

Cannabinoid receptor expression in the bladder is altered in detrusor overactivity

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Abstract

Introduction:

Immunohistochemical (IHC) evidence shows cannabinoid receptors (CB) are expressed in human bladders and cannabinoid agonists are known to inhibit detrusor contractility. However, the mechanism for this inhibition remains unknown. In addition, the role of CB in detrusor overactivity (DO) is under investigated. The aim of this study was to compare CB expression in normal and DO human bladders and to further characterise these receptors.

Methods:

PCR was used to detect differences in CB transcripts in bladder samples. Differences in CB protein expression was assessed by IHC. Immunofluorescence (IF) was used to evaluate co-localisation of CB with nerve fibres. Receptor density and binding affinity were measured using the cannabinoid radioligand [³H]-CP-55,940.

Results:

There were higher levels of CB1 transcripts in the urothelium of patients with DO and lower levels in the detrusor, compared to normal bladders. Radioligand binding revealed CB density of 421 ± 104 fmol/mg protein in normal human bladders. IHC confirmed these findings at the protein level. IF staining demonstrated co-localisation of CB1 with Choline Acetyltransferase-(ChAT) positive nerves in the detrusor and co-localisation with PGP9.5 in both urothelium and detrusor. CB2 was co-localised with both ChAT and PGP9.5 in the urothelium and the detrusor.

Conclusions:

Cannabinoid receptor expression is reduced in the detrusor of patients with DO, which may play a role in the pathophysiology of the disease. Co-localisation of CB receptors with cholinergic nerves may suggest that CB1, being localised on pre- and postsynaptic terminals, could influence neurotransmitter release. Our findings suggest a potential role for cannabinoid agonists in overactive bladder pharmacotherapy.

Key words:

Cannabinoids, cholinergic nerves, detrusor overactivity, urinary bladder, immunofluorescence, radioligand binding,

Brief summary:

CB1 receptor expression was lower in the detrusor and higher in the urothelium of patients with detrusor overactivity compared to normal bladders.

Introduction

There has been increased interest in the function of the endocannabinoid system in the lower urinary tract following a study which demonstrated the beneficial effects of cannabis on multiple sclerosis (MS) patients-related lower urinary tract symptoms (LUTS) [1,2]. The endocannabinoid system consists of cannabinoid (CB) receptors, their endogenous ligands, and related enzymes for biosynthesis and degradation. Cannabinoids are lipophilic molecules with anti-nociceptive and anti-hyperalgesic properties, which activate specific G-protein-coupled CB1 and CB2 receptors. Endocannabinoids are synthesized “on demand” upon sensitization, and their “effect” can be enhanced by inhibitors of fatty acid-amido hydrolase (FAAH), an enzyme that regulates endocannabinoid homeostasis [3].

Both cannabinoid receptors have been localised in the urinary bladder [4-7]. However, there are discrepancies in the available morphological studies regarding the exact location and function of CB. The available data indicate that CB are located in the bladder mucosa and in nerves expressing various sensory markers [7-9]. CB1 has been localised in the urothelium and nerve fibre structures of the suburothelium and detrusor [7-9]. In another study, CB1 receptors were not localised in the urothelium and nerve fibres, but CB2 immunoreactivity was found in these structures [5].

In addition, the majority of available studies have only looked at efferent functions of the bladder and have found electrically-evoked contractions of bladder strips to be

reduced after the addition of a CB1 agonist [4,10,11]. Cystometric studies supporting the role of cannabinoids on efferent functions have shown that CB agonists increased micturition threshold and voiding interval [12-14]. A study by Walczak *et al.* [15] supports the assumption that cannabinoids may have effects directly on nociceptive nerve endings as local instillation of cannabinoids directly into the bladder attenuated hyperactivity of bladder afferent nerves seen after production of experimental cystitis [15]. Furthermore, expression of CB1 is increased in sensory neurons after inflammation [16]. These data support the assumption of possible involvement of cannabinoid receptor-mediated functions in local regulation of mechanoafferent activity [17].

