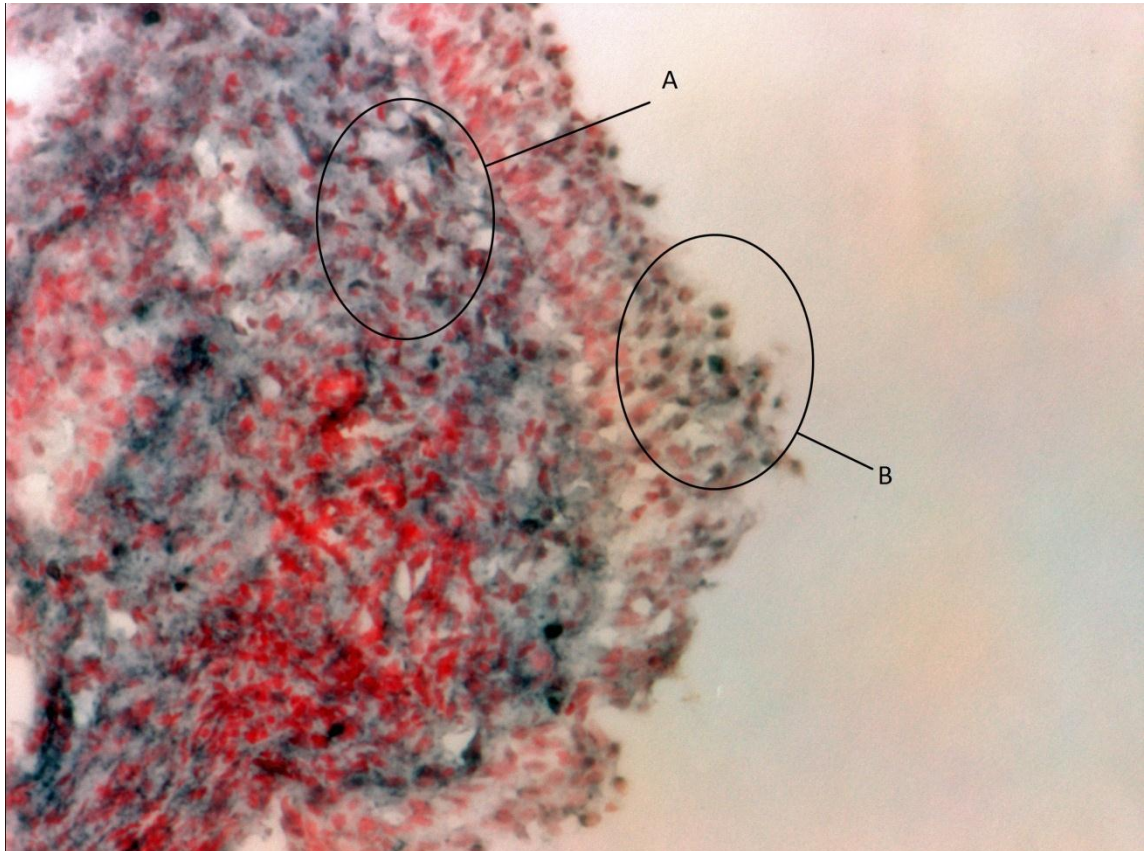


Figure 31: M3 Immunoreactivity in the urothelium and suburothelial layer. Cellular staining is seen in the suburothelium (A). The apical urothelium is also immunoreactive (B).

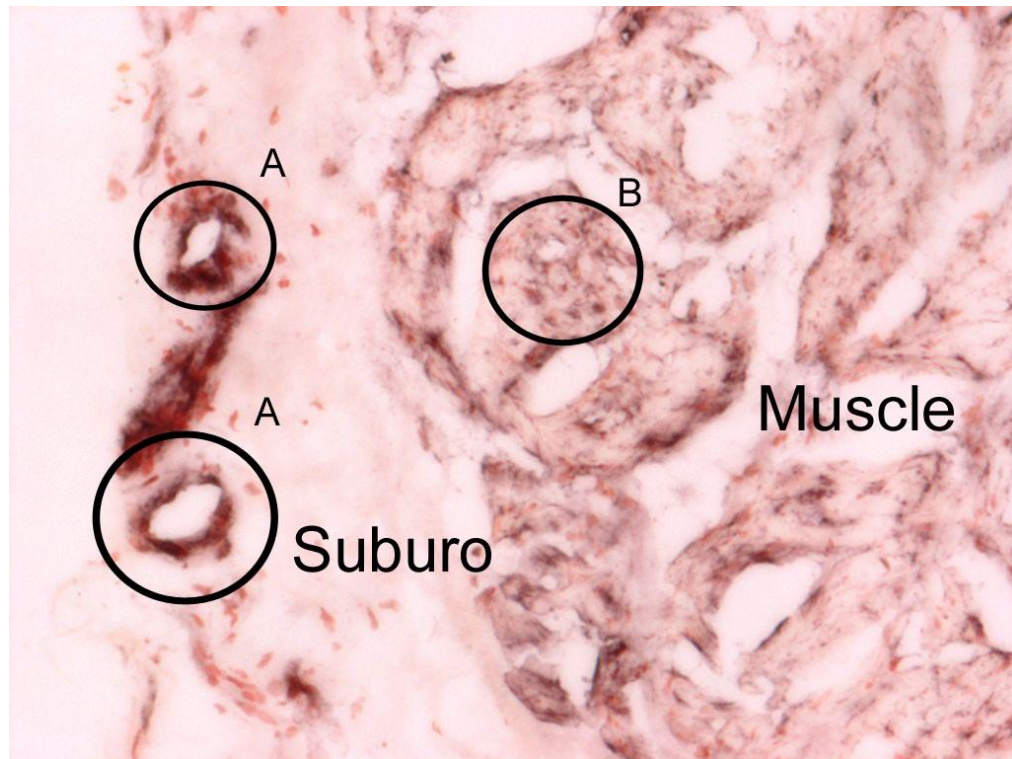


Figure 32: M3 immunoreactivity present in suburothelial endothelial cell (A) and cellular staining within the muscle layer (B) x20 HPF.

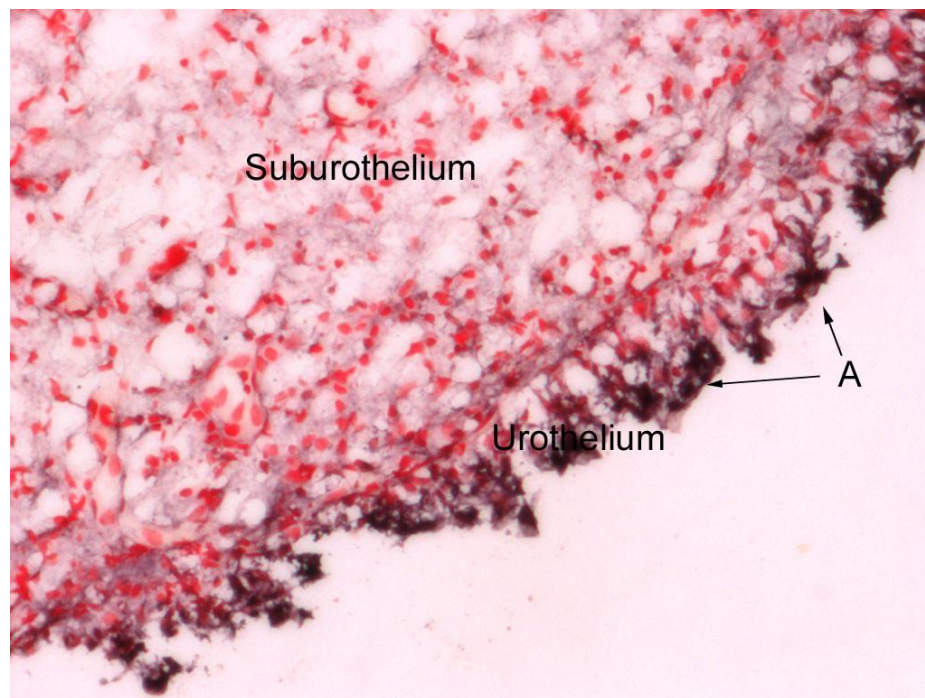


Figure 33: M3 receptor staining in the apical layer of the urothelium (A) (x20 HPF).

Positive controls from mouse species were used to ensure antibody specificity (Figure 34 and Figure 35).

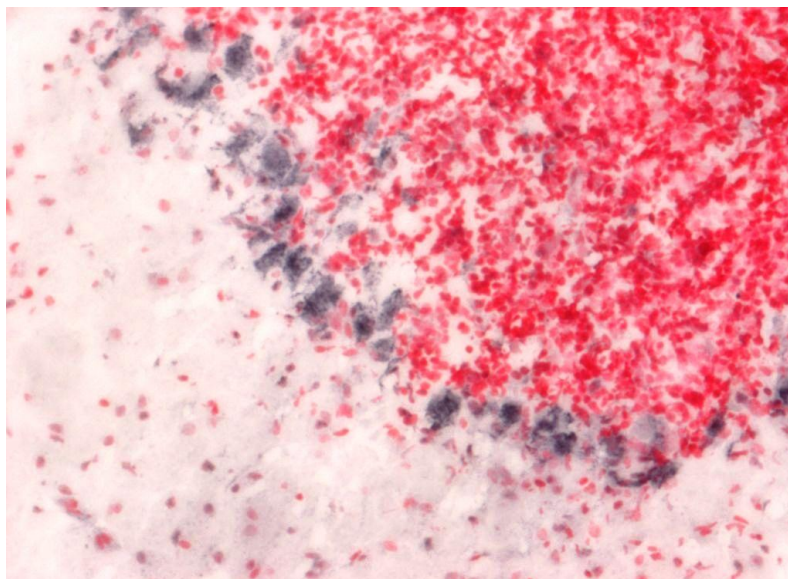


Figure 34: Mouse cerebellum showing positive M3 staining of the Purkinje cells (x10 image)

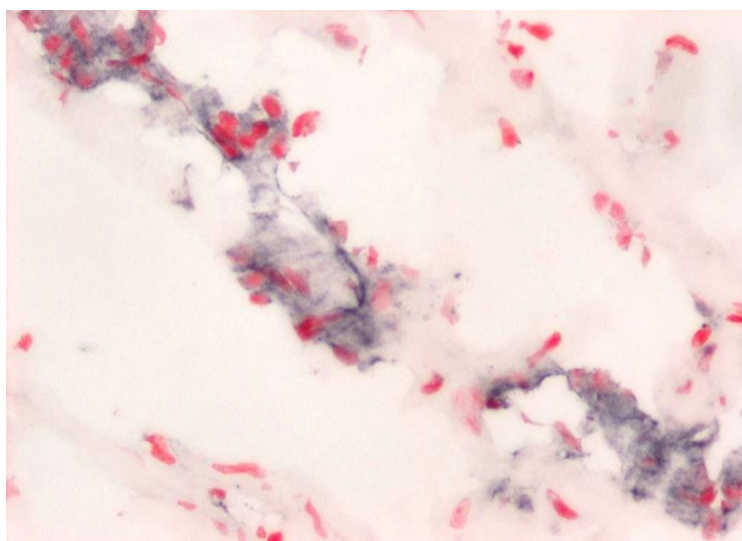


Figure 35: Mouse heart muscle demonstrating M3 immunoreactivity (x40 image)

A total of 332 HPF images of urothelium and suburothelium were taken from the 47 slides and analysed using computer image quantification based on DAB-nickel chromogen. There were differences in suburothelial M3 receptor IR between control subjects (5.97%) and baseline DO specimens (1.62%) ($p=0.015$) (Figure 37). Subgroup analysis showed these differences were present in the baseline NDO and IDO populations (Figure 36, both groups, $p=0.03$), when compared to controls.

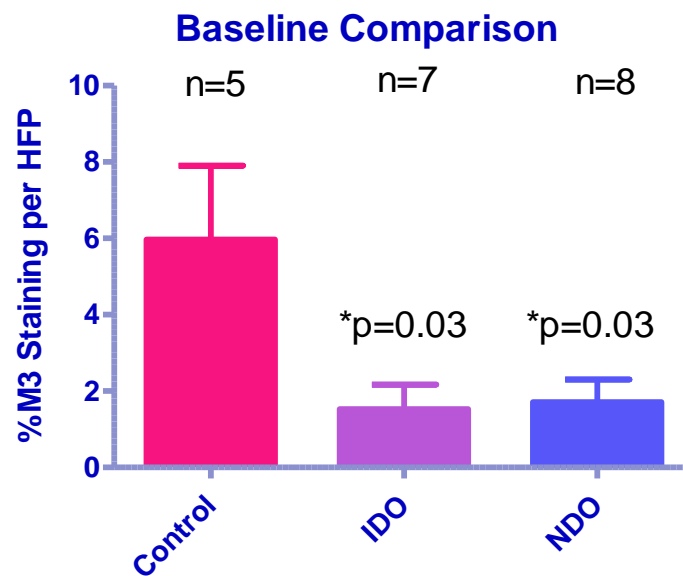


Figure 36: Comparison of M3 receptor suburothelial immunoreactivity between groups. Differences were found between controls and IDO and NDO subjects (both $p=0.03$).

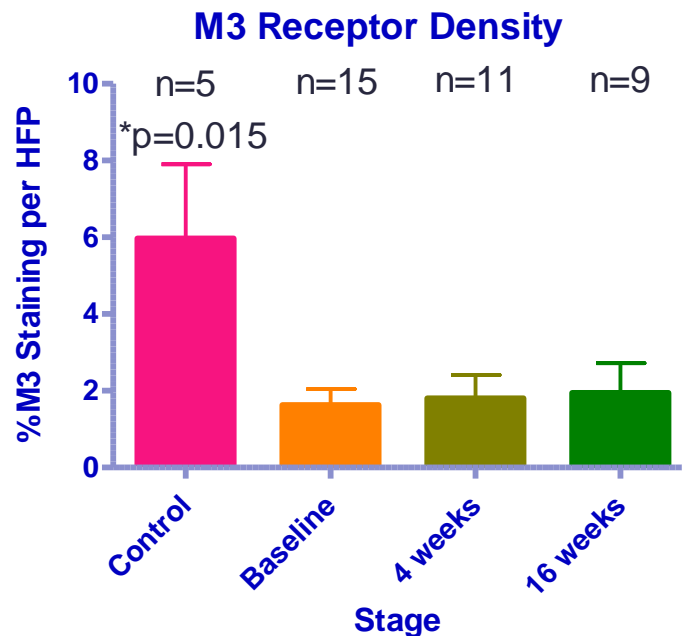


Figure 37: M3 receptor suburothelial immunoreactivity density per HPF for the total DO population. A difference was seen between controls and baseline value ($p=0.015$). No change was noted after BoNT/A treatment.

Suburothelial M3 receptor IR remained unchanged at both follow-up time points following injections of BoNT/A (Figure 37).

Urothelial M₃ staining

There was significant M3 receptor IR present within the urothelium (Figure 31 and Figure 33), which was analysed separately from the suburothelium. 19 out of 35 patients' slides had urothelium present suitable for analysis (IDO=8, NDO=11). This showed a significant increase in immunoreactivity following BoNT/A at 16 weeks ($p=0.03$, Figure 38). There was insufficient control M3 stained urothelial tissue to make a valid comparison.

