DEDICATION

I dedicate this work to my loving parents Chantale and Laurent who have always given me all the encouragement and support I could ever wish for.
ACKNOWLEDGEMENTS

I am so grateful to have had the opportunity to learn and work with Professor Andersson and to have him become, not only my advisor, but my mentor. Your passion and motivation in scientific research have driven during the last few years. I would also like to thank each member of my committee who has provided so much insight and interest to my thesis work.

I have also been fortunate to join the Department of Physiology and Pharmacology, which has provided me with high standards of education. I could not thank enough my laboratory colleagues at Wake Forest Institute for Regenerative Medicine and my collaborators who have helped me throughout the way and who have taught me so much. Your hard work and dedication have played a major part in my success. I am also very grateful for the invaluable friendships that have grown from our work together.

I am thankful for the scholarships and funds that have allowed me to complete my training (American Urological Association Foundation Research Scholar Fellowship, Royal College of Physician and Surgeons of Canada Detweiler Travelling Fellowship, Quebec Urological Association Training Scholarship)

I am blessed with wonderful parents and brothers who have always encouraged me and provided the most needed guidance. I also want to thank my closest friends, who despite the distance, have made every effort to brighten up my days and bring a smile to my face. And lastly, I owe so much gratitude to my boyfriend who has been with me every step of the way, even at the darkest moments. Words cannot express how much your dedication and loyalty has meant to me.
# TABLE OF CONTENT

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES AND TABLES</td>
<td>v</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER I – Introduction</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER II – Bladder dysfunction and parkinsonism: Current pathophysiological understanding and management strategies</td>
<td>7</td>
</tr>
<tr>
<td>CHAPTER III – Effects of allogeneic bone marrow-derived mesenchymal stromal cell therapy on voiding function in a rat model of Parkinson’s disease</td>
<td>32</td>
</tr>
<tr>
<td>CHAPTER IV – Cannabinoid system contribution to control of micturition</td>
<td>61</td>
</tr>
<tr>
<td>CHAPTER V – Role of cannabinoid and TRPV1 receptors in the spinal mechanisms of micturition in the normal rat</td>
<td>78</td>
</tr>
<tr>
<td>CHAPTER VI – Cannabinoid receptor type 1 (CB1) is important for normal micturition – results from in vitro and in vivo bladder evaluation of a CB1 knock-out mouse model</td>
<td>98</td>
</tr>
<tr>
<td>CHAPTER VII – Characterization of Bladder Function in a Cannabinoid Receptor Type 2 Knockout Mouse in vivo and in vitro</td>
<td>118</td>
</tr>
<tr>
<td>CHAPTER VIII – Discussion and Perspectives for the Future</td>
<td>137</td>
</tr>
<tr>
<td>APPENDIX I – Stem cell therapy ameliorates bladder dysfunction in an animal model of Parkinson's disease</td>
<td>147</td>
</tr>
<tr>
<td>APPENDIX II – Role of spinal cord fatty acid amide hydrolase (FAAH) in normal micturition control and bladder overactivity in awake rats</td>
<td>169</td>
</tr>
<tr>
<td>Curriculum Vitae</td>
<td>189</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS AND TABLES

CHAPTER I

Figure 1: Components of micturition reflex_____________________________ 3

CHAPTER II

Table 1: Prevalence of lower urinary tract symptoms in patients
with Parkinson’s disease____________________________________________ 11

Figure 1: The nigrostriatal dopaminergic pathway________________________ 11

Figure 2: 7T Functional MRI in the 6-OH dopamine rat model______________ 22

Figure 3: Tyrosine hydroxylase staining after injection of
6-hydroxydopamine (6-OHDA) into de medial forebrain bundle (MFB) in rats__ 22

Figure 4: Representative traces of cystometric records in a rat model of
Parkinson’s disease 3, 14, and 28 days after lesion of the MFB______________ 23

Figure 5: Expression of SOD-2 immunofluorescence______________________ 26

Figure 6: Representative tracings of cystometric records at 14 and 28 days
after the injection treatments__________________________________________ 27

CHAPTER III

Figure 1: Cell population after EGFP lentiviral infection___________________ 43

Table 1: Body weight in Vehicle treatment, rBMSC and ErBMSC
at all time-points___________________________________________________ 44
Table 2: Urodynamic parameters in Vehicle treatment, rBMSC and ErBMSC at all time-points

Figure 2: Representative urodynamic tracings 42 days after injection of vehicle (A), rBMSC alone (B) and ErBMSC (C)

Figure 3: Cystometric data at 7, 14, 28 and 42 days

Figure 4: Representative confocal images of right SNpc at 10x magnification

Figure 5: Effect of unilateral 6-OHDA lesion and treatment on SNpc TH-positive neurons at 42 days

Figure 6: Representative confocal images of right SNpc at 20x magnification examined by immunohistological staining using an antibody against nestin

Figure 7: Representative confocal images of right SNpc at 20x magnification examined by immunohistological staining using an antibody against GFAP

Figure 8: Representative confocal images of right SNpc at 20x magnification examined by immunohistological staining using an antibody against IBA-1

Figure 9: Representative confocal images of right SNpc at 40x magnification examined by immunohistological staining using an antibody against IBA-1

CHAPTER IV

Figure 1: Endocannabinoid metabolism and sites of action

Figure 2: Representative 10-minute urodynamic recordings in PUO rats

Figure 3: Protein expression in spinal cord
CHAPTER V

Table 1: Cystometric parameters with IT administration of MA__________ 87

Table 2: Cystometric parameters with IT administration of WIN 55,212-2____ 88

Figure 1: Cystometric data following CB agonist________________________ 89

Table 3: Cystometric parameters with administration of IT vehicle, followed by systemic OeTA and IT MA______________________________ 90

Table 4: Cystometric parameters with administration of IT SB 366791,
followed by systemic OeTA and IT MA______________________________ 90

Figure 2: Cystometric data following TRPV1 antagonist________________ 90

CHAPTER V1

Figure 1: Carbachol concentration response curves (CCRCs) of bladder
strips from wildtype and cannabinoid type 1 receptor knock-out__________ 108

Figure 2: Electrical field stimulation (EFS) of bladder strips from wildtype
and cannabinoid type 1 receptor knock-out___________________________ 109

Figure 3: 15 minutes representative urodynamic (cystometric) tracings______ 109

Table 1: mean values (±SEM) of urodynamic parameters of KO and WT mice__ 110

Table 2: mean values (±SEM) of urodynamic parameters of WT mice (n = 5)
before and after administration of Rimonabant (CB1 receptor antagonist)______ 110

CHAPTER VII

Table 1: Age, body and bladder weight of mice__________________________ 126

Table 2: Baseline cystometric parameters______________________________ 127
Figure 1: Representative tracings of cystometric records of WT and CB2RKO __ 127

Table 3: Cystometric parameters in WT _______________________________ 128

Figure 2: Bladder strips contractile responses __________________________ 129

APPENDIX I

Figure 1: Experimental design timeline _______________________________ 153

Figure 2: Cystometric data __________________________________________ 156

Figure 3: Expression of SOD-2 – immunofluorescence ___________________ 157

Figure 4: Expression of IL6 – immunofluorescence ______________________ 157

Figure 5: Expression of GDNF – immunofluorescence ____________________ 158

Figure 6: Survival of substantia nigra dopaminergic cells and striatal density__ 159

APPENDIX II

Table: Urodynamic parameters in healthy nonPUO rats after increased OEtA and with ongoing intravesical PGE2 before and after 30 nmol OEtA, and in PUO rats after increased OEtA ________________________________________ 177

Figure 1: Representative 10-minute urodynamic recordings in healthy rats____ 178

Figure 2: Representative 10-minute urodynamic recordings in PUO rats______ 179

Figure 3: Western blot bands of FAAH, CB1, CB2 and GAPDH in 4 normal____ 180

and 4 PUO rats
**LIST OF ABBREVIATIONS**

2-AG = 2-arachidonoylglycerol  
6-OHDA  = 6-hydroxydopamine  
Δ9-THC = Δ9-tetrahydrocannabinol  
AD = Alzheimer’s disease  
AUC = area under the curve  
BC = bladder capacity  
Bcom = bladder compliance  
BMSC = bone marrow-derived mesenchymal stromal cells  
BO = bladder overactivity  
BP = basal pressure  
BPH = benign prostatic hyperplasia  
BPO = bladder prostatic obstruction  
CB = cannabinoid  
CB₁ = cannabinoid type 1  
CB₂ = cannabinoid type 2  
CB₁R = cannabinoid receptor type 1  
CB₂R = cannabinoid receptor type 2  
CB₂RKO = cannabinoid receptor type 2 knockout  
CGRP = calcitonin gene-related peptide  
CNS = central nervous system  
COM = compliance  
CSF = cerebrospinal fluid
DA = dopamine
Dan-PSS = Danish prostate symptom score
DO = detrusor overactivity
EC$_{50}$ = half maximal effective concentration
EFS = electrical field stimulation
EGFP = enhanced green fluorescent protein
ErBMSC = microencapsulated rat bone marrow mesenchymal stromal cell
FAAH = fatty acid amide hydrolase
FACS = fluorescent activated cell sorting
GABA = $\gamma$-aminobutyric acid
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GFAP = glial fibrillary acidic protein
GPR55 = G-protein coupled receptor 55
HBSS = Hank's balanced salt solution
IBA-1 = ionized calcium binding adaptor molecule 1
ICI = intercontraction intervals
IP = intermicturition pressure
IPSS = international prostate symptom score
IT = intrathecal
KO = knockout
LUT = lower urinary tract
LUTS = lower urinary tract symptoms
LVM = low viscosity high mannuronic acid
MA = methanandamide
MFB = medial forebrain bundle
MP = maximum pressure
MS = multiple sclerosis
MV = micturition volume
NMS = non-motor symptoms
OAB = overactive bladder
OeTA = oleoyl ethyl amide
PAG = periaqueductal gray
PGE2 = prostaglandin E2
PD = Parkinson’s disease
PTNS = posterior tibial nerve stimulation
PUO = partial urethral obstruction
rBMSC = rat bone marrow mesenchymal stromal cell
RV = residual volume
SA = spontaneous activity
TP = threshold pressure
TRPV1 = transient receptor potential cation channel subfamily V member 1
WT = wildtype
ABSTRACT

Lysanne Campeau

CENTRAL NERVOUS SYSTEM CONTROL OF MICTURITION IN RODENTS

THROUGH NEUROPROTECTION AND ENDOCANNABINOIDS –
DOPAMINERGIC AND CANNABINOID CONTRIBUTION

Dissertation under the direction of

Karl-Erik Andersson, MD, PhD

Wake Forest Institute for Regenerative Medicine

Micturition consists of reflexes involving the central and peripheral nervous system controlling the bladder and urethra. A better understanding of the underlying contribution of the dopaminergic and cannabinoid systems to the control of micturition could help direct therapeutic modalities for related voiding dysfunction.

Disruption of the central control of the voiding reflex, and bladder dysfunction, commonly occur with neurodegenerative disorders such as Parkinson’s disease (PD), possibly due to an imbalance in the dopaminergic system. Chronological development of bladder dysfunction has been shown after producing a unilateral lesion in the medial forebrain bundle with 6-OHDA. Rat bone marrow-derived mesenchymal stem cells (rBMSC) or cells protected by microencapsulation (ErBMSC) were transplanted into the substantia nigra pars compacta (SNpc). Urodynamic effects of the 6-OHDA lesion persisted up to 42 days after vehicle injection. Transplantation of rBMSC alone improved the urodynamic pressures at 42 days after treatment more markedly than ErBMSC. This was associated with a higher number of TH-positive neurons in the treated SNpc of rBMSC animals, suggesting that functional improvements require a juxtacrine effect.

Systemic administration of cannabinoid (CB) receptor agonists affects bladder function, but the main site of action (peripheral tissues versus central nervous system) or the contribution of individual CB receptors (type 1 [CB1R] and type 2 [CB2R]) to normal micturition has not been clearly defined. Central application of CB receptor agonist localized to the spinal cord with intrathecal administration was found to increase bladder capacity (BC) during conscious cystometry, possibly mediated by the activation of spinal TRPV1 channels, and likely through an afferent pathway. The overall involvement of CB1R and CB2R in micturition was examined individually by characterizing the in vivo and in vitro bladder function in their respective KO mouse. The absence of CB1R was associated with a smaller BC and more spontaneous activity during cystometry and a lower response to electrical stimulation of nerves. On the other hand, lack of CB2R was linked with lower BC and higher bladder compliance than when CB2R is present.

In conclusion, these studies have determined that the dopaminergic and CB system play a significant role in the CNS control of micturition.
CHAPTER I

Introduction
Micturition consists of reflexes involving the central and peripheral nervous system controlling the bladder and urethra. Most symptomatic disturbances of voiding function are directly or indirectly related to pathologies involving the nervous system, while many etiologies remain undefined. A recent initiative of the National Institute of Diabetes and Digestive and Kidney Disease funded the Symptoms of Lower Urinary Tract Dysfunction Network (LURM) with its primary goal to better characterize the individuals affected by lower urinary tract dysfunction. A better understanding of the underlying contribution of the dopaminergic and cannabinoid (CB) systems to the control of micturition could help direct therapeutic modalities for related voiding dysfunction.

Micturition is organized in two distinct phase. During the storage phase, the bladder slowly fills and sends afferent signals via the Aδ fibers in the pelvic nerve through the spinal cord. Afferent activity reaches the pontine continence center, located in the dorsolateral pontine tegmentum. Efferent signaling of the sympathetic nervous system via the hypogastric nerve tonically contracts the bladder outlet to maintain continence. Somatic output through the pudendal nerve contracts the external sphincter. Efferent sympathetic output also inhibits parasympathetic activation of the bladder detrusor muscle.

The voiding phase is mediated by the spinobulbospinal reflex. With bladder distension, urothelial signaling occurs and bladder afferent activity reaches the central periaqueductal gray (PAG) through afferent nerves and dorsal root ganglia.
The PAG also receives tonic suppression from higher centers, namely the medial prefrontal cortex, for cognitive inhibition of voiding in socially unacceptable situations. Neural integration occurs in the PAG, and its lateral region sends excitatory signals to the premotor pontine micturition center. Direct efferent output to parasympathetic preganglion neuron causes detrusor contraction, and inhibits the sympathetic and somatic outflow to the bladder outlet. The pontine micturition center projects descending spinal fibers containing glutamate as an excitatory neurotransmitter to the sacral parasympathetic nucleus and fibers containing γ-amino-butyric acid (GABA) and glycine as inhibitory neurotransmitter to Onuf’s nucleus. (Figure 1)
Voiding dysfunction as a result of neurologic injury manifests itself with symptoms such as urinary frequency, urgency and incontinence. This condition is referred to as neurogenic detrusor overactivity (NDO) on urodynamic assessment and is a consequence of disruption of the normal voiding reflexes involving the peripheral and central nervous system. It affects a heterogeneous population with underlying conditions such as Parkinson’s disease (PD), multiple sclerosis (MS), spinal cord injury (SCI) and children with myelomeningocoele. Approximately 70-84% of patients with SCI have some form of impaired bladder function.(1) Urinary disturbances are present in 38-71% of PD patients, with the most common symptoms being nocturia and urgency (2), and up to 40% of MS patients suffer NDO.(1) Oral antimuscarinic medications are currently used for treatment of this condition. They improve bladder compliance, but are mainly palliative for symptoms. These medications are poorly tolerated due to unpleasant side-effects, and have compliance rates as low as 32% at 6 months.(3) Other therapeutic modalities are being investigated for the treatment of this condition. For example, whole plant cannabis extract and Δ9-tetrahydrocannabinol significantly decreased the number of urge incontinence episodes in multiple sclerosis patients in a randomized placebo controlled trial.(4)
There appears to be a strong relationship between the CB and dopaminergic system, through direct or indirect pathways, affecting receptor expression or neurotransmitter release (5, 6). Dopamine and endocannabinoids may therefore both be involved in control of micturition through interrelated pathways. We will therefore study the underlying contribution of the dopaminergic and CB system to the control of micturition as a way to develop novel therapeutic strategies such as allogeneic stem cell replacement therapy and CB compounds using an integrated approach with animal models.
References

CHAPTER II

Bladder dysfunction and parkinsonism: Current pathophysiological understanding and management strategies

Sections of the following chapter has been published in Current Urology Reports
**Parkinson’s disease**

Neurodegenerative disorders progress in severity over time as their accompanying symptoms worsen. Current management not only involves palliation of life-threatening neurological and motor deterioration, but also improvement of accompanying symptoms significantly affecting the quality of life of patients with the disorder. Parkinson’s disease (PD), the most common neurodegenerative disorder after Alzheimer’s disease affecting 1.4% of adults over the age of 55 years, is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc).(1) Dopamine (DA) deficiency leads to cardinal motor abnormalities that include tremor at rest, rigidity, bradykinesia, and axial symptoms. It is becoming more evident that other lesions can develop in these patients and be responsible for the associated autonomic dysfunctions. Autonomic symptoms such as lower urinary tract symptoms (LUTS) become more prominent with disease progression and significantly impair the quality of life of affected individuals. LUTS occur frequently and their management remains challenging.