The available evidence for 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 CB with other structures. Currently, research on the role of the endocannabinoid system in bladder dysfunction has increased but there are little available data that examine detrusor overactivity (DO).

In this study we compared differences in CB expression in patients with DO and normal bladders and further characterized these receptors by co-localization studies with two nerve markers (PGP 9.5 and ChAT). PGP 9.5 is a neurone specific protein found in neurons at all levels of the central and peripheral nervous system while ChAT is the enzyme responsible for synthesising acetylcholine (ACh), and its presence in a cell is thought to indicate the ability to synthesise and release ACh [18]. Furthermore, radioligand-binding experiments in human and rat bladder were performed to evaluate affinity (K_d) and receptor density (B_{max}) and strengthen the

evidence that the cannabinoid receptors are present in the urinary bladder.

Materials and Methods

Tissue source and handling

Leicestershire and Rutland Ethics Committee approval was obtained, and patients gave informed written consent.

Bladder biopsies were taken from 17 women (age 45-76) (6 samples used for immunohistochemistry (IHC) and immunofluorescence (IF), 5 samples for qRT-PCR and 6 samples for radioligand-binding) without urinary symptoms at rigid cystoscopy who were undergoing elective gynaecological procedures and from 9 women (5 samples used for IHC and 4 samples for qRT-PCR) with DO demonstrated by urodynamics [19]. Full thickness 1 cm square bladder samples (away from the tumour margins) were taken from 4 men undergoing cystectomies for bladder cancer and these tissues were used for radioligand-binding assays. Patients with a history of cannabis use within three months of surgery were excluded. Samples that were used for quantitative PCR had the mucosa separated from the detrusor using microdissection and the separated tissue were stored in RNAlater. Biopsies used for IHC and IF were fixed in 4% (w/v) paraformaldehyde for 3 days and embedded in paraffin for IHC and IF analyses. Blocks were cut in transverse sections (5 μ m) on a Leica (model RM2035) microtome and allowed to air dry for 3–5 days.

Immunohistochemistry and Immunofluorescence

Bladder specimens were fixed and further processed for IHC as previously described

[4]. Sections were incubated overnight at room temperature with antibodies raised in rabbits against CB1 (Cayman Chemicals, UK, Cat No: 10006591, 1:50 dilution) or CB2 (Abcam, UK, Cat No: ab45942, 1:500 dilution). Positive control tissues were rat brain for CB1 and rat spleen for CB2 (data not shown). Blocking peptides for CB1 and CB2 were used to confirm specificity of antibodies. For the simultaneous demonstration of co-localisation of CB and neurones, antibodies to CB1 with either mouse anti-choline acetyltransferase (ChAT) antibody clone 28C4, (Chemicon International, Germany, Cat No:MAB5350, dilution 1:100) or mouse protein gene product 9.5 (PGP 9.5) (Abcam, UK Cat No:Ab8189 1:50 dilution) and CB2 with either ChAT or PGP 9.5 were incubated as cocktails and anti-rabbit FITC conjugate (Sigma-Aldrich, UK Cat No:F9887, 1:160 dilution) was used to display CB fluorescence. After rinsing, the slides incubated with ChAT antibody had goat anti-mouse IgG conjugated with Alexa Fluor 594 (Life Technologies, UK Cat No:A-11032, 1:160 dilution) applied to the sections for 60 min. Sections incubated with PGP9.5 antibody had goat anti-mouse IgG2A conjugated with Texas Red (Abcam, Cat No:Ab51410, 1:160 dilution) applied for 60 min. 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.