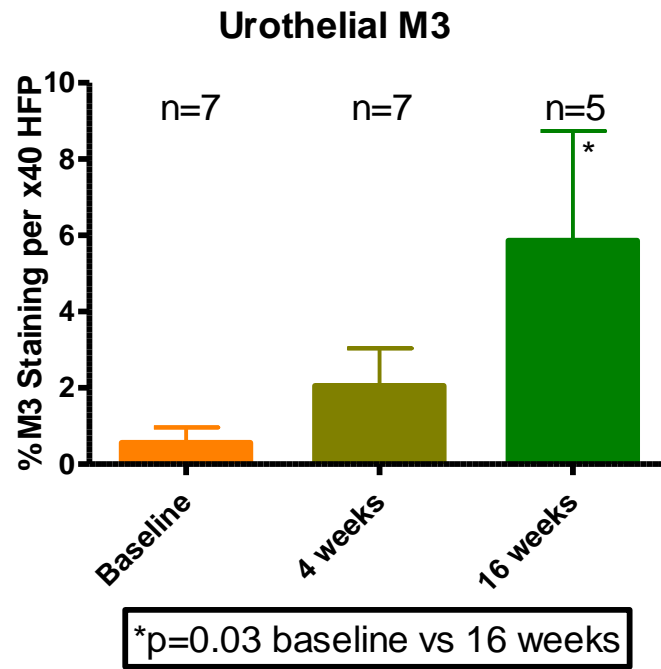


Figure 38: Increase in M3 urothelial immunoreactivity density following BoNT/A

Chapter 11 - SNAP-25

40 patients (controls=8, IDO=12, NDO=20) with 73 biopsies were stained for SNAP-25 protein. There were seven controls (mean 60.8, range 32.4 – 77.9 years), 27 baseline biopsies (mean 48.1, range 27.3 – 66.8 years), 21 4-week follow-up biopsies (mean 46.7, range 27.3 – 66.3 years) and 17 16-week (mean 44.6, range 27.3 – 55.3 years) follow-up subject biopsies. Neuronal like structures immunoreactive for SNAP-25 were clearly visible in the suburothelial layer, but no IR was present within the urothelium (Figure 40).

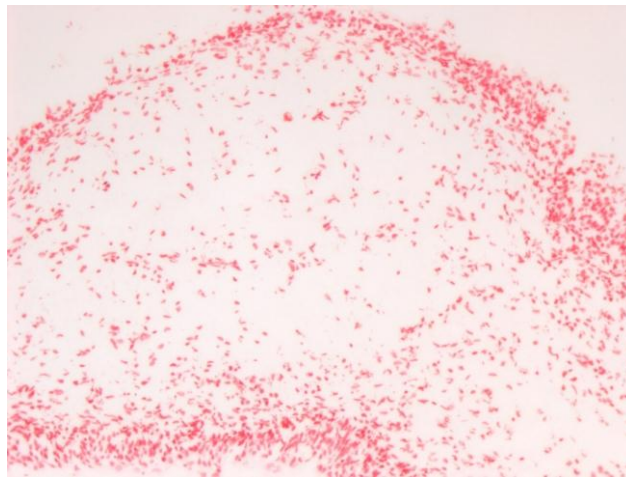


Figure 39: SNAP-25 Human Bladder Negative Control with no primary antibody

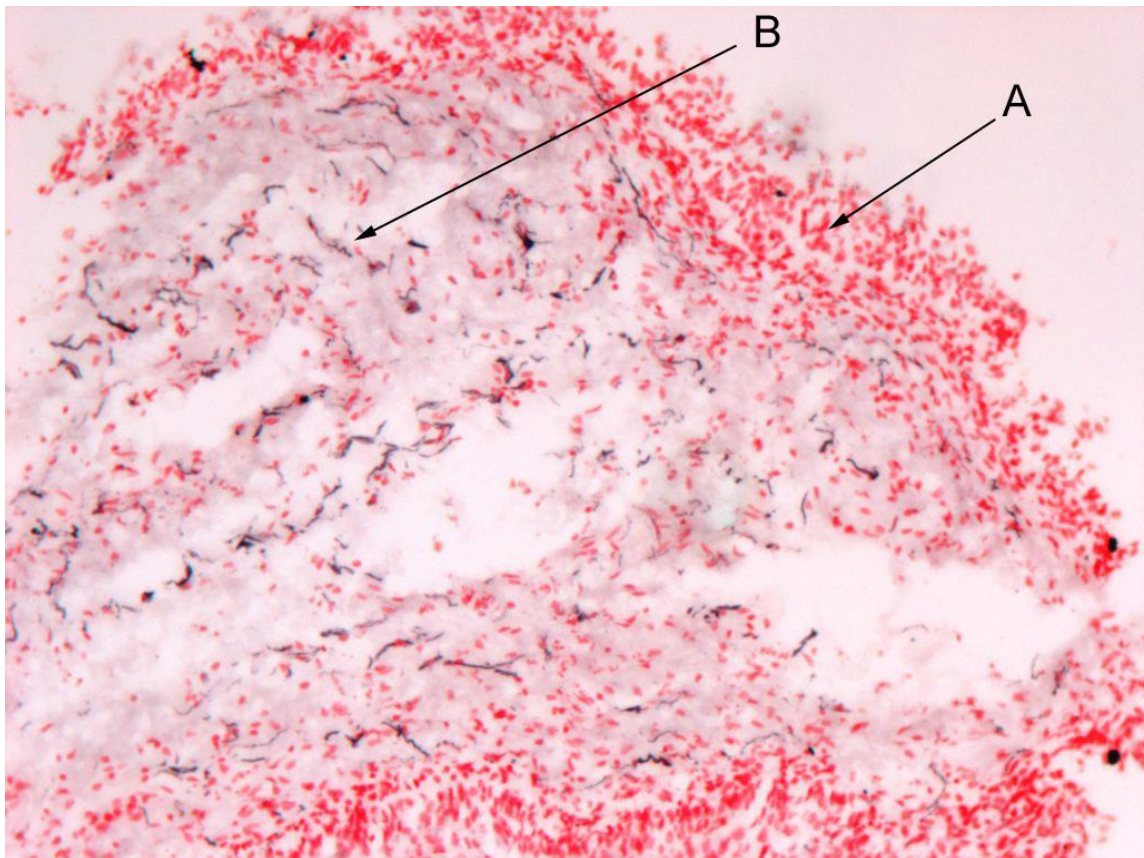


Figure 40: Urothelial staining was not present (A). SNAP-25 staining throughout sub-urothelium (B) on fibre-like structures (x10 HPF image).

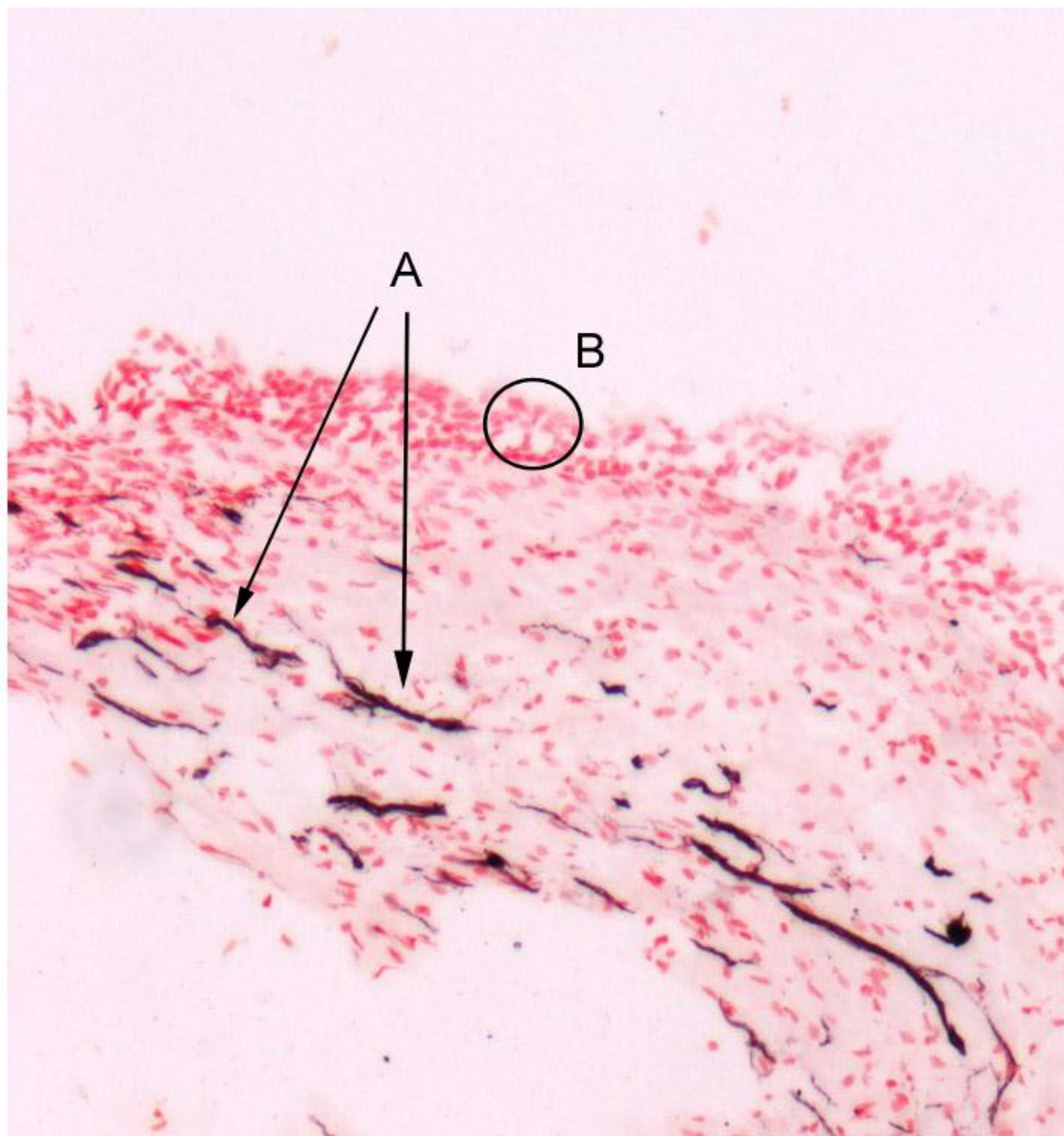


Figure 41: Neuronal SNAP-25 staining in the suburothelium (A). No staining was present within the urothelium (B) (x20 image).

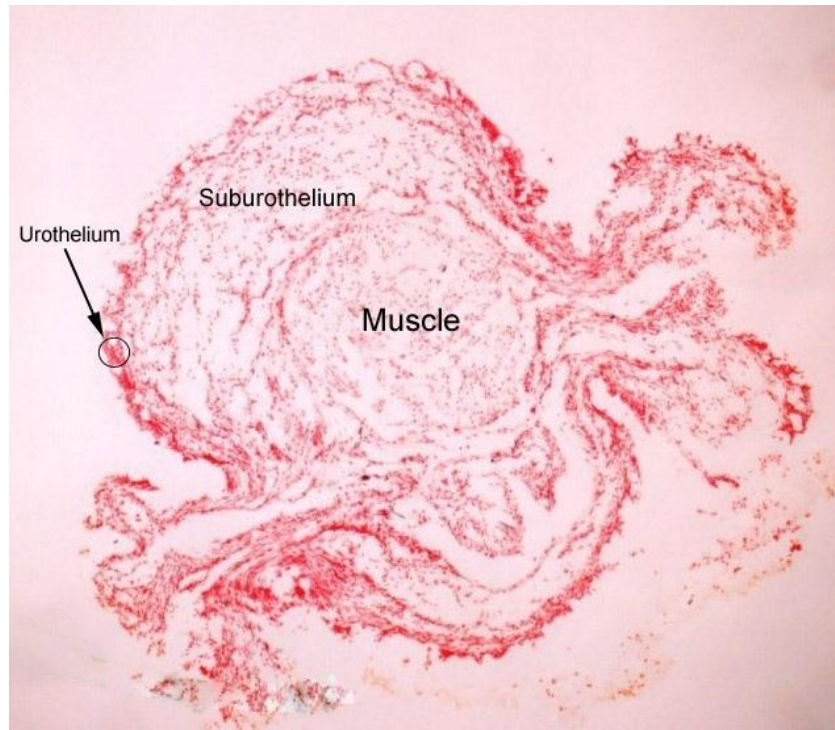


Figure 42: Low power (x2.5) photo of biopsy. Layers are labelled.

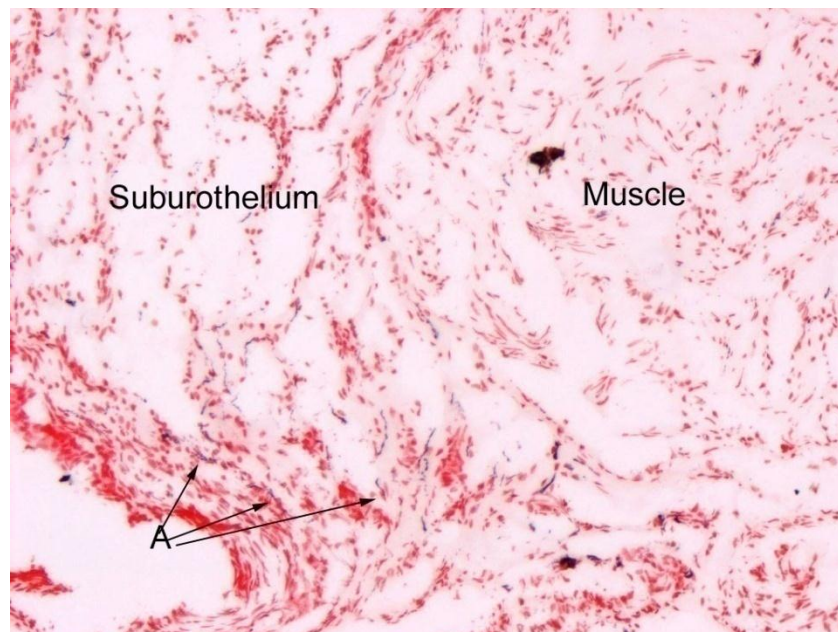


Figure 43: Same section as above but at x20 magnification. Neuronal SNAP-25 immunoreactivity present in suburothelium (A), with minimal immunoreactivity observed in muscle (B)

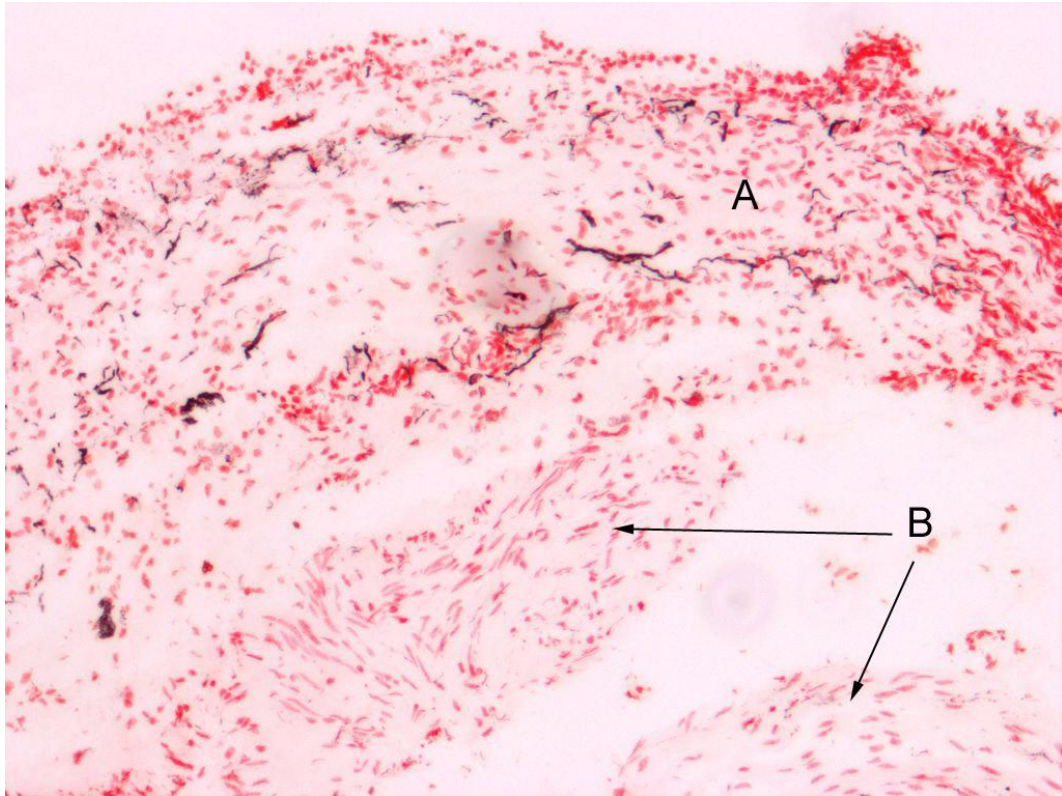


Figure 44: Section demonstrating SNAP-25 suburothelial(A) and muscle (B) immunostaining.

