The role of endocannabinoids and their receptors on the regulation of bladder function and detrusor overactivity

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by

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June 2015
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Wellbeing of Women Research Training Fellowship

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ABSTRACT

The endocannabinoid system (ECS) has been identified in the urinary bladder of humans and rats and is purported to play a modulatory role in detrusor overactivity (DO). Clinical studies demonstrated that cannabis improves urgency in patients with neurogenic DO. However, how endocannabinoids are regulated in vivo as well as the pathophysiological relevance of deregulation of the system in the aetiopathogenesis of DO remain unexplored. This thesis explores the clinical relevance of the ECS in the aetiopathogenesis of DO, by evaluating the effects of cannabinoid agonists on bladder contractions in vitro and in vivo.

Cannabinoid receptor (CBR) expression was localised in human and rat bladders. In vitro, ACEA (CB1 agonist) had both pre- and postsynaptic effects on bladder contraction, while GP1a (CB2 agonist) only had postsynaptic effect. CB1 receptor was co-localised with ChAT (choline acetyltransferase enzyme) in human detrusor but not in the urothelium, while CB2 co-localised with ChAT in both urothelium and detrusor. qRT-PCR showed that CB1 receptor was significantly upregulated by 2.8-fold in the urothelium of DO patients and downregulated by 3.2-fold in the detrusor of DO patients compared to normal.

Rat cystometry experiments demonstrated CP55,940 (CBR non-selective agonist) significantly increased micturition interval (MI) and bladder capacity (BC) by 52±5.5% and induced a 25 ± 2.9% decrease in maximal pressure (MP). Addition of CP55,940 to rats with induced bladder irritation, showed an increase in MI by 78% ± 16.7% and a 22% ± 5.8% reduction in MP.

In conclusion, cannabinoid receptors are involved in normal micturition at both peripheral and CNS sites, data supported by in vitro studies where CB1 reduced neuronal activity and where both cannabinoid receptors modulated bladder contractility. CB1 receptors expressed in afferent fibre endings in the urothelium could influence bladder function. CB1 receptor agonists may be useful for future treatment of DO as there is altered CB1 receptor expression in these bladders.
AUTHOR’S DECLARATION

I state that this thesis represents my own unaided work, except where acknowledged in the text, and has not been submitted previously in consideration for a degree at this, or any other university.

------------------------------------------
(08/05/2015)
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May 2015
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I am deeply indebted to my supervisors Prof. David G Lambert (Department of Cardiovascular Sciences, University of Leicester) and Prof. Douglas G. Tincello (Department of Health Sciences, University of Leicester) for their unwavering support, patience and continuous encouragement throughout my PhD studies. I especially want to thank Prof. Lambert for ‘adopting’ me as one of his students and for the guidance he has provided me throughout my studies. Without him I would not have been able to complete this degree. I would like to extend my heartfelt thanks to my former supervisors Dr Ruth Elliott and Dr Anthony Taylor who retired halfway through my degree, for their continuous support, expert advice and tutorage. I would also like to thank Dr John McDonald for his help and support with qRT-PCR and radioligand binding experiments.

I would also like to acknowledge Dr Yvonne Mbaki and Dr Rob Mason (School of Life Sciences, University of Nottingham) for our collaboration in this study and their extensive hand-on help for the in vivo experiments and for generously allowing me to utilise their lab and all their equipment.
DEDICATION

To my husband Panayiotis for his patience and support which made this hard work come to fruition and to my son Mario for brightening my days and always managing to put a smile on my face which gave me the determination to complete this work. Thanks for your love, understanding and encouragement in all my endeavours.

To my Mum and Dad for their enduring love and support and for teaching me the values of education.
AWARDS RELATED TO THIS THESIS

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Peer reviewed journals
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Abstract publications

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Presentations

Podium presentations

International Meetings


**National Presentations**


Bakali E, Elliott R, Willets J, Konje J, Tincello D. Distribution and function of the endocannabinoid system in the rat. *UKCS Bristol UK April 2011*


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Poster Presentations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin-Biotin Complex</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AEA</td>
<td>N-arachidonylethanolamide (anandamide)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BC</td>
<td>Bladder Capacity</td>
</tr>
<tr>
<td>BoNT-A</td>
<td>Botulinum toxin serotype A</td>
</tr>
<tr>
<td>BP</td>
<td>Basal Pressure</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAMK</td>
<td>Ca(^{2+})/calmodulin-dependant kinases</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CAMS</td>
<td>Cannabis in Multiple Sclerosis Study</td>
</tr>
<tr>
<td>CB1</td>
<td>Cannabinoid receptor 1</td>
</tr>
<tr>
<td>CB2</td>
<td>Cannabinoid receptor 2</td>
</tr>
<tr>
<td>CBₐₑ</td>
<td>Endothelial cannabinoid receptor</td>
</tr>
<tr>
<td>CCh</td>
<td>Carbachol</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>ChAT</td>
<td>Anti-choline acetyltransferase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca(^{2+})-induced Ca(^{2+}) release</td>
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<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CNG</td>
<td>Cyclic nucleotide-gated</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CP55,940</td>
<td>3-(2-hydroxy-4-(1,1-dimethylheptyl)phenyl)-4-(3-hydroxypropyl)cyclo-hexanol</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DAGL</td>
<td>Diacylglycerol lipase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide (</td>
</tr>
<tr>
<td>DO</td>
<td>Detrusor Overactivity</td>
</tr>
<tr>
<td>ECS</td>
<td>Endocannabinoid system</td>
</tr>
<tr>
<td>EFS</td>
<td>Electrical field stimulation</td>
</tr>
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<td>EL</td>
<td>Extracellular</td>
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<td>Exchange protein directly activated by cAMP</td>
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<td>Extracellular signal-regulated kinases</td>
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<td>G protein-coupled receptor-55</td>
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<td>Guanosine triphosphate</td>
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<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl-1-piperazine ethanesulfonic acid</td>
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<td>HIER</td>
<td>Heat-induced epitope retrieval</td>
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<td>Hour</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methyl xanthine</td>
</tr>
<tr>
<td>ICS</td>
<td>International Continence Society</td>
</tr>
<tr>
<td>IDO</td>
<td>Idiopathic detrusor overactivity</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Intracellular</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial Methylated Spirit</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>IPHFO</td>
<td>Intraluminal pressure high frequency oscillations</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LPI</td>
<td>Lysophosphatidylinositol</td>
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<td>LUTS</td>
<td>Lower urinary tract symptoms</td>
</tr>
<tr>
<td>MAGL</td>
<td>Monoacylglycerol lipases</td>
</tr>
<tr>
<td>MCLP</td>
<td>Myosin light chain phosphatase</td>
</tr>
<tr>
<td>MI</td>
<td>Micturition Interval</td>
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min  Minutes
MLCK  Myosin light chain kinase
MP  Maximum voiding pressure
MS  Multiple Sclerosis
NADA  N-arachidonoyl dopamine
NAPE  N-arachidonoyl-phosphatidylethanolamine
NAPE-PLD  N-arachidonoylphosphatidylethanolamine phospholipase D
NDO  Neurogenic detrusor overactivity
NGF  Nerve growth factor
NGS  Normal goat serum
NICE  National Institute for Health and Care Excellence
noladin ether  2-arachidonylglycerol ether
NSB  Non-specific binding
OAB  Overactive Bladder
OLDA  N-oleoyl dopamine
PAA  Palmitoylthanolamide-preferring acid amidase
PBS  Phosphate buffer solution
PCr  Phosphocreatinine
PCR  Polymerase chain reaction
PFM  Pelvic floor muscle
PFMT  Pelvic floor muscle training
PGP 9.5  Protein gene product 9.5
PIER  Proteolytic-induced epitope retrieval
PKA  Protein kinase A
PLC  Phospholipase C
PMC  Pontine micturition centre
PTX  Pertussis toxin
QoL  Quality of life
qRT-PCR  Quantitative Real-time reverse transcription PCR
RCT  Randomized control trial
RIPA  Radio Immune Precipitation Assay Buffer
ROCK  Rho-kinase
RT  Room temperature
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SD</td>
<td>Sprague-Dawley</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
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<tr>
<td>sec</td>
<td>Seconds</td>
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<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Synaptosomal-associated protein 25</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid-1</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
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<tr>
<td>UI</td>
<td>Urinary incontinence</td>
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<td>UTI</td>
<td>Urinary tract infection</td>
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<tr>
<td>UUI</td>
<td>Urge urinary incontinence</td>
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<tr>
<td>VACHT</td>
<td>Vesicular acetylcholine transporter protein</td>
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<tr>
<td>virodhamine</td>
<td>O-arachidonoyl ethanolamine</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
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<tr>
<td>(\Delta^9)-THC</td>
<td>(\Delta^9)-tetrahydrocannabinol</td>
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</table>
Chapter 1

General Introduction
Chapter 1 General Introduction

1.1 Overactive bladder and detrusor overactivity

1.1.1 Definitions

Lower urinary tract symptoms (LUTS) include urge urinary incontinence (UUI) and overactive bladder (OAB). OAB is defined as urgency, urge incontinence, frequency and nocturia, which the International Continence Society (ICS) recognises as a symptom syndrome suggestive of underlying detrusor overactivity (DO) (Haylen et al. 2010). UUI is an involuntary loss of urine combined with a sudden sensation of urgency with or without detrusor overactivity (Abrams et al. 2010). DO is a urodynamic observation and is one of the most common causes of both incontinence and storage symptoms in men and women and is characterised by spontaneous and/or provoked contractions of the detrusor muscle during bladder filling, demonstrable during urodynamic testing, something that cannot be suppressed by the patient (Abrams et al. 2010). DO is further divided into idiopathic DO (IDO), i.e., DO with no clear cause, and neurogenic DO (NDO), in which the patient has an underlying neurological condition (commonly multiple sclerosis, or after spinal injury).

1.1.2 Prevalence and health economics

Two large population-based studies, EPIC and NOBLE, have examined OAB in the context of urgency and UUI. The prevalence of urgency varied between 10.4% and 11.8% and was equal across sexes in both studies. The prevalence of UUI was significantly different between the sexes and varied between 1.8% to 2.6% for men and 3.9% to 13.4% for women. OAB symptoms in both studies were shown to increase with age independent of sex (Stewart et al. 2003; Irwin et al. 2006a). Calculations from the EPIC study estimate that in 2013 approximately 273 million women worldwide will have experienced urge symptoms and 30 million women worldwide will have had UUI (Milsom 2009). Furthermore, Cheung et al. (2009) indicated a prevalence of 82.9% of urinary incontinence (UI) in OAB patients.
A number of large trials using survey instruments have demonstrated that OAB is associated with significant personal and health-related burdens. Stewart et al. (2003) demonstrated that both men and women with OAB with or without UUI have significantly poorer scores on health-related quality of life measures. Patients with OAB with UUI had statistically more depression, stress and concern about urinary accidents especially in social situations, than patients without UUI (Irwin et al. 2006b). Female patients with urodynamically proven detrusor overactivity and urinary incontinence were found to have significantly worse sexual function using a validated questionnaire than women with normal urodynamics (Cohen et al. 2008). In addition, OAB is associated with more co-morbid conditions. A retrospective claims analysis comparing patients with OAB to age-matched controls demonstrated significantly increased prevalence of depression (10.5 % vs. 4.9 %), skin infections (3.9 % vs. 2.3 %), vulvo-vaginitis (4.7 % vs. 1.8 %), falls and fractures (25.3 % vs. 16.1 %), and urinary tract infection (UTI) (28 % vs. 8.4 %) (Darkow et al. 2005). Furthermore, analyses of the EPIC, EpiLUTS, and NOBLE databases have all demonstrated significant declines in work productivity in these patients, which in turn puts a burden on society and has significant cost implications (Sexton et al. 2009).

Given the significant prevalence of OAB, association with personal and societal burdens, poor health-related outcomes, and significantly more comorbid conditions, it is no surprise that there are significant costs associated with the diagnosis and treatment of OAB. OAB costs are related to pad use, pharmacotherapy, catheters, physician visits, outpatient and inpatient attendances and associated intangible costs, such as time and loss of productivity (Turner et al. 2004). The economic burden is significant and comparable to the cost of breast cancer, osteoporosis, and diabetes (Hu and Wagner 2000) with one study in Germany demonstrating the cost of OAB to be €3.57 billion per year, close to the €5.1 billion per year cost of diabetes and €5.6 billion per year cost of dementia (Klotz et al. 2007). Similarly, another study in the US estimated that the annual attributable costs of OAB were $825 per person with an aggregate annual total of $1.8 billion (Cisternas et al. 2009). Another study by Ganz et al., calculated annual per patient and total US costs for direct medical care (i.e., physician visits, laboratory tests), direct non-medical care (i.e., diapers, gloves) and indirect costs (i.e., lost productivity). They found that in 2007 the average annual per patient costs were $1925 ($1433 in direct medical, $66 in direct
non-medical, $426 in indirect costs) with total national costs of $65.9 billion ($49.1 billion direct medical, $2.3 billion direct non-medical, $14.6 billion indirect). They projected calculations for the future and found that annual per patient costs would be between $1944 and $1969 in 2015 and 2020 respectively, and that national costs would be $76.2 billion and $82.6 billion, respectively (Ganz et al. 2010).

1.1.3 Causes of OAB and DO

Depending on the underlying cause, two types of DO have been identified. NDO, which is a result of an upper motor neuron lesion leading to hyper-reflexia and increased tone in the reflex seen in patients with multiple sclerosis. IDO occurs when there is no defined cause with an incidence of symptomatic IDO estimated to occur in 10% of the general population aged between 20 and 60 years (Swami and Abrams 1996).

1.1.3.1 Neurological theory

Neurological causes of clinical significance predominantly involve failure of the storage phase and have been classified by Fowler (2006) as follows:

1. Central failure of inhibition of the pontine micturition centre (PMC): Central disorders, which disrupt the normal inhibition of the PMC during the filling phase will allow bladder emptying at inappropriate times, causing incontinence. Some causes include, dementia, frontal lobe diseases, stroke, multiple system atrophy and Parkinsonism. Focal brain disease may affect neural bladder control. It has been established that in stroke the most common cystometric finding is DO with normally coordinated voiding (Fowler 2006).

2. Spinal lesions that permit emergence of a segmental reflex, causing DO: it has been shown in cats that following reorganization of synaptic connections in the spinal cord, previously quiescent C-fibres form the afferent limb of the reflex, which mediates DO (de Groat et al. 1990). These C-fibres express transient potential vanilloid 1 (TRPV1) receptor on their surface and are sensitive to capsaicin. Changes in the properties of the C-fibres occur so that they become mechanosensitive and display increased electrical excitability. Provided some spinal cord function remains intact and bladder
sensation is preserved, the DO that develops following a spinal lesion is preceded by the sensation of urgency. Patients with multiple sclerosis develop NDO due to demyelination in the posterior and lateral columns of the cervical spinal cord (Nathan and Smith 1958).

3. Abnormal intrinsic bladder reflexes causing bladder overactivity, where the urothelium and sub-urothelium play a major role in initiating detrusor contractions (Birder and Andersson 2013). The urothelium is now recognised as having substantial sensory properties (Birder and Andersson 2013). The suburothelium and the lamina propria are known to be extensively innervated (Andersson and Arner 2004; Birder and Andersson 2013). Communication with the detrusor muscle through intrinsic bladder reflexes may be the basis for autonomous bladder activity (Fowler 2006).

1.1.3.2 Myogenic theory

It is proposed that smooth muscle cells of the detrusor should be considered as a syncytium and they have spontaneous contractile properties (Fry et al. 2004). However, during filling this spontaneous activity is not synchronised as the contact between the cells is not extensive. On the other hand, during voiding, a synchronous contraction occurs as a result of activation of a dense neural innervation, which is able to stimulate a large number of smooth muscle cells (Elbadawi et al. 1993). Studies employing electron microscopy in patients with IDO revealed a ‘dysjunction pattern’ where smooth muscle cells had an excessive number of distinctive protrusion junctions where cells closely abut each other (Elbadawi et al. 1993).

Furthermore, myofibroblasts found in the lamina propria have been shown to have close contact with unmyelinated axons and it has been proposed that interaction of these two structures may play a role in the response to bladder stretch and may act as part of the bladder volume sensing system (Wiseman et al. 2003). Fowler proposes that communication of this system with the detrusor smooth muscle through intrinsic bladder reflexes may be the basis for autologous bladder activity (Fowler 2006).
C-fibres located in the detrusor smooth muscle layer, may also be involved in the sensation of urgency as they are thought to be activated mainly by bladder filling to functional capacity and in pathologic situations where the functional or anatomic capacity is reduced. C-fibre input may thus have an important role in the generation of involuntary detrusor contractions and LUTS symptoms in patients with IDO (Andersson and Arner 2004).

### 1.1.3.3 Post-surgical DO

DO can occur following surgery for stress urinary incontinence with an incidence varying between 8 and 29% (Botros et al. 2006; Huang et al. 2006). Different hypotheses have been proposed for the cause of DO after surgery for stress incontinence, including nerve damage to the bladder during dissection, increasing patient age, and reaction to mesh (Tsui et al. 2008). It has been suggested that women undergoing stress incontinence surgery who had an elevated detrusor pressure during the filling phase of cystometry prior to surgery, were more likely to develop urgency and urge incontinence postoperatively (Alperin et al. 2008). It was hypothesised that patients with abnormally high filling pressure may be at an early stage in the DO disease process and are consequently at an increased risk for symptomatic urge incontinence postoperatively (Alperin et al. 2008). It has also been proposed that an immune hyper-reactivity caused by bacterial infection could be a causative factor for the symptoms associated with DO following surgery (Wang et al. 2008).

### 1.1.4 Evaluation of OAB and DO

Evaluation includes a detailed history supplemented with a voiding diary and preferably with a validated incontinence questionnaire. Parsons et al. (2007) analysed bladder diary patterns in women with DO and found that DO is characterised by high voiding frequency, low voided volume and symptoms of urgency with more ‘urge-than-activity’ related leaks. A physical examination of patients with urinary incontinence is important to check for aetiological conditions, such as a pelvic mass, that may contribute to urinary incontinence and that might affect the choice of treatment. A general examination including body mass index, an abdominal examination looking for scars and palpable bladder and a neurological examination concentrating on the sacral segments (S2-4)
should be performed. A perineal/vaginal examination to exclude pelvic organ prolapse and a rectal examination to assess anal tone should be performed. All patients should have a standard urinalysis to exclude urinary tract infection or suspected conditions, such as diabetes, that may cause urinary incontinence (UI). Urine culture for cytology may be needed if a test is positive for infection or if blood is detected. As recommended by National Institute for Health and Care Excellence (NICE), an assessment of residual urine should be made by measuring post-void residual volume by bladder scan or catheterisation in women with symptoms suggestive of voiding dysfunction or recurrent UTI (Smith et al. 2013).

After undertaking a detailed clinical history and examination, multi-channel filling cystometry can be considered. If they have symptoms of OAB leading to a clinical suspicion of DO, or if symptoms suggestive of voiding dysfunction or anterior compartment prolapse or previous surgery for stress incontinence, then voiding cystometry is recommended for all women who are awaiting surgery (Smith et al. 2013). Cystometry is not recommended by NICE prior to starting conservative treatment and ambulatory urodynamics or video urodynamics is recommended if the diagnosis is unclear after conventional urodynamics (Smith et al. 2013). Although not recommended by NICE (Smith et al. 2013), a pad test can be considered as an option for evaluation of UI.

1.1.4.1 Urodynamics in DO

DO is an urodynamic diagnosis made during the filling phase of cystometry, and is characterised by phasic detrusor contractions (Figure 1.1). Involuntary detrusor contractions occur in up to 76% of non-neurological patients with irritative bladder symptoms and in 100% of neurologically impaired patients with irritative bladder symptoms (Romanzi et al. 2001). These involuntary contractions are clinically relevant once the patient can simultaneously perceive urgency with those abnormal detrusor contractions.
Figure 1.1: Cystometry trace demonstrating detrusor overactivity in a patient undergoing multi-channel filling and voiding cystometry. Phasic contractions demonstrating DO are annotated on the detrusor pressure-measurement column. Image taken from www.sghurol.demon.co.uk.

1.1.5 Management of DO and OAB

All treatment interventions for DO and OAB are based on the information gathered during patient assessment, investigations conducted on the individual and specifically on answers to quality of life (QoL) questions. Three different treatment modalities are available; conservative, pharmacological or surgical, and each of these will be discussed in turn.

1.1.5.1 Conservative management

Changes to various lifestyle factors may have a role in improving OAB symptoms, such as reduction in caffeine (Creighton and Stanton 1990) intake and smoking (Hannestad et al. 2003). Weight loss (Hannestad et al. 2003; Subak et al. 2005) and timed voiding may
improve symptoms (Ostaszkiewicz et al. 2004b). Pelvic floor physiotherapy is well known for its use in stress incontinence (Bø et al. 1999; Dumoulin and Hay-Smith 2010) but may also be used in the management of urge incontinence (Lagro-Janssen et al. 1992; Albertsen 2005). The rationale is that detrusor muscle contraction can be reflex or voluntarily inhibited by pelvic floor muscle (PFM) contraction (Dumoulin and Hay-Smith 2010). Therefore, a single or repeated voluntary PFM contraction may be used to control urgency and prevent urge incontinence (Dumoulin and Hay-Smith 2010). Patients undergoing PFM therapy should be followed up by a physiotherapist to ensure compliance and that PFM therapy is performed correctly (Dumoulin and Hay-Smith 2010).

Behavioural strategies are designed to reduce the frequency of incontinence episodes by relearning a more appropriate behaviour (Ostaszkiewicz et al. 2004a; Albertsen 2005) as certain cases of OAB are thought to result from maladaptive learning behaviour (Wallace et al. 2004; Albertsen 2005). A Cochrane Database Systematic Review of published studies has demonstrated the effectiveness of bladder training in reducing urgency or urge urinary incontinence symptoms (relative risk 17; 95% confidence interval 1.13–256) (Wallace et al. 2004). Bladder retraining is the most commonly used technique whereby the patient is taught to suppress the dominant stimuli acting on the pontine micturition centre, using higher centres in the brain (Ashok and Wang 2010). This helps increase bladder capacity and reduce the number of episodes of incontinence but requires the patient’s active participation. If there is no reduction in incontinence episodes after three weeks of bladder re-training then the patient should be reassessed and further treatment offered (Wallace et al. 2004) A combination of lifestyle modification, bladder retraining and pelvic floor muscle training (PFMT) has an impact on improving urge incontinence episodes and improving the overall QoL (Wyman et al. 1998).

1.1.5.2 Pharmacological Treatment

Pharmacotherapy is the mainstay of treatment for urgency and urge incontinence and anti-muscarinics are still the standard first line therapy for OAB (Smith et al. 2013). However, the mechanism by which they may improve urgency is not well understood. Anti-muscarinics act during the storage phase where they compete with acetylcholine
(Ach) on muscarinic receptors leading to a decrease in the ability of the detrusor to contract (Andersson et al. 2008). During the micturition phase the parasympathetic activity leads to the release of large amounts of ACh (Andersson et al. 2008) and the action of anti-muscarinics is overwhelmed (Andersson et al. 2008). In addition, it is speculated that the mucosa could be affected by anti-muscarinics via the bloodstream as well as affecting luminal muscarinic receptors (Andersson et al. 2008). Therefore, anti-muscarinic preparations may target urothelial muscarinic receptors or urothelial release mechanisms. The main anti-muscarinics used are oxybutynin, tolterodine, trospium, propiverine, and solifenacin (Madhuvrata et al. 2012). All anti-muscarinics are associated with a good efficacy/tolerability profile (Buser et al. 2012) with the main side effects being dry mouth, constipation, tachycardia, palpitations, cognitive dysfunction and blurred vision (Buser et al. 2012).

When anti-cholinergic drugs are contraindicated or clinically ineffective then Mirabegron, a β3 agonist, can be used. β3 agonists act on a different receptor pathway than the anti-muscarinics (Andersson et al. 2008) by binding to and activation of the β-adrenoreceptors. Three subtypes of β-adrenoreceptors (β1, β2, β3) have been identified (Andersson et al. 2008) in the detrusor muscle and urothelium, with the β3 subtype most common in the human detrusor (Andersson et al. 2008). Stimulation of β3 adrenoreceptors results in direct relaxation of the detrusor by activation of Gs proteins and adenylate cyclase which results in an increase in cAMP levels (Aizawa et al. 2012). A phase 3 randomized control trial (Khullar et al. 2013) reported a statistically significant improvement in incontinence episodes in patients taking both 50 mg and 100 mg of Mirabegron over a 12 week period, when compared to placebo or 4 mg of Tolderidine. The long-term safety and efficacy for Mirabegron was assessed by another randomized control trial (RCT) (Chapple et al. 2013), where the prevalence of adverse effects were reported to be mild to moderate, (60% in each study arm for Mirabegron and placebo) with discontinuation due to these effects being 6% in both arms. Efficacy data demonstrated that improvements were maintained throughout the 12-month treatment period (Chapple et al. 2013). Mirabegron appears to be a potential second-line agent for the treatment of OAB with a distinct efficacy/tolerability balance (Chapple et al. 2013; Khullar et al. 2013). Recommendation for the use of these drugs has been published by NICE (Smith et al. 2013).
1.1.5.3 Surgical treatment

Surgical management of OAB/DO includes intravesical botulinum toxin, sacral neuromodulation, with augmentation cystoplasty and urinary diversion being reserved for end-stage disease.

1.1.5.4 Botulinum toxin

For refractory cases of OAB/DO, Botulinum toxin serotype A (BoNT-A) can be administered via an intravesical injection into the detrusor at 20-30 sites during either flexible or rigid cystoscopy under local or general anaesthesia. BoNT-A is a purified neurotoxin from Clostridium botulinum (Dykstra et al. 1988) that has recently been licenced for OAB in USA, Europe (including the UK). However, NICE recommends the use of BoNT-A for women with OAB caused by proven DO that has not responded to conservative and drug treatment (Smith et al. 2013). Women need to be counselled appropriately and need to have been trained and have performed clean intermittent catheterisation successfully prior to starting treatment with BoNT-A (Smith et al. 2013).

BoNT-A works by blocking the presynaptic release of acetylcholine at the neuromuscular junction by inactivating the synaptosomal-associated protein 25 (SNAP-25), a SNARE protein component produced by enzymatic cleavage (Dolly 2003). The resulting paralysis of the injected muscle is permanent, and regrowth of new motor end plate units is required to re-establish neuromuscular connection. In clinical terms, this can take anywhere between 3–24 months, at which point retreatment is required. Additionally, it is proposed that BoNT-A causes peripheral afferent desensitization through inhibition of vesicular release of ACh, ATP and substance P, and also by inhibition of axonal TRPV1 expression on the urothelium and sub-urothelial nerve endings. BoNT-A also decreases the expression of contractile gap junctions (Apostolidis et al. 2006).

The most commonly studied BoNT-A preparations for the treatment of DO are Botox® (Allergan Pharmaceuticals, Irvine, CA, USA; available in ampules of 100 units (U) or 200 U each); and Dysport® (Ipsen Biopharm Ltd, Slough, UK; available in ampules of 500 U each) (Mangera et al. 2011). Treatment with BoNT-A, has shown that complete continence occurs in approximately 31% of patients after a single BoNT-A treatment, which lasts for about 6 months and hence repeat treatments may be necessary (Tincello
et al. 2012). There is some evidence in refractory cases to anti-muscarinics, that BoNT-A has beneficial effects on symptom relief, urodynamic parameters and improvement in QoL (Apostolidis et al. 2009). NICE recommends that if BoNT-A treatment is effective, a follow-up treatment injection should be offered at six months, or sooner if symptoms return (Smith et al. 2013).

1.1.5.5 Sacral nerve neuromodulation and posterior tibial nerve stimulation

Patients with idiopathic detrusor overactivity who have failed all conservative and medical treatments and who cannot use intermittent self-catheterisation, can be offered sacral nerve neuromodulation. This works by the principle that activation of the afferent neurons in the pudendal nerve leads to inhibition of micturition by suppressing inter-neuronal pathways in the spinal cord as well as directly inhibiting parasympathetic preganglionic neurons (de Groat et al. 1981). Thus, modulation of the afferent input to the spinal cord segments involved in micturition may provide some control over the reflex arc (Ashok and Wang 2010). Sacral nerve and trans-cutaneous posterior tibial nerve stimulation act by stimulating the S3 nerve root using an implanted electrical pulse generator in order to provide relief from OAB symptoms and chronic non-neurogenic retention. During sacral nerve stimulation, this lead electrode is inserted into the third sacral foramina and a pulse generator is placed subcutaneously in the superior gluteal region. It has been shown that 70% of patients receiving sacral nerve stimulation have an improvement in urgency, frequency and urge incontinence compared to 4% in the placebo group (Leng and Morrisroe 2006).

Posterior tibial nerve stimulation is less commonly used and evaluation of its efficacy and safety still needs to be performed. Briefly, it involves a percutaneous insertion of a 34 G needle to the posterior tibial nerve behind the medial malleolus and a stimulation current (0-10 mA) at a fixed frequency of 20 Hz and pulse width of 200 ms, is applied until curling of the big toe is seen. Vandoninck et al. (2003) reported that 70% of patients showed a more than 50% reduction in the number of total leakage episodes, with 46% of these patients completely cured of urge incontinence after 12 sessions.
### 1.1.5.6 Augmentation cystoplasty/ Autoaugmentation

Augmentation cystoplasty is aimed to increase functional bladder capacity and improve bladder compliance, indicated for patients who do not respond to other forms of treatment and who present with severe symptoms. This is not commonly performed and is considered a “last resort” procedure. Augmentation can be done using a segment of the ileum, colon or even ureter (George et al. 1991) to achieve a low-pressure bladder with an increased functional capacity. The mean cure rate is 77.2% (George et al. 1991), but this procedure is associated with significant morbidity including recurrent UTIs, stone formation, persistent mucous production, metabolic acidosis, and tumour formation (George et al. 1991).

Autoaugmentation, or myectomy, can also be performed where excision of the detrusor over the entire dome is performed in order to reduce the intravesical pressure created by involuntary detrusor contraction. Finally, urinary diversion is an end-stage operation offered to those in whom all other alternatives have either failed or are inappropriate (Ashok and Wang 2010).

### 1.2 Anatomy and physiology of the urinary bladder

#### 1.2.1 Anatomy of the urinary bladder

The urinary bladder is an expandable smooth muscle organ whose function is the storage and disposal of urine. Storage of urine occurs when the bladder slowly distends under low pressure to contain a large volume of urine, whilst emptying is achieved by coordinated contraction of the bladder and relaxation of the urethral sphincter.

The anatomy of the urinary bladder is demonstrated in Figure 1.2a. It consists of the bladder dome, which is the region above the ureters, and this contains the detrusor smooth muscle. The region extending from the ureters to the urethra is called the bladder base or neck, which consists of the trigone, urethrovessical junction, deep detrusor, and anterior bladder wall (Gosling 1979). The bladder is lined by an epithelium (Figure 1.2b), that is continuous above, through the ureters with the lining epithelium of the renal tubules, and
below with that of the urethra. The transitional epithelium covering it, varies in shape and size according to the contraction and distention of the bladder (Andersson et al. 2008).

**Figure 1.2:** Anatomy of the female urinary bladder. Panel A shows the different structures of the urinary bladder while panel B is a histological image of the urothelium demonstrating the transitional epithelium, lamina propria and submucosa. Image taken from [www.wikipedia.com](http://www.wikipedia.com).

The muscular layer (detrusor) (**Figure 1.3**) consists of three layers of smooth muscle fibres. The fibres of the external layer (on the walls of the bladder) are longitudinal and are arranged obliquely, intersecting one another. The fibres of the middle circular layer are irregularly scattered, whilst the internal longitudinal layer is thin and its fasciculi have a reticular arrangement. The orientation and interaction between the different smooth muscle cells in the bladder determine changes in the bladder wall and affect bladder shape and intraluminal pressure (Andersson and Arner 2004).
1.2.1.1 **Key features of detrusor smooth muscle cells and smooth muscle contraction**

The bladder wall undergoes large changes in extension during normal filling and emptying (Andersson and Arner 2004). During filling of the bladder, the smooth muscle cells have to relax, elongate and rearrange in the wall over a very large length interval (Andersson and Arner 2004). In their relaxed state, cells are several hundred microns long, and five to six microns in diameter at their widest (Andersson and Arner 2004). During micturition, shortening is initiated, synchronized and fast over a large length range (Andersson and Arner 2004). The smooth muscle cells are arranged in muscle bundles and contraction of the smooth muscle results from the interaction between the contractile proteins, actin and myosin (Andersson and Arner 2004). The thin actin filaments are anchored to the membrane or cytoplasm and interact with the thick filaments through cross bridges formed by the heads of the myosin filaments (Andersson and Arner 2004).
For this contractile system to be initiated, a rise in intracellular calcium concentration \([\text{Ca}^{2+}]_i\) is needed and ATPase activity in the myosin heads needs to be activated by phosphorylation of one of the two light chains on the cross bridge (Andersson and Arner 2004). The enzyme responsible is myosin light chain kinase (MLCK), which is activated by a \(\text{Ca}^{2+}\)-dependent calmodulin complex (Arner and Pfitzer 1999). Calcium can enter the cytoplasm via L- and T-type calcium channels on the cell membrane or be released from intracellular stores, such as the sarcoplasmic reticulum (SR) (Wu et al. 1995). An increase of 50-100 nM from basal levels of sarcoplasmic \([\text{Ca}^{2+}]_i\) initiates a contraction and half maximal activation is achieved at about 1 \(\mu\text{M}\) (Wu et al. 1995). Calcium enters the cytoplasm during membrane depolarization, mechanical stretch or stimulation by agonists. Intracellular stores release calcium as a second means of increasing cellular calcium concentrations and it is an important step in the activation of detrusor muscle contraction (Andersson and Arner 2004). The release of \(\text{Ca}^{2+}\) from the SR is triggered by inositol trisphosphate (IP₃) via IP₃ receptors and by calcium (\(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release) via ryanodine receptors (Wu et al. 2002).

Dephosphorylation of the myosin light chain induces relaxation of the smooth muscle via the myosin light chain phosphatase (MCLP) (Gallagher et al. 1997). Sarcoplasmic \([\text{Ca}^{2+}]_i\) is reduced to resting levels by re-uptake into the SR using an adenosine triphosphate (ATP) \(\text{Ca}^{2+}\) pump (SERCA) (Ganitkevich and Isenberg 1992b). \(\text{Ca}^{2+}\) is either removed from the cell via the Na⁺/\(\text{Ca}^{2+}\) exchange and an ATP-dependent \(\text{Ca}^{2+}\)-pump, or re-accumulated in intracellular stores via a SERCA pump, which is inhibited by phospholamban and depletion of this protein leads to altered contractility (Nobe et al. 2001). The main pathways of cellular contractile activation are shown in Figure 1.4.

In addition, an important characteristic of detrusor smooth muscle is the ability of myogenic activity; defined as the ability of smooth muscle cells to generate contractile activity independent of external stimuli (Andersson and Arner 2004). Detrusor muscle exhibits spontaneous rhythmic activity both in vivo and in vitro (Brading 1992; Herrera et al. 2000; Drake et al. 2003). Myogenic activity may be exaggerated in conditions where an increased afferent activity leads to conditions of DO (Andersson and Arner 2004).
**Figure 1.4:** Intracellular signalling pathways involved in detrusor contraction via muscarinic M₃ receptors. Activation of the M₃ receptor by acetylcholine (ACh) activates phospholipase C (PLC) generating the second messengers inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to receptors on the sarcoplasmic reticulum (SR) and releases intracellular Ca²⁺ into the sarcoplasm. A rise of [Ca²⁺] near the surface of Ca²⁺ stores releases further Ca²⁺ through ryanodine receptors (RyR) by a process of Ca²⁺-induced Ca²⁺ release (CICR) to accelerate the increase of the sarcoplasmic [Ca²⁺]. Ca²⁺ binds to calmodulin (CaM) to activate myosin light chain (MLC) kinase and phosphorylate and activate myosin to bind to actin. M₃ receptor activation may also activate the rho-kinase pathway, which phosphorylates and reduces the activity of myosin light chain (MLC) phosphatase. Shown also is the influx of cations through the P2X₁ receptor ion channel and the L-type Ca²⁺ channel. Adapted from Fry et al. (2010).
1.2.1.2 Key features of the urothelium

The urothelium is a transitional epithelium, which influences detrusor function (Ikeda, and Kanai 2008), extending from the renal pelvis to the urethra. It is composed of three layers: a basal cell layer attached to the basement membrane, an intermediate layer, and a superficial apical layer, also called “umbrella cells” (Apodaca 2004). The umbrella cells are interconnected by tight junctions composed of multiple proteins including claudins, (Apodaca 2004) and are covered on their apical surface by crystalline proteins called uroplakins (Apodaca 2004). Umbrella cells act as a barrier to most substances found in urine (Apodaca 2004) and when damaged due to inflammation or injury, allow toxic substances to pass into the neural/muscle layers (Birder 2005). This may result in symptoms of urgency, frequency, and painful voiding (Birder 2005). Below the urothelium is a sub-urothelial layer containing a dense network of capillaries and afferent nerves, and also a network of interstitial cells connected by gap junctions (Sui et al. 2004). Interstitial cells are found throughout the muscle bundles within the interstitium of the bladder, but are mainly concentrated in the outer fibromuscular coat, where they form a dense interconnecting network (Smet et al. 1996). During bladder filling, interstitial cells may be important for the communication between urothelium and detrusor smooth muscle (Figure 1.5).
Figure 1.5: Schematic model of urothelial signalling and possible role of interstitial cells for detrusor and urothelial communication. Stretch of the bladder during filling may evoke activity in afferent nerves directly or by the interstitial cells. The interstitial cells may also mediate signals from other agents generated or released from the urothelium (e.g. PTX3 receptors: ATP; NO - soluble guanylyl cyclase, sGC). Stimulation of vanilloid receptors (VR1) on the urothelium, afferent nerves, and/or interstitial cells may also have effects on the detrusor muscle. Afferent nerves register changes in smooth muscle cells (force) induced by stretch, myogenic activity (contraction-relaxation), or transmitters (P2X1: ATP; muscarinic M2 and M3 receptors: acetylcholine (ACh)). Adapted from Andersson and Arner (2004)
1.2.1.3 Sensory function of the urothelium

The urothelium is thought to be a critical player in the sensory process of the bladder. Urothelial cells are able to communicate with underlying cells including bladder nerves, smooth muscle and inflammatory cells (Birder and Andersson 2013), through bladder afferent and efferent nerves localised in close proximity and within the urothelium (Birder et al. 2002). The sub-urothelium contains a network of capillaries, afferent nerves and interstitial cells, which are different to those in the detrusor (Davidson, and McCloskey 2005). Interstitial cells are extensively linked by gap junctions and have close contacts with nerves, which can respond to neurotransmitters such as ATP released from nerves or urothelial cells (Birder and Andersson 2013). This suggests they can act as intermediates in urothelial-nerve interactions (Ikeda et al. 2007). In addition, these cells are increased in number in DO bladders (Kubota et al. 2008).

Furthermore, supporting the hypothesis that urothelial cells play a role in sensation, is the expression of numerous receptors/ion channels found in nociceptors and mechanoreceptors (Birder 2010). Some of the “sensor molecules” identified in the bladder include the purinergic receptors (P2X1-7, P2Y1,2 and 4) (Burnstock 2001; Chopra et al. 2008) acetylcholine (muscarinic and nicotinic) (Chess-Williams 2002; Beckel et al. 2006), bradykinin (B1 and B2) (Chopra et al. 2005) and various TRP channels (TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1) (Birder et al. 2002). The expression of these receptors enables the urothelium to respond to stimuli, such as stretching, during bladder filling (Wang et al. 2005). This stimulates the release of neurotransmitters, such as ATP, acetylcholine, prostaglandins, prostacyclin, nitric oxide (NO) and several cytokines, which are capable of modulating sensory nerves, smooth muscle and cells of the immune and inflammatory systems (Apodaca et al. 2007). ATP, for example, can directly depolarise and initiate firing in sensory nerves by activating P2X or P2Y receptors on afferent nerves and on the urothelium (Cockayne et al. 2005) and stimulate intracellular second messenger pathways that will modulate other ion channels.

It has also been shown, that removal of the urothelium from in vitro detrusor preparations can significantly increase detrusor contraction as a result of acetylcholine stimulation.
(Hawthorn et al. 2000). This suggests that the urothelium has an inhibitory effect on bladder smooth muscle contractility possibly through the release of a factor not yet identified (Hawthorn et al. 2000; Birder and Andersson 2013). Consequently, the mechanism by which the release of chemical mediators occurs from the urothelial cells is poorly understood.

1.2.2 Physiology of the urinary bladder

1.2.2.1 Smooth muscle cell metabolism

ATP is the immediate substrate for the mechanisms leading to contraction and relaxation in the detrusor muscle (Andersson and Arner 2004). Cellular ATP is maintained by mitochondrial respiration, glycolysis and conversion of the high energy compound phosphocreatinine (PCr) and is rapidly decreased during contraction (Hellstrand and Vogel 1985). During bladder contraction cellular ATP concentration is maintained due to the 10-30% decrease in PCr (Hellstrand and Vogel 1985). Another important factor in smooth muscle metabolism is the movement of glucose through the GLUT1 glucose transporter (Mann et al. 2003). A large fraction of glucose is metabolised to lactate under aerobic conditions (Haugaard et al. 1987). Oxidative metabolism is the main source of ATP under normal conditions, as the ATP yield is higher for the aerobic metabolism of glucose.

The resting membrane potential of rat detrusor muscle cells has been reported to be 47 mV (Creed and Malmgren 1993), while in isolated human detrusor cells it was found to be between 47 and 55 mV (Montgomery and Fry 1992). Human detrusor demonstrates spontaneous action potentials associated with slow waves of depolarisation (Creed and Malmgren 1993). The frequency of the spontaneous action potentials is voltage sensitive, with depolarisation increasing and hyper-polarisation decreasing the rate of firing (Mostwin 1986). In the human detrusor, the depolarising phase of the action potential occurs by an inward Ca\(^{2+}\) influx while re-polarisation involves inactivation of the Ca\(^{2+}\) current and activation of an outward K\(^{+}\) current that is calcium dependent (Montgomery and Fry 1992) (Figure 1.6).
An increase in $[Ca^{2+}]$, is the main determinate of detrusor contraction, but it is not yet clear whether this is due to influx from extracellular stores and/or release from intracellular stores (Andersson and Arner 2004). Ganitkevich and Isenberg (1992a) found that Ca$^{2+}$ transport through voltage-gated Ca$^{2+}$ channels is the main factor in depolarisation-mediated changes in $[Ca^{2+}]$. In addition, they found that Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels resulting from depolarisation, results in a release of intracellular stores. Furthermore, Wu et al. (2002) suggested that Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels replenishes intracellular stores. A fall in [Ca$^{2+}$], reduces the activity of Ca$^{2+}$-activated K$^+$ channels, causing cell depolarisation and an enhancement of L-type Ca$^{2+}$ channel conductance. Another process of Ca$^{2+}$ influx is via Na$^+$-Ca$^{2+}$ exchange (Wu and Fry 1992). This exchanger can move calcium in or out of the cell depending on the cell’s resting potential.