RNA isolation and real-time PCR

Separated tissue pieces of human detrusor and mucosa were dissected from biopsies of patients with normal and DO bladders and stored in RNeasy® at 4 °C prior to RNA isolation. RNA was extracted from bladder tissue using a preparatory RNA isolation kit *mirVana™* (Applied Biosystems), briefly this consisted of homogenizing tissue samples in a lysis/binding solution, using a Qiagen tissue ruptor following which a combination of both organic and solid phase extraction methodologies were used to isolate total RNA which was finally re-suspended into PCR-grade water. RNA mass was determined using a Nanodrop and purity assessed from both 260/280 and 260/230 nm ratios which were >1.8. Extracted RNA was treated using a Turbo DNA-free® kit. Subsequently samples were reverse-transcribed using a high-capacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qRT-PCR) using commercially available TaqMan gene expression assays (Applied Biosystems) was used to assay samples for expression of RNA transcripts which encode for human CB1 (identifier Hs00275634_m1) and CB2 (Hs00275635_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, identifier 4326317E-1110043- which was used as a reference gene for the study). The thermal profile for qRT-PCR reactions in the StepOne instrument (Applied Biosystems) was 2 min at 50 °C, 10min at 95 °C, 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Data for qRT-PCR experiments are presented as ΔC_t , which represents the difference between the C_t (cycle threshold) value of the target gene of interest and the endogenous control, GAPDH. Results are reported as mean \pm SEM of five normal human bladders and four from patients with DO with all experiments run in duplicate. Mann-Whitney test was performed to assess significance between groups and p-value <0.05 was considered significant.

Fold change of CBr expression between normal and DO bladder samples was calculated using $2^{\Delta\Delta Ct}$.

Radioligand binding

Drugs and solutions

CP55,940, a synthetic cannabinoid, purchased from Tocris, was diluted to a stock concentration of 10 mM with DMSO and stored at -20 °C. [³H]-CP-55,940 (specific activity 100-180 Ci (3700-6660 GBq)/mmol) was purchased from Perkin Elmer.

Membrane preparation

Membrane fragments were prepared separately from 4 normal human bladder sections of patients undergoing cystectomies and from pooled bladder biopsies collected from 6 normal patients undergoing gynaecological surgery. The cerebellum and bladder were dissected from 6 female Wistar rats (250-300 g), killed by cervical dislocation, and these tissues were used as control samples. All rats were used under schedule 1 procedure of the Animal (Scientific Procedures) Act 1986.

Dissected tissues were separately homogenised using an Ultra Turrax homogeniser in ice-cold buffer consisting 50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgSO₄, p.H.7.2. Membrane suspensions were centrifuged at 20,374 g for 10 min at 4 °C, and the supernatant discarded and membrane pellets re-suspended in ice-cold buffer, then homogenized and centrifuged similarly twice more. Membrane pellets were finally re-

suspended in buffer and protein concentration determined using the Lowry method [20].

Saturation receptor binding assay

45-300 μg , 7.5-25 μg and 60-100 μg of rat bladder, rat cerebellum and human bladder membrane homogenates, respectively, were used for saturation binding experiments. Tissues were incubated in buffer containing 50 mM Tris-HCl, 2.5 mM EDTA, 5 mM MgSO_4 , which was supplemented with 1mg/ml bovine serum albumin (BSA) and between 2pM-10 nM of [^3H]-CP-55,940; experiments were incubated for 60 min at 30 °C with gentle shaking. Non-specific binding was defined in the presence of 30 μM of the non-radioactive CP55,940. Reactions were terminated and bound/free radioactivity separated by vacuum filtration through polyethylenimine (0.5%)-soaked Whatman GF/B filters (Fisher Scientific, UK), using a Brandel harvester and bound radioactivity determined using liquid scintillation spectrophotometry (Packard 1900TR) [21]. K_d (equilibrium dissociation constant) and B_{max} (maximal binding) values were determined by analyzing the saturation binding data by nonlinear regression and fitted to sigmoid function using GraphPad Prism 6.0 software (GraphPad, San Diego, CA).

Results

qRT-PCR

The relative transcript level for the CB1 receptor was higher in mucosa of patients with DO compared to normal 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 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 mucosa of DO patients when compared to normal mucosa. 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. Changes for the CB2 receptor were not statistically significant.