**Epidemiology**

Urinary complaints are commonly reported by both men and women affected with PD. Although this patient population presents a greater risk of overactive bladder (OAB) symptoms due their age, the frequency of LUTS is higher in PD patients than in unaffected adults. In a prevalence study, LUTS were evaluated using questionnaires (Dan-PSS and IPSS) in a cohort of PD patients and compared to a “healthy control group”. The Dan-PSS correlated with the stage of PD. The time of onset of PD to the onset of LUTS was in average 5.0 years. The most frequent symptoms were nocturia,
with 86% of patients reporting it on IPSS, followed by frequency (IPSS: 71%) and urgency (IPSS: 68%). (2) Another study assessed urinary symptoms in a cohort of patients with neurodegenerative disease: 15 with dementia with Lewy bodies, 15 with PD and 16 with Alzheimer’s disease (AD). Urge episodes and urge incontinence were present in 53% and 27% of patients with PD, which was less than patients with dementia with Lewy bodies (93% and 53%) but more than patients with AD (19% and 12%). Detrusor overactivity (DO) on urodynamics was more common in patients with dementia with Lewy bodies (92%) than in patients with PD (46%) or AD (40%). (3)

The Priamo study is a multicenter assessment of non-motor symptoms (NMS) and their impact on the quality of life of 1,072 enrolled patients with PD in 55 Italian centers. Patients were thoroughly assessed with interviews and questionnaires. The majority of patients were treated with levodopa and DA receptor agonists (58.9%). Almost all patients reported at least one NMS (98.6%), with NMS in the psychiatric domain being the most frequent (67%). Urinary symptoms of urgency (35%) and nocturia (35%) were amongst the most common. NMS in the urinary domain were present in 57.3% of PD patients. The presence of urinary NMS was associated with significantly longer disease duration than in patients without urinary NMS. (4)

Using the International Continence Society questionnaire, Sammour et al. correlated the LUTS of 110 PD patients (84 men) with age, gender, disease duration, degree of neurological impairment, and impact on quality of life. Neurological impairment measured by the Unified Parkinson’s Disease Rating Scale (UPDRS) was significantly
associated with LUTS. The latter were not associated with age or disease duration. 63 patients (57.2%) were symptomatic, with nocturia being the most common symptom (80.9%). Both men and women were equally affected by their LUTS.(5)

As male PD patients age, their likelihood of developing bladder prostatic obstruction (BPO) also increases. The treatment of their LUTS becomes more challenging as it relies on the proper diagnosis of its etiologies. A detailed history and physical examination accompanied by a proper urodynamic evaluation will determine the presence or absence of bladder outlet obstruction (BOO). If a diagnosis of BOO is made, it is also necessary to differentiate detrusor-sphincter dyssynergia from BPO with the use of electromyography synchronous with cystometry and pressure-flow study. Pharmacotherapy with alpha-receptor blockers should first be attempted in the cases of BPO before considering prostatic surgery. Roth et al. demonstrated a 70% success rate after transurethral resection of the prostate (TURP) in 23 PD patients, with no cases of de novo incontinence.(6) Overall, studies consistently report nocturia and urinary frequency as the most common storage (irritative) symptoms causing distress in PD patients. (Table 1) LUTS can present years after the initial diagnosis of a motor disorder and usually worsen with disease severity.
Figure 1 legend: The substantia nigra pars compacta (SN) contains the cell bodies of the dopaminergic neurons, projecting to the basal ganglia, which are part of a closed loop connecting all cortical areas, through the nigrostriatal neurons (NS neurons), and synapse in the striatum (putamen (Pu) and caudate nucleus (Ca Nu)), forming the nigrostriatal pathway. Parkinson’s disease is a neurodegenerative disorder characterized by a loss of dopaminergic neurons mainly in the SNpc leading to a reduction of the dopamine input to the target structure of the nigrostriatal pathway, the striatum.


Table 1

<table>
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<tr>
<th>Symptoms</th>
<th>Nocturia</th>
<th>Frequency</th>
<th>Urgency</th>
<th>Urge incontinence</th>
<th>Intermittency</th>
<th>Incomplete emptying</th>
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</thead>
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<td>Winge 2006 (2)</td>
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<td>71%</td>
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<td>26%</td>
<td>35%</td>
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<td>Sammour 2009 (5)</td>
<td>80.9%</td>
<td>35.4%</td>
<td>36.3%</td>
<td>20.9%</td>
<td>44.5%</td>
<td>20%</td>
<td>37.3%</td>
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</table>

Pathophysiology

It is a well established fact that neurodegeneration of the nigrostriatal dopaminergic pathway occurs in Parkinson’s disease. (Figure 1) This neurochemical deficiency explains many of the motor disturbances encountered in patients afflicted with this disease. However, the underlying cause of urinary dysfunction present in over half of patients with PD is poorly understood.(7, 8) The urinary symptoms experienced by patients with PD would lead one to hypothesize that DA modulates a normal micturition reflex, and thereby a lack of this neurotransmitter would cause a dysregulation of the voiding reflex. In fact, the micturition reflex is under the influences of dopamine receptors (both inhibitory D1 and...
facilitatory D2 receptors) and GABA (γ-aminobutyric acid) (inhibitory). The SNpc neuronal firing and the DA in the striatum seem to activate GABA release via the dopamine D1 receptor direct pathway. This inhibits the basal ganglia output nuclei, but also the micturition reflex via GABAergic collaterals to the PAG.(9) The disruption of this pathway in PD pathophysiology may be responsible for the DO and resultant LUTS.

A very interesting and elegant study by Kitta et al. tried to elucidate the role of extracellular DA, glutamate and GABA by in vivo microdialysis in the PAG during cystometry, as well as to evaluate the role of the dopaminergic system in the micturition reflex of an animal model of PD. DA and glutamate levels increased during micturition while GABA levels decreased. D1 receptor blockade in the PAG increased the GABA levels during micturition, while DA and glutamate levels were unaltered. D1 receptor antagonist also increased the maximal voiding pressure and decreased the intercontraction interval (ICI). D2 agonist did not cause any changes in cystometric parameters or microdialysis findings. The same experiments were performed in 6-OHDA lesioned animals, and they elicited decreased ICI. Glutamate levels increased during micturition as in sham animals, but the GABA increased in a similar fashion as with D1 receptor blockade. The authors concluded that the micturition-induced decrease of PAG extracellular GABA is prevented by D1 receptor antagonism and DA depletion in 6-OHDA lesioned rats. Therefore, absence in dopaminergic tone causes a dysfunction in GABA regulation and bladder hyperactivity.(9)
Although the dopaminergic deficiency likely plays a role in the accompanying LUTS, it is important to point out that PD involves other brain locations and neurotransmitters that can affect the micturition reflex.

**Brain imaging**

Evolving brain imaging technologies offer a unique opportunity to understand the complex structural and neurochemical basis for the development of LUTS in PD patients. Brain activity was monitored by blood flow in a positron emission tomography study in nine male patients with PD during bladder filling and emptying while being treated with levodopa or DA receptor agonist. DO during bladder filling was accompanied by activation of several brain regions such as cerebellar vermis, thalamus, PAG, putamen and insula.(10) As compared to younger healthy men, the pons and the anterior cingulate gyrus were not activated in men with PD during the storage phase.(11)

Winge et al. investigated the correlation between dopaminergic denervation and urinary symptoms in 14 PD patients using single-photon emission computerized tomography (SPECT) imaging of the DA transporter with [123I]-FP-CIT. Bladder symptoms and function were assessed with the Dan-PSS and a complete urodynamic examination. They did not demonstrate any significant direct correlation between Dan-PSS and DA transporter-receptor uptake measured with SPECT. However, patients with at least one urinary symptom stated in the questionnaire (Dan-PSS > 1, n = 11) had a significantly lower specific uptake of [123I]-FP-CIT in striatum. They observed a statistically significant correlation between the severity of urinary symptoms based on the Dan-PSS
and the relative degeneration of the caudate nucleus in the sub-group with prominent bladder symptoms (Dan-PSS > 10, n = 7). SPECT findings did not correlate with urodynamic parameters.\(^{(12)}\)

The most recent brain imaging studies demonstrate functional differences between cerebral structures in PD patients associated with DO. Studies involving more detailed analysis in a larger population are, however, required to make more solid conclusions regarding functional deficits in these patients.

**Pharmacotherapy**

The effects on voiding function of levodopa and DA agonists used for the treatment of motor symptoms in PD patients are not clearly understood, as studies conclude with conflicting results. The controversial clinical effects in patients may possibly be related to dose and time-dependent drug mechanisms. In animal studies, D1 and D2 receptor stimulation have different effects on the micturition reflex. Activation of D1 receptors in rats inhibits voiding, while D2 receptor stimulation activates the micturition reflex.\(^{(13)}\)

Uchiyama et al. studied the urodynamic changes caused by the administration of three different doses of apomorphine, a non-selective DA agonist, in rats. A single low-dose (0.01 mg/kg) of apomorphine initially decreased frequency and increased micturition volume (MV). Conversely, the medium (0.05 mg/kg) and high-dose (0.5 mg/kg) of apomorphine induced biphasic changes in frequency and MV. An initial rise in frequency was followed by a decrease, while the MV initially decreased only to rise thereafter. The
biphasic effects of apomorphine demonstrate its dose-dependent DA receptor selectivity.(14) These findings may reflect the conflicting results observed in human studies of anti-parkinsonian medications on bladder function.

Urodynamic evaluation was performed in 87 patients with mild idiopathic Parkinson’s disease and overactive bladder syndrome after acute carbidopa/levodopa (50/200 mg) administration. A subgroup of 70 patients underwent urodynamic evaluation after either the co-administration of D2 receptor antagonistic central and peripheral effects (l-sulpiride) with carbidopa/levodopa (50/200 mg) or the co-administration of a D2 receptor antagonist of peripheral effects (domperidone). Acute administration of levodopa worsened DO, while the co-administration of l-sulpiride reversed these findings. No changes were found in the domperidone group. The authors concluded that the deterioration of bladder function observed with levodopa is mediated through the D2 central receptor stimulation.(15)

Brusa et al. studied the effects of acute and chronic levodopa administration in DA-naïve patients with mild PD reporting urinary urgency. The first dose of levodopa significantly worsened the urodynamic parameters of bladder overactivity (neurogenic overactive detrusor contractions threshold [NDOC-t; 32% of worsening] and bladder capacity [BC; 22% of worsening]). A chronic administration of levodopa over two months improved the first sensation of bladder filling (FS; 120% of improvement), NDOCT-t (93% improvement), and BC (33% of improvement) compared to the values after acute
administration. The irritative symptoms on IPSS also significantly improved after chronic administration compared to the washout period (p < 0.0001).(16)

The urodynamic effect of a single dose of bromocriptine (DA agonist) and a single dose of levodopa were evaluated in eight PD patients. Urinary urgency aggravated in 50% of patients after levodopa and in 25% of patients after bromocriptine. DO was aggravated after both medication, but slightly more pronounced after levodopa. However, bromocriptine improved bladder emptying.(17)

Forty PD patients were submitted to the OAB-q, a voiding diary and urodynamic evaluation before and after antimuscarinic medication. Improvement in OAB-q scores were noted in 24/32 patients following antimuscarinic therapy, with a significant reduction in urinary urgency rate (46%) and of nocturnal micturition episodes. The presence of DO on urodynamic studies in all patients correlated with the OAB-q scores.(18)

The management of motor symptoms of PD consists of levodopa and DA agonists. However, the motor improvements are not always associated with LUTS improvement. Antimuscarinics can moderately alleviate them, but involve adding another drug to the regimen with associated side-effects. Recent studies demonstrate that D2 receptor activation cause deterioration of bladder function. Animal studies also show that pharmacoselectivity and pharmacokinetics of DA modulators can explain the inconsistent findings on bladder function in human studies. A clearer understanding of the implication
of chronic administration of these compounds on micturition will allow better pharmacological therapy strategies.

**Deep Brain Stimulation**

The overall unsatisfying results of pharmacotherapy for the treatment of motor symptoms in PD have led to the development of high frequency stimulation of the subthalamic nucleus (STN). When first studied in patients, it allowed the improvement of the main three motor symptoms, akinesia, rigidity and tremor, while reducing the administration of dopaminergic drugs and the related drug-induced dyskinesia. (19, 20) To investigate the possible application of this therapeutic modality in the treatment of bladder dysfunction in PD, Sakakibara et al. performed electrical stimulation and extracellular single unit recording in the STN during rhythmic isovolumetric bladder contractions in anesthetized adult male cats. They recorded tonic activity in a total of 10 neurons in the STN during urinary storage/micturition cycles. Electrical stimulation in the STN caused termination and subsequent inhibition of micturition reflex, concluding that STN may be involved in neural control of micturition. (21) The effect of deep brain stimulation (DBS) of the STN were investigated in 16 PD patients using questionnaires (International Prostate Symptom Score and Dan-PSS) and urodynamic assessment. A clinical improvement of the motor symptoms using UPDRS rating was observed following electrode placement. The total score of each urinary symptom questionnaires was not significantly changed 3 and 6 months after electrode placement. However, the storage (irritative) bladder symptoms score (IPSS irritative) after implantation and stimulation in the STN were significantly
decreased. Similar results were found with the Dan-PSS score. The urodynamic parameter did not significantly change after surgery.\(^{(22)}\)

Deficient perception of sensory information occurs in PD and can manifest in visceral pathways such as the sensation of bladder filling.\(^{(23)}\) Herzog et al. sought to clarify the influence of STN-DBS on sensory processing in PD patients by investigating the sensation of bladder filling using PET and concomitant urodynamic evaluation. Increased regional cerebral blood flow was observed in the PAG, the posterior thalamus, the insular cortex as well as in the right frontal cortex and the cerebellum bilaterally during bladder filling. The posterior thalamus and the insular cortex interacted more during the ON state of the STN-DBS as compared to the OFF state. This neural activity was also modulated by the PAG during the ON state of the STN-DBS. Therefore, it is postulated that STN-DBS enhances the processing of afferent urinary bladder information.\(^{(24)}\)

Fritsche et al. described the case report of two male patients who developed urinary retention following urethral catheter removal two days after undergoing STN-DBS. They had a significant improvement postoperatively in their motor symptoms. Both patients denied desire to void after intravesical instillation of 600 ml of saline. Due to persistent urinary retention, they had a suprapubic catheter inserted for a period of 8 and 12 weeks, after which sensory function and detrusor motility recovered spontaneously. The authors hypothesize that this adverse effect may be caused by the previously reported STN-DBS modulation of afferent bladder information.\(^{(24, 25)}\) This adverse reaction could possibly be explained by the inhibition of the micturition reflex by HFS-STN observed in cats.\(^{(21)}\)
This therapeutic modality was found to be very effective for the treatment of motor symptoms in certain patients with parkinsonism. Patients with manifestation of parkinsonism may have variable underlying neurological etiologies and affected pathways. Combining this technique with advanced brain imaging may provide a clearer understanding of the circuitry involved in micturition in a normal or diseased state, and therefore a more targeted approach to the treatment of urinary symptoms.

**Other therapies**

Without a clear understanding of the underlying pathophysiological and neurochemical mechanism of LUTS associated with PD, treatment strategies are mainly empirical in nature. Several other therapies have been studied for the treatment of bladder dysfunction in PD patients. A 2-week course of low frequency 1 Hz repetitive transcranial magnetic stimulation was given to 8 patients with idiopathic PD and stable urinary symptoms. Their changes in urinary function were then assessed with urodynamic evaluation and IPSS questionnaire. They recorded an immediate significant improvement in IPSS score irritative symptoms that persisted until 2 weeks after the cessation of treatment. First sensation at bladder filling and BC significantly increased following repetitive transcranial magnetic stimulation.(26) The mechanism of action of this treatment effect is yet to be understood.

A study by Kulaksizoglu and Parman aimed to evaluate the effects of intravesical injection of Botulinum toxin for the treatment of LUTS in PD patients. After injecting
500 units of botulinum toxin-A in 16 patients, transient improvement in functional bladder capacity were observed at 3, 6 and 9 months. These improvements were also noted in the number of incontinence episodes per 24 hours.(27) Giannantoni et al. also observed improvement in urinary frequency and quality of life in four PD patients who received 200 units of botulinum Toxin-A injection in the bladder.(28)

Posterior tibial nerve stimulation (PTNS) is another therapy that was investigated for the treatment of storage symptoms in PD patients. Urodynamic evaluation was performed before and during PTNS. Mean first involuntary detrusor contraction (145.2 ± 41.1 [55–265] ml) and mean cystometric capacity (204.8 ± 40.5 [115–320] ml) both significantly increased during PTNS (p = 0.001), respectively 244.7 ± 51.7 (145–390) ml and 301.2 ± 51.5 (230–395) ml. PTNS also improved the findings of pseudo-dyssynergia in 5 of the 7 patients.(29)

Although their mechanism of action are not fully understood, these therapies have been used to successfully treat patients with both idiopathic and neurogenic detrusor overactivity (NDO) of many different etiologies. It is therefore reasonable to expect a response in patients with PD who suffer from a subtype of NDO, as demonstrated by these studies.

**Animal models of PD**

In order to adequately explore the pathogenesis and evaluate potential therapeutic options, animal models of PD have been developed with either administration of
neurotoxins or genetic modifications. Experimental PD can be induced in animal models using several different methods. In a meta-analysis of over 250 studies, ten different models were used, with the most common being 6-hydroxydopamine (6-OHDA) striatal lesioning in 136 publications, followed by MPTP (105 publications) and reserpine (22 publications).(30) Lesions can be done unilaterally or bilaterally and can be performed in the striatum or the medial forebrain bundle (MFB) or the substantia nigra. In the same systematic review, the neurobehavioural outcome included motor activity, spontaneous activity, rotational behaviour, balance and gait. 6-OHDA is a neurotoxin that causes mixed neuronal cell death by e.g., activation of the intrinsic pathway of apoptotic programmed cell death through caspases.

Animal models of the PD-related motor disturbances have been widely developed and investigated, by creating lesions in the nigrostriatal dopaminergic pathway. (Figure 1) However, the other autonomic symptoms including voiding dysfunction do not have established models, and therefore have not been explored in this arena. Based on the animal models of PD looking at neurobehavioral changes, we developed an animal model of PD that demonstrated measurable changes in micturition. Female Sprague-Dawley rats underwent a unilateral MFB with the neurotoxin 6-OHDA. (Figure 2, 3)
Different animal groups were then examined at multiple time points with cystometry where bladder pressure and volume voided are measured while the animals are awake in metabolic cages. Our group has developed and demonstrated urodynamic changes indicative of DO, with higher bladder pressures and micturition frequencies, lasting up until 28 days. (31) (Figure 4)
Stem cell application

Cell replacement therapy can offer a restorative and regenerative approach to neurodegenerative disorders. Most animal and clinical human studies of stem cell application for PD are aimed at improving motor symptoms. The first open-label transplantation of allogeneic fetal tissue transplant showed promising results, but the more recent double-blinded trials did not show improvement in the primary outcome of motor symptoms, however, demonstrating that dopamine neurons survived and grew (32). Intrastriatal grafts of human fetal ventral mesencephalic tissue can reinnervate the striatum and provide lasting symptomatic relief in some patients, but the results have been inconsistent. (33) A clinical trial using bilateral fetal nigral transplantation into the postcommissural putamen revealed no significant overall treatment effect for the primary
end point (change between the score at baseline and final visits in the motor component of the UPDRS in the practically defined off state). Adverse events of urinary incontinence were reported in two patients in the one donor/side, and in two patients in the four donor/side group, but none in the placebo group. Similarly, urinary frequency was reported in one patient in the one donor/side, and in two patients in the four donor/side, but none in the placebo group.(34)

Human embryonic stem cells can be geared towards differentiation into dopaminergic neurons using several morphogens. Yet, multiple ethical issues arise from the use of fetal tissue to harvest those cells for clinical investigation in PD patients.