The important K$^+$ channels in the detrusor muscle cells are ATP-sensitive (K$_{ATP}$) and Ca$^{2+}$-activated (BKCa), however, their role is less clear than the calcium channels.
described above and respond to intracellular ATP. Petkov et al. (2001) showed that activation of <1% of $K_{\text{ATP}}$ channels significantly inhibits action potentials and phasic contractions, showing how these channels regulate bladder contraction. In addition, activation of BKCa channels during an increase in intracellular Ca$^{2+}$, hyperpolarise the cell membrane and limit subsequent Ca$^{2+}$ entry (Fry et al. 2002), thus controlling basal tension and membrane potentials. Furthermore, ligand-gated ion channels in the detrusor muscle utilize a number of transmitters/modulators, however ACh and extracellular ATP are of immediate interest (Andersson and Arner 2004). Activation of P2X receptors opens a channel, which is non-selective to cations, generating an inward current carried mainly by Na$^+$ and Ca$^{2+}$. This leads to depolarisation, which is sufficient to activate L-type Ca$^{2+}$ channels, generating an action potential (Inoue and Brading 1991).

In detrusor muscle cells, mechanical stretch of the cell membrane has been suggested to activate non-specific cation channels (Wellner and Isenberg 1993; Wellner and Isenberg 1994; Wellner and Isenberg 1995). If a quiescent cell is stretched by >20% of its resting length, the Ca$^{2+}$ influx through the channel is sufficient to raise the [Ca$^{2+}$], significantly, and the total current will be enough to depolarise the cell sufficiently to open L-type Ca$^{2+}$ channels, and provoke Ca$^{2+}$ influx (Fry et al. 1998). These stretch-activated channels have the potential to act as length detectors in the bladder wall (Fry et al. 1998). It is suggested that activation of stretch-induced signalling in detrusor cells may have an important role in myogenic regulation of detrusor contractility during bladder filling and that inhibition of stretch-activated channels may improve bladder compliance (Tertyshnikova et al. 2003).

1.2.2.2 Neurological and hormonal control

Bladder contraction is mediated by both cholinergic and non-cholinergic (Ambache and Zar 1970; Taira 1972) mechanisms. Figure 1.7 shows the distribution of the adrenergic and muscarinic receptors in the urinary bladder.
Figure 1.7: Schematic representation of the distribution of α-adrenergic, β-adrenergic, and muscarinic cholinergic receptors throughout the lower urinary tract. The bladder body contains high density of β-adrenergic receptors, stimulation of which results in relaxation and urine storage, but contains only a few α-adrenergic receptors. In contrast, the bladder base and urethra contain a high density of α-adrenergic receptors, stimulation of which results in contraction, and only a small number of β-adrenergic receptors. Cholinergic muscarinic receptors are found throughout the bladder, however, the density of the muscarinic receptors is greater in the bladder body than the base. Adapted from Caine (1984).

1.2.2.2.1 Cholinergic mechanism

The cholinergic nervous system is primarily responsible for voiding (Longhurst and Uvelius 2001). Cholinergic muscarinic receptors are found throughout the smooth muscle of the bladder (Longhurst and Uvelius 2001). Experiments using cholinesterase inhibitors and muscarinic antagonists, such as atropine, suggested that in normal human detrusor muscle, the emptying contraction and the contraction resulting from electrical field stimulation is mediated mainly by activation of the muscarinic receptors, since these responses can be completely blocked by atropine (Kinder and Mundy 1985; O'Reilly et al. 2002; Palea et al. 1993). The muscarinic receptors predominately found in the human bladder are of the M² (80%) and M³ (20%) subtypes (Sigala et al. 2002) but M₁, M₄ and M₅ subtypes have also been identified, all of which are coupled to G proteins (Caulfield and Birdsall 1998). In humans, the M₃ receptors are believed to be the most important for
inducing detrusor contraction (Chess-Williams 2002). The function of M_{2} receptors has not yet been clarified and their action may increase in some pathological conditions (Braverman et al. 1999). M_{1}, M_{3}, and M_{5} receptors couple to G_{q11}, (Figure 1.4) activating phospholipase-C (PLC) (Andersson and Arner 2004). Activation of this enzyme generates the second messengers IP\(_3\) and diacylglycerol (DAG) from membrane phosphoinositides (PIP\(_2\)). IP\(_3\) binds to receptors on the sarcoplasmic reticulum and releases intracellular Ca\(^{2+}\) into the sarcoplasm (Berridge 1993). M_{2} and M_{4} receptors couple to a pertussis toxin sensitive G_{i/o}, leading to inhibition of adenylate cyclase (AC), resulting in a reduction of cAMP (Andersson and Arner 2004). This process leads to an increase of Ca\(^{2+}\)-sensitivity of the contractile proteins (Andersson and Arner 2004).

1.2.2.2 Adrenergic Mechanisms

Activation of β-adrenoreceptors results in smooth muscle relaxation (Anderson 1993). In contrast, activation of α-adrenoceptors leads to smooth muscle contraction (Anderson 1993). The distribution of the α- and β-adrenoreceptors varies in the bladder. β-adrenoreceptors have been shown to predominate over α-adrenoreceptors so the response of normal detrusor to norepinephrine is relaxation during bladder filling (Perlberg and Caine 1982). There are three types of β-adrenoreceptors present in the detrusor with β\(_{3}\) being the most abundant (Nomiya and Yamaguchi 2003). The β-adrenoreceptors are coupled to the G\(_{s}\)-protein, which leads to a rise in cAMP (Badawi et al. 2005) when stimulated by norepinephrine, resulting in detrusor relaxation (Perlberg and Caine 1982). Conversely, the bladder base and urethra contain large numbers of α-adrenoreceptors, which contract when stimulated by noradrenaline, and a small number of β-adrenoreceptors (Longhurst and Uvelius 2001).

1.2.2.3 Purinergic mechanisms

ATP is co-released with ACh from parasympathetic nerves, and activates purinergic receptors to initiate a contraction. There are two types of purinergic receptors that respond to ATP identified in the bladder, P2X (ion channel) and P2Y (G-protein coupled receptors) (Burnstock 2007). They have been located at or near the luminal surface of the urinary bladder, suggesting that ATP has an important role in chemical communication (Burnstock 2007). P2X receptors are ligand-gated cation channels and when activated, generate a depolarising Na\(^{+}\) and Ca\(^{2+}\) current that activates L-type Ca\(^{2+}\) channels to
generate an action potential and therefore cause a further rise in [Ca^{2+}] inducing a smooth muscle contraction (Wu et al. 1999). In adult human bladders, O’Reilly et al. (2001) found that P2X_1 was the predominant purinergic receptor at the mRNA level). P2X_1 receptor activation generates an inward, depolarising current of Na^+ and Ca^{2+} sufficient to activate L-type Ca^{2+} channels to generate an action potential and further Ca^{2+} influx (Wu et al. 1999) (Figure 1.4). The influx of Ca^{2+} can either directly activate the contractile proteins, or more effectively, raise the intracellular [Ca^{2+}] further by Ca^{2+}-induced Ca^{2+} release (CICR) from Ca^{2+} stores. On the other hand, P2Y are G-protein coupled receptors (Fry et al. 2010), which are believed to result in relaxation of the detrusor when activated via cAMP-dependent PKA activity (McMurray et al. 1998).

In regards to the urothelium, Chopra et al. (2008) showed the existence of functional urothelial P2Y_3/P2Y_4 receptors and activation of these receptors could have a role in autocrine/paracrine signalling throughout the urothelium. In addition, Fry et al. (2007) described functional P2Y_6 receptor expression within the suburothelial myofibroblasts, which may play an important role in integrating or amplifying the response to bladder distention. By activation of a population of sub-urothelial bladder nerves, urothelial-derived ATP release during distension could trigger sensations of fullness and pain or induce changes in bladder activity (Gur et al. 2007).

1.2.2.3 Micturition reflex and voiding

The urinary bladder is innervated by sympathetic, parasympathetic, and somatic afferent and efferent nerves. The sympathetic nerves originate in the thoraco-lumbar spinal cord and travel through the hypogastric nerves to allow contraction of the smooth muscle of the urethra and bladder base (Longhurst and Uvelius 2001). The sympathetic nerves also inhibit detrusor contraction through an action on parasympathetic ganglia (Longhurst and Uvelius 2001). The parasympathetic (pelvic) nerves originate from the sacral spinal cord (S2-4) having an important role in the coordination of micturition also referred to as the ‘spinal micturition centre’ resulting in contraction of the detrusor muscle (de Groat and Wickens 2013). The somatic motor activation to the striated urethral sphincter originates from Onuf’s nucleus in the sacral spinal cord and travels in the pudendal nerve (Thor and de Groat 2010). Activation of the somatic nerves results in contraction of the urethral
external sphincter (Fry et al. 2009; Thor and de Groat 2010). Finally, the afferent sensory nerves send sensory signals from the bladder wall and the urethra to the spinal cord connecting with structures in the brain (Fry et al. 2009). The sensory fibres detect the degree of stretch in the bladder wall and also carry sensations of temperature and pain (Fry et al. 2009).

Urinary storage and voiding are two distinct phases of the lower urinary tract. The storage phase is driven by the sympathetic system where during filling, afferent activity from stretch receptors increase and pass through the posterior roots of the sacral cord and the lateral spinothalamic tracts to the brain mediating the desire to void. Activity in the striated component of the urethral sphincter is increased keeping the external urethral sphincter contracted. During the voiding phase the sympathetic system is inhibited and there is increased parasympathetic neuronal activity which lead to removal of the central inhibition acting on the sacral centres. This leads to detrusor contraction and relaxation of the external urethral sphincter.

The Pontine Micturition Centre (PMC) is located in the brainstem and coordinates the micturition process (de Groat and Wickens 2013). PMC is under the control of the brain centres, which may switch the PMC to storage or micturition modes depending on afferent signals (de Groat and Wickens 2013). The brain centres controlling the PMC are critical in delaying voiding until socially convenient. This is achieved by inhibitory influences over the PMC arising from the prefrontal cortex and the periaqueductal gery area in the hypothalamus (Fry et al. 2009). When the PMC is switched to the storage mode, the PMC induces inhibition of the parasympathetic innervation and activation of the somatic nerves (Fry et al. 2009). In micturition mode, the parasympathetic nerves are activated allowing contraction of the detrusor muscle and inhibition of the somatic nerves leads to relaxation of the external urethral sphincter (Fry et al. 2009).

### 1.3 Cannabinoids

Over the past decade, interest in the role of endocannabinoids in regulating many mammalian processes has increased and has been proposed to be involved in the signal transduction mechanism regulating micturition (Gratzke et al. 2010; Mukerji et al. 2010). In a sub-analysis of a multi-centre, randomized controlled trial of “Cannabis in Multiple
Sclerosis” (CAMS) (Freeman et al. 2006), the effect of cannabinoids on reducing urge incontinence episodes without affecting voiding in patients with MS and NDO was tested. The 630 patients were randomized to receive an oral administration of the cannabis extract, Δ⁹-tetrahydrocannabinol (Δ⁹-THC) or matched placebo. Based on incontinence diaries, there was a 25% reduction (p=0.005) in the cannabis extract group and Δ⁹-THC showed a 19% reduction (p=0.039) in urinary incontinence episodes relative to placebo (Freeman et al. 2006), suggesting cannabis may modulate detrusor function. This clinical effect of cannabis is supported by the localisation and increased density of sub-urothelial CB1 nerve fibres in patients with idiopathic detrusor overactivity and painful bladder syndrome compared with controls (p=0.0123 and p=0.0013 respectively) (Mukerji et al. 2010). However, there are several possible CB receptor isoforms and subtypes and their anatomical distribution, through which the Δ⁹-THC effect is mediated, remains unknown. Since Δ⁹-THC acts on the brain, improvement in urgency and urinary incontinence episodes observed in the CAMS study might be attributed to the effects of Δ⁹-THC at any point in the peripheral nervous system and/or in the micturition centres of the central nervous system.

Cannabis consists of the aerial and root parts of Cannabis sativa, Cannabis indica and Cannabis ruderalis, which is an annual herb indigenous to central and western Asia and is cultivated in other tropical and temperate regions for the fibre used to produce ropes and carpets (Mechoulam 1986). There have been more than 60 cannabinoids identified in Cannabis extracts (Samuelsson 2010) of which the major psychotropic compound is Δ⁹-THC (Samuelsson 2010). Other constituents include cannabinol, cannabidiol, cannabigerol, cannabichromene and related acids (Samuelsson 2010). Cannabis has been mentioned in early Hindu and Chinese medicine and its use spread through Persia to Arabia at around the time of the 10th century (Abel 1980). The therapeutic effects of cannabinoids were studied in the early 19th Century by Irish physician Sir William B. O’Shaughnessy, who demonstrated the potential efficacy in a range of disorders including cholera, rheumatic diseases, delirium and infantile convulsions (O’Shaughnessy 1843). Historically cannabis has been used in obstetrics and gynaecology for the treatment of menstrual irregularity, dysmenorrhoea, hyperemesis gravidarum, childbirth, postpartum haemorrhage, menopausal symptoms and urinary symptoms (Hollister 1986). More common therapeutic applications of cannabis extract
include analgesia, migraine, muscle spasms, seizures, attenuation of nausea and vomiting of cancer chemotherapy, anti-rheumatic and antipyretic actions (Hollister 1986; Ben Amar 2006).

1.3.1 Cannabinoid receptors

The pharmacological effects of cannabinoids are mediated by three types of G protein-coupled receptors (GPCR) called CB1, CB2, G protein-coupled receptor-55 (GPR55). GPCRs are molecules that transverse the plasma cell membrane and activate intracellular signal transduction pathways and, ultimately, cellular responses (Wang and Ueda 2009). They are also called seven-transmembrane receptors because they pass through the cell membrane seven times (Figure 1.8) (Ji et al. 1998). GPCRs are characterised by an extracellular N-terminus, followed by seven transmembrane (7-TM) α-helices connected by three intracellular (IL-1 to IL-3) and three extracellular loops (EL-1 to EL-3) and an intracellular C-terminus (Ji et al. 1998). When a ligand binds to the GPCR it causes a conformational change that leads to its interaction with the residues of the extracellular loops and TM domains, allowing it to act as a guanine nucleotide exchange factor (GEF) (Northup et al. 2012). The GPCR can then activate an associated G-protein by exchanging its bound guanosine diphosphate (GDP) for a guanosine triphosphate (GTP) (Lodish et al. 2000). The G-protein’s α-subunit, together with the bound GTP, can then dissociate from the β/γ subunit complex to further affect intracellular signalling proteins or target functional proteins directly depending on the α subunit type (G_{αs}, G_{αi/o}, G_{αq/11}, G_{α12/13}) present (Wettschureck and Offermanns 2005).

Most GPCRs are capable of activating more than one G_{α}-subtype, but they show a preference for one subtype over another (Wang and Ueda 2009). The effector of both the G_{αs} and G_{αi/o} pathways is the enzyme adenylylate cyclase (AC), which catalyses the conversion of cytosolic ATP to cyclic-adenosine monophosphate (cAMP) (Lodish et al. 2000). This mechanism is stimulated by G-proteins of the G_{αs} class and conversely, interaction with G_{α} subunits of the G_{αi/o} type inhibits AC from generating cAMP (Lodish et al. 2000). The effector of the G_{αq/11} pathway is phospholipase C-β (PLCβ), which catalyses the cleavage of membrane-bound phosphatidylinositol 4,5-biphosphate (PIP2) into the second messengers inositol (1,4,5) trisphosphate (IP3) and diacylglycerol (DAG) (Lodish et al. 2000). IP3 acts on IP3 receptors found in the membrane of the endoplasmic
reticulum (ER) to elicit Ca\(^{2+}\) release from the ER, while DAG diffuses along the plasma membrane where it may activate any membrane localised forms of a second serine/threonine (ser/thr) kinase called protein kinase C (PKC) (Lodish et al. 2000). Since many isoforms of PKC are also activated by increased intracellular Ca\(^{2+}\), both these pathways can also converge on each other to signal through the same secondary effector (Massotte and Kieffer 2005). Elevated [Ca\(^{2+}\)], also binds and allostERICALLY activates the calmodulins, (Hwangpo and Iyengar 2005), which in turn bind and activate enzymes such as the Ca\(^{2+}\)/calmodulin-dependant kinases (CAMKs) (Hwangpo and Iyengar 2005). Finally, the effectors of the \(G_{\alpha12/13}\) pathway are three RhoGEFs (p115-RhoGEF, PDZ-RhoGEF, and LARG), which, when bound to \(G_{\alpha12/13}\) allosterically activate the cytosolic small GTPase, Rho (Hwangpo and Iyengar 2005). Once bound to GTP, Rho can then go on to activate various proteins responsible for cytoskeleton regulation, such as Rho-kinase (ROCK) (Hwangpo and Iyengar 2005). Most GPCRs that couple to \(G_{\alpha12/13}\) also couple to other sub-classes, often \(G_{\alphaq/11}\).

CB1 was first identified in 1988 (Devane et al. 1988) and subsequently cloned from rat cerebral cortex in 1990 (Matsuda et al. 1990). It is most widely expressed in central nervous system regions involved with pain transmission and is the most abundant cannabinoid GPCR in the brain (Pertwee and Ross 2002). It has also been located at a considerably lower density on neurons of peripheral tissues including the heart, vas deferens, urinary bladder and small intestine (Pertwee and Ross 2002). The CB2 receptor was cloned from human promyelocytic leukaemia cells (HL-60 cells) in 1993 (Munro et al. 1993) and is mainly expressed in immune tissues but is also expressed in low levels in the CNS in both microglia and some neurons (Gong et al. 2006). The localisation of CB2 receptors in immune tissues implies that some cannabinoid-induced immunosuppression involves a receptor-mediated process. The cannabinoid receptors are activated by natural ligands with arachidonylethanolamine (anandamide; AEA) being the first endogenous ligand to be isolated (Devane et al. 1988). Anandamide mimics the effects of \(\Delta^9\)-THC by binding to CB receptors, but lacks the psychoactive effects, probably because it is highly susceptible to enzymatic hydrolysis (Deutsch and Chin 1993). In smooth muscle the principal signal transduction pathway involving the cannabinoid receptors is via the phosphatidylinositol signal pathway, which is mediated by the various subunits of G-proteins (Wang and Ueda 2009).
Figure 1.8: The seven-transmembrane α-helix structure of a CB1 and CB2 G-protein-coupled receptors in humans. Each circle in the diagram represents one amino acid. The shaded bar represents the cell membrane. THC, anandamide, and other known cannabinoid receptor agonists bind to the extracellular portion of the receptor, thereby activating the signal pathway inside the cell. The CB1 receptor is larger than CB2. The receptor molecules are most similar in four of the seven regions where they are embedded in the cell membrane (known as the transmembrane regions). The intracellular loops of the two cannabinoid receptor sub-types are quite different, which might affect the cellular response to the ligand, because these loops are known to mediate G-protein signalling. Receptor homology between the two receptor sub-types is 44% for the full length protein, and 68% within the seven transmembrane regions. Image adapted from https://www.icmag.com.
Calcium acts as an intracellular messenger where it plays a key role in regulating basic cellular responses, such as migration and proliferation (Shabir and Southgate 2008). Under resting conditions, cytoplasmic calcium concentration is maintained at approximately 100 nM (Shabir and Southgate 2008). When stimulated, calcium enters the cell from extracellular stores via ion channels in the plasma membrane or it is released from intracellular stores through channels and receptors in the endoplasmic reticulum (Shabir and Southgate 2008). These channels may be activated and modulated by second messengers including IP₃, which is produced by binding of ligands to GPCRs, such as ATP (Galione and Churchill 2002).

The CB1 receptor is a member of the rhodopsin subfamily of GPCRs (Demuth and Molleman 2006). The three cytosolic loops and a putative fourth loop formed by palmitoylation at the juxtamembrane C-terminal region contribute to the activation of the G-proteins (Nie and Lewis 2001). The proximal CB1 receptor intracellular C-terminal domain is critical for G-protein coupling and the distal C-terminal tail domain modulates signal transduction (Nie and Lewis 2001). Most cannabinoid effects are sensitive to pertussis toxin (PTX) (Howlett et al. 1986) implicating CB1 and CB2 receptors couple to a Gᵢₒ protein. The binding of endocannabinoids and exocannabinoids to CB1 and CB2 results in a decrease of intracellular cAMP levels (Howlett and Fleming 1984) and activation of mitogen-activated protein kinase through the coupled Gᵢₒ proteins (Howlett et al. 1986; Bidaut-Russell et al. 1990). Cannabinoid-mediated inhibition of cAMP has been demonstrated in slices of rat hippocampus, striatum, cerebral cortex and cerebellum (Bidaut-Russell et al. 1990). CB1 can also stimulate the formation of cAMP through Gₛ under certain conditions (Howlett 2005). It may also be that CB1 receptors can exist as two distinct sub-populations, one coupled to Gᵢₒ proteins and the other to Gₛ (Bonhaus et al. 1998; Calandra et al. 1999) (Figure 1.9).

Additionally, activation of CB1 receptor modulates ion channels through Gᵢₒ proteins (Mackie and Hille 1992; Mackie et al. 1995), leading to the activation of A-type and inwardly rectifying potassium channels (Deadwyler et al. 1995; Henry and Chavkin 1995; Kobayashi et al. 2001). This is due to decreased phosphorylation of the channels,
as protein kinase A activity is decreased due to cannabinoid-induced inhibition of AC (Deadwyler et al. 1995). In addition, activation of CB1 causes a cAMP-independent, but $G_{i/o}$-dependent inhibition of N-type and P/Q-type calcium channels and activation of inwardly rectifying potassium channel proteins (e.g. GIRK1, GIRK2), leading to decreased calcium influx and increased potassium efflux (Mackie and Hille 1992; Mackie et al. 1995; Henry and Chavkin 1995).

The MAP kinase pathway is a key signalling mechanism that regulates many cellular functions, such as cell growth, transformation, differentiation, gene expression and apoptosis (Pearson et al. 2001). Activation of the MAP kinase pathway is associated with the activation of a tyrosine kinase-linked receptor, (Demuth and Molleman 2006) that activates the intracellular G-protein Ras and sets up a signalling cascade beginning with the activation of the serine/threonine kinase Raf (MAP kinase kinase kinase) (Demuth and Molleman 2006). Raf kinase activates MEK leading to phosphorylation and activation of MAP kinase, which phosphorylates other cytoplasmic and nuclear proteins (Demuth and Molleman 2006). CB1 receptors have been shown to link positively to MAP kinase via G-protein dependent and independent systems (Galve-Roperh et al. 2002). However, in contrast to CB1, CB2 receptor stimulation is believed not to modulate ion channel function, as demonstrated in AtT-20 cells transfected with CB2 receptors (Felder et al. 1995) and Xenopus oocytes transfected with CB2 (McAllister et al. 1999). Additionally, unlike CB1 receptors, CB2 receptors do not appear to couple to $G_s$, suggesting there is a difference between CB1 and CB2 receptor signalling (Glass and Felder 1997).

There is evidence that GPR55 is a novel cannabinoid receptor (Mackie and Stella 2006) that has a different signalling pathway to that of CB1 and CB2 (Lauckner et al. 2008). GPR55 is also a rhodopsin-like GPCR, and has been implicated in diverse physiological and pathological processes, such as inflammatory and neuropathic pain, bone development and cancer (Brown and Hiley 2009). However, GPR55 shares only low amino acid sequence identity with CB1 (13.5%) and CB2 (14.4%) (Pertwee 2007) and lacks the typical functional response elicited by these receptors. Activated GPR55 receptors couple to the $G_{i/o}$, $G_{12}$, RhoA, actin, phospholipase C pathway and triggers the release of Ca$^{2+}$ from IP$_3$R-gated stores, which leads to increased intracellular Ca$^{2+}$
(Lauckner et al. 2008) (Figure 1.10). GPR55 can be activated by putative endogenous agonist, lysophosphatidylinositol (LPI), and can be antagonized by CP55,940 and cannabidiol.

Figure 1.9: Schematic representation of CB1 signalling pathways. Activation of the CB1 receptor coupled to $G_{i/o}$ triggers a series of signalling pathways that involve: (1) inhibition of adenylate cyclase (AC) mediated conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and as a consequence inhibition of intracellular cAMP accumulation and inhibition of protein kinase A (PKA). (2) inhibition of N or P/Q type Ca$^{2+}$, (3) opening of K$^+$ channels and activation of inwardly rectifying potassium channels and (4) activation of the mitogen activated protein kinase (MAPK) pathways responsible for activation of extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (p38) to control cell proliferation, migration and apoptosis. Modified from Guindon and Hohmann (2011).
Figure 1.10: Suggested signalling mechanism of GPR55 showing antagonism by CP55,940. Adapted from Brown and Hiley (2009).
1.3.3 *The Endocannabinoid System*

The endocannabinoid system (ECS) consists of the cannabinoid receptors, their endogenous ligands, the enzymes involved in the synthesis and degradation of these ligands and the transport systems involved in the transfer of these ligands across the cell membrane. After the cannabinoid receptors were identified as the molecular targets for Δ⁹-THC, the endogenous ligands that bind to these receptors, were discovered (Devane *et al.* 1992). This group of bioactive lipid signalling molecules were collectively referred to as endogenous cannabinoids or endocannabinoids. *N*-arachidonoyl ethanolamide (anandamide, AEA) was the first endogenous ligand identified for the cannabinoid receptors in 1992, following isolation from porcine brain (Devane *et al.* 1992). Since then, a number of bioactive lipid signalling molecules with divergent affinities have been identified. Additional endocannabinoids include, *N*-docosatetra-7,10,13,16-enylethanolamine, 2-arachidonoylglycerol (2-AG), 2-arachidonylglyceryl ether (noladin ether), O-arachidonoyl ethanolamine (virodhamine), *N*-dihomo-γ-linoenooyl ethanolamine, *N*-docosatetraenoyl ethanolamine, oleomide, *N*-arachidonoyl dopamine (NADA) and *N*-oleoyl dopamine (OLDA).

### 1.3.3.1 Biosynthesis and degradation of N-acylethanolamides

AEA synthesis involves a series of enzymatic reactions, the final stage of which involves the enzyme *N*-arachidonoylphosphatidylethanolamine specific phospholipase D (NAPE-PLD). NAPE-PLD can be stimulated by Ca²⁺, Mg²⁺, Co²⁺, Mn²⁺, Ba²⁺ and Sr²⁺ and other organic cations (Liu *et al.* 2002). Spermine, spermidine, and putrescine are also stimulatory (Liu *et al.* 2002). Initial characterisation of NAPE-PLD revealed the enzyme to be membrane associated and it lacks the ability to catalyse a transphosphatidylation reaction, which is a common feature of other PLDs (Petersen and Hansen 1999). NAPE-PLD is the first PLD-type phosphodiesterase which belongs to the metallo-β-lactamase family (Wang *et al.* 2006) and unlike classical neurotransmitters and neuropeptides, its primary product, AEA is not stored in vesicles but synthesised and released “on demand”. This occurs in response to physiological and pathological stimuli, hormones (Piomelli 2003), neurotransmitters and depolarizing agents from its direct biosynthetic precursor *N*-arachidonoyl-phosphatidylethanolamine (NAPE) (De Petrocellis *et al.* 2004), a
phospholipid commonly found in biological membranes. Figure 1.11 shows an outline of the major pathways through which anandamide and 2-AG are produced and degraded.

Fatty acid amide hydrolase (FAAH) is the enzyme primarily involved in the hydrolysis of AEA, but can also degrade other endocannabinoids (Pertwee 2005). FAAH was first cloned and purified from rat liver microsomes (Cravatt et al. 1996), but is present in many other tissues and often in tissues containing CB1 and CB2 receptors. In addition to FAAH, AEA can also be degraded by palmitoylethanolamide-preferring acid amidase (PAA), cyclooxygenase-2, lipoxygenases and cytochrome P450 (Pertwee 2005).

2-AG was the second member of the endogenous cannabinoid family to be identified, and it too binds to both CB1 and CB2 receptors with similar affinities to AEA, although 2-AG has a higher affinity for CB2 receptors than CB1 (Pertwee and Ross 2002). The synthesis of 2-AG depends on the conversion of 2-arachidonate-containing phosphoinositides to diacylglycerols and their subsequent transformation to 2-arachidonoylglycerol by the action of two diacylglycerol lipase (DAGL) isozymes, DAGLα and DAGLβ (Pertwee and Ross 2002). Following their synthesis and release, these endocannabinoids are removed from their sites of action by cellular uptake and degraded by the enzymes monoacylglycerol lipase (MAGL) with a small amount being degraded by FAAH.
**Figure 1.11:** Major pathways for the synthesis and degradation of 2-AG and anandamide.
Cannabinoid agonists are classified by chemical structure into four main groups: classical; non-classical; aminoalkylindoles; and eicosanoids (Figure 1.12) (Nocerino et al. 2000). Classical cannabinoids are dibenzopyrane derivatives and include $\Delta^9$-THC, while non-classical cannabinoids consist of a bicyclic and tricyclic analogue of $\Delta^9$-THC that lack a pyran ring (Nocerino et al. 2000). One major practical difficulty associated with cannabinoid research both in vivo and in vitro, is the high lipophilicity and low water solubility of most CB1 and CB2 receptor ligands. This necessitates the use of a non-aqueous vehicle such as ethanol, dimethyl sulphoxide (DMSO), polyvinylpyrrolidone, Tween 80, Cremophor, Emulphor, bovine serum albumin, or the water soluble emulsion Tocrisolve 100, which is a mixture of soya oil, Pluronic F68 and water (Howlett et al. 2002) so that the compound of interest can interact with the cell surface. Their inherent lipophilicity means that these compounds “stick” to equipment during treatment, which needs to be taken into consideration during experimental procedures.
Figure 1.12: Chemical structure of some cannabinoid ligands.
1.3.5 Interactions of cannabinoids with other receptor systems

1.3.5.1 Opioid receptors

Like cannabinoid receptors, opioid receptors are seven-transmembrane GPCRs coupled to $G_{i/o}$, which participate in a number of biological responses. Just like cannabinoid receptors, their activation stimulates intracellular signalling mechanisms including activation of inwardly rectifying potassium channels and inhibition of voltage N-type Ca$^{2+}$ channels and AC activity (Waldhoer et al. 2004). Opioids induce their pharmacological effects by activating $\mu$-, $\delta$-, and $\kappa$-opioid receptors (Mansour et al. 1995) and share several actions with cannabinoids including hypothermia, hypotension, motor depression and anti-nociception (Bloom and Dewey 1978). An interaction between the cannabinoid and opioid systems in the caudate putamen has been suggested (Corchero et al. 1999). Rats administered repeated doses of $\Delta^9$-THC showed a 50% down regulation of CB1 mRNA levels in the caudate putamen after 7 days compared to the vehicle control (Corchero et al. 1999). After 14 days, a decrease of 40% was still observed but there was a concomitant increase of proenkephalin gene expression and $\mu$-opioid receptor activation of G-proteins (Corchero et al. 1999) suggesting a cannabinoid-mediated increase in opioid peptide synthesis. Cannabinoids and opioids might thus interact at the level of their signal-transduction mechanisms since opioid and cannabinoid receptors are coupled to similar intracellular signalling mechanisms (Manzanares et al. 1999).

1.3.5.2 The transient receptor potential vanilloid-1 (TRPV1) receptor

TRPV1 is a nonselective cationic channel that induces Ca$^{2+}$ influx and facilitates depolarization of neurons (Birder et al. 2002). In the urothelium, it behaves as a sensor molecule, which has an important role in nociception and urinary bladder function (Szallasi 2001). It can be activated by heat above 43 ºC, acid pH <6.0 and capsaicin; a compound found in chilli peppers, which can cause painful sensations (Caterina et al. 1997). TRPV1 has been localised throughout the afferent aspect of the micturition reflex pathway, including the urinary C-fibre nerves, which detect bladder distention (Chancellor and de Groat 1999; Birder et al. 2002), but also on other non-neural cells
(Ost et al. 2002). In addition to TRPV1 being expressed in the nerve fibres of the muscular, submucosal and mucosal layers of the bladder, it has also been localised in urothelial cells (Birder et al. 2002). When TRPV1 is activated by capsaicin or resiniferatoxin, there is a rise in intracellular Ca\(^{2+}\) levels evoking transmitter (NO or ATP) release (Birder et al. 2001). Clinically, activation of this receptor is known to cause a burning pain sensation and increased reflex activity of organs, such as the urinary bladder (Ishizuka et al. 1994). Studies have shown that TRPV1 has an important role in bladder pathophysiology in relation to painful bladder syndrome (Vizzard 2000) and bladder overactivity accompanying cystitis (Sculptoreanu et al. 2005). In addition, patients with overactive bladder have shown increased TRPV1 mRNA expression in the trigonal mucosa (Everaerts et al. 2008).

The CB1 receptor and TRPV1 are co-expressed in a significant proportion of nociceptive primary sensory neurons, which innervate both somatic and visceral tissues (MacNaughton et al. 2004). The cannabinoid system interacts with the TRPV1 via anandamide. Anandamide is structurally related to capsaicin, so it is also known as an agonist for TRPV1 and so could be an endovallinoid (Smart et al. 2009). Interestingly, AEA binds to an intracellular domain of TRPV1 (De Petrocellis et al. 2001), whereas the binding site for AEA for CB1 is on the extracellular side. Avelino and Cruz (2006) postulated that anandamide contributes to the development of bladder overactivity via activation of TRPV1. They found that application of a high concentration of exogenous anandamide to normal bladders increased the frequency of bladder contractions (Avelino and Cruz 2006). Furthermore, it is speculated that the contractile response of bladder tissue mediated by anandamide is partly due to activation of prostanoid EP1 receptors due to production of prostaglandins (Saitoh et al. 2007), but this is yet to be confirmed. In addition to anandamide, cannabidiol has been shown to be a TRPV1 agonist and to exert antipsychotic and analgesic effects through TRPV1 activation (Izzo et al. 2009), suggesting that TRPV1 is important to endocannabinoid signalling in the bladder.

### 1.3.6 Cannabis and the urinary bladder

Although the processes involved in the pathogenesis of overactive bladder as discussed above are now much better understood, the molecular and signal transduction
mechanisms involved including the endocannabinoid system remain poorly characterised (Mukerji et al. 2010).

### 1.3.6.1 Cannabinoid Receptor distribution in the urinary bladder

The effect of cannabis on NDO symptoms (Freeman et al. 2006) is probably mediated through a mechanism that depends on endocannabinoids. The mechanism of this effect is far from clear, and published data on the expression and functional sites of cannabinoid receptors in the bladder are contradictory. It is thought that endocannabinoids bind to CB1 and CB2, resulting in relaxation of the detrusor muscle during the filling phase (Gratzke et al. 2009). CB1 receptors are mainly found at the central and peripheral neuron terminals of the bladder, inhibiting neurotransmitter release (Gratzke et al. 2009). Several studies have localised both cannabinoid receptors in the urinary bladder of humans (Tyagi et al. 2009; Mukerji et al. 2010) rats (Hayn et al. 2008; Merriam et al. 2008), mice (Walczak et al. 2009), and monkeys (Gratzke et al. 2009). The localisation of CB1 receptors is in the urothelium and nerve fibres of the sub-urothelium and in human and rat detrusor muscle (Hayn et al. 2008; Walczak et al. 2009; Mukerji et al. 2010). However, other studies failed to detect the CB1 receptor in rat urothelium or nerve fibres but reported immunoreactivity for CB2 in these structures and in ganglion cells of the outflow region (Gratzke et al. 2009; Gratzke et al. 2010). In addition, human bladder studies identifying the presence of gene transcripts by quantitative polymerase chain reaction (qRT-PCR) and tissue expression and localisation by immunohistochemistry (IHC), revealed a higher abundance of the CB1 receptor in the urothelium compared to the detrusor (Tyagi et al. 2009). Similar results were found for CB2 but receptor protein expression was much lower when compared to CB1 receptor protein expression (Tyagi et al. 2009).

### 1.3.6.2 Cannabinoid Receptor function in the urinary bladder

Studies have demonstrated that the activation of presynaptic CB1 and CB2 receptors inhibits electrically evoked contractions in isolated mammalian bladder tissue with \( \Delta^9 \)-THC and the non-selective CB receptor agonists CP55940, CP55244, JWH015, (Pertwee and Fernando 1996; Martin et al. 2000; Gratzke et al. 2009), which corresponds to the localisation of CB1 receptors in nerve fibres of the detrusor muscle (Walczak et al. 2009).
In isolated mouse bladder, several cannabinoid receptor agonists, including WIN 55212-2, Δ⁹-THC and anandamide, inhibited electrically-evoked bladder contractions in a concentration-dependent manner (Pertwee and Fernando 1996). In the same study, it was shown that the inhibitory effect was not post-synaptic because contractile responses to muscarinic or purinergic receptor agonists were unaffected by pre-treatment with Δ⁹-THC (Pertwee and Fernando 1996). In rat detrusor muscle, cannabinor (a CB₂ selective agonist) did not have any effects on nerve-induced contractions (Gratzke et al. 2010). Similarly, in a study where human bladder muscle strips were used, there was no inhibitory effect of the non-selective CB agonist, WIN 55212-2, on electrical field stimulation (EFS) evoked contractions (Martin et al. 2000). In contrast, another study found an attenuation of EFS-evoked human detrusor contraction in the presence of both CB₁ (ACEA) and CB₂ (GP1A) agonists (Tyagi et al. 2009). These findings suggest cannabinoids act on pre-junctional nerve endings attenuating contractile responses (Tyagi et al. 2009).

Data supporting that cannabinoids act on pre-junctional nerve endings to attenuate a contractile response was provided by Gratzke et al. (2009), who demonstrated co-localisation of vesicular acetylcholine transporter protein (VACHT) nerve structures and CB₂ immunoreactive terminal varicosities. They also showed inhibitory effects of CP55,940 on nerve-mediated contractions but not on carbachol-induced contractions in detrusor preparations, suggesting a modulatory function of CB₂ on cholinergic neurotransmission (Gratzke et al. 2009). Similarly, cannabinor did not attenuate carbachol-induced contractions in isolated rat detrusor tissue, suggesting that the action of the CB₂ receptor is not directly involved in post-junctional regulation of smooth muscle contractility (Gratzke et al. 2010). A study by Capasso et al. (2011) showed that both pure cannabibidiol (CBD) and Cannabis sativa extract enriched with CBD also termed “CBD botanic drug substance” (CBD BDS), which are devoid of psychotropic activity, inhibited human and rat bladder contractility via a postsynaptic site of action.

The differences seen between the results of these studies may be due to inter-species differences in cannabinoid receptor expression and distribution, or the effect of these receptors on the release of contractile transmitters and anatomical variations in bladder innervation. Inter-species differences in the neuroanatomy of the mammalian bladders
are known to exist (Martin et al. 2000). For example, there are several parasympathetic ganglia in isolated bladder tissue from guinea pigs and humans while there are none in the urinary bladders of mice and rats (Gilpin et al. 1983; Gabella 1990).

Cystometric studies have shown an increase of the micturition threshold in rats receiving systemic cannabinoids in normal and inflamed conditions induced by acetic acid, cyclophosphamide (Hiragata et al. 2007) or turpentine oil (Dmitrieva and Berkley 2002). In rats, anandamide, WIN 55212-2 (synthetic CB non-selective agonist), and ajulemic acid (IP-751) (a synthetic THC analogue), suppress normal bladder activity and urinary frequency induced by bladder irritation, suggesting the inhibitory effects are least in part mediated by CB1 receptors (Jaggar et al. 1998a; Dmitrieva and Berkley 2002; Hiragata et al. 2007). A study by Gratzke et al. (2010) showed that CB2 receptor-mediated signals using the high affinity CB2 receptor selective agonist, cannabinor at 3.0 mg/Kg, increased micturition intervals and volumes by 52% (p<0.05) and 96% (p<0.01), respectively, and increased threshold and flow pressures by 73% (p<0.01) and 49% (p<0.001), respectively, in conscious rats during cystometry. It has not been clarified if these actions are related to CB receptors in the central nervous system, at peripheral sites in the lower urinary tract, or both. Furthermore, it is not known which of the two CB receptor subtypes is mainly responsible for the regulation of micturition in the different species.

1.3.7 Cannabinoid receptors as therapeutic targets

The most studied cannabinoid compound as a therapeutic agent is cannabidiol (CBD), which exerts a number of pharmacological effects, such as analgesia, anti-inflammatory, antioxidant, and antitumor (Izzo et al. 2009). It has been clinically evaluated for the treatment of anxiety, psychosis, and movement disorders and has been found to have a safe clinical profile (Izzo et al. 2009). CBD is one of the components of Sativex, which also contains Δ⁹-THC, a cannabis-derived drug used for the treatment of pain and spasticity associated with multiple sclerosis (Wade et al. 2006; Kavia et al. 2010). In a clinical survey, administration of Δ⁹-THC improved nocturia and detrusor overactivity in patients with multiple sclerosis (Consroe et al. 1997). Clinical studies have shown that Sativex reduces urgency, the number of incontinence episodes, frequency and nocturia in patients with multiple sclerosis (Wade et al. 2004; Brady et al. 2004a). Other cannabinoid
receptor agonists are already used clinically to suppress nausea and vomiting provoked by anticancer drugs (nabilone) (Ben Amar 2006) (see Table 1.1).

**Table 1.1:** Potential therapeutic applications of cannabinoid drugs

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Use</th>
</tr>
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<tbody>
<tr>
<td>CB1 receptor agonists</td>
<td>Treatment of cancer/post operative pain</td>
</tr>
<tr>
<td></td>
<td>Anticonvulsant</td>
</tr>
<tr>
<td></td>
<td>Anti-spastic in multiple sclerosis or spinal injury</td>
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<tr>
<td>Peripheral CB1 agonists</td>
<td>Appetite booster</td>
</tr>
<tr>
<td></td>
<td>Treatment of gut motility disorder</td>
</tr>
<tr>
<td>CB2 agonists</td>
<td>Treatment of peripheral ‘inflammatory’ pain immunosuppression</td>
</tr>
<tr>
<td>CB1 antagonists</td>
<td>Treatment of memory deficits</td>
</tr>
<tr>
<td></td>
<td>Treatment of obesity</td>
</tr>
<tr>
<td></td>
<td>Treatment of alcohol dependence</td>
</tr>
<tr>
<td>Peripheral CB1 antagonists</td>
<td>Treatment of gut hyper-motility disorders</td>
</tr>
</tbody>
</table>

*Data taken from Nocerino et al.(2000)*

### 1.4 Hypothesis

The presence of the ECS in the urinary bladder has led to speculation that endocannabinoid-signalling is involved in the signal transduction pathways regulating bladder relaxation and may be involved in bladder pathophysiology. On the basis of this, it was hypothesised that the binding of cannabinoids to CB1 and CB2 may result in relaxation of the urinary bladder during the filling phase and that dysregulation of the ECS in human bladder may be responsible for the aetiopathogenesis of OAB and DO.

#### 1.4.1 Main Objective

This hypothesis was tested by the performance of experiments designed to:

(a) Evaluate the expression and distribution of the endocannabinoid system in human and rat bladders and

(b) To determine the functional responses of cannabinoid receptors, *in vitro* and *in vivo*, in regulating bladder function after exposure to cannabinoid agonists
1.4.2 Aims

The experiments undertaken to achieve these objectives are presented in the following chapters:

Chapter 3
The aim of this chapter was to localise the cannabinoid receptors and the modulating enzymes in human and rat bladder tissue and assess their function in rat bladder strips in vitro.

Chapter 4
The aim of this chapter was to characterise human and rat urothelial cell lines in order to determine their suitability as potential models to study cannabinoid signalling. Changes in induced intracellular Ca\(^{2+}\) levels in the cell lines after activation of the cannabinoid receptors was investigated.