Immunohistochemistry

Differential mRNA levels were verified by CB protein expression using IHC of human bladder biopsies from patients with normal bladders and those with DO. IHC revealed positive staining for CB1 and CB2 receptors in normal human detrusor and mucosa. The staining in the detrusor is primarily in the smooth muscle cells although some staining of the endothelial cells is also obvious. 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 DO patients (**Figure 1**). Furthermore, denser staining was seen for both receptors in the urothelium and suburothelium of patients with DO relative to the detrusor muscle and compared to the normal control biopsies (**Figure 1**). In summary, CB1 and CB2 receptor immunoreactivity was denser in the mucosa of patients with DO and less dense in the detrusor compared to controls.

Immunofluorescence

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 mucosa and detrusor with both cannabinoid receptors (**Figure 2**). 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 mucosa (**Figure 3**). CB2 receptor was also co-localised with ChAT in both mucosa and detrusor (**Figure 3**). Negative controls were in the absence of primary antibodies and incubation with non-immunised IgG2A (data not shown).

Saturation Binding assays

Saturation binding experiments in human cystectomy samples, rat bladder and cerebellum showed that the binding of [³H]-CP55,940 (a synthetic cannabinoid) was concentration dependent and saturable (**Figure 4**). The B_{max} for human bladder was 421.4 fmol[³H]-CP55,940/mg protein and the K_d 1.26 nM. For rat bladder, B_{max} was 429.7 fmol[³H]-CP55,940/mg protein with the K_d being 0.39 nM. Binding of [³H]-CP55,940 to rat cerebellum (positive control) demonstrated a higher B_{max} of 1974 fmol[³H]-CP55,940/mg protein and a similar K_d (0.45 nM), which is in agreement with a previous report [22].

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 recent study using bladder biopsies from MS patients with neurogenic DO and normal bladders, found a lower RNA expression of CB1 receptor and a higher expression of CB2 receptor in bladders with neurogenic DO [23]. Similarly, we showed, using qRT-PCR, that CB1 receptor was significantly upregulated 2.8 fold in the urothelium of DO samples and down regulated 3.2 fold in the detrusor of DO

samples compared to normal bladder samples. Although a similar pattern was seen for CB2 receptor, those differences were not statistically significant. Our patient selection was based on the presence of idiopathic DO demonstrated by urodynamics, while in the above study, patients had neurogenic DO following MS. This may suggest differences in expression of CB in the bladders of these patient groups. Another explanation for the discrepancy seen between our data for CB2 receptor expression and those of Katagigiotis *et al.* [23] is that they processed the bladder biopsies as whole tissue, which could easily mask localised differences in expression, while we compared differences in receptor expression between separated mucosa and detrusor muscle. This provided a more accurate assessment of receptor transcript levels in the different tissue types rather than using homogenates of the entire bladder, because differences in cellularity and presumed function are controlled.

Furthermore, our IHC findings depicted a greater CB receptor immunoreactivity in mucosa of patients with DO compared to detrusor, corroborating the findings by Mukerji *et al.* [8] where they reported increased densities of CB1 immunoreactive nerve fibres in the suburothelium 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 our results where we observed reduced immunoreactivity of CB1 receptor in detrusor of DO compared to controls, could be explained; we made comparisons using the entire detrusor section rather than focusing on nerve fibres only. It will be interesting for future studies to clarify this by using co-localisation of nerve fibres with CB1 receptors to identify the type of nerve fibres altered in the suburothelium and detrusor of patients with DO. A

role for CB2 receptors in bladder dysfunction has been suggested by the finding of upregulation of receptor expression in rat bladders with acute and chronic inflammation [24] and in MS patients with neurogenic DO [23]. Similarly, we found increased CB2 receptor expression in the mucosa of patients with DO and a decrease in receptor expression in the detrusor of these patients, 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 [25], and despite thoroughly washing the tissue, the effects reported could be a result of CB2 detection from contaminating blood .

Our result demonstrating increased expression of CB1 in the urothelium of patients with DO does not explain the clinical symptoms associated with DO in accordance to the current understanding of cannabinoid receptor signalling (activation of cannabinoid receptors results in relaxation). However, recently there has been discussion of an endothelial cannabinoid receptor (CBe) that is distinct from CB1 and CB2 [26] that causes vasorelaxation when activated. It may be that in the urothelium some form of this receptor exists in pathological states such as DO, this receptor is upregulated either as a result of DO or could be the cause of it.