**Mesenchymal stromal cells**

Another possible therapeutic avenue that brings less ethical concern is the use of somatic mesenchymal stromal cells. Mesenchymal stromal cells have the ability to differentiate into different mesenchymal and non-mesenchymal cell lineage without being teratogenic (35). Progressive functional motor improvements were demonstrated from 4 weeks to 3 months following transplantation of human BMSC in a hemiparkisonian rat model (36). Unilateral transplantation of autologous bone marrow mesenchymal stem cells (BMSC) in an open-labeled clinical study showed significant improvements in the Unified Parkinson’s Disease Rating Scale (UPDRS) and symptoms such as gait and facial expression in three out seven PD patients at a follow-up of 10 to 36 months (37). There were no adverse events, but graft survival or improvement of dopaminergic striatal function was not assessed.
In order to investigate the effects of stem cell implantation for LUTS, we evaluated the effect of cell therapy with human amniotic fluid stem cells and bone marrow derived mesenchymal stem cells on bladder dysfunction using that PD model. We also studied some general markers of (anti)inflammation, oxidative stress and neurogenesis to determine the direction of future studies.

A nigrostriatal lesion was induced by 6-hydroxydopamine in 96 athymic nude female rats divided into 3 treatment groups. After 2 weeks, the groups were injected with human amniotic fluid stem cells, bone marrow derived mesenchymal stem cells and vehicle for sham treatment, respectively. At 3, 7, 14 and 28 days the bladder function of 8 rats per group was analyzed by conscious cystometry. Brains were extracted for immunostaining.

The nigrostriatal lesion caused bladder dysfunction, which was consistent in sham treated animals throughout the study. Several cystometric parameters improved 14 days after human amniotic fluid stem cell or bone marrow derived mesenchymal stem cell injection, concomitant with the presence of human stem cells in the brain. (38) (Figure 5, Appendix I) At 14 days only a few cells could be observed, in a more caudal and lateral position. After 28 days, the functional improvement subsided and human stem cells were no longer seen. Human stem cell injection improved the survival of dopaminergic neurons until 14 days. Human stem cells expressed superoxide dismutase-2 and seemed to modulate the expression of interleukin-6 and glial cell-derived neurotrophic factor by host cells. (Figure 6)

Cell therapy with human amniotic fluid stem cells and bone marrow derived mesenchymal stem cells temporarily ameliorated bladder dysfunction in a Parkinson
disease model. In contrast to integration, cells may act on the injured environment via cell signaling. Our demonstrated transient urodynamic improvements may be due to cell migration or cell death, as the transplanted cells were sparse at 14 days and absent at 28 days. Lasting improvements may be achieved by prolonging cell survival and preventing migration. Therefore, we decided to change our approach on three different aspects. First, we would use allogeneic BMSC, namely Sprague-Dawley rat-derived cells to avoid potential immunological destruction. Second, we would inject the cells in the SN, which may be less hostile to the cells, and avoiding the lesion site. Considering that our lesion is in the MFB and therefore primarily causes the death of SN neurons, our goal should be to promote the regeneration of SN neurons. And third, we transplanted microencapsulated cells which allows nutrients and growth factors to transit back and forth and avoid migration or cell death. Our goal was to demonstrate longer lasting cystometric improvements in our animal model.

Figure 5
Expression of SOD-2–immunofluorescence. Red is SOD-2, blue is DAPI (nuclei) and green is GFP-labeled human stem cells. Orange/yellow represents co-localization (red+green). A,D and G: some expression of SOD-2 by the rat brain cells was seen in a uniform distribution at day 3, 7 and 14 in sham animals. B,E and H: Expression of SOD-2 was seen in the hAFS cells and in the surrounding rat brain cells at days 3 and 7. At day 14 the hAFS cells no longer expressed SOD-2 . C,F and I: hBM-MSC expressed SOD-2 at days 3 and 7, but did not at day 14. Rat brain cells surrounding the hSC expressed SOD-2. Representative pictures of the 28 day endpoint are not presented, because no hSC were observed at this point and no changes in SOD-2 expression was seen.
Ultimately, continued research in this field strives to discover a possible cure and regeneration of involved structures that would therefore improve motor and non-motor symptoms together.

**Summary and future directions**

LUTS in patients with PD greatly decreases their QOL and does not respond to pharmacological therapeutics. Significant advancement in the understanding of pathophysiology of bladder dysfunction in PD has been witnessed in the past decade of research. There is a clear associated higher prevalence of LUTS in this patient population, but the causality has not yet been fully explained. Pharmacotherapy using levodopa and DA agonist allows limited relief of LUTS in PD patients, while antimuscarinics add another drug to their regimen with their associated side-effects. STN-DBS can offer additional improvements for both LUTS and motor symptoms. However,
no therapeutic modality currently can offer a chance for cure. A better appreciation of the neurochemical mechanism of LUTS associated with PD and ongoing development of stem cell therapy may lead to a more targeted approach for each patient suffering from LUTS associated with PD. Lasting improvements may be achieved by prolonging cell survival and preventing migration. We have attempted to induce longer lasting urodynamic effects in an animal model of Parkinson’s disease by transplanting allogeneic rat bone marrow stem cells (rBMSC) in the SNpc, and by microencapsulating them in an alginate construct to avoid migration and cell death by immunological attack. A better appreciation of the neurochemical mechanisms of LUTS associated with PD and optimization of stem cell therapy in PD by increasing the survival and effect of implanted allogeneic BMSC in an animal model may provide translational information. This could be useful for the development of targeted approach for individual patients suffering from LUTS associated with PD.
References:


CHAPTER 3

Effects of allogeneic bone marrow-derived mesenchymal stromal cell therapy on voiding function in a rat model of Parkinson’s disease

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The following chapter has been submitted to Journal of Urology

Literature citations and figure formats are adherent to the guidelines set forth in Journal of Urology

Lysanne Campeau is responsible for almost all data collection and analysis.
Abstract

Introduction: Cellular therapy induced transient urodynamic improvement in a rat model of Parkinson’s disease where bladder dysfunction was demonstrated after unilateral injection of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle (MFB). We attempted to prolong the effect by injecting allogeneic rat bone marrow mesenchymal stromal cells before (rBMSC) and after microencapsulation (ErBMSC) into the substantia nigra pars compacta (SNpc).

Methods: Female rats underwent a unilateral stereotactic injection of 6-OHDA in the MFB. Treatment injection was performed in the ipsilateral SNpc of vehicle alone, or with either rBMSC or ErBMSC. Animals were evaluated by cystometry at four different time points after treatment: 7, 14, 28, and 42 days. Brains were extracted for immunostaining.

Results: At 42 days, the rBMSC group had lower threshold pressure (TP), intermicturition pressure, spontaneous activity (SA) and area under the curve than vehicle treated animals. The ErBMSC animals had lower TP at 28 days and lower SA at 42 days compared to vehicle treated animals. ErBMSC and rBMSC were demonstrated in the SNpc up to 42 days following transplantation. At 42 days, tyrosine hydroxylase (TH) positive neurons were more numerous in the SNpc of rBMSC, followed by ErBMSC, and vehicle treated animals.

Conclusion: Urodynamic effects of the 6-OHDA lesion persisted up to 42 days after vehicle injection. Transplantation of rBMSC improved urodynamic pressures at 42 days after treatment more markedly than ErBMSC. This was associated with a higher number
of TH-positive neurons in the treated SNpc of rBMSC animals, suggesting that functional improvements require a juxtacrine effect.
Introduction

Parkinson’s disease (PD), the most common neurodegenerative disorder after Alzheimer’s disease, is caused by neurodegeneration of the nigrostriatal dopaminergic pathway. Dopamine deficiency leads to cardinal motor abnormalities that include tremor at rest, rigidity, bradykinesia, and axial symptoms. Autonomic symptoms such as lower urinary tract symptoms (LUTS) become more prominent with disease progression and significantly impair quality of life. Urinary disturbances are present in 38-71% of PD patients, the most common symptoms being nocturia and urgency. Voiding dysfunction does not respond well to dopamine replacement for treatment of motor symptoms, and current treatment modalities provide only symptomatic relief without offering neuroprotection or neurorestoration. Neurotrophic factor delivery and stem cell therapy have progressed from preclinical to clinical trials. While the first open-label transplantation of fetal tissue transplant showed promising results, somatic mesenchymal stromal cells can be more easily obtained and are more generally ethically acceptable.

Our previous work revealed that we can reproduce urodynamic abnormalities, similar to those seen in humans, in a rat model of PD. Transplantation of human bone marrow-derived stromal cells (BMSC) significantly improved urodynamic parameters 14 days after treatment, but improvements did not persist at 28 days. Injected cells were sparse and with altered morphology 14 days after, and disappeared after 28 days. In this study, using the same model, we tried to prolong the urodynamic effects by transplanting
allogeneic rat bone marrow mesenchymal stromal cells without (rBMSC) and with microencapsulation (ErBMSC). Cells were injected into the substantia nigra pars compacta (SNpc) to initiate an endogenous process of regeneration of damaged neurons.
Methods

Design

Female Sprague Dawley rats (weight: 150-230 g) were separated into two treatment groups (40 animals each) and one sham treatment group (20 animals). Experiments were approved by the local Animal Care and Use Committee and conducted in accordance with the National Research Council publication Guide for Care and Use of Laboratory Animals. Animals were housed in pairs, and then individually after surgery, in a 12 hour light-dark cycle at Wake Forest University Health Sciences. Food and water were available ad libitum. Rats first underwent a stereotactic procedure to produce a unilateral MFB lesion by injecting 8µg of 6-hydroxydopamine (6-OHDA) in 4µl of saline in the right MFB. Two weeks after the lesion, the animals underwent an ipsilateral treatment: Group A received a saline injection (sham control), Group B received an injection of rBMSC, and Group C received an injection of ErBMSC.

The time points of observation were 7, 14, 28 and 42 days after treatment. At each time point, cystometry was performed three days after inserting a suprapubic bladder catheter. After cystometry, the animals were euthanized with transcardial formaldehyde perfusion under isoflurane. Brains were extracted and processed for immunohistochemistry.
Stem cell preparation

The rBMSC were obtained from a commercial source (Gibco®, Invitrogen, Grand Island NY USA). They were expanded in Dulbecco’s Modified Eagle Medium (1X), low glucose with GlutaMAX™-I (Invitrogen, Grand Island NY USA), with 10% MSC Qualified Fetal Bovine Serum (Invitrogen, Grand Island NY USA) and 1% gentamicin (Invitrogen, Grand Island NY USA). A subgroup of the rBMSC were infected at fifth passage with an enhanced green fluorescent protein (EGFP) tagged lentivirus, created from the Addgene plasmid 12257.

Flow cytometry

After expansion, non-infected cells were harvested using TrypLE™ Express Dissociation Enzyme (Invitrogen, Grand Island NY USA) at sixth passage. A total of $2 \times 10^6$ cells were exposed for each antibody in the dark for 10 minutes, then washed three times with cold Hank's balanced salt solution (HBSS) by centrifugation at 400 x g for two minutes at room temperature. Expression of the corresponding cell surface proteins were assayed by a flow cytometer for CD90 (Anti-CD90 Mouse monoclonal IgG2a, FITC, 1:100, eBioscience), CD34 (Anti-CD34 Mouse monoclonal IgG1, AF488, 1:5, Santa Cruz) and CD45 (Anti-CD45 Mouse monoclonal IgG1, FITC, 1:50, BD). Our secondary antibodies were mouse monoclonal against IgG1 and IgG2a (Anti-IgG1 and Anti-IgG2a, FITC, 1:50, eBioscience). Fluorescence-activated cell sorting confirmed and selected cells expressing EGFP.
Cell microencapsulation

A portion of the rBMSCs was microencapsulated on the day of injection using a modification of a procedure previously described by Darrabie et al. 8 Briefly, rBMSCs were suspended in low viscosity (20-200 mPa-s) ultra-pure sodium alginate with high mannuronic acid (LVM) content (Nova-Matrix Sandvika, Norway) at a concentration of approximately 40,000 cells/µl. LVM alginate is reported to have molecular weights 75-200kDa and G/M ratios of ≤1 by the manufacturer. Solutions for alginate microcapsules were made using HEPES and CaCl2 (Acros, Geel, Belgium). Using 1.5% LVM alginate containing rBMSCs, microspheres of about 100 μm in diameter were extruded through a 3D-microfluidic device 9 at a flow rate of 0.1 ml/minute with an air jacket pressure of 4 psi. Microspheres were received into a dish containing 100 mM CaCl2 solution, and cross-linked for 15 minutes in this solution before being centrifuged at 250 x g for 2 minutes. The supernatant was replaced with HBSS (with calcium) and centrifuged again at 250 x g for 2 minutes, and repeated three times. Microcapsules were stained with live/dead cell reduced biohazard assay (Gibco®, Invitrogen, Grand Island NY USA) to assess viability following encapsulation.

The Parkinsonian animal model

Using a stereotactic frame, three-dimensional coordinates targeting the MFB (coordinates anteroposterior −0.4, mediolateral −1.6 and dorsoventral −7 from the bregma) were used
based on published data for rats.\textsuperscript{6, 7, 10} Following a midline incision exposing the bregma, as a starting landmark for the coordinates, we injected 8 µg of 6-OHDA in 4 µl of saline in the right MFB.

\textit{Unilateral treatment injection}

Two weeks after the lesion, 4 µl of the rBMSC suspension containing approximately 100,000 cells was injected in a similar fashion into the right SNpc (coordinates anteroposterior −5, mediolateral −1.6 and dorsoventral −8.2 from the bregma), at a rate of 1 µl/minute \textsuperscript{7, 10, 11}. The second group of rats received an injection of 100,000 ErBMSC in an alginate construct within a comparable volume. The sham treated animals underwent the same procedure, using 4 µl of saline.

\textit{Cystometry}

Cystometric investigations were performed without anesthesia three days after bladder catheterization as previously described.\textsuperscript{6} The following cystometric parameters were investigated: maximum pressure (MP) (maximum bladder pressure during a micturition cycle), threshold pressure (TP) (pressure at initiation of voiding contraction), basal pressure (BP) (lowest pressure in between voids), intermicturition pressure (IP) (average calculated pressure between voids), spontaneous activity (SA) (IP minus BP), intercontraction intervals (ICI), bladder capacity (BC), micturition volume (MV) (volume of the expelled urine), and residual volume (RV) (BC minus MV), area under the curve.
(AUC) as a proxy for non-voiding contractions. AUC is a calculated integral that correlates with overall bladder pressures between voiding contractions and has no particular threshold.

*Free-floating immunohistochemistry*

Rats were perfused intracardially with 4% formaldehyde before decapitation under isoflurane inhalation anesthesia. Free-floating brain sections (40µm) were incubated overnight with specific primary antibodies against tyrosine hydroxylase (TH; 1:1000; Abcam), a marker of dopaminergic and noradrenergic neurons, nestin (1:500; Millipore), a marker of neuroprogenitor cells, ionized calcium binding adaptor molecule 1 (IBA-1; 1:1,000; Wako chemicals), a marker of microglia and glial fibrillary acidic protein (GFAP; 1:1,000; Dako), a marker of astrocytes. Sections were incubated with biotinylated or Alexa Fluor® 594 labeled secondary antibodies (1:200; Invitrogen) for 2 hours at room temperature. Sections were mounted on slides and counterstained with either hematoxylin (TH) or DRAQ5. Images were obtained for the mounted sections by confocal microscopy.

*Analysis*

For the final time-point (42 days), TH positive neurons in the outlined SNpc were counted using Stereo Investigator System (MicroBrightfield, Williston, Vermont) every fourth section, for a minimum of three sections, and expressed as the percentage of TH-
positive neurons in the lesioned SNpc with respect to the contralateral, intact side. Comparisons among the groups were carried out by performing One Way ANOVA, followed by Bonferroni, Tukey and Newman-Keuls posthoc corrections.

Cystometric variables were compared between the different groups at different time points using two-way ANOVA followed by the Bonferroni, Tukey and Sidak posthoc corrections, using IBM SPSS Statistics 19 software. All values are expressed as the mean ± standard error of the mean (SEM). A probability of $p < 0.05$ was considered significant. Immunohistochemistry findings were qualitatively assessed and compared between each group and time-points.
Results

_In vitro rBMSC characterization and encapsulation_

Flow cytometry showed cells to be positive for CD90 (100%) and negative for CD34 (0.5%) and CD45 (0.9%). We confirmed EGFP lentiviral infection of rBMSC using fluorescent activated cell sorting enrichment and selected for treatment injection. In all events, 90.4% were confirmed rBMSC and 89.8% were EGFP positive. Following encapsulation, cellular viability of 85% was given by analysis of green (live) and red (dead) channels via Image J software in the live/dead cell reduced biohazard assay after confocal microscopy. (Figure 1)
Functional evaluation

A total of 100 animals underwent the 6-OHDA lesion, with four animals dying in the postoperative period. Three animals were excluded from the study due to macroscopic hematuria and dribbling voiding, suggesting bladder catheter complications during cystometry. Body weights were only statistically different at 42 days between the vehicle and both rBMSC and ErBMSC. (Table 1)

Table 1 Body weight in Vehicle treatment, rBMSC and ErBMSC at all time-points

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
<td></td>
</tr>
<tr>
<td>Vehicle (n=5)</td>
<td>234.2 ± 10.2</td>
</tr>
<tr>
<td>rBMSC (n=9)</td>
<td>224.3 ± 16.2</td>
</tr>
<tr>
<td>ErBMSC (n=8)</td>
<td>214.7 ± 12.8</td>
</tr>
<tr>
<td>14 days</td>
<td></td>
</tr>
<tr>
<td>Vehicle 14 days (n=5)</td>
<td>239.6 ± 12.3</td>
</tr>
<tr>
<td>rBMSC 14 days (n=9)</td>
<td>236.6 ± 11.3</td>
</tr>
<tr>
<td>ErBMSC 14 days (n=10)</td>
<td>230.4 ± 13.1</td>
</tr>
<tr>
<td>28 days</td>
<td></td>
</tr>
<tr>
<td>Vehicle 28 days (n=4)</td>
<td>258.1 ± 34.4</td>
</tr>
<tr>
<td>rBMSC 28 days (n=10)</td>
<td>237.6 ± 21.9</td>
</tr>
<tr>
<td>ErBMSC 28 days (n=10)</td>
<td>265.3 ± 21.6</td>
</tr>
<tr>
<td>42 days</td>
<td></td>
</tr>
<tr>
<td>Vehicle 42 days (n=4)</td>
<td>295.8 ± 22.9 *</td>
</tr>
<tr>
<td>rBMSC 42 days (n=9)</td>
<td>251.6 ± 13.6</td>
</tr>
<tr>
<td>ErBMSC 42 days (n=10)</td>
<td>252.7 ± 23.1</td>
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</tbody>
</table>

* p < 0.05 Between Vehicle and rBMSC, between Vehicle and ErBMSC
When pooling all time-points together for each treatment group, BC was significantly lower in the ErBMSC animals than both vehicle treatment (0.64 ± 0.05 vs 0.93 ± 0.07 ml, p<0.05) and rBMSC treatment (0.64 ± 0.05 vs 0.87 ± 0.05 ml, p<0.05). Also, the overall RV of the ErBMSC animals for all time-points was significantly lower than the rBMSC animals (0.08 ± 0.02 vs 0.16 ± 0.02 ml, p<0.05).