Chapter 5
The purpose of this chapter was to optimise the methodology to study the effects of cannabinoid agonists on carbachol-induced changes in intracellular Ca\(^{2+}\) levels in human bladder biopsies and to compare these results with those from bladder samples taken from patients with DO. Differences in expression of cannabinoid receptors between DO and normal human bladders were also studied. In addition, cannabinoid receptors were characterised further, to investigate their co-localisation with nerve terminals in the bladder muscle and urothelium.

Chapter 6
The aim of this chapter was to develop a robust and reproducible binding assay to measure cannabinoid receptor density in the urinary bladder.

Chapter 7
The aim of this chapter was to explore the influence of a non-selective cannabinoid agonist (CP55,940) on bladder function in vivo by cystometry in whole animals. In addition, the effects of the non-selective cannabinoid agonist on an overactive bladder model in whole animals was determined.
Chapter 2

General Methods
Chapter 2  Materials and Methods

2.1  Source of materials

All suppliers of materials are listed in alphabetical order.

**ABCAM** (Cambridge, UK): IHC/IF/WB- see Table 2.1 for the antibodies and fluorescent conjugates used and their catalogue numbers.

**Applied Biosystems** (Warrington, Cheshire, UK): qRT-PCR-RNAlater, mirVANA™ isolation kit, TURBO™ DNase, cDNA Reverse Transcription Kit, gene expression master mix, custom synthesised TaqMan™ probes for receptors of interest (Table 2.1).

**BDH** (Poole, Dorset, UK): Activated charcoal

**Bio-Rad Ltd.** (Hemel Hempstead, Hertfordshire, UK): **Protein estimation WB**- Bio-Rad Protein Assay, **WB**- Immun-Star Western C Chemiluminescent kit.

**Calbiochem** (UK): **WB**-phosphatase inhibitor cocktail

**Cayman Chemicals** (Cambridge, UK): see Table 2.1 for antibodies and catalogue numbers.

**Chemicon International** (Germany): see Table 2.1 for antibodies and catalogue numbers.

**Fisher Scientific** (Loughborough, Leicestershire, UK): **General reagents**-Potassium chloride, Potassium dihydrogen phosphate, Sodium chloride, TRIS-HCl, Folin’s reagent, HEPES, EGTA, Whatman G/F B filters, **scintillation fluids for radioligand binding assays**- ‘HiSafe3’, OptiPhase Safe.

**GIBCO/Invitrogen** (Paisley, UK): **cell culture**- Foetal bovine serum (FBS), albumin, Fungizone, L-Glutamine, Penicillin/streptomycin, Trypsin-EDTA, Tissue culture media
(RPMI advance 1640, DMEM, HAM’s F-12), epidermal growth factor, insulin, transferrin; **confocal microscopy**- Oregon Green 488 BAPTA-1 AM, Fluro-4

**Life Technologies** (UK): see Table 2.1 for the antibodies and fluorescent conjugates used and their catalogue numbers. Fluo4-AM powder

**Perkin Elmer** (Boston, USA): **radiolabelled compounds**- $[^{3}]$H-CP55,940 (100-180 Ci/mmol), $[^{3}]$H-cAMP (30-50 Ci/mmol)

**Sigma Aldrich** (Poole, Dorset, UK): see Table 2.1 for the antibodies and fluorescent conjugates used and their catalogue numbers. **General reagents**- H$_2$O$_2$, BSA, Calcium chloride, EDTA, Glucose, HEPES, Hydrochloric acid, Magnesium sulphate. **cAMP assay**- CyclicAMP, Triton X-100, Forskolin, 3-isobutyl-1-1methyl xanthine (IBMX); **cell culture**- selenium, hydrocortisone, triiodothyronine, MEM, Accutase; **IHC**-DPX; **WB**- protease inhibitor cocktail; **IF**- DAPI; **confocal microscopy**- pluronic F-127

**Thermoscientific** (Cramlington, Northumberland, UK): **WB**- Precise™ Protein Gels, protein molecular weight markers.

**Tocris Bioscience/R&D Systems** (Abingdon, Oxfordshire, UK): **Agonists and antagonists**- ACEA, GP1a, AM251, AM630, anandamide, CP55,940, Carbachol, ATP

**Vector Laboratories** (Peterborough, Cambridgeshire, UK): Vectastain® Elite ABC kit, 3,3’-diaminobenzidine substrate (DAB)- SK-4100, normal goat serum (NGS), Vectashield® Mounting Media
Table 2.1: Primary and secondary antibodies, blocking peptides and fluorescent conjugates used. All primary antibodies were raised in rabbit unless stated otherwise.

<table>
<thead>
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<th>Antibody/ Peptide</th>
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<th>Working dilution /Experiment</th>
<th>Manufacture/ Catalogue no.</th>
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<td>1:50/ICC</td>
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<td>1:100/ WB</td>
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<td>1:500/ WB</td>
<td></td>
</tr>
<tr>
<td>CB2 blocking peptide</td>
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<td>1:500/ ICC</td>
<td>ABCAM,UK/ ab5361</td>
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<td></td>
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<tr>
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<td>1:500/ ICC</td>
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<td>Cayman,UK/ 301600</td>
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<td></td>
<td>1:500/ WB</td>
<td></td>
</tr>
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<td>1:50 / IF</td>
<td>ABCAM, UK/ ab52870</td>
</tr>
<tr>
<td>Cytokeratin 20</td>
<td>Polyclonal</td>
<td>1:50 / IF</td>
<td>ABCAM, UK/ ab97511</td>
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<tr>
<td>mouse anti-choline acteyltransferase (ChAT) antibody clone 28C4</td>
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</tr>
<tr>
<td>mouse protein gene product 9.5 (PGP 9.5)</td>
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<td>1:50/IF</td>
<td>Abcam, UK/ Ab8189</td>
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<tr>
<td>secondary antibody (goat anti-rabbit horseradish peroxidase (HRP))</td>
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<td>secondary antibody goat, anti-rabbit IgG conjugated with FITC</td>
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<td>Sigma-Aldrich, UK/ F9887</td>
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<tr>
<td>secondary antibody Goat, anti-mouse IgG conjugated with Alexa Fluor 594</td>
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<td>secondary antibody goat anti-mouse IgG2A conjugated with Texas Red</td>
<td>Polyclonal</td>
<td>1:160/IF</td>
<td>Abcam, UK/ Ab51410</td>
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2.2 Buffer compositions

<table>
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<tr>
<th>Buffer Composition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP assay buffer</td>
<td>50 mM Tris-HCl, 4 mM EDTA, to pH 7.4 with NaOH</td>
</tr>
<tr>
<td>cAMP binding protein</td>
<td>stock prepared from bovine adrenal glands obtained from a local slaughterhouse. Working solution- 0.5 ml stock extract in 9.5 ml cAMP assay buffer</td>
</tr>
<tr>
<td>Charcoal suspension</td>
<td>250 mg activated charcoal, 100 mg BSA in 25 ml cAMP assay buffer</td>
</tr>
<tr>
<td>Krebs’/HEPES wash buffer</td>
<td>143 mM NaCl, 10 mM HEPES, 12 mM Glucose, 4.7 mM KH₂PO₄, 1.2 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgSO₄, to pH 7.4 with NaOH</td>
</tr>
<tr>
<td>Suspension assay buffer</td>
<td>0.5% BSA in Krebs’/HEPES wash buffer</td>
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</tbody>
</table>

Organ Bath and Confocal Microscopy

<table>
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<tr>
<th>Buffer Composition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs’ buffer</td>
<td>119 mM NaCl, 4.4 mM KCl, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 20 mM NaHCO₃, 11 mM glucose, to pH 7.4 with HCl</td>
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</tbody>
</table>

Fluorimetry

<table>
<thead>
<tr>
<th>Buffer Composition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvest buffer</td>
<td>154 mM NaCl, 10 mM HEPES, 1.7 mM EDTA, to a pH 7.4 with NaOH</td>
</tr>
<tr>
<td>Krebs’-HEPES buffer</td>
<td>143 nM NaCl, 11.7 mM Glucose, 10 mM HEPES, 4.7 mM KCl, 12 mM KH₂PO₄, 2.6 mM CaCl₂, 1.2 mM MgSO₄, to pH 7.4 with NaOH</td>
</tr>
</tbody>
</table>

Immunofluorescence (IF) and Immunohistochemistry (IHC)

<table>
<thead>
<tr>
<th>Buffer Composition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-T (0.2% Tween-20) (IF)</td>
<td>200 µM of Tween-20 in 9800 µM of PBS</td>
</tr>
<tr>
<td>PBS-T + 10% normal goat serum (IF)</td>
<td>1 ml of normal goat serum in 9 ml of PBS-T</td>
</tr>
<tr>
<td>TBS/ 0.05% Tween 20 (IHC)</td>
<td>25 mM Tris Base, 143 mM NaCl, 500 µl Tween 2, to a pH 7.5 with HCl</td>
</tr>
</tbody>
</table>
Radioligand Binding assays

*Binding assay buffer*  
Wash buffer plus BSA 1 mg/ml.

*Wash buffer*  
50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgSO₄, pH 7.4 with KOH

**Western Blots (WB)**

*Blocking solution (5% NGS)*  
5 ml of normal goat serum in 95 ml TBS-0.05% Tween20

*HEPES running buffer*  
100 mM tris base, 10 mM HEPES, 1 g of SDS

*Lysis buffer*  
10 μL of protease inhibitor cocktail per 1 ml of TBS-T buffer

*Protease inhibitor cocktail*  
4-(-2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEFSF), Aprotinin, Bestatin hydrochloride, N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64), Leupeptin hemisulfate salt and Pepstatin A

*Radio Immune Precipitation Assay Buffer (RIPA-modified)*  
150 mM NaCl, 0.1% SDS, 50 mM Tris, 1% IGEPAL CA-630, to a pH of 8.0

*Sample buffer*  
0.3 M HCl, 5% SDS, 50% glycerol, 100 mM dithiothreitol (DTT) and a pink tracking dye

*TBS/0.05% Tween 20*  
25 mM Tris Base, 143 mM NaCl, 500 μl Tween 2, to a pH 7.5 with HCl

*Transfer buffer*  
25 mM Tris base, 190 mM glycine, 0.4 g of SDS, 200 ml of 99.6% methanol in 1 L of distilled H₂O

### 2.3 Samples

#### 2.3.1 Human Bladder Biopsies

The collection of human bladder tissue was approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee (see appendix for letter) and all patients gave informed written consent.
Of the bladder biopsies taken from 34 women (45-76 years of age), 27 were taken from women who had no urinary symptoms at cystoscopy and who were undergoing elective gynaecological procedures (7 samples used for immunohistochemistry IHC and IF, 7 samples used for WB 5 samples for qRT-PCR and 6 samples for radioligand-binding). Biopsies were also taken from 9 women with DO demonstrated by urodynamics (Haylen et al. 2010). (5 samples were used for IHC and 4 samples for qRT-PCR). Samples were either stored in RNAlater to be processed for qRT-PCR or fixed in 4% (w/v) paraformaldehyde for 3 days before being embedded in paraffin for IHC and IF analyses. Tissues in paraffin blocks were cut as transverse sections (5 μm) on a Leica (model RM2035) microtome, floated onto Silane-coated slides and allowed to air dry for 3–5 days. Additionally, full thickness 1 cm² bladder samples (far away from the tumour margins) were taken from 4 men undergoing cystectomies for bladder cancer and these tissues were used for radioligand-binding assays. A further, 6 biopsies from normal female bladders were pooled and also used for radioligand binding assays. Patients admitting to using cannabis within three months prior to surgery were excluded.

2.3.2 Rat Brain and Bladder Samples

2.3.2.1 Animal Ethics and Housing

Female Wistar rats were housed at the University of Leicester in accordance with the Animals (Scientific Procedures) Act 1986. Animals were housed under standard conditions (12-hr light-dark cycle) with food and water ad libitum.

Following schedule I euthansia (pre-stunning followed by cervical dislocation), dissection of cortical tissue was made by removing the brainstem, cerebellum and midbrain areas, from 6 female Wistar rats (250-300 g) whilst the urinary bladder was removed through a midline abdominal incision. The cortex, cerebellum and urinary bladder from 6 rats were suspended in the relevant buffer, dependent on the post-analysis to be carried out. A further, 6 bladders were snap-frozen and 15 were fixed in paraformaldehyde as described above. Additionally, 24 bladders were placed in Krebs’ buffer solution and processed immediately for muscle contraction experiments.
2.4 Cell culture

Cells were maintained in sterile T75 culture flasks incubated at 37 °C with 5% CO$_2$ in humidified air. When confluent, cells were sub-cultured with trypsin-EDTA using an aseptic technique.

### 2.4.1 Cell lines

**UROtsa:** This cell line, derived from normal human urothelium lining the ureter, was immortalized using a Simian virus 40 large T antigen gene construct (Petzoldt et al. 1995). This cell line was a gift from Dr S. Garrett, University of North Dakota, USA and was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% (v/v) foetal bovine serum and 1 mg/ml glucose. Confluent flasks were sub-cultured at 1:4 ratio.

**HCV 29:** This cell line, derived from normal human urothelium, was a gift from Professor Helen McPherson, University of Leeds, Yorkshire, UK. HCV 29 cells were grown in RPMI 1640 medium containing 10% (v/v) foetal bovine serum and 1% L-glutamine. Confluent flasks were sub-cultured at 1:3 ratio.

**MYP 3:** The MYP 3 cell line was obtained from a small nodule that developed in a heterotopically transplanted rat urinary bladder after treatment with N-methyl-N-nitrosourea (Sen et al. 2007) and was a gift from Dr Sam Cohen, Department of Pathology, University of Nebraska, USA. MYP 3 cells were cultured in Ham’s F-12 supplemented with selenium (5 ng/ml), insulin (5 g/ml), transferrin (5 g/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and epidermal growth factor (EGF) (10 ng/ml), all dissolved in MEM. Confluent flasks were sub-cultured at 1:4 ratio.

### 2.4.2 Subculture and propagation

Cell medium was aspirated using a Pasteur pipette from the confluent monolayer and rinsed with 10 ml of sterile PBS. The monolayer was incubated with 2 ml of trypsin-EDTA or 5 ml of Accutase for either 2 min (HCV 29), 5 min (UROtsa) or 10 min (MYP 3) at 37 °C / 5% CO$_2$. The monolayer was detached from the culture flask by gentle agitation and 10 mL of fresh culture medium was added to the cell suspension.
Cells were fed by removing the spent medium and replacing it with 20 ml of fresh selection medium. The culture was then maintained as above until next subculture or use in experiments.

2.5 Reverse transcriptase PCR

2.5.1 Theory

Polymerase chain reaction (PCR) is one of the most widely used molecular techniques for making multiple copies of a gene. The core principle of PCR is the use of the enzyme DNA polymerase to make a copy of a single DNA strand by applying heat to first denature the double-stranded DNA followed by annealing of specific primers and then elongation of the second strand by the enzyme to produce double stranded DNA (dsDNA). PCR was made possible through the discovery of a heat stable DNA polymerase enzyme from the bacterium *Thermus Aquaticus* (Taq DNA polymerase) isolated from hot springs. This allowed the repeated and rapid replication of DNA strands at high temperatures at which human DNA polymerase is unsuitable. In addition, the invention of thermal cyclers, capable of heating and cooling to specific temperatures at rapid rates have made PCR almost routine.

PCR occurs in three stages facilitated by repeated thermal cycles (Figure 2.1). The first stage of each cycle is known as ‘denaturation’ where double stranded DNA is separated by heating the reaction to 94-98 °C. This disrupts the hydrogen bonds between complementary bases therefore yielding single-stranded DNA molecules. The ‘annealing’ second step occurs at a temperature 50-65 °C to allow the binding of primers (short portions of the DNA code chemically synthesised for both strands of the sequence) to the single-stranded DNA template. The polymerase binds to the primer-template hybrid and begins DNA formation. The final extension/elongation’ stage depends on the action of DNA polymerase which produces DNA strands in a 5” to 3” direction.
Figure 2.1: Three major steps in a PCR, which are repeated for 30 or 40 cycles using an automated cycler. Step 1 is Denaturation at 94 °C where the double strand melts and opens to a single stranded DNA. Step 2 is Annealing at 54 °C where the primers bind to the single-stranded DNA. Step 3 is Extension which occurs at 72 °C, in which nucleotides are added to the primers in the 5' to 3' direction to form a double-stranded copy of the target DNA. Temperatures and times stated are for illustration only, and do not apply to all cases. Figure adapted from Andy Vierstraete (www.chdiagnostic.com).
Traditional PCR may be used to detect a gene, however, reverse transcription PCR (RT-PCR) allows relative gene expression to be measured by measuring the amount of transcripts produced. RT-PCR is a PCR process that uses reverse transcriptase enzymes to make DNA from an RNA template. It detects and quantifies messenger RNA (mRNA), which is important for the study of gene expression and sequences. The mRNA can be isolated from a cell by the use of a phenol/chloroform extraction method and converted to complementary DNA (cDNA) by the action of the reverse transcription enzyme. There is only a small amount of mRNA in the cell, which is usually destroyed after translation so it never accumulates to very high concentrations, consequently RT-PCR can be used to make cDNA libraries of many thousand- to a million-fold.

Real-time reverse transcription PCR (qRT-PCR) allows the collection of data throughout the PCR process, combining the amplification and detection processes in a single step. This is achieved using a variety of fluorescent probes (e.g. SYBR Green, TaqMan) that correlate DNA concentration to fluorescence intensity (relative fluorescence units RFU or Rn). The fluorescence that is emitted is normalised against a passive dye, ROX, contained in the PCR master mix, which allows the change in fluorescence to be calculated (ΔRn) as shown in Figure 2.2. The greater the quantity of the target gene in the sample, the faster the rise in fluorescent signal, that will be detected thus leading to a lower cycle threshold (Ct) (Heid et al. 1996). An endogenous control, also known as ‘house-keeping gene’ is needed as it has a stable Ct because its expression does not change as a consequence of the experimental conditions. The relative quantification of a gene of interest (GOI) is achieved by normalising the Ct value to that of an endogenous control (EC), which corrects sample-to-sample variation. Normalisation of a GOI is achieved using the following formula: ΔCt = Ct^{GOI} - Ct^{EC}
**Figure 2.2**: C\textsubscript{t} and R\textsubscript{n} determination in a real time reaction. When ΔR\textsubscript{n} is plotted against the cycle number then the C\textsubscript{t} for the target gene is determined. The threshold is automatically determined by the PCR machine and the C\textsubscript{t} is the point at which fluorescence is detected when the reaction crosses over the threshold. 

C\textsubscript{t} = cycle threshold, R\textsubscript{n} = relative fluorescence

TaqMan™ probes are hydrolysis probes, which in addition to the traditional forward and reverse primers, rely on a third primer that binds downstream to the forward primer. This is called the TaqMan™ probe (Figure 2.3), which is a dual-labelled molecule with two fluorophores at either end of the probe. A reporting fluorophore (FAM or VIC in this study) is found at the 5’ end and a quencher (TAMRA) fluorophore is attached to the 3’ end. In the resting state the quencher fluorophore absorbs the reporter’s fluorescence. The probe is hydrolysed during the amplification stage, which separates the two fluorophores therefore allowing the free reporter fluorophore to fluoresce. Each amplification cycle will result in a release of more reporter dye and a greater fluorescence. These probes utilise the 5’exonuclease activity of Taq polymerase (Heid et al. 1996) that hydrolyse the probe during complimentary strand extension. Unlike SYBR Green, Taqman™ probes are very specific as they only bind to the gene of interest thus minimising the chances of non-specific products being detected.
**Figure 2.3:** A representation demonstrating fluorescence signal generation with the TaqMan PCR system. Both primers and probe anneal to the template DNA, fluorescence is low due to the proximity of the quencher to the probe. Extension of the complementary strands occurs in a 5” to 3” direction caused by Taq polymerase. The probe is irreversibly cleaved from the quencher by the 5” nuclease activity of the polymerase and a fluorescent signal is generated. Image taken from www.en.wikipedia.org.

### 2.5.2 Methodology

#### 2.5.2.1 Cell culture and RNA extraction

HCV 29, UROtsa, and MYP 3 cell lines were maintained in T75 flasks until they were 80% confluent as described in Section 2.4.2. Cells were washed twice with PBS before
addition of 1.2 ml of lysis/binding solution from the mirVANA™ isolation kit to one T75 flask. A homogenate lysate was formed through vigorous pipetting and placed in PCR-grade microfuge tubes to be used either immediately or stored at -80 °C until needed. Organic extraction was achieved by adding 1/10th of miRNA homogenate additive to the lysates, and incubated on ice for 10 min. A volume equal to that of lysate of acid-phenol:chloroform was added and the three phases (aqueous, interphase, protein) separated under centrifugal force (10,000 x g/ 5 min / at room temperature (RT)). The upper aqueous phase was measured and transferred to new PCR-grade microfuge tube. The RNA was precipitated with ethanol at 1.25 x the volume of the aqueous phase. Precipitates were transferred to filter cartridges and centrifuged (10,000 x g/ 20 sec/ RT) with the flow-through discarded. The RNA bound to the filter cartridge was subjected to three washes (solution 1 and solution 2 of the mirVana isolation kit) and then eluted from the filter using 100 μl of heated (90 °C) PCR-grade water. The mass of RNA was determined using an Eppendorf BioPhotometer and purity assessed from the 260/280 nm ratio, which was >1.75 for all samples. RNA samples were stored at -80 °C until DNase treatment.

2.5.2.2 DNase treatment

TURBO™ DNase was used to degrade genomic DNA (gDNA) in 5 μg of total extracted RNA. Briefly, the RNA sample was mixed with 5 μl DNase buffer, 1 μl DNase enzyme supplemented with PCR grade water to make a total reaction of 50 μl and the mixture incubated for 30 min at 37 °C. The DNase enzyme activity was then stopped by the addition of 5 μl DNase inactivating agent. The mixture was incubated at room temperature for 5 min and centrifuged (10,000 x g/ 1.5 min). The supernatant was removed and either stored at -80 °C or kept on ice to carry out the reverse transcription step.

2.5.2.3 Reverse transcription and cDNA synthesis

The synthesis of complimentary DNA from DNase-treated RNA was performed using a high-capacity cDNA Reverse Transcription Kit. The reverse transcription (RT) mixture was prepared according to the manufacturer’s instructions (Table 2.2). Two mixtures were prepared; one labelled RT+ and one labelled RT-. The RT+ mixture contained the
reverse transcriptase enzyme to allow gene expression to be quantified during PCR. The RT- reaction did not include the reverse transcriptase and was included as a negative control. An amplification signal detected on PCR from RT- sample indicates contamination or failure to remove all of the gDNA. 10 µl of DNase-treated RNA was added to 10 µL of RT mix in microfuge tubes and incubated according to the following thermal cycler program (Table 2.3). Samples were stored at -20 ºC to be processed for PCR.

**Table 2.2:** Components of high-capacity cDNA reverse transcription kit’s master mix shown as volume per reaction (µL).

<table>
<thead>
<tr>
<th>Component</th>
<th>RT+</th>
<th>RT-</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x RT buffer</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>25x dNTP mix</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>10x RT random primers</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Multiscribe™ reverse transcriptase</td>
<td>1.0</td>
<td>X</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>PCR- grade water</td>
<td>3.2</td>
<td>4.2</td>
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</table>

**Table 2.3:** Thermal cycler program for the high-capacity cDNA reverse transcription kit

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
<td>1</td>
<td>25 ºC</td>
<td>10 min</td>
</tr>
<tr>
<td>Reverse transcription</td>
<td>2</td>
<td>37 ºC</td>
<td>120 min</td>
</tr>
<tr>
<td>Enzyme inactivation</td>
<td>3</td>
<td>85 ºC</td>
<td>5 sec</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>4 ºC</td>
<td>indefinite</td>
</tr>
</tbody>
</table>

**2.5.2.4 Real-time PCR with StepOne thermocycler**

Reactions were prepared using the gene expression master mix and custom synthesised TaqMan™ probes for receptors of interest (Table 2.4). The gene expression master mix is an optimised mix that contains all the necessary constituents required for carrying out a PCR, namely: Amplitaq Gold® DNA polymerase UP (ultrapure), uracil-N-glycosylase (UNG), deoxyribonucleotide triphosphates (dNTPs) with deoxyuridine triphosphate (dUTP) replacing dTTP (deoxythymidine triphosphate), ROX passive reference dye and
the other buffer components required for carrying out a reaction. Eighteen µl of the reaction mix (Table 2.5) was added to 2 µl of cDNA template in a microtube strip, which was placed in the real-time PCR StepOne instrument (Applied Biosystems). The thermal profile of a reaction using the StepOne software is shown in Table 2.6.

### Table 2.4: Taqman™ probes for receptors of interest.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Species</th>
<th>Identifier</th>
<th>Dye Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH- (glyceraldehyde-3-phosphate dehydrogenase)</td>
<td>Human</td>
<td>4326317E-1110043</td>
<td>VIC®</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>4352338E-0906011</td>
<td></td>
</tr>
<tr>
<td>CB1 receptor</td>
<td>Human</td>
<td>Hs00275634_m1</td>
<td>FAM™</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Rn02758689_s1</td>
<td></td>
</tr>
<tr>
<td>CB2 receptor</td>
<td>Human</td>
<td>Hs00275635_m1</td>
<td>FAM™</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Rn03993699_s1</td>
<td></td>
</tr>
<tr>
<td>GPR 55 receptor</td>
<td>Human</td>
<td>Hs00271662_s1</td>
<td>FAM™</td>
</tr>
<tr>
<td>M1</td>
<td>Human</td>
<td>Hs00265195_s1</td>
<td>FAM™</td>
</tr>
<tr>
<td>M2</td>
<td>Human</td>
<td>Hs00265208_s1</td>
<td>FAM™</td>
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<td>FAM™</td>
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<tr>
<td>M4</td>
<td>Human</td>
<td>Hs00265219_s1</td>
<td>FAM™</td>
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<tr>
<td>M5</td>
<td>Human</td>
<td>Hs00255278_s1</td>
<td>FAM™</td>
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<td>Human</td>
<td>Hs00175686_m1</td>
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</tr>
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<td>P2X3</td>
<td>Human</td>
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<td>Human</td>
<td>Hs0041764_s1</td>
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<tr>
<td>P2Y4</td>
<td>Human</td>
<td>Hs00267404_s1</td>
<td>FAM™</td>
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### Table 2.5: Reaction mixture for Taqman™ PCR. Volumes are expressed in µL per sample reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene expression master mix</td>
<td>10</td>
</tr>
<tr>
<td>GOI TaqMan probe</td>
<td>1</td>
</tr>
<tr>
<td>House keeping gene, GAPDH</td>
<td>1</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>6</td>
</tr>
<tr>
<td>cDNA template</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 2.6: Thermal cycling program for OneStep PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>Stage</th>
<th>Description</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Holding</td>
<td>Uracil-DNA glycosylase incubation</td>
<td>2 min</td>
<td>50 °C</td>
</tr>
<tr>
<td>2</td>
<td>Holding</td>
<td>Polymerase activation</td>
<td>10 min</td>
<td>95 °C</td>
</tr>
<tr>
<td>3</td>
<td>Cycling</td>
<td>Denaturation</td>
<td>15 sec</td>
<td>95 °C</td>
</tr>
<tr>
<td>4</td>
<td>Cycling</td>
<td>Annealing/extension*</td>
<td>1 min</td>
<td>60 °C</td>
</tr>
</tbody>
</table>

*Fluorescence measured at the end of this stage

2.5.2.5 Statistical analysis

Fold change of the GOI expression was calculated by the formula: Fold change= $2^{\Delta \Delta Ct}$ assuming 100% efficiency of the PCR reaction. Unpaired Student’s t-test was used to compare the significance in mRNA expression of CB1 and CB2 receptors for patients with DO and normal bladder samples.

2.6 Immunohistochemistry

2.6.1 Theory

IHC allows the study of the distribution and localisation of specific cellular components in cells or tissues. Similar to western blotting and immunofluorescence, IHC uses antibodies to trace antigen-antibody interactions; the antibody can be detected by several methods. Most commonly, a secondary antibody conjugated to an enzyme (horseradish peroxidase or alkaline phosphatase) is used; this binds to the primary antibody and indirectly stains the antigen of interest. The first step to IHC is preparation of the sample. There is no universal method for tissue fixation, however, in this thesis formalin-fixed paraffin-embedded tissue sections were used to maintain the natural shape and architecture of the tissue during long-term storage and sectioning for IHC. Alternatively, sections may be prepared by snap-freezing in liquid nitrogen encased in a cryogenic embedding medium and sectioned with a cryostat, followed by fixation with cold acetone or alcohol, if samples are too sensitive for either chemical fixation or the solvents used to remove the paraffin.
Formalin-fixed, paraffin-embedded tissues were sectioned into slices as thin as 2 to 5μm with a microtome. These sections were then mounted onto glass slides and coated with an adhesive. In this thesis, this adhesive was added by surface-treating glass slides with 3-aminopropyltriethoxysilane (APTS) which leaves amino groups on the surface of the glass to which the tissue directly couples. After mounting, the sections were dried in an oven at 100 °C in preparation for deparaffinization. The paraffin from formalin-fixed, paraffin-embedded sections must be completely removed for the antibodies to reach the target antigens. Xylene was used to remove the paraffin from IHC slides. Formaldehyde fixation generates methylene bridges that crosslink proteins in tissue samples; these bridges can mask antigen presentation and prevent antibody binding. Formalin-fixed, paraffin-embedded sections commonly require a treatment to unmask the antibody epitopes, either by application of heat (heat-induced epitope retrieval; HIER) or use of digestive enzymes (proteolytic-induced epitope retrieval; PIER).

There are different direct and indirect staining methods used in IHC, however, the indirect Avidin-Biotin Complex (ABC) method was employed in this thesis. Avidin, a large glycoprotein, can be labelled with peroxidase or fluorescein and has a very high affinity for biotin. Biotin, a low molecular weight vitamin, can be conjugated to a variety of biological molecules, such as antibodies. The technique involves the addition of three layers as shown in Figure 2.4.
2.6.2 Methodology

Tissue sections were dewaxed with xylene three times at 3 min each and rehydrated through graded alcohols (99% industrial methylated spirit (IMS) two times at 3 min each and 95% IMS for 3 min, plus 3 min dH2O). Endogenous peroxidases were suppressed by incubation of the slides for 10 min at RT in 6% H2O2 then washed in running tap water for 5 min. Slides were subjected to a further wash with Tris-buffered saline (TBS)-Tween/0.05% for 5 min prior to addition of blocking serum. An avidin–biotin complex immunoperoxidase system (Vectastain® Elite ABC kit) was used to detect immunoreactivity according to the manufacturer’s instructions. Two drops of the

Figure 2.4: Illustration of the indirect Avidin-Biotin Complex (ABC) method. Step 1 is the addition of an unlabelled primary antibody followed by the addition of biotinylated secondary antibody. A third layer is added with a complex of avidin-biotin peroxidase. The horseradish peroxidase is then developed by the DAB or other substrate in the presence of H2O2 to produce different colorimetric end products. Image taken from http://blog.ptglab.com/wpcontent/uploads/2010/08/Immunoh111.jpg
Vectastain blocking serum was added to each slide and incubated at RT for 20 min. The blocking serum was made up by addition of 150 μl of stock supplied in the ABC kit to 10 ml of TBS-Tween/0.05%. The serum for blocking is prepared from the same species in which the biotinylated secondary antibody is raised. Sections were incubated overnight at 4 °C in a humid chamber with the antibodies mentioned in Table 2.1.

Next day, sections were washed in TBS-Tween/0.05% for 20 min prior to addition of 100 μl secondary antibody (anti-rabbit raised in goat). Vectastain secondary antibody was used, provided in the ABC kit, which was made up by mixing 150 μl of normal blocking serum stock to 10 ml of buffer with 50 μl of biotinylated antibody. Sections were incubated for 30 min at RT. Slides were then washed with TBS-Tween/0.05% for 20 min and then incubated with 100 μl of Vectastain ABC reagent (avidin/biotinylated enzyme macromolecular complex) for a further 30 min at RT. Slides were washed again with TBS-Tween/0.05% prior to staining with 100 μl DAB for 5 min in RT. Slides were then washed in dH2O for 5 min prior to counterstaining with haematoxylin for 2 min. Slides were washed for 5 min under running tap water prior to dehydration in IMS (95% IMS for 3 min, twice 99% IMS for 3 min) and cleared in xylene (once for 3 min and once for 6 min). Coverslips were mounted with DPX. Images were taken on an Axioplan transmission microscope with a Sony®DXC-151P analogue camera connected to a computer running Axiovision, v4.4 image capture and processing software.

2.7 Western blots

2.7.1 Theory

Detection of intracellular RNA for receptors using the qRT-PCR method described above does not always correlate with receptor protein expression and function, so further characterisation of the receptor is required. Western blotting provides information on the amount of protein expressed and also gives an indication of molecular weight. This is achieved by first denaturing the proteins by adding sample buffer that contains sodium dodecyl sulfate (SDS) and heating them to 100 °C. SDS is an anionic detergent that helps denature the proteins and coats them with an overall negative charge. The remaining components of the sample buffer are Tris which provides an appropriate pH, glycerol to
make the samples sink into the bottom of the wells and a dye to allow visualization of the samples as they move down the gel. The sample is placed in wells at the top of the gel, which is a cross-linked polymer matrix and its density can be controlled by varying the monomer concentration. Gels can have a constant density or have variable density also known as gradient gels. The appearance of bands, progressing down the lanes of the gel, are based on the sizes of the different proteins. Smaller proteins move faster and can be found towards the bottom of the gel. A molecular weight marker, which contains proteins of known sizes, produces bands in different positions along the length of the gel and is used to identify the size of the proteins of interest.

Proteins separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), provide information about the potential expression of different isoforms of the proteins of interest in addition to their relative molecular weights. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, therefore the amount of bound SDS is relative to the size of the protein. During electrophoresis an electric current applied to the gel, forces the molecules to move towards the positive electrode. In this study, separated proteins were transferred to a nitrocellulose membrane using an electric current applied at 90 degrees to the gel, which causes the proteins to migrate out of the gel into the membrane.

There are two types of electrophoretic transfer, wet and semi-dry. Semi-dry transfer is generally faster but wet transfer is less prone to failure due to drying of the membrane and is especially recommended for large proteins, >100 kDa. In wet transfer, the gel and membrane are sandwiched tightly together between sponge and filter paper (sponge/paper/gel/membrane/paper/sponge). The sandwich is covered in transfer buffer to which an electrical field is applied. The negatively-charged proteins travel towards the positively-charged electrode, but the membrane stops them, binds them, and prevents them from continuing on through all of the different layers. In semi-dry transfer, a sandwich of paper/gel/membrane/paper is placed initially in transfer buffer and then positioned directly between positive and negative electrodes.

Western blotting can now be used to detect a target protein in a sample using either a polyclonal or monoclonal antibody specific to that protein. The recognition sites of an
antibody, target a small region on the antigen called an epitope (Figure 2.5) and this interaction is the key to a successful Western blot.

Monoclonal and polyclonal antibodies are created when an antigen, usually a protein or peptide of interest, is injected into an animal and its immune system responds by producing antibodies specifically targeted against that antigen and specifically various epitopes found on that antigen. Polyclonals are usually produced in rabbits, donkeys, sheep, and goats, and are purified from serum. They are produced by B-lymphocytes, which produce IgG immunoglobulin specific for the antigen that was injected in the animal. Polyclonal antibodies consist of a mixed pool of immunoglobulin molecules that bind to several different epitopes found on a single antigen (Figure 2.6). In contrast, monoclonal antibodies bind to a single epitope within a target antigen. Monoclonals are usually made by fusing myeloma cells with spleen cells typically from a rabbit or mouse that has been immunised with the desired antigen (Figure 2.7).
**Figure 2.6**: Panel A demonstrates a monoclonal antibody showing specificity for a single epitope. Panel B shows a polyclonal antibody (in blue) showing specificities to multiple epitopes.

**Figure 2.7**: A general representation of the method used to produce monoclonal antibodies. Image taken from http://upload.wikimedia.org/wikipedia/commons/9/9a/Monoclonals.png
2.7.2 Methodology

2.7.2.1 Sample preparation

Snap-frozen bladder tissue was homogenised in modified RIPA buffer containing 10 IU/ml protease inhibitor cocktail and phosphatase inhibitor cocktail 20 IU/ml with an Ultra Turrax-T8. The resultant mixture was centrifuged on a standard centrifuge at 12,000 rpm at 4 °C for 20 min and the supernatant protein concentration determined using Bio-Rad Protein Assay. Samples were stored at −20 °C.

2.7.2.2 Protein estimation

The Bio-Rad protein assay (50000001) was employed to determine the amount of protein in the bladder samples. Several dilutions of protein standards were prepared containing 1 to 25 µg/ml (see Table 2.7). A standard curve was prepared each time the assay was performed with 0.8 ml of standards and appropriately diluted tissue samples placed in dry tubes. Simultaneously, 0.8 ml of dH₂O was used for the standards blank and 0.8 ml of diluted buffer (diluted the same as the tissue samples) was used for the sample blank. To this 0.2 ml of dye reagent concentrate was added to each tube and incubated for 5 min, at which point OD₅₉₅ versus blanks was measured. The OD₅₉₅ of the different concentration of standards was plotted and unknowns were determined from the standard curve using Prism software’s interpolation algorithm.

Table 2.7: Shows protein assay dilutions used in the Bio-Rad assay.
Protein standard=1.5 mg/ml.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Dilution</th>
<th>Protein Standards</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td>µg/ml</td>
</tr>
<tr>
<td>25</td>
<td>1:60</td>
<td>0.1 ml</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>1:75</td>
<td>0.1 ml</td>
<td>16</td>
</tr>
<tr>
<td>15</td>
<td>1:100</td>
<td>0.1 ml</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>1:150</td>
<td>0.01 ml</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>1:300</td>
<td>0.01 ml</td>
<td>4</td>
</tr>
<tr>
<td>1.25</td>
<td>1:1200</td>
<td>0.01 ml</td>
<td>1</td>
</tr>
</tbody>
</table>
2.7.2.3 SDS PAGE

Laemmli sample buffer was added to bladder samples before heating at 100 °C for 5 min to denature the protein mixture. Five, 10 and 20 μg of protein were separated by 10% Tris-hydroxyethyl-1-piperazine ethanesulfonic acid (HEPES) sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. 20-40 μl of tissue proteins were loaded per well of the protein gels and 10 μl of Pierce chemiluminescent blue pre-stained peroxidase-labelled protein molecular weight marker was loaded in the first well of the gel. Gels were placed in a Mini Protean tank filled with HEPES running buffer and electrophoresis commenced at 150 V and 300 mA (adjusted according to the number of gels as per manufacturers’ instructions for a total time of 45-60 min.

2.7.2.4 Western Blot

A wet electroblotting protocol was used. Nitrocellulose membrane, filter paper, and sponge were soaked in cold transfer buffer and maintained at 4 °C for 10 min. Gels were removed from the cassette after electrophoresis and also soaked for 10 min in cold transfer buffer. The transfer sandwich was assembled and firmly packed as follows:

Cathode (---)
Sponge pad
Filter paper
Gel
Transfer membrane
Filter paper
Sponge pad
Anode (+++)

The cassette was placed in an electro-blotting module and the tank filled with cold transfer buffer. Protein transfer was initiated at 40 V and 140 mA (adjusted according to the number of gels as per manufacturer’s instructions) and allowed to continue for 90 min.

Nitrocellulose membrane was separated from the gel and incubated at RT, under gentle
agitation, in 5%-normal goat serum (NGS) in TBS (pH 7.6) containing 0.05% Tween 20 (TBS-T) blocking buffer for 1 hr to prevent non-specific background binding of the primary and secondary antibodies to the membrane. Membrane was then incubated with the primary antibody or antibody plus blocking peptide (CB1 1:100; CB2 1:500; TRPV1 1:3,000; NAPE-PLD 1:2,000 and FAAH 1:500 dilution, see Table 2.1) overnight at 4 °C. The membrane was washed three times with TBS-T for 15 min to remove any residual primary antibody. The membrane was then incubated for 1 hr at RT with gentle agitation with secondary antibody (goat anti-rabbit horseradish peroxidase (HRP) at 1:20,000 dilution in TBS-T containing 5% normal goat serum. The membrane was washed three times for 15 min with TBS-T to remove residual secondary antibody and then incubated for 5 min with Super Signal Chemiluminescent Substrate™ (Immuno-Star Western C Chemiluminescent kit). The membrane was placed between two cellophane sheets and placed into a developing cassette for subsequent exposure to X-ray film. After variable exposure to X-ray film that was dependent upon relative protein expression levels, the X-ray film was developed in an automated developer.

2.8 Organ Bath

2.8.1 Theory

Tissue-organ baths were used to maintain the integrity of the bladder tissue for several hours, in a temperature-controlled and oxygenated environment, while physiological measurements are performed. Typical experiments involve the addition of drugs to the organ bath or by direct electrical field stimulation of the tissue. The tissue reacts by contracting/relaxing and an isometric or isotonic transducer is used to measure force or displacement, respectively. From the experimental results concentration-response curves are generated. A perfusion organ bath provides a stable and easily adjustable way of not only temperature control and pH but allows timely interventions. Substrates and other nutrients that are required to sustain tissue function are provided via a physiological solution, which allows the study of evoked tissue responses to:

1. Pharmacological drug/agents (concentration-response studies)
2. Electrical field stimulation
3. Both pharmacological and electrical stimulation
Tissues were usually prepared in a Petri-dish containing physiological solution (i.e. Krebs’ solution). The ends of the tissue were then attached to the mounting hook and transducer using fine silk thread or fine wire (non-compliant) to mount the tissue. Tension is then applied and a period of acclimatization is allowed to permit the tissue to adjust to its new tension and environment. This period can vary depending on the tissue used. For rat bladder smooth muscle, a 60 min equilibration period was allowed. During this time, the tissue may be gradually stretched or undergo pre-treatment with selected agonists or antagonists (to limit or increase physiologic and pharmacological responses) or be exposed to probes or radio chemicals that are taken up into the tissues. This acclimatisation period allows tissues to recover from the effects of cooling and/or surgical trauma and to wash out metabolites, anaesthetics and other products used or produced during these periods.

To measure muscular activity the muscle strip is attached by a silk suture to a force transducer, which converts the force generated by the contracting muscle into an electrical signal that can then be detected on a chart recorder or a computer based data acquisition system. The muscle is usually pre-loaded with a weight or pre-stretched using a tissue tensioner or micrometer. The suture attaching the muscle to the transducer should not touch the bath walls and should be in line with the transducer. The force transducer can make an isometric measurement where the muscle length remains constant, as force changes and is measured as grams or milliNewtons.

Some limitations to this method are due to the damage occurring during removal of the sample from the body and the difficulty in exactly reproducing its natural environment. In addition, tissue and organ preparations will not last indefinitely and their response may not be identical to in situ or in vivo preparations. Other problems may arise due to imbalances in oncotite pressure or trace elements caused by the use of synthetic media, overstretching or mechanical damage to cell layers due to mechanical attachments, photo bleaching or metal chelation that occurs when indicators are used.