A total of 660 (x20 magnification) HPF images were quantified for SNAP-25 immunoreactivity. There was no overall difference in SNAP-25 immunoreactivity density between control, baseline and follow-up groups (Figure 45). This did not change when subgroup analysis was performed (Figure 46).

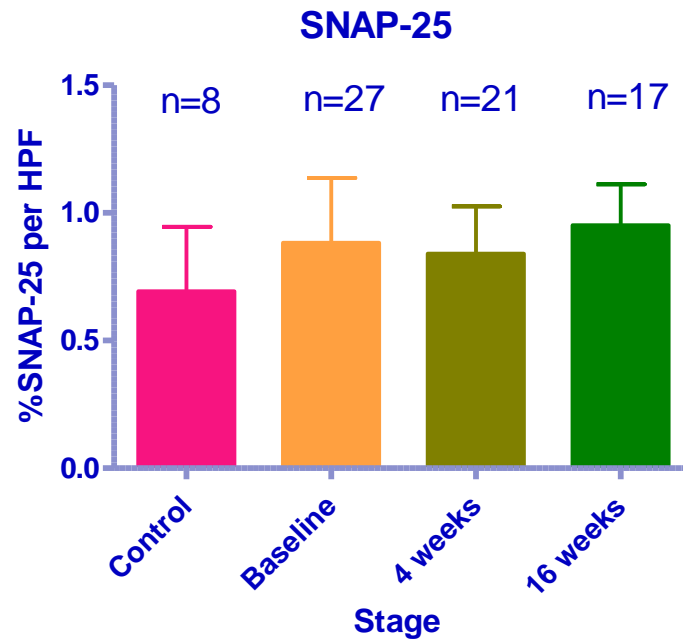


Figure 45: No difference was observed in SNAP-25 immunoreactivity between any of the groups.

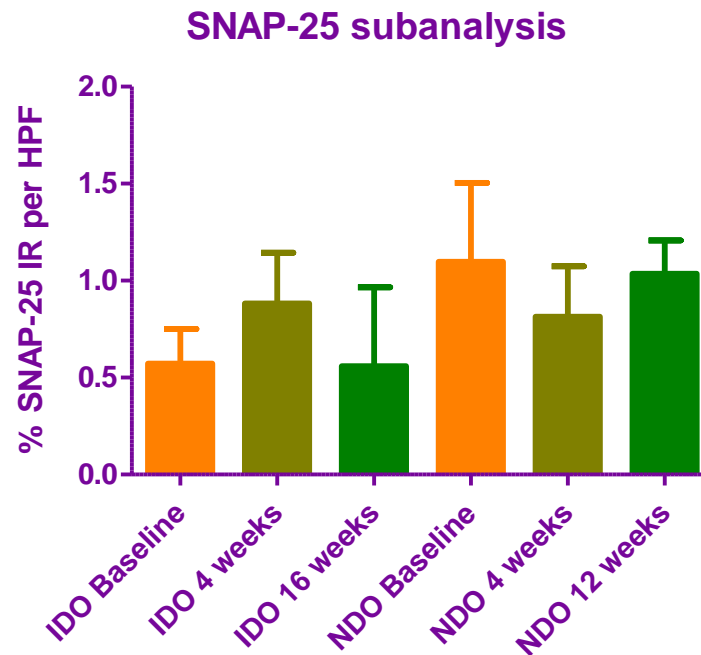


Figure 46: No significant change in SNAP-25 IR within IDO / NDO subgroups following BoNT/A

There were 13 biopsies which included muscle below the suburothelium. Paired sampling of suburothelium and muscle in those specimens showed less SNAP-25 immunoreactivity in the muscle (0.583%) compared to the suburothelial layer (0.831%) (One way ANOVA, $P < 0.0001$) (Figure 43, Figure 44, Figure 47).

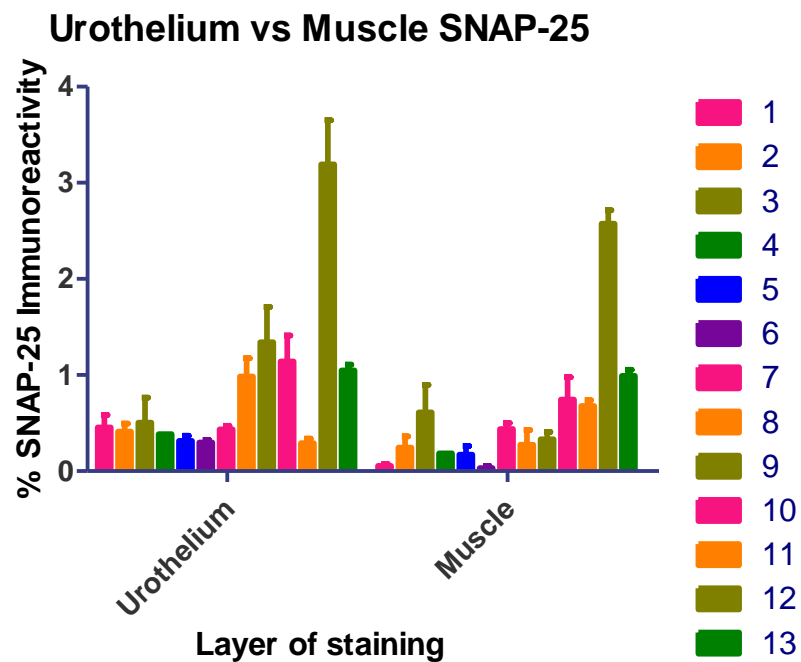


Figure 47: Comparison of Suburothelial and Muscle Immunoreactivity for SNAP-25 protein. Muscle IR was significantly less (One way ANOVA, $p < 0.0001$)

Chapter 12 - Neuropeptide Y

40 patients (controls=9, DO=31) were included with 70 biopsies stained with antibody for NPY. 10 biopsies were rejected for poor quality of sections (folded, minimal tissue and dropped off sections). There were nine controls (mean 62.1, range 32.4 – 77.9 years), 21 baseline (mean 47.9, range 27.3 – 66.3 years), 16 4-week follow-up (mean 49.1, range 29.2 – 66.3 years) and 14 16-week follow-up (mean 42.6, range 27.3 – 55.3 years) subject biopsies.

NPY immunoreactivity was present in the apical layer of the urothelium. Fibre like structure IR was present within the suburothelium, with no fibre like staining present in the muscle layer (limited sections of tissue with muscle present).

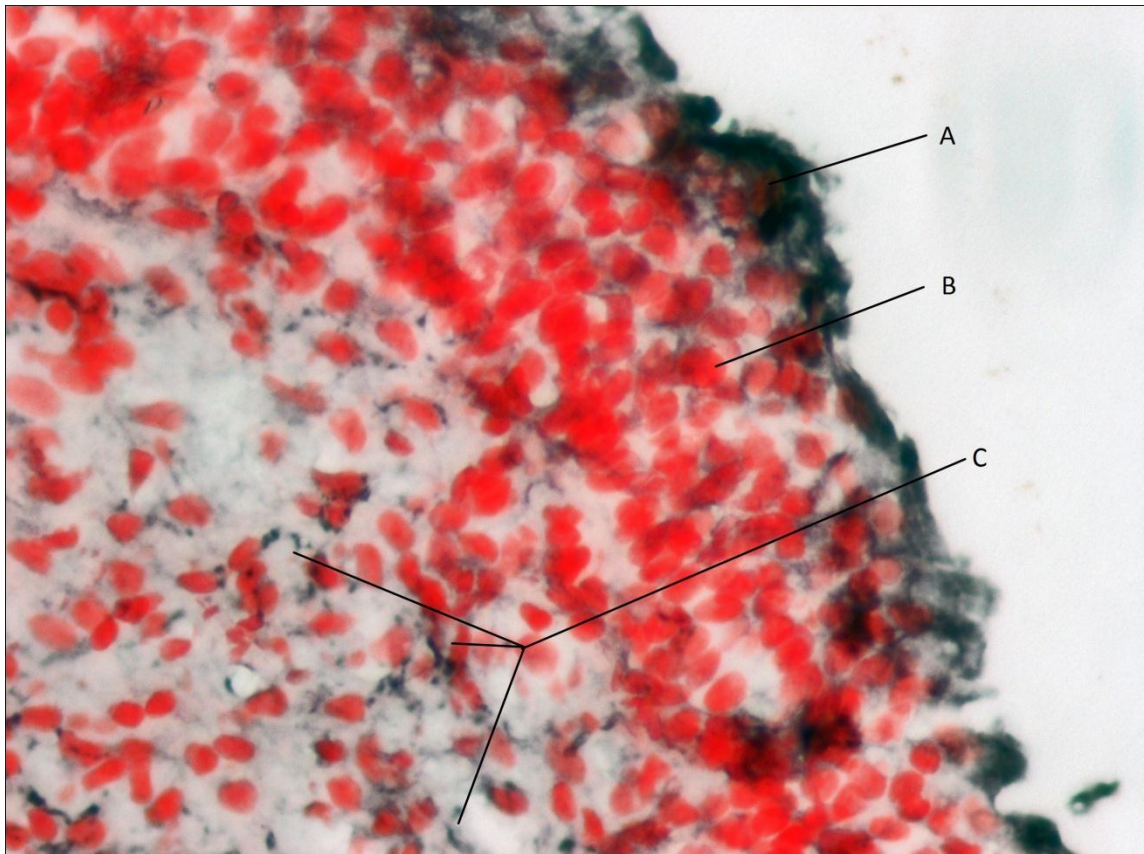


Figure 48: NPY immunoreactivity in apical layer of the urothelium (A). Very little IR was observed in the basal or middle layers (B). Fibre like staining was present in the suburothelium (C) (x 40 magnifications).

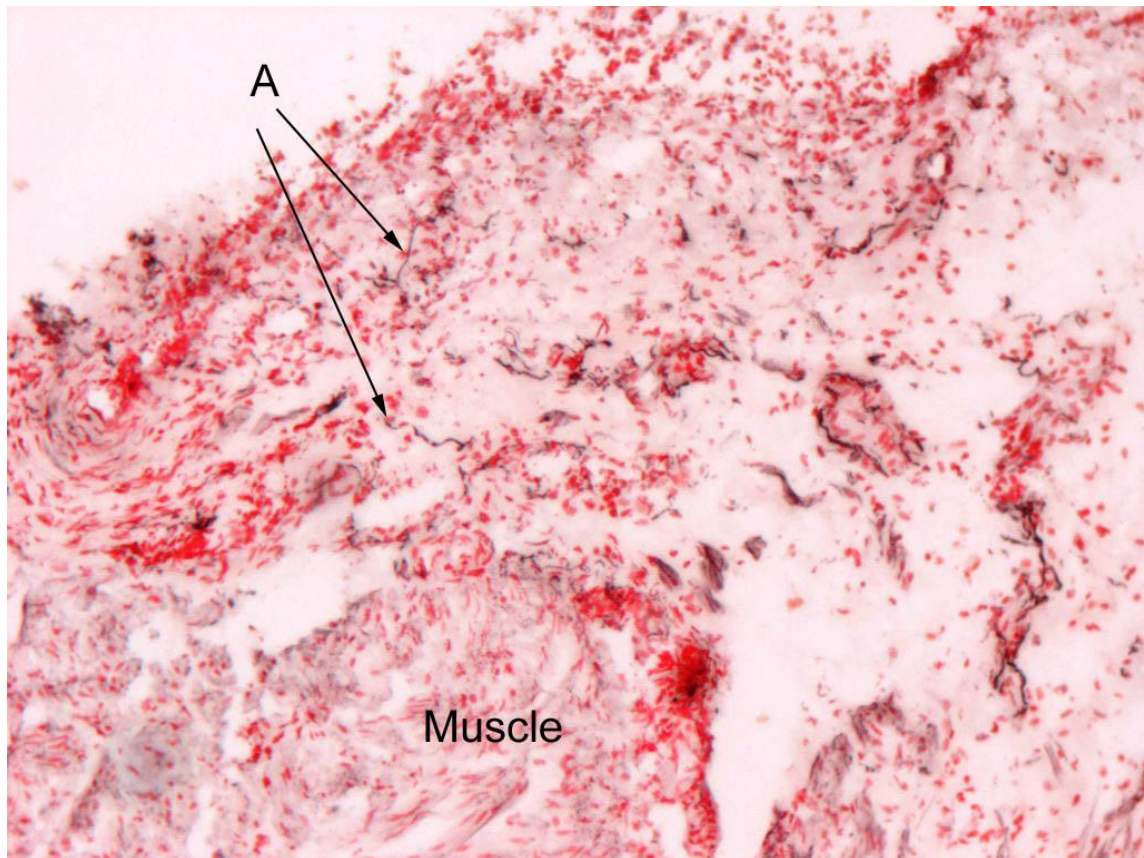


Figure 49: Suburothelial NPY immunostaining was observed in fibre-like structures (A). No specific fibre like NPY immunostaining was seen in the muscle layer (x10)

Over 600 images were quantified for NPY immunoreactivity. An increase in NPY immunoreactivity was observed between controls (0.29%), compared to patients with DO (1.00%) ($p=0.02$, Figure 51). Subgroup analysis of controls compared to IDO and NDO baseline patients did not show significant difference, due to lower numbers (Figure 50). Following administration of BoNT/A there were reductions in mean immunoreactivity towards control values, especially at 16 weeks (0.38%), but this did not achieve statistical significance on the Kruskal-Wallis test (Figure 51).

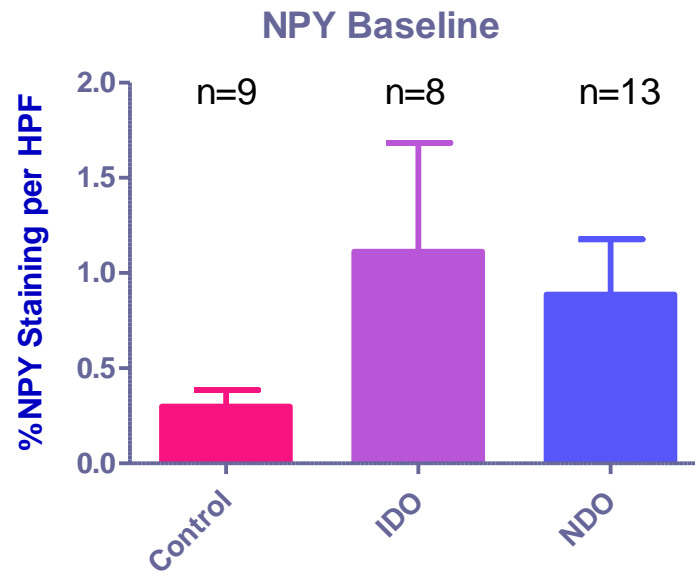
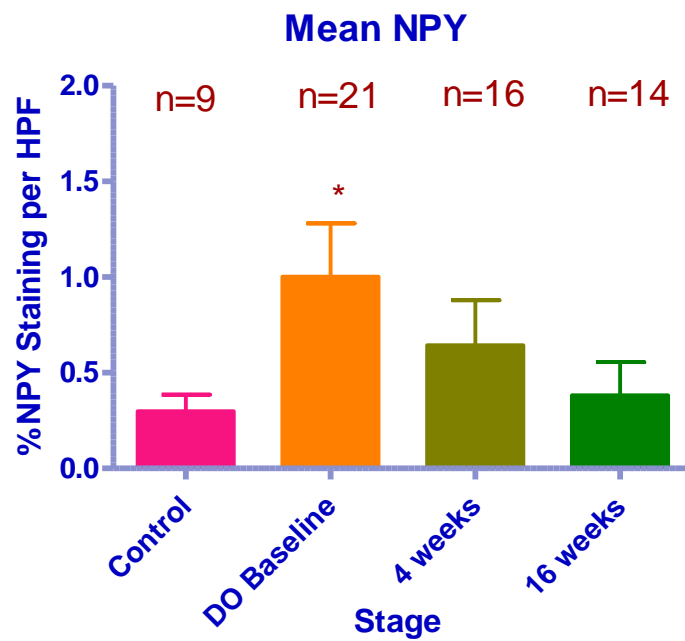


Figure 50: Subgroup analysis of controls compared to IDO and NDO baseline patients.