At 7 days MV was lower in ErBMSC animals compared to rBMSC animals (0.45±0.06 vs 0.93±0.10 ml, p<0.01).

At 14 days MV was lower in both rBMSC and ErBMSC as compared to vehicle treated animals (rBMSC vs vehicle: 0.71±0.08 vs 1.28±0.17 ml, p<0.01; ErBMSC vs vehicle: 0.75±0.09 vs 1.28±0.17 ml, p<0.05).

At 28 days, ErBMSC treatment significantly decreased the TP compared to vehicle treatment (20.35±3.40 vs 43.84±6.00 cm H2O, p<0.05).

At 42 days (Figure 2), rBMSC treatment significantly decreased TP, IP, SA and AUC compared to vehicle treatment (TP: 16.65±2.47 vs 37.10±8.22 cm H2O, p<0.05; IP: 11.70±2.52 vs 39.56±10.07 cm H2O, p<0.01; SA: 3.57±0.87 vs 22.43±10.59 cm H2O, p<0.01; AUC: 12.46±2.64 vs 39.91±9.44, p<0.01). Similarly, ErBMSC treatment significantly decreased SA compared to vehicle treatment (7.71±1.77 vs 22.43±10.59 cm H2O, p<0.05), but also decreased their MV (0.52±0.09 vs 1.04±0.14 mL, p<0.05). The animals treated with rBMSC alone had a lower BP compared to ErBMSC treated animals (8.13±1.77 vs 17.16±2.55 cm H2O, p<0.05). (Table 2, Figure 3)
Figure 2
Representative urodynamic tracings 42 days after injection of vehicle (A), rBMSC alone (B) and ErBMSC (C). Upper curves indicate bladder pressure. Lower curve indicate micturition volume. Pressure and volume curve scale is the same in all groups.

Figure 3
Cystometric data at 7, 14, 28 and 42 days. Values expressed as mean ± SEM parameters in sham treated animals, rBMSC treated animals, and microencapsulated rBMSC (ErBMSC) treated animals.

Figure 3 Legend
* p < 0.05 between sham and rBMSC
** p < 0.01 between sham and rBMSC
† p < 0.05 between rBMSC and ErBMSC
‡ p < 0.05 between sham and ErBMSC
Brain histomorphology and cell survival

The rBMSC were present up to 42 days following the treatment injection and did not migrate, but decreased in number over time. The site of injection of the ErBMSC within the right SNpc were marked with small cavities lined with EGFP positive cells, that became smaller with increasing time points, as were the cell number surrounding them. (Figure 4)

Brain immunohistochemistry

The percentage of TH-positive cells in the lesioned SNpc was significantly different between groups, with the highest in rBMSC (n=5; 89.0 ± 1.7 %), followed by ErBMSC (n=5; 71.6 ± 1.6 %), then vehicle treated animals (n=3; 61.5 ± 3.2 %). (Figure 5) Minimal nestin immunoreactivity was identified around the site of saline injection at all time points but with decreasing intensity. Host cells surrounding injected rBMSC strongly expressed nestin at 7 and 14 days, with decreasing
expression at 28 and 42 days. Host cells surrounding the graft site of the ErBMSC also expressed nestin, but with less intensity than those surrounding rBMSC. (Figure 6)
GFAP expression was present around the graft site of rBMSC and ErBMSC at all time-points, with decreasing intensity at later time-points, indicating the presence of activated astrocytes around graft sites. Astrocytic presence was not as conspicuous at the saline injection site. (Figure 7)

<table>
<thead>
<tr>
<th>Sham</th>
<th>rBMSC</th>
<th>ErBMSC</th>
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<tbody>
<tr>
<td>A</td>
<td>B</td>
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*Figure 7*
Representative confocal images of right SNpc at 200x magnification following injection of vehicle (7 days, A; 14 days, D; 28 days, G; 42 days, J), rBMSC alone (7 days, B; 14 days, E; 28 days, H; 42 days, K) and ErBMSC (7 days, C; 14 days, F; 28 days, I; 42 days, L) at different time points. Brain slices were examined by immunohistological staining using an antibody against GFAP (red) The EGFP+ cells are seen in green and the DRAQ5 stained nucleus in blue. GFAP is expressed in all treatment groups with decreasing intensity over time.

IBA-1 expression was demonstrated at the injection site in all three groups at 7 and 14 days. Microglia infiltration was present around the injected rBMSC and ErBMSC. IBA-1 expression was no longer present at 28 and 42 days in all groups. (Figure 8 and 9)
Figure 8
Representative confocal images of right SNpc at 200x magnification following injection of vehicle (7 days, A; 14 days, D; 28 days, G; 42 days, J), rBMSC alone (7 days, B; 14 days, E; 28 days, H; 42 days, K) and ErBMSC (7 days, C; 14 days, F; 28 days, I; 42 days, L) at different time points. Brain slices were examined by immunohistological staining using an antibody against IBA-1 (red) The EGFP+ cells are seen in green and the DRAQ5 stained nucleus in blue. IBA-1 is expressed in all treatment groups with decreasing intensity over time.
Figure 9
Representative confocal images of right SNpc at 400x magnification following injection of vehicle (7 days, A; 14 days, D; 28 days, G; 42 days, J), rBMSC alone (7 days, B; 14 days, E; 28 days, H; 42 days, K) and ErBMSC (7 days, C; 14 days, F; 28 days, I; 42 days, L) at different time points. Brain slices were examined by immunohistological staining using an antibody against IBA-1 (red) The EGFP+ cells are seen in green and the DRAQ5 stained nucleus in blue. IBA-1 is expressed in all treatment groups with decreasing intensity over time.
Discussion

We attempted to prolong the voiding function improvement seen in our previous investigation of a rat model of Parkinson’s disease by changing our approach in several ways. We first selected to transplant allogeneic rBMSC instead of a xenogenic cells, and then transplanted them in the SNpc as a potentially less hostile site. Lastly, we microencapsulated cells to allow a paracrine effect while avoiding destruction or migration. We demonstrated that: 1) the unilateral MFB 6-OHDA lesion induced persistent urodynamic effects for up to 8 weeks; 2) the injection of both rBMSC and ErBMSC produced urodynamic pressure improvements compared to vehicle treated animals (rBMSC better than ErBMSC); 3) rBMSC survival decreased over time, but cells were still present 6 weeks after injection and did not migrate; 4) the lesioned SNpc of rBMSC had a greater percentage of TH-positive neurons in respect to the intact hemisphere, compared to ErBMSC and vehicle-treated animals 5) host cells surrounding rBMSC expressed more markedly a neural stem cell marker, nestin, at earlier time-points; while microglia and astrocytic infiltration was present in all three groups and decreased over time.

Our study was designed to study functional urodynamic parameters following 6-OHDA lesion and transplantation of rBMSC and ErBMSC, and therefore we did not perform motor or other behavioral assessments. We found characteristic urodynamic changes in our vehicle treated animals at all time points for up to 8 weeks. Functional improvements were conspicuous at 42 days following treatment injection, with rBMSC treated animals
having lower TP, IP, SA and AUC than vehicle treated animals. It may be speculated that higher pressures during micturition (TP) in vehicle treated animals could be related to a component of sphincter bradykinesia, similar to what is observed in patients with PD. However, our model and study design does not allow examination of sphincter effects. If decreases in detrusor pressures during (TP) and in between voids (IP, SA) can be translated to patient scenario, they may be clinically relevant since the therapeutic goal in management of neurogenic voiding dysfunction is to decrease detrusor pressures while maintaining emptying efficacy.\textsuperscript{12} Provided that SA is considered clinically analogous to involuntary detrusor contractions and/or urgency episodes\textsuperscript{13}, the lower SA in rBMSC treated animals could be translated into quality of life improvement due to a decrease in urgency and urgency incontinence episodes seen with detrusor overactivity.\textsuperscript{14} However, translation of these experimental findings to clinical reality should be done with caution.

The delay of 4 to 6 weeks before demonstrable improvements may be explained by a required regeneration or restorative period, initiated by rBMSC injection. Behavioral improvements following transplantation of human embryonic stem cells in 6-OHDA lesioned animals have been observed with most significant results at 6 and 8 weeks.\textsuperscript{15, 16} Although we cannot determine if this response would be sustained longer as our study was not designed for further time-points, our findings indicate that allogeneic rBMSC injection in the SNpc provides more delayed urodynamic improvements than those obtained with human BMSC injected in the MFB of the same animal model.\textsuperscript{7} We demonstrated prolonged survival of rBMSC up to 6 weeks following transplantation. Allogeneic mesenchymal stromal cells may immunologically be better tolerated than
human cells due to their immunosuppressive capabilities.\textsuperscript{17} The site of injection likely influences cell survival. Injection into the brain somewhat isolates the cells from serum complement components involved in the immunological response.\textsuperscript{18} Transplantation of the rBMSC in closer proximity to cell bodies in the SNpc that underwent apoptosis following 6-OHDA lesion, avoids the injury site in the MFB. The rBMSC seemed not to integrate or mature within the brain, although we did not specifically intend to study these mechanisms. However, functional improvements may be caused by juxtacrine factors promoting neurogenesis.\textsuperscript{19} Stem cell transplantation can stimulate endogenous neural stem/precursor cells-induced neurogenesis, causing a “niche” effect.\textsuperscript{20, 21} The higher percentage of TH-positive neurons in animals injected with rBMSC in the lesioned SNpc in respect to the intact side, may correspond to a recovery or regeneration of lesioned dopaminergic cells. The ratio of TH-positive neurons in vehicle-treated animals is similar to findings in the literature several weeks after a lesion.\textsuperscript{22} Other studies have described a similar recovery in dopaminergic cells within the SNpc after BMSC transplantation into animal models, postulating a prevention of progressive neuronal death through neurotrophic factors.\textsuperscript{23, 24} Although our primary goal was to demonstrate physiological differences in voiding function of treated animals, we also attempted to explain functional effects between groups by studying distinct immunohistochemical brain stainings that would illustrate the role of a neuroproliferative (nestin) and glial (IBA-1 and GFAP) reaction. The nestin expression pattern suggests that rBMSC recruit surrounding cells with neural stem cell properties. Activated astrocytes can also express nestin in the graft vicinity.\textsuperscript{25} Nestin is an intermediate filament protein important for survival and self-renewal of
neuroprogenitor cells, and can track their migration and differentiation. The increased nestin expression in host cells surrounding rBMSC suggests that cell-to-cell apposition is necessary to induce host neurogenesis, as compared to the mere presence of cells within alginate constructs. According to GFAP and IBA-1 staining, microglia and astrocytic infiltration was similar across all groups, and may not explain functional differences observed. Although we can only speculate on the mechanism of action of rBMSC transplantation on differences in cystometric parameters, they may induce a neuroprotective and neuroregenerative effect on host cells via a juxtacrine interaction and thereby normalize neural control of micturition. Also, free-floating immunohistochemistry could not allow visualization of microcapsule morphology ex vivo, as PBS dissolved the alginate construct. However, injection of microcapsules generated small cavities within the SNpc that may be responsible for functional differences.
Conclusion

We confirmed persistent urodynaminc effects of the 6-OHDA lesion up to 42 days after vehicle injection. Transplantation of rBMSC alone improved urodynamic pressures at 42 days after treatment more markedly than ErBMSC. This marked improvement in the rBMSC alone animals was associated with a higher number of TH-positive neurons in the treated SNpc, suggesting that functional improvements may require a juxtacrine effect.

Acknowledgements: This work was supported in part by a grant from the AUA Foundation Research Scholars Program and the AUA Southeastern Section Research Scholar Endowment Fund.
References:


18. Li Y and Lin F: Mesenchymal stem cells are injured by complement after their contact with serum. Blood 2012; 120: 3436.


Table 2 Urodynamic parameters in Vehicle treatment, rBMSC and ErBMSC at all time-points

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CHAPTER IV

Cannabinoid system contribution to control of micturition
Dopaminergic and endocannabinoid interaction

The previous chapters address the role of the dopaminergic system in the central nervous system control of micturition, by studying the effect of stem cell transplantation on voiding following nigrostriatal lesion. The subsequent dopamine depletion caused measurable voiding changes in our animal model, suggesting a contribution of dopamine in the normal circuitry involved in normal voiding. The endocannabinoid tone can be altered in animal models of PD. The effect of endogenous cannabinoid in normal or pathological conditions on micturition has not been clearly established. This chapter will review the current literature on the endocannabinoid system as related to effects on the lower urinary tract and its role in normal and abnormal urinary tract function.

Endocannabinoid system

Voiding dysfunction related to neurological lesions is particularly challenging to treat with our current pharmacological armamentarium due to the limited number of drugs that have efficacy and the adverse effect profile meeting criteria for approval and clinical use. Currently, the most commonly used drugs target the cholinergic (muscarinic acetylcholine receptors) and adrenergic systems (β3-adrenoceptors), or affect both autonomic and somatic nerves (botulinum toxin). As the different pathophysiological processes of lower urinary tract symptoms (LUTS) are under investigation, the understanding of the contribution of other endogenous systems to the control of micturition will expand our therapeutic options.
The endocannabinoid system plays a prominent role in several normal and pathological conditions, and has generated significant interest as a novel target in the academic and pharmaceutical fields. Phytocannabinoids can be extracted from the cannabis plant (marijuana). The main psychoactive compounds are Δ9-tetrahydrocannabinol (Δ9-THC), cannabidiol and cannabinol. The chemical and pharmacological investigation of these compounds led to the discovery of two G-protein coupled cannabinoid (CB) receptors type 1 (CB₁R) and type 2 (CB₂R). A third receptor has recently been established to be sensitive to CB, called the G-protein coupled receptor 55 (GPR55; for review see (2)).

The endocannabinoid system is composed of at least two major arachidonate-derived ligands, N-arachidonylethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG), that mediate their effects by binding to CB₁R and CB₂R. (Figure 1)

Both ligands are synthesized post-synaptically on demand and delivered in a retrograde fashion to bind to presynaptically localized CB₁R in the CNS.(3) Activation of presynaptic CB₁R in the brain or on primary afferents prevents neurotransmitters release by diminishing calcium conductance and by increasing
potassium conductance (4), leading to modulation of GABAergic and glutamatergic synapses and post-synaptic transmission of norepinephrine and dopamine (DA). Activation of both receptors inhibits adenylate cyclase by coupling to the α-subunit of the G protein of the G_{i/o} family.

In the nervous system, anandamide and 2-AG are primarily metabolized by the serine hydrolase enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase, respectively. Preventing their degradation with inhibitors of these enzymes can enhance their endogenous actions and avoid the deleterious side-effects of direct agonists of CB receptors. Anandamide and other exogenous CB compounds are known to also act at other receptors such as the vanilloid TRPV1 channel. The vanilloid TRPV1 channel is a nonselective cation channel activated by naturally occurring vanilloids, capsaicin and resiniferatoxin. CB_{1}R are located in a much higher density within than outside the CNS. CB_{2}R are present in peripheral cells such as lymphocytes and macrophages, and in organs such as spleen and thymus. In the nervous system, they are found on infiltrating immune cells and resident microglia/macrophages. CB_{2}R are located on peripheral nerve terminals, but are also present on post-synaptic neurons in several regions of the brain and on non-neuronal cells of the CNS, such as infiltrating immune cells and resident microglia/macrophages.

**CB receptors in the lower urinary tract**

Both CB_{1}R and CB_{2}R have been localized to the rat bladder, particularly on the urothelium. In human whole bladders obtained from male organ donors, both CB_{1}R and CB_{2}R were found to be expressed twice as much in the urothelium than in the
detrusor, and were localized to the cell membranes. Overall CB_1R expression was higher than that of CB_2R. Bakali et al. also demonstrated that both human and rats expressed CB_1R, TRPV1 and FAAH in their bladder. CB_2R were found to be expressed in higher density in rat, monkey and human bladder mucosa (urothelium and suburothelium) than in the detrusor and were also co-localized with TRPV1 channels and CGRP. In the detrusor wall, CB_2R immunoreactive fibers were identified on VAchT-positive nerve fibers. CB_2R, but not CB_1R, were up-regulated in the bladder after acute and chronic inflammation induced by intra-vesical acrolein in rats. CB_1R immunoreactive fiber density was significantly increased in the suburothelium of bladder specimen from patients with painful bladder syndrome (PBS) and idiopathic detrusor overactivity (IDO), and in the detrusor of patients with IDO, and correlated with their symptom scores, as compare to control. Bladder CB_2R possibly mediated the effects of oral CB agonist in a placebo-controlled study on MS patients. CB_2R mRNA expression was higher in the bladder of MS patients, and decreased after active treatment. CB_1R and CB_2R were identified in the spinal cord and dorsal root ganglia of rats, but bladder inflammation did not affect their expression. Spinal cord and dorsal root ganglia CB_2R expression was significantly up-regulated in inflammatory and neuropathic pain conditions in rats, and may help mediate analgesic effects. Most studies identify CB_1R and CB_2R in the bladder urothelium and detrusor along with other related proteins such as FAAH or TRPV1, with variable density across species. The spinal cord also expresses CB receptors. Pathological processes related with inflammation or pain conditions can cause an up-regulation of these receptors, particularly CB_2R.
**CBs in clinical trials**

The first clinical study of potential effect of CB on bladder function was seen in 1997, in a questionnaire based study where patients with MS using cannabis reported an improvement in urinary symptoms (urinary urgency in 64%, urinary hesitancy in 58.5%, and urinary incontinence in 54.7%). (20) Whole plant cannabis extract was studied in an open-label trial in patients with advanced multiple sclerosis (MS) and severe lower urinary tract symptoms (LUTS), and a significant decrease in urinary urgency, number and volume of incontinence episodes, frequency and nocturia was demonstrated. (21) These findings were followed by a randomized multicenter placebo-controlled clinical trial where oral administration of cannabis extract, Δ9-THC, or placebo was given to patients with MS. Both active compounds significantly decreased urgency incontinence episodes compared to placebo. (22) There are now three available medications that activate the CB₁R / CB₂R in the clinic: Cesamet (nabilone), Marinol (dronabinol; Δ9-THC) and Sativex (Δ9-THC with cannabidiol).