2.8.2 Methodology

Full-thickness rat bladder strips (5 x 2 x 0.5 mm) were suspended using fine silk sutures
in a 0.2 ml volume Perspex® organ-bath chamber perfused at 1 ml/min with Krebs’ solution aerated with 95% oxygen (O₂) and 5% carbon dioxide (CO₂) at 37 °C (see Figure 2.8). The strip was tied between the chamber base and an isometric force transducer connected to a four channel PowerLab® running Chart v7.1 (AD Instruments). Equilibration for 1 hr under 1 g tension was allowed before experimentation. Electrical field stimulation (EFS) was delivered as repeated square pulses at 5, 10, 20, 40 Hz delivered for 10 sec at 1 min intervals to establish the submaximal response. Pulse width was set to 0.05 ms, 50 V and 10 ms delay. Ten Hz was used in all subsequent experiments as this was where a submaximal response was obtained. Once the correct conditions had been established, a control contractile response was obtained, followed by contractile responses in the presence of ACEA (10⁻⁸ M to 10⁻⁴ M) or GP1A (10⁻⁹ M to 10⁻⁶ M). The frequency, amplitude and pulse width used stimulates the release of both acetylcholine and adenosine triphosphate (ATP) from the intrinsic nerves. Contractile responses to carbachol (CCh) (concentration–response curves 10⁻⁷ M to 10⁻³ M) were obtained before and after treatment with ACEA (10⁻¹⁰ M to 10⁻⁶ M) or GP1A (10⁻⁹ M to 10⁻⁶ M). Each concentration of CCh was perfused for 30 s, allowing 5 min between each contraction. The organ bath was perfused with either CB1 or CB2 agonists for 30 min before repeat CCh concentration response curves were obtained.

2.8.2.1 Statistical analysis

Statistical analysis was performed using repeated measures analysis of variance (ANOVA), followed by Dunnett’s multiple comparison on GraphPad Prism5.0®, with p<0.05 considered significant for organ bath studies.
Figure 2.8: Set up of organ bath chamber used for rat bladder contractility experiments. One of the 4 chambers is magnified to demonstrate where the rat bladder strip was tied between two platinum electrodes which delivered the electrical field stimulation and changes in force were detected by an AD Instruments PowerLab system running Chart software.

2.9 Immunofluorescence

2.9.1 Theory

Fluorescence is the property of emitting electromagnetic radiation in the form of light as the result of (and only during) the absorption of light from another source. The emission of light occurs extremely rapidly after the absorption of light that raises the absorbing molecule to a higher excitation shell. It is a three-stage process that occurs in molecules called fluorophores or fluorescent dyes. The three stages of fluorescence are:
Stage 1- excitation
Stage 2- excited-state lifetime
Stage 3- fluorescence emission
The emitted fluorescence has a lower energy than the absorbed light, so the wavelength of the emitted light is longer than that of the excitation light. A range of wavelengths can result in excitation of a fluorochrome. Fluorescein isothiocyanate (FITC) will fluoresce when hit by light with any wavelength between 450 nm and 520 nm with an emission peak approximately 515 nm. If the excitation wavelength is close to 495 nm, there will be more fluorescence produced. When FITC is excited at a wavelength other than its peak excitation, the shape of the emission curve stays the same, but the intensity is reduced. The shift in wavelength from short to long during fluorescence is called “Stokes shift” (Figure 2.9).

![Fluorescence Diagram](image)

**Figure 2.9:** A shift in wavelength is described as the Stokes shift.

The use of a fluorochrome, such as FITC, has the major limitation of photobleaching, which is the destruction of the fluorophore due to the generation of reactive oxygen species. These occur as by-products of fluorescent excitation and can be minimized by decreasing the excitation light in both intensity and duration. The use of fluorophores to label antibodies or antigens in immunofluorescence, can be detected with a confocal...
There are two main methods of immunofluorescent labelling, direct and indirect. The direct method involves the primary antibody being chemically conjugated to the fluorophore. On the other hand, the indirect method employs the use of two antibodies; an unlabelled primary and a secondary anti-immunoglobulin antibody directed toward the constant portion of the first antibody (Figure 2.10). The secondary antibody is tagged with a fluorescent dye. Advantages of using the direct method of immunofluorescence include shorter staining times and simpler labelling procedures. However, there is usually a lower signal and a higher cost.

The indirect method is most commonly used as it offers greater sensitivity than direct immunofluorescence. There is a higher amplification signal compared to the direct method, as more than one secondary antibody can attach to each primary antibody (Figure 2.10). In addition, commercially produced secondary antibodies are inexpensive and quality controlled. There is the potential for cross-reactivity when using the indirect method and the need to use primary antibodies that are not raised in the same species. Samples with endogenous immunoglobulin may exhibit a high background so a further negative control should be used with an immunoglobulin antibody.

**Figure 2.10:** Principles of direct and indirect immunofluorescence (IF). Direct IF uses a single antibody directed against the target of interest (known as a primary antibody). The antibody is directly conjugated to a fluorophore. Indirect IF uses two antibodies. The primary antibody is unconjugated and a fluorophore-conjugated secondary antibody directed against the primary antibody is used for detection. Image taken from [www.abcam.com](http://www.abcam.com).
2.9.2 Methodology

2.9.2.1 Cell lines

One 16 mm diameter circular glass coverslip was placed in each well of a 12-well plate and sterilized with 0.5 ml of methanol for 10 min. The methanol was then aspirated and the coverslips washed with sterile phosphate buffer solution (PBS). Cells were subcultured as described previously (Section 2.4.2) and plated (1.25 x 10^5 cells/well depending on cell type) to the 12-well plates containing sterile coverslips. Each well contained 1 ml, consisting of the calculated volume of cells and additional growth medium. Cells were incubated overnight at 37 ºC with 5% CO₂ humidified air, in order to attach to the coverslips. The next day, the culture medium was aspirated and the cells washed with ice cold PBS. Cells were then incubated at -20 ºC for 10 min with 0.5 ml of ice-cold methanol (previously kept at -20 ºC) to fix and permeabilise.

Fixed and permeabilsed cells were washed three times with 0.5 ml of PBS, the PBS aspirated and the cells incubated with 0.5 ml of PBS-T for 5 min. Cells were incubated for 5 min in 1 ml of PBS-T with 10% goat serum to block non-specific binding of the primary antibody. Cells were incubated overnight at 4 ºC with 250 μl of either primary antibody diluted in PBS-T 10% NGS (see Table 2.1 for antibody dilutions), PBS-T 10% NGS as a negative control, or IgG as an additional negative control. IgG was used at the same concentration as the primary antibody.

The following day, cells were washed three times with 0.5 ml of PBS and then incubated for 10 min in PBS-T. Cells were incubated for 1 hr at RT in the dark with FITC-conjugated goat, anti-rabbit IgG (1:160) secondary antibody (see Table 2.1). The unbound reagents were removed by washing with PBS three times and one wash in PBS-T for 5 min. Cell nuclei were visualised by incubation with DAPI (4',6-diamidino-2-phenylindole) for 30 min at RT. Excess DAPI was removed by washing with PBS-T and the coverslips inverted and mounted onto slides with Vectashield® Mounting Media with the edges secured with clear nail varnish.
Human bladder specimens were fixed and further processed as for IHC described in Section 2.3.1. Sections were incubated overnight at room temperature with antibodies raised in rabbits against CB1 or CB2. For the simultaneous demonstration of co-localisation of cannabinoid receptors and neurones, antibodies to CB1 with either mouse anti-choline acetyltransferase (ChAT) antibody clone 28C4, or mouse protein gene product 9.5 (PGP 9.5) and CB2 with either ChAT or PGP 9.5 were incubated as cocktails and anti-rabbit FITC conjugate was used to display cannabinoid receptor fluorescence (see Table 2.1 for source of antibodies). Blocking peptides for CB1 and CB2 were used to confirm specificity of antibodies. After rinsing, the slides were incubated with ChAT antibody and subsequently had goat anti-mouse IgG conjugated with Alexa Fluor 594 applied to the sections for 60 min. Sections incubated with PGP 9.5 antibody had goat anti-mouse IgG2A conjugated with Texas Red applied for 60 min, instead.

Sections were visualized using a Nikon C1Si confocal laser-scanning microscope. Images for IHC analysis were taken on an Axioplan-transmission microscope with a Sony® DXC-151P analogue camera connected to a computer running Axiovision, version 4.4 image capture and processing software. Negative control staining was performed either in absence of primary antibodies, primary antibody pre-incubated with blocking peptide or with isotype controls; IgG and IgG2A.

2.10 Measurement of intracellular [Ca$^{2+}$]:

2.10.1 Theory

Calcium is a universal second messenger involved in specific and selective regulation of cellular processes such as muscle contraction and synaptic transmission. Ca$^{2+}$ cannot be visualized directly in living cells so to measure changes in [Ca$^{2+}$], specific fluorescent molecules are used that have optical properties which change upon interacting with Ca$^{2+}$. There are different methods to detect fluorescent molecules but all fluorescence instruments contain three basic elements: a source of light, a sample holder, and a detector. The system used must be equipped with adjustable monochromators that can
accurately select excitation and emission wavelengths. It is also essential to monitor and correct any fluctuations in source intensity. The Perkin-Elmer Model Luminescence Spectrometer measures the fluorescence intensity of cell suspensions at selected excitation and emission wavelength pairs using a cuvette based system (Figure 2.11).

**Figure 2.11:** The cuvette reader excites the sample over the entire path length and reads the emitted light at right angles.

Fura-2 AM (pentaacetoxymethyl) was used in this thesis with the Perkin-Elmer Model spectrometer. Fura-2 AM is a ultraviolet excitable ratiometric dye which binds to free intracellular calcium. When added to cells, Fura-2 AM crosses cell membranes and once inside the cell, the acetoxyethyl groups are removed by cellular esterases leading to a cell-impermeant indicator.
Upon binding to Ca$^{2+}$, Fura-2 undergoes a shift in absorption rather than emission peak. In the presence of Ca$^{2+}$, Fura-2 excitation spectrum peaks at 340 nm. In the absence of Ca$^{2+}$, there is a broad spectrum with maximum at 380 nm. The ratio of 340 to 380 is proportional to Ca$^{2+}$ concentration (Figure 2.12). Regardless of the presence of calcium, Fura-2 emits at 510 nm. The use of the ratio accounts for confounding variables such as local differences in Fura-2 concentration or cell thickness that would otherwise lead to artefacts when attempting to measure calcium concentration of cells.

When ratiometric dyes are used to measure changes in [Ca$^{2+}$], the rise in Ca$^{2+}$ is recorded as a number that has no unit. In order to express this number with a unit of concentration it is necessary to calibrate the dye. The change in [Ca$^{2+}$] is calculated from the ratio at dual excitation using the Grynkiewicz equation (Grynkiewicz et al. 1985).

$$[\text{Ca}^{2+}]_i = K_d \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \times \frac{F_{380\text{max}}}{F_{380\text{min}}}$$

[Ca$^{2+}$]$_i$ represents the intracellular calcium concentration, $K_d$ is the equilibrium constant of Ca$^{2+}$ for Fura-2 and is equal to 225 nM (for Ca$^{2+}$ binding to Fura-2 at 37 °C), R is the fluorescence ratio (F340 nm/F380 nm), $R_{\text{min}}$ and $R_{\text{max}}$ represents the fluorescence ratio at minimum (EGTA) and maximum (Triton-X) [Ca$^{2+}$] respectively and $F_{380\text{max}}$/$F_{380\text{min}}$ is the fluorescence ratio at minimum saturated [Ca$^{2+}$]. The $K_d$ for Ca$^{2+}$ binding to Fura-2 decreases with decreasing temperature (Patel et al. 2013).
Cells were cultured in T175 flasks as previously described (Section 2.4). At confluence, the cell medium was removed and replaced by 5 mL of Harvest buffer (see Section 2.2 for components). The Harvest buffer was replaced three times and then incubated 37 °C for 10 min (for HCV 29), 20 min (UROtsa and MYP 3) at 37 °C/ 5% CO₂ in order for the cells to detach. Cells were dislodged from the flask with gentle agitation and transferred to a centrifuge tube. Cells were sedimented under centrifugal force (3 min / 1600 x g) (Heraeus Instruments Labofuge 400R), the supernatant was removed and the pellet of cells re-suspended in 20 ml of Krebs’-HEPES buffer. The cells were washed twice with Krebs’-HEPES before re-suspending the pellet in 2 ml of Krebs’-HEPES buffer. The cell suspension was loaded with 10 μl Fura-2/AM (1 mM stock in DMSO) to give a loading concentration of 5 μM, for 30 min at 37 °C. The loaded cells were re-suspended in Krebs’-HEPES buffer and incubated in the dark at room temperature for 20 min to allow for ester hydrolysis. The cells were sedimented and re-suspended in Krebs’-HEPES buffer a total of three-times (1800 x g). [Ca²⁺]ᵢ was measured in 2 ml suspension of cells placed in
quartz cuvette containing a mini magnetic stirrer, using a Perkin–Elmer LS50-B fluorimeter (Beaconsfield, UK) at 340/380 nm excitation and 510 nm emission at 1 sec intervals.

The temperature of the cuvettes was maintained at 37 ºC by external silicon tubing connected to a water bath. Cell suspensions were allowed to equilibrate for 180 sec prior to addition of drugs (at 180 sec). Cells were stimulated with either carbachol (1 mM) or ATP (1 mM). HCV 29 cells were pre-incubated with the CB1 agonist ACEA (10^{-5} M diluted in 100% ethanol) for 3 min and then stimulated with ATP. As a control, cells were incubated with ethanol for 3 min and stimulated with ATP. UROtsa cells were stimulated with the GPR55 activator LPI (10^{-6} M) and ATP to confirm the cells contained functional receptors. Data collection was carried out using the FLDM program integral to the instrument (Perkin Elmer).

Calibrations and subsequent calculation of [Ca^{2+}]_i were determined using 0.1% Triton-X-100 and 4.5 mM EGTA (pH >8.0) (to obtain the R_{max} and R_{min} respectively). [Ca^{2+}]_i was calculated from the 340/380 ratio according to Grynkiewicz et al. (1985).

2.11 Confocal Microscopy

2.11.1 Theory

Confocal laser scanning microscopy (CLSM) enables high-resolution optical images with depth selectivity to be obtained by a process known as optical sectioning. A laser light beam is focused onto a fluorescent specimen through the objective lens. The mixture of reflected and emitted light is captured by the same objective and is sent to the dichroic mirror. The reflected light is deviated by the mirror while the emitted fluorescent light passes through a confocal aperture (pinhole) to reduce the “out of focus” light (Figure 2.13). The pinhole obstructs light coming from object points outside the focal plane and excludes it from detection. The focused light then passes through the emission filter and proceeds to the photomultiplier. In order to generate an entire image, the single point is scanned in an X-Y manner as the laser focus is moved over the specimen. To obtain information about the entire specimen, the laser beam needs to be guided across the
specimen or to move the specimen relative to the laser beam in process known as scanning.

Confocal microscopes are also available with an upright objective that allows optical sectioning where imaging of a thin “optical slice” out of a “thick” specimen (typically up to 100 µm) is possible. This is an advantage over conventional fluorescent microscopes where the image of a “thick” biological specimen will only be in focus if its Z dimension is not greater than the wave-optical depth of focus specified for the respective objective.

Figure 2.13: Confocal imaging. The laser beam irradiated from a light source passes through the object lens, which moves rapidly up and down, based on the movement of the tuning fork, and focuses on the object. The reflected light from the target object passes through a half mirror and a pin hole, and reaches the light-receiving element. Fluorescent light (i.e. signal) passes back through the dichroic reflector and then passes through the second (exit) pinhole which is confocal. The exit pinhole can be made small enough that any light emanating from regions away from the vicinity of the illuminated point will be blocked by the aperture, thus providing yet further attenuation of out-of-focus interference. A photomultiplier detector (PMT) provides a signal of the light passing as the specimen is scanned. A computer is used to control the sequential scanning of the sample and to assemble the image for display. Image taken from www.snipview.com.

2.11.2 Single cell confocal microscopy methodology

HCV 29 cells were grown on large glass coverslips in 6 well plates for 48 hours until they reached 70% confluency. Cells were then perfused with Krebs’ solution at a rate of
5 ml/min. Cells were washed with Krebs’ solution and then incubated with Fluo4-AM for 30 min in darkness. The cells on the coverslip were then transferred to a perfusion chamber and 1 ml of Krebs’ solution was added. Cells were then stimulated with 100 μM carbachol by perfusing the drug for 30 sec. At this point, 1 μM ACEA was directly pipetted onto the coverslip containing the cells and the perfusion was stopped. Cells were incubated with ACEA for 3 min. The perfusion with Krebs’ solution was re-started and the cells were then stimulated with 100 μM carbachol by perfusing the drug for 30 secs. Control experiments were performed in a similar manner where ACEA was replaced with 0.1% ethanol. The cells were imaged every 2 sec for 500-600 sec. Changes in fluorescence were analysed using NIC Elements Software.

2.11.3 Upright confocal microscopy methodology

2.11.3.1 Calcium indicators used

- 10 mM Oregon Green 488 BAPTA-1 AM in 1% DMSO/0.2% pluronic F-127.
- Fluro-4

2.11.3.2 Tissue sample and drug preparation

Human bladder biopsies taken at cystoscopy as described in Section 2.3.1 were transferred in Krebs’ solution (for constituents see Section 2.2.). As calcium indicators, both Oregon Green 488 BAPTA-1 AM and Fluro-4 were used to optimise the methodology. Tissue was incubated in bubbling Krebs’ (rate of 1 bubble per second, 95% O₂/5% CO₂) loading solution with a calcium indicator for 1 hr in darkness. Experiments were commenced with Oregon Green 488 but no calcium signal was detected. On transferring to another lab, Fluro-4 was used so the majority of experiments were carried out with this calcium indicator. Serial concentrations of carbachol 10⁻⁵ M -10⁻³ M were prepared in Krebs’ and ACEA 10⁻³ M.
2.11.3.3 Leica TCS SP2 Confocal microscopy

2.11.3.3.1 Tissue Preparation for confocal microscopy

Tissue was rinsed in bubbling (95% O\textsubscript{2}/5% CO\textsubscript{2}) Krebs’ solution (pH 7.4) for at least 10 min to remove any extracellular dye and then positioned with the luminal side of the urothelium facing downwards on a glass coverslip positioned on the confocal stage (Leica SP2; Leica Microsystems, Milton Keynes, UK). A weight was placed on the biopsy to keep it in position. A perfusion pump was used to continuously perfuse the tissue with bubbling Krebs’ solution at a rate of 100 ml/h with the temperature maintained at 36 °C using a rack-mounted heater unit. A temperature probe and inflow and outflow tubes were positioned on the preparation dish (affixing each to the metal strip by the magnet on its base). The tissue was allowed to equilibrate for 15 min in the dark.

2.11.3.3.2 Selection of image site and confocal microscopy

A low power objective (10x or 20x) was placed directly onto the tissue and epifluorescence (excitation with a blue light) was used to view the smooth muscle until a suitable region to monitor was identified. At this point, the x40 objective was lowered into the bath and the stage adjusted so the objective was directly above the specimen. Oregon Green-488 BAPTA-1AM and Fluro-4 were excited with laser light set at 488 nm and emission was collected through a prism and shutters set to allow wavelengths longer than 510 nm to pass. A series of 100 frames (256 x 256 pixels; approx. 100 x 100 μm) were captured at approximately 5 Hz, to generate one image set. Such sets were acquired once every minute. Ten sets were generated for each region of the preparation, with at least three regions sampled per preparation. Images were acquired with a Leica SP2 upright confocal microscope (Leica Microsystems, Milton Keynes, UK) using the associated software (Leica LCS) which generates each series as a single viewable ‘movie’ within the software, but saves the whole set as individual frames.

2.11.4 Digital fluorescence camera

Simultaneous measurements of calcium were obtained by using an excitation wavelength of 490 nm with emitted light (515–545 nm) being collected with a charge-coupled device camera (C4880-81, Hamamatsu) attached to an upright microscope (BX50 WI,
Olympus). The monochromator and camera were controlled with Openlab Software (Improvision) and data processed offline with Igor Pro Software (Wavemetrics Inc.) using customized procedures. Regions of interest (ROI) were identified and average changes in fluorescence intensity for each ROI were calculated for the duration of the experiment.

2.12 Radioligand Binding

2.12.1 General theory on receptor ligand binding

Receptor binding assays rely on the binding of ligand molecules to receptor molecules and provide a measure of the interactions that occur between two molecules. This binding interaction is illustrated as follows:

\[
K_+ + [L] + [R] \rightleftharpoons [LR] \\
K_-
\]

\([L] = \text{ligand concentration} \quad [R] = \text{concentration of free receptors} \]

\([LR] = \text{concentration of ligand/receptor complexes} \]

\(K_+ = \text{association rate constant} \]

\(K_- = \text{dissociation rate constant} \]

At any given time there are a proportion of receptors that are ligand bound together with a proportion of receptors that are in a non-bound (free) state. A ligand, [L], binds to its receptor, [R], to become the ligand-receptor complex, [LR]. The rate of the formation of the receptor-ligand complex (association rate; \(K_+\)) and the rate of the reverse (dissociation rate; \(K_-\)) are expressed as constants. This reaction proceeds in the forward direction at a rate of \(K_+[L][R]\), while the rate of dissociation of the ligand-receptor complex is determined by the formula \(K_- [LR]\). If this system is at equilibrium i.e. there is no net movement of the drug onto or off the receptor, then these two rates are equal and, \(K_+[L][R] = K_- [LR]\)

\(K_d\) is the equilibrium dissociation rate constant for this ligand-receptor interaction and represents the concentration of the ligand, which will bind to half of the total number of receptors at equilibrium. It is determined by the following equation:
\[ K_d = \frac{K_1}{K_{+1}} = \frac{[L][R]}{[LR]} \]

When receptor occupancy is at 50% \([R] = [LR]\) so that \(K_d = [L]\)

Typically data are presented as pK<sub>d</sub>, which is the negative log<sub>10</sub> of the concentration required to occupy 50% of the receptors and therefore giving an indication of the affinity of a drug/ligand for a particular receptor. Another value determined from saturation experiments is the B<sub>max</sub>, expressed as a mass of the radiolabel bound per milligram of tissue. These two values, K<sub>d</sub> and B<sub>max</sub>, are derived from a graphical representation of a ligand’s specific binding to a receptor in the form of a Langmuir isotherm, which plots the concentration of a radiolabelled drug against the amount bound to the receptor, as shown in Figure 2.14 (Bylund and Toews 1993). Data are sometimes transformed to a semilogarithmic plot (Figure 2.15).

**Figure 2.14:** The Langmuir absorption isotherm. When the amount of bound radioligand reaches a point of saturation this is characterised by the hyperbolic saturation curve showing specific binding in fmol/mg<sup>-1</sup> of protein vs radioligand concentration. B<sub>max</sub> is the maximal receptor binding sites and K<sub>d</sub> the dissociation constant of the radioligand where 50% of the receptors are occupied. Isotherm plots are shown for total and non-specific binding from which specific binding can be calculated by subtraction of non-specific binding from total binding.
**Figure 2.15:** Semi-logarithmic plot of log drug concentration vs. specific binding, (fmol•mg⁻¹ protein versus log[radioligand]), where the $K_d$ can be determined as 50% of $B_{\text{max}}$

2.12.2 **Membrane preparation**

Membrane fragments were prepared separately from four normal human bladder sections of patients undergoing cystectomies and from pooled bladder biopsies collected from 6 normal patients undergoing gynaecological surgery. Rat tissue was used as control samples as described in Section 2.3.

Dissected tissues were separately homogenised using an Ultra Turrax homogeniser in ice-cold wash buffer. Membrane suspensions were then centrifuged (20,374 g at 4 °C for 10 min) and the supernatant discarded. Membrane pellets were re-suspended in fresh ice-cold wash buffer, homogenized and centrifuged again twice. Membrane fractions were finally re-suspended in a volume buffer dependent on the starting mass of tissue and the protein concentration was determined using the method described by Lowry *et al.* (1951). Membrane suspensions were aliquoted and stored at -80 °C.
2.12.3 Protein estimation - Lowry assay

The Lowry protein assay was used to determine the concentration of the membrane preparations (Lowry et al. 1951) with BSA (0 to 250 µg protein/ml in 0.1 M NaOH) as standards. Samples were also diluted in 0.1 M NaOH and both standards and samples (0.5 mL) were incubated for 10 min with 2.5 ml solution consisting of solution A (NaHCO$_3$ in 0.1 M NaOH), solution B (1% CuSO$_4$) and solution C (2% Na$^+$ K$^+$ tartrate) mixed to a ratio of 100:1:1 and after the addition of Folin’s reagent (diluted 1:4 in dH$_2$O) incubated at RT for 30 min. The absorbance at 750 nm was determined using a benchtop spectrophotometer. Linear regression of the known BSA protein concentrations was used to produce a standard curve (Figure 2.16) from which the concentration of protein in the different membrane fractions was determined.

![Figure 2.16: Example of protein assay standard curve used to determine the protein concentration of unknown samples.](image-url)
2.12.4 Time Course Experiments

2.12.4.1 Theory

These experiments are used to determine how long it takes for a ligand to reach binding equilibrium. Equilibrium is reached when the rate of the forward binding reaction equals the rate of the reverse dissociation reaction. Therefore at the steady state, the rate of forward binding reaction is equal to the rate of the reverse dissociation reaction obeying the law of mass action.

\[ K_d = \frac{K_{-1}}{K_{+1}} = \frac{[L][R]}{[LR]} \]

Such experiments are carried out using a fixed mass of radioligand, and binding is measured at various times intervals.

2.12.4.2 Methodology

Time course experiments were performed using a high (1.7 nM) and a low (0.66 nM) concentrations of \( ^{3}H \)-CP55,940. Non-specific binding (NSB) was determined using 30 \( \mu \)M of unlabelled CP55,940. Rat cerebellum membranes were used and prepared as described previously (Section 2.12.2) and were added to the mixture at various time points between 0 and 4 hr (0, 5, 10, 15, 30, 45 min, 1, 1.5, 2, 3, and 4 hr).

2.12.5 Displacement Experiments to determine ligand used for NSB

2.12.5.1 Theory

Displacement binding assay are used to determine a ligand’s binding affinity for the receptor. In a displacement assay, various concentrations of unlabelled ligand compete with fixed concentration of a radiolabelled ligand for the receptor. As the concentration of the unlabelled ligand increases, the amount of radiolabelled ligand bound to the receptor decreases. From this experiment the concentration of unlabelled ligand, which displaces 50% of the radioligand can be determined. This value represents the IC\(_{50}\) for the non-radiolabelled ligand (Figure 2.17), and is dependent upon three factors:
- The affinity of the competing ligand for the receptor (i.e. the non-radiolabelled ligand)
- The concentration of the radiolabelled ligand
- The affinity of the radiolabelled ligand for the receptor, its $K_d$ 

The $IC_{50}$ is a measure of the affinity of the non-radiolabelled ligand for the receptor. From the $IC_{50}$ the $K_i$ can be calculated, where $K_i$ is an estimation of the concentration of ligand required to occupy 50% of the receptors in the absence of the radiolabelled ligand. The Cheng-Prusoff equation is used to calculate the $K_i$ for a ligand from the derived $IC_{50}$ value, correcting for the mass and affinity of the radiolabel (Cheng and Prusoff 1973).

Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{Ligand}]}{K_d}}$$

$IC_{50} = IC_{50}$ of competing non radiolabelled ligand

$[\text{Ligand}] = $ concentration of radiolabelled ligand

$K_d = $ equilibrium dissociation constant of radiolabelled ligand
2.12.5.2 Method

NSB represents the binding of radiolabel to sites other than the receptor, the percentage of the NSB will vary with different radiolabels, factors such as the lipophilic nature of the ligand will be important. The filtration process can also affect the amount of NSB since radiolabel binding to filters will form an element of the NSB, for this reason (in binding experiments) filters are treated with PEI, a detergent shown to reduce NSB (Dooley et al. 2000).

In order to determine which unlabelled ligand would best determine non-specific binding, and at what concentration, displacement experiments (n=6) using serial dilutions of non-radioactive ligand were performed. From previous studies using myometrial cells, anandamide and CP55,940 were explored as suitable ligands for determining the non-specific binding as both bind to CB1 and CB2 receptors. In order to quantify NSB, an unlabelled ligand is used at a saturating concentration that will occupy all available receptor sites. Anandamide and CP55,940 were used over a series of concentrations in the presence of a fixed concentration of $[^3$H]-CP-55,940 (0.5 nM) to define this
concentration. Rat cerebellum and cortex are known to express high concentrations of CB receptors and were used in these preliminary experiments, necessary to define the correct assay conditions. Membranes were prepared as described in Section 2.12.2 and added to a total reaction volume of 0.5 ml. Anandamide and CP55,940 were added in increasing concentrations (10^{-11}-10^{-4} M) displacing the specific binding of radiolabel from receptor sites. Concentrations where no further displacement of the radiolabel took place defined the NSB.

2.12.6 Saturation binding assay

2.12.6.1 Theory

Saturation binding assays measure the affinity of a radiolabelled drug for a receptor (K_d) and also give a measure of the receptor density (B_{max}) (Kenakin 1997). Specific binding is calculated by subtracting NSB of the radioligand from the total binding (Figure 2.14). In a saturation assay, increasing concentrations of a radiolabelled ligand known to bind to a specific receptor, are added to a membrane suspension until saturation is reached. Following the removal of any excess unbound radioligand by washing, the fraction of radioactivity bound to receptors is calculated to provide a binding profile such that the maximum binding and affinity of the ligand for the receptor can be derived. A requirement of the saturation binding assay is that less than 10% of the added radioligand is bound in order to prevent ligand depletion from the assay and therefore an appropriate amount of protein needs to be determined.

2.12.6.2 Method

Rat cerebellum, bladder or human bladder membranes were prepared as described in Section 2.12.2. 45-300 µg of rat bladder, 7.5-25 µg of rat cerebellum or 60-100 µg of human bladder protein were incubated in wash buffer (see Section. 2.2 for buffer constituents) containing a range of [^3H]-CP55,940 concentrations (2 pM-10 nM) (Table 2.8). The final reaction volume was 0.5 ml and experiments were incubated for 1 hr at 30 °C with gentle shaking. NSB was defined in the presence of 30 µM of the non-radioactive CP55,940.
Table 2.8: Contents of tubes for saturation binding assay. All volumes are in μl.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Binding buffer</th>
<th>NSB (30μM CP55,940)</th>
<th>[³H]- CP-55,940</th>
<th>Membrane suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>300</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NSB</td>
<td>200</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

2.12.7 Cyclic AMP assay

2.12.7.1 Theory

Adenosine 3,5-cyclic monophosphate (cyclic AMP, cAMP) is a second messenger generated by the enzyme adenylate cyclase (AC), which is regulated by G-protein coupled receptors, resulting in the conversion of ATP to cAMP and pyrophosphate (Tang and Hurley 1998) (Figure 2.18). cAMP is then hydrolysed by phosphodiesterases (PDEs) into AMP. cAMP signals through three main effectors; protein kinase A (PKA), nucleotide exchange factors Epac (exchange protein directly activated by cAMP) and cyclic nucleotide-gated (CNG) ion channels (Houslay and Kolch 2000). The activation of adenylate cyclase is initiated by the binding of hormones, cytokines, or neurotransmitters and all membrane-bound ACs can also be directly activated by forskolin. PDEs are inhibited by the inhibitor 3-isobutyl-methylxanthine (IBMX).
Confluent cultures of HCV 29 cells were washed with 5 ml of Harvest buffer (see Section 2.2 for components) followed by gentle dissociation with 5 ml of Accutase (A6964 SIGMA). Harvested cells were collected at 1600 g for 3 min (Heraeus Instruments Labofuge 400R), the supernatant discarded and cells re-suspended in 20 ml of Krebs’-HEPES buffer. The cells were “washed” twice with this buffer before a final re-suspension in 4 ml of Krebs’-BSA buffer. In each assay tube, 200 μl cell suspension, was incubated (37 °C, 15 min, with gentle agitation). Whole cell suspensions were incubated in the presence of IBMX (1 mM), forskolin (1 μM) and ACEA (10^-6 M) in various combinations for 15 min (see Table 2.9).

Accumulation of intracellular cAMP was stopped by the addition of 20 μl of 10 M HCl, and the mixture neutralised with 20 μl of 10 M NaOH before being buffered to a pH 7.4 with 200 μl 1 M Tris-HCL. Cellular debris was collected at 16100 x g for 5 min and the concentration of cAMP present in the supernatant was determined using a specific protein binding assay.

In the assay, 50 μl of supernatant was incubated (4 °C, >2.5 h) with 100 μl of [³H]-cAMP (in ratio of 1 μl stock (Amersham) to 2.5 ml assay buffer) and 150 μl binding protein in a competitive binding assay. Pre-aliquoted cAMP standards (0-10 pmol in 50 μl and NSB at 250 pmol/50 μl, all in cAMP assay buffer) were used to create a standard curve. Where

**Figure 2.18:** Diagram showing the formation of Cyclic AMP (cAMP). cAMP serves as a second messenger to activate or inactivate proteins within the cell. Taken from www.figures.boundless.com
necessary, samples were pre-diluted with cAMP assay buffer to ensure bound radiolabel lay within the standard curve. All assay tubes were vortexed and incubated at 4 °C overnight to allow equilibration (Brighton et al. 2009).

After incubation, 250 μl of charcoal mixture (250 μg charcoal, 100 mg bovine serum albumin (BSA) per 25 ml of cAMP assay buffer) was added to all tubes and allowed to stand for 1 min (charcoal mixture binds any free/unbound [³H]-cAMP). The tubes were centrifuged for 1 min at 16100 x g at room temperature and 200 μl of the supernatant added to 1 ml of “Hi-safe3” scintillation fluid and was thoroughly mixed. This was immediately counted for 2 min, and the resulting data were interpreted using Riasmart software on the Packard 1900TR scintillation counter.

Table 2.9: Contents of tubes for cAMP inhibition assay. All volumes are in μl

<table>
<thead>
<tr>
<th>Tube label</th>
<th>Assay buffer (μl)</th>
<th>Forskolin (1 μM)</th>
<th>Agonist (1 mM)</th>
<th>IBMX (1 mM)</th>
<th>Cells (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>Forskolin</td>
<td>40</td>
<td>20</td>
<td>-</td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>Agonist</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>40</td>
<td>200</td>
</tr>
</tbody>
</table>

2.12.8 Extraction and counting

For the [³H]-CP55,940 binding protocols, experiments were terminated by vacuum filtration onto Whatman G/F-B filters (trapping bound radioactivity) using a Brandel harvester. For [³H]-CP55,940, filters were pre-soaked in 0.5% polyethylenimine (PEI) to reduce NSB prior to being loaded onto the harvester. After filtration, the filters were placed in scintillation vials to which 4.5 ml of Optiphase safe scintillation fluid was added. Filter bound radioactivity was quantified after an 8 hr delay to allow for extraction, and the amount of radioactivity in the vials were counted for 3 min each using a scintillation counter (Packard 1900TR) (Williams et al. 2007; Brighton et al. 2009).
2.12.9 Statistical analysis

All data are expressed as mean ± SEM. N refer to the number of individual experiments with data plots determined individually for each separate experiment. All curve fitting was performed using GraphPad Prism v6.0 (GraphPad, San Diego, CA).

For saturation experiments $K_d$ and $B_{max}$ values were determined by analysing data by nonlinear regression and fitted to a one-site binding model. The cAMP data are presented as percentage inhibition of the forskolin-stimulated response. Statistical analysis (paired/unpaired Student’s t-test and ANOVA with Bonferroni correction for multiple comparison, where appropriate) was performed using GraphPad Prism v6.0 and p<0.05 was considered significant.

2.13 Rat Cystometry

2.13.1 Theory

The rat model has been used to investigate urinary storage and voiding function with cystometry as a common investigative methodology. Cystometric recording techniques in awake or anesthetized rats provide valuable tools for analysing the neural control of the urinary bladder (Andersson et al. 2011). A variety of techniques have been used, including acute (Takagi and Matsumoto et al. 2004) and sub-acute (Peng et al. 2006) suprapubic catheterisation in anesthetised (Maggi 1990) and awake (Angelico et al. 2006) animals and acute transurethral catheterisation in anesthetised animals (Nishijima et al. 2007). Transurethral cystometry appears to be obstructive and may activate nociceptive reflexes (Smith et al. 2008) so when possible suprapubic catheterisation should be preferred. The effect of bladder and urethral instrumentation as well as retrograde bladder infusion in rat cystometric studies might be considered to be a source of deviation of measured parameters from normal physiologic responses. However, a study has shown that other than volume-related parameter changes probably related to surgical compromise of bladder capacity, suprapubic catheterisation does not alter the cystometric and physiologic responses to voiding when compared to normal, non-instrumented voiding (Smith et al. 2008).
While a variety of anaesthetic agents have been used, urethane is commonly employed when bladder and urethral functions are studied due to its predictable and relatively minor impact on the micturition reflex (Maggi 1990; Cannon and Damaser 2001). Micturition in rodents is produced by sequential activation of reflexes whose co-ordination is essential for efficient voiding (Andersson et al. 2011). In addition to detrusor contraction and urine flow, rat voiding includes abdominal wall contraction (Cruz and Downie 2006) and an elevation in blood pressure (Matsumoto et al. 2004) As abdominal wall contraction is a significant component of rat voiding, it has been termed the voiding-associated abdominal wall response (Smith et al. 2007) which has been demonstrated to be preserved under urethane anaesthesia in rats (Sivarao et al. 2007).

The rat bladder demonstrates some morphological and functional differences compared to the human bladder. For example, the rat bladder wall is devoid of intramural ganglia (Uvelius and Gabella 1995) and the major part of normal rat bladder contraction both in vitro and in vivo is mediated via ATP, and not as in humans by acetylcholine (Brading and Williams 1990; Andersson and Arner 2004; Streng et al. 2004b). Streng et al. (2004b) demonstrated in rats that ATP was most important for the initial, rapid part of the voiding contraction, whereas acetylcholine was more important for the latter part. In addition, the striated muscle of the proximal urethra (rhabdosphincter) plays an important part in the micturition process in rats (Streng et al. 2001; Streng et al. 2004a). There are four phases that have been distinguished in rat micturition, as measured by transvesical cystometry (Figure 2.19). There is an initial increase in the intravesical pressure (phase 1) followed by intraluminal pressure high frequency oscillations (IPHFOs, phase 2) leading to a pulsatile flow of urine. In the third phase there is a rebound increase in intra-vesical pressure, as the pulsatile flow ends. In the fourth phase there is a rapid pressure decline to the level before the micturition contraction. The IPHFOs consist of a series of urethral openings and closings, which is different from what is found in humans. Without the IPHFOs the rat bladder does not empty effectively and is associated with pulsatile flow of urine demonstrated on cystometry (Streng et al. 2002). In addition, there are minor gender differences in the voiding between female and male rats as reported by Streng et al. (2002). Male rat voiding consists of a fast spike-like urine flow, whereas female rat voiding is ongoing but interrupted for short periods when bladder pressure is increased.
Figure 2.19: Phases of female rat micturition. Phase 1—initial increase of intravesical pressure. Phase 2—intraluminal pressure high frequency oscillations (IPHFOs) (pulsatile flow of urine). Phase 3—rebound increase in intravesical pressure (end of pulsatile flow). Phase 4—rapid pressure decline to the level before the micturition contraction.

Several cystometric parameters measured in rats, such as threshold pressure, intermicturition pressure, spontaneous activity, and micturition frequency, are usually not used in human cystometry. The parameters measured in this study are described below. It is difficult to establish a normality range, since several factors can influence urodynamic recording. A control group must be part of every study involving cystometric analysis, in order to establish a comparison baseline for the different parameters. Basal (or baseline) pressure (BP) is the minimum pressure between two micturition episodes (Figure 2.20). This parameter is important as an internal control for the procedure as high basal pressures may mean technical issues, such as catheter occlusion, bladder stones, bladder clots, or equipment issues. Maximum voiding pressure (MP) also known as peak pressure, is the maximum pressure during a micturition cycle (Figure 2.20) (Yaksh et al. 1986; Chang and Havton 2008). This pressure does not however, correspond to the voiding pressure in humans, since voiding in rats starts with the appearance of IPHFOs, and rat MP often occurs after the end of urine flow. Some intravesical pressure
measurements such as basal pressure, maximum pressure, and abdominal pressure recordings should be comparable in human and rats (Andersson et al. 2011). The micturition interval (MI) also known as the intercontraction interval is defined as the time period between two maximum voiding pressures (Figure 2.20) (Masuda et al. 2007; Chang and Havton 2008). Cystometric bladder capacity is defined by ICS as ‘‘the bladder volume at the end of the filling cystometrogram, when ‘permission to void’ is usually given’’ (Abrams et al. 2002) however, it is not possible to give permission to void to rats. In rats, bladder capacity (BC) can be determined as infusion rate multiplied by the micturition interval.

![Figure 2.20](image)

**Figure 2.20:** A magnification (5:1) of two micturition contractions demonstrating cystometric parameters in a female rat. MI, micturition interval; BP, basal pressure; MP, maximal voiding pressure; TP, threshold pressure; IPHFOs, intraluminal pressure high-frequency oscillations. TP occurs synchronously with the start of IPHFOs and represents the pressure just before the release of fluid.

### 2.13.2 Methodology

#### 2.13.2.1 Animals

Adult female Wistar (n=7 weighing between 200 and 250 g) and Sprague-Dawley rats (n=5-6 per group weighing between 250 and 300 g), were purchased from Charles River (UK) and housed in an environmentally-controlled room with a 12-hour light/dark cycle and unlimited access to food and water. All experiments on these animals were performed in accordance with the UK Home office regulations and the Animals (Scientific
Procedures) Act, 1986. The results of all these studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny et al. 2010).

2.13.2.2 Drugs and solutions

CP55,940 was dissolved in DMSO to make up stock solutions. The dose of 0.005 mg/Kg of CP55,940 was based on evidence from a previous study by Gratzke et al. (2009), who showed a significant increase in the micturition interval following intravesical infusion of the drug. AM251, the selective CB1 receptor antagonist, and AM630, the selective CB2 antagonist, were dissolved in DMSO. The dose for AM630 (3 mg/kg) and AM251 (1 mg/Kg) was chosen based on previous published studies (Malan et al. 2001; Hiragata et al. 2007; Ueda et al. 2014).

2.13.2.3 General Preparation

Anaesthesia was induced and maintained with isoflurane (induction dose 4%; maintenance dose 1.5-3%) throughout. The femoral vein was cannulated with an intravenous infusion set containing an injection port and safety device. At this point, urethane (1.2 g/Kg i.v.) was slowly administered until a suitable depth of anaesthesia (assessed by an absence of hind limb withdrawal in response to paw pinch and absence of a corneal reflex) was achieved. Supplementary doses of urethane (0.1 g/Kg i.v.) were administered as required. Body temperature was maintained at 37 °C by placing the animal on a homeothermic blanket system (Harvard Apparatus, Holliston, MA) and changes in temperature monitored using a rectal temperature probe.

2.13.2.4 Cystometry

The ureters were exposed by retroperitoneal incisions and the distal ends tied and cut to ensure no backflow and hence no leakage of saline from the bladder. The abdomen was opened through a midline incision and a single cuffed polyethylene catheter (PE-90 1.02 mm, Portex, Hythe, Kent, UK) was inserted through a small incision into the bladder dome. The catheter was secured with a suture around the top of the bladder dome and this was followed by closure of the abdominal incision. The catheter was connected via a three-way stopcock to a pressure transducer (DTX plus transducer, Digitimer Ltd.,
Singapore) to record bladder pressure and a syringe pump (Pump 33, Harvard Apparatus, Holliston, MA) that supplied continuous infusion (0.15 ml/min) to evoke the micturition reflex.

During cystometry, data was captured using the data acquisition software (LabChart v7.3, AD Instruments, UK) and cystometric parameters, such as micturition interval (MI), baseline pressure (BP), maximum voiding pressure (MP) and bladder capacity (BC), recorded.