Our previous findings [4] showed ACEA, a CB1 agonist, had an inhibitory effect on electrical field stimulation (EFS)-induced rat bladder strip contractions. EFS stimulates the intrinsic nerves, suggesting cannabinoids affect efferent functions of the bladder, leading us to explore the expression of CB on nerve fibres. Both CB were co-localized with PGP 9.5 (general antibody for neurons) in detrusor and mucosa. Cannabinoid receptors were found to co-localize with acetylcholine

neuronal markers, with CB1 co-localizing with ChAT in detrusor muscle of normal human bladder but not in the mucosa, while CB2 co-localized with ChAT in both mucosa and detrusor. These results correlate with the findings of others who have localized both CB with nerve fibres in the urothelium and detrusor. Veress et al. [27] showed partial co-localization of CB1 with calcitonin gene-related peptide (CGRP, a marker for sensory nerves) in the muscular layer of rat bladders but there was no co-localisation in the urothelium. In our study we detected co-localisation of CB1 with the generic neuronal marker PGP 9.5 and ChAT in the mucosa, which supports the findings of the above study. Since ChAT antibody detects the presence of acetylcholine, one possibility is that the nerves detected in this study are sensory nerves releasing acetylcholine as their neurotransmitter. However, in a study by Gratzke *et al.* [5] there was no immunoreactivity seen for CB1 on nerve structures using CRGP and VACHT antibodies but they did detect CB2 positive nerve fibres. With further co-localization studies they located CB2 on the sensory and cholinergic nerves of the bladders of rats and humans, corroborating our findings of co-localization of the CB2 receptor on cholinergic nerves.

The co-localization of CB receptors with cholinergic nerves may in part explain the functional results obtained from *in vitro* experiments where activation of the CB1 receptor has both a pre- and post-synaptic effects on bladder contraction [4, 6, 11] while activation of CB2 receptor with GP1A only has a post synaptic effect [4]. We want to emphasize that co-localization of the cannabinoid receptors with cholinergic nerves, described in this study, does not necessarily mean that these receptors have a functional role in the development of DO as further studies need to be undertaken to confirm a functional association.

Taken together, with the co-localization data, it may be speculated that a likely localization for the CB1 receptor is in pre- and postsynaptic nerve terminals of the detrusor, and for the CB2 receptor in the postsynaptic nerve terminals. A possible theory for how cannabinoid receptors inhibit detrusor contractility *in vitro* is illustrated in **figure 5**. It can be speculated from knowledge acquired in other organs, particularly the brain, that in the detrusor, postsynaptic neurons synthesize membrane-bound endocannabinoid precursors and cleave them to release active endocannabinoids following an increase of cytosolic free Ca^{2+} concentrations after the binding of neurotransmitter acetylcholine to muscarinic receptors on post-ganglionic nerves or increased ATP binding to P2X receptors. Endocannabinoids subsequently act as retrograde messengers by binding to presynaptic CB1 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 [28]. This will reduce membrane depolarization and exocytosis, thereby inhibiting the release of acetylcholine and affecting the ability of the cholinergic system to initiate a detrusor contraction. Activation of the cannabinoid receptors therefore, would result in an inhibition of detrusor contraction. This theory needs to be explored further by conducting co-localisation studies of the cannabinoid receptors with both cholinergic and noradrenergic nerves using microscopy that can delineate details of pre and post-ganglionic nerves. Furthermore, studies to identify the signalling of endocannabinoids needs to be conducted using inhibitors of the modulating enzymes and to measure changes in the levels of endocannabinoids, possibly through the use of mass spectrometry or a fluorescent label that can be designed to detect specific endocannabinoids such as anandamide.