**CB in pain**

Sativex is now licensed in Canada and in the UK for symptomatic relief of cancer pain and/or management of neuropathic pain and spasticity in adults with multiple sclerosis. The antinociceptive action of CB receptors is likely related to their peripheral spinal and supraspinal anatomical location relevant to pain in the brain, spinal dorsal horn, dorsal root ganglia and peripheral afferent neuron. (23) CB agonists have been extensively investigated in animal studies and in clinical trials related to pain, but their therapeutic effect has been limited by their psychoactive components. This has prompted the interest
in investigating compounds that inhibit the metabolism of endocannabinoids, or compounds that are peripherally restricted.

**CB in micturition**

The presence and activity of CB₁R in the bladder was first suggested with an inhibition of electrically-evoked contractions of the mouse urinary bladder in the presence of CB₁R agonist. In the same study, the selective CB₁R antagonist SR141716 caused a rightward shift in the concentration-response curve with CB₁R agonist electrically evoked bladder contraction.(24) Martin et al. demonstrated some species differences for the effect of CB₁R agonist on neuronally evoked bladder contractions, with a higher inhibitory effect in mouse than in rats. SR141716 potentiated electrically evoked contraction through an undetermined mechanism.(25) Anandamide application produced slowly developing contractions in muscle strips isolated from the rat urinary bladder. These responses were attenuated by previous capsaicin sensitization.(26) The presence of either anandamide or CP55,940 did not affect carbachol-induced contractions in neither rat, monkey nor human bladder preparations. However anandamide increased EFS-induced contractions while CP55,940 decreased them at all frequencies.(14) ACEA, a selective CB₁R agonist attenuated the EFS and carbachol-induced contractions of rat bladder. GP1A, a CB₂R agonist only decreased carbachol-induced contractions in rat bladder.(13) Application of ajulemic acid, a mixed CB₁R/ CB₂R agonist, to rat bladder preparation significantly decreased the CGRP release as compared to control, presumably from sensory afferent fibers.(11) Cannabidiol decreased the carbachol-induced contractions in both rat and
human bladder preparations, but this effect was attenuated by TRPV1 antagonists ruthenium red and capsazepine in the rat.\(^{(27)}\)

CB receptor activation reduced afferent activity in an electrophysiological ex vivo preparation under normal conditions.\(^{(28)}\) A non-selective CB receptor agonist was found to decrease afferent activity from inflamed bladders at certain intravesical pressures, an effect which was blocked by a selective CB\(_1\)R antagonist.\(^{(29)}\)

These studies almost consistently show the lack of direct effect of CB agonists on bladder contractility. However, there are significant conflicting findings between the different CB receptor agonist and their action on carbachol- or electrically-induced bladder contractions. CB receptor activation decreases contractility in vitro, as seen in several studies. The effect CB receptor agonists on carbachol-induced contractions have been less convincing, and may be mediated through other receptors such as TRPV1.

The N-acylethanolamides, anandamide (via CB\(_1\)R) and palmitoylethanolamide (putative endogenous CB\(_2\)R agonist), caused analgesia in models of viscerovisceral hyper-reflexia induced by inflammation of the urinary bladder.\(^{(30, 31)}\) These agents were found to decrease the expression of spinal cord c-fos at L6 following intravesical NGF instillation.\(^{(32)}\) Cyclophosphamide injection increase the anandamide content in the rat bladder, while its intravesical instillation increased c-fos expression in the spinal cord and increased the bladder reflex activity, which was blocked by TRPV1 antagonist capsazepine and resiniferatoxin. The authors concluded that anandamide is partly responsible for the hyperreflexia and hyperalgesia in cystitis, via TRPV1.\(^{(33)}\)

Intraperitoneal administration of GP1a, a highly selective CB\(_2\)R agonist, decreased the
mechanical sensitivity in a mouse model of acrolein-induced cystitis, possibly by preventing phosphorylation of ERK1/2 via MAPK activation. (34) Treatment with a selective CB₂R agonist (O-1966) following spinal cord injury improved bladder recovery in rats by modifying the inflammatory response. (35) CB₂R agonism appears to decrease viscera-visceral pain caused by bladder inflammation, possibly by modulating afferent signaling in the spinal cord and promoting an anti-inflammatory effect. CB₂R activation has immunomodulatory function that can limit the endothelial inflammatory response, chemotaxis, and inflammatory cell adhesion and activation in atherosclerosis and reperfusion injury. (36)

Administration of CP55,940 and methanandamide during cystometry in cats decreased micturition volume threshold at all doses, but did not change the frequency of spontaneous detrusor contractions. (37) Intravesical anandamide increased threshold pressure (TP) and decreased micturition interval in rats, while CP55,940 increased both TP and micturition interval. (14) Intra-arterial WIN55212 in rat cystometry significantly increase micturition threshold at all doses, and was particularly enhanced following turpentine induced bladder inflammation or bilateral hypogastric neurectomy. (38) Cannabinor, a highly selective CB₂R agonist increased micturition intervals and TP during conscious cystometry. (15) The chronic administration of this compound during two weeks following partial urethral obstruction in rats decreased post void residual and number of non voiding contractions, and increased bladder compliance as compared to controls. (39) Strittmatter et al. demonstrated that FAAH is expressed in the bladder of rats, mice and humans. They also demonstrated that systemic or intravesical
administration of a FAAH inhibitor, oleoyl ethyl amide (OeTA), during conscious
cystometry significantly increased intercontraction interval, micturition volume, bladder
capacity and TP in rats. These effects were abolished with the concomitant use of
SR144528, a CB2R antagonist, showing that FAAH inhibition mediated its effect on
micturition via CB2R.(40)

Overall, CB agonists have an inhibitory effect on micturition, by increasing TP and
decreasing frequency, possibly through afferent signaling. Anandamide has a more
controversial mechanism of action, as it seems to influence micturition differently,
demonstrated by in vitro and in vivo studies. The endocannabinoid anandamide is known
to also activate TRPV1 channels, potentially via the release of CGRP.(41) Studies have
found that a higher concentration of anandamide is required to evoke a TRPV1 channel
mediated release of this neuropeptide as compared to that mediated via the CB
receptor.(42)

Although there is a significant body of data demonstrating that CBs affect micturition,
there is very little known about the site of action that is primarily responsible for their
effects. As most CB agents easily cross the blood-brain barrier because of their
lipophilicity, systemic administration cannot determine how much of their voiding effects
are due to peripheral or central activation.

Intrathecal administration of compounds provides several applications. It allows the
investigation of localized drug delivery of minimal concentration to distinguish their
action at the spinal level. Also, restricting the distribution of active concentrations of
these compounds to the spinal cord makes it possible to avoid deleterious psychoactive side-effects from brain CB receptor activation. As the micturition reflex involves the spinal cord and ganglia, this approach may allow the development of new management strategies for the treatment of intractable detrusor overactivity.

Using intrathecal administration technique, we assessed whether spinal inhibition of the cannabinoid degrading enzyme FAAH would have urodynamic effects in normal rats and rats with bladder overactivity induced by partial urethral obstruction or prostaglandin E₂. We also determined the expression of FAAH, and the receptors CB₁ and CB₂ in the sacral spinal cord. Functional assessment with conscious cystometry was performed after the FAAH inhibitor oleoyl ethyl amide (OeTA; 3 to 300 nmol) was administered intrathecally (subarachnoidally) or intravenously. The expression of FAAH and CB₁/CB₂ receptors was determined by Western blot.

Intrathecal OeTA affected micturition in normal rats and rats with bladder overactivity but effects were more pronounced in the latter. It decreased micturition frequency in normal rats, and also decreased overall bladder pressures in rats with bladder overactivity, in a dose-dependent fashion, without affecting behavior. (Figure 2) The same doses did not affect the cystometric parameters when given systemically.
Intrathecal OeTA decreased micturition frequency in normal rats, and also decreased overall bladder pressures in rats with bladder overactivity, in a dose-dependent fashion, without affecting behavior. The same doses did not affect the cystometric parameters when given systemically. FAAH, CB₁R and CB₂R were expressed in the rat sacral spinal cord, while CB₁R and CB₂R were only increased in obstructed rats. (43) (Figure 3, Appendix II)

Therefore, we demonstrated that FAAH inhibition in the sacral spinal cord by OeTA resulted in urodynamic effects in normal rats and rats with bladder overactivity. The spinal endocannabinoid system may be involved in normal micturition control and it appears altered when there is bladder overactivity. Anandamide and other exogenous CB compounds are known to also act at other receptors such as the vanilloid TRPV1 channel. (6) It would therefore be of interest to determine the involvement of spinal TRPV1 receptors on the effect on micturition in the presence of endocannabinoids.

Figure 2 Representative 10-minute urodynamic recordings in PUO rats. A and B, before drug. C and D, after 30 nmol intrathecal OeTA. A and C, intravesical pressure in cm H2O. B and D, voided volume in ml.
Summary and future direction

The endocannabinoid system is a relatively new field of study in the physiology and pharmacology of the lower urinary tract. The literature reports that most CB receptor agonists have an effect on micturition through a yet unknown mechanism, as demonstrated by clinical trials, and *in vivo* and *in vitro* animal studies. There are likely interactions with other receptors or channels to ultimately inhibit micturition. Previous animal studies have focused on administration of oral or intravesical compounds and their effects on urodynamic parameters.(29, 39, 40) Using an intrathecal catheter delivery system, we administered different CB compounds in the lumbosacral area of the spinal cord in an animal model, and studied the changes that occur in their voiding function with
urodynamic assessment, to determine the contribution of central CB receptors in the
control of voiding function. We aimed to demonstrate that activation of either CB1R or
CB2R in the spinal cord can modulate afferent signaling in normal voiding, without the
effect of peripheral receptor activation.

Selective CB agonists and antagonists have provided valuable information to understand
their action in the control of micturition. However, their selectivity and potency are
relative and cannot completely obviate their action at other sites. Knockout (KO) mouse
technology can provide very powerful means of determining gene function in vivo. We
assessed the voiding function in CB1R and CB2R KO mice by quantitatively measuring
urodynamic parameter at baseline and after administering different CB compounds. This
methodology has not yet been used for the assessment of lower urinary tract function, and
therefore will gather very valuable information in order to develop novel therapeutic
strategies.

These studies will allow us to understand the contribution of CB receptors at the level of
subtype in the central and peripheral nervous system control of micturition. This could
translate into the application of localized delivery of selective compounds to treat NDO.
References:

CHAPTER V

Role of cannabinoid and TRPV1 receptors in the spinal mechanisms of micturition in the normal rat

Lysanne Campeau, Claudius Füllhase, Norifumi Sawada, Petter Hedlund, Allyn Howlett, Karl-Erik Andersson

The following chapter will be submitted to Neurourology and Urodynamics

Literature citations and figure formats are adherent to the guidelines set forth in Neurourology and Urodynamics

Lysanne Campeau is responsible for all data collection and analysis.
Abstract

AIMS: Systemic administration of cannabinoid (CB) receptor agonists and fatty acid amide hydrolase (FAAH) inhibitors affects bladder function, but whether the main site of action is peripheral tissues or the central nervous system is unknown. Intrathecal (IT) CBs have been shown to produce antinociception in neuropathic pain animal models. Many of these compounds also act at the transient receptor potential cation channel subfamily V member 1 (TRPV1). Our goal was to determine the effects of IT CB receptor agonists and the impact of TRPV1 channel activation on bladder function of normal rats when spinal degradation of endogenous CBs is inhibited.

METHODS: In female rats, bladder and IT catheters were inserted prior to cystometric evaluation. Urodynamic parameters were recorded in conscious animals at baseline, and after each drug. The first part of the study involved two groups of animals treated with incremental doses, preceded by vehicle, of methanandamide (MA) (5, 10, 20, 40 µg), a selective CB₁R agonist, and WIN 55212-2 (10, 20, 40 µg), a non-selective CB receptor agonist. The second part involved two other groups of animals: the first received IT SB366791 (200 nmol), a selective TRPV1 channel antagonist, followed by intraperitoneal oleoyl ethyl amide (OeTA) (0.75 mg/kg), a FAAH inhibitor, and finally IT MA (100 µg); the second group received IT vehicle (DMSO) for SB366791, followed by OeTA, and IT MA.

RESULTS: The micturition pressures did not change after vehicle or drug administration across all groups. In the MA only group, bladder capacity (BC) significantly increased from baseline after 40 µg administration. BC also significantly increased after administration of 40 µg of WIN 55212-2 when compared to baseline and to vehicle.
Micturition volume (MV) increased from baseline after 20 µg administration of WIN 55212-2. In the animals that received vehicle prior to OeTA and MA, we observed a significant increase from baseline in the BC and MV following both systemic OeTA and IT MA (p<0.05), while in the animals that were given SB366791, there were no significant changes from baseline in BC or MV following systemic OeTA and IT MA.

CONCLUSION: IT CB receptor agonist administration increases BC in normal rats. Both drugs used may activate TRPV1 channels along with CB receptors. IT TRPV1 channel antagonist administration abolished the effects of both MA and OeTA on BC and MV. This suggests that spinal TRPV1 channel activation is involved in effects by FAAH substrates and MA on afferent signaling in micturition.
Introduction

Treatment of voiding dysfunction such as overactive bladder has been challenging due to the limited number of drugs that have an efficacy and adverse effect profile sufficient for approval and clinical use. Currently used drugs target the cholinergic (muscarinic acetylcholine receptors) and adrenergic systems (β3-adrenoceptors). As the different pathophysiological processes of lower urinary tract symptoms (LUTS) are under investigation, the understanding of the contribution of other endogenous systems to the control of micturition will expand our therapeutic armamentarium. Whole plant cannabis extract was studied in an open label trial in patients with advanced multiple sclerosis (MS) and severe LUTS, and a significant decrease in urinary urgency, the number and volume of incontinence episodes, frequency and nocturia was demonstrated. These findings were followed by a randomized multicenter placebo-controlled clinical trial where oral administration of cannabis extract, Δ9-THC, or placebo was given to patients with MS. Both active compounds significantly decreased urgency incontinence episodes compared to placebo.

The endocannabinoid system is composed of at least two major arachidonate-derived ligands, anandamide and 2-arachidonoylglycerol, that mediate their effects by binding to the cannabinoid (CB) receptors type 1 (CB1R) and type 2 (CB2R). In the nervous system, anandamide and 2-arachidonoylglycerol are primarily metabolized by the serine hydrolase enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase, respectively. Preventing their degradation with inhibitors of these enzymes can enhance their endogenous actions and avoid the deleterious side-effects of direct agonists.
of CBs. Anandamide and other exogenous CB compounds are known to also act at other receptors such as the vanilloid TRPV1 channel. (4) The vanilloid TRPV1 channel is a nonselective six trans-membrane domain cation channels of the large TRP superfamily and more specifically, the TRPV channel subfamily, activated by naturally occurring vanilloids, capsaicin and resiniferatoxin. CB1R are located pre-synaptically and in a much higher density within than outside the central nervous system (CNS). (5) CB2R are located on peripheral nerve terminals (6) but are also present on post-synaptic neurons in several regions of the adult rat brain and on non-neuronal cells of the CNS. (7) Both CB1R and CB2R are also located in the peripheral nervous system and have been localized to the rat bladder, particularly on the urothelium. (8)

As most CB agents easily cross the blood brain barrier because of their lipophilicity, systemic administration cannot determine how much of their voiding effects are due to peripheral or central activation. Localized drug delivery to the CNS by means of an intrathecal (IT) catheter could separate experimentally their effects on the spinal cord, potentially without peripheral and psychoactive brain effects. CB receptors have been localized to the spinal cord (9, 10) and IT CB receptor agonists have been shown to produce antinociception in neuropathic pain animal models. Our goal was to determine the effects of IT CB agonists and the impact of TRPV1 channel activation on bladder function of normal rats when spinal degradation of endogenous CB is inhibited.
Methods

Study design

A total of 32 Sprague-Dawley female rats (Weight: 220-270 gm) were involved in this study. Experiments were approved by the local animal care and use committee and conducted in accordance with the National Research Council publication Guide for Care and Use of Laboratory Animals. Animals were housed in pairs, and then individually after surgery, in a 12 hour light-dark cycle at Wake Forest University Health Sciences. Food and water were available ad libitum. Cystometry was performed in conscious animals, three days after inserting a suprapubic bladder catheter and an IT catheter. The first part of the study involved two groups of animals with incremental doses, preceded by vehicle, of methanandamide (MA) (Tocris Biosciences, Minneapolis, MN), a selective CB$_1$R agonist, or WIN 55212-2 (Tocris Biosciences, Minneapolis, MN), a non-selective CB receptor agonist. The second part involved two other groups of animals: the first received IT SB366791 (Cayman Chemical, Ann Arbor, Michigan, USA), a selective TRPV1 channel antagonist, followed by intraperitoneal (IP) oleoyl ethyl amide (OeTA; Cayman Chemical, Ann Arbor, Michigan, USA), a FAAH inhibitor, and finally IT MA; the second group received IT vehicle (DMSO) for SB366791, followed by OeTA, and IT MA.

Catheter implantation

Under inhalation anesthesia (3% isoflurane), the abdomen was opened through a mid-line incision. A polyethylene catheter (Clay-Adams PE-50, Parsippany, NJ, USA) with a cuff
was inserted into the dome of the bladder and held in place with a purse string 6-0 silk suture. The catheter was tunneled subcutaneously and anchored to the skin of the back with a 3-0 Vicryl suture. The free end of the catheter was heat-sealed. Wounds were closed in layers and the animals were allowed to recover.

At the same anesthesia setting, the animal was repositioned prone and the atlanto-occipital membrane was exposed after making a midline incision behind the head of the animal and splitting the muscular attachments. The membrane was incised and a 32 gauge polyethylene IT catheter (ReCathCo, Allison Park, PA, USA) was slowly introduced within the subarachnoid space. The length of the catheter was adjusted for the end of the lumen to be 3 centimeters above the hip joints. Adequate catheter location at L6-S2 was confirmed at necropsy.