### 2.13.2.5 Experimental protocol

A stabilization period of 30-45 min, with continuous intravesical infusion (0.15 ml/min) of 0.9% (w/v) sterile saline into the rat bladder was undertaken. Following the acquisition of stable contractile responses, saline was infused for a further 1 hr to evaluate bladder activity and this was designated as the ‘control period’.

To control for the effects of solvents, the vehicle (0.2% DMSO), was subsequently infused for another hour, followed by intravesical infusion of the cannabinoid mimetic CP55,940 (0.005 mg/Kg). In experiments examining the effects of CB receptor antagonists, intravesical CP55,940 infusion was commenced five min after intravenous administration of the CB1 receptor antagonist AM251 (1 mg/Kg), or the CB2 receptor antagonist AM630 (3 mg/Kg).

In the studies examining the effect of CP55,940 on irritated bladders (the acetic acid-treated group), 0.25% acetic acid (Hino et al. 2010; Mally et al. 2013) was infused intravesically for 30 min to induce bladder irritation (Hiragata et al. 2007) before intravesical CP55,940 (0.005 mg/Kg) infusion was commenced for 1 hr.

### 2.13.3 Statistical Analysis

For data comparison, the cystometric parameters were measured for one hour from the application of the drug. All data values are expressed as mean ± SEM. Paired Student’s t-test was used to compare the cystometric parameters between control with saline and after application of CP55,940 in the same group and also used to compare CP55,940 and
acetic acid-exposed rats. Kruskal-Wallis one way ANOVA with Dunn's multiple comparisons test was used to compare cystometric parameters between CP55,940 and the CB receptor antagonists. P-values <0.05 were considered significant. GraphPad Prism v6.0 was used for statistical analysis.
Chapter 3

Distribution and function of the endocannabinoid system in the rat and human bladder
Chapter 3 Distribution and function of the endocannabinoid system in the rat and human bladders

3.1 Introduction

Although both cannabinoid receptor isoforms have been identified in human and rat bladder, the published data show conflicting observations for CB1 receptor localisation, with human and rat urothelium and detrusor (Hayn et al. 2008; Walczak et al. 2009; Mukerji et al. 2010) being positive, but undetectable in another study examining CB1 receptor in the rat urothelium (Gratzke et al. 2009; Gratzke et al. 2010). Although the localisation and expression of FAAH in the urinary bladder has only recently been reported (Strittmatter et al. 2012), NAPE-PLD has not. These enzymes have been studied extensively in the brain (Egertová et al. 2008), where the distribution of FAAH co-localises with CB1 receptors, suggesting that FAAH plays a key role in cannabinoid signalling mechanisms (Tsou et al. 1998).

Furthermore, the transient receptor potential vanilloid type 1 (TRPV1) (a well-characterised receptor in the rat and human bladder) is also activated by anandamide and activation of this receptor may contribute to the development of an overactive bladder, since its activation regulates the frequency of bladder reflex contraction. This was demonstrated using intravesical administration of the TRPV1 agonist capsaicin, which resulted in an increase in bladder capacity and decrease in urinary incontinence. However, administration of capsaicin was abandoned due to the intense neuronal excitation it induced (Geirsson et al. 1995). TRPV1 is expressed in the nerve fibres within the bladder muscularis, sub-mucosal and mucosal layers and in the urothelium (Birder et al. 2001). The hypothesis being tested in this study is that the endocannabinoid system is present in the urinary bladder and has a functional role.

3.2 Aims

The main objectives described in this chapter are to characterise the expression and distribution of CB1, CB2 and TRPV1 receptors, and the cannabinoid-modulating enzymes, FAAH and NAPE-PLD (components of the endocannabinoid system) in human and rat bladder using immunohistochemistry and Western blots. Secondly, to
examine the effects of a CB1 receptor agonist (ACEA) and a CB2 receptor agonist (GP1A), on isolated bladder strips obtained from rats and thirdly to determine the post synaptic responses of parasympathetic nerves to stimulation with the acetylcholine receptor agonist carbachol (if any) in the presence and absence of the cannabinoid receptor agonists and determine the possible pathways involved.

3.3 Results

3.3.1 Immunohistochemistry

To show that the antibodies used in this study were specific for their respective antigens, positive control tissues (rat brain for CB1 and NAPE-PLD, rat spleen for CB2 and FAAH, human skin for TRPV1) and for negative controls IgG or blocking peptide for all of the antigens, were used. Rat tongue is not known to express the cannabinoid receptors, so tongue was used as a negative control where the antibody of interest was incubated on the tissue. As expected, no immunostaining was detected in the negative controls. Thus, the data indicated that the immunohistochemistry was specific for each antibody used (Figure 3.1). The concentration used for each antibody was determined by optimisation of the methodology in rat tissue repeating each experiment 5-6 times. Two to three concentrations were used for the various antibodies, except for TRPV1, whose optimal concentration had already been established in the laboratory (CB1 1:50, 1:100; CB2 1:500, 1:800; FAAH 1:500, 1:1000; NAPE-PLD 1:1000, 1:5000, 1:6000; TRPV1 1:1000) before deciding on the final concentration shown in Table 2.1.
Figure 3.1: Immunohistochemical staining showing positive controls for the cannabinoid receptors and their modulating enzymes. Panel A shows the presence of CB1 receptor (brown staining) in rat brain indicated by the red arrows. Mayer’s haematoxylin was used to stain nuclei. Rat brain is known to express high levels of CB1 receptors, so was used as a positive control. Panel B shows negative control where rat brain was incubated with CB1 antibody plus the blocking peptide to ensure the specificity of the antibody. Panel C shows positive immunostaining for CB2 in rat spleen where CB2 receptors are known to be present in high numbers. Panel D shows incubation with CB2 antibody plus the blocking peptide used as a negative control. Panel E shows positive immunostaining for the FAAH enzyme in the spleen and Panel F demonstrates specificity of the FAAH blocking peptide as no immunostaining is detected. Panel G is a section of rat brain incubated with NAPE-PLD antibody confirming the presence of this antigen in rat brain and Panel H demonstrates no staining when a brain section is incubated with IgG instead. Panels I and J demonstrate further negative controls for FAAH and NAPE-PLD, respectively, using rat tongue, which is known not to express the endocannabinoid system. For these sections, slides were incubated with the respective antibody for the enzyme of interest and no staining was detected suggesting the absence of the two enzymes in this tissue. Panel K shows a section of human skin known to express abundant amounts of TRPV1 receptors, and therefore an effective positive control. Panel L, shows a section of rat tongue used as a negative control, where the section was incubated with TRPV1 antibody but no staining was detected. Images were captured 200x magnification.
Figures 3.2 and 3.3 (A, B, I, J) show that immunoreactive CB1 and TRPV1 receptors were detected in the urothelium and detrusor of rat and human bladders. In the rat bladder, CB2 receptor immunostaining was observed only in detrusor muscle (Figure 3.2 E, F) whereas in the human bladder CB2 immunoreactivity was observed both in the urothelium and smooth muscle (Figure 3.3 E, F). The intensity of staining in the human urothelium was more pronounced when compared to the staining observed in the detrusor muscle.

Immunoreactivity for the modulating enzymes of the cannabinoid system, FAAH and NAPE-PLD, were observed in human and rat urothelium and detrusor muscle (Figures 3.4 and 3.5). The staining was more intense throughout the three layers of the urothelium for these enzymes than that observed in smooth muscle suggesting greater protein expression.
Figure 3.2: Immunohistochemical staining of rat bladder urothelium and detrusor sections depicting cannabinoid and TRPV1 receptors. Each image is a representative of 15 rat samples. Panel A shows the presence of CB1 receptor (brown staining) using CB1 antibody (1:50) on the urothelium indicated by the red arrows. Mayer’s Haematoxylin was used to stain nuclei. Panel B shows CB1 immunoreactivity in the detrusor muscle indicated by the red arrows. Panels C and D are negative controls where sections were incubated with CB1 antibody plus the blocking peptide (1:50) for urothelium and detrusor, respectively. Panel E demonstrates no CB2 (1:500) staining in rat urothelium while CB2 immunoreactivity was detected in detrusor muscle as shown in panel F. Panels G and H are negative controls where sections were incubated CB2 antibody plus the blocking peptide (1:500). Panels J and I demonstrate the presence of TRPV1 receptor using TRPV1 antibody (1:3000) in both the urothelium and detrusor of rat bladder as indicated by the red arrows. Panels K and L are negative controls where sections were incubated with IgG isotype. Images were captured at 100 or 200x magnification. U; urothelium, SU; suburothelium; D, Detrusor muscle.
Figure 3.3: Immunohistochemical staining of human bladder urothelium and detrusor sections depicting cannabinoid and TRPV1 receptors. Each image is a representative of 6 human samples. Panel A shows the presence of CB1 receptor on the urothelium indicated by the red arrows showing brown staining. Mayer’s Haematoxylin was used to stain nuclei. Panel B shows CB1 immunoreactivity in the detrusor muscle indicated by the red arrows. Panels C and D are negative controls where sections were incubated with CB1 antibody plus the blocking peptide for urothelium and detrusor, respectively. Panels E and F demonstrate CB2 staining in urothelium and detrusor muscle. Panels G and H are negative controls where sections were incubated CB2 antibody plus the blocking peptide. Panels I and J demonstrate the presence of TRPV1 receptor in both the urothelium and detrusor as indicated by the red arrows. Panels K and L are negative controls where sections were incubated with IgG isotype. Images were captured at 100 or 200x magnification. U; urothelium, SU; suburothelium; D, Detrusor muscle.
Figure 3.4: Immunohistochemical staining of rat bladder urothelium and detrusor sections depicting the modulating enzymes for the cannabinoid system; FAAH and NAPE-PLD. Each image is a representative of 15 rat samples. Panels A and B show the presence of FAAH enzyme which degrades cannabinoids, using FAAH antibody (1:500) on the urothelium and detrusor indicated by the red arrows showing brown staining. Mayer’s Haematoxylin was used to stain nuclei. Panels C and D are negative controls where sections were incubated with FAAH antibody plus the blocking peptide (1:500) for urothelium and detrusor, respectively. Panels E and F demonstrate synthesizing enzyme, NAPE-PLD (1:5000) staining in rat urothelium and detrusor muscle. Panels G and H are negative controls where sections were incubated with IgG isotype. Images were captured at 200x magnification. U; urothelium, SU; suburothelium; D, Detrusor muscle.
Figure 3.5: Immunohistochemical staining of human bladder urothelium and detrusor sections depicting the modulating enzymes for the cannabinoid system; FAAH and NAPE-PLD. Each image is a representative of 6 human samples. Panels A and B show the presence of degrading enzyme FAAH, on the urothelium and detrusor indicated by the red arrows showing brown staining. Mayer’s Haematoxylin was used to stain nuclei. Panels C and D are negative controls where sections were incubated with FAAH antibody plus the blocking peptide for urothelium and detrusor, respectively. Panels E and F demonstrate synthesizing enzyme, NAPE-PLD staining in urothelium and detrusor muscle. Panels G and H are negative controls where sections were incubated with IgG isotype. Images were captured at 200x magnification. U; urothelium, SU; suburothelium; D, Detrusor muscle.
3.3.2 Western blot analysis

The antibody concentrations used for the immunoblots were obtained through optimising the methodology. Each optimisation experiment was performed 5-6 times using numerous concentrations of each antibody in the presence of different blocking buffers (5%-milk-TBS-T, 2%-NGS-TBT-T, 5%-NGS-TBS-T, 10%-NGS-TBS-T) in order for the background noise detected on developing the film to be reduced to a minimum. For experiments using CB1 antibody, concentrations of 1:100 and 1:200 were used, with 1:100 for both antibody and peptide being considered to be optimal. For the CB2 antibody, 1:500 was optimal from the start for both antibody and peptide but optimisation was required regarding use of blocking buffer. Similarly, FAAH antibody and peptide were used at 1:500 but optimisation was required with regards to the use of the correct blocking buffer. Various concentrations (1:2000; 1:3000; 1:4000) of NAPE-PLD antibody were tested with 1:2000 being optimal. Similarly, for the TRPV1 antibody, a concentration of 1:3000 was found to be optimal when antibody dilutions 1:1000, 1:2000 and 1:3000 were tested.

To determine whether the antibodies used were specific for the CB1, CB2 or TRPV1 antigens, isolated rat and human bladder proteins were first separated on SDS-PAGE gels together with representative positive control tissue proteins, transferred to nitrocellulose membranes and immunostained with respective antibodies (see Section 2.7.2). Probing such blots with CB1 antibody produced specific bands of 53 kDa in the rat bladder and brain and 40 kDa in the human bladder (Figure 3.6 Panel A). A reason for the difference in size of the bands detected could be due to glycosylation, species differences or detection of a different isoform of CB1. By contrast, CB2 immunoblots showed identical 45 kDa molecular mass bands in rat bladder and spleen, but a smaller 43 kDa protein in human bladder (Figure 3.6 Panel B), whereas immunoreactive bands for TRPV1 receptors were observed as a specific band of 104 kDa in all tissues (Figure 3.6 Panel C).
Figure 3.6: Western blots showing protein expression for CB1, CB2 and TRPV1 receptors in rat and human bladders with the respective positive controls. Panel A shows a representative immunoreactive CB1 (1:100) blot using various amounts of protein, as shown below the immunoblot. The upper blot in panel A demonstrates an immunoreactive band at 57 kDa for rat bladder (RB) and a band at 53 kDa for rat brain (Br). The lower blot shows a band at 40 kDa for human bladder (HB) and 53 kDa for rat Br. Both bands were detected in CHO cells transfected with CB1 (used as a further positive control). Panel B displays immunoblots using the CB2 antibody (1:500). The upper blot shows a band at 45 kDa for both RB and rat spleen (Sp) using various concentrations of protein (shown under the relevant band). In the lower blot using HB protein, a band was detected at 43 kDa for HB and 45 kDa for the positive control Sp. Panel C shows immunoblots using the TRPV1 antibody (1:3000). A single band at the known molecular weight (104 kDa) was detected in Br, HB and RB.
Immunoblots for FAAH protein (Figure 3.7 Panel A) showed specific bands at 55 kDa in both the human and rat bladder and 56 kDa in rat brain. These observations are lower than the expected molecular weight (60 kDa), possibly due to proteolytic cleavage of the protein or possibly that there are different isoforms of FAAH. Specific bands to the NAPE-PLD antibody were seen at 53 kDa in the rat brain, 44 kDa in the rat bladder and 45 kDa in the human bladder (Figure 3.7 Panel B).

**Figure 3.7:** Western blots showing protein expression for FAAH and NAPE-PLD enzymes in rat and human bladders with the respective positive controls. Panel A shows immunoreactive FAAH binding using increasing amounts of protein as shown below the immunoblot. The upper blot in panel A demonstrates an immunoblotted band at 55 kDa for rat bladder (RB) and a band at 56 kDa for rat brain (Br). The lower blot shows a band at 44 kDa for human bladder (HB) and 56 kDa for rat Br. Panel B displays immunoblots using NAPE-PLD antibody. The upper blot shows a more prominent band at 45 kDa and a band at 50 kDa for rat brain and a band at 53 kDa for rat bladder using different concentrations of protein (shown under the relevant band). In the lower immunoblot, a band was detected at 45 kDa for HB and 53 kDa for the positive control Br.
3.3.3 Quantitative PCR

The relative transcript levels for the CB1 receptor was significantly higher in detrusor muscle compared to normal urothelial samples (p=0.004). Mean ± SEM of the different groups are shown in Table 3.1.

Table 3.1: Cannabinoid receptor transcript levels in human urinary bladder expressed as ΔCt values for the gene of interest (mean ± SEM) using GAPDH as a housekeeping gene. Data were produced by qRT-PCR from tissue lysates of biopsies from normal human bladders.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Normal Urothelium (n=5)</th>
<th>Normal Detrusor (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Ct values ±SEM</td>
<td>ΔCt relative to GAPDH</td>
</tr>
<tr>
<td>CB1</td>
<td>35.1 ± 0.5</td>
<td>8.4 ± 0.3</td>
</tr>
<tr>
<td>CB2</td>
<td>36.4 ± 0.4</td>
<td>10.7 ± 1.0</td>
</tr>
</tbody>
</table>

3.3.4 In vitro functional studies

Once the presence of CB1 and CB2 receptors had been established in both rat and human bladders, organ bath studies were performed to determine if those receptors were functional. Full thickness rat bladder tissue was suspended in an organ-bath chamber perfused at 1ml/min with Krebs’ solution (see Section 2.2. for constituents) at 37 °C. Strips were tied between the chamber base and an isometric force transducer connected to a four channel PowerLab® running Chart v7.1 (AD Instruments) that measured changes in muscle tension, as described in Section 2.8. Electrical field stimulation (EFS) was delivered as repeated square pulses at 5, 10, 20, and 40 Hz to determine where a submaximal response occurred. This was found to be 10 Hz. The effects of different concentrations of the CB1 agonist ACEA (10⁻⁸ M to 10⁻⁴ M) or the CB2 agonist GP1A (10⁻⁹ M to 10⁻⁶ M) on muscle contractile response as a result of EFS were obtained. Figure 3.8 shows that ACEA (10⁻⁴ M) significantly reduced bladder muscle contractile response by 35 ± 5.4% (p<0.001) while the CB2 agonist GP1A had no effect on EFS-induced contractions.
Figure 3.8: *In vitro* functional studies using rat bladder strips. The effects of the CB1 agonist (ACEA) and the CB2 agonist (GP1A) on contractile response of rat bladder strips subjected to electrical field stimulation (EFS) are shown. The data are presented as the mean ± SD of n=6 separate experiments with *p<0.05. Repeated measures ANOVA followed by Dunnett’s multiple comparison test, (compared to absence of agonist), was performed.

Carbachol response curves were established by adding increasing concentrations of carbachol (10⁻⁷ M to 10⁻³ M) to produce a control curve both in the presence and absence of CB agonist. Rat bladder strips were pre-treated with either ACEA (10⁻¹⁰ M to 10⁻⁶ M) or GP1A (10⁻⁹ M to 10⁻⁶ M) for 30 min prior to stimulation with increasing concentrations of carbachol. The data showed that the EC₅₀ (Figure 3.9) values were significantly shifted to the right by both ACEA and GP1A (Table 3.2) indicating that both CB receptor agonists inhibited the effect of carbachol.
Figure 3.9: Functional *in vitro* studies showing the effects of CB agonists on carbachol-induced contractions. Concentration-response curves are shown for rat bladder strips treated with carbachol (10^{-7} M to 10^{-3} M) alone and after the administration of the CB1 agonist ACEA as shown in panel A or the CB2 agonist GP1A as seen in Panel B. Both CB agonists significantly attenuated the carbachol-induced contractions, suggesting a post-synaptic inhibitory effect of the cannabinoid agonists. Data are presented as % of the maximum contraction induced by carbachol alone and are the mean ± SEM for 6 independent samples.
Table 3.2: Carbachol concentration EC$_{50}$ for ACEA and GP1A. P-values were calculated using one-way ANOVA followed by Dunnett’s multiple comparison test.

<table>
<thead>
<tr>
<th></th>
<th>pEC$_{50}$ ± SEM</th>
<th>Shift in EC$_{50}$ (Fold increase)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control- Cch</td>
<td>5.24 ± 0.05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ACEA 10$^{-10}$ M</td>
<td>4.94 ± 0.06</td>
<td>2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ACEA 10$^{-9}$ M</td>
<td>4.84 ± 0.07</td>
<td>2.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACEA 10$^{-8}$ M</td>
<td>4.80 ± 0.07</td>
<td>2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACEA 10$^{-7}$ M</td>
<td>4.75 ± 0.05</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACEA 10$^{-6}$ M</td>
<td>4.76 ± 0.07</td>
<td>3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GP1a 10$^{-9}$ M</td>
<td>4.84 ± 0.07</td>
<td>3.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GP1a 10$^{-8}$ M</td>
<td>4.81 ± 0.09</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>GP1a 10$^{-7}$ M</td>
<td>4.85 ± 0.10</td>
<td>3.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GP1a 10$^{-6}$ M</td>
<td>4.73 ± 0.09</td>
<td>5</td>
<td>&lt;0.001</td>
</tr>
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</table>
Table 3.3: Summary of results for CB1 and CB2 receptor identification in human urinary bladder

<table>
<thead>
<tr>
<th>Receptor</th>
<th>PCR</th>
<th>IHC</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uro</td>
<td>Det</td>
<td>Uro</td>
</tr>
<tr>
<td>CB1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>CB2</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 3.4: Summary of results for CB1 and CB2 receptor identification in rat urinary bladder.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>IHC</th>
<th>WB</th>
<th>Pre-synaptic effect</th>
<th>Post-synaptic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uro</td>
<td>Det</td>
<td>kDa</td>
<td></td>
</tr>
<tr>
<td>CB1</td>
<td>✓</td>
<td>✓</td>
<td>57</td>
<td>✓</td>
</tr>
<tr>
<td>CB2</td>
<td>↔</td>
<td>✓</td>
<td>45</td>
<td>↔</td>
</tr>
</tbody>
</table>

3.4 Discussion

The data presented in this Chapter not only confirms the presence of both CB1 and TRPV1 receptors in the urothelium and detrusor muscle of both human and rat urinary bladders, but also that the endocannabinoid modulating enzymes NAPE-PLD and FAAH are also present in these tissues. In addition, CB2 expression in the rat was observed only in the detrusor muscle, but in human bladder was found in both the urothelium and detrusor muscle. This is an interesting finding as previous studies examining the expression of CB2 receptors in Sprague-Dawley rats localised the receptor in both the urothelium and detrusor (Hayn et al. 2008; Gratzke et al. 2009; Gratzke et al. 2010) indicating a possible difference in CB2 receptor expression between different rat strains (Wistar rats were used in this study). This is important for researchers working with the rat model when investigating bladder function to bear in mind when interpreting their results.

Western blot analysis confirmed protein expression for both receptors and enzymes in both species but revealed lower molecular sizes for proteins of the endocannabinoid system in the human samples compared to that of the rat. The reason for this is unclear,
but may be due to the possible proteolytic truncation of CB receptor proteins, presence of splice variants, post-translational modification, relative charge of the proteins or even the presence of different CB receptor isoforms in the two species. This is something that may need to be investigated in the future by using antibodies developed that are specific to the different isoforms. qRT-PCR data showed mRNA transcripts for both receptors in the urinary bladder thus strengthening the evidence that the receptors are synthesised in the bladder rather than the proteins being sequestered from other sites. Tables 3.3 and 3.4 summarise the results obtained for CB1 and CB2 receptors in human and rat bladders.

NAPE-PLD is one of the synthesizing enzymes that mediates the release of N-acylethanolamides from a phospholipid precursor (N-acylphosphatidylethanolamide, NAPE). This enzyme has been localised to several brain regions (Egertová et al. 2008) and in human colonic tissue (Marquéz et al. 2009) with similar results to the data shown here. In contrast to NAPE-PLD, FAAH plays an important role in terminating the endocannabinoid signalling process in the central nervous system and other peripheral tissues (Ueda et al. 2000), with the highest FAAH activity observed in liver, brain and testis (Desarnaud et al. 1995; Katayama et al. 1997). In humans, the distribution of FAAH activity is very different to that of the rat, with the enzyme being more abundantly expressed in the pancreas, brain, kidney and skeletal muscle, with relatively lower levels in the liver and placenta, with it being undetectable in the heart and lung (Giang and Cravatt 1997). Immunohistochemical detection of FAAH protein in the rat brain often complements the pattern of CB1 receptor expression (Tsou et al. 1998).

Strittmatter et al. (2012) reported FAAH immunoreactivity in the urothelium but not the detrusor of Sprague-Dawley rats and human bladder. They showed that FAAH is co-distributed with CB2 in the urothelium. This differs from the findings reported in this study where FAAH was detected in both the urothelium and detrusor in rat and human bladders. They showed that FAAH is co-distributed with CB2, which would explain the differences seen in rat bladder, as CB2 was not detected in the urothelium of Wistar rats and only in the detrusor muscle in this study. This, however, does not explain the detection of FAAH in the detrusor of human bladders reported in this study and not by Strittmatter. Other groups have localised CB2 in the detrusor of
human and rat bladders (Gratzke et al. 2009; Tyagi et al. 2009), so if the theory proposed by Strittmatter is correct, that FAAH is co-distributed with CB2, then the findings in this study support his. It may also be the case that FAAH is co-distributed with CB1, as described in the brain (Tsou et al. 1998). This would therefore explain the differences between the distribution of FAAH reported in this study and the results reported by Strittmatter. Unfortunately, these disparate observations do not resolve the issue because they are based on one published study and thus further experiments are required to confirm or refute these findings. Nevertheless, the additional presence of this degrading enzyme in the human and rat bladder suggests that production and degradation of the endocannabinoids occurs in the bladder. This is important, because the use of the FAAH inhibitor URB597 (FAAH inhibitor) reduces inflammation (Storr et al. 2008) in the colon, where FAAH has been localised (Marquéz et al. 2009), these observations suggest that a similar mechanism might occur in the bladder, where URB597 would possibly increase endocannabinoid levels resulting in relaxation of the detrusor muscle. The use of a FAAH inhibitor needs to be explored in the bladder both experimentally and clinically, as it may be the way forward for the treatment of OAB symptoms as it avoids the psychoactive side effects of some CB agonists.

It has been known for some time that bladder TRPV1 is activated by anandamide, causing an excitatory effect, while anandamide also causes an inhibitory effect through CB1 receptor activation (Dinis et al. 2004a; Dinis et al. 2004b). Activation of TRPV1 results in increased reflex activity of the urinary bladder and a burning sensation (Ishizuka et al. 1994). The identification of TRPV1 immunoreactivity and localisation in both the urothelium and smooth muscle in both human and rat bladders supports previous observations (Lazzeri et al. 2004; Brady et al. 2004b). TRPV1 is expressed in both normal and neurogenic overactive human bladders (Ost et al. 2002), with the latter having increased TRPV1 immunoreactive sub-urothelial nerve fibres (Brady et al. 2004b). In addition, it has been suggested that hyperalgesia and hyper-reflexia often experienced in inflammatory conditions is attributed to the activation of TRPV1 by anandamide (Dinis et al. 2004a). The presence of some components of the endocannabinoid system in the rat and human bladder thus suggests a possible role for endocannabinoids in the modulation of bladder sensation and nociception.
To determine what effect cannabinoid receptor activation would have on bladder contractility, CB1- and CB2-specific agonists were used on both EFS-induced and carbachol-induced bladder strip contractions. The data showed that 10 Hz EFS-induced contractions in rat bladder strips were significantly attenuated in the presence of the CB1 receptor selective agonist ACEA. These data support previous observations that activation of the CB1 receptor, appears to have both pre- and post-synaptic inhibitory effects on rat bladder contraction, (Pertwee and Fernando 1996; Martin et al. 2000; Gratzke et al. 2009; Tyagi et al. 2009); an effect that can be explained by either the direct activation of CB1 receptors on the intrinsic nerves to modulate neurotransmitter release, (Walczak et al. 2009) or through activation of urothelial cells, which have been described as having a critical role in the sensory function of the rat bladder (Birder 2010). Contrary to previous published results using male Sprague-Daley rats (Martin et al. 2000) and mice (Pertwee and Fernando 1996), a statistically significant attenuating effect of ACEA on carbachol-induced contractions was observed in rat bladder strips, suggesting an additional post-synaptic effect of the CB1 receptor agonist. A reason for the discrepancy seen in the experiments reported in this study compared to previous published literature is that female Wistar rats were used in this study, while a different strain of rodents were used in previous studies. In addition, a CB1 selective agonist was used in this study (ACEA), while non-selective CB agonists, WIN-55212-2 (Martin et al. 2000) or Δ⁹-tetrahydrocannabinol (Pertwee and Fernando 1996) were used in the previous published studies.

In contrast, GP1A, which is a selective agonist for CB2 receptors, did not show any effect on nerve-induced contractions in the rat bladder. As such, the effects of this cannabinoid receptor ligand cannot be ascribed to modulating pre-synaptic neurotransmitter release, supporting the recently published results using a different CB2 agonist, cannabinor, which had no effect on EFS-induced contractions (Gratzke et al. 2010). In contrast, a similar study using GP1A reported a decrease in EFS-induced contractions of the human bladder (Tyagi et al. 2009). These data suggest that there are functional differences in cannabinoid receptor mechanisms between rat and human bladders (Martin et al. 2000). Furthermore, examination of post-junctional contractile effects of other CB2 agonists on rat bladders, demonstrated that the present
data differed to those previously published (Tyagi et al. 2009; Gratzke et al. 2010), as GP1A attenuated carbachol-induced contractions in rat bladder strips.

3.5 Conclusion

Immunohistochemical and molecular data supporting the presence and expression of the endocannabinoid system (receptors and modulating enzymes) in the human and rat bladder is presented in this chapter. The receptors are functional in that selected cannabinoid receptor agonists affect detrusor muscle contractility, indicating a possible functional role for endocannabinoids in the bladder. These observations provide preliminary evidence of the mechanism through which the clinical effects of cannabis derivatives may be mediated, but further understanding of this mechanism at a cellular level is required. This will be explored in chapter 4.
Chapter 4

Human urothelial cell lines as potential models for studying cannabinoid and excitatory receptor interactions in the urinary bladder
Chapter 4 Human urothelial cell lines as potential models for studying cannabinoid and excitatory receptor interactions in the urinary bladder

4.1 Introduction

Bladder urothelial cells were originally believed to function as a barrier protecting the underlying stroma and detrusor, however, they are now known to secrete a number of signalling molecules including acetylcholine (ACh) (Hanna-Mitchell et al. 2007), ATP (Kullmann et al. 2008) and nitric oxide (Birder et al. 1998), which may influence abnormalities in the bladder where both paracrine and autocrine modulators of smooth muscle and afferent nerve function occur (Apodaca et al. 2007). Many of these neuronal stimuli activate specific G-protein coupled receptors (GPCRs) on the urothelial cell surface to modulate urothelial function (Birder et al. 1998; Bidaut-Russell et al. 1990), suggesting that manipulation of these GPCRs could provide potential targets for treatment of various bladder pathologies, such as overactive bladder syndrome (OAB) and interstitial cystitis (Ochodnický et al. 2012). Specific mechanisms linking signalling molecules to specific urothelial GPCRs and regulation of these receptors in both normal and diseased conditions remains unknown.

Of all the studies that have focused on the signalling mechanism of the cannabinoid system in the bladder smooth muscle, cannabinoid signalling in the urothelial cell has been generally ignored. A major limitation in studying any potential interaction between receptor signalling pathways is the lack of appropriate model systems; a fact that is mainly due to the complexities of acquiring isolated human urothelial cells and placing them into culture. As a result of this limitation, a suitable urothelial cell culture model that would help to explore cannabinoid signalling in the urothelium is required.

Although human urothelial cancer-derived cell lines, that appear to be fully characterised, were easy to identify in the literature, the same was not possible for cell lines that retain the features of normal urothelium. The desired features in a cell culture model include: retention of known features of normal urothelium; appropriate gene expression patterns identified in situ; appropriate responses to muscarinic purinergic and cannabinergic
signalling. Additionally, the cell culture model should be immortal and derived from human tissue, but not be tumorigenic (Rossi et al. 2001). The hypothesis of this chapter is that the cell lines acquired for this research express the cannabinoid receptors and therefore can be used as urothelial models to study cannabinoid signaling. In addition, it is hypothesised that these cell lines can be used as models to study any potential signalling interaction(s) between the cannabinoid receptor signalling and purinergic or muscarinic signalling pathways in the urothelium.

4.2 Aims

The aim of this chapter is to characterise two human urothelial cell lines (UROtsa and HCV 29 cells) and one rodent urothelial cell line (MYP 3) by providing information on protein expression and mRNA transcripts of the cannabinoid receptors. In addition, muscarinic and purinergic expression and signalling interactions with the cannabinoid system will be determined using fluorimetry. Finally, a conclusion on the suitability of these cell lines as model systems to study cannabinoid function in the urothelium will be explored.

4.3 Results

The cell lines examined were human HCV 29 and UROtsa and the rat urothelial cell line MYP 3. The HCV 29 cells were derived from normal human urothelium and their growth in culture is shown in Figure 4.1. UROtsa cells were derived from normal human urothelium lining the ureter and immortalized using a Simian virus 40 large T antigen gene construct. Their growth in culture is seen in Figure 4.2. The MYP 3 cell line was obtained from a small nodule that developed in a heterotopically transplanted rat urinary bladder after treatment with N-methyl-N-nitrosourea and their growth is seen in Figure 4.3.
Figure 4.1: Images of the HCV 29 cell line grown in culture and photographed at different stages of growth. Magnification 40 x for all images. These cells were grown in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and 1% L-Glutamine. Confluent flasks were subcultured at 1:3 a ratio. Panel A shows the cells on day 1, panel B shows the cells on day 2 and panel C shows the cells on day 3 when they were 80% confluent and used for experiments.

Figure 4.2: Images of the UROtsa cell line grown in culture and photographed at different stages of growth. Magnification 40 x for all images. These cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% (v/v) fetal bovine serum and 1 mg/ml glucose. Confluent flasks were sub-cultured at 1:4 ratio. Panel A shows the cells on day 1, panel B shows the cells on day 2 and panel C shows the cells on day 3 when they were 80% confluent and used for experiments.
Figure 4.3: Images of the MYP 3 cell line grown in culture and photographed at different stages of growth. Magnification 40 x for all images. Cells were cultured in Ham’s F-12 supplemented with selenium (5 ng/ml) insulin (5 g/ml), transferrin (5 g/ml), hydrocortisone (36 ng/ml), tri-iodothyronine (4 pg/ml), epidermal growth factor (EGF) (10 ng/ml), and MEM. Confluent flasks were sub-cultured at a 1:4 ratio. Panel A shows the MYP 3 cell on day 1. The red arrow indicates one alive MYP 3 cell while the white arrows indicate dead cells. Panel B shows the cells (indicated by red arrow) on day 2 and panel C shows the cells (indicated by red arrow) on day 3. Panel D shows the MYP 3 cells on day 4 while panel E shows the cell growth on day 5. Panel F shows the cells on day 6 when they are 70% confluent.
4.3.1 Expression of urothelial markers and cannabinoid, muscarinic and purinergic receptors

In order to confirm the epithelial purity of these cell lines, cytokeratin 7 and 20 staining using commercially available antibodies in immunofluorescence (IF) was employed (method described in Section 2.9). Cytokeratin 7, which is normally expressed in all urothelial subtypes (i.e. umbrella, intermediate and basal cells), but not cytokeratin 20 (a specific marker for umbrella cells), was detected by IF in both the UROtsa and HCV 29 cells (Figure 4.4), thereby confirming the urothelial origin of both cell lines. A range of concentrations were used for the primary antibodies (1:50, 1:100, and 1:200) but these do not alter the findings shown in Figure 4.4.

To determine the presence of the cannabinoid receptors (CB1, and CB2) and the putative cannabinoid receptor (GPR55), two independent methods were used. RT-PCR was used to determine the presence of transcripts for these genes, and IF was used to confirm the expression of the corresponding proteins.

4.3.1 Transcript levels in urothelial cell lines

qRT-PCR using TaqMan probes for cannabinoid, muscarinic and purinergic receptors (see chapter 2, Table 2.4 for specific details of each probe) were performed on HCV 29 and UROtsa cell lines. The two cannabinoid receptors were only evaluated in the MYP 3 cell line. Amplified transcript levels are expressed as ΔCt (cycle threshold) values (i.e. difference between the Ct value of the gene of interest and the Ct value of the housekeeper GAPDH) and are shown in Table 4.1.

CB1 mRNA transcripts were detected only in the HCV 29 cells while neither the UROtsa nor MYP 3 cell lines expressed CB2 mRNA transcripts (Table 4.1). Despite the UROtsa cells expressing neither of the two cannabinoid receptors, they produced a GPR55 gene transcript while the HCV 29 cells did not. CB2 and GPR55 transcripts were detected in peripheral blood mononuclear cells (PBMC), used as a positive control and CB1 in ULTR cells and myometrial cells previously identified as positive controls for the relevant receptors (Table 4.2).
Figure 4.4: Immunofluorescence images showing staining after the use of antibodies to detect cytokeratin 7 (1:100) and 20 (1:100) to confirm the epithelial nature of HCV 29 and UROtsa cells. The Images taken at 60 x magnification are a representative of n=5 for HCV 29 and n=6 for UROtsa cells. Panel A shows the presence of cytokeratin 7 on the cell membrane of HCV 29 cells indicated by the white arrows confirming that they are indeed epithelial cells. The DAPI (blue fluorescence) stains the cell’s nucleus. In panel B, there is no cytokeratin 20 staining, which indicates that HCV 29 cells are not umbrella cells. Panel C is the negative control where HCV 29 cells were incubated without the cytokeratin antibody. Panel D demonstrates cytokeratin 7 staining of the cell membrane in UROtsa cells indicated by the white arrows, confirming their epithelial nature. In panel E, there is no cytokeratin 20 staining in the UROtsa cells so they are not umbrella cells. Panel F is the negative control where the UROtsa cells were incubated without the cytokeratin antibody.
Table 4.1 shows the relative expression levels of cannabinoid, muscarinic and purinergic receptor mRNA in the HCV 29, UROtsa and MYP 3 cell lines and expression levels of all transcripts relative to GAPDH are reported as calculated ΔCt relative to the Ct of the GAPDH reference gene. The first important observation was that qRT-PCR confirmed the presence of transcripts for muscarinic receptors M₂-M₅, but not M₁ in HCV 29 cells.

The UROtsa cells were different to the HCV 29 cells and expressed transcripts for M₁, M₄ and M₅ but not for the M₂ or M₃ receptor, which are considered to be the most abundant in human urothelium (Birder and Andersson 2013). All purinergic (P₂X₁, P₂X₃, P₂Y₂, P₂Y₄) receptor transcripts were present in both cell lines with the UROtsa cells having a greater abundance of these purinergic receptor transcripts when compared to the HCV 29 cells. The MYP 3 cell line was not screened for any of the muscarinic or purinergic receptors, as it did not express any of the cannabinoid receptors of interest.

4.3.2 Demonstration of protein in selected cell lines by immunofluorescence

HCV 29, UROtsa and MYP 3 cells were exposed to commercially available antibodies to CB₁ and CB₂ as described in section 2.9. Consistent with findings from qRT-PCR, CB₁ was expressed only in the HCV 29 cells as shown in Figure 4.5A and no cell line demonstrated immunoreactivity for the CB₂ receptor (Figures 4.5-4.7)
Table 4.1 Relative expression levels of cannabinoid, muscarinic and purinergic receptor mRNA in the HCV 29, UROtsa and MYP 3 cell lines. ΔCt values (Ct GOI – Ct GAPDH) for all cell lines (GOI: gene of interest), as produced from their respective amplification plots by analysis of qRT-PCR data are presented. The higher the ΔCt value, the lower the amount of RNA of the GOI detected in the sample. Average (± SEM) GAPDH value for HCV 29 cells was 21.35±0.45 and for UROtsa cells 20.23±0.31.

<table>
<thead>
<tr>
<th></th>
<th>HCV 29 (n=6)</th>
<th>UROtsa (n=5)</th>
<th>MYP 3 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Ct values ±SEM</td>
<td>ΔCt relative to GAPDH</td>
<td>Average Ct values ±SEM</td>
</tr>
<tr>
<td><strong>Cannabinoid receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CB1</td>
<td>35.2±0.79</td>
<td>15.0±1.0</td>
<td>Not detected</td>
</tr>
<tr>
<td>CB2</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>GPR55</td>
<td>Not detected</td>
<td></td>
<td>36.3±0.29</td>
</tr>
<tr>
<td><strong>Muscarinic receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>Not detected</td>
<td></td>
<td>34.7±1.25</td>
</tr>
<tr>
<td>M2</td>
<td>36.1±0.48</td>
<td>16.9±0.26</td>
<td>Not detected</td>
</tr>
<tr>
<td>M3</td>
<td>36.3±0.63</td>
<td>18.4±1.99</td>
<td>Not detected</td>
</tr>
<tr>
<td>M4</td>
<td>36.7±0.72</td>
<td>15.1±1.27</td>
<td>31.7±1.00</td>
</tr>
<tr>
<td>M5</td>
<td>34.9±0.78</td>
<td>16.6±1.90</td>
<td>36.2±0.83</td>
</tr>
<tr>
<td><strong>Purinergic receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2X1</td>
<td>37.0±0.88</td>
<td>19.9±0.98</td>
<td>34.9±0.95</td>
</tr>
<tr>
<td>P2X3</td>
<td>33.9±0.51</td>
<td>14.8±0.88</td>
<td>37.4±0.11</td>
</tr>
<tr>
<td>P2Y2</td>
<td>32.0±1.59</td>
<td>12.3±1.07</td>
<td>25.9±0.23</td>
</tr>
<tr>
<td>P2Y4</td>
<td>34.4±1.65</td>
<td>12.9±0.72</td>
<td>35.7±0.14</td>
</tr>
</tbody>
</table>
Table 4.2: Relative expression levels of cannabinoid receptor mRNA in ULTR, Myometrial and PMBC cells used as positive controls.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>ULTR cells (n=3)</th>
<th>Myometrial cells (n=2)</th>
<th>PMBC cells (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Ct values ±SEM</td>
<td>Average GAPDH values ± SEM</td>
<td>ΔCt relative to GAPDH</td>
</tr>
<tr>
<td>CB1</td>
<td>26.0±0.5</td>
<td>15.8±0.2</td>
<td>10.2±0.7</td>
</tr>
<tr>
<td>CB2</td>
<td>Not detected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPR55</td>
<td>Not detected</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.5: Immunofluorescence images demonstrating expression of the cannabinoid receptors in the HCV 29 cell line. FITC was used as the conjugate to the secondary antibody (anti-rabbit raised in goat) and appears bright green indicating the presence of the receptor of interest and DAPI was used to stain nuclei, which appear blue. Images taken at 60x magnification and are representative of n=6. Panel A. Cells were incubated with CB1 primary antibody and the white arrows show positive immunoreactivity of the receptor on the cell membrane. Panel B represents a negative control where the CB1 antibody was omitted and replaced with non-immunised rabbit IgG. Panel C. shows an additional negative control where CB1 antibody was omitted and the cells incubated with buffer. Panel D. does not demonstrate CB2 protein expression in the HCV 29 cells as no immunoreactivity was detected. Panels E and F are negative controls incubated with either rabbit IgG or buffer, respectively.
Although total immunoreactivity with CB1 antibody in the UROtsa and MYP 3 cells was frequently bright and clear, it was no greater in intensity than the non-specific staining seen in the absence of the primary antibody with exposure to the secondary antibody (anti-rabbit raised in goat), plus FITC conjugate only, and incubation with non-immunised IgG (used at the same concentration of the antibody). Figures 4.6A and 4.7A show confocal images of the UROtsa and MYP 3 cells, exposed to the primary antibody CB1 and demonstrating some FITC staining in both cases. No clear staining of CB1 receptors is seen as fluorescence with rabbit IgG in panels B of Figures 4.6 and 4.7 show similar staining intensity, demonstrating lack of specific staining. Similarly, there is no specific staining for CB2 receptor as shown in Figures 4.5 (D-E), 4.6 (D-F) and 4.7 (D-F), which show confocal images for HCV 29, UROtsa and MYP 3 cells respectively, with and without primary antibody. As discussed in the methods section, a range of concentrations for the primary antibodies and secondary antibodies with the appropriate conjugate were used. The results indicated that the difference between specific and non-specific staining remained unaltered.
Figure 4.6: Immunofluorescence images of UROtsa cells after incubation with CB1 and CB2 primary antibodies. FITC was used as the conjugate to the secondary antibody (anti-rabbit raised in goat) and should appear bright green to indicate the presence of the receptor of interest and DAPI was used to stain nuclei, which appear blue. Images taken at 60x magnification and are representative of n=6. Panel A. Cells were incubated with CB1 primary antibody but there is no pronounced fluorescent green staining along the cell membrane to indicate expression of the receptor. The green staining that is evident on the cell membrane and within the cytoplasm is non-specific as suggested by panel B. Panel B demonstrates some staining even though the cells were incubated with non-immunised IgG as a negative control. Similar staining patterns are seen as in Panel A indicating the staining seen in the cells incubated with the primary antibody is non-specific, and therefore does not indicate the presence of the receptor in this cell line. Panel C. shows an additional negative control where the CB1 antibody was omitted and the cells were incubated with buffer. Panel D. does not demonstrate CB2 protein expression in the UROtsa cells as no immunoreactivity was detected. Panels E and F are negative controls incubated with either IgG or buffer, respectively.
Figure 4.7: Immunofluorescence images of MYP 3 cells after incubation with CB1 and CB2 primary antibodies. FITC was used as the conjugate to the secondary antibody (anti-rabbit raised in goat) and should appear bright green to indicate the presence of the receptor of interest and DAPI was used to stain nuclei, which appear blue. Images taken at 60x magnification and are representative of n=7. Panel A. Cells were incubated with CB1 primary antibody but there is no pronounced fluorescent green staining along the cell membrane to indicate expression of the receptor. The green staining that is evident on the cell membrane and within the cytoplasm is due to background staining as evidenced in panel B. Panel B demonstrates increased staining when the cells were incubated with IgG as a negative control compared to panel A. This indicates that the staining seen in the cells incubated with the primary antibody is non-specific, and therefore does not indicate the presence of the receptor in this cell line. Panel C. shows an additional negative control where CB1 antibody was omitted and the cells incubated with buffer only. Panel D. does not demonstrate CB2 protein expression in the MYP 3 cells as the staining seen in not specific. Panels E and F are negative controls with incubation of either IgG or buffer, respectively.
4.3.3 Functional Studies

Carbachol and ATP-induced changes in \([Ca^{2+}]_i\) in HCV 29, UROtsa and MYP 3 cells

In cuvette based calcium assays conducted at 37 °C, carbachol (CCh) when used at 1 mM, did not increase \([Ca^{2+}]_i\) in UROtsa, HCV 29 or MYP 3 cells (Figures 4.8 and 4.9). In contrast, ATP (1 mM) significantly increased \([Ca^{2+}]_i\) in all three cell lines. \([Ca^{2+}]_i\) was increased by 395 ± 61 nM (p<0.05) for HCV 29, by 705 ± 100 nM (p<0.05) for UROtsa cells (Figure 4.8 B,D), and by 3232 ± 815.2 nM (p <0.05) for MYP 3 cells (Figure 4.9B).