*p=0.02 between Control and DO Baseline

Figure 51: DO baseline biopsies showed an increase in NPY immunoreactivity density when compared to control subjects (p=0.02). There was a trend towards normalisation following BoNT/A, but this did not achieve significance.

Table 8: Summary of immunohistochemistry results.

Layer and Receptor	Control Subjects	Baseline DO	4 weeks post BoNT/A	16 weeks post BoNT/A
Suburothelial M1	1.711% (±0.4409)	0.7935%* (±0.1025)	1.612%** (±0.3354)	1.734%** (±0.3608)
Suburothelial M2	1.191% (±0.3119)	0.7422% (±0.1909)	1.628%** (±0.4251)	1.353%** (±0.2546)
Suburothelial M3	5.973% (±1.935)	1.625%* (±0.4222)	1.801% (±0.6151)	1.948% (±0.7709)
Urothelial M1	19.52% (±5.490)	3.161%* (±0.7602)	4.576% (±1.278)	8.211%** (±1.811)
Urothelial M3	No Data	0.5623% (±0.4017)	2.0555% (±0.9837)	5.864%** (±2.875)
SNAP-25	0.8884% (±0.3482)	0.9445% (±0.2321)	0.9840% (±0.2183)	0.9840% (±0.1805)
NPY	0.2984% (±0.08732)	1.002%* (±0.2786)	0.6440% (±0.2357)	0.3819% (±0.1740)

Mean percentage staining per HPF (±Standard Error of the Mean)

*= significant difference between Baseline DO and Control biopsy immunoreactivity.

**= significant difference between Baseline DO and post BoNT/A immunoreactivity.

Chapter 13 – Immunohistochemistry correlations with clinical symptoms.

Inverse correlations were found between suburothelial IR levels of each receptor in the whole DO population, and the number of urgency episodes before and after treatment (M1 receptor $r = -0.34$, $p = 0.012$, M2 receptor $r = -0.34$, $p = 0.048$ and M3 receptor $r = -0.39$, $p = 0.039$, Figure 52, a, c and e). Inverse correlations were also noted between suburothelial M1 and M3 receptor levels, and the number of voids per 24 hours before and after treatment ($r = -0.30$, $p = 0.024$ and $r = -0.44$, $p = 0.020$, respectively, Figure 52, b and f).

A weak trend was noted for a correlation between M2 receptor levels and micturition frequency ($r = -0.31$, $p = 0.094$, Figure 52, d). Urothelial M3 receptor levels inversely correlated with urgency ($r = -0.51$, $p = 0.049$). Urothelial M1 receptor levels showed only a trend toward correlation with urgency ($r = -0.32$, $p = 0.082$). Stronger correlations were noted between urothelial receptor levels and micturition frequency (M1 receptor $r = -0.41$, $p = 0.017$ and M3 receptor $r = -0.64$, $p = 0.006$) (Figure 53).

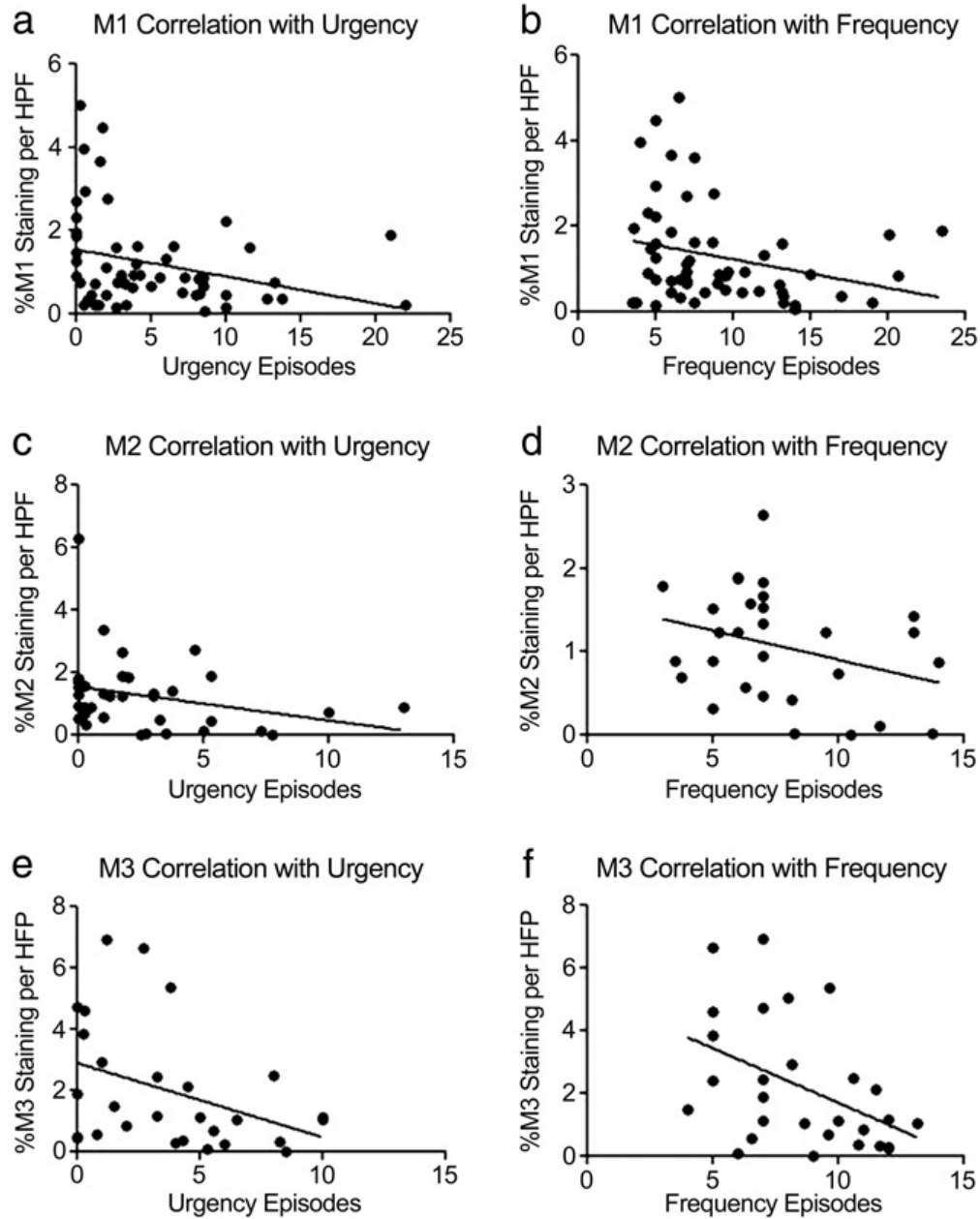


Figure 52: Receptor suburothelial IR by number of urgency and micturition episodes per 24 hours in whole DO population using Spearman r test, including values at baseline, and 4 and 16 weeks. Inverse associations were found between quantified urgency and IR for M1 ($r=-0.34$, $p=0.012$) (a), M2 ($r=-0.34$, $p=0.048$) (c) and M3 ($r=-0.39$, $p=0.039$) (e). M1 ($r=-0.30$, $p=0.024$) (b) and M3 ($r=-0.44$, $p=0.020$) (f) receptor levels also correlated inversely with 24-hour micturition frequency. Weak trend was noted for M2 receptor association with micturition frequency ($r=-0.31$, $p=0.094$) (d). Urothelial receptor levels were also inversely associated with patient symptoms.

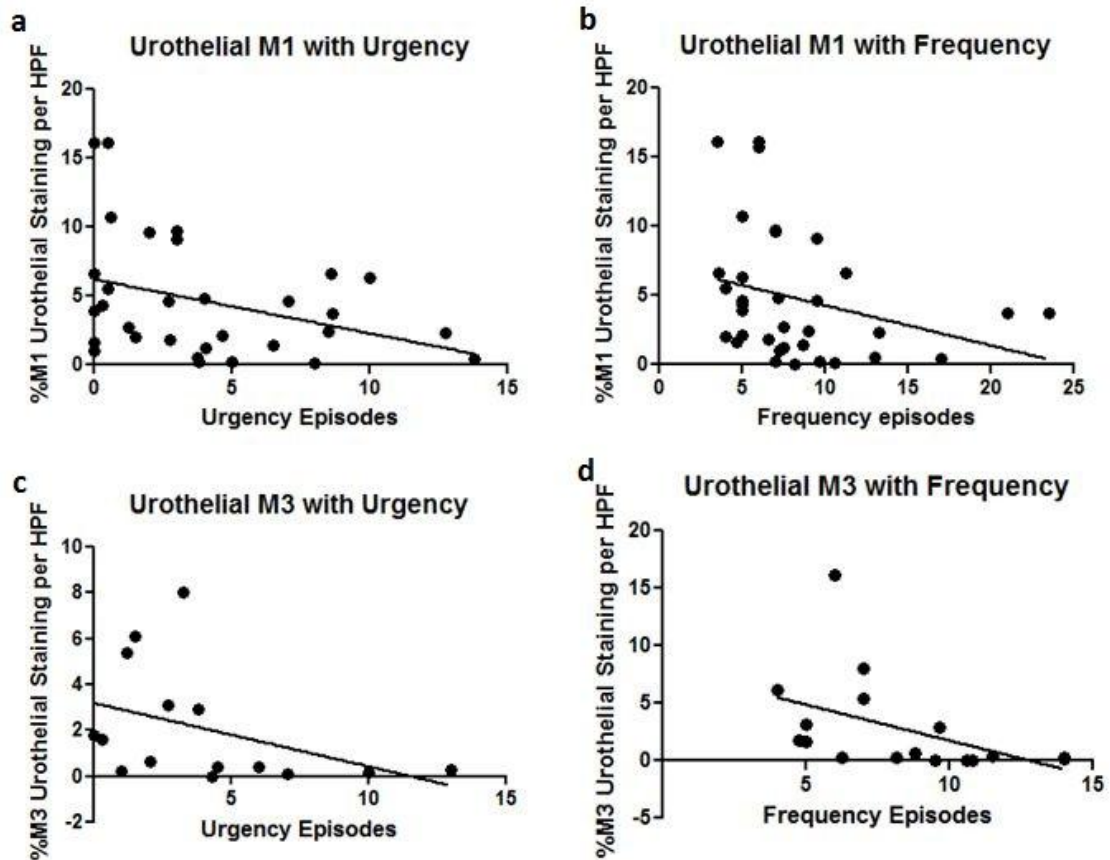


Figure 53: Receptor urothelial IR by number of urgency and micturition episodes per 24 hours in whole DO population using Spearman r test, including values at baseline, and 4 and 16 weeks. Inverse associations were found between quantified urgency and IR for M3 ($r = -0.5143$, $p = 0.0498$) (c). M1 ($r = -0.4064$, $p = 0.0171$) (b) and M3 ($r = -0.6384$, $p = 0.0058$) (d) urothelial receptor levels also correlated inversely with 24-hour micturition frequency.

Chapter 14 – Subgroup analysis on the effect of antimuscarinics.

To assess a possible synergistic effect of antimuscarinics we separated results at each time point and analysed for antimuscarinic use versus non-use by applying the Mann-Whitney test.

The incidence of patients on antimuscarinics decreased from 52.8% at baseline to 27.8% 4 weeks and 30.6% 16 weeks after treatment. Clinical outcomes were independent of antimuscarinic use at all time-points (Figure 54). Muscarinic receptor IR was no different in antimuscarinic users versus nonusers at all time-points (Figure 55). However, with very few antimuscarinic users at certain time points, we were not adequately able to compare urothelial M3 receptor at all time-points or suburothelial M3 and urothelial M1 receptors at 16 weeks.

We observed no statistical differences in clinical parameters or immunoreactivity between subjects that took antimuscarinics and those that did not.

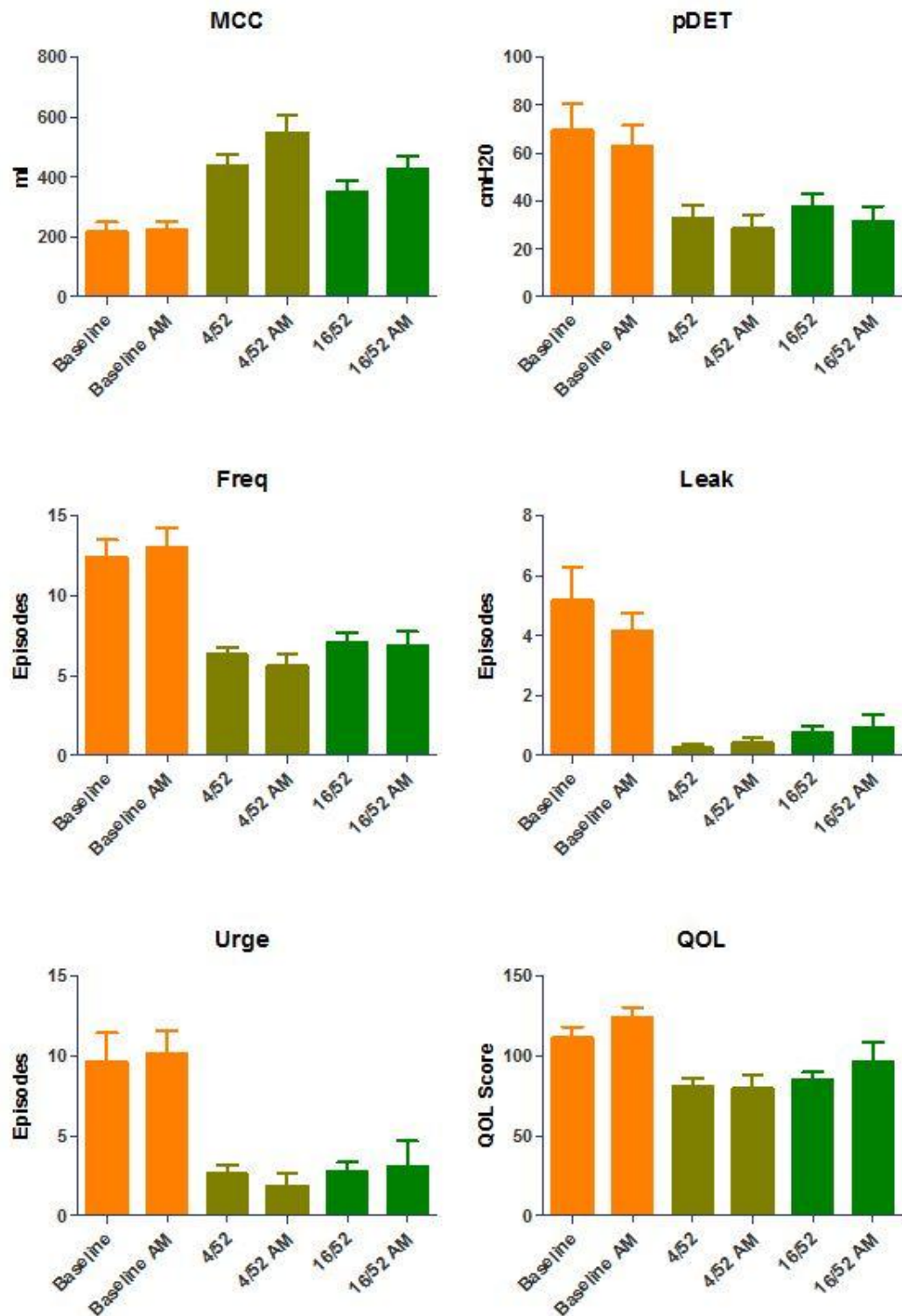


Figure 54: Clinical outcomes of BoNT/A were independent of antimuscarinic (AM) use at all time-points.