**Cystometry**

Cystometric investigations were performed without anesthesia three days after the bladder catheterization. Catheters were connected via a T-tube to a pressure transducer (P23 DC, Statham Instruments Inc; Oxnard, California) and to a micro injection pump (CMA 100, Carnegie Medicine AB, Solna, Sweden). The conscious rat was placed without any restraints in a metabolic cage and room temperature saline was infused into the bladder at a rate of 10 ml/hour. Intravesical pressures and voided volumes were recorded by having the pressure and volume transducers connected to an ETH 400 (CD Sciences, Dover, NH) transducer amplifier and, consequently, connected to a PowerLab/8e (Analog Digital Instruments, Castle Hill, NSW, Australia) data acquisition board. Three days after catheterization, baseline cystometry was recorded for 30 minutes
after the animals stabilized and got accustomed to their new environment (approximately 30 minutes). For the first part of the study, a group of animals were then given an IT injection of 10 µl vehicle, followed by incremental doses of MA (5, 10, 20, 40 µg). Cystometric data were recorded for 30 minute periods after each IT injection. The second group received an IT injection of vehicle, followed by incremental doses of WIN 55,212-2 (10, 20, 40 µg).

For the second part of the study, the animals were evaluated in two different groups. Both groups of animals underwent baseline conscious cystometry for 30 minutes after the animals stabilized. The first group then received an IT injection of SB366791 (200 nmol), followed by IP OeTA (0.75 mg/kg), and finally IT MA (100 µg). Cystometric data were recorded for 30 minute periods after each drug administration. The second group instead received the vehicle for SB366791, followed by IP OeTA (0.75 mg/kg), and finally IT MA (100 µg).

The following cystometric parameters were investigated: maximum pressure (MP) (maximum bladder pressure during a micturition cycle), threshold pressure (TP) (pressure at initiation of voiding contraction), basal pressure (BP) (lowest pressure in between voids), intermicturition pressure (IP) (average calculated pressure between voids), spontaneous activity (SA) (IP minus BP), intercontraction intervals (ICI), bladder capacity (BC), bladder compliance (Bcom) (BC divided by TP minus BP), micturition volume (MV) (volume of the expelled urine), residual volume (RV) (BC minus MV), and area under the curve (AUC) as a proxy for non-voiding contractions. AUC is a calculated integral that correlates with overall bladder pressures between voiding contractions and has no particular threshold.
**Statistical analyses**

Cystometric variables were compared between periods of drug administration using ANOVA of repeated measures, followed by Bonferroni post-hoc testing. All statistical analysis and calculations were obtained using GraphPad Prism. All values are expressed at the mean ± standard error of the mean. A probability of p < 0.05 was considered significant.

**Drugs and Chemicals**

For stock solution preparation, MA and WIN 55,212-2 were diluted in Tocrisolve (Tocris Biosciences, Minneapolis, MN), and maintained at 4° Celsius and -20° Celsius respectively. For the 10 µl IT injection, the compounds were diluted in a vehicle preparation of 40% Tocrisolve and 60% artificial cerebrospinal fluid (ACSF). The ACSF was made of 142 mM NaCl, 5 mM KCl, 10 mM glucose and 10mM HEPES Na. Stock solution for OeTA was done in ethanol, and was then diluted in phosphate buffered saline for IP injection. SB366791 stock solution was in DMSO and then diluted in ACSF for injection. Both stock solutions were maintained at -20° Celsius. The vehicle for SB366791 was done with the same proportion of DMSO and ACSF.
Results

Out of the 32 rats that underwent bladder and IT catheter implantation, two were excluded due to non-analyzable cystometry tracings from movement artifacts. Three animals were excluded due to one mortality and two lower limb neurological deficits.

The last dose of 40 µg of MA after cumulative increment of the drug caused a significant increase in BC and ICI compared to baseline period. (Table 1, Figure 1A) The dose of 20 µg of WIN 55,212-2 after cumulative increment significantly increased the MV compared to baseline period. Following the administration of 40 µg of WIN 55,212-2, the BC and ICI significantly increased from baseline and vehicle periods. (Table 2, Figure 1B, 1C)

Table 1: Cystometric parameters with IT administration of MA

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<td>310.3 ± 49.4</td>
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<td></td>
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Each value represents mean ± SEM. Values compared with ANOVA of repeated measures, followed by Bonferroni post-hoc testing.

* p<0.05 Baseline vs 40 µg
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Each value represents mean ± SEM. Values compared with ANOVA of repeated measures, followed by Bonferroni post-hoc testing.
* p<0.05 Baseline vs 40 µg
** p<0.05 Vehicle vs 40 µg
† p<0.05 Baseline vs 20 µg
For the second part of the study, the last period following the administration of IT MA following IT vehicle and systemic OeTA caused a significant increase in the MV. (Table 3, Figure 2A) However, the animals that received IT MA, following the administration of IT SB366791 instead of vehicle and systemic OeTA, did not have a significant change in their ICI, BC, nor MV. (Table 4, Figure 2B)
Table 3: Cystometric parameters with administration of IT vehicle, followed by systemic OeTA and IT MA

<table>
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<tr>
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<th>n = 6</th>
<th>Baseline</th>
<th>Vehicle</th>
<th>OeTA</th>
<th>MA</th>
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<tbody>
<tr>
<td>ICI</td>
<td></td>
<td>286 ± 40</td>
<td>274 ± 39</td>
<td>292 ± 39</td>
<td>317 ± 39</td>
</tr>
<tr>
<td>BC</td>
<td>mL</td>
<td>0.79 ± 0.11</td>
<td>0.76 ± 0.11</td>
<td>0.81 ± 0.11</td>
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</tr>
<tr>
<td>MV</td>
<td>mL</td>
<td>0.68 ± 0.08</td>
<td>0.74 ± 0.12</td>
<td>0.77 ± 0.12</td>
<td>0.92 ± 0.14</td>
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</tbody>
</table>

Each value represents mean ± SEM. Values compared with ANOVA of repeated measures, followed by Bonferroni post-hoc testing.

* p<0.01 Baseline vs MA
** p<0.05 Vehicle vs MA

Table 4: Cystometric parameters with administration of IT SB 366791, followed by systemic OeTA and IT MA

<table>
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<td>316 ± 19</td>
<td>333 ± 25</td>
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<td>BC</td>
<td>mL</td>
<td>0.88 ± 0.05</td>
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<td>MV</td>
<td>mL</td>
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<td>0.96 ± 0.09</td>
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</tbody>
</table>

Figure 2
Cystometric data following TRPV1 antagonist. Values are expressed as mean ± SEM parameters. Micturition volume at different periods following administration of intrathecal vehicle (A) and intrathecal SB 366791 (B).

* p<0.05 Baseline vs 40 µg
Discussion

This study’s results suggest that IT administration of CB receptor agonists MA and WIN 55,212-2 produce a significant increase in ICI and BC of female rats during conscious cystometry. Our results also suggest that activation of spinal TRPV1 channels is required to mediate this effect, and likely do so through an afferent pathway.

The potential interaction between CB receptor agonists and the TRPV1 channel has been demonstrated and discussed in other studies, particularly related to pain. CB₁R activation inhibits adenylate cyclase activity and Ca²⁺ currents in the CNS. (11) The endocannabinoid, anandamide, is known to also activate TRPV1 channels, potentially via the release of CGRP. (12) Studies have found that a higher concentration of anandamide is required to evoke a TRPV1 channel mediated release of this neuropeptide as compared to that mediated via the CB₁R. (13) TRPV1 channels are located on primary afferents and co-localized with FAAH in the lumbar spinal cord. (14) CB₁R agonists added to primary sensory neurons reduce depolarization and the release of glutamate and nociception-related peptides such as substance P and CGRP activated by TRPV1. (15, 16) The deep dorsal horn of L4-5 of rats was found to have strong immunopositivity for CB₁R, and was also immunopositive for CGRP and IB4. (17) Other studies have identified CB₁R within the dorsal horn. (18, 19) These findings could provide some additional mechanisms by which CB receptor agonists reduce TRPV1 channel mediated pain. If administered directly to the spinal cord, CB compounds have been shown to induce significant analgesia in animal models of neuropathic or inflammatory hyperalgesia. (20) Using a
spinal slice preparation, Morisset et al. demonstrated that CB$_1$R-mediated reduction in synaptic input from nociceptive primary afferents to the spinal cord was produced by the activation of presynaptically expressed CB$_1$R.(15) The effect of an IT FAAH inhibitor, URB597, on analgesia in a neuropathic pain model was uniquely blocked by TRPV1 channel antagonism.(14) Similarly, Sagar et al. demonstrated that the effect of spinal administration of UCM707, a CB$_2$R agonist, was significantly blocked by the TRPV1 channel selective antagonist iodo-resiniferatoxin.(21)

Both pain and bladder control mechanisms are influenced by spinal afferent signaling. Systemic administration of a CB$_2$R agonist caused a decrease in intermicturition interval and in threshold pressure required for normal micturition.(22) Walczak et al. demonstrated in an electrophysiological ex vivo preparation that stimulation of CB receptors reduced afferent activity under normal conditions.(23) Pathological conditions are known to alter the expression pattern of CB receptors. Patients with chronic bladder pain syndrome had higher CB$_1$R mRNA transcription in their bladder than normal adults.(24) A non-selective CB receptor agonist was found to decrease afferent activity from inflamed bladders at certain intravesical pressures, an effect which was blocked by a selective CB$_1$R antagonist.(25) However, these studies do not address the exact site of action of CB receptor agonists to cause changes in micturition. As these compounds are very lipophilic, they are easily distributed both peripherally and centrally when administered systemically. If the micturition effects could be achieved with IT delivery of relatively small doses, this could avoid potential stimulation of central brain CB$_1$R and accompanying psychogenic consequences.
Stritmatter et al. demonstrated that FAAH is expressed in the bladder of rats, mice and humans. They also demonstrated that systemic or intravesical administration of OeTA during conscious cystometry significantly increased ICI, MV, BC and TP in rats. These effects were abolished with the concomitant use of SR144528, a CB2R antagonist, showing that FAAH inhibition was mainly mediated by the CB2R. (26) Füllhase et al. studied the effects of IT OeTA on normal rats and rats with bladder overactivity induced by partial urethral obstruction or intravesical prostaglandin E2. IT OeTA decreased micturition frequency in normal rats, and also decreased overall bladder pressures in rats with bladder overactivity. FAAH and CB1R / CB2R were expressed in the rat sacral spinal cord, but only CB1R / CB2R were increased in obstructed rats. (27)

IT administration of small concentration of compounds reduces their action at peripheral sites of action such as at the bladder. Therefore, the observed increase in bladder capacity and micturition volume in animals following MA and WIN 55,212 IT administration suggests that it is caused by the presence of receptors in the spinal cord and that they impact afferent signaling in micturition control. We could not assess the impact of their action in the brain, but considering the location of the catheter end, the amount of these compounds reaching the supraspinal areas is likely minimal. Although we demonstrated that TRPV1 channel activation plays a role in mediating afferent signaling in micturition, we did not assess the individual contribution of either CB1R or CB2R by using antagonists. Therefore, it is probable that the activation of these different receptors has in a synergistic effect, and therefore we cannot exclude the possibility that antagonism of any of the CB receptors may have generated similar findings. While providing some
clarifying explanation in the role of TRPV1 receptors on micturition, our results may have potential translational application. Thus, localized IT delivery of CB receptor agonists may be useful for the treatment of refractory LUTS or severe intractable pelvic pain.
Conclusion

IT administration of agonists of both CB₁R and CB₂R increases BC and MV in normal rats during conscious cystometry. The increase in BC and MV with systemic administration of a FAAH inhibitor and IT MA was abolished in the presence of a specific TRPV1 channel antagonist. Although we cannot rule out the potential synergistic action of the CB receptors and TRPV1 channels in the micturition reflex, our findings suggest that spinal TRPV1 channel activation is involved in effects by FAAH substrates and MA on afferent signaling in micturition.
References:

CHAPTER VI

Cannabinoid receptor type 1 (CB₁) is important for normal micturition – results from in vitro and in vivo bladder evaluation of a CB₁ knock-out mouse model

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† both authors contributed equally to the work

The following chapter has been accepted for publication in

British Journal of Urology International

Literature citations and figure formats are adherent to the guidelines set forth in

British Journal of Urology International

Lysanne Campeau is responsible for most of data collection and analysis.
Abstract

Objective
- To evaluate bladder function in an established cannabinoid type 1 (CB1) receptor knock-out mouse model via organ bath (in vitro) and urodynamic (cystometric; in vivo) experiments.

Materials & Methods
- Twenty 8-week-old female wildtype (WT) mice (C57BL/6) and 20 age-matched CB1 knock-out (KO) mice were used.
- Six animals of each group were used for organ bath experiments where the contractile responses of bladder tissue strips following carbachol exposure (CCRC, carbachol concentration response curve) (myogenic contraction) and during electrical field stimulation (EFS) (neurogenic contraction) were assessed.
- Fourteen animals per group were used for cystometric experiments without any anesthesia, in which standard urodynamic parameters were assessed three days following bladder catheterization.

Results
- The CCRC of bladder strips from CB1 KO mice was similar to that of WT animals. However, during EFS the bladder strips from CB1 KO mice had a significantly lower contractile response than WT preparations, indicating that in CB1 KO animals the neuronal component of bladder contraction was different.
- In cystometric experiments CB1 KO mice showed a higher micturition frequency (shorter inter-micturition interval: 3.24 ±0.29 vs. 7.32 ±0.5 min), a lower bladder
capacity (0.09 ±0.01 vs. 0.18 ±0.01 mL) and micturition volume (0.07 ±0.01 vs. 0.14 ±0.01 mL), a lower bladder compliance (0.007 ±0.001 vs. 0.02 ±0.002 mL/cmH₂O), and a higher spontaneous bladder activity (5.1 ±0.5 vs. 2.6 ±0.6 cmH₂O) than WT mice (all p < 0.05 using Student’s t-test).

- In WT animals, systemic administration of rimonabant (SR141716), a CB₁ receptor antagonist, resulted in urodynamic changes similar to those in CB₁ KO mice.

**Conclusions**

- In vitro, bladder strips from CB₁ KO mice responded to muscarinic receptor stimulation similar to WT controls, but were less responsive to electrical stimulation of nerves. In vivo, CB₁ KO mice had a higher micturition frequency and more spontaneous activity than WT animals. The present findings suggest that CB₁ receptors are involved in peripheral and central nervous control of micturition.
Introduction

Cannabinoid type 1 (CB₁) and type 2 (CB₂) receptors have been shown to be present in the lower urinary tract (LUT) of various species (1, 2). Based on promising findings in preclinical experiments, it has been suggested that taming the cannabinoid system might be clinically useful for treatment of bladder dysfunctions such as the overactive bladder (OAB) syndrome or benign prostatic hyperplasia (BPH) related lower urinary tract symptoms (LUTS) (3, 4). There are clinical studies reporting on the beneficial effects of cannabis or cannabis extracts on patients with multiple sclerosis (MS) related bladder dysfunction (5). However, due to the psychotropic side effects of cannabis and cannabis extracts, a routine use of these compounds is not possible. The use of selective cannabinoid receptor agonists seems attractive, but to the best of our knowledge, there are no clinical data on the effects of selective CB₁ and/or CB₂ receptor agonists and/or antagonists in regards to bladder function.

The results from preclinical experiments seem conflicting. Whereas some authors report a predominance of CB₂ receptor binding sites in the LUT (2), others found a higher expression of CB₁ than of CB₂ receptors (6). Some authors report that CB₁, but not CB₂ receptor agonists, relax bladder tissue in organ bath experiments (1), whereas others report the opposite, i.e., that only CB₂, but not CB₁ receptor agonists, relax the bladder muscle (2). In in vivo experiments with chemically induced bladder overactivity (BO) in rodents, some authors report an up-regulation of bladder CB₂ but not CB₁ receptors (7), whereas others report that only CB₁, but not CB₂, receptors seem to be of functional relevance in BO (8). These seemingly contrasting findings of different studies might be explained by use of different compounds or antibodies, different dosages or dilutions, and
different experimental set-ups or species. Thus, according to currently available information, the exact role of CB₁ and CB₂ receptors in micturition remains unclear.

A reliable way to examine the role of a specific gene, and in consequence, a specific protein, is the use of knock-out (KO) models (9). In 2002, a CB₁ KO mouse model was created (10), which since has been used and well characterized in many respects (11-13). However, to the best of our knowledge, it has never been studied with respect to bladder function. The aim of the present study was to characterize bladder function in this CB₁ KO mouse model by in vitro (organ bath) and in vivo (urodynamic; cystometry) experiments.
Materials & Methods

Animals

Twenty female eight-week-old wildtype (WT) mice (C57BL/6) and 20 female age-matched knock-out (KO) mice were obtained from a collaborating research group with extensive experience of the model (11, 14, 15). Animals were housed and breed according to standard conditions at the Walter-Brendel-Center for Experimental Medicine of the Ludwig-Maximilians-University, Munich. Experiments were approved by the local Animal Care and Use Committee (ACUC) and conducted in accordance with the National Research Council publication Guide for Care and Use of Laboratory Animals (16).

In vitro experiments

Six WT and six KO mice were euthanized by CO₂ suffocation followed by cervical dislocation. Bladders were removed via a lower mid abdominal incision. The resection margin was the bladder neck. Adherent fat was removed. The bladder was swing opened and two circular strips were prepared, measuring approximately 2 x 5 mm. All strips were used immediately after removal. The strips were mounted in an organ bath (750 TOBS, DMT Danish Myotechnology, Aarhus, Denmark) containing 37°C warm, 95% oxygenated Krebs solution (mM: 119 NaCl, 4.6 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 15 NaHCO₃, 1.2 NaH₂PO₄, and 5 glucose); resulting in a pH of 7.4. The strips were suspended between the hooks using silk ligatures. The strips were stretched to a tension of 0.5 g, allowed to equilibrate for 45 minutes, and washed every 15 minutes with fresh Krebs solution. Data were collected via DMT compatible data acquisition hard- and software (Polygraph 7E, Grass Technologies, West Warwick, RI, USA and PowerLab,
AD Instruments, Colorado Springs, CO, USA). The force transducer of the organ bath as well as acquisition software was calibrated before each experiment. Krebs solution was tested with pH-meter to verify pH 7.4.