Since transcripts for the CB1 receptor (qRT-PCR) and its protein were demonstrated using IF for HCV 29 cells, and they robustly responded to ATP stimulation, an attempt to distinguish an interaction between the purinergic and cannabinoid receptor systems was made. HCV 29 cells were pre-incubated for 3 min with CB1 agonist ACEA (10^-5 M diluted in 100% ethanol) and then stimulated with ATP. Incubation with ethanol was used in the control group. Figure 4.10 shows that ACEA did not alter ATP-induced \([Ca^{2+}]_i\) levels.
Figure 4.8: Carbachol and ATP induced changes in $[\text{Ca}^{2+}]_i$ in HCV 29 and UROtsa cells. Panel A demonstrates a sample calcium response curve (from n=6), of HCV 29 cells stimulated with carbachol or ATP. The bar indicates time of addition of the drug and its duration in the cuvette. Panel B indicates the mean calcium response of n=6 independent cultures of HCV 29 cells stimulated with carbachol or ATP. Panel C shows a sample calcium response curve (from n=6) of UROtsa cells stimulated with carbachol or ATP. Panel D indicates the mean calcium response (n=6) when stimulated with carbachol or ATP. A one-way ANOVA with Bonferroni’s post-test was used to calculate the p values for both cell lines with p considered significant if <0.05.

$B_1$ = basal $[\text{Ca}^{2+}]_i$ for carbachol (CCh), $B_2$ = basal $[\text{Ca}^{2+}]_i$ for ATP
Figure 4.9: Carbachol and ATP induced changes in \([\text{Ca}^{2+}]_i\) in MYP 3 cells. Panel A demonstrates a sample calcium response curve (from n=6) of MYP 3 cells stimulated with carbachol or ATP. The bar indicates time of addition of the drug and its duration in the cuvette. Panel B indicates the mean calcium response of n=6 independent cultures of MYP 3 cells stimulated with carbachol or ATP. A one-way ANOVA with Bonferroni’s post test was used to calculate the p values with p considered significant if <0.05. 

B1 = basal \([\text{Ca}^{2+}]_i\) for carbachol (CCh), B2 = basal \([\text{Ca}^{2+}]_i\) for ATP

Figure 4.10: Effects of ACEA on HCV 29 cells in cuvette based fluorimetry experiments. Panel A shows a representative calcium response curve from n=3 where cells were either incubated for 3 min with ACEA or ethanol. Cells were then stimulated with ATP. The bar indicates time of addition of the drug and its duration in the cuvette. Panel B shows the mean ± SEM \([\text{Ca}^{2+}]_i\) levels (n=3), with paired Student’s t-test being used to compare the change in \([\text{Ca}^{2+}]_i\) between control (ethanol) and the test group (cells incubated with ACEA) when stimulated with ATP. ACEA had no significant effect on \([\text{Ca}^{2+}]_i\) when compared to the control group (p = 0.45).

Since the UROtsa cells only expressed GPR55 mRNA transcripts the effect of a GPR55 agonist on \([\text{Ca}^{2+}]_i\) was explored using lysophosphatidylinositol (LPI, 10⁻⁶ M). Figure 4.11 demonstrates that there was no increase \([\text{Ca}^{2+}]_i\) in UROtsa cells indicating a relative lack
(or ultra-low abundance) of GPR55 protein in these cells or that the receptors were uncoupled from downstream signalling molecules and pathways. To confirm these cells were functional from a purinergic standpoint, cells were stimulated with ATP, which resulted in a rise of $[Ca^{2+}]_i$ (Figure 4.11 A&B).

**Figure 4.11**: Effects of LPI on $[Ca^{2+}]_i$ levels in UROtsa cells. Panel A shows a sample of $n=6$ calcium response in cultured cells when activated with LPI or ATP. The bar indicates time of addition of the drug and its duration in the cuvette. Panel B indicates the mean calcium response of $n=6$ independent cultures of UROtsa cells stimulated with either LPI or ATP. Statistical analysis using one-way ANOVA with Bonferroni’s post-test was performed with a p value <0.05 considered significant.

B1 = basal $[Ca^{2+}]_i$ prior to stimulation with LPI, B2 = basal $[Ca^{2+}]_i$ prior to stimulation with ATP

**4.3.4 Confocal Microscopy**

Having detected muscarinic receptor transcripts in HCV 29 and UROtsa cells using RT-qRT-PCR but not having detected any calcium changes in fluorimetry experiments when CCh was added, a method studying individual cells was sought. Confocal microscopy with a perfusion system using HCV 29 cell line (as described in section 2.11.2) was employed to investigate whether the effect observed in the fluorimetry experiments was in fact due to the muscarinic receptors not being functional in these cell lines, whether it was due to low expression of the muscarinic receptors or whether muscarinic receptor expression was only on a subset of the cells.

HCV 29 cells were grown on cover slips and incubated with Fluo4-AM so that the emitted fluorescence, as a result of changes in $[Ca^{2+}]_i$, could be detected by the confocal
microscope. As described in section 2.1.12, cells were perfused with Krebs’ solution containing calcium. Cells were stimulated with carbachol (100 μM) and the effects of the cannabinoid agonist, ACEA (1 μM) were examined. Some single cells responded to carbachol, but this was sporadic in the pool of cells tested (see video 2.1 (control) and video 2.2 (ACEA) supplementary material). Due to the lipophilic nature of cannabinoids and the possibility of the ACEA sticking to the tubes of the perfusion system, ACEA was pipetted directly on to the cells and the perfusion was halted for 180 secs. Control experiments were conducted using 0.1% ethanol (Figure 4.12) and the data shows the mean fold-change of fluorescence in 19 HCV 29 cells, used for control and ACEA. As seen in Figure 4.12, after the perfusion was stopped the levels of intracellular calcium did not return to baseline in both the control group (ethanol) and ACEA. Figure 4.13 shows raw traces from individual experiments for both ACEA and control demonstrating there is no pattern and no consistency in whether a carbachol response occurs or for ACEA inhibition. An observation that can be made is that ACEA may display an element of inhibition to the general oscillations observed in the control group. Statistical analysis to compare changes in fluorescence intensity in ACEA-exposed cells with the control group could not be performed due to changes in the baseline after the perfusion was stopped.

Figure 4.12: Confocal microscopy measuring changes in fluorescence intensity equating to intracellular calcium levels in individual HCV 29 cells. The mean fold increase of fluorescence emitted by 19 cells when stimulated with carbachol (CCh) is shown. In panel A, cells were stimulated with carbachol then incubated with ethanol while the perfusion with Krebs’ solution was stopped. Cells were then stimulated with carbachol and this was used as a control. In panel B, cells were stimulated with CCh then incubated with ACEA while the perfusion was stopped and then stimulated again with CCh.
Figure 4.13: Raw traces obtained from confocal microscopy of individual HCV 29 cells. The left hand column shows control samples with ethanol while the right hand column shows cells pre-incubated with ACEA (CB1 agonist). These traces demonstrate that there is no pattern or consistency for each individual experiment.
4.3.5 Cyclic AMP assay

To further assess the function of the CB1 receptor in HCV 29 cells, cAMP assays were performed. Forskolin activates adenylate cyclase and increases intracellular levels of cAMP. As seen in Figure 4.14, ACEA and CCh had no effect on forskolin-stimulated cAMP formation in HCV 29 cells.

![Figure 4.14](image)

**Figure 4.14**: Shows changes in cAMP levels (treated:control) at 1 x fold basal, when cell cultures (n=7) were treated with Carbachol (CCh) or ACEA (A) in the presence of forskolin (FSK/F). One-way ANOVA with Bonferroni’s post-test was used to assess statistical significance p<0.05 was considered significant.

4.4 Discussion

The urothelium is considered to be a critical player in sensory function of the bladder, with that sensory function being regulated by GPCRs expressed on the cell membrane (Ochodnický et al. 2012). In Chapter 3, it was demonstrated that the cannabinoid receptor agonists ACEA and GP1A inhibit detrusor contractility suggesting that the urothelium may respond to these agonists in isolation. However, removal of the urothelium can increase detrusor contraction as a result of acetylcholine stimulation (Hawthorn et al. 2000). These findings suggest the urothelium has an inhibitory effect on bladder smooth muscle contractility and this is mediated by the release of an as yet unidentified factor (Hawthorn et al. 2000). Therefore, useful information could be gathered by studying a human urothelial cell line isolated from the interaction of the bladder smooth muscle cell.
The aim of the present study therefore, was to determine if the UROtsa, HCV 29 and MYP 3 urothelial cell lines could be used as potential models for studying the functional activities of CB1 and CB2 receptors and their possible interactions with muscarinic and purinergic receptor pathways, that are known to be important in normal bladder urothelial physiology (Birder and Andersson 2013).

Although previous studies have identified cannabinoid receptors in human urothelium and detrusor muscle (Tyagi et al. 2009; Mukerji et al. 2010)(see Chapter 3), the expression of these receptors in human urothelial cell lines has not been previously reported. Furthermore, utility of these cell lines as potential models to study cannabinoid signalling mechanisms was unknown. Although HCV 29 cells expressed CB1 receptors and UROtsa cells expressed the GPR55 receptor, functional studies did not show that receptor activation resulted in the expected decreases of carbachol raised \([Ca^{2+}]_i\). This may be due to either the apparently low expression of the cannabinoid receptors in the cell lines or to the fact that receptor activation may not be linked to downstream events. Another reason for these findings could be the sensitivity of the main technique used to detect calcium changes. HCV 29 cells did not respond to carbachol in cuvette-based fluorimetry experiments but some cells may have responded in confocal microscopy experiments.

Despite detecting possible calcium changes in response to carbachol (indicated by the green flashes demonstrated on the attached video) with the use of confocal microscopy, the results obtained from the addition of ACEA could not be interpreted. Due to the perfusion setup of the confocal microscope, ACEA had to be added directly to the cells and the perfusion halted whilst the drug or ethanol control was added. After the perfusion was stopped the levels of intracellular calcium did not return to baseline in both the control group (ethanol) and ACEA. Thus, it is questionable whether ACEA indeed significantly lowered intracellular calcium in HCV 29 cells. In addition, the response to carbachol shown in the raw traces are hypervariable and it may be possible that some of the CCh peaks are due to artefacts. Corroborating the findings of the fluorimetry experiments, HCV 29 cells did not respond to carbachol in cAMP assays. Furthermore, ACEA did not reduce intracellular cAMP concentrations when HCV 29 cells were
stimulated with forskolin, suggesting that CB1 signalling does not occur by the translocation of the $G_{i/o}$ protein to the membranes of this cell line.

Binding of cannabinoids to CB1 and CB2 normally results in a decrease of intracellular cAMP levels and activation of mitogen-activated protein kinase through the coupling of $G_{i/o}$ proteins in neuroblastoma cells (Howlett et al. 1986). Cannabinoid-mediated inhibition of cAMP has been demonstrated in slices of rat hippocampus, striatum, cerebral cortex and cerebellum (Bidaut-Russell et al. 1990). CB1 can also stimulate the formation of cAMP through $G_s$ under certain conditions (Howlett 2005), which may explain the effects of cannabinoids on muscle relaxation previously demonstrated in the urinary bladder (Martin et al. 2000; Gratzke et al. 2009; Tyagi et al. 2009) and rat muscle strips (Chapter 3). However, in this study an increase in intracellular cAMP was not demonstrated, suggesting a possible coupling of CB1 to the $G_s$ protein in the HCV 29 cell line also does not occur. Since CB1 receptors may exist as two distinct subpopulations, one coupled to $G_{i/o}$ proteins and the other to $G_s$ (Calandra et al. 1999), further studies need to be undertaken in primary human urinary bladder tissue to explore this possible association.

It is uncertain why both cell lines did not respond to carbachol, even though the presence of transcripts for $M_3$ and $M_5$ for the HCV 29 cells, and $M_1$ and $M_5$ subtype in UROtsa cells, could be demonstrated. As these receptor subtypes couple to $G_q$ proteins that elevate $[Ca^{2+}]_i$ via inositol trisphosphate and diacylglycerol, one would expect elevated $[Ca^{2+}]_i$ to have occurred. A similar finding was noted in a study measuring intracellular calcium in response to carbachol in four bladder urothelial cancer cell lines, which expressed $G_q$-coupled muscarinic receptors. Even after transfection of the J82 cell line with the $M_3$ receptor, only some single cells responded to carbachol (Tully et al. 2009). A possible explanation for this apparent discrepancy could be that although these cells express these transcripts as demonstrated by qRT-PCR, the corresponding protein is either; not expressed, expressed at ultra-low levels or endogenously blocked by unknown factors. This would also explain the findings that carbachol did not reduce intracellular cAMP levels in HCV 29 cells despite the $M_3$ subtype transcript being detected. Another question is whether the cell lines no longer have an epithelial phenotype and might have become stromal in nature. This possibility was not supported by the finding of positive cytokeratin
7 immunofluorescence in both cell lines, supporting the idea that these urothelial cell lines retain an epithelial lineage in culture. Another possibility is that these cells need to be in close proximity to detrusor smooth muscle cells in order to function properly. Although transcripts for all muscarinic receptor subtypes have been reported in human urothelium (Tyagi et al. 2006) and M2, M3, and M4 subtypes in UROtsa cells (Ochodnický et al. 2012), the UROtsa cells used here only contained M1, M4 and M5 transcripts whilst HCV 29 cells only contained transcripts for the M2-M5 subtypes. These observed differences could be due to differences in immortalisation or in methods of culturing of the cell lines, which have been shown to affect the phenotype of urothelial cells (Georgopoulos et al. 2011), therefore contributing to altered expression profile.

Both cell lines expressed purinergic receptors, which are known to be abundant in the human bladder and activation with ATP significantly increased \([\text{Ca}^{2+}]_i\). ATP is co-released with acetylcholine from parasympathetic nerves, and activates purinergic receptors to initiate a contraction (Andersson and Arner 2004). P2X receptors are ligand-gated non-specific cation channels and when activated, generate depolarising \(\text{Na}^{+}\) and \(\text{Ca}^{2+}\) currents that open L-type \(\text{Ca}^{2+}\) channels to generate an inward flux of \(\text{Ca}^{2+}\) causing a further rise in \([\text{Ca}^{2+}]_i\) (Wu et al. 1999). On the other hand, P2Y receptors are G-protein coupled receptors, which when activated are believed to cause relaxation of the detrusor muscle when activated via cAMP-dependent PKA activity (McMurray et al. 1998). The CB1 agonist ACEA, did not have an effect on \([\text{Ca}^{2+}]_i\) levels, suggesting the cannabinoid system does not interact with the purinergic system in these cells. However, ACEA is not known to inhibit P2X receptors, which may be predominately responsible for the rise in \([\text{Ca}^{2+}]_i\) observed in the two cell lines.

The two urothelial-derived cell lines described here may nevertheless represent a valuable model system to study some aspects of purinergic receptor signalling, but this conclusion may need to be considered with caution because both cell lines exhibit markedly reduced expression of the muscarinic and cannabinoid GPCRs, and therefore lack the full receptor repertoire of native urothelial cells (Birder and Andersson 2013). It is on this basis that it is recommended from the results of this study that a careful comparison of expression of the receptor of interest with that of the normal tissue or with
primary urothelial cells is important when planning to use cell lines as models of the normal urothelium.

4.5 Conclusions

Although HCV 29, UROtsa and MYP 3 cell lines showed evidence of functional purinergic receptors, none of these cell lines showed that their muscarinic receptors are capable of elevating \([\text{Ca}^{2+}]_i\). HCV 29 cells showed expression of CB1 but activation with ACEA did not modify the ATP-induced rise in \([\text{Ca}^{2+}]_i\) levels, indicating a lack of interaction between these two signalling pathways in this cell line.

The HCV 29 cells may prove to be a suitable model to examine isolated CB1 expression but a poor model to examine cannabinoid and muscarinic signalling interactions. Since these cells lines could not be used for further experiments to achieve the objective of this thesis, full thickness bladder tissues (as described in the next chapter) were used to explore the effect of cannabinoids on calcium signalling.
Chapter 5

Cannabinoid receptor expression in bladders from patients with detrusor overactivity
Chapter 5 Cannabinoid receptor expression in bladders from patients with detrusor overactivity

5.1 Introduction

In chapter 3 it was described that the cannabinoid receptors are present in the urinary bladder of both rats and humans. The data from that chapter suggests that the rat bladder could be a good model for studying endocannabinoid physiology for normal bladders. Since the cannabinoid receptors were also expressed in the human bladder, human bladder biopsies could be used to study endocannabinoid physiology in DO bladders.

The available evidence of cannabinoid-mediated effects on bladder function does not discern the exact site of action and little is known of the significance of co-localisation of the CB receptors within other structures. Although the amount of research on the role of the endocannabinoid system in bladder dysfunction has increased, there is little available data specifically examining the role of the endocannabinoid system in DO. In Chapter 4, the idea that a specific urothelial cell model could be used to study cannabinoid-induced calcium signalling was proven to be ineffective as none of the cell lines expressed all of the receptors of interest. This prompted a different approach and the one used and presented in this chapter is a novel method that examines calcium signalling in full thickness bladder biopsies using an upright confocal microscope. The hypothesis of the study presented here, is that cannabinoid receptors are coupled to G_{i/o} so the addition of cannabinoid agonists should result in reduction of detrusor contractility, as seen by a reduction in calcium release. In addition, it is hypothesised that there will be differences in cannabinoid receptor expression and distribution in patients with DO when compared to that of normal bladders and that cannabinoid receptors are co-localised with acetylcholine nerves in the urinary bladder.

5.2 Aims

The aims of this chapter are therefore to develop an experimental protocol to investigate calcium signalling in human normal bladder biopsies and compare results to biopsies from patients with DO. In addition, differences in cannabinoid receptor expression and distribution in the bladders of patients with DO is investigated using
immunohistochemistry and quantitative qRT-PCR and compared to that of patients with normal bladders. Finally, the expression of the cannabinoid receptors are further characterised by co-localisation studies with two nerve cell markers, PGP 9.5 and ChAT.

5.3 Results

5.3.1 Quantitative PCR

The relative transcript levels for the CB1 receptor was higher in urothelium of patients with DO compared to normal urothelial samples (p=0.002). In contrast, patients with DO had lower levels of CB1 receptor in the detrusor compared to normal detrusor samples (p=0.0012). Table 5.1 shows that the transcript levels for both the CB1 and CB2 receptors increased by 2.8 to 3.0-fold, respectively, in the bladder urothelium of DO patients when compared to normal urothelium. By contrast, the transcript levels for CB1 and CB2 receptor decreased by 3.2 and 2.0-fold in the detrusor samples of DO bladders when compared to normal detrusor samples.

5.3.1 Immunohistochemistry

To determine if the differential transcript levels reported in Table 5.1 also applied to differential protein expression, CB receptor protein expression for biopsies from patients with normal bladders and those with DO was determined using IHC. The data revealed positive staining for both CB1 and CB2 receptors in normal human detrusor and urothelium as expected and complementing data already described in chapter 3. The intensity of immunoreactivity observed for CB1 (Figure 5.1) and CB2 (Figure 5.2) receptor expression appeared similar in both urothelium and detrusor of normal bladders. The qRT-PCR results showing lower CB1 and CB2 transcript levels in the detrusor of patients with DO relative to normal detrusor was corroborated by minimal staining in detrusor samples from these patients (Figures 5.1 B, E and 5.2 B, E). At the same time, the qRT-PCR data predicted a 2.8-fold increase in CB1 receptor and 3.0-fold increase in CB2 expression in the urothelium of DO patients. The data (Figures 5.1 A, D and 5.2 A, D) support this with denser staining for both receptors in the urothelium of patients with DO relative to the detrusor muscle and compared to the normal control biopsies.
<table>
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<th></th>
<th>Normal Urothelium (n=5)</th>
<th>DO Urothelium (n=4)</th>
<th>Fold change urothelium</th>
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<td></td>
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<td>ΔCt relative to GAPDH</td>
<td>Average Ct values ±SEM</td>
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<tr>
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<table>
<thead>
<tr>
<th></th>
<th>Normal Detrusor (n=5)</th>
<th>DO Detrusor (n=4)</th>
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<td>ΔCt relative to GAPDH</td>
<td>Average Ct values ±SEM</td>
</tr>
<tr>
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</tr>
<tr>
<td>CB2</td>
<td>33.5±0.4</td>
<td>10.4±0.4</td>
<td>37.0±1.0</td>
</tr>
</tbody>
</table>

Table 5.1: Cannabinoid receptor transcript levels in human urinary bladder expressed as ΔCt values for the gene of interest (mean ± SEM) using GAPDH as a housekeeping gene. Data were produced by qRT-PCR from tissue lysates of biopsies from normal human bladders and those of patients with detrusor overactivity. Significant effects on transcript regulation (p<0.01, unpaired Student’s t-test) are indicated with an up-arrow (↑) for increased expression (lower ΔCt) and a down-arrow (↓) for decreased expression (higher ΔCt) compared with normal urinary bladder. Data are presented as mean ± SEM of different groups.
Figure 5.1: Immunohistochemistry photomicrographs showing CB1 receptor expression in human bladder. A. Brown staining indicates CB1 receptor protein expression in normal urothelium. B. Shows positive staining in the detrusor of normal bladder tissue. C. CB1 negative controls in sections incubated with primary CB1 antibody adsorbed onto blocking peptide. D. Positive staining for CB1 receptor in urothelium of patients with DO. E. Note the reduced staining intensity for CB1 receptor in the detrusor muscle of patients with DO compared to normal detrusor seen in B. F. CB1 negative controls with rabbit IgG. Images captured at 200x magnification. Scale bars at 10 μm. U= urothelium, SU= suburothelium, D= detrusor muscle.
Figure 5.2: Immunohistochemistry photomicrographs showing CB2 receptor expression in human bladder. A. Brown staining shows positive for CB2 receptors in normal urothelium. B. Shows positive staining in the detrusor of normal bladder tissue. C. CB2 negative controls in sections incubated with primary CB2 antibody adsorbed onto blocking peptide. D. Positive staining for CB2 receptor in the urothelium of patients with DO. E. Decreased staining intensity for CB2 receptor in detrusor of patients with DO compared to normal detrusor in B. F. CB2 negative controls with rabbit IgG. Scale bars at 10 μm. Images captured at 200x magnification. U = urothelium, SU = suburothelium, D = detrusor muscle.
Double IF staining of normal human bladders was employed to co-localise CB1 and CB2 in nerves. PGP 9.5, a marker for neural cells, was co-localised in both urothelium and detrusor with both cannabinoid receptors (Figure 5.3 and 5.4). In order to determine which nerves are co-localised with the CB receptors, ChAT, a cholinergic nerve marker was used. Co-localisation of CB1 receptor with ChAT was detected in detrusor muscle but not in the urothelium (Figure 5.5). CB2 receptor was also co-localised with ChAT in both urothelium and detrusor (Figure 5.6). Negative controls were in the absence of primary antibodies and incubation with non immunised IgG as shown in Figures 5.7 and 5.8.
Figure 5.3: Confocal microscopy images showing tissue sections after incubation with CB1 antibody and PGP 9.5 antibody in normal human bladder. Top row (A-D) shows staining in the detrusor muscle of a normal human biopsy section. A. nuclear staining with DAPI, B. CB1 antibody staining with anti-rabbit secondary antibody with FITC conjugate, C. indicates PGP 9.5 positive staining with anti-mouse secondary antibody with Texas Red conjugate, D. is a merged image of A-C with the arrows indicating yellow/orange staining which signifies co-localisation of CB1 receptors on nerve fibres, E. nuclear staining with DAPI in urothelium, F. CB1 receptor protein expression in urothelium indicated by FITC staining, G. shows PGP 9.5 staining in urothelium, H. is a merged image of E-G indicating co-localisation of CB1 with PGP 9.5. Images captured at 60x magnification.
Figure 5.4: Double staining immunofluorescence with CB2 antibody and PGP 9.5 antibody in normal human bladder. Top row (A-D) shows staining in the detrusor muscle of a normal human biopsy section. A. nuclear staining with DAPI, B. CB2 antibody and anti-rabbit secondary antibody with FITC conjugate staining, C. indicates PGP 9.5 positive staining with anti-mouse secondary antibody with mouse conjugate, D. is a merged image of A-C with the arrows indicating to yellow/orange staining that signifies co-localisation of CB2 receptors on nerve fibres, E. nuclear staining with DAPI in urothelium, F. CB2 receptor protein expression in urothelium using FITC, G. shows PGP 9.5 staining in urothelium, H. is a merged image of E-G indicating co-localisation of CB2 with PGP 9.5 (arrowheads). Images captured at 60x magnification.
Figure 5.5: Double staining immunofluorescence with CB1 antibody and ChAT antibody in normal human bladder. Top row (A-D) shows staining in the detrusor muscle of a normal human biopsy section. A. nuclear staining with DAPI, B. CB1 antibody staining with anti-rabbit secondary antibody FITC conjugate, C. indicates ChAT positive staining with anti-mouse secondary antibody with Alexa Fluor conjugate, D. is a merged image of A-C with the arrows indicating yellow/orange staining which signifies co-localisation of CB1 receptors with nerve fibres, E. nuclear staining with DAPI in urothelium, F. CB1 receptor protein expression in urothelium shown as FITC staining FITC, G. shows no ChAT staining in urothelium, H. is a merged image of E-G indicating no co-localisation of CB1 with ChAT. Images captured at 60x magnification.
Figure 5.6: Confocal microscopy images showing double staining immunofluorescence using CB2 antibody and ChAT antibody in normal human bladder. Top row (A-D) shows staining in the detrusor muscle of a normal human biopsy section. A. nuclear staining with DAPI, B. CB2 antibody staining with anti-rabbit secondary antibody FITC conjugate, C. indicates ChAT positive staining with anti-mouse secondary antibody with Alexa Fluor conjugate, D. is a merged image of A-C with the arrows indicating yellow/orange staining which signifies co-localisation of CB2 receptors with nerve fibres, E. nuclear staining with DAPI in urothelium, F. CB2 receptor protein expression in urothelium indicated by FITC staining, G. shows ChAT staining in urothelium, H. is a merged image of E-G indicating co-localisation of CB2 with ChAT indicated by the arrows. Images captured at 60x magnification.
Figure 5.7: Confocal microscopy images showing double staining immunofluorescence controls for CB receptors with PGP 9.5 antibody. These stained sections represent negative controls where the tissue was incubated in the absence of the primary antibodies rows (A-D), or incubated with IgG2a (rows E-D). Panel A shows nuclear staining with DAPI in the absence of CB and PGP 9.5 primary antibodies. Panels B and C were incubated with anti-rabbit secondary antibody with FITC conjugate, or anti-mouse secondary antibody with Texas Red conjugate, respectively, without any primary antibodies, and no fluorescence was detected. Panel D is a merged image of panels A-C. Panel E shows nuclear staining with DAPI in the presence of IgG2a but no primary antibodies. Panels F and G have been incubated with secondary antibody conjugates (FITC and Texas Red, respectively) in the presence of IgG2a. Panel H is a merged image of panels E-G confirming no non-specific staining was detected with IgG2a incubation. Images captured at 60x magnification.
**Figure 5.8:** Confocal microscopy showing double staining immunofluorescence for controls of CB receptors with ChAT antibody. These stained sections represent negative controls where the tissue was incubated in the absence of the primary antibodies rows (A-D), or incubated with IgG (rows E-D). Panel A shows nuclear staining with DAPI in the absence of CB and ChAT primary antibodies. Panels B and C were incubated with anti-rabbit secondary antibody FITC conjugate, or anti-mouse secondary antibody with Alexa Fluor conjugate, respectively, without any primary antibodies, and no fluorescence detected. Panel D is a merge of panels A-C. Panel E shows nuclear staining with DAPI in the presence of IgG but no primary antibodies. Panels F and G have been stained with secondary antibody conjugates (FITC and Alexa Fluor, respectively) in the presence of IgG. Panel H is a merge of panels E-G confirming no non-specific staining was detected with IgG incubation. Images captured at 60x magnification.
5.3.2 Confocal Microscopy

Once it was established that the cannabinoid receptors co-localised with cholinergic nerves in human bladder biopsies, it was decided to determine if any interaction between cannabinoid and cholinergic signalling might occur in this model system. Upright confocal and digital imaging microscopes were used in an attempt to visualize changes in calcium levels in smooth muscle cells when carbachol was added to the human bladder biopsy through a perfusion system (Section 2.11.3). Samples were loaded with the calcium indicator Fluro-4 for 1 hour in darkness and the tissue was rinsed for 5 min in bubbling (95% O₂/5% CO₂) Krebs’ solution to remove extracellular dye. Images were taken with a Leica SP2 upright confocal microscope as described in Section 2.11.3 using the Leica LCS software package.

In order for images to be taken, movement of the imaged site needs to be minimized. This was attempted using various specially designed weights that were placed on the tissue. Spontaneous contractions were minimised and did not pose a problem in capturing an image. When the experimental protocol was initiated, however, and carbachol reached the bladder biopsy, the tissue contracted so quickly and violently that it repeatedly became out of focus, or individual fibres contracted so much that they left the field of view (see video 5.1 supplementary material). Therefore, subcellular Ca²⁺ transients were impossible to measure as they could not be viewed by the confocal microscope, which needs to have a motorised focussing facility coupled with the ability to move with the tissue. Nevertheless, it was possible to delineate nerves and smooth muscle cells in a bladder biopsy (Figure 5.9) but only prior to stimulation with carbachol.
Figure 5.9: Upright confocal microscopy images of human bladder biopsies loaded with Fluro-4 calcium indicator. All images are taken prior to stimulation with carbachol and captured at 40x magnification. Panels A and B show nerves and nerve bundles within the detrusor muscle as indicated by the arrows. Panels C and D show smooth muscle cells shown by the white arrows.

An attempt to measure calcium changes in bladder biopsies was then made using an upright microscope connected to a digital camera, to take images while the tissue was perfused with Krebs’ solution. The details for this are described in chapter 2, Section 2.11.4. With the addition of carbachol, the same problem was experienced with confocal microscopy, where the muscle contracted and the biopsy moved out of focus. Figure 5.10, shows a time lapse series of photos taken of a human bladder smooth muscle cell prior to addition of carbachol. Addition of carbachol in panel D of Figure 5.10 indicates a release of calcium, but also the smooth muscle cell simultaneously moves out of focus. An
attempt to analyse the data is shown in Figure 5.11 where graphs were generated using the associated IGOR software. It appears that there was a change in intracellular calcium but interpretation is difficult because a shift in the tissue was also found to result in a change in fluorescence, and thus misleading the analysis that there was an increase in intracellular calcium. Without the ability to control for movement of the tissue, interpretation of the results was thus unreliable and the protocol was abandoned.

![Figure 5.10: Magnified images of a smooth muscle cell from a human bladder biopsy section taken with a digital camera attached to an upright microscope. The black arrow indicates the smooth muscle cell in panel A while being perfused with Krebs’ solution. Panel B and C show minimal changes to the cell due to spontaneous contraction. In Panel D, carbachol has been added to the biopsy and the tissue has contracted releasing its intracellular stores, and moved out of focus.](image)
Figure 5.11: Intracellular calcium changes in human bladder tissue detected using a digital camera connected to an upright microscope. Panel A is a representative trace from one of four (n=4) samples showing changes in fluorescence over time indicating the average absolute fluorescence intensity. The dotted line indicates the point where carbachol was added to the perfusion system. Panel B is the same trace as shown in Panel A but controlled for movement by the software program. (The IGOR software has the ability for the user to outline the movement of the image and then compensates the analysis for the movement defined by the user. The program calculates the fluorescence intensity ratio against time. The difficulty with the current experiment is that the movement was significant and it is unlikely that the software can make accurate analysis in this situation). Panel C shows another representative trace from a different bladder biopsy, where changes in fluorescence intensity is plotted against time. The dotted line indicates addition of carbachol and unlike results in Panel A, there is a decrease in fluorescence instead of the expected rise suggesting a release of intracellular calcium. This occurred because the tissue moved out of focus. The graph in Panel D, is calculated from the same biopsy as in Panel C where movement is accounted for so the fluorescence intensity ratio is plotted over time.
5.4 Discussion

Studies investigating differences in the expression and distribution of cannabinoid receptors between normal human bladders and those from patients with DO remain scarce. A study using bladder biopsies from multiple sclerosis (MS) patients with neurogenic DO and normal bladders, found a lower expression of CB1 receptor transcripts and a higher expression of CB2 receptor transcripts in patients with neurogenic DO (Katagigiotis et al. 2012). Similarly, in this chapter, a lower expression of CB1 in the detrusor muscle of patients with DO, and a higher expression in the urothelium was reported. However, in this study, CB2 expression followed the same pattern as for CB1 and generally, lower receptor transcripts were detected when compared to CB1 receptor in both detrusor and urothelium. Patients approached in this study were selected based on proven idiopathic DO on urodynamics, while those patients used in the above study, had neurogenic DO following MS. This may suggest differences in expression of cannabinoid receptors between these patient groups. Another explanation for the discrepancy seen for the increased CB2 receptor expression in the study by Katagigiotis et al. (2012) compared to the findings described in this chapter, is the fact that they processed the bladder biopsies as whole tissue, while in this thesis, receptor expression was determined on isolated urothelium and detrusor before being compared. This provides a more accurate assessment of receptor transcript expression than using homogenates of the entire tissue, because differences in cellularity are controlled.

Furthermore, the IHC findings reported in this chapter depicted a greater CB receptor immunoreactivity in the urothelium of patients with DO compared to detrusor, corroborating the findings by Mukerji et al. (2010) who reported increased densities of CB1 immunoreactive nerve fibres in the sub-urothelium compared to controls. However, they also reported increased CB1-positive detrusor nerve fibres in patients with overactive bladder disorder compared to normal bladder. The disparity with the results described in this chapter where reduced immunoreactivity of CB1 receptor in detrusor of DO compared to controls was observed, can be explained; in this study comparisons using the entire detrusor section were made rather than focusing on nerve fibres only. It will be interesting for future studies to clarify co-localising nerves with CB1 receptors to identify the type of nerve fibres altered within the suburothelium and detrusor of patients with DO. A role for CB2 receptors in bladder dysfunction has been suggested by the
finding of increased receptor expression in rat bladders with acute and chronic inflammation (Merriam et al. 2008) and in MS patients with neurogenic DO (Katagigiotis et al. 2012). Similarly increased CB2 receptor expression in the urothelium of patients with DO and a decrease in receptor expression in the detrusor muscle of these patients is reported in this chapter, suggesting altered CB2 function in the urinary bladder that is related to disease. However, one should bear in mind that CB2 is heavily expressed in immune cells and blood cells (Munro et al. 1993; Gong et al. 2006), and despite thoroughly washing the tissue, the effects reported could be a result of CB2 detection in blood cells in the bladder.

In the urinary bladder, there is only one study showing direct evidence of a peripheral action of cannabinoids on the sensory innervation (Walczak and Cervero 2011), as all other available studies have measured the effects of cannabinoids on its afferent activity indirectly using behavioural assessment (Farquhar-Smith and Rice 2001; Farquhar-Smith et al. 2002) or cystometry (Jaggar et al. 1998a; Dmitrieva and Berkley 2002; Hiragata et al. 2007). Walczak and Cervero (2011) reported that local activation of CB1 receptors reduce the inflammation-induced hypersensitivity of sensory afferent fibres in the bladder after co-localising CB1 expressing nerve fibres with TRPV1 and showing that intravesical administration of AZ12696915 (non-selective CB agonist) reduced sensitisation of mouse bladder afferent fibres. Furthermore, expression of CB1 receptor is increased in sensory neurons after inflammation (Amaya et al. 2006). These data support the conclusion that there remains a possible involvement of CB receptor-mediated functions in the local regulation of mechanoafferent activity (Hedlund 2014).

In chapter 3, the CB1 agonist ACEA was shown to produce an inhibitory effect on electrical field stimulation-induced contractions of intact bladder biopsies, an effect which is unique to the stimulation of the intrinsic nerves, suggesting cannabinoids affect efferent functions of the bladder. This led to the exploration of the expression of cannabinoid receptors in nerve fibres. Cannabinoid receptors were co-localised with PGP 9.5 antibody, which is a neurone specific protein found in neurons at all levels of the central and peripheral nervous system. Since both CB receptors were co-localised with PGP 9.5, further characterisation was performed using ChAT antibody to examine cannabinoid receptor co-localisation with cholinergic nerves. The finding described in
this chapter that both CB receptors were co-localised with the generic neuronal marker in the urothelium and detrusor, correlate with the findings of others who have localised both CB receptors with nerve fibres in the urothelium and detrusor. Veress et al. (2013) showed partial co-localisation of CB1 with calcitonin gene-related peptide (CGRP, a marker for sensory nerves) in the muscular layer of rat bladders but no co-localisation in the urothelium. Findings reported in this chapter where co-localisation of CB1 with the generic neuronal marker PGP 9.5 was detected in the urothelium, supports the findings of Veress et al. (2013). However, in a study by Gratzke et al. (2009) there was no immunoreactivity seen for CB1 on nerve structures but they did detect CB2 positive nerve fibres. Further co-localisation studies, located CB2 on the sensory and cholinergic nerves of bladders of rats, rhesus monkeys and humans (Gratzke et al. 2009) supporting observations reported in this chapter of co-localisation of the CB2 receptors with cholinergic nerves. The current co-localisation of ChAT and cannabinoid receptor immunoreactivity, and the inhibitory effects of cannabinoid agonists in vitro described previously in chapter 3 on carbachol induced contractions in detrusor preparations, imply a modulatory function of cannabinoid receptors on cholinergic neurotransmission. Furthermore, the expression of CB1 has been reported to be increased in sensory neurons after inflammation (Amaya et al. 2006), thus supporting the notion that CB receptors could possibly be involved in the local regulation of mechanoafferent activity (Hedlund 2014).

Upright confocal microscopy using a continuous perfusion system was employed to compare cannabinoid receptor effect on calcium signalling in human bladder biopsies with the development of a novel experimental protocol. It was hoped that using this method, a better understanding of the relationship between the urothelium and detrusor would be achieved and DO bladders could be compared to normal bladders to complement IHC and qRT-PCR data described in earlier chapters. The main difficulty with this method, however, was the fact that as soon as carbachol was perfused and reached the biopsy, tissue would contract and move out of the field of view. In confocal microscopy, the section of the tissue that has been focused on is continuously scanned by a laser in order to capture images throughout the different layers. If the section moves out of focus, the upright microscope does not have the ability to automatically re-focus. Since this manipulation needs to be done manually, by which time the section is back in focus,
any calcium changes will have happened and will not have been recorded. Different attempts to prevent the section of the tissue moving out of focus were tried of which, all failed. Different types of weights of varying sizes were used, placing grease on the coverslip was also attempted and using a digital upright microscope was also explored as an alternative option. Although, using upright confocal microscopy has been described previously in rat bladder strips (Young et al. 2009) where they pinned out the bladder strip on a Sylgard-lined organ bath that was mounted on the stage of the confocal microscope, a similar methodology could not be used in this study. The same physical confocal microscope setup was unavailable at both the University of Leicester and University of Birmingham (where incidentally, the microscope stage did not permit the Sylgard-lined organ bath to be used). After six months of experiments both in laboratories at the University of Leicester and the University of Birmingham, it was decided to explore cannabinoid function in the urinary bladder using an in vivo model, which will be described in chapter 7.

5.5 Conclusions

A significant increase of CB receptor expression in the urothelium of patients with DO and a decrease in receptor expression in the detrusor muscle of these patients compared to normal bladders has been demonstrated. This chapter complements the qRT-PCR, IHC and WB findings in human tissue described in chapter 3. Furthermore, the demonstration of co-expression of ChAT with CB1 immunoreactivity and with CB2 immunoreactivity in detrusor muscle together with co-localisation of ChAT with CB2 immunoreactivity in the urothelium, suggests that further studies into the potential interaction between CB receptor signalling and cholinergic signalling is essential (once the technical problems are resolved).
Chapter 6

Binding of $^{3}\text{H}$-CP55,940 to probe bladder CB receptor expression
Chapter 6 Binding of \[^{3}\text{H}\]-CP55,940 to probe bladder CB receptor expression

6.1 Introduction

The pharmacology of cannabinoid ligands may be studied using a number of different models, from cell lines expressing cannabinoid receptors to whole organs and animals. In chapter 4 it was demonstrated that none of the urothelial cell lines expressed both cannabinoid receptor isoforms and functional muscarinic receptors and therefore were not suitable models to study urothelial signalling mechanisms. As a result, human bladder biopsies were used to study the pharmacology of cannabinoid ligands using confocal imaging. However, as described in chapter 5, this proved to be ineffective as the biopsies would contract and shift out of focus making the experiments unworkable. It was then decided to develop a robust assay to determine cannabinoid receptor density in human bladder tissue and the binding affinity of \[^{3}\text{H}\]-CP55,940 in the urinary bladder. Radioligand binding assays can provide useful information on cannabinoid receptor expression, such as the total number of binding sites (\(B_{\text{max}}\)), the equilibrium binding constant (\(K_d\)) and with the judicious use of cannabinoid receptor ligands a receptor-dependent pharmacological profile. The findings from these experiments will complement the results from chapters 3 and 5 where the cannabinoid receptors were localised in the urinary bladder using qRT-PCR, IHC, and WB and strengthen the evidence that the cannabinoid receptors are present in the urinary bladder.