There is extensive literature available on the pharmacokinetics of various cannabinoid agonists in the central nervous system, which has further characterized these receptors by providing the affinity in various tissues for the different CB agonists. [³H]-CP-55,940 has been used in a number of radioligand binding assays and the reported binding parameters in rat cerebellum was 1.3 nM (K_d) [29], which is consistent with our findings. The K_d value obtained for CB receptors in the human bladder (1.26 nM) was larger than the K_d value found in the rat bladder and cerebellum (0.39 and 0.45 nM respectively), but was within the range 0.5-5 nM previously reported [30]. Since the binding affinity of CP55, 940 in the urinary bladder has not been reported before, we cannot directly compare the K_d value calculated in this study to others. However, the small difference in K_d values seen between rat and human bladders suggests there may be an element of species differences in the binding affinity of CP55,940 to cannabinoid receptors. Another possibility for the lower affinity in the human bladder compared to rat bladder may be due to the presence of endogenous CB ligands shifting the human bladder towards a desensitised and low affinity state.

Receptor densities in human and rat bladders were very similar, which correlates with our previous reported immunohistochemistry and western blot findings that showed a similar expression and distribution of CB [4]. The receptor density found in the urinary bladder in human and rat tissue was significantly less than detected in rat cerebellum where CB1 receptors are known to be highly expressed. This study demonstrates for the first time that the synthetic cannabinoid agonist CP55,940 binds with high nanomolar affinity to cannabinoid receptors in the urinary bladder.

While the present study reveals that CP55,940 has the ability to act at cannabinoid receptors in whole bladder tissue, further *in vitro* analysis will be required with cannabinoid receptor-specific ligands to determine the relative distribution of CB1 and CB2 receptors in the urothelium and detrusor.

At the start of the study we set out to compare binding affinity and receptor density in patients with DO and normal bladders to complement our findings from IHC and qRT-PCR, which showed differences in receptor expression between these two groups. However, large pieces of tissue were required to determine receptor density, and this was obtained from whole rat bladders and bladder tissue from patients undergoing cystectomies. For one experiment we pooled 6 biopsies, as each biopsy contained insufficient tissue to use alone. Tissue from patients diagnosed with DO could only be obtained from biopsies, which on average were less than 0.5 mm in length and approximately 0.2 mm in width, resulting in insufficient amount of tissue to complete the experiments. Another limitation of this study was that mixed samples from males and females were used for the radioligand-binding assays. Cystectomy samples from male patients were used for the majority of the radioligand-binding experiments and one experiment yielded results from pooled biopsies from women. One may argue that there may be differences in cannabinoid receptor density and expression in the bladder of females and males. There is currently no evidence available comparing differences in cannabinoid receptor function in the bladder by gender. We do not feel that the use of bladders from males and females has affected our data as the K_d and B_{max} values obtained in individual experiments from pooled female biopsies was similar to the values obtained from male cystectomy samples. In addition, we have used only female samples for qRT-PCR, IHC and IF studies

where we explored cannabinoid receptor expression. Furthermore, by using CP55,940, which is non-selective for CB receptors, the specific characteristics for one receptor were not obtained, as this was beyond the scope of this study. However, the present work is the first to describe receptor density and binding affinity in rat and human bladders and further studies are needed to elucidate differences between CB1 and CB2 receptors in the bladder.

Conclusions

We have demonstrated a significant increase in cannabinoid receptors in the mucosa of patients with DO and a decrease in receptor expression in the detrusor of these patients, compared to normal bladders. Whether these changes are the cause or the consequence of DO can only be speculated upon, and further studies are needed to answer this question. Co-localisation of ChAT and cannabinoid receptor immunoreactivity, and the inhibitory effects of CB agonists that have previously been described *in vitro*, imply a modulatory function of cannabinoid receptors on cholinergic neurotransmission. Finally, the present study is the first to report the results of [³H]-CP55,940 saturation binding assays in both human and rat bladders and also confirms the presence of cannabinoid receptors in the bladder. Our findings suggest a potential role for cannabinoid agonists in overactive bladder pharmacotherapy.