Each experiment started by exposing strips to a high K⁺ (80mM) Krebs solution until two reproducible contractions were obtained. Strips from one WT mouse had to be excluded due to non-reactivity; the remaining strips from five WT and six KO were all adequately responding, and used for analysis. Between exposures strips were washed five times with changes of fresh Krebs solution. Carbachol concentration response curves (CCRCs) were obtained by cumulative addition of increasing concentrations of the drug (half log increments: 10⁻⁷ to 10⁻⁴ M). After pharmacological stimulation, strips were washed with several changes of fresh Krebs solution and allowed to rest for 45 minutes before electrical stimulation. Electrical Field Stimulation (EFS) was performed by parallel platinum electrodes positioned on either side of the strips and connected to a square pulse stimulator (S88, Grass Instruments, Quincey, Massachusetts, USA). The square pulse duration was 0.5ms, the voltage 20V and the train duration 10 seconds. Between the stimuli the strips were allowed to rest for two minutes. Contractile responses to EFS were studied at frequencies of 1, 2, 4, 8, 16 and 32 Hz. After the experiments, the viability of the tissue was assessed by a new exposure to 80mM KCl. Measured contraction force of bladder tissues following CCRC or EFS was expressed as percent of the maximum KCl-induced contraction.
In vivo experiments

Bladder catheter implantation

Fourteen WT and 14 KO mice were given preemptive buprenorphin (0.03mg/kg) and metamizol (200mg/kg) subcutaneously (s.c.), and anaesthetized with 3% isoflurane inhalation. A low midline abdominal incision was made and the bladder was identified. A small incision was made in the bladder dome and a polyethylene catheter (PE-10, Clay Adams, Parsippany, New Jersey, USA) with a cuff was inserted into the bladder. A 6-0 silk purse string suture was placed around the catheter to anchor it and to close the bladder incision. Saline was injected via the catheter to exclude bladder leakage. Then the catheter was tunneled subcutaneously to the neck of the animal, extracted via a small incision, and anchored with a 4-0 vicryl suture. The free end was sealed thermally. The abdominal wall was closed using a 4-0 vicryl suture. Postoperative pain medication (metamizol 200mg/kg s.c.) was given 6, 12, and 18 hours postoperatively. Two animals had to be excluded due to postoperative catheter dislocation.

Cystometry

Cystometric investigations were performed without anesthesia three days after bladder catheterization. The bladder catheter was unsealed and connected via a T-tube to a pressure transducer (BP100, iWOrx Systems Inc., Dover, NH, USA) and an infusion pump. Room temperature saline was infused into the bladder at a rate of 2 mL/hour. The pressure transducer was connected via a transducer amplifier (ETH-401, iWorx) to a data acquisition system (MP150 using AcqKnowledge 3.9.1 software, Biopac Systems Inc., Goleta, CA, USA). The conscious mice were placed in metabolic cages without any restraint. This also enabled measurement of urine volumes by means of a fluid collector.
connected to a force displacement transducer (FT-302, iWorx), which was connected via the transducer amplifier to the same data acquisition software. In consequence intravesical pressure and micturition volumes were recorded synchronously and continuously. After an equilibration phase of 30 minutes, data were collected for a 60 minutes period. The pressure and force transducers as well as the analog-to-digital interface were calibrated before each experiment. Four animals had to be excluded due to movement artifacts and subsequent non-usable urodynamic tracings. In the final analysis 11 WT and 11 KO mice could be used.

The following cystometric parameters were investigated: micturition pressure (MP, maximum bladder pressure during micturition), threshold pressure (TP, bladder pressure at onset of micturition), basal pressure (BP, minimum bladder pressure between two micturitions), intermicturition pressure (IMP, mean bladder pressure between two micturitions), spontaneous activity (SA, intermicturition pressure minus basal pressure), intermicturition interval (IMI), bladder capacity (BCap, calculated: saline infusion rate x IMI), micturition volume (MV, measured), residual volume (RV, BCap minus MV), and bladder compliance (BCom, BCap divided by TP minus BP).

After 90 minutes of cystometry (baseline data) WT animals (not KO animals) received rimonabant (= SR141716) (Cayman Chemical Company, Ann Arbor, MI, USA), a CB₁ receptor antagonist, in a dose of 0.3mg/kg bodyweight intraperitoneally (i.p.), then cystometry was continued for another 60 minutes.

After the experiments, mice were euthanized by CO₂ suffocation followed by cervical dislocation, and then weighed immediately. Bladders were removed and weighed.
**Statistical Analysis**

Results are given as mean ± standard error of the mean (SEM). Comparisons between groups were carried out by Student’s t-test, within groups by paired Student’s t-test. Statistical significance was considered when p<0.05. SigmaPlot 11.0 software (Systat Inc., Chicago, IL, USA) was used for statistical calculations.
Results

Age-matched WT mice were bigger than CB₁ KO mice (25.2 ±2.4 vs. 18.9 ±0.8 g, p<0.05), what is intrinsic to the model (17). However, bladder weights were not significantly different (100.4 ±4.0 vs. 96.3 ±5.2 mg). The bladder-to-body ratio was not different between groups (4.1 ±0.4 vs. 5.3 ±0.1).

Organ bath experiments

Carbachol induced concentration-dependent contractions of WT bladder tissue with a maximum at 10µM, reaching 108.1 ±19.4% of maximum KCl-induced contraction force. The bladder tissue of KO mice showed no different contraction force development, reaching 101.6 ±8.7% of maximum KCl-induced contraction force at 10 µM carbachol (Figure 1).

Even though the bladder tissue of WT and KO mice did not react differently upon carbachol exposure, there was a significant difference at the EFS. Whereas in WT tissue EFS induced frequency-dependent contractions with a maximum at 32Hz (111.2 ±20.7% of maximum KCl-induced contraction force), the response of KO tissue was lower (at

Figure 1: Carbachol concentration response curves (CCRCs) of bladder strips from wildtype (WT; black; n = 2 x 5) and cannabinoid type 1 receptor knock-out (CB₁ KO; grey; n = 2 x 6) mice following cumulative addition of increasing concentrations of carbachol (Cch) by half log increments from 10⁻⁷ to 10⁻⁴ M (x-axis). Contraction force of bladder strips is presented in reference (in percent; %) to maximum KCl-induced contraction force (y-axis). Student’s t-test was used for statistical comparisons, no statistical differences between WT and CB₁ KO bladder strips in CCRC experiments.
32Hz: 78.9 ±11.6% of maximum KCl-induced contraction force). The lower contraction force of KO tissue upon EFS was statistically significant for 2, 4, and 8 Hz (Figure 2).

**Cystometry**

In the cystometric experiments (Figure 3; Table 1) CB₁ KO mice showed a higher micturition frequency (shorter inter-micturition interval: 3.24 ±0.29 vs. 7.32 ±0.5 min), a lower bladder capacity (0.09 ±0.01 vs. 0.18 ±0.01 mL) and micturition volume (0.07 ±0.01 vs. 0.14 ±0.01 mL), a lower bladder compliance (0.007 ±0.001 vs. 0.02 ±0.002 mL/cmH₂O), and a higher spontaneous bladder activity (5.1 ±0.5 vs. 2.6 ±0.6 cmH₂O) than WT mice (all p < 0.05 using Student’s t-test).

**Figure 2**: Electrical field stimulation (EFS) of bladder strips from wildtype (WT; black; n = 2 x 5) and cannabinoid type 1 receptor knock-out (CB₁ KO; grey; n = 2 x 6) mice at frequencies of 1, 2, 4, 8, 16 and 32 Hz (x-axis). Contraction force of bladder strips is presented in reference (in percent; %) to maximum KCl-induced contraction force (y-axis). Student’s t-test was used for statistical comparisons, statistical differences between WT and CB1 KO bladder strips at 2, 4, and 8 Hz (p < 0.05).

**Figure 3**: 15 minutes (x-axis) representative urodynamic (cystometric) tracings with intravesical pressure on top (y-axis; cm H₂O) and voided volume below (y-axis; mL) under artificial bladder filling (2mL/h) without any anesthesia of a WT mouse (3a) and a CB₁ KO mouse (3b).
When normal WT mice were given rimonabant i.p., they showed a significant decrease of IMI, BCap, MV, and BCom. There was no effect of rimonabant on any other urodynamic parameter assessed (Table 2).

### Table 1: mean values (±SEM) of urodynamic parameters of KO and WT mice; IMI = Intermicturition Interval; Bcap = Bladder Capacity; MV = Micturition Volume; RV = Residual Volume; BP = Basal bladder Pressure; IMP = InterMicturition bladder Pressure; TP = Threshold bladder Pressure; MP = Maximum Micturition bladder Pressure; SA = Spontaneous bladder Activity; BCom = Bladder Compliance; Student’s t-test, * = p < 0.05

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<th>MP</th>
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<td>(n = 11)</td>
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### Table 2: mean values (±SEM) of urodynamic parameters of WT mice (n = 5) before and after administration of Rimonabant (CB1 receptor antagonist); IMI = Intermicturition Interval; Bcap = Bladder Capacity; MV = Micturition Volume; RV = Residual Volume; BP = Basal bladder Pressure; IMP = InterMicturition bladder Pressure; TP = Threshold bladder Pressure; MP = Maximum Micturition bladder Pressure; SA = Spontaneous bladder Activity; BCom = Bladder Compliance; paired Student’s t-test, * = p < 0.05

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<td></td>
<td>3.4</td>
<td>±0.9</td>
<td>±0.01</td>
<td>±0.9</td>
<td>±1.3</td>
<td>±3.0</td>
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<td>±0.01</td>
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110
Discussion

CB₁ receptors have been demonstrated in the bladder as well as in the central nervous system (CNS) (1,2,7). Consequently, these receptors may be involved in micturition control both peripherally and in the CNS (spinal and supraspinal structures). In the present organ bath experiments there was no difference between KO and WT mice with respect to pharmacological stimulation with carbachol, indicating that the bladder smooth muscle of CB₁ KO and WT mice has the same ability to contract. This finding was supported by the urodynamic data, where there was no significant difference in bladder pressures between KO and WT mice. Age-matched WT mice were bigger than CB₁ KO mice, but bladder weights and bladder-to-body ratio were not significantly different. This apparent discrepancy might be explained by the fact that bladder weights were assessed 3 days after bladder catheterization, when the bladder showed different degrees of edema, which increased the variation of the measurement.

The present findings on bladder tissue responses to muscarinic receptor stimulation in eight week old mice, are in line with results reported by others (18). However, during organ bath EFS, which is believed to assess neurogenic contractions (19), the bladders of KO animals showed a significantly weaker response, suggesting that the ‘overactive’ voiding pattern of CB₁ KO mice might be due to a different or altered neuronal control of transmitter release. Similarly to the organ bath data presented here, Gratzke et al. reported that in rat, monkey, and human bladder tissue neither anandamide nor CP55,940, a CB₁ CB₂ receptor agonist, had any influence on carbachol induced contractions, however, both modulated nerve induced contractions (2). Together with their in vivo data showing that CP55,940 increased IMI and TP (as indicators of afferent bladder function), the
authors suggested that CB receptors might be relevant for the mechanoafferent bladder function. Pertwee and Fernando also reported that activation of CB\textsubscript{1} receptors reduced electrically evoked mouse bladder contractions in the organ bath, but had no influence on acetylcholine induced responses (20). Tyagi and colleagues reported that activation of presynaptic CB\textsubscript{1} receptors in the human bladder reduced the EFS induced release of acetylcholine (6). Similarly, Hayn et al. reported that ajulemic acid, a CB\textsubscript{1} CB\textsubscript{2} receptor agonists, reduced calcitonin gene-related peptide release in the rat bladder (a marker of afferent sensory neuronal activity), and this effect could be blocked by AM251, a CB\textsubscript{1} receptor antagonist (21). In an electrophysiological recording study Walczak at al. showed that activation of CB receptors of the mouse bladder with AZ12646915, a non-subtype selective CB receptor agonist, reduced afferent nerve firing, whereas this effect could be blocked by AM251 (22). In a similar experiment this effect could also be blocked by AM251, but not by AM630, a CB\textsubscript{2} receptor antagonist (8).

In this study it was shown by in vivo cystometry in non-anesthetized animals that with respect to several urodynamic parameters CB\textsubscript{1} KO mice differed significantly from age-matched WT mice. The WT mice showed a voiding pattern similar to that reported by others who assessed normal mice urodynamically (18, 23, 24). In comparison with the WT mice, CB\textsubscript{1} KO mice had a significant shorter micturition interval with a subsequent reduced micturition volume and bladder capacity. Additionally, the KO mice showed lower bladder compliance and higher spontaneous bladder activity than WT mice. Several urodynamic studies using anesthetized chemical cystitis models showed that CB\textsubscript{1} (but not CB\textsubscript{2}) receptor activation reduces micturition threshold pressures (25, 26). As such, urodynamic data support the idea that CB\textsubscript{1} KO mice have a different voiding
pattern compared to normal mice. Frequency is believed to be a marker for bladder neuronal control (27), whereas pressures depend rather on the integrity of the bladder muscle.

The CB₁ KO mouse is known to have an ‘anxious’ phenotype (13). This might lead to a higher level of stress during the experiments, and consequently to a higher micturition frequency, since it is known that psychological stress can lead to bladder overactivity (28). This would suggest that the CB₁ receptors within the CNS mediate an inhibitory effect on micturition. However, if a lack of CB₁ receptors would affect voiding only via CNS sites of action, it should not affect the reactivity towards EFS in the bladder strips (which was observed here). In analogy to the ‘overactive’ voiding pattern of CB₁ KO mice, it has also been reported that CB₁ KO mice have an increase colonic propulsion (29), and it has been speculated that there might be a relevant synergy between bowel and bladder overactivity (30).

The role of CB₁ receptors for normal micturition was confirmed by administration of rimonabant, a CB₁ receptor antagonist, which caused urodynamic changes similar to those found in the CB₁ KO mouse. Since rimonabant is known to cross the blood-brain-barrier (31), it can only be speculated on whether or not the CB₁ receptor related changes in micturition are mainly related to a CNS or peripheral nervous site of action (or both).

The importance of afferent bladder activity in the pathogenesis of bladder dysfunction is becoming increasingly evident (32). Mukerji and colleagues showed that patients with idiopathic detrusor overactivity (IDO) had a significant higher CB₁ immunoreactivity in bladder nerve fibres than healthy controls (33). Whether the increase of CB₁ receptors in their study was the cause or consequence of IDO can only be speculated on. However,
the authors concluded, based on the existing literature, that CB$_1$ receptor agonists might be useful in the treatment of IDO. Drugs for the treatment of LUTS and OAB, might exert their *clinically relevant* effects via a CNS site of action as well as acting on the peripheral organ. Since peripheral bladder nerves (ending within the bladder wall) and relay stations, such as the dorsal root ganglia and spinal cord interconnection centers, are lying outside the blood-brain-barrier (BBB) (34), a relevant nervous site of action might be possible and true also for drugs which do not cross the BBB.
Comments

The presented data suggest that the CB₁ receptor is relevant for normal micturition – most likely at both peripheral and CNS sites. CB₁ KO mice showed an ‘overactive’ voiding pattern compared to WT mice, and it may be speculated that CB₁ receptor agonists can be useful for treatment of bladder disturbances characterized by a high micturition frequency, such as OAB and/or storage LUTS. Designing and testing peripheral CB₁ receptor agonists, which do not cross the blood-brain-barrier (but still reach peripheral nerves and neuronal relay centers), might be a way to harness the beneficial effects of the cannabinoid system on voiding without inducing the well-known CNS psychotropic side effects.

Acknowledgements

The study was partly supported by a grant of the Deutsche Forschungsgemeinschaft (DFG) GR 3333/2-1
References

8. Walczak JS, Cervero F. Local activation of cannabinoid CB(1) receptors in the urinary bladder reduces the inflammation-induced sensitization of bladder afferents. *Mol Pain*; 7: 31
13. Pape HC, Pare D. Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiol Rev*; 90: 419-63
22. Walczak JS, Price TJ, Cervero F. Cannabinoid CB1 receptors are expressed in the mouse urinary bladder and their activation modulates afferent bladder activity. *Neuroscience* 2009; 159: 1154-63
31. Wu YK, Yeh CF, Ly TW, Hung MS. A new perspective of cannabinoid 1 receptor antagonists: approaches toward peripheral CB1R blockers without crossing the blood-brain barrier. *Curr Top Med Chem*; 11: 1421-9
32. Kanai A, Andersson KE. Bladder afferent signaling: recent findings. *J Urol*; 183: 1288-95
CHAPTER VII

Characterization of Bladder Function in a Cannabinoid Receptor Type 2 Knockout Mouse in vivo and in vitro

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†Both authors contributed equally to the work

The following chapter has been accepted for publication in Neurourology and Urodynamics

Literature citations and figure formats are adherent to the guidelines set forth in Neurourology and Urodynamics

Lysanne Campeau is responsible for almost all data collection and analysis.
Abstract

AIMS: The contribution of individual CB receptors (CB1R and CB2R) to normal micturition has not been clearly defined. Our goal was to study if differences in urodynamic parameters or *in vitro* bladder contractility can be demonstrated between CB2R knockout (CB2RKO) and C57BL/6J control (WT) mice.

METHODS: Female WT and CB2RKO mice underwent bladder catheterization and cystometry was performed after two and three days. Cystometric evaluations were performed in awake animals without drug administration, and WT were also given HU-308 (CB2R agonist) followed by AM630 (CB2R antagonist). Bladders were removed for in vitro assessment of contractile responses to carbachol and electrical field stimulation (EFS).

RESULTS: CB2RKO mice had significantly higher intercontraction intervals (ICI), bladder capacity (BC) and compliance (Bcom) than WT controls (p<0.05). In WT mice, BC and ICI were increased from baseline by HU-308 exposure, and then returned to baseline levels after AM630 administration (p<0.05). There were no differences in contractility after carbachol or EFS between the groups.

CONCLUSIONS: Lack of CB2R was associated with longer ICI and higher BC and Bcom than its presence (WT controls). This was unexpected since in WT, an increase in BC and ICI from baseline was observed after CB2R agonist administration, and this action was reversed by a CB2R antagonist. Since there were no differences in the *in vitro* responses to carbachol and EFS in bladder strips, it may be speculated that the urodynamic differences are caused by a change in the central nervous micturition control in CB2RKO animals.
Introduction

Treatment of voiding dysfunction such as overactive bladder has been clinically restricted to a few pharmacological targets, including the cholinergic (muscarinic acetylcholine receptors) and adrenergic systems ($\beta_3$-adrenoceptors). Extensive studies have demonstrated that several other systems, such as purinergic and vanilloid receptors or potassium channels, play a significant role in the control of micturition, but have yet to render approved pharmacotherapeutics. The contribution of other endogenous systems to the control of micturition is under investigation with the goal to expand our therapeutic armamentarium.