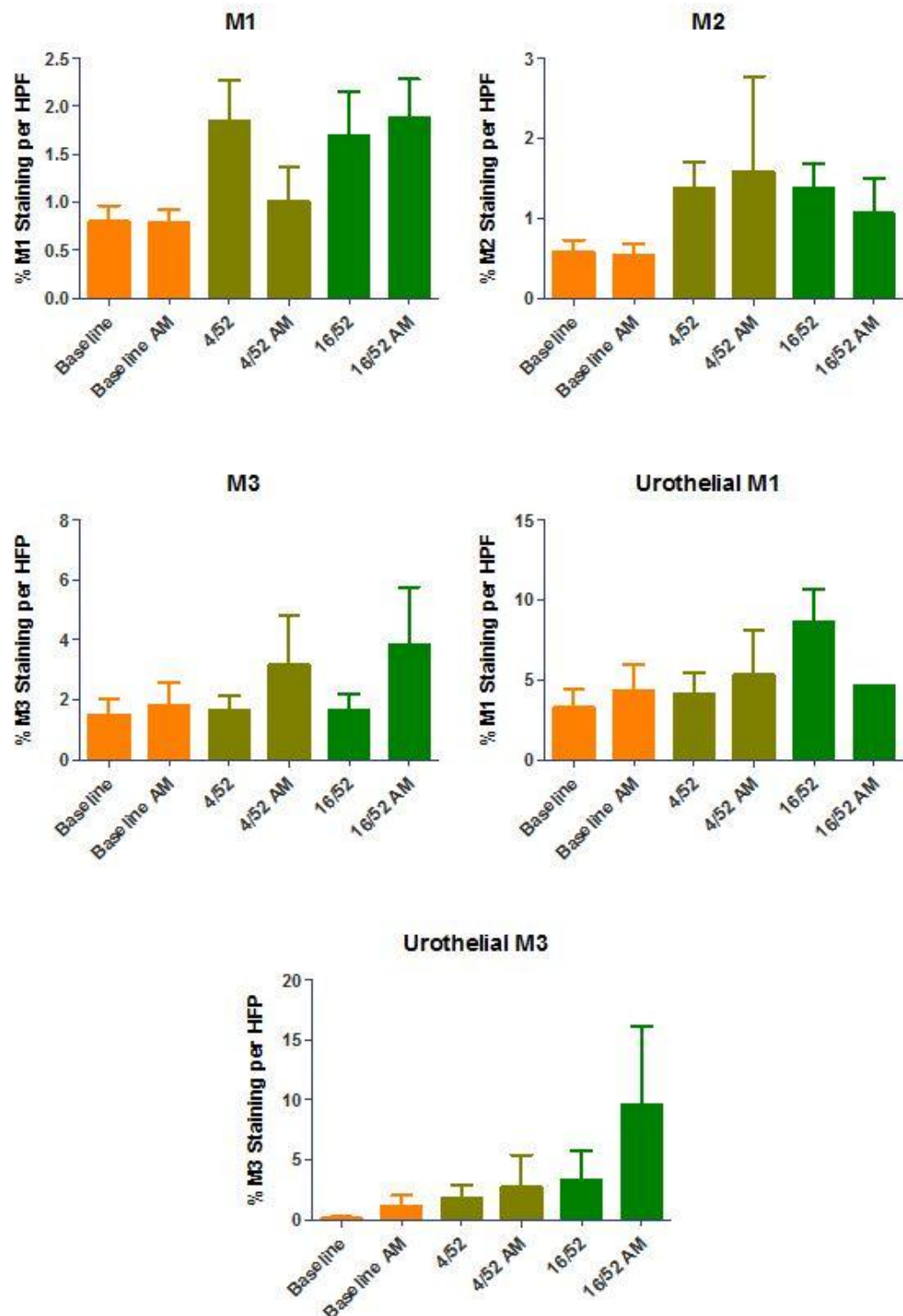


Figure 55: No statistical differences were seen in immunoreactivity in antimuscarinic users versus nonusers at all time-points. Large standard errors are reflective of smaller numbers in the AM groups.

IV - Discussion

Chapter 15

This is the first study, to our knowledge, to assess the effect of BoNT/A on the 3 muscarinic ACh receptors M₁, M₂, M₃ and NPY in the human bladder in both idiopathic and neurogenic DO. It is also the first study to investigate M₁ receptors in human overactive bladder conditions. The pattern of muscarinic receptor IR in the suburothelium and in muscle layers was similar to that reported by other groups (Mukerji et al., 2006; Jositsch et al., 2008).

There is current controversy regarding the mechanism of action of intra-detrusor BoNT/A; whether this acts in the urothelium / suburothelium, the detrusor muscle layer, or possibly both. This is in addition to the different proposed theories surrounding the pathophysiology of DO and where antimuscarinic drugs may be acting. Kuo showed no difference in outcome in a randomised study looking at BoNT/A injection either into the suburothelium or in the detrusor layer (Kuo, 2007). It is likely that BoNT/A diffuses through to both layers once injected (Mehnert et al., 2009). Initially it was thought that BoNT/A would produce a paralysis of the detrusor smooth muscle (Schurch et al., 2000), similar to its action in skeletal muscle (Dolly, 2003). However others have suggested that the urothelium and suburothelial complex has been shown to be crucially involved in a sensory mechanism and can release chemical mediators that may be involved in the pathogenesis of OAB and DO (Birder and de Groat,

2007). BoNT/A with its remarkable clinical efficacy at ameliorating symptoms of urgency (Kalsi et al., 2007) and frequency has led others to propose that at least part of its action is via modulation of afferent pathways (Apostolidis et al., 2006).

There is complex crosstalk at the urothelial, suburothelial and myofibroblast interface with involvement of ATP, ACh, NO and SP neurotransmitters modulating peripheral afferent input and contributing to a sensory web (Apostolidis et al., 2005b; Apostolidis et al., 2006; Birder and de Groat, 2007; Hanna-Mitchell et al., 2007).

Discovery of BoNT/A effects on TRPV₁ and P2X₃ receptors has prompted research into its effects on other putative afferent receptors found in the urothelium and suburothelium (Apostolidis et al., 2005b).

Following detrusor BoNT/A injections, urodynamic results showed significant improvements in all clinical parameters with decreases in frequency, urgency and incontinence episodes. There were also improvements in bladder capacity and quality of life. These results are comparable to those observed in other studies (Schurch et al., 2005; Sahai et al., 2007).

Suburothelial muscarinic receptor IR was decreased in patients with DO, significantly in M₁ and M₃, and to a lesser degree in M₂. Following treatment with BoNT/A, M₁ and M₂ IR were increased to levels close to control values, but M₃

immunoreactivity remained unchanged. Urothelial M₁ immunoreactivity followed the suburothelial pattern of changes. Similar post-BoNT/A increases were noted in the urothelial expression of M₃ receptors.

There was inverse correlation between patients symptoms of urgency and frequency and muscarinic receptor density.

NPY IR was increased in DO patients compared to healthy controls. Following BoNT/A application, there was a decreasing trend in NPY IR. SNAP-25 IR was found abundantly in the suburothelial layer in fibre like structures, with no changes following treatment.

Muscarinic receptors

It has been postulated that urothelial M₁ receptors respond to the paracrine / autocrine release of acetylcholine (Hanna-Mitchell et al., 2007). M₁ receptors in the rat urinary detrusor are thought to be autocrine facilitatory presynaptic receptors which enhance the release of ACh (Somogyi et al., 1994). The activation of the M₁ receptors is thought to modulate the neural input for bladder contraction during voiding. In Bschleipfer's study, M₁ receptors were shown to be localised to the basal layer of the urothelium (Bschleipfer et al., 2007), contrary to the finding in our study.

Acetylcholine (ACh) is a phylogenetically ancient molecule involved in cell-to-cell signalling in almost all life-forms on earth (Kawashima and Fujii, 2008). Cholinergic components, including ACh and choline acetyltransferase have been identified in keratinocytes, cancer cells, immune cells, urinary bladder, airway epithelial cells, vascular endothelial cells and reproductive organs. It is thought to be an important molecule in signal transduction.

There is a basal release of ACh from the bladder, which increases with age and decreases with removal of the mucosa (Yoshida et al., 2004; Yoshida et al., 2006). Choline acetyltransferase, an enzyme required for ACh synthesis, has been shown to be present within the urothelium (Yoshida et al., 2008).

Our results show for the first time immunohistochemical evidence for the presence of M₁ muscarinic receptors in the urothelium and suburothelium of human overactive bladders as well as healthy controls. We demonstrated apical urothelial staining, whereas previously this was shown only in the basal layer (Bschleipfer et al., 2007). Differences in the fixation protocol and in the use of commercial antibodies may account for the discrepancies noted in the presence and localisation of muscarinic receptor IR in the urothelium, compared with previous studies. The differences observed by Bschleipfer et al. may have been partly due to their use of patients' tissue with different pathological conditions (bladder cancer, carcinoma-in-situ and interstitial cystitis), where there may have been pathological urothelial turnover. It has been shown that bladder

urothelial cancer cells have altered muscarinic signalling and receptor expression (Tully et al., 2009). Their study was also limited to 8 older female patients. Our study showed the presence of M₁ receptors in apical urothelial cells in patients with DO. This finding could be related to the pathophysiology of DO, where M₁ receptors may appear throughout the urothelium.

Urothelial IR quantification could only be established for M₁ and M₃. In contrast to previous publications where the investigators used a different M₂ receptor antibody (Mukerji et al., 2006), we only observed non-evaluable urothelial M₂ immunoreactivity in patients with DO.

Muscarinic M₁ IR could be identified in the suburothelium on fibre-like structures and on cells with bipolar processes, morphologically resembling nerve fibres and interstitial cells/myofibroblasts, although double-staining experiments were not performed to confirm this. In the detrusor, IR was seen on fibre-like structures and endothelial cells.

Relative decreases were noted in urothelial as well as suburothelial M₁ IR in biopsies from DO patients prior to treatment, when compared to control subjects. The relative decrease was noted in both IDO and NDO groups. Whether M₁ receptors in the urothelium perform this same facilitatory role as noted in the detrusor muscle is unknown.

It is well established that prolonged exposure of G protein coupled receptors to agonists results in a reduction in receptor mediated responses, through regulatory mechanisms that involve phosphorylation, G protein uncoupling, internalization and down-regulation (Wess, 1996; Ferguson, 2001; van Koppen and Kaiser, 2003). The M₁ and M₃ receptors have been shown to be phosphorylated by protein kinase C (PKC) both in-vitro and in-vivo in an agonist independent manner (Uchiyama et al., 1990; Richardson and Hosey, 1992; Haga et al., 1996). Phosphorylation of muscarinic receptors by PKC results in receptor protein downregulation and a reduction in their mRNA levels, with significant loss of cell surface muscarinic receptors (Liles et al., 1986; Rousell et al., 1995). This PKC phosphorylation is more prominent in M₁ and M₃ than in M₂ receptors (Haga et al., 1993), in line with the findings of our study. The decrease in M₁ and M₃ IR in DO observed in our study may result from a compensatory mechanism to reduce facilitation, in a system with an increased ACh release from the urothelium.

There is evidence that there is plasticity in muscarinic receptor expression and this has been shown in spinal cord transected rats, where other receptor subtypes (M₃) take on a facilitatory role from low affinity M₁ receptors (Somogyi and de Groat, 1999). An increase in non-neuronal ACh could be the underlying primary pathology of DO, which has been noted to occur with age and detrusor stretch (Yoshida et al., 2004; Yoshida et al., 2006).

Following successful treatment of DO with BoNT/A, there is a normalisation of suburothelial IR for M_1 both at 4 and 16 weeks. Urothelial and suburothelial increases in M_1 IR after BoNT/A may occur due to reduced amounts of ACh now able to activate the receptor, resulting in reduced PKC phosphorylation. It is known that receptor downregulation is reversed when PKC is inhibited (Liles et al., 1986).

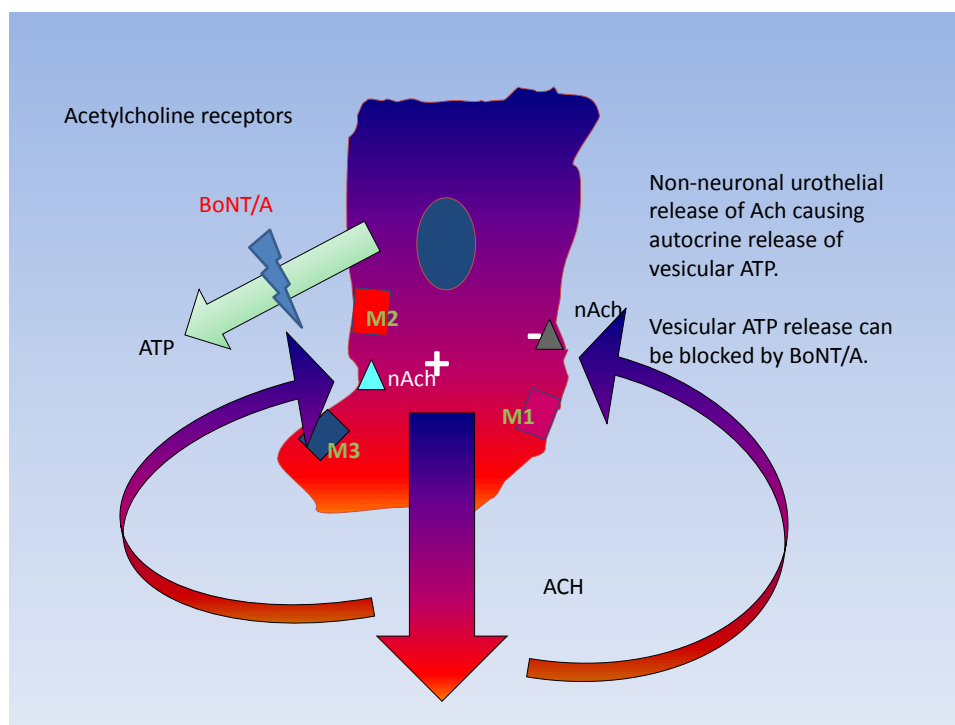
Normalisation changes with BoNT/A have been reported in urothelial ATP and NO neurotransmitter release (Smith et al., 2008). Similarly suburothelial P_2X_3 and TRPV₁ receptors have normalised with BoNT/A treatment (Apostolidis et al., 2005b). In the case of P_2X_3 and TRPV₁ receptors, levels decreased, but with muscarinic receptors there appeared to be an increase following successful BoNT/A treatment. This may illustrate the opposing actions of the neurotransmitter systems in the suburothelium.

In the urothelium the increase in M_1 receptor is only significant at 16 weeks, which may indicate a slower pattern of change as compared to the suburothelium. In addition, this increase in IR was not as pronounced as the normalisation seen in the suburothelium, suggesting that BoNT/A may not have such a pronounced effect in the urothelium, or that the effect is indirect.

Although ACh is released from the urothelium, it is not thought to be stored in vesicles. There is also release of neuronal vesicular ACh from cholinergic

nerves that have been detected in close proximity to the urothelial cells (Hanna-Mitchell et al., 2007). Cholinergic agonists applied to cultured urothelial cells can elicit an increase in intracellular calcium concentration and evoke the release of NO and ATP (Beckel et al., 2006; Kullmann et al., 2008a). Currently the known mechanism of action of BoNT/A, is its activity to disable the vesicle docking system of exocytosis. In the urothelium, BoNT/A may not directly influence ACh release, but may indirectly influence the downstream effect of ACh by inhibiting the vesicular release of ATP (Figure 56). The observed change in muscarinic receptors following BoNT/A in our study may result partly from a decrease in ATP release as well as suburothelial ACh release.