6.2 Aims

The aim of this chapter is to use the binding of the non-selective CB receptor radioligand \[^{3}\text{H}\]-CP55,940 to determine receptor density in human bladder. Rat brain (cerebellum and cortex) and rat bladder were used as control tissues for assay validation. The cerebellum and cortex are noted as tissues with high CB receptor expression and bladder (rat) a tissue of primary interest.
6.3 Results

6.3.1 Time course experiments

The binding of [³H]-CP55,940 in rat cerebellum and cortex was measured over a series of time points and the time to reach equilibrium binding determined. The time course was performed with rat cerebellum and showed a 60 min incubation as being sufficient to reach equilibrium using both low (0.66 nM) and high (1.7 nM) concentrations of [³H]-CP55,940. This incubation time was subsequently used for all saturation and displacement experiments.

6.3.2 Displacement experiments to determine the optimal ligand for NSB

A series of competition binding assays were undertaken in rat cerebellum and cortex using [³H]-CP55,940 as the radioligand, with anandamide and CP55,940 as displacing ligands. The purpose of these experiments was to identify an appropriate ligand and concentration for determining NSB. Both anandamide and CP55,940 caused a concentration dependent displacement of [³H]-CP55,940 in rat cortex and cerebellum (Figure 6.1). Anandamide displaced the binding of [³H]-CP55,940 with an IC₅₀ of 89.9 nM in the rat cerebellum and 450.5 nM in rat cortex and a maximum displacement of 25%. CP55,940 produced a lower IC₅₀ of 2.25 nM in rat cerebellum and 4.5 nM in rat cortex when compared to anandamide but with a similar maximum displacement (29%). Because of the lower IC₅₀ for CP55,940, its greater solubility, and the observation that anandamide did not form a homogenous solution at higher concentrations even in the presence of organic solvents, made CP55,940 the optimum ligand for determining NSB (Table 6.1 summarises characteristics for each ligand). From these displacement experiments 30 μM of CP55,940 was chosen because this concentration caused the maximum displacement of [³H]-CP55,940 from the cannabinoid receptors.
Figure 6.1: Determination of NSB using rat cerebellar and cerebrocortical membranes. Panel A shows displacement experiments using different concentrations of anandamide with the displacing concentration required to reach equilibrium 100 μM. Panel B shows displacement experiments using various concentrations of CP55,940 and the displacing concentration required to reach equilibrium is 30 μM. Data are presented as the mean ± SEM of 6 independent observations.

Table 6.1: Summary comparing characteristics to determine the ideal NSB. Values are generated from Figure 6.1.

<table>
<thead>
<tr>
<th>Cerebellum</th>
<th>Log IC₅₀ (nM)</th>
<th>Kᵢ (nM)</th>
<th>Solubility</th>
<th>Conc. at equilibrium (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP55,940</td>
<td>8.3±0.3</td>
<td>2.25</td>
<td>Soluble in DMSO, stayed in solution</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(5 nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anandamide</td>
<td>6.7±0.2</td>
<td>89.9</td>
<td>Soluble in ethanol, difficult to keep in solution</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>(199.5 nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortex</th>
<th>Log IC₅₀ (nM)</th>
<th>Kᵢ (nM)</th>
<th>Solubility</th>
<th>Conc. at equilibrium (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP55,940</td>
<td>8.0±0.3</td>
<td>4.5</td>
<td>Soluble in DMSO, stayed in solution</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(10 nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anandamide</td>
<td>6.0±0.2</td>
<td>450.5</td>
<td>Soluble in ethanol, difficult to keep in solution</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>(1000 nM)</td>
<td></td>
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</table>

6.3.3 Saturation Binding assays

Saturation binding experiments in human cystectomy samples, rat bladder and cerebellum showed that the binding of [³H]-CP55,940 was concentration dependent and saturable (Figure 6.2), with the Bₘₐₓ and Kᵦ values for human and rat bladder and rat
cerebellum summarised in Table 6.2. The $B_{\text{max}}$ for human bladder was 421.4 fmol $[^{3}\text{H}]$-CP55,940/mg protein and the $K_{d}$ of 1.26 nM. For rat bladder, $B_{\text{max}}$ was 429.7 fmol $[^{3}\text{H}]$-CP55,940/mg protein with the $K_{d}$ being 0.39 nM. Binding of $[^{3}\text{H}]$-CP55,940 to rat cerebellum (positive control) demonstrated a higher $B_{\text{max}}$ of 1974 fmol $[^{3}\text{H}]$-CP55,940/mg protein and a similar $K_{d}$ (0.45 nM), which is similar to a previous report (Thomas et al. 2004).

![Figure 6.2](image-url)

**Figure 6.2:** Saturation-binding experiments of $[^{3}\text{H}]$-CP55,940 using rat and human bladder and rat cerebellum membranes. Log-transformed specific binding plots were used to determine the maximum receptor binding capacity ($B_{\text{max}}$) and the equilibrium dissociation constant ($K_{d}$) in each of the respective membranes. Panel A shows representative curves from rat bladder (n=6) and rat cerebellum (n=7) experiments. Panel B depicts a sample curve using human urinary bladder (n=5).

<table>
<thead>
<tr>
<th></th>
<th>$K_{d}$</th>
<th>$B_{\text{max}}$ ($[^{3}\text{H}]$-CP55,940 fmol /mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human bladder</strong></td>
<td>8.9 ± 0.2 (1.26 nM)</td>
<td>421.4 ± 104.2</td>
</tr>
<tr>
<td><strong>Rat Bladder</strong></td>
<td>9.4 ± 0.1 (0.39 nM)</td>
<td>429.7 ± 102.1</td>
</tr>
<tr>
<td><strong>Rat cerebellum</strong></td>
<td>9.3 ± 0.1 (0.45 nM)</td>
<td>1974 ± 308.2</td>
</tr>
</tbody>
</table>

**6.4 Discussion**

To the author’s knowledge, this is the first study where radioligand binding assays were conducted using urinary bladder tissue. As such, the methodology required optimisation using rat tissue prior to the use of scarce human bladder tissue. The time course
experiments identified that CP55,940 reached equilibrium at 60 min. In addition, a series of experiments were performed to identify the best ligand that could be used as an NSB and CP55,940 was selected. This is a bicyclic non-classical cannabinoid and a full agonist at both CB1 and CB2 receptors and reported to be 10–50 times more potent than Δ²-THC (Howlett et al. 2002). Anandamide on the other hand, is an endocannabinoid and not only a full agonist for both CB receptors but also for the TRPV1 receptor.

The radioligand (³H]-CP-55,940) used in this study has been used in a number of radioligand binding assays (Brents et al. 2011) and the reported binding parameters in mouse brain membranes are 2336 fmol/mg (Bₘₐₓ) and 2.31 nM (Kₐ) (Thomas et al. 2004) and 1.3 nM (Kₐ) (Gatley et al. 1997) in rat cerebellum, which are consistent with findings reported in this chapter. The Kₐ of [³H]-CP55,940 binding to human bladder membranes (1.26 nM) was larger than that found in the rat bladder and cerebellum (0.39 and 0.45 nM respectively), but was within the range 0.5-5 nM as reported in previous studies (Aung et al. 2000; Pertwee et al. 2010; Hill et al. 2013). Since the binding affinity of CP55,940 in the urinary bladder has not been reported before, the Kₐ value reported in this chapter cannot be directly compared with other studies, however, the small difference in Kₐ values seen between rat and human bladders suggests there may be an element of species difference in the binding affinity of CP55,940 to cannabinoid receptors. In addition, another possibility for the lower affinity in the human bladder compared to rat bladder may be due to a result of endogenous CB ligands in the human shifting the bladder cannabinoid receptors towards a desensitised and low affinity state.

Receptor density in human and rat bladders was very similar, which correlates with findings described in chapter 3 that showed a similar expression and distribution of CB receptors in human and rat bladders. The receptor density found in the urinary bladder in both human and rat tissue was significantly less than that detected in rat cerebellum where CB1 receptors are known to be highly expressed. This study demonstrates for the first time in the urinary bladder that synthetic cannabinoid agonist CP55,940 binds with high nanomolar affinity to cannabinoid receptors in vitro. While the present study reveals the ability of CP55,940 to bind to CB receptors in whole bladder tissue extracts, further in vitro analysis is required, using different CB ligands, to employ this radioligand to
determine the relative numbers of CB1 and CB2 receptors in the urothelium and detrusor muscle.

NSB represents binding of a radiolabel to sites other than the receptor of interest, which varies according to factors, such as the lipophilic nature of the ligand. Cannabinoid ligands are notoriously difficult to work with, due to their lipophilic nature. In saturation experiments there was a high level of non-specific binding as exemplified by the observation that only ~30% of total $[^3H] \text{-CP-55,940}$ binding was to specific displaceable sites, which resulted in difficult data analysis.

The objective at the start of the study was to compare receptor densities of the CB receptor isoforms in patients with DO and normal bladders to complement the findings described in chapter 5, which showed differences in receptor expression between these two groups. However, large pieces of tissue were required to determine receptor density, and these were obtained from whole rat bladders and cystectomies. Tissue from patients diagnosed with DO could only be obtained from biopsies taken during cystoscopy, which on average are less than 0.5 cm in length and approximately 0.2 cm in width, resulting in insufficient amount of tissue to complete the planned experiments. Another limitation to this study was that by using CP55,940, which is non-selective for the CB receptors, the specific characteristics for one receptor were not obtained, as this was beyond the scope of this study. Thus, further studies using CB antagonists and selective agonists need to be performed.

6.5 Conclusions

The results of this study produced important information on the binding properties of CP55,940 and confirm the presence of the cannabinoid receptors in rat and human urinary bladders. These results corroborate the findings described in chapter 3, where the presence of the CB receptors was demonstrated in the bladder using qRT-PCR, IHC, and WB. As a result from the findings presented in this chapter, CP55,940 was used as the CB agonist of choice for the following in vivo experiments that investigate the effect activation of CB receptors on cystometric parameters presented in chapter 7.
Chapter 7

Effects of CP55,940 on normal and irritation-induced bladder overactivity in rats
Chapter 7  Effects of CP55,940 on normal and irritation-induced bladder overactivity in rats.

7.1  Introduction

The presence of the endocannabinoid system (ECS) in the urinary bladder has led to speculation that endocannabinoid-signalling is involved in the signal transduction pathways regulating bladder relaxation and may be involved in pathophysiological processes of the bladder (Freeman et al. 2006). It has been suggested that activation of cannabinoid receptors (CB1 and CB2) might promote relaxation of the urinary bladder during the filling phase. Dysregulation of the ECS in human bladder may be responsible for the pathogenesis of overactive bladder syndrome (OAB) and DO. In chapter 3 the components of the endocannabinoid system were localised in both rat and human bladder and functional in vitro studies showed that CB1 receptors have both pre- and post-synaptic inhibitory effects on rat bladder contraction, whereas CB2 acts only post-synaptically.

Although the processes involved in the pathogenesis of overactive bladder are now much better understood, the molecular and signal transduction mechanisms involved have not been fully explored. The endocannabinoid system has been suggested as one of these mechanisms (Mukerji et al. 2010). Cystometric studies have shown an increase of the micturition threshold in rats receiving systemic cannabinoids in an overactive bladder model induced by acetic acid, cyclophosphamide (Hiragata et al. 2007) or turpentine oil (Dmitrieva and Berkley 2002). These effects were stronger when cannabinoids were administered through a close-arterial route rather than systemically through the tail vein of the rat, supporting the hypothesis of a local regulatory role of the cannabinoid system in the micturition reflex (Dmitrieva and Berkley 2002). Intravesical infusion of acetic acid has been shown to alter the micturition reflex in rats (Kakizaki and de Groat 1996) by increasing the frequency of contractions, and decreasing bladder capacity, with the micturition pressure unaffected or increased (Zhang et al. 2003). In that study, acetic acid was used as an overactive rat bladder model (Hiragata et al. 2007; Hino et al. 2010; Mally et al. 2013) to assess the effects of intravesical CP55,940 in a pathophysiological state, in order to determine its potential use as a therapeutic agent.
CP55,940 displays similar affinity for both central and peripheral cannabinoid receptors ($K_i = 0.5-5.0$ and $0.7-2.6$ nM at CB1 and CB2, respectively) (Griffin et al. 1998; Thomas et al. 1998). The hypothesis of this study was that intravesical administration of cannabinoid agonists will alter urodynamic parameters by activation of either CB1 or CB2 receptor and have a reversal effect on bladder hyperactivity induced in a rat model.

### 7.2 Aims

The aim of the current chapter was to characterise the receptor subtype (CB1 or CB2) responsible for mediating normal rat bladder function following intravesical administration of CP55,940. Additionally, the effects of CP55,940 on a pathophysiological overactive rat bladder was also investigated.

### 7.3 Results

#### 7.3.1 Rats with normal bladders

Initially, all planned in vivo experiments were to use female Wistar rats, however, after 9 initial cystometry experiments, there was a problem and it was decided to change to female Sprague-Dawley (SD) rats because this strain appeared to be less sensitive to urethane anaesthesia and therefore there was a lower mortality rate. Wistar rats (200-250 g) were smaller and lighter than the Sprague-Dawley (250-300 g) rats and technically it was more difficult to cannulate the femoral vein. The higher number of deaths using Wistar rats upon initiation of urethane anaesthesia, could have therefore been due to the increased length of time taken to cannulate the femoral vein (approximately 45 min compared to 5-10 min in the Sprague-Dawley rats). The increased length of time needed to initiate urethane anaesthesia meant that the Wistar rats were subjected to isoflurane anaesthesia for longer when compared to the Sprague-Dawley possibly making them more sensitive to the urethane and contributing to the increased number of deaths observed with this strain. In addition, the bladder reflexes did not appear to have a consistent pattern when infused with saline control making it difficult to analyse and draw conclusions because any comparisons with CP55,940 or vehicle would be unreliable, as demonstrated in Figure 7.1. Figure 7.2 shows representative cystometry traces in SD rats where the pattern of the micturition reflexes is more regular than that observed in the
Figure 7.1: Original cystometry pressure recordings obtained from female Wistar rats. Panel A. Shows a section of the trace from infusion with intravesical saline as a control and a section showing the addition of intravesical DMSO (vehicle). Panel B. Shows a representative trace showing control and then the addition of intravesical CP55,940 (0.005 mg/Kg). In both traces the contractions are irregular unlike those observed with the Sprague-Dawley rats seen in Figure 7.2. Scale bars indicate 60 seconds.
Figure 7.2: Representative cystometry pressure recordings obtained from female Sprague-Dawley rats. Scale bars indicate 60 sec intervals. Panel A. Shows a section of the trace from infusion with intravesical saline as control and a section showing the addition of intravesical CP55,940 (0.005 mg/Kg) infusion. The trace is annotated to demonstrate maximal voiding pressure (MP), micturition interval (MI), and basal pressure (BP). Panel B. Representative trace showing control and then the addition of DMSO (vehicle). Panel C. A cystometry trace showing infusion with saline (control) and then CP55,940 infusion after administration of intravenous AM251 (1 mg/Kg). Panel D. A cystometry trace showing infusion with saline (control) and then intravesical infusion of CP55,940 infusion after administration of intravenous AM630 (3 mg/Kg). Panel E. Representative sections of a cystometry recording showing saline (control), the effects of intravesical 0.25% acetic acid infusion and the effects of intravesical CP55,940 on the irritated bladder.
Using Sprague-Dawley rats, CP55,940 (0.005 mg/Kg) increased MI and BC by 52 ± 5.5% (both p<0.05, Table 7.1 and Figures 7.2A, 7.3, 7.4, 7.5) compared to the saline-treated control. Although there was a 25 ± 2.9% decrease in MP recorded after intravesical CP55,940 administration (p<0.05, Table 7.1 and Figures 7.2A, 7.3, 7.5), there was no reproducible change in baseline pressure compared to intravesical saline infusion. Addition of DMSO (vehicle) had no effect on any of the cystometric parameters when compared to the saline-treated control (Table 7.1, Figure 7.2B).
Figure 7.3: Results of cystometry using female Sprague-Dawley rats (n=5). Panels A and B show a significant increase in MI and BC, respectively, on addition of CP55,940. BC was calculated by rate of infusion x MI. Panel C shows that CP55,940 significantly reduced MP but did not affect BP. Control was performed with a saline infusion (0.15 ml/hr) for 1 hour and then CP55,940 (0.005 mg/Kg) infusion for an additional hour. Data are presented as the mean ± SEM for 5 separate experiments; **p<0.01 Student’s paired t-test.

MI, Micturition interval; BC, Bladder capacity; MP, Maximal voiding pressure; BP, basal pressure.
Intravenous pre-treatment with AM251 (1 mg/Kg) or AM630 (3 mg/Kg) 5 min prior to intravesical infusion of CP55,940, inhibited the previously observed increases in MI and BC, as well as the decreases in MP (Table 7.1 and Figure 7.3). Representative cystometry traces showing the effects of the addition of either AM251 or AM630 prior to infusion of CP55,940 are displayed in Figure 7.2 C and D, respectively. This suggests a clear functional role for CB1 and CB2 receptors in bladder function.
Figure 7.4: Continuous cystometry using female Sprague-Dawley rats. Baseline measurements (saline infusion for 1 hour are shown (control). Panel A shows that addition of the CB1 antagonist AM251 before infusion of CP55,940 reduced MI when compared to the addition of CP55,940 alone, however, this reduction was not statistically significant, but BC was significantly inhibited (Panel B). Panel C, shows inhibitory effects of CP55,940 were significantly attenuated by AM251 for MP, but no significant effect was seen for BP. Panels D and E demonstrate comparable data for the effects of AM631 on MI and BC, respectively. These data indicate that the addition of AM630 decreases the MI and BC induced by CP55,940, but this was only statistically significant for BC. Panel F shows that AM630 significantly attenuated the effect of CP55,940 on MP but again had no effect on BP. The data are presented as mean ± SEM of 5 separate experiments; *p<0.05; **=p<0.01 Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparisons test. MI, Micturition interval; BC, Bladder capacity; MP, Maximal voiding pressure; BP, basal pressure.
Table 7.1: Effects of CP55,940 on bladder function in the absence and presence of either AM251 or AM630 using Sprague-Dawley rats.

<table>
<thead>
<tr>
<th></th>
<th>MI (secs)</th>
<th>BP (mmHg)</th>
<th>MP (mmHg)</th>
<th>BC (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td><strong>Vehicle</strong></td>
<td>39.1 ± 5.8</td>
<td>33.8 ± 5.7</td>
<td>-12.7 ± 6.4</td>
<td>-12.7 ± 6.4</td>
</tr>
<tr>
<td><strong>CP55,940</strong></td>
<td>58.0 ± 3.6</td>
<td>87.8 ± 4.5†</td>
<td>-3.2 ± 0.9</td>
<td>-3.3 ± 0.6</td>
</tr>
<tr>
<td><strong>AM 251</strong></td>
<td>50.1 ± 8.4</td>
<td>49.9 ± 3.9</td>
<td>-8.1 ± 8.7</td>
<td>-4.7 ± 8.4</td>
</tr>
<tr>
<td><strong>AM 630</strong></td>
<td>65.0 ± 22.9</td>
<td>60.0 ± 21.4</td>
<td>-2.8 ± 1.9</td>
<td>-3.0 ± 2.2</td>
</tr>
</tbody>
</table>

**Ml=Micturition interval, BP=basal pressure, MP=maximum pressure, BC=bladder capacity.**

†=p<0.01 Student’s paired t-test between before addition of drug using saline as a control and after addition of drug.

*=p<0.01 Kruskal-Wallis with Dunn's multiple comparisons test, comparing CP55,940 to AM251 and AM630.

**=p<0.05 Kruskal-Wallis with Dunn's multiple comparisons test comparing CP55,940 to AM251 and AM630.
Figure 7.5: The cystometry results using Sprague-Dawley rats are shown as a percentage of the saline control. Micturition interval (MI), bladder capacity (BC) and maximum voiding pressure (MP) for vehicle, CP55,940 with or without addition of AM251 or AM630 (n=5 for each group). Values are given as mean ± SEM. For comparisons between CP55,940 and AM251 or AM630, Kruskal-Wallis with Dunn's multiple comparisons test was used. * p<0.05, ** p<0.01.

7.3.2 Acetic acid induced bladder hyperactivity

Control was performed for each experiment with an intravesical saline infusion (0.15 ml/hr) for 1 hour and then acetic acid was infused intravesically for 30 min to induce bladder hyperactivity. Changes in cystometric parameters were compared to the saline control to ensure that acetic acid had indeed induced bladder hyperactivity. CP55,940 (0.005 mg/Kg) was then infused intravesically for 1 hr and its effects on cystometric parameters were compared to changes induced by acetic acid. Intravesical infusion of acetic acid (0.25%) induced bladder irritation, as shown by a significant 41 ± 6.3% reduction in MI (p<0.01) compared to values obtained from the saline control. For the other cystometric parameters, acetic acid did not cause any significant changes in MP, BP or BC when compared to saline control. Addition of intravesical CP55,940 in the same rats increased MI by 78 ± 16.7% (p<0.05) and reduced MP by 22 ± 5.8% (p<0.05). Table 7.2, Figures 7.2 E and 7.6.
**Figure 7.6:** Effects of CP55,940 on a hyperactive rat bladder model induced by 0.25% acetic acid. Continuous rat cystometry was performed on anaesthetised female Sprague-Dawley rats (n=6) using urethane anaesthesia. Panel A shows that acetic acid-induced bladder hyperactivity by significantly decreasing the MI when compared to saline control. On addition of CP55,940, the MI significantly increased, therefore fully reversing the effects induced by acetic acid. **Panel B:** There were no significant effects on BC after infusion of either acetic acid or CP55,940. As shown in **panel C,** acetic acid did not have an effect on MP, however, infusion of CP55,940 significantly decreased MP. Neither acetic acid nor CP55,940 had any effect on BP. The data are presented as mean ± SEM; n=5;

^p<0.01, Student’s paired t-test performed between saline control group and acetic acid;
*p<0.05, Student’s paired t-test performed between acetic acid group and CP55,940.

MI, Micturition interval; BC, Bladder capacity; MP, Maximal voiding pressure; BP, basal pressure.
Table 7.2: Effects of intravesical CP55,940 infusion on cystometric parameters after administration of acetic acid infusion in Sprague-Dawley rats.

<table>
<thead>
<tr>
<th></th>
<th>Control Mean ± SEM</th>
<th>Acetic Acid Mean ± SEM</th>
<th>CP 55,940 Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI (sec)</td>
<td>52.9 ± 9.9</td>
<td>29.2 ± 4.8**</td>
<td>52.6 ± 10.9*</td>
</tr>
<tr>
<td>BP (mm Hg)</td>
<td>-11.4 ± 4.6</td>
<td>-10.1 ± 5.3</td>
<td>-10.5 ± 4.6</td>
</tr>
<tr>
<td>MP (mm Hg)</td>
<td>17.5 ± 1.0</td>
<td>18.8 ± 1.3</td>
<td>14.4 ± 0.9*</td>
</tr>
<tr>
<td>BC (µL)</td>
<td>114.7 ± 17.9</td>
<td>128.2 ± 22.0</td>
<td>103.0 ± 25.0</td>
</tr>
</tbody>
</table>

MI= Micturition interval, BP= basal pressure, MP= maximum pressure, BC= bladder capacity.

** = p < 0.01, Student’s paired t test performed between saline control group and acetic acid.
* = p < 0.05, Student’s paired t test performed between acetic acid group and CP55,940.
7.4 Discussion

The results of these experiments demonstrate that intravesical infusion of CP55,940 suppressed bladder activity, as well as urinary frequency induced by bladder irritation. The inhibitory effects of CP55,940 appeared to be mediated by both CB1 and CB2 receptor subtypes. Since, MI and BC cystometric parameters can be considered indicators for afferent function of the micturition reflex and MP and BP are indicators of motor activity (Gratzke et al. 2009), then the main function of CP55,940 seemed to be mediated by modulation of both afferent and motor activity as it not only induced an increase in MI and BC but also affected MP. In a similar study, which utilised an intravesical 0.005 mg/Kg bolus of CP55,940, an increase in MI and BC was reported, with no changes in MP (Gratzke et al. 2009). Furthermore, the authors did not report the CB receptor subtype involved in mediating these responses (Gratzke et al. 2009). One reason for the difference between the results presented in this chapter and those reported previously could be differences in methodologies and/or anaesthetic agents used. In this study, isoflurane and i.v. urethane were used to anaesthetise the rats, whereas Gratzke et al. (2009) used intraperitoneal ketamine and xylazine and the cystometry was performed on conscious rats. It has been reported that ketamine inhibits the micturition reflex (Castroman and Ness 2002; Ozkurkcugil and Ozkan 2010). Castroman and Ness (2002) reported that ketamine inhibits the reflexive responses of the bladder to the acute stimulus of distension in a dose-dependent manner. The findings described in this chapter show an effect of CP55,940 on motor activity, which is in accordance with the in vitro studies described in chapter 3, where activation of both CB receptors reduced the detrusor muscle contractions. Taken together, it may be speculated that CP55,940 activates CB receptors directly on the muscle resulting in inhibition of contractility.

The observed effects of CP55,940 on bladder function demonstrated in this chapter are in agreement with the effects of cannabinoid agonists reported in human clinical trials. In a large randomised controlled trial, patients with neurogenic DO, who were randomised to the THC and cannabidiol (CBD) treatment groups, reported a significant reduction in urge incontinence episodes compared to placebo (Freeman et al. 2006). Similarly, in another study, patients with neurogenic DO given a cannabis extract containing THC and CBD, demonstrated a reduction in urinary urgency, the number of

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incontinence episodes, frequency and nocturia. In addition, there was an increase in the maximum cystometric capacity in these patients (Brady et al. 2004a), supporting the cystometric observations described in this chapter, where CP55,940 increased MI and BC.

With the knowledge that cannabinoid agonists are negatively coupled to calcium channels, and their inhibition of adenylate cyclase, the mixed picture of cystometric effects described in this chapter and those reported in the literature is understandable. The increased micturition interval and the decrease in maximal pressure of the contractions could be due to inhibition of adenylate cyclase, which may be involved in relaxation of detrusor smooth muscle during urine collection. The alteration of calcium fluxes may decrease neurotransmitter release required for detrusor contractions, and that decrease could be manifested as an increase in the volume requirement (demonstrated as an increase in bladder capacity). More experiments must be performed to clarify these preliminary results.

It appears from the data presented in this chapter, that the activation of both CB1 and CB2 receptors have inhibitory effects on the bladder, as demonstrated by both CB antagonists blocking the actions of CP55,940. This is supported by a study that suggested a CB2-mediated effect in the rat urinary bladder (Gratzke et al. 2010) where cannabinor (a selective high-affinity CB2 receptor full agonist) was used and resulted in a 44% increase in MI. In another study, using the non-selective CB receptor agonist WIN55212, an increase in the threshold for micturition was reported in anaesthetised rats, and this effect was counteracted by the CB1 receptor antagonist SR141716A, suggesting a CB1-mediated effect (Dmitrieva and Berkley 2002). Similarly, yet another study using a synthetic analogue of THC, IP-751 at 10 mg/Kg, reported an increase in MI by 63% and threshold pressure by 53%. These effects were counteracted by the CB1 antagonist AM251 but not the CB2 antagonist AM630 (Hiragata et al. 2007). In this chapter both AM251 and AM630 inhibited the effects of CP55,940 suggesting a role for both CB receptors in urinary bladder function. A reason for the discrepancy between the results presented in this chapter and that of Hiragata, could be the route of administration of the cannabinoid agonist. In this chapter, CP55,940 was administered intravesically while Hiragata administered IP-751 systemically and therefore the effects of IP-751 on the
central nervous system cannot be excluded. Another possibility for these differences could be related to the receptor subtype that is activated when using different agonists suggesting differences in receptor activation between the urothelium and detrusor muscle. It has been reported that CB1 and CB2 receptor expression is different in the urothelium and detrusor (Tyagi et al. 2009) and so different agonists may differentially activate the endocannabinoid receptor response in each of these specific tissues resulting in different downstream events. In addition, there may be an element of CB receptor turnover that depends on the type of agonist used, which could explain this discrepancy. This can be assumed since full reversal of CP55,940 effects seen on some cystometric parameters occurred with both CB1 and CB2 antagonists.

The mechanism by which cannabinoid receptors modulate the micturition reflex is by their presence in the afferent nerve fibre endings located in the suburothelial layer, which is supported by *in vitro* studies using CB1 or CB2 receptor agonists. *In vitro* studies using CB1 receptor agonists have been shown to reduce neuronal activity (Di Marzo et al. 2004) and attenuate bladder contractility as a result of electrical field stimulation in isolated mouse bladder strips (Pertwee and Fernando 1996). In rats, anandamide, WIN 55212-2 (a synthetic CB non-selective agonist), and IP-751 also known as Ajulemic acid), suppressed normal bladder activity and urinary frequency induced by bladder irritation suggesting that the inhibitory effects are least in part mediated by CB1 receptors (Jaggar et al. 1998a; Dmitrieva and Berkley 2002; Hiragata et al. 2007). Cannabinor, a selective CB2 receptor agonist, increased the micturition intervals and volumes by 52% (p<0.05) and 96% (p<0.01), respectively, and increased threshold and flow pressures by 73% (p<0.01) and 49% (p<0.001), respectively, in conscious rats during cystometry (Gratzke et al. 2010). To support the findings described and the results presented in this chapter, ATP-evoked calcitonin gene-related peptide release (CGRP; a neurotransmitter released by sensory nerve endings) was reported to be inhibited by the activation of both CB1 and CB2 receptors after an alteration in the afferent cystometric parameters from the infusion of a CB receptor agonist (Daly et al. 2011). However, it is possible that some of the effect could be centrally mediated and whether these actions are related to CB receptors in the central nervous system, at peripheral sites in the lower urinary tract, or both needs to be clarified. Furthermore, it is not known which of the two CB receptor subtypes is primarily responsible for the regulation of micturition in different species.
Cannabinoid receptor expression appears to be altered in pathological conditions, as has been shown in a clinical study where CB1 receptor immunoreactive nerve fibres were increased in the suburothelium of patients with painful bladder syndrome and detrusor overactivity (Mukerji et al. 2010). In addition, CB1 mRNA transcripts were increased in patients with chronic bladder pain syndromes (Sanchez Freire et al. 2010) and CB2 transcripts and protein expression was increased in rats with bladder inflammation induced by acrolein (Merriam et al. 2008). In vivo studies have shown an increase in micturition interval by cannabinoid agonists in rat bladders with induced bladder hyperactivity (Jaggar et al. 1998a; Dmitrieva and Berkley 2002; Hiragata et al. 2007; Walczak and Cervero 2011). Taken together with the results presented in this chapter, these data support a regulatory role for the CB1 and CB2 receptor subtypes in both normal bladder function and bladder dysfunction.

### 7.5 Conclusions

CP55,940 decreased rat bladder activity and urinary frequency induced by bladder nociceptive stimuli, probably by suppression of bladder afferent activity. These effects are modulated by activation of both CB1 and CB2 receptors and these data implicate a role for the endocannabinoid system in bladder mechanoafferent functions in rats. In addition, the results of this study show that intravesical CP55,940 reverses urinary frequency exemplified in an overactive bladder model, suggesting it could potentially be an effective treatment for patients with lower urinary tract symptoms.
Chapter 8

General Discussion
Chapter 8  General Discussion

Substantial evidence accumulated over several years indicates a regulatory role for the endocannabinoid system in bladder function (Hedlund 2014). Clinical studies have demonstrated that cannabis use causes an improvement in urgency and urge incontinence in patients with neurogenic DO (Brady et al. 2004a; Freeman et al. 2006). Although these observations support the proposition that the endocannabinoid system may play a significant regulatory role in micturition and bladder function and also possibly is involved in the pathogenesis of detrusor overactivity and other lower urinary tract symptoms (LUTS); how the endocannabinoids are regulated in vivo as well as the pathophysiological relevance of deregulation of the system in the aetiopathogenesis of detrusor overactivity and overactive bladder remained largely unexplored (Hedlund 2014). This deficiency precipitated the studies presented in this thesis, which explored the clinical relevance of the endocannabinoid system in the urinary bladder by evaluating the effects of cannabinoid agonists on bladder contractions in vitro and in vivo.

8.1 Summary of main findings

Figure 8.1 summarises the overall findings of the thesis by presenting the key results with each of the different models used. These findings will be discussed in detail.
Figure 8.1: Illustration of the key findings of this thesis, showing the sequence of the models used; rat and human bladder, cell lines, and whole animal in vivo and the chapters where each finding is presented.
8.1.1 Expression and distribution of the endocannabinoid system in the urinary bladder

In chapter 3 the endocannabinoid system and TRPV1 receptors were localised in human and rat bladder using qRT-PCR, immunohistochemistry and immunoblotting. Both cannabinoid receptor mRNA transcripts were detected in the urothelium and detrusor of normal human bladders suggesting that the receptor proteins are synthesised in the bladder rather than sequestered from blood or lymph. CB1 and TRPV1 receptors were detected in both urothelium and detrusor of rat bladder while CB2 receptor was only detected in the detrusor of Wistar rats. In the human bladder, both CB receptors and TRPV1 were localised in urothelium and detrusor, whilst the endocannabinoid modulating enzymes NAPE-PLD and FAAH were detected in both urothelium and detrusor of both human and rat bladders.

Furthermore, probing blots with CB1 antibody produced specific protein bands of 53 kDa in the rat bladder and 40 kDa in the human bladder while CB2 immunoblots revealed a 45 kDa protein band in rat bladder and a smaller 43 kDa protein in human bladder. Immunoreactive bands for TRPV1 receptors were observed as a specific band of 104 kDa in all tissues. Immunoblots for degrading enzyme, FAAH, showed specific bands at 55 kDa in both the human and rat bladder. Specific bands to the synthesising enzyme, NAPE-PLD, were seen at 44 kDa in the rat bladder and 45 kDa in the human bladder. Table 8.1 summarises the WB data reported in this thesis compared to results published in the literature.

The difference in the molecular weights reported in this thesis compared to the predicted values by Abcam (supplier of the antibody) or reported in other studies may be due to the presence of splice variants, post-translational modification, relative charge of the proteins or for the CB receptors; the presence of different isoforms in the two species. In addition, the use of different antibodies in the different studies could be a reason for the discrepancies reported, where the antibodies used in previously reported studies may have been specific for a different region of the receptor than the antibody used in this thesis. The rationale for requiring region-specific antibodies is driven partly by experimental design and partly by the consideration that a single antibody is unlikely to
detect all receptor species (i.e. post-translationally modified, splice-variants, receptor oligomers, and degradation products) (Grimsey et al. 2008). The detection of cannabinoid receptors has been the subject of some debate, because the available antibodies may not be as specific as one would like. The antibodies used herein are specific for CB1 and CB2 having been validated in numerous other systems (Taylor et al. 2010; den Boon et al. 2012; Gebeh et al. 2013; Karasu et al. 2014). In addition, positive controls for each antibody were used for each experiment in this thesis.

The results in this thesis show that classical cannabinoid receptors CB1, CB2, and the endocannabinoid synthetic enzyme NAPE-PLD and degradation enzyme FAAH are expressed in human and rat urinary bladder, as demonstrated by qRT-PCR, IF, IHC and WB. These data are supported by findings reported previously (Tables 8.2 and 8.3). However, the expression pattern of CB1 and CB2 is still under debate as conflicting results have been published in the literature. Some studies have claimed that CB1 is predominantly expressed in human bladder (Tyagi et al. 2009) while others have reported a greater expression of CB2 in human bladder (Gratzke et al. 2009). Although there is no clear consensus, it appears that both receptors are broadly expressed in the bladder of both humans and animals as described in this thesis and as summarised in Tables 8.2 and 8.3. The apparent disparity in some of the findings may result from several possibilities, including species differences, underlying pathophysiology, use of different compounds or antibodies, different dosages or dilutions and different methods of quantitation.

The co-localisation of CB1 and CB2 receptors in nerve fibres of the muscular layer is in line with results from in vitro experiments presented in chapter 3, where administration of cannabinoids to preparations of bladder strips decreased the contractility of the bladder. CB1 receptor mRNA and protein are expressed in cell bodies of neurons in the dorsal root ganglion (Bridges et al. 2003). Afferent fibre endings are located adjacent to the urothelium, in the sub-urothelial layer (Yoshimura 2007). The localisation of CB1 receptors in nerve fibres located in the sub-urothelial layer of the bladder wall, and therefore putative afferent fibres, could be the substrate for the effects observed in the electrophysiological experiments, as it is known that activation of CB1 receptors can reduce neuronal activity (Di Marzo et al. 2004).
Table 8.1: Western Blot data as published in literature and compared to results in chapter 3 of this thesis and the known expected weight. All molecular weights are in kDa.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Abcam</td>
<td>Rat</td>
<td>Human</td>
<td>Human</td>
<td>Rat</td>
</tr>
<tr>
<td>CB1</td>
<td></td>
<td></td>
<td>60</td>
<td>57</td>
<td>40</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>CB2</td>
<td></td>
<td></td>
<td>40</td>
<td>45</td>
<td>43</td>
<td>43</td>
<td>60</td>
</tr>
<tr>
<td>TRPV1</td>
<td></td>
<td></td>
<td>96</td>
<td>104</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td></td>
<td></td>
<td>63</td>
<td>55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAPE</td>
<td></td>
<td></td>
<td>46</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 8.2: Expression and distribution of the CB1 receptor in the urinary bladder from different species. Ab control=control experiment performed for antibody (Ab) specificity.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Species</th>
<th>Method</th>
<th>Ab control</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detrusor/urothelium</td>
<td>Human</td>
<td>WB</td>
<td>Omitting primary ab</td>
<td>Gratzke et al. (2009)</td>
</tr>
<tr>
<td>Whole bladder</td>
<td>Rat</td>
<td>WB, PCR</td>
<td>-</td>
<td>Li et al. (2013)</td>
</tr>
<tr>
<td>Whole bladder</td>
<td>Rat</td>
<td>IF</td>
<td>Omitting primary ab</td>
<td>Hayn et al. (2008)</td>
</tr>
<tr>
<td>Detrusor/urothelium</td>
<td>Human</td>
<td>WB, IHC</td>
<td>Omitting primary ab/block peptide</td>
<td>Tyagi et al. (2009)</td>
</tr>
<tr>
<td>Detrusor</td>
<td>Mouse</td>
<td>PCR, IF</td>
<td>Omitting primary ab/block peptide</td>
<td>Walczak et al. (2009) (2011)</td>
</tr>
<tr>
<td>Detrusor/urothelium</td>
<td>Rats</td>
<td>WB, PCR</td>
<td>-</td>
<td>Merriam et al. (2008)</td>
</tr>
<tr>
<td>Detrusor/urothelium</td>
<td>Humans</td>
<td>IHC</td>
<td>-</td>
<td>Mukerji et al. (2010)</td>
</tr>
<tr>
<td>Urothelium</td>
<td>Rat, mouse</td>
<td>PCR/WB/IF</td>
<td>Omitting primary ab/CB1 KO mice</td>
<td>Veress et al. (2013)</td>
</tr>
</tbody>
</table>

Table 8.3: Expression and distribution of the CB2 receptor in the urinary bladder from different species.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Species</th>
<th>Method</th>
<th>Ab control</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urothelium</td>
<td>Human, monkey, rat</td>
<td>IHC</td>
<td>Omitting primary ab</td>
<td>Gratzke et al. (2009)</td>
</tr>
<tr>
<td>Whole bladder</td>
<td>Rat</td>
<td>WB, PCR</td>
<td>-</td>
<td>Li et al. (2013)</td>
</tr>
<tr>
<td>Whole bladder</td>
<td>Rat</td>
<td>IF</td>
<td>Omitting primary ab</td>
<td>Hayn et al. (2008)</td>
</tr>
<tr>
<td>Detrusor/urothelium</td>
<td>Human</td>
<td>WB, IHC</td>
<td>Omitting primary ab/block peptide</td>
<td>Tyagi et al. (2009)</td>
</tr>
<tr>
<td>Urothelium</td>
<td>Rat</td>
<td>IHC</td>
<td>Omitting primary ab</td>
<td>Gratzke et al. (2010)</td>
</tr>
<tr>
<td>Detrusor/urothelium</td>
<td>Rats</td>
<td>WB, PCR</td>
<td>-</td>
<td>Merriam et al. (2008)</td>
</tr>
<tr>
<td>Urothelium</td>
<td>Mouse</td>
<td>WB</td>
<td>-</td>
<td>Wang et al. (2013)</td>
</tr>
</tbody>
</table>
The presence of both CB receptors and FAAH in the same tissue (urothelium/detrusor) suggests that the presence of FAAH, the enzyme that degrades endocannabinoids, may be necessary to establish the optimal endocannabinoid tone to regulate bladder function. Strittmatter et al. (2012) demonstrated co-localisation of FAAH with CB2 in human urothelial cells and suggested that the CB2 on urothelial cells or afferent nerve terminals could be targets for FAAH substrates. FAAH is of interest, because a study using transgenic mice without peripheral FAAH had increased levels of AEA in peripheral tissues but not in the CNS, and they still exhibited a preserved anti-inflammatory phenotype (Cravatt et al. 2004). These findings suggest that central and peripheral FAAH may separately regulate endocannabinoid turnover and may be targeted as a therapeutic option (Cravatt et al. 2004). In addition, a second FAAH enzyme (FAAH-2) was identified and shown to be expressed only in higher mammals but not in rats and mice (Wei et al. 2006). It was found that FAAH but not FAAH-2, was highly expressed in the brain (Wei et al. 2006) promoting this enzyme as a drug target for manipulating endocannabinoid activity, without the undesirable CNS side-effects. FAAH-2 has been localised in the human bladder and future studies may show that this enzyme has a role in micturition and bladder dysfunction (Strittmatter et al. 2012). This suggests that rats and mice might be not good candidates for studying the role of endocannabinoids in bladder function where regulation by FAAH-2 is important.

8.1.2 Functional effects of cannabinoid receptors on urinary bladder

The signalling of these the classical cannabinoid receptors is complex and may involve inhibition of adenylate cyclase activity, activation of various mitogen-activated protein kinases and modulation of various Ca$^{2+}$ and K$^+$ channels (Pacher et al. 2006; Pertwee 2010) and other signalling pathways. In vitro and in vivo studies have shown that acute cannabinoid treatments produce effects on efferent/afferent bladder nerve systems resulting in a neurotransmitter release, in which CB1 receptor activation decreases visceral cholinergic neurotransmission in both inflamed and normal bladder (Hiragata et al. 2007; Gratzke et al. 2010; Walczak and Cervero 2011). Furthermore, the inhibition of amplitude of phasic activity by CB agonists described in this thesis could be attributable to blockade of the amplitude of voltage-gated Ca$^{2+}$ currents. This is important because smooth muscle contraction is regulated by an elevation of cytosolic Ca$^{2+}$ resulting to myosin light chain phosphorylation, which is controlled by the balance between Ca$^{2+}$
entry into the cell/release from intracellular stores, and Ca\textsuperscript{2+} sequestration/extrusion from the cell (Suzuki et al. 2010). Under physiological conditions, bladder smooth muscle contractions are reported to depend upon extracellular Ca\textsuperscript{2+} secondary to L-type Ca\textsuperscript{2+}-channel opening (Wegener et al. 2004). It is well established that signal transduction by CB1 and CB2 occurs through an interaction with G\textsubscript{i/o} proteins to inhibit N, L and P/Q type voltage-gated channels (Gebremedhin et al. 1999). It is likely, therefore that treatment with the CB agonists presented in this thesis stimulate the CB receptors to cause inhibition of voltage-gated Ca\textsuperscript{2+} channels to suppress detrusor smooth muscle contraction.