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Figure Legends

Figure 1. Immunohistochemistry micrographs showing CB1 and CB2 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. **G.** Brown staining shows positive for CB2 receptors in normal urothelium. **H.** Shows positive staining in the detrusor of normal bladder tissue. **I.** CB2 negative controls in sections incubated with primary CB2 antibody adsorbed onto blocking peptide. **J.** Positive staining for CB2 receptor in urothelium of patients with DO. **K.** decreased staining intensity for CB2 receptor in detrusor of patients with DO compared to normal detrusor in B. **L.** CB2 negative controls with rabbit IgG. Scale bars at 10 μ m. **U=** urothelium, **SU=** suburothelium, **D=** detrusor muscle

Figure 2. Confocal microscopy images showing tissue sections after incubation with CB1 or 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.** CB1 antibody staining with anti-rabbit secondary antibody with FITC conjugate, **C.** indicates PGP9.5 positive staining with anti-mouse secondary antibody with Texas Red conjugate, **D.** is a merge of A-C with the arrows indicating to 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 using FITC, **G.** shows PGP 9.5 staining in urothelium, **H.** is a merge of E-G indicating co-localisation of CB1 with PGP9.5. Row (**I-L**) shows staining in the detrusor muscle of a normal human biopsy section. **I.** nuclear staining with DAPI, **J.** CB2 antibody staining with anti-rabbit secondary antibody with FITC conjugate, **K.** indicates PGP9.5 positive staining with anti-mouse secondary antibody with Texas Red conjugate, **L.** is a merge of A-C with the arrows indicating to yellow/orange staining which signifies co-localisation of CB2 receptors on nerve fibres, **M.** nuclear staining with DAPI in urothelium, **N.** CB2 receptor protein expression in urothelium using FITC **O.** shows PGP9.5 staining in urothelium, **P.** is a merge of M-) indicating co-localisation of CB2 with PGP9.5.

Figure 3. Double staining immunofluorescence with CB1 or 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.** 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 using FITC, **G.** shows no ChAT staining in urothelium, **H.** is a merge of E-G indicating no co-localisation of CB1 with ChAT. Row (**I-L**) shows staining in the

detrusor muscle of a normal human biopsy section. **I.** nuclear staining with DAPI, **J.** CB2 antibody staining with anti-rabbit secondary antibody FITC conjugate **K.** indicates ChAT positive staining with anti-mouse secondary antibody with Alexa Fluor conjugate, **L.** is a merged image of I-K with the arrows indicating to yellow/orange staining which signifies co-localisation of CB2 receptors on nerve fibres, **M.** nuclear staining with DAPI in urothelium, **N.** CB2 receptor protein expression in urothelium using FITC, **O.** shows ChAT staining in urothelium, **P.** is a merge of M-O indicating co-localisation of CB2 with ChAT indicated by the arrows. Images captured at 60x magnification.

Figure 4. Saturation-binding experiments of [3 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_{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).

Figure 5. The metabolic pathways of the two major endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are shown, with their most likely localization in presynaptic and postsynaptic neurons. Anandamide biosynthesis occurs from a phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine (NAPE), which is synthesized from phosphatidylethanolamine (PE) and another phospholipid by an N-acyl-transferase (NAT). NAPE is then hydrolyzed to anandamide by a specific phospholipase D (NAPE-PLD). These enzymes are localized in intracellular membranes, although it is not known whether they are presynaptic or postsynaptic. The biosynthesis of 2-AG occurs through the formation from phospholipids of a diacylglycerol (DAG) precursor, which is catalyzed by a phospholipase C (PLC), followed by the hydrolysis of DAG by DAGLs [31]. Similar to PLC, it can be speculated according to evidence in the human brain that DAGLs are in the plasma membrane (postsynaptic in the adult brain [31]). In the brain, degradation of anandamide by fatty acid amide hydrolase (FAAH) occurs postsynaptically at intracellular membranes, whereas degradation of 2-AG by monoacylglycerol lipases (MAGLs) occurs presynaptically in the cytosol and at intracellular membranes [32].

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 characterized [32]. The endocannabinoid system is a regulatory apparatus that is present in the urinary bladder and is activated ‘on demand’.

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