Figure 56: Autocrine / paracrine action of ACh to release ATP from urothelial cells. BoNT/A is able to block ATP release and may indirectly alter afferent signalling.



ATP can also act in an autocrine manner to facilitate its own release from urothelial cells (Wang et al., 2005). Once released, ATP can alter the threshold for activation of ion channels such as TRPV1 (Birder, 2009). Future functional studies looking at changes in ATP concentration and its effect on ACh release will be needed to establish if there is crosstalk between different transmitter systems.

In bladder efferent nerves, it has been shown that different subtypes of muscarinic receptors mediate inhibitory (M_2 – M_4) and facilitatory effects (M_1) on ACh release (Somogyi et al., 1994; Andersson and Yoshida, 2003), however this has not been shown in the urothelium. Future experiments looking at different subtypes of muscarinic receptors and their effect on ACh release in the urothelium would help elucidate their functional roles in non-neuronal cholinergic transmission.

Despite being the predominant subtype of muscarinic receptor, the functional role of M_2 receptor in the detrusor muscle and urothelium is unclear (Abrams et al., 2006). We found consistent immunostaining of M_2 receptors in the suburothelium and in muscle layers, similar to that shown by other groups (Mukerji et al., 2006; Bschiepfer et al., 2007; Jositsch et al., 2008). Suburothelial IR was seen in fibre like structures and on cells with bipolar processes, which has also been seen in previous studies. Other authors have shown the presence of M_2 receptor mRNA in the urothelium and suburothelium,

via Reverse Transcription Polymerase Chain Reaction (RT-PCR) (Bschleipfer et al., 2007; Mansfield et al., 2007). Although limited urothelial M₂ IR was seen, we were not able to evaluate this separately from the suburothelial immunostaining.

Physiological studies have suggested that M₂ receptors take on a functional role in disease states such as surgically denervated rat bladder (Braverman et al., 1998) and NDO (Pontari et al., 2004), but this has been disputed by others who looked specifically for this in IDO and NDO, finding no change in the receptor subtype mediating contraction (Stevens et al., 2007). RT-PCR experiments by Mansfield et al. also showed no difference in M₂ mRNA expression between controls and women with IDO, both in the mucosa (urothelium and suburothelium) and in the detrusor (Mansfield et al., 2007). Our results are in keeping with these studies, showing no statistical change in M₂ IR between control subjects and patients with IDO or NDO. The lesser effect of PKC phosphorylation on downregulation of M₂ receptors may also contribute to our findings.

One other group has looked at samples of bladder smooth muscle between OAB patients and patients undergoing cystectomy for cancer, and found no difference in M₂ mRNA expression at the dome, but higher expression in the non-OAB group at the trigone (Hinata et al., 2004). That study used bladder cancer patients as controls. Cancer of the bladder is most frequent in the trigone with patients requiring endoscopic surgery for staging prior to radical surgery, both of which may have affected receptor expression.

The study by Mukerji et al. looked at M₂ myofibroblast-like IR in the suburothelium and found increases in biopsies from patients with IDO or Painful Bladder Syndrome compared to controls, but no change in the urothelium (Mukerji et al., 2006). These studies differ in methodology; different antibodies were used, capturing potentially different M₂ epitopes. Further to this, Mukerji et al. quantified MF-like staining, whereas in our study we quantified all suburothelial IR.

Following successful administration of BoNT/A in patients with DO, we showed an increase in suburothelial M₂ IR. As with M₁ receptor change, there was normalisation of the receptor density values at both 4 and 16 weeks post-BoNT/A. Separate analysis of the two subgroups showed the main change was in the NDO subgroup, whilst the IDO subgroup did not show significant change, due to the small IDO subgroup sample size.

It is difficult to explain the pharmaco-physiology behind the increase in M₂ receptor IR following BoNT/A, but a direct effect is unlikely since its mechanism of action is to inhibit vesicular release. An indirect consequence to the change of various afferent pathways should also be considered. From our results, it is apparent that there is considerable plasticity of receptor expression in the superficial layers of the bladder, with changes occurring in a relatively short period of time. Imaging of efferent pathways in the neuro-muscular junction in

the mouse, following application of BoNT/A, has shown that affected motor nerve endings undergo extensive nerve sprouting with end plate remodelling (de Paiva et al., 1999).

Following prolonged muscarinic receptor stimulation, G protein-coupled Receptor Kinase (GRK) phosphorylate agonist occupied receptors, leading to binding of arrestins which sterically suppress G protein interaction. Human M₂ receptors have been shown to undergo rapid agonist-dependent phosphorylation (Haga et al., 1994; Pals-Rylaarsdam et al., 1995). We speculate that BoNT/A reduces ACh release and relieves this agonist-dependent phosphorylation and internalisation, resulting in greater numbers of M₁ and M₂ receptors. Similarly, muscarinic antagonists have been shown to be able to upregulate muscarinic receptor expression (Milligan and Bond, 1997; Dowling et al., 2006). Future experiments looking at ACh release following stretch or other stimuli, before and after BoNT/A, would help understand the normal physiology of the urothelium.

The M₃ receptor IR showed significant immunostaining of both urothelium and suburothelial structures (neurones and cell bodies), similar to previous studies using different antibodies (Mukerji et al., 2006). Some biopsies had sections of muscle present which also showed cellular M₃ IR. Areas of strong cellular staining in the suburothelium were noted close to nerve fibres.

We noted a reduction in M₃ IR in subjects with DO, which was also significant when they were analysed separately as IDO and NDO groups. This is contrary to the findings of a previous IHC study, which showed an increased IR in DO patients (Mukerji et al., 2006). Reduced M₃ mRNA expression in accord with our results has been previously demonstrated by PCR in the urothelium and suburothelium of patients with IDO, as compared to controls (Mansfield et al., 2007).

Much of the previous literature has concentrated on the detrusor muscle being the site of action of antimuscarinic drugs and potentially the site of pathophysiology in OAB and DO. However, the clinical observation that patients benefitted during the filling phase, with a reduction of their symptoms of urgency, frequency and nocturia could not be explained by an effect on efferents alone (Andersson and Yoshida, 2003). Furthermore, the majority of patients taking antimuscarinics are able to void to completion. All these facts called into question whether the main site of action was just the detrusor muscle (Andersson, 2004).

An afferent hypothesis is supported by the inverse associations found between muscarinic receptor IR levels, and patients' degree of urgency and frequency in our study. This suggests that changes in urothelial and suburothelial muscarinic receptor expression in patients with DO have clinical relevance. Previous correlation with clinical outcomes have been shown with M2 receptors but this

was contrary to inverse relationship that we have described (Mukerji et al., 2006).

Recent radioligand binding studies looking at antimuscarinic binding in urothelium, suburothelium and detrusor have demonstrated that antimuscarinics exhibit equal affinity for both urothelium/suburothelium and detrusor (Mansfield et al., 2008). Our results show that muscarinic receptors are present in the urothelium and suburothelium with decreases occurring in patients with DO, with normalisation of M_1 and M_2 and increased urothelial M_1 and M_3 receptors after BoNT/A. The therapeutic efficacy of antimuscarinic drugs may similarly be based on their ability to antagonise urothelial and suburothelial muscarinic receptors, preventing overstimulation by ACh. The greater efficacy of BoNT/A may be due to its effect on multiple pathways as well as its inhibition of ACh release, whereas anticholinergics reduce ACh binding to its receptor.

Our finding of changes in the superficial layer fits with the emerging hypothesis that the urothelium acts as a mechanosensor, releasing ATP and ACh that activates purinergic and muscarinic receptors in suburothelial nerves. This in turn conveys information on bladder fullness to the central nervous system (Andersson and Arner, 2004; Apostolidis et al., 2006; Chancellor et al., 2008). In conditions of increased sensory nerve transmission such as OAB, IDO and NDO, there is increased release of ATP and ACh from the urothelium and suburothelium, which activate $P2X_3$ and muscarinic receptors to increase

afferent nerve activity. This would account for the higher frequency of bladder contractions reported in human and animal models of spinal cord injury (Smith et al., 2003; Khera et al., 2004; Smith et al., 2008). We speculate that BoNT/A reduces the release of ATP and ACh and therefore helps to normalise P2X₃, suburothelial M₁, M₂ and urothelial M₁ and M₃ populations.

Researchers who studied bladder contraction with carbachol found that greater contractions are seen when the urothelium is removed. They have proposed that a UDIF modulates the contractility of the detrusor muscle (Hawthorn et al., 2000; Templeman et al., 2002; Chaiyaprasithi et al., 2003). It is known that muscarinic agonists mediate the release of this factor. A reduction of muscarinic receptors in the urothelium as seen in DO patients may inhibit the release of UDIF predisposing towards uninhibited reflex detrusor contractions.

Activation of superficial muscarinic receptors in the rat bladder wall by an agonist (Oxotermorine methiodide) showed it could both inhibit as well as excite frequency of voiding, depending on its concentration (Kullmann et al., 2008a). Low concentrations of the agonist inhibited voiding frequency, whereas high concentrations elicited excitatory effects. Capsaicin pre-treatment prevented any effect by the agonist. The inhibitory effect seemed to be mediated by NO (effect diminished by L-NAME), whereas the excitatory effects were mediated partly by ATP. This study indicates that an increase / decrease in muscarinic receptors could give rise to increased afferent activity.

Daly et.al showed stimulation of mouse bladder muscarinic receptors with bethanechol, carbachol and indirectly with physostigmine significantly inhibited the afferent response to bladder distention (Daly et al., 2010). These effects were reversed with atropine but not by 4-diphenylacetoxy-N-methylpiperidine (4-DAMP), a M_3 receptor antagonist, suggesting other muscarinic receptors are responsible for the inhibition of afferent responses. This is in line with our findings of an increase of M_1 and M_2 receptors after BoNT/A, with a reduction in clinical urgency symptoms and an increase in mean cystometric capacity.

Further investigation of the underlying muscarinic modulatory mechanisms, specifically whether a particular mAChR subtype (M_1 – M_5) or a mAChR subtype located at a particular site (urothelium or nerves) is involved, might help to identify new targets for the treatment of overactive bladder (Kullmann et al., 2008a). Afferent M_1 and M_2 receptors may facilitate the therapeutic response seen in response to antimuscarinics and BoNT/A.

The administration of BoNT/A did not change the suburothelial M_3 IR at any time point, but did show an increase in the urothelial M_3 IR at 16 weeks. These results are in contrast to decreases in suburothelial $P2X_3$ and $TRPV_1$ IR after BoNT/A, but did not show in the urothelium. We know from this previous study that BoNT/A does not cause a significant change in the suburothelial neuronal population, as measured by the IR of the pan-neuronal marker PGP 9.5

(Apostolidis et al., 2005b) and a further study showed no evidence of apoptosis (Kessler et al., 2009).

The increase in urothelial M₃ IR cannot be explained by a direct action of BoNT/A, as SNAP-25 has not been shown to be found in the urothelium in our study or in previous studies (Chancellor et al., 2008). This observation may come about by a compensatory paracrine mechanism in a low-frequency functioning bladder with reduced release of neuronal ACh in the suburothelium. BoNT/A reduction of some sensory receptor levels (P₂X₃ and TRPV₁) and increase in muscarinic receptor levels (M₁, M₂ and urothelial M₃) points to a complex cascade mechanism of action, with several indirect changes resulting from the suburothelial and detrusor vesicular neurotransmitter blockade. Future work is needed to help us understand how these occur.

Other potential targets of BoNT/A include arachidonic acid, a potent fusogen, which facilitates vesicular fusion and exocytosis (Karli et al., 1990), enabling ACh release (Nishio et al., 1996). BoNT/A has been shown to block arachidonic acid release from membrane stores (Ray et al., 1993), which may partly prevent ACh release from the urothelium.

From the multiple changes noted in muscarinic receptors, the most plausible explanation for the clinical efficacy of BTX-A in the treatment of human detrusor

overactivity is that it impairs the release of ATP and ACh in the superficial layers of the bladder.

SNAP-25

When injected, the diffusion of BoNT/A is visually apparent in the superficial layer by the addition of indigo carmine (Schulte-Baukloh and Knispel, 2005). In our study, SNAP-25 IR was seen in the suburothelium and muscle layers as an extensive network of SNAP-25 fibres. However SNAP-25 fibres were not seen within the urothelium, implicating the suburothelium being the main site of action within the afferent layers of the bladder. BoNT/A uses secretory vesicle protein 2 (SV2) as its protein receptor on the neurone surface (Dong et al., 2006) to become internalised. Without SNAP-25 being present, BoNT/A could not act and consequently SNAP-25 is a good marker to delineate the site of action of BoNT/A.

Comparison of SNAP-25 IR in suburothelium and detrusor in our study showed that there was 43% more IR density within the suburothelial layer. This suggests that suburothelial layer may play a critical role in the mechanism of action of BoNT/A. Studies have shown that BoNT/A inhibits release of ATP from the urothelial side of the bladder, implying that part of its action is in the urothelial/suburothelial layer (Khera et al., 2004) and supporting our hypothesis that at BoNT/A has an effect on urothelium and suburothelium. A limitation of our study is that we could only examine the inner superficial detrusor layer due

to the limited size of the flexible cystoscope biopsies, therefore we do not know if this finding applies to the larger outer part of the detrusor muscle.

Following BoNT/A administration, there was no change in SNAP-25 IR at 4 or 16 weeks. The antibody we used was not able to differentiate between SNAP-25 and the cleaved product SNAP-25_A. This limitation has been noted by other authors, and they were able to demonstrate the cleavage products by immunoprecipitation studies in one patient 11 months after injection (Schulte-Baukloh et al., 2007). They found detection of SNAP-25_A challenging due to its minute concentration and the prolonged interval (between 1-30 months) following injection and follow-up biopsies being taken, resulting in it being detectable in only 4 out of 7 patients analysed. Repeating this work in the suburothelium was not possible, as commercial antibodies are currently unavailable for SNAP-25_A.

BoNT/A acts on ubiquitous intracellular targets required for any SNARE dependent neurotransmitter release (Ashton and Dolly, 1988). Our current understanding of the afferent pathways in the bladder is just beginning. There are numerous neurotransmitters systems in the urothelium/suburothelium; those that are potentially affected by BoNT/A that have yet to be identified.

Animal studies have shown BoNT/A reduces distension evoked urothelial release of ATP in a rat model of NDO (Khera et al., 2004). Other

neurotransmitter systems, including CGRP, have similarly been inhibited by BoNT/A (Chuang et al., 2004). Human urinary NGF levels have been found to be elevated in IDO and NDO, with these levels falling after successful treatment with BoNT/A (Liu et al., 2008). These examples all illustrate the complex interactions of BoNT/A on the afferent pathways, as well as its multifactorial mechanism of action in DO.

Our study was able to identify the location and density of SNAP-25 in the superficial layers of the bladder and suggests that part of BoNT/A clinical effectiveness may be due to its action on SNAP-25 in the suburothelium.

Neuropeptide Y

NPY is a key co-transmitter of ACh in the urinary tract. It has been found to be present with nerves positive for VACHT immunostaining (Dixon et al., 2000), both in the suburothelium as well as on the detrusor of the human bladder. Our results have shown NPY IR present within the apical urothelium and in a suburothelial nexus of fibre like structures. Findings similar to ours have been seen in previous studies looking at suburothelium (Iwasa, 1993; Dixon et al., 2000).

This study demonstrated a significant increase in NPY IR in the suburothelium in patients with DO compared to asymptomatic controls. Given the close association of ACh and NPY, this would be in accordance with previous studies

showing an increased urothelial / suburothelial ACh release with age and stretch (Yoshida et al., 2006; Yoshida et al., 2008). Similarly, men with bladder neck dyssynergia have been shown to have a greater density of suburothelial NPY immunoreactive neurones (Crowe et al., 1995).

Rat bladders have shown depletion of NPY IR following prolonged distension, suggesting that NPY is a neurotransmitter involved in signal transduction (Lasanen et al., 1992). Postganglionic denervation of the bladder in rats resulted in a decrease in NPY nerves both in the suburothelium and detrusor, whereas preganglionic lesions with decentralisation resulted in an increase in NPY IR fibres in the bladder wall (Mattiasson et al., 1985). Radiation injury (>25Gy) has shown significant increases in the density of rat detrusor NPY IR nerves (Crowe et al., 1996).