8.1.3 In vitro studies of cannabinoid agonists

In chapter 3, the effect of ACEA and GP1A on EFS- and CCh-induced contractions was studied in vitro using rat bladder strips suspended in an organ bath. ACEA significantly reduced EFS-induced contractions by 35% while GP1A had no effect. Both ACEA and GP1A significantly shifted EC\textsubscript{50} values of a CCh concentration-response curve to the right indicating that both CB receptor agonists partially inhibited the carbachol effect.

Other studies reporting pharmacological in vitro experiments have shown that cannabinoid agonists can modulate bladder contractility in isolated bladder preparation by pre- and post-synaptic effects. Pertwee and Fernando (1996) found that cannabinoids (CP55,244, WIN55212-2, THC, and nabilone) can interact to inhibit nerve-evoked contractions in the mouse urinary bladder in a concentration-related manner and that the effects were counteracted by the CB1 antagonist SR141716A but not by the CB2 antagonist AM630. Furthermore, as the CB agonists did not exert an effect on agonist-induced contractions, pre-synaptic CB1-mediated modulatory activity of nerve-induced mouse bladder contractions was suggested (Pertwee and Fernando 1996). Tyagi et al. (2009) reported that activation of pre-synaptic CB1 receptors in the human bladder reduced the EFS-induced release of acetylcholine. Similarly, in this thesis activation of CB1 receptors with ACEA attenuated EFS-induced contractions but CB2 agonist GP1A, had no effect. In contrast, another study reported a decrease in EFS-induced contractions of human bladder using GP1A, however, quantification of the effect by GP1A or control experiments with vehicle (DMSO) were not presented (Tyagi et al. 2009). Conversely, Cannabis sativa enriched in cannabidiol extracts or pure cannabidiol (CBD) attenuated

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acetylcholine-induced contractions of the isolated rat and human bladder, but not contractions induced by electrical field stimulation (Capasso et al. 2011).

The non-selective CB receptor agonists CP55,940, CP55,244, JWH015, and THC have been shown to produce inhibitory effects on nerve-induced contractions of mouse, rat, monkey or human detrusor preparations (Pertwee and Fernando 1996; Gratzke et al. 2009) In the mouse detrusor, effects of CB receptor agonists could be attributed to CB1 receptor activation, whereas the effects of JWH015 on the rat detrusor were consistent with possible actions at both CB1 and CB2 receptors (Pertwee and Fernando 1996; Martin et al. 2000; Walczak et al. 2009). Varying effects by WIN55212-2 on nerve-induced contraction of isolated detrusor from dogs, pigs, monkeys, or humans has been reported (Martin et al. 2000). However, similar to anandamide, WIN55212-2 has been reported to interact with the function of the capsaicin receptor TRPV1, which is co-expressed with CB receptors on nerves and urothelium in rat and human bladder (Gratzke et al. 2009; Weinhold et al. 2010). It has been reported that most bladder sensory fibres are immunoreactive for TRPV1 and CGRP (Avelino et al. 2002). Studies have shown that CGRP can be released from sensory axons by nerve stimulation and by chemical agents such as capsaicin (Avelino et al. 2002). Capsaicin-sensitive bladder afferents contribute to pain and to bladder overactivity associated with cystitis (Chancellor and Yoshimura 2004).

8.1.4 Cellular model to study cannabinoid signalling

A cellular model was sought to study cannabinoid urothelial signalling (Chapter 4) and three urothelial cell lines were characterised using immunofluorescence and qRT-PCR. CB1 receptor was detected and localised in HCV 29 cells along with most of the muscarinic and purinergic receptors. UROtsa cells only expressed GPR55 receptor and most of the muscarinic and purinergic receptors, whilst the rodent urothelial cell line, MYP 3 did not express any of the cannabinoid receptors so further characterisation was not performed. Muscarinic and purinergic signalling interactions with the cannabinoid system was investigated using fluorimetry and cAMP assays in the case of HCV 29 cells. ATP significantly increased [Ca\(^{2+}\)]\(_i\) in HCV 29, UROtsa and MYP 3 cells by 395, 705, and 3232 nM, respectively. However, ACEA did not have an inhibitory effect on [Ca\(^{2+}\)]\(_i\) in HCV 29 cells and LPI did not have a stimulatory effect in UROtsa cells. In addition,
ACEA did not reduce cAMP accumulation when HCV 29 cells were stimulated with forskolin. Binding of cannabinoids to CB1 and CB2 receptors results in a decrease of intracellular cAMP levels and activation of mitogen-activated protein kinase through the coupling of G_{i/o} proteins in neuroblastoma cells (Howlett et al. 1986). Cannabinoid-mediated inhibition of cAMP has been demonstrated in slices of rat hippocampus, striatum, cerebral cortex and cerebellum (Bidaut-Russell et al. 1990) and activation of CB1 can also stimulate the formation of cAMP through G_{s} under certain conditions (Howlett 2005), which may explain the effects of cannabinoids on muscle relaxation described in chapter 2 and previously demonstrated in the urinary bladder (Martin et al. 2000; Gratzke et al. 2009; Tyagi et al. 2009). However, in chapter 4, an increase in intracellular cAMP was not demonstrated, suggesting coupling of CB1 to the G_{s} protein in the HCV 29 cell line may not occur or that expression of the CB1 receptor is very low. Since CB1 receptors may exist as two distinct subpopulations, one coupled to G_{i/o} proteins and the other to G_{s} (Calandra et al. 1999), further studies need to be undertaken in primary human urinary bladder tissue to explore this potential association.

After characterising the three urothelial cell lines, other cell models were also investigated. An attempt to culture primary urothelial cells from human biopsies using an explant method was made but the technique abandoned due to lack of time. Several months were spent trying to optimise the methodology but the cells were growing at a very slow rate. The rate was such that completion of the other objectives described in this thesis and use of limited funding resources would have been compromised. Ideally, this method would be the most desirable to study urothelial signalling as it would be the closest representative to the natural occurrence of these receptors. Furthermore, a commercially available primary detrusor smooth muscle cell line was purchased from CELLnTEC (Berne, Switzerland) as an alternative model to study cannabinoid signalling. This method was also abandoned as these cells also took a very long time to reach confluence. In addition, the main interest was to study urothelial cell signalling, so the use of this cell line was not continued.

8.1.5 Human bladder biopsy model

Since the cell lines did not prove to be suitable models to study cannabinoid signalling, human bladder biopsies were used to explore the effect of cannabinoids on calcium signalling and explore differences between normal and DO bladders. These data are
shown in chapter 5. Quantitative PCR showed that the CB1 receptor was significantly upregulated by 2.8 fold in the urothelium of DO samples and downregulated by 3.2 fold in the detrusor of DO samples compared to normal bladder samples. Although a similar pattern was seen for the CB2 receptor those differences were not statistically significant. IHC confirmed these results as the intensity of staining for CB1 receptor was greater in the urothelium of patients with DO compared to normal bladders while the staining was less in the detrusor of patients with DO compared to normal bladders. The finding that there is increased expression of CB1 in the urothelium of patients with DO may be seen after loss of endogenous ligands in these patients. In addition, recently there has been discussion of an endothelial cannabinoid receptor (CBe) that is distinct from CB1 and CB2 (Stanley and O'Sullivan 2014) that causes vasorelaxation when activated. It may be the case in the urothelium that some form of this receptor exists or in pathological states such as DO, this receptor is upregulated either as a result of DO or to cause it.

Both CB receptors were co-localised with anti-PGP 9.5 (general antibody for neurons) in detrusor and urothelium. CB receptors were found to co-localise with acetylcholine neuronal markers, with CB1 co-localising with ChAT (choline acetyltransferase enzyme) in detrusor muscle of normal human bladder but not in the urothelium, while CB2 co-localised with ChAT in both urothelium and detrusor. This enzyme is responsible for synthesising acetylcholine (ACh) from choline, and its presence in a cell is thought to indicate the ability to synthesise and release ACh from cholinergic nerves (Gillespie et al. 2006). The fact that CB1 was not co-localised with ChAT in the urothelium, when IHC, WB and qRT-PCR data have demonstrated CB1 to be present in the urothelium is interesting. The significance of this observation, at present, is unknown but it may be speculated that it has a causative role in pathophysiological states in the bladder. The qRT-PCR and IHC data described in chapter 5 showed an increase in CB1 in the urothelium of patients with DO; it would be interesting for future studies to explore changes in co-localisation with ChAT in the urothelium of DO patients.

To complement these findings, upright confocal microscopy was employed to compare cannabinoid receptor effects on calcium signalling in human bladder biopsies and results compared with DO by the development of a novel experimental protocol. Frustratingly, although the model could be created and could be tested, it was not very useful because
after the addition of carbachol, the tissue would contract and move out of focus. Different attempts to prevent the section of the tissue moving out of focus were tried, of which, all failed. Different types of weights of varying sizes were used, placing grease on the coverslip was also attempted. If this experimental route is to be pursued, then a confocal microscope where a Sylgard-lined preparation dish with the tissue pinned on it needs to be incorporated on the microscope stage. Furthermore, if there had not been time or funding restraints, then a motorized focusing module could have been devised that would be inserted on the confocal microscope stage and once set would have the speed and capacity to ‘follow’ the contraction preventing the image going out of focus.

Since these experiments did not perform as expected, radioligand binding assays were utilised (Chapter 6) using a radiolabeled non-selective cannabinoid agonist (CP55,940) to perform saturation binding assays. The binding of $[^3H]$-CP55,940 to human and rat bladder membranes was concentration-dependent and saturable. The $B_{\text{max}}$ for human bladder was 421.4 fmol $[^3H]$-CP55,940/mg protein and the $K_d$ was 1.26 nM. In rat bladder, the $B_{\text{max}}$ was found to very similar to that of the human bladder at 429.7 fmol $[^3H]$-CP55,940/mg protein and the $K_d$ was 0.39 nM. The main limitation to these studies was the amount of tissue required to do even the most basic experiments and often exceeded the amount available. This limited the amount of work that could be completed, as tissue from patients diagnosed with DO could only be obtained from punch biopsies, which (on average) are less than 0.5 cm in length and approximately 0.2 cm in width. These conditions resulted in insufficient amounts of tissue being available to perform radioligand binding assays using DO samples resulting in not enough data to compare to that obtained from normal human bladder samples.

### 8.1.6 In vivo Cystometry

In chapter 7, *in vivo* data of the effects of intravesical CP55,940 infusion on cystometric parameters in Sprague-Daley rats was presented. In addition, the effects of intravesical CP55,940 on bladder irritation-induced rat model with acetic acid were examined. CP55,940 significantly (p<0.05) increased MI and BC by 52 ± 5.5% compared to the saline control and induced a 25 ± 2.9% decrease in MP. Intravenous pre-treatment with CB1 or CB2 antagonists prior to infusion of CP55,940, inhibited the previously observed increases in MI and BC, as well as the decreases in MP. Addition of intravesical
CP55,940 in rats that had bladder irritation, induced by acetic acid infusion, showed an increase in MI by 78% ± 16.7% and a reduction in MP by 22% ± 5.8%.

In some *in vivo* experiments with chemically-induced bladder overactivity in rodents, authors report an up-regulation of bladder CB2 receptor expression but not CB1 receptor expression (Merriam *et al.* 2008), whereas others report that only CB1, but not CB2 receptors, seem to be of functional relevance in bladder overactivity (Walczak and Cervero 2011). In this thesis, it appeared that both CB1 and CB2 may be involved in micturition control, as addition of both CB1 and CB2 antagonists inhibited the effect of CP55,940. Furthermore, the effect of CP55,940 seen on urodynamic parameters, were such that a significant increase in BC could be explained by an alteration of calcium fluxes, which may decrease the neurotransmitter release required for detrusor contractions and that decrease could be manifested as an increase in the bladder volume required to produce a micturition contraction.

Non-selective CB agonists, such as WIN55212 and CP55,940 have been demonstrated to increase the threshold for micturition and micturition intervals in rat urodynamic models (Jaggar *et al.* 1998b; Dmitrieva and Berkley 2002; Gratzke *et al.* 2009). In anaesthetised rats, IP-751 (10 mg/Kg), a synthetic analogue of THC, increased the micturition intervals by 63% and threshold pressures by 53%, and these effects were counteracted by AM251 but not by AM630. At doses of >10 mg/Kg, IP-751 exhibited overt central effects, affecting motor performance and resulting in catalepsy in the rats (Hiragata *et al.* 2007). It has not been established if this is a result of CB receptor activation in the central nervous system, at peripheral sites in the lower urinary tract, or in both sites. Furthermore, Hayn *et al.* (2008) evaluated bladder afferent activity indirectly by measuring calcitonin gene-related peptide release (CGRP; a marker of afferent sensory neuronal activity) from dissected bladder domes stimulated with a mix of capsaicin and ATP. They observed a reduction of CGRP release after administration of a cannabinoid agonist and that this reduction was absent in the presence of CB1 or CB2 antagonists. CGRP release depends on afferent activity but also on the control of exocytosis of neuronal vesicles (Hayn *et al.* 2008).
Taken together, the results of urodynamic rat model studies, with the data presented in this thesis, suggest that CB receptors might be relevant for mechanoafferent bladder function. Furthermore, it is not known which of the two CB receptor isoforms is of primary importance for the regulation of micturition, as described in this thesis, where both CB1 and CB2 antagonists inhibited the effects of CP55,940 in equal measure. As AM251 and AM630 are known to cross the blood-brain barrier (Pertwee et al. 1995; Gatley et al. 1997), it can only be speculated as to whether or not the CB1 and CB2-receptor-related changes in micturition are mainly related to a CNS or peripheral nervous site of action (or both). It should be kept in mind that genes can compensate for each other, especially as CB1 and CB2 share intracellular pathways (Gi/o protein). Therefore, discrepancies in reports of CB receptor subtype involvement in regulation of micturition may very well suggest their multiple functions in different regulatory units of lower urinary tract control, reached via systemic or local administration of drugs.

8.2 Possible theories on how the cannabinoid system regulates bladder function

8.2.1 Inhibition of cholinergic nerve neurotransmission

The significance of the co-localisation of CB receptors on parasympathetic cholinergic nerves may explain the functional results obtained from the in vitro experiments described in chapter 3. Activation of CB1 receptor with ACEA had both a pre- and post-synaptic effect on bladder contraction in rats while activation of CB2 receptors with GP1A only had a post-synaptic effect. Figure 8.2 describes the most likely localisation for CB1 in pre- and post-synaptic neurons in the detrusor and the post-synaptic localisation for CB2. The effect seen in vitro upon activation of these receptors is also illustrated. It can be speculated that in the detrusor, post-synaptic neurons synthesise membrane-bound endocannabinoid precursors and cleave them to release active endocannabinoids following an increase of cytosolic free Ca\(^{2+}\) concentrations after the binding of ACh to nicotinic receptors on post-ganglionic nerves or the increase of ATP via P2X receptors. Endocannabinoids subsequently act as retrograde messengers by binding to presynaptic CB1 cannabinoid receptors, which are coupled to the inhibition of voltage-sensitive Ca\(^{2+}\) channels and the activation of K\(^+\) channels, as has been demonstrated in the brain (Wilson and Nicoll 2001). This will blunt membrane depolarisation and exocytosis, thereby inhibiting the release of ACh and in turn affect the
ability of the cholinergic system to initiate a detrusor contraction. Activation of the cannabinoid receptors would then inhibit detrusor contraction as demonstrated in vitro and in vivo in this thesis. This theory needs to be explored further by conducting co-localisation studies of the cannabinoid receptors with both cholinergic and noradrenergic nerves using specialised microscopy that can delineate details of pre- and post-ganglionic nerves. Furthermore, studies to identify the signalling of endocannabinoids need to be conducted using inhibitors for both MAGL and FAAH and by measuring changes in the levels of endocannabinoids, possibly through the use of mass spectrometry or a fluorescent label that can be designed to detect AEA or 2-AG. The presence of neurons needs to be confirmed using specific neuronal markers.
Figure 8.2: Cannabinoid signalling in pre-synaptic nerve terminals. The metabolic pathways of the two major endocannabinoids, anandamide (AEA) and 2-arachidonylgllycerol (2-AG) are shown, with their most likely localisation in pre and post-synaptic neurons. Anandamide biosynthesis occurs from a phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine (NAPE), which is synthesised from phosphatidylethanolamine (PE) and another phospholipid by an N-acyl-transferase (NAT). NAPE is then hydrolysed to anandamide by a specific phospholipase D (NAPE-PLD) (Di Marzo et al. 1994; Okamoto et al. 2004). These enzymes are localised in intracellular membranes, although it is not known whether they are pre or post-synaptic. The biosynthesis of 2-AG occurs through the formation from phospholipids of a diacylglycerol (DAG) precursor, which is catalysed by a phospholipase C (PLC), followed by the hydrolysis of DAG by DAGLs (Bisogno et al. 2003). Similar to PLC, it can be speculated according to evidence in the human brain that DAGLs are in the plasma membrane (post-synaptic in the adult brain (Bisogno et al. 2003)). In the brain, degradation of anandamide by fatty acid amide hydrolase (FAAH) occurs post-synaptically at intracellular membranes (Cravatt et al. 1996), whereas degradation of 2-AG by monoacylglycerol lipases (MAGLs) occurs pre-synaptically in the cytosol and at intracellular membranes (Dinh et al. 2002).

Endocannabinoids diffuse through the plasma membrane depending on their intracellular–extracellular concentration gradient by an endocannabinoid membrane transporter or binding protein (EMT) that is still to be characterised (Fowler et al. 2005; Fowler 2013). The endocannabinoid system is a regulatory apparatus that is present in the urinary bladder (as demonstrated in chapter 3) and has been extensively studied in the brain. It is activated ‘on demand’ to re-establish transient perturbations in the homeostasis of other mediators (neurotransmitters, hormones and cytokines) whose levels and actions are modulated by endocannabinoids and which, in turn, control endocannabinoid levels (Piomelli 2003; Di Marzo et al. 2004).

Solid arrows denote either activation or movement, blunted arrows denote antagonism, thick blue arrows denote enzymatic reactions and dashed arrows denote degradation pathways. AA; Arachidonic acid, ET; ethanolamine
8.2.2 Co-localisation of cannabinoid receptors with sensory nerves in the bladder

There is supporting evidence to show that CB receptors are co-localised with sensory afferent nerves in the bladder, as demonstrated in chapter 5, using immunofluorescence experiments employing antibodies to PGP 9.5 and ChAT. The co-localisation with ChAT may demonstrate co-localisation of CB receptors with sensory nerves where the neurotransmitter is ACh and therefore not necessarily cholinergic nerves as described in the theory above. To further support the immunofluorescence findings, in vivo experiments described in chapter 7 demonstrated that activation of CB receptors with the non-selective CB agonist CP55,940, had an effect on urodynamic parameters resulting from involvement of afferent nerve fibres. This is further supported by studies demonstrating expression of both CB receptors, and FAAH in nerves that co-express sensory markers, including P2X₃, TRPV1, TRPV4 and CGRP (Bridges et al. 2003; Mitrirattanakul et al. 2006; Veress et al. 2013). Inhibitory effects by cannabinoids on nerve-mediated bladder contractions and bladder afferent nerve activity have been demonstrated (Dmitrieva and Berkley 2002; Hiragata et al. 2007; Gratzke et al. 2009; Gratzke et al. 2010), but there is a lack of consensus on the involvement of specific CB receptor subtypes. Systemic and intravesical administration of cannabinoids in the urinary bladder of rat animal models demonstrate consistent inhibitory effects on afferent pathways underlying micturition.

The urothelium plays an active role in bladder physiology, possessing an array of ion channels and receptors normally associated with neuro-transmission (Birder and Andersson 2013). The close proximity of the urothelium to sensory nerve terminals allows it to influence neural afferent activity, which has led to the emerging concept that bladder afferent transmission is a multifactorial process involving cross-talk between the urothelium and the sensory nerves (Birder and Andersson 2013). It may be the case that in patients with DO, endocannabinoids may directly modulate afferent activity in the urothelium, because there was increased CB receptor expression in these patients (described in chapter 5). In addition, in vivo studies (described in chapter 7), demonstrated that activation of CB receptors significantly reversed the hyperactivity induced by acetic acid. This finding is in agreement with previous urodynamic studies.
conducted in rats showing that bladder hyperactivity induced by infusion of chemical agents, such as cyclophosphamide or acetic acid, could be attenuated by activation of cannabinoid receptors (Hiragata et al. 2007). Together these findings suggest that endocannabinoids could potentially play a prominent role in controlling bladder filling and micturition by modulating the excitability of bladder afferent nerves. Moreover, these data may also indicate that alterations in cannabinoid signalling could be a key factor in the generation of hypersensitivity disorders of the bladder, such as DO.

8.2.3 Cannabinoid receptors act directly on the detrusor muscle

The other possible mechanism of action could be that cannabinoid receptors are located on the smooth muscle and exert their effects directly on the detrusor contractile apparatus resulting in relaxation (Figure 8.3). In chapter 3 both CB receptors were localised in the detrusor muscle and activation of both receptors resulted in inhibition of CCh induced contractions in vitro. In addition, rat cystometry experiments demonstrated that CP55,940 significantly decreased maximal voiding pressure, which is a urodynamic parameter controlled by the motor component of bladder innervation.

Several mechanisms dictate smooth muscle tone; for example, elevation of cAMP levels through Gs protein coupling, leads to smooth muscle relaxation, which could be one possible cannabinoid signalling route in the detrusor muscle. Smooth muscle tone is also regulated by membrane potential, a property regulated by the activity of various ion channels. Activation of CB receptors may induce detrusor relaxation by inhibiting L-, N-, and/or P/Q-type calcium channels and activation of potassium channels (Figure 8.3) via pertussis toxin-sensitive Gi/o protein coupling. This would induce hyperpolarization and therefore inhibition of intracellular Ca2+ store release, leading to muscle relaxation (Gebremedhin et al. 1999). The detrusor muscle expresses many K+ channels and also L-type Ca2+ channels (Andersson and Arner 2004), and a similar interaction between CB receptors and these channels may explain the ability of endocannabinoids and synthetic cannabinoid agonists to induce relaxation.

Endocannabinoids may be synthesised in the detrusor on demand, as evidenced by IHC and WB data described in chapter 3, where the modulating enzymes (NAPE-PLD and FAAH) were localised in the detrusor and urothelium. However, further experiments
need to be undertaken to explore this theory further for example; using Ultra performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) one can measure the levels of anandamide (AEA) produced after incubating bladder extracts/tissues or cells with NAPE or NAT. Another way to demonstrate where the endocannabinoids are being synthesised is by using isolated urothelium/detrusor and measure production of AEA using UPLC-MS/MS. This would also provide further information to support one of the above theories describing the possible location where endocannabinoids act e.g. directly on the muscle; or nerve fibres. Finally, experiments using inhibitors for FAAH, FAAH-2 and NAPE-PLD can be used to manipulate production of endocannabinoids
Cannabinoid signalling in the detrusor muscle. The metabotropic CB1 receptor exhibit 48% amino acid sequence identity with CB2 receptors and both of them are negatively coupled to adenylate cyclase to inhibit cyclic AMP that indirectly inhibits L-type Ca$^{2+}$ channels. Another mechanism in which detrusor muscle relaxation may occur is by CB receptor coupling via pertussis toxin-sensitive G$_{i/o}$ proteins where the G$\beta\gamma$ complexes couple to and inhibit N-, and P/Q-type Ca$^{2+}$ channels. Furthermore, indirect inhibition of voltage-sensitive Ca$^{2+}$ channels by cellular hyperpolarisation, as a result of K$^+$ channel activation, may occur resulting to muscle relaxation. Hyperpolarisation of the muscle cell may occur by G$\beta\gamma$ complexes coupling to and activating K$^+$ channels. In addition, CB receptors may be coupled to G$_s$ protein and on activation would stimulate adenylate cyclase, which in turn, will stimulate formation of cAMP. cAMP can then act as a second messenger that can interact with and activate protein kinase A (PKA), which in turn, inhibits calcium channels leading to muscle relaxation.
8.3 The role of cannabinoids in detrusor overactivity

During pathological conditions, there is a change in the expression of cannabinoid receptors, as described in chapter 5. CB1 expression has been found to be increased in patients with chronic bladder pain syndromes (Sanchez Freire et al. 2010) and overactive bladder (Mukerji et al. 2010), whilst CB2 mRNA and protein expression increased in rats with bladder inflammation due to acrolein (Merriam et al. 2008). In addition, the concentration of anandamide is increased in the bladder after acute or chronic inflammation (Dinis et al. 2004a). Thus, the increased expression of CB1 receptors in urothelium of patients with DO, may contribute to suppression of the inflammatory response and may be of use in developing new treatments for inflammation-associated bladder disorders.

The importance of afferent bladder activity in the pathogenesis of bladder dysfunction is becoming increasingly evident (Kanai and Andersson 2010). Recent studies have reported that dual CB1/CB2 non-selective cannabinoid agonists increase the threshold for micturition and reduce the inflammation-induced hypersensitivity of sensory afferent fibres in preclinical urodynamic models (Hiragata et al. 2007; Walczak and Cervero 2011), however, the therapeutic role of cannabinoids in DO remains unclear. In previous studies, and as reported from in vivo and in vitro experiments described in this thesis, cannabinoid agonists produce effects on both efferent and afferent bladder nerves in which activation of CB1 receptor decreases visceral cholinergic neurotransmission similarly in inflamed and in normal bladder (Hiragata et al. 2007; Gratzke et al. 2010; Walczak and Cervero 2011). Therefore, it is not clear if there is a ameliorating effect using these compounds, if there is a suppression of the inflammatory process, or if these effects are related to the ability of cannabinoids to modulate smooth muscle contractility (Howlett et al. 2002).

Furthermore, Mukerji et al. (2010) showed that patients with idiopathic detrusor overactivity had a significantly higher CB1 immunoreactivity in bladder nerve fibres than that found in healthy controls. Similarly, in this thesis an increase in CB1 receptors was seen in the urothelium of patients with DO. The changes in CB1 expression in DO patients and the co-localisation of CB1 with neurofilament-positive nerve fibres in
bladder tissue (chapter 5), could be mediated in part, by neurotrophic factors, such as nerve growth factor (NGF). NGF levels have been found to be increased in both the urothelium and the urine of patients with painful and overactive bladder disorders (Lowe et al. 1997; Zvara and Vizzard 2007; Liu et al. 2009; Liu and Kuo 2009). CB1 is expressed by NGF-dependent primary afferent neurones, which comprise most of the visceral primary afferent input (Friedel et al. 1997; Hohmann and Herkenham 1999). Furthermore, local instillation of cannabinoids directly into the bladder reduced bladder-induced hyperactivity, an effect, which was prevented by a CB1 antagonist (Walczak and Cervero 2011). This suggests that cannabinoids may have effects directly on nociceptor nerve endings where NGF and TRPV1 are perceived to produce their nociceptive and hyper-analgesic effects and is consistent with studies that show that the CB1 receptor is co-expressed with TRPV1 (Ahluwalia et al. 2000; Binzen et al. 2006; Agarwal et al. 2007; Anand et al. 2008), Substance P, and CGRP (two neuropeptides that are highly correlated with NGF responsiveness) (Ahluwalia et al. 2002; Agarwal et al. 2007). Moreover, expression of CB1 receptors is increased in sensory neurones after inflammation, particularly in those neurones that also express TRPV1 (Amaya et al. 2006). Levels of endogenous cannabinoids, such as anandamide, are also increased in the bladder after inflammation (Guerios et al. 2006).

Whether the increase in CB1 receptors reported in this thesis and the study by Murkerji et al. (2010) was the cause or consequence of DO can only be speculated upon, and further studies need to be conducted to answer this question, however, based on the existing literature and results of this thesis, CB1 receptor agonists might be useful in the treatment of DO. Drugs for the treatment of LUTS and DO might exert their clinically relevant effects via a CNS site of action, as well as acting on the peripheral organ. As peripheral bladder nerves (ending within the bladder wall) and relay stations, such as the dorsal root ganglia and spinal cord interconnection centres, are lying outside the blood-brain barrier (Bartanusz et al. 2011), a relevant neuronal site of action might be possible and also for drugs that do not cross the blood-brain barrier.

8.3.1 Clinical studies

Few clinical studies have assessed the effects of cannabinoids on the bladder (summarised in Table 8.4). Some of these studies are small pilot investigations and
therefore would not be adequately powered to provide statistically significant differences. An open-label pilot study on bladder dysfunction found consistent and statistically significant improvement in urgency, daytime frequency, incontinence episodes and nocturia (Brady et al. 2004b). On urodynamic investigation, maximum cystometric capacity increased, but this was not significant. Comparisons in this study are among individuals, expressed as change over time (before and after treatment). The sample size required for such comparisons is typically far smaller than for comparisons between groups. Maximum cystometric capacity results showed large individual variation (45-608 ml at baseline and 103-665 ml after treatment) making comparisons with other studies almost meaningless. Freeman et al. (2006) reported as part of the sub-study of the CAMS trial that patients that received cannabis extract (cannabidiol) and THC showed a significant reduction (p<0.01) in incontinence episodes compared to placebo. These findings are suggestive of a clinical effect of cannabis on incontinence episodes in patients with MS. In another multicentre double-blind randomised clinical study, Sativex (containing tetrahydrocannabinol (THC) and cannabidiol (CBD)) showed a reduction in the number of nocturia episodes, number of voids daily and number of daytime voids in patients with neurogenic detrusor overactivity due to MS (Kavia et al. 2010).
Table 8.4: Clinical trials in which the effects of cannabis/cannabinoid on bladder symptoms have been investigated.

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Drug treatment</th>
<th>Bladder outcome measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kavia et al. (2010)</td>
<td>Randomized, double-blind, placebo-controlled, multicentre study (n=130)</td>
<td>Sativex (nabiximols) (oromucosal spray delivering a dose of 2.7 mg THC and 2.5 mg CBD) maximum 48/d for 10 weeks</td>
<td>No significant change in daily incontinence episodes compared to placebo, however, total number of voids in 24 h, number of daytime voids, nocturia episodes and void urgency episodes were significantly reduced in the Sativex group. Patient global impression of change showed an 84% improvement using Sativex while 58% reported improvement in the placebo group.</td>
</tr>
<tr>
<td>Freeman et al. (2006)</td>
<td>Randomized, double blind, placebo controlled, parallel group multicentre study (n=630)</td>
<td>THC (dronabinol) or THC/CBD (2:1 ratio) (oral cannabis extract) or oral placebo capsules to maximum 25 g/d of THC for 13 wk.</td>
<td>Urge incontinence episodes, all 3 groups showed significant reduction at the end of treatment; 38% cannabis extract, 33% THC, and 18% placebo. Both active treatments showed significant effects over placebo.</td>
</tr>
<tr>
<td>Wade et al. (2006)/2004</td>
<td>Open-label, long term follow-up of Wade et al. 2004 (n=137)</td>
<td>THC/CBD (2.7 mg-2.5 mg ratio) for average 434d oromucosal spray to maximum 48 sprays/d</td>
<td>Those completing at least 1yr showed consistent reduction in VAS scores for bladder symptom.</td>
</tr>
<tr>
<td>Wade et al. (2004)</td>
<td>Randomized, double blind, placebo controlled, parallel group study (n=80/group)</td>
<td>THC/CBD (1:1 ratio) or placebo for 6 wk oromucosal spray to maximum 120 mg each CME/day</td>
<td>Bladder symptom change, by VAS and questionnaire, did not show significant differences between CME and placebo.</td>
</tr>
<tr>
<td>Brady et al. (2004a)</td>
<td>Open-label pilot, 15 of 21 patients evaluated for bladder dysfunction</td>
<td>THC/CBD (1:1 ratio) for 8 wk followed by THC only for 8wk oromucosal spray to maximum 120 mg each/d</td>
<td>Number of incontinence episodes, volume of leakage, nocturia, and daytime frequency all decreased significantly; both treatments comparable.</td>
</tr>
<tr>
<td>Wade et al. (2003)</td>
<td>Randomized, double blind, placebo-controlled, single-patient crossover (n=24)</td>
<td>THC/CBD (1:1 ratio), THC, CBD, or placebo for 4 by 2 wk periods oromucosal spray to maximum of each CME of 120 mg/d.</td>
<td>Frequency, incontinence severity, urgency, and nocturia; reported improvement of impaired bladder control in some patients, changes not statistically significant.</td>
</tr>
<tr>
<td>Martyn et al. (1995)</td>
<td>N-of-1 crossover trial:4 period</td>
<td>1mg of nabilone or placebo every second day, each period 4 wk.</td>
<td>Frequency of nocturia; striking reduction corresponding to taking nabilone.</td>
</tr>
</tbody>
</table>

CAMS- cannabinoids in multiple sclerosis; CBD-cannabidiol; CME-crude marijuana extract; THC- Δ9-tetrahydrocannabinol; VAS-visual analogue scale
8.4 Limitations of study and suggestions for further experiments

While the urothelium has historically been viewed as a simple barrier separating the bladder wall from urine, increasing evidence also suggests that the urothelium plays a critical role in physiological and pathophysiological processes in the bladder (Birder and Andersson 2013). Specifically, urothelial cells have the capacity to secrete a variety of signalling molecules, such as PGE2, nerve growth factor, nitric oxide, and cytokines in response to various stimuli (Birder and Andersson 2013). An attempt to study cannabinoid signalling in the urothelium at a cellular level was made in this thesis but a suitable useable urothelial cell line was not found. The cell lines screened did not consistently express the cannabinoid receptors. Urothelial cell explant culture and primary cell culture were utilised as alternative model systems but they too did not prove feasible to complete the experiments planned. In addition, another limitation of the experiments presented in chapter 4, using the HCV 29 and UROtsa cells, was that expression profiling for the muscarinic and purinergic receptors was performed at the mRNA level only, but these data suggests the presence of functional proteins with further experiments conducted to measure changes in intracellular calcium and cAMP. No attempt was made to carry out immunofluorescence or immunoblotting experiments to demonstrate expression of muscarinic and purinergic receptors because commercially available antibodies for some of these receptors lack specificity and have not been properly validated. Studies with commercially available antibodies to muscarinic receptors (M1-M5) found a lack of specificity (Pradidarcheep et al. 2008; Pradidarcheep et al. 2009) when tested with knockout mice models. Similarly some purinergic receptor antibodies appear to lack receptor isotype specificity (Ashour et al. 2006; Michel et al. 2009; Yu and Hill 2013).

Altered CB1 expression was observed in patients with DO, but this was not correlated to the symptoms of these patients. The significance of these findings needs to be explored further with a larger number of patients and combined with an accurate method of clinical measurement. In addition, the functional significance of these findings was not assessed because the methodology used in this thesis could not be optimised. Confocal microscopy using full thickness tissue was not feasible as the biopsy would move out of focus after the addition of carbachol. If this experimental route is to be pursued, then a confocal
microscope where a Sylgard-lined preparation dish with the tissue pinned on it needs to be incorporated on the microscope stage.

Finally, in the cystometry experiments, the effects seen on urodynamic parameters could have been influenced by CNS actions of CP55,940 and so central effects cannot be excluded. Furthermore, it is possible that CP55,940 has effects on efferent signals of the mechano-afferent pathway, i.e. transmitter release from the urothelium or sensory nerves locally in the bladder, may have influenced the observations obtained.

8.5 Future work

Only one study has been published showing FAAH co-distribution with CB2. Further studies are required to investigate the co-distribution of FAAH with CB1 in the urinary bladder as CB1 was detected in both the urothelium and detrusor in this thesis and also in other published literature. In addition, potential targeting of FAAH-2 for inhibition of the homeostasis of endocannabinoids for LUTS may be a future direction for cannabinoid research, but studies would be limited to human tissue because rats and mice do not express this gene product. Links between FAAH activity and development of bladder dysfunction, as well as studies of the effect of FAAH inhibition in urodynamic models for experimental bladder overactivity, should be pursued.

Diverging information on the expression and/or activity of CB1 and CB2 in the urinary bladder of humans and other mammals, calls for additional investigations to clarify the function of these receptors in the urinary bladder. A reliable way to examine the role of each specific gene and in consequence, a specific protein, is the use of knockout models, which is possibly the way forward to study cannabinoid function in the urinary bladder. Although there was a change in CB1 mRNA levels in patients with DO, this was not entirely reflected at the protein level. Further functional studies are required to establish if this observation is clinically relevant. In vitro organ bath assays may be the approach to take as radioligand assays using GTPγS may prove difficult to obtain functional results due to the lipophilic properties of cannabinoid ligands and the small size of human tissue obtained from biopsies. Another method to compare differences in cannabinoid function in normal and DO human bladder samples is by using primary cell cultures from
biopsies of the two patient groups. An explant method established for smooth muscle cells could be optimised and would serve as an important tool in clarifying some of the discrepancies about the cannabinoid receptors discussed in this thesis.

Since there is no urothelial cell model available at present to study cannabinoid signalling, an adequate cell model needs to be established. This ideally should be a human cell line that expresses both cannabinoid receptors, in order to study the effect of cannabinoids on intracellular calcium, which was the original aim of this thesis. Ideally, establishing a human cell line that has immortality, expresses the receptors of interest, would allow the study of cannabinoid signalling. Furthermore, it would be possible to test interactions between receptor signalling pathways of cannabinoids and the purinergic and muscarinic pathways and examine the results in the context of relevant clinical or physiological importance.

One of the key challenges for the clinical use of cannabinoids is that they should be devoid of the psychoactive effects of cannabis. The use of FAAH inhibitors with the design of CB antagonists that target cannabinoid receptors without penetrating the brain, should be an aim for future research.

A clinical trial with a local intravesical administration of selective, peripherally-restricted cannabinoids would provide a novel strategy in the treatment of overactive and painful bladder disorders. Large, high powered studies are needed to study the effects of cannabis on idiopathic OAB as all the clinical studies published to date were taken from neurological conditions. In addition, evaluation of the safety of the long-term use of cannabinoids requires further work. Designing and testing peripheral CB1 receptor agonists, which do not cross the blood-brain barrier (but still reach peripheral nerves and neuronal relay centres), might be a way to harness the beneficial effects of the cannabinoid system on voiding, without inducing CNS psychoactive side-effects.

8.6 Conclusion

This thesis describes an experimental continuum of cannabinoid receptor expression and distribution, basic pharmacology, and cystometry with the intention of building a complete picture of the properties of these receptors and some of their ligands. The data
presented suggest that both CB1 and CB2 receptors are involved in normal micturition, most probably at both peripheral and CNS sites. This has been supported by in vitro pharmacologic experiments, which have shown that CB1 receptor activation can reduce neuronal activity and both CB receptors modulate bladder contractility in isolated bladder strips. The presence of CB1 receptors in the afferent fibre endings localised in the urothelium could be the location for the effects observed in these experiments. It may be speculated that CB1 receptor agonists can be useful for the treatment of DO, because there is altered CB1 receptor expression in the bladder of these patients, or that more data on how CB1 receptor expression is regulated in these tissues may result in a method of treatment to ameliorate DO.
Appendices
Appendices

Glossary

**Affinity**
Is a measure of how tightly a drug binds to the receptor. If the drug does not bind well, then the action of the drug will be shorter and the chance of binding will also be less. This can be measured numerically by using the dissociation constant $K_d$.

**Agonist**
A drug that binds to and activates a receptor. Agonists can be full, partial and inverse in nature. A full agonist produces a full response while binding to a low proportion of receptors. A partial agonist exhibits lower efficacy than a full agonist and can only evoke a submaximal response while occupying a population of receptors. An inverse agonist evokes responses opposite to an agonist by binding to the same receptor.

**Antagonist**
A drug that is capable of inhibiting the effect of an agonist.
Antagonists can be competitive or non-competitive. A competitive antagonist will bind to the same site as an agonist without evoking any biological effect thereby preventing agonist driven activation. A non-competitive antagonist binds to a site different to the agonist site, thereby preventing the activation of the receptor when the agonist binds. Both classes of compound can exist as reversible or irreversible; where the former can be washed off while the latter cannot be displaced by washing or competing ligands.

**$B_{max}$**
The receptor density in a given preparation, which is determined by saturation binding experiments. Receptor density is measured in the unit mol/g of protein.

**Cheng and Prusoff Equation**
A formula that is used to calculate the inhibition constant ($K_i$) of an antagonist in relation to the IC$_{50}$. The latter value is obtained by carrying out competition binding experiments.

**Denaturation**
A process in which proteins or nucleic acids lose the quaternary structure, tertiary structure and secondary structure which is present in their native state, by application of some external stress or compound such as a strong acid or base, a concentrated inorganic salt, an organic solvent (e.g., alcohol or chloroform), radiation or heat.

**Desensitisation**
A phenomenon that occurs when a receptor is unable to evoke a response upon secondary challenge by an agonist after an initial primary stimulus.

**EC$_{50}$**
The molar concentration of an agonist that produces 50%
of the maximum possible response for a given agonist. It is also referred to as the pEC\textsubscript{50}, which is the negative logarithm of the EC\textsubscript{50} is a measure of potency.

**Efficacy**

A term used to describe the manner in which an agonist response varies according to the number of receptor it occupies. A high efficacy agonist will produce a maximal response while occupying a low proportion of receptors whilst a low efficacy agonist will only evoke a lower response even when occupying a greater receptor population.

**IC\textsubscript{50}**

The molar concentration of an antagonist which causes 50% maximal inhibition or in binding studies defined as the molar concentration of competing ligand which reduces specific binding of a radiolabel by 50%.

**K\textsubscript{D}**

Equilibrium dissociation constant that represents the molar concentration of radioligand that occupies 50% of a receptor population. This value is determined by saturation binding experiments.

**K\textsubscript{i}**

A measurement obtained through binding studies. It is the molar concentration of competing ligand that would occupy 50% of receptor population if no radioligand was occupying the receptor population. It is also expressed as pK\textsubscript{i}. The negative logarithm of the K\textsubscript{i} value.

**Non specific binding**

Binding of the tracer (radiolabel) to sites/components other than the experimental system.

**Potency**

The molar concentration range over which a drug produces a response. It is usually defined as EC\textsubscript{50} or pEC\textsubscript{50}, where the latter is the negative logarithm of the EC\textsubscript{50} value.

**Scatchard analysis**

A linear transformation method employed to saturation binding data

**Specific binding**

A proportion of radiolabel tracer that can be displaced by ligands specific for a given receptor.
Ethics Approval Letter

Health Research Authority

NRES Committee East Midlands - Leicester
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20 August 2012

Dr Douglas Tincello
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University of Leicester
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LE7 2LX

Dear Dr Tincello

Study title: Research projects relating to bladder function
REC reference: 09/H0406/97
Protocol number: version 1 (generic)
Amendment number: 4
Amendment date: 31 July 2012

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

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<tr>
<td>Notice of Substantial Amendment (non-CTIMPs)</td>
<td>4</td>
<td>31 July 2012</td>
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<td>Covering Letter</td>
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Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

A Research Ethics Committee established by the Health Research Authority

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NRES Committee East Midlands - Leicester

Attendance at Sub-Committee of the REC meeting on 15 August 2012

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<tr>
<td>Mr John Baker</td>
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<tr>
<td>Dr Carl Edwards</td>
<td>Implementation Fellow</td>
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