Previous studies looking at human detrusor muscle had found decreases in NPY IR nerve fibres in patients with meningocele and spinal cord injury associated with partial nerve degeneration, as compared to women with stress urinary incontinence (Haferkamp et al., 2006). Our biopsies have not shown any evidence of degeneration, with no change in PGP 9.5 (Apostolidis et al., 2005b).

There is variability in the findings of NPY IR nerves and how they change in disease states. Although they have been noted in suburothelium, few studies have tried to quantify these nerves, especially in humans. This study is the first

to describe changes in suburothelial NPY with DO. As almost all nerves that contain ACh contain NPY (Dixon et al., 2000), it would be reasonable to presume that ACh containing nerves in the suburothelium would also be increased in DO.

The role of NPY in the urothelium is speculated to involved detection of stretch, however functional studies involving NPY (> 1 nM) have shown efferent effects by enhancing the force and frequency of spontaneous contractions and generating a rise in the resting tone of the detrusor; at higher concentrations NPY initiated detrusor contraction (Iravani and Zar, 1994). Pharmacological studies show NPY having two opposing actions, inhibition of adrenergic transmission and prevention of cholinergic disinhibition of NA release (Tran et al., 1994).

Following successful BoNT/A, our results showed a steady decrease in NPY IR towards control levels, although this was not statistically significant (baseline vs. 16 weeks, student t-test, $p=0.07$). NPY is found in dense core vesicles in suburothelial nerves and are released with neuronal activity however it is not known if they are SNARE dependent. It seems possible that BoNT/A decreases NPY IR similarly to its effect on ACh and ATP release, through its inhibitory action on SNARE vesicle release.

Although changes in NPY IR were noted in patients with DO and after BoNT/A, it is difficult to speculate about its role in the pathophysiology and treatment of DO without understanding normal NPY function. Future studies looking at the functional role of NPY or studies of KO mice will give us a greater understanding of its physiological function within the bladder.

Chapter 16 - Summary and Conclusions

Our key experimental findings show that:

- i. M_1 and M_3 muscarinic receptor IR density is decreased in patients with IDO and NDO, compared to control subjects.
 - ii. Muscarinic receptors M_1 , M_2 and urothelial M_3 IR increase following BoNT/A.
 - iii. No SNAP-25 IR is present within the urothelium.
 - iv. SNAP-25 IR is present extensively in the suburothelium, suggesting this may be the afferent site of action of BoNT/A.
 - v. There is a greater SNAP-25 IR density in the suburothelium compared to muscle.
 - vi. NPY, a cholinergic co-transmitter, has an increased IR density in DO compared to control subjects.
 - vii. NPY IR decreases after BoNT/A.
 - viii. There is a negative correlation between frequency and urgency and suburothelial muscarinic receptor density.
-
1. Our findings support the hypothesis that the urothelium and suburothelium play a crucial role in the afferent innervation of the bladder. It is likely that there is significant cross talk between different neurotransmitter systems, allowing modulation of the afferent signal to the CNS. We have identified key differences in these structures between

control subjects and patients, implying that they have a role in the pathophysiology of DO.

2. We had hypothesised that SNAP-25 would be present in the superficial layers of the bladder due to BoNT/A clinical efficacy in ameliorating urinary sensory symptoms. We were able to show the relative abundance of SNAP-25 labelled nerves in the suburothelium and in the muscle layers. SNAP-25 IR was not present in the urothelium and as expected its IR did not change in BoNT/A treated patients or in the control group.
3. It is clear from our experimental findings that BoNT/A has a significant effect on afferent pathways via its interactions with neuropeptides, receptors and neurotransmitters. BoNT/A does not just provide motor blockade at the smooth muscle layer, as originally proposed.

A. Detrusor Overactivity

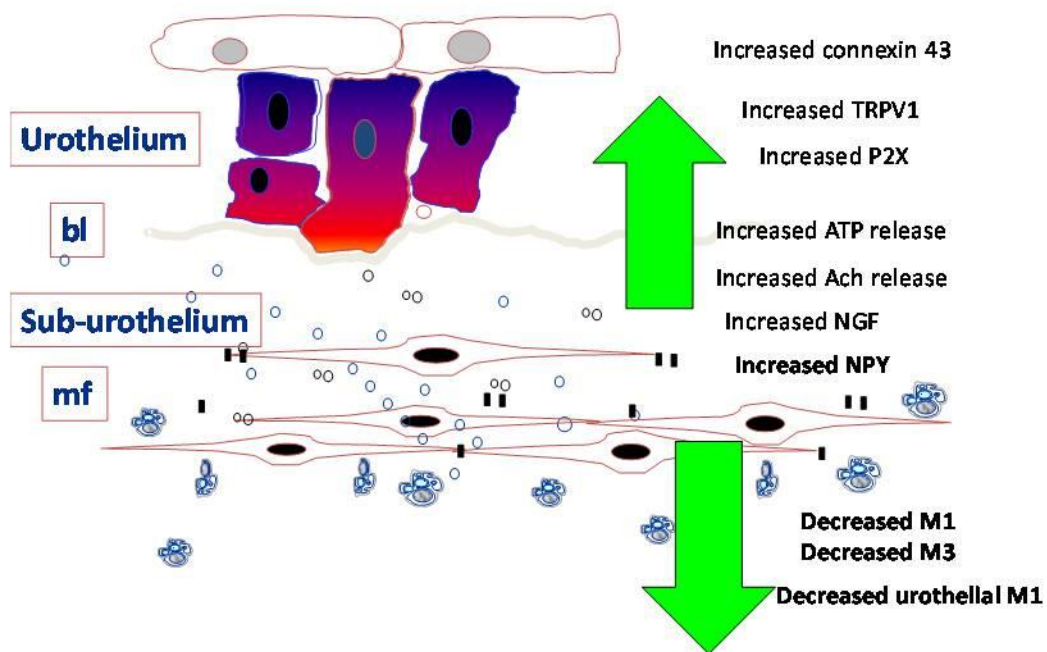
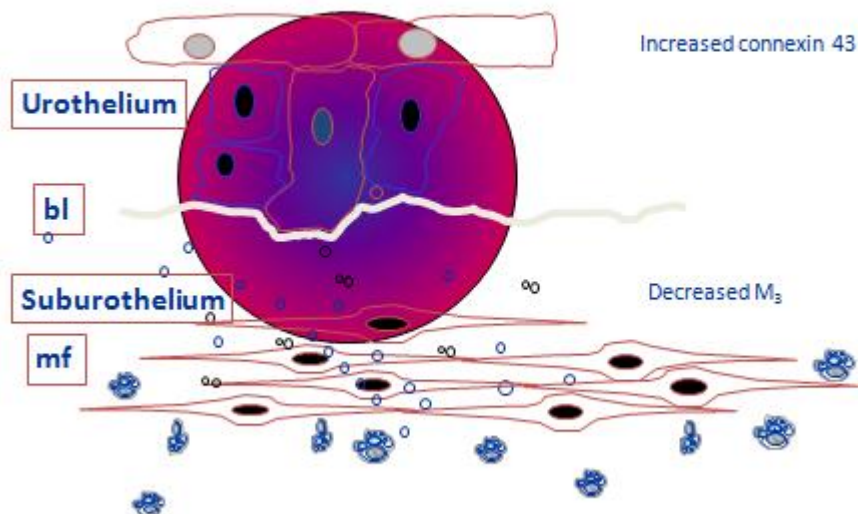


Figure 57: A) Summary of findings in Detrusor Overactivity found in the urothelium and suburothelium. B) Following BoNT/A treatment, all of neurotransmitters and receptors return towards control levels apart from M3 and connexin 43. Bl = basement layer, mf = myofibroblast.

B. After BoNT-A



Chapter 17 - Limitations

Our current experimental work was confined to the superficial layers of bladder due to the samples available for study. Possible future work looking at the outer muscle layer would help understand the effect of BoNT/A on the entire bladder. The effect on the efferent layer is likely to be considerable given the wide mechanism of action of BoNT/A.

Immunohistochemistry

We are aware of the methodological restrictions of semi-quantitative immunohistochemistry – but defend the position that it is the only available method to quantitatively examine the suburothelial space selectively. Slight differences in staining intensities are regarded as non-specific, due to the limited accuracy of the method.

Quantification of elongated structures is particularly difficult, as their orientation in the section determines their representation either as punctate or as fibre-like structures. Therefore, we used standardised procedures for tissue processing, staining and analysis during the entire study. Myofibroblast density increases towards the bladder neck (van der et al., 2004), so biopsies were taken consistently from same area. Theoretical limitations of the size of biopsies obtained via flexible cystoscopy have been addressed in several previous studies using identical protocols (Brady et al., 2004a; Apostolidis et al., 2005b;

Apostolidis et al., 2008) but the clinical ease of obtaining these specimens has enabled this work which would not otherwise have been possible.

Concerns have recently been raised about the specificity of muscarinic receptor antibodies used for immunohistochemistry (Jositsch et al., 2008; Pradidarcheep et al., 2009). It has been proposed that if multiple antibodies raised against different epitopes of the receptor show a similar pattern of IR, this could be considered evidence of their selectivity (Michel et al., 2009), supporting partly the specificity of at least our suburothelial immunostaining. Moreover, the use of negative controls and the apparently specific cellular immunostaining of mouse cerebellar and heart tissue (positive controls), together with a similar trend of muscarinic receptor mRNA changes seen in our biopsies (results not included here) and other studies, add to the argument for specificity of our results.

Antimuscarinic medication

At least half our patient population were on antimuscarinic medication when the study began; this percentage dropped to about one third of the patients at the 4- and 16-week follow-up time points. Although a synergistic effect of the concomitant use of antimuscarinic medication with BoNT/A was a theoretical possibility, our study showed both the expression of receptors and the clinical parameters examined before and after treatment were found to be no different between patients who use and those who did not use antimuscarinics. This may be in accordance with in-vitro animal experiments showing that the blockade of

urothelial and suburothelial muscarinic receptors had no effect on afferent discharge or response to bladder distension (Daly et al., 2010). Larger, specifically designed studies are needed to examine whether antimuscarinics have a synergistic effect with BoNT/A.

We understand that a further confirmatory technique would be useful, and we are currently looking at other experimental techniques like RT-PCT to detect any changes in muscarinic mRNA expression before and after BoNT/A.

Future Work

The use of PCR or western blot gel electrophoresis would provide confirmation of the findings of our study and would be the next logical step. We were limited by the biopsy sizes that could be taken from patients who were involved in our study. Double staining experiments would help elucidate whether muscarinic receptors are present on interstitial cells / myofibroblasts and would be a good future study to determine if these cells are involved in the afferent transduction process.

The site and role of the other muscarinic receptors M_4 and M_5 in the urothelium / suburothelium are yet to be elucidated.

As we learn more about the superficial layers of the bladder, we will develop a greater understanding about the individual systems and the sensory modalities

they convey, and be able to compare these with structures like the dermis which have been studied more extensively. Studying conditions like OAB and DO with therapeutic drugs like BoNT/A allow us to gain insight into the normal physiology of the bladder. The afferent neurotransmitter pathways of the bladder are yet to have their functional roles fully elucidated. The transduction pathways responsible for sensing heat, cold, stretch or pain are still unknown. Whether upregulation in one sensory pathway in the bladder can affect others is a key question in the patho-physiology of disease state. There are many questions yet to be answered.

Although understanding the physiology is important, manipulating it to help our patients improve their symptoms and quality of life is the ultimate goal for clinicians. Drug treatments for OAB and DO run into many billions of pounds and until BoNT/A (currently unlicensed), there had been little progress in new classes of medication. Currently our therapies are limited to antimuscarinic medications or major surgery, where efficacy is often limited and side-effects considerable. Targeting of urothelial/suburothelial receptors and ACh release mechanisms will permit testing of new drugs and allow us to develop novel strategies, eventually allowing development of targeted treatments efficacious solely on the sensory afferents.

The bladder gives an impression of simplicity, but just under the surface is a remarkably intricate sensory organ.

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Appendix A

University College London Hospitals

NHS Trust

Dr N Hirsch - Chair

Please address all correspondence to:
Ms Doreen Sharpe – Senior Ethics Administrator
Email: doreen.sharpe@uclh.org

The National Hospital for Neurology and
Neurosurgery and the Institute of Neurology
Joint Research Ethics Committee
Research & Development
1st Floor, Vezey Strong Wing
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30th April 2003

Our Ref: NH/ds/03L247

Professor Clare Fowler
Dept of Uro-Neurology
NHNN
Queen Square

Dear Professor Fowler

REC Ref No: 02/N023 (please quote in all correspondence)

Study Title: The mechanism of action of botulinum toxin in the treatment of the overactive bladder

Further to Dr Hirsch's letter of 24th July 2002, I can confirm that the decision of the Chair and Vice Chair to approve the above study was ratified at the ethics committee meeting on 15th August 2002. The committee had no comments.

Your application has been given a unique reference number please use it on all correspondence with the REC

Yours sincerely



Doreen Sharpe
Senior Ethics Administrator



UCL Hospitals is an NHS Trust incorporating the Eastman Dental Hospital, Elizabeth Garrett Anderson and Obstetric Hospital, Hospital for Tropical Diseases, The Heart Hospital, The Middlesex Hospital, National Hospital for Neurology & Neurosurgery and University College Hospital.



**The National Hospital for Neurology and Neurosurgery &
Institute of Neurology Joint REC**

(Research and Development)
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Our ref: NH/vih/05L 172

6 May 2005

Professor C J Fowler
Dept of Uro-Neurology
Mailbox 71
The National Hospital for Neurology and Neurosurgery
Queens Square
London WC1N 3BG

Dear Prof Fowler

Study title: *The mechanism of action of botulinum toxin in the treatment of the overactive bladder.*
REC reference: 02/N023

The REC gave a favourable ethical opinion to this study on 6 May 2005.

Notification(s) have now been received from local site assessor(s), following site-specific assessment. On behalf of the Committee, I am pleased to confirm the extension of the favourable opinion to the sites listed on the attached form.

Management approval

The Chief Investigator or sponsor should inform the local Principal Investigator at each site of the favourable opinion by sending a copy of this letter and the attached form. The research should not commence at any NHS site until management approval from the relevant NHS care organisation has been confirmed.

Statement of compliance


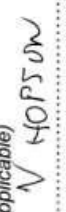
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference number: 02/N023	Please quote this number on all correspondence
-------------------------------	--

Yours sincerely,

Virginia Hopson
Temporary Administrator
Enclosure: Site approval form (SF1)

The National Hospital for Neurology and Neurosurgery & Institute of Neurology Joint REC				
LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION				
For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.				
REC reference number:	02/N023	Issue number:	1	Date of issue:
Chief Investigator:	Professor C J Fowler			
Full title of study:	The mechanism of action of botulinum toxin in the treatment of the overactive bladder			
This study was given a favourable ethical opinion by The National Hospital for Neurology and Neurosurgery & Institute of Neurology Joint REC on 01 January 0001. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.				

Principal Investigator	Post	Research site	Site assessor	Date of favourable opinion for this site	Notes ⁽¹⁾
Mr Tom Rosenbaum		Ealing Hospital NHS Trust Uxbridge Road Southall Middlesex UB1 3HW	Ealing Local Research Ethics Committee	06/05/2005	
<p>Approved by the Chair on behalf of the REC:</p> <p>  (Signature of Chair/Administrator*) (*delete as applicable)  (Name) </p>					

⁽¹⁾ The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension or termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.

Appendix B

Department of Health

MEDICINES CONTROL AGENCY

Market Towers 1 Nine Elms Lane London SW8 5NQ

Telephone 020 7273 0327

Facsimile 020 7273 0443

Room 12-241



Professor C J Fowler

UCLH

Department Uro-Neurology

National Hospital for Neurology and Neurosurgery

Queen Square

LONDON

WC1N 3BG

Our Ref: MF8000/11826

30 April 2002

Dear Professor Fowler

THE MEDICINES (EXEMPTION FROM LICENCES) (SPECIAL CASES AND MISCELLANEOUS PROVISIONS) ORDER 1972

PRODUCT: BOTOX® (Botulinum toxin A)

I am writing in connection with your notification under the Medicines (Exemption from Licences) (Special Cases and Miscellaneous Provisions) Order 1972 which relates to a proposed trial using **BOTOX® (Botulinum toxin A)** supplied by **Allergan Ltd.**

This exemption is effective from 30 April 2002; the above-named supplier may lawfully supply the product for the purpose outlined in your notification: the Licensing Authority have decided not to issue a direction under article 4(2)(v) of the Order. There is no need for a marketing authorisation or for a clinical trial certificate for the purpose of the trial.

Please note that all serious unexpected adverse reactions occurring during the trial should be notified to the Licensing Authority.

In coming to its decision the Licensing Authority has not evaluated the safety, quality and efficacy of the product, and this notice should not be taken to imply approval of the product in terms of safety, quality or efficacy.

We shall be pleased to see a copy of any report which is produced as a result of this trial.

Yours sincerely

Mrs S Syed

CLINICAL TRIALS UNIT

ddxappro

visit our website at <http://www.mca.gov.uk>



INVESTOR IN PEOPLE



**Medicines and Healthcare products
Regulatory Agency**

Market Towers
1 Nine Elms Lane, London SW8 5NQ

Direct Line 020 7084 2456
Direct Fax 020 7084 2443
Room 12- 242

CTA Number: 17022/0017/001-0001
Eudract Number: n/a

Professor Clare J Fowler
University College London Hospitals NHS Trust
National Hospital for Neurology and Neurosurgery
Dept of Uro-Neurology, Internal Mailbox 71
Queen Square
London WC1N 3BG

25 July 2005

Dear Professor Fowler

**THE MEDICINES FOR HUMAN USE (CLINICAL TRIALS) REGULATIONS
2004 S.I. 1031**

Product: BOTOX® (Botulinum toxin A)
Previously DDX No: MF8000/11826

Thank you for your letters dated 18 June 2004 and 20 July 2005, respectively.

Please accept my sincere apologies for any inconvenience the delay in responding to your request may have caused you.

Remark:

*Please note that your CTA Number is: 17022/0017/001-0001.

Yours sincerely

Dr Martyn Ward
Head of Clinical Trials Unit

RECEIVED 27 JUL 2005

An Executive Agency of the Department of Health
www.mhra.gov.uk



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Appendix C

Copy

University College London Hospitals

NHS Trust

Professor Allyson Pollock - Director of R&D
Dr Nick McNally - Directorate Manager
Mr Graham Petty - Finance Manager (R&D)
Ms Doreen Sharpe - Directorate Secretary
Mr Philip Diamond - Research Administrator
Ms Sabrina Balendra - Ethics Administrator

Research & Development
1st Floor, Vezey Strong Building
112 Hampstead Road
London NW1 2LT

Tel: 020 7380 9833/9940/9579
Fax: 020 7380 9937
Website: www.uclh.org

24 July 2002

R+D ethical
24/07/02

Professor Fowler
Department of Neuro-Urology
NHNN

Dear Professor Fowler,

Ref: 02/N023 (Please quote in all correspondence)
Title: The mechanism of action of botulinum toxin in the treatment of the overactive bladder

Thank you for registering the above study with the R&D Directorate. I am pleased to give the approval of UCL Hospitals NHS Trust for the study to proceed.

You will be aware that as principal investigator you have various responsibilities under the Department of Health's *Research Governance Framework for Health and Social Care*. Please note that you are required:

- to comply with the UCLH Information Security Policy (the R&D Directorate's data protection toolkit "Consent and Security" will help you meet the requirements of the Data Protection Act and is available at http://www.uclh.org/services/r_and_d).
- to ensure that any co-investigator who is not an employee of UCLH has in place an up-to-date honorary contract.
- to keep copies of all consent forms with your project documentation. UCLH will be carrying out audits of informed consent and if your project is selected for audit, you will need to provide access to the consent forms.

Please ensure that you have addressed any outstanding issues raised by the ethics committee before you start your project and to ensure that you comply with all the requirements of the ethics committee regarding progress reports and notification of protocol amendments and adverse events.

You are strongly recommended to use an investigator file to store all the documentation relating to this research project. This will help facilitate the research audit process which is now a research governance requirement. The attached list of headings is designed to help you assemble your investigator file.

Yours sincerely

P. O. Pollock

Professor Allyson Pollock
Director of R&D, UCL Hospitals NHS Trust



UCL Hospitals is an NHS Trust incorporating the Eastman Dental Hospital, Elizabeth Garrett Anderson and Obstetric Hospital, the Heart Hospital, Hospital for Tropical Diseases, The Middlesex Hospital, National Hospital for Neurology & Neurosurgery and University College Hospital.

Appendix D

The Mechanism of Action of Botulinum Toxin in Treatment of the Overactive bladder

Patient information sheet

Botulinum toxin is a neurotoxic drug safely used for the past 20 years to control various muscular disorders of a neurological origin. It is licensed for use in treating muscle spasms (in the neck and shoulders as well as eyelid and face), excessive sweating of the armpits and also foot deformities in children with cerebral palsy. It has also been shown to be useful in the treatment of spasticity, visual squints and some digestive tract problems. Botulinum toxin is not yet licensed for use in the bladder, however, in recent years physicians in both Europe and America have reported its successful use in the treatment of bladder overactivity, which is one of the commonest causes of urinary incontinence.

We are planning to study the effects of botulinum toxin treatment in patients with bladder overactivity and are interested in determining which factors are important in achieving improved continence whilst minimising the risk of urinary retention. We would like to invite you to undergo treatment with botulinum toxin as part of this study.

If you are interested in taking part in this study, we would like to take biopsies from your bladder before and after your treatment in order to examine the bladder nerve endings under a microscope. We hope this will give us useful information about how botulinum toxin is working in your bladder. Botulinum toxin is a very safe product, but as with any drug there may be side effects. In other studies, less than 1% of patients have complained of a rash, a transient flu-like illness and drowsiness. There have been two reported cases in literature of muscle weakness in the legs following treatment. There is also a small risk of needing to empty the bladder by catheterising yourself intermittently.

200-300 units of botulinum toxin will be injected into your bladder here in our department using a special bladder telescope (fibre-optic flexible cystoscope), which does not require general anaesthesia. Before the injections, 3-4 tiny pieces of bladder lining will be removed (biopsies) using special forceps introduced down the cystoscope. You will probably experience some minor discomfort during the procedure and you may notice a little blood in your urine, which is to be expected. We will give you antibiotics before the procedure to minimise the risk of infection. We will telephone you after 1 week to assess any side effects and ask you to return after approximately 4 and then 16 weeks to enable us to assess your symptoms and to take further bladder biopsies.

Your participation in this study is entirely voluntary. You are free to decline to enter or to withdraw from the study at any time and without having to give a reason. If you choose not to enter the study or to withdraw once entered, this will in no way affect your future medical care. All information regarding your medical records will be treated as strictly confidential and will only be used for medical purposes. Your medical records may be inspected by competent authorities and properly authorised persons, but if any information is released this will be done so in coded form so that confidentiality is strictly maintained. Participation in this study will in no way affect your legal rights.

The doctors involved in this study are Professor CJ Fowler, Mr V Kalsi and Mr P Dasgupta. You can contact them through the hospital switchboard at any time (number above) on extensions 3643, 3418 or 3259.

This research project has been reviewed by the National Hospital for Neurology and Neurosurgery and the Institute of Neurology Ethics Committee.

PATIENT INFORMATION SHEET –
14/12/04
Version 1.0 Study Number
02/N023

Uxbridge Road
Southall
Middlesex
UB1 3HW

Tel: 020 8967 5000

1. Research Title.

"Biopsies for examination of the nerves of the urinary bladder"

2. You are being invited to take part in clinical research.

Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Please take as much time to read the following information carefully and discuss it with friends, relatives and your doctor if you wish. Ask us if there is anything that is not clear or if you would like more information. We appreciate you taking the time to read, ask about, and understand the following information.

3. What is the purpose of the research?

We are conducting a study of nerve endings just beneath the surface lining of the bladder. We are comparing bladder samples of patients who have had a bladder injection treatment with normal healthy individuals. We will be using the bladder samples (biopsies) to look for specific nerves.

4. Why have I been chosen?

You will shortly be having a bladder examination using a special bladder telescope called a "flexible cystoscope" as part of your evaluation for blood in your urine. If your bladder is found to be healthy, we would like to take 2 very small biopsies (1-2mm) from your bladder. You may well have biopsies taken as part of the investigative procedure.

5. Do I have to take part?

No, it is up to you to decide whether or not to take part. You will be given this information sheet to keep. If you do decide to take part you will be asked to sign a consent form, and you will receive a copy of your signed form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

Whether you decide to take part in this study or not, your standard of care will not be affected.

6. What will happen to me if I take part?

Biopsies of the bladder will be taken with specially designed fine forceps, which will be inserted through the telescope already lying inside your bladder. There will, therefore, be no extra discomfort except for the sensation of a pinch in your bladder. Usually it is not painful.

Although there is no direct clinical benefit to you from taking part in the study, the results of this study are expected to be important in understanding normal and abnormal bladder function.

7. Are its side-effects?

Rarely, there may be minimal blood staining of your water the first time you pass urine, however this clears rapidly. Any mild soreness resulting from the procedure also wears off



Ealing Hospital is an Associated University Hospital of the University of London

quickly. There is a very small risk of an infection in your bladder following the procedure. You **will be given antibiotics** as an additional precaution against this.

8. What should I do after the procedure?

There will be no specific restrictions in your lifestyle, but you will be required to drink plenty of fluids for a day or two. **You should continue taking your regular medication, unless this includes aspirin, clopidogrel or warfarin. In this case, you should inform your doctor in advance, as this may affect your participation in the study.**

You will also be able to drive, take part in sport or give blood. If there is a little bleeding when you pass urine, it is best to avoid any stressful physical activities.

Your participation in the study will be strictly limited to this procedure and you will not be asked to visit the hospital again for purposes of this study.

9. Will my taking part in this research be kept confidential?

Your family doctor (General Practitioner) will be told that you have decided to take part in this research. Your health records will remain strictly confidential at all times. By signing the consent form you agree to this research procedure

10. Who has reviewed the research?

This study has been reviewed by The National Hospital of Neurology and Neurosurgery and Institute of Neurology Joint Research Ethics Committee together with Ealing Hospital's Ethics Committee.

11. Data Protection: What use will be made of the data and tissue collected from this research?

All information which is collected about you during the course of research will be kept strictly confidential.

Personal data, which may be sensitive, (e.g. date of birth) will be collected and processed, but only for research purposes.

The tissue samples from your bladder are being considered as a donation and parts of them or results from their examination may be used also for future research. Any new research project for which they would be used will be again reviewed by a research ethics committee. However, consent for use of your biopsies in future studies will only be required from you if the committee considers that the study is likely to affect your interests substantially.

YOUR RIGHTS UNDER ANY APPLICABLE DATA PROTECTION LAWS ARE NOT AFFECTED.

12. Contact for further information

Thank you for taking the time to read (or have read to you) the information about this trial. If you have any questions or concerns now or at any time about the trial, your safety or your rights, please contact any of the staff below if you would like further information on the following telephone number: **020 7837 3611**.

Mr Sam Datta	Research Registrar	020 78373611 x3169
Mr Shafi Chowdhury	Registrar	020 89675778
Mr T Rosenbaum	Consultant Urologist	020 89675778
Prof Clare Fowler	Professor of Uro-neurology	020 78373611 x3418

Appendix E

University College London Hospitals

NHS Trust

CONSENT FORM

National Hospital for Neurology and Neurosurgery
Department of Uro-Neurology
Internal Mailbox 71
Queen Square
London WC1N 3BG

Confidential

Telephone: 020 7837 3611 Ext 3418
Direct Line: 020 7676 2024
Fax: 020 7813 4587

The Mechanism of action of botulinum toxin in the treatment of the overactive bladder

Principal Investigator: Professor C J Fowler
Project no: 02/N023

Patient name:
Hospital no:
Date of birth:

1. I confirm that I have read and understood the information sheet the above study and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from Allergan Ltd. UK or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.
4. I agree to take part in the above study.

☐☐☐☐

Name of patient Date Signature

Name of Person taking consent Date Signature

If you have any comments or concerns you may discuss these with the investigator. If you wish to go further and complain about any aspect of the way you have been approached or treated during the course of the study, you should write or get in touch with the Complaints Manager, UCL hospitals. Please quote the UCLH project number at the top this consent form.

1 form for Patient; 1 to be kept as part of the study documentation, 1 to be kept with hospital notes

9 Jul 2004



UCL Hospitals is an NHS Trust incorporating the Eastman Dental Hospital, Elizabeth Garrett Anderson & Obstetric Hospital, The Heart Hospital, Hospital for Tropical Diseases, The Middlesex Hospital, National Hospital for Neurology & Neurosurgery, The Royal London Homoeopathic Hospital and University College Hospital.



Version 2

CONFIDENTIAL

Name:
Patient Number:
DOB :

Uxbridge Road
Southall
Middlesex
UB1 3HW

Tel: 020 8967 5000

Trial Title: Investigation into nerves of urinary bladder from control patient's biopsies.
Study Number 02/N023
Principal Investigator: Professor Fowler & Mr T Rosenbaum

CONSENT FORM

AGREEMENT TO PARTICIPATE IN A CLINICAL TRIAL

Your Consent	(Subject Initials)
1. I confirm I have read and understand the information sheet dated 14 December 04 for the above study and have had the opportunity to ask questions. I am aged 18-65.	
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3. I understand that sections of my medical records may be looked at by researchers and regulatory authorities where it is relevant to my taking part in research. I agree to this access. Your confidentiality will be kept at all times.	
4. I confirm that I have had sufficient time to consider whether or not I want to be included in the study.	
5. I agree not to restrict the use of any data or results, which arise from this study.	
6. I agree to take part in the above Study.	

Subject's Name (print): _____

Signed by Subject: _____ Date: _____
(Please date your own signature at the time of signing)

Doctor's Name (print): _____

Signed by Investigator: _____ Date: _____

1 form for Patient; 1 to be kept as part of the study documentation, 1 to be kept with hospital notes
Version 1 Date: 14.12.2004



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Appendix F

Abstracts from this thesis have been presented at the following meetings

1. *Cholinergic signalling pathways in the superficial layers of the human bladder; comparing health, disease and the effect of Botulinum Toxin Type A. ***Prize Winning Presentation* (Best of AUA - Incontinence section) AUA Meeting - Chicago May 2009***
2. *Superficial muscarinic receptors (M2 and M3) in patients with detrusor overactivity (DO) and the effect of Botulinum toxin type A (BoNT/A)*
BAUS Meeting - Manchester June 2008
3. *Suburothelial muscarinic receptors M1, M2, M3 in patients with detrusor overactivity (DO) and the effect of Botulinum toxin type A (BoNT/A).*
Prize winning presentation
European Association of Urology Meeting - Milan March 2008
4. *M1 muscarinic receptor immunohistochemistry in detrusor overactivity before and after treatment with Botulinum toxin*
British Association of Urological Surgeons Meeting – Glasgow - Podium presentation June 2007

Appendix G

The muscarinic receptor experiments have been published in the Journal of Urology in December 2010.

Immunohistochemical expression of muscarinic receptors in the urothelium and suburothelium of neurogenic and idiopathic overactive human bladders, and changes with botulinum neurotoxin administration.

Datta SN, Roosen A, Pullen A, Popat R, Rosenbaum TP, Elneil S, Dasgupta P, Fowler CJ, Apostolidis A.

J Urol. 2010 Dec;184(6):2578-85. Epub 2010 Oct 28.