Whole plant cannabis extract was studied in an open label trial in patients with advanced multiple sclerosis (MS) and severe lower urinary tract symptoms (LUTS), and a significant decrease in urinary urgency, the number and volume of incontinence episodes, frequency and nocturia was demonstrated.(1) These findings were followed by a randomized multicenter placebo controlled clinical trial where oral administration of cannabis extract, $\Delta_9$-tetrahydrocannabinol, or placebo was given to patients with MS. Both active compounds significantly decreased urgency incontinence episodes compared to placebo.(2)

The endocannabinoid system is composed of at least two major arachidonate-derived ligands, anandamide and 2-arachidonoylglycerol, that mediate their effects by binding to the cannabinoid (CB) receptors type 1 (CB1R) and type 2 (CB2R). Both CB1R and CB2R have been localized to the rat bladder, particularly on the urothelium.(3) CB1 immunoreactive fiber density was significantly increased in the suburothelium of bladder specimens from patients with painful bladder syndrome and idiopathic detrusor
overactivity, and correlated with symptom scores. (4) Bladder CB2R possibly mediated the effects of oral cannabinoid agonist treatment in a placebo-controlled study on MS patients. CB2R mRNA expression was higher in the bladder of MS patients, and decreased after active treatment. (5) Spinal cord and dorsal root ganglia CB2R expression was significantly up-regulated in inflammatory and neuropathic pain conditions in rats, and may help mediate analgesic effects. (6) CB2R have also been localized in sensory and cholinergic bladder nerves of rats. In the same study, specific CB2R agonism was found to increase micturition interval and threshold pressure, suggesting a role in the sensory function of voiding. (7) Although evidence indicates that both CB2R and CB1R are involved in micturition, their defined role in normal or pathological voiding is yet to be elucidated. A possible way to define the individual roles of the receptors would be to study mice with deletions of CB1R or CB2R. We used a mouse that is deficient for the CB2R generated by inactivating the gene through homologous recombination with a mutation removing part of intracellular loop 3, transmembrane domains 6 and 7 and the carboxy-terminus. The homozygous mice were generated with the expected Mendelian frequency and found to be as healthy and of similar morphology as their wild type littermates. (8)

In this investigation we wanted to study if there were differences between CB2R knockout (CB2RKO) and C57Bl/6J wild type (WT) mice with respect to urodynamic parameters, and in vitro bladder contractility.
Materials and Methods

Study design

A total of 15 C57BL/6J female mice (WT) and 15 B6.129P2-Cnr2tm1Dgen/J female mice (CB2RKO) with a targeted KO mutation at the CB2R gene, aged between 8 and 10 weeks, were involved in this study. The gene was disrupted by homologous recombination and the animals were obtained from Deltagen (San Mateo, CA). Experiments were approved by the local Animal Care and Use Committee and conducted in accordance with the National Research Council publication Guide for Care and Use of Laboratory Animals. The animals were housed in groups, and then individually after surgery, in a 12 hour light-dark cycle at Wake Forest University Health Sciences. Food and water were available ad libitum. Cystometry was performed in conscious animals, two and three days after inserting a suprapubic bladder catheter. Bladders were extracted for in vitro assessment of contractility to carbachol and electrical field stimulation (EFS).

Catheter implantation

Under inhalation anesthesia, the abdomen was opened through a mid-line incision. A polyethylene catheter (Clay-Adams PE-10, Parsippany, NJ, USA) with a cuff was inserted into the dome of the bladder and held in place with a purse string 6-0 silk suture. The catheter was tunneled subcutaneously and anchored to the skin of the back with a 5-0 polyglactin suture. The free end of the catheter was heat-sealed. Wounds were closed in layers and the animals were allowed to recover.
Cystometry

Cystometric investigations were performed without anesthesia two and three days after the bladder catheterization. Catheters were connected via a T-tube to a pressure transducer (P23 DC, Statham Instruments Inc; Oxnard, California) and to a micro injection pump (CMA 100, Carnegie Medicine AB, Solna, Sweden). The conscious mouse was placed without any restraints in a metabolic cage and room temperature saline was infused into the bladder at a rate of 1.5 ml/hour. Intravesical pressures were recorded by having the pressure transducers connected to an ETH 401 (CD Sciences, Dover, NH) transducer amplifier and, consequently, connected to a PowerLab/8e (Analog Digital Instruments, Castle Hill, NSW, Australia) data acquisition board. Voided volumes were collected, but could not be reliably measured for each micturition, and were therefore not recorded. During the first evaluation two days after catheterization, baseline cystometry was recorded for 30 minutes after the animals stabilized and got accustomed to their new environment (approximately 30 minutes). WT animals were then given an intraperitoneal dose of 5 mg/kg of HU-308 (CB₂R agonist) (Tocris Biosciences, Minneapolis, MN), based on tolerated dose range used in pain studies and other animal models (9, 10), and cystometric data were recorded for another 30 minute period. This was followed by the animals receiving an intraperitoneal dose of 5 mg/kg of AM630 (Cayman Chemicals), based on tolerated and efficacious antagonism of CB₂R in previous pain studies and other animal models (11, 12), and a 30 minute period of cystometric recording. During the second evaluation three days after catheterization, conscious cystometry was performed without drug administration. The following cystometric parameters were investigated: maximum pressure (MP) (maximum bladder pressure during a micturition cycle),
threshold pressure (TP) (pressure at initiation of voiding contraction), basal pressure (BP) (lowest pressure in between voids), intermicturition pressure (IP) (average calculated pressure between voids), spontaneous activity (SA) (IP minus BP), intercontraction intervals (ICI), bladder capacity (BC), bladder compliance (Bcom) (BC divided by TP minus BP), area under the curve (AUC) as a proxy for non-voiding contractions. AUC is a calculated integral that correlates with overall bladder pressures between voiding contractions and has no particular threshold.

Organ bath

Bladders were dissected and placed in Krebs solution on ice. Two longitudinal strips for each bladder were suspended on hooks in 15 ml organ baths containing the Krebs solution, maintained at 37°C and gassed with 95% O_2 - 5% CO_2, pH 7.4. The upper hook was attached to a Grass FT03 isometric transducer, and force was recorded on a real-time data-acquisition system DMT organ bath system (750TOBS) and ChartLab software (v1.1). Tissues were allowed to equilibrate under a resting tension of 1.5 g for at least 60 min, during which time the Krebs solution was replaced regularly. Contractions were recorded as changes in tension from baseline in response to pharmacological and EFS. The strips were first bathed in a Krebs solution containing 60 mM KCl (NaCl exchanged for KCl). This was followed by a carbachol concentration-response curve, generated by adding increasing concentrations of carbachol at 0.5 logarithmic increments starting at 3 nM up to 100 μM. EFS was performed where the strips were placed between two platinum electrodes in the organ chamber. An electrical pulse (0.1 ms pulse width, 20 V in the bath) was delivered, lasting 10 seconds at increasing frequencies (1, 2, 4, 8, 16 and
32Hz), using an S88 stimulator (Grass Instruments). This initial frequency response curve was followed by an incubation of 30 minutes with 10 μM of atropine. The frequency-response curve was repeated in order to pharmacologically dissect the neurogenic responses. All tissue responses were normalized to gram of tissue weight. Drug concentrations were expressed as final concentration in the bath.

Statistical analyses

Cystometric variables were compared between both groups using independent two-tailed Student’s T-test and compared between periods of drug administration using ANOVA of repeated measures, followed by Bonferroni post-hoc testing. The carbachol dose-response, half maximal effective concentration (EC50) and EFS frequency-response obtained for each strip were compared using independent two-tailed Student’s T-test. All statistical analysis and calculations were obtained using GraphPad Prism. All values are expressed as the mean ± standard error of the mean. A probability of p < 0.05 was considered significant.

Drugs and Chemicals

For stock solution preparation, HU-308 and AM630 were initially diluted in DMSO, and maintained at –20 Celsius. The stock solutions were diluted in sterile saline at the appropriate concentration for intraperitoneal injection. Drugs and chemicals for organ bath studies were obtained from Sigma Chemicals (St. Louis, MO).
Results

A total of 30 mice (15 C57BL/6J female mice and 15 B6.129P2-Cnr2tm1Dgen/J female mice) underwent a bladder catheter implantation. There was no morbidity or mortality following the procedure. The CB2RKO mice were slightly younger (2.3 days) than the WT animals, and had a lower body weight. However, there was no statistically significant difference between their mean bladder weight and bladder/body weight ratio (Table 1).

Table 1
Age, body and bladder weight of mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CB2RKO</th>
<th>p</th>
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<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>15</td>
<td></td>
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<tr>
<td>Age (days)</td>
<td>70.0 ± 1.8</td>
<td>72.3 ± 1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>17.5 ± 0.4</td>
<td>19.1 ± 0.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bladder weight (g)</td>
<td>0.039 ± 0.001</td>
<td>0.040 ± 0.003</td>
<td>0.53</td>
</tr>
<tr>
<td>Bladder / body weight ratio</td>
<td>-</td>
<td>0.0022 ± 0.0001</td>
<td>0.0021 ± 0.0001</td>
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Cystometric evaluation

We obtained analyzable cystometric tracings without drug administration for a total of 13 animals in each group. Cystometry curves from two animals in each group could not be analyzed due to movement artifacts. CB2RKO mice had significantly longer ICI and higher BC and Bcom than WT animals (p<0.05; Table 2). Figure 1 illustrates representative baseline tracings of animals during conscious cystometry without drug administration.
Table 2
Baseline cystometric parameters

<table>
<thead>
<tr>
<th></th>
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<th>CB₂RKO</th>
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<tbody>
<tr>
<td>n</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>ICI</td>
<td>140.0 ± 19.8</td>
<td>209.9 ± 21.7  *</td>
</tr>
<tr>
<td>Bcap</td>
<td>0.058 ± 0.008</td>
<td>0.087 ± 0.009  *</td>
</tr>
<tr>
<td>MP</td>
<td>29.5 ± 1.6</td>
<td>28.4 ± 1.5</td>
</tr>
<tr>
<td>TP</td>
<td>21.6 ± 1.8</td>
<td>20.5 ± 1.2</td>
</tr>
<tr>
<td>BP</td>
<td>10.2 ± 1.6</td>
<td>8.3 ± 1.3</td>
</tr>
<tr>
<td>IP</td>
<td>15.5 ± 1.5</td>
<td>13.2 ± 1.1</td>
</tr>
<tr>
<td>SA</td>
<td>5.3 ± 0.04</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>AUC</td>
<td>16.5 ± 1.4</td>
<td>14.3 ± 1.1</td>
</tr>
<tr>
<td>Bcom</td>
<td>0.0054 ± 0.0006</td>
<td>0.0074 ± 0.0007  *</td>
</tr>
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</table>

Each value represents mean ± SEM. Values compared with Student’s T-test. * p < 0.05 CB₂RKO vs WT.

The cystometric evaluation with drug administration provided analyzable tracings for 9 WT mice. Six of the animals’ cystometry curves could not be analyzed due to movement artifacts. In WT mice, ICI and BC was increased from baseline by HU-308 (p<0.05), and then returned to baseline after AM630 administration (Table 3).
Table 3
Cystometric parameters in WT

<table>
<thead>
<tr>
<th></th>
<th>n = 9</th>
<th>Baseline</th>
<th>HU-308</th>
<th>AM630</th>
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<tbody>
<tr>
<td>ICI</td>
<td>seconds</td>
<td>97.9 ± 11.2</td>
<td>119.5 ± 15.9 *</td>
<td>98.2 ± 18.7 **</td>
</tr>
<tr>
<td>Bcap</td>
<td>mL</td>
<td>0.041 ± 0.005</td>
<td>0.050 ± 0.007 *</td>
<td>0.041 ± 0.008 **</td>
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<tr>
<td>MP</td>
<td>cm H₂O</td>
<td>31.8 ± 1.4</td>
<td>32.8 ± 1.2</td>
<td>31.8 ± 1.2</td>
</tr>
<tr>
<td>TP</td>
<td>cm H₂O</td>
<td>23.8 ± 1.4</td>
<td>26.5 ± 1.0 *</td>
<td>24.6 ± 1.0</td>
</tr>
<tr>
<td>BP</td>
<td>cm H₂O</td>
<td>14.5 ± 1.5</td>
<td>13.7 ± 0.9</td>
<td>14.0 ± 1.4</td>
</tr>
<tr>
<td>IP</td>
<td>cm H₂O</td>
<td>19.2 ± 1.8</td>
<td>19.6 ± 1.3</td>
<td>19.0 ± 1.6</td>
</tr>
<tr>
<td>SA</td>
<td>cm H₂O</td>
<td>4.7 ± 0.3</td>
<td>5.8 ± 0.5</td>
<td>4.9 ± 0.8</td>
</tr>
<tr>
<td>AUC</td>
<td></td>
<td>20.0 ± 2.1</td>
<td>20.2 ± 1.3</td>
<td>19.9 ± 1.6</td>
</tr>
<tr>
<td>Bcom</td>
<td>mL/cm H₂O</td>
<td>0.0044 ± 0.0004</td>
<td>0.0039 ± 0.0004</td>
<td>0.0039 ± 0.0006</td>
</tr>
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</table>

Each value represents mean ± SEM. Values compared with ANOVA of repeated measures, followed by Bonferroni post-hoc testing. * p < 0.05 Baseline vs HU-308. ** p < 0.05 HU-308 vs AM630.

Organ bath study

There was no significant difference in contractile response of bladder strips from CB2RKO (n=15) and WT mice (n=15) to KCl stimulation (Figure 2A). The carbachol concentration-response curve in CB2RKO mice was not significantly different from the one of WT mice (Figure 2B). The log [EC50] values for bladder strips from CB2RKO and WT mice were, respectively -5.784 ± 0.115 and -5.868 ± 0.082. The contractile response to EFS was not different between the bladder strips of CB2RKO and WT mice in the absence (Figure 2C) or presence of atropine (Figure 2D). No differences were
observed when values were expressed as per cent of maximum KCl-induced contraction (data not shown).

**Figure 2 Bladder strips contractile responses**  
Contractile response to 60 mM KCl (A), concentration-response curve to carbachol (B), frequency-response to EFS (C), and frequency-response to EFS in the presence of 10 µM atropine (strips from WT n=30, strips from CB2RKO n=30)
Discussion

The presented results suggest that compared to WT, CB2RKO mice had a larger urine storage capacity, reflected in a higher BC and Bcom and longer ICI than the WT animals. Interestingly, this is opposite to what has been previously shown in mice deficient for the CB1R which had significantly lower BC and shorter ICI than WT littermates (data submitted). The findings in the CB2RKO animals were surprising since in WT mice the selective CB2R agonist, HU-308, increased ICI and BC, and that this effect was reversed by AM630. Gratzke et al. also found an increase in the ICI following CB2R activation in normal rats (7, 13), which implies that CB2R agonism can be expected to increase storage function in normal animals.

Since there were no differences in the in vitro responses to carbachol and EFS in bladder strip preparations, it may be speculated that the differences between CB2RKO and WT mice are caused by differences in the central nervous control of micturition.

The CB2RKO mouse has been extensively studied and characterized. (14) The strain was generated by inactivating the gene through homologous recombination with a mutation removing part of intracellular loop 3, transmembrane domains 6 and 7 and the carboxy terminus. (8) CB2RKO mice do not have any gross morphological differences from their wild type counterparts and do not seem to have altered CB1R expression. (8) However, they do display a delay in embryonic development and absence of CB2R can be associated with certain changes in CNS development. (15) For example, Palazuelos et al. demonstrated that CB2RKO mice had significantly decreased neural progenitor cell proliferation in the dentate gyrus of the hippocampus. (16) Both CB1R and CB2R are
also involved in mouse stem cell proliferation.(17) The basal response to nociceptive pain in three nociceptive models with sham surgery were similar in CB2RKO and WT littermates.(18)

It may be speculated that the observed higher BC and longer ICI in the CB2RKO compared to WT mice are caused by developmental changes in the CNS with consequences for the regulation of the micturition reflex, and that congenital lack of CB2R may lead to up-regulation of mechanisms producing changes similar to those of acute CB2R stimulation in normal mice. There is evidence that chronic CB2R activation following an injury can change voiding function. A selective CB2 agonist administered after spinal cord injury allowed a faster recovery of spontaneous voiding.(19) Also, CB2R agonists improved voiding efficiency after partial urethral obstruction in rats.(20)

Therefore, the absence of CB2R mediated activity during development may, possibly through CNS changes, modulate voiding function in the adult CB2RKO mouse.

Peripherally, CB2R have been localized to the detrusor in rats and in urothelium and detrusor muscle in humans. (21) Other studies have identified the receptor in higher density in urothelium than in the detrusor of rats (13), and a role for CB2R in the sensory function of voiding has been suggested.(7) We found in WT mice that CB2R stimulation increased significantly BC and ICI as compared to baseline. It cannot be excluded that at least part of this effect is caused by CB2R mediated decrease in afferent activity.

Although we observed significant differences in cystometric parameters between CB2RKO and WT mice, differences were not seen in the organ bath studies, which suggested similar responses to muscarinic receptor stimulation and EFS of nerves. In a
previous study, CP55,940, a CB2R agonist, did not change the baseline tone or carbachol induced contractions of isolated detrusor strips from rats, monkey and human. However, the drug decreased the contractions evoked by EFS in all species.(13) Tyagi et al. reported similar results, showing a decreased detrusor contraction amplitude in response to EFS in human bladder strips in the presence of GP1A, a CB2R agonist.(22) Bakali et al. showed contradictory findings of decreased EC50 values in the carbachol response curve of rat bladder preparations in the presence of GP1A, while no effect was seen on EFS-induced contractions.(21) These different findings may relate to species and drug variations. Our findings suggest that the absence of CB2R in mice does not alter the responses to muscarinic receptor activation or the responses to EFS of nerves. Considering our observed cystometric differences, a more detailed evaluation of the peripheral contribution of other CB receptors in the CB2RKO mouse could be done in the future by assessing contractility to EFS in the presence of specific CBR agonists.

Our results with the CB2RKO mice can be particularly useful in predicting the role of CB2R up-regulation caused by different pathologies in an animal model. As previously mentioned, CB2R was found to be upregulated in bladder of patients with MS, and decreased after treatment.(5) These receptors are up-regulated in the spinal cord in neuropathic pain and inflammatory states (6, 23), and in the bladder following acute or chronic inflammation(24). The abundant CB2R likely has immunomodulatory activity, with its stimulation causing immune response from inflammation to neuroprotection.(25) Therefore, the CB2RKO mouse could help establish how CB2R activation following inflammatory conditions affecting the bladder may change voiding function. Selective
CB2 activation, with compounds such as HU-308, provides anti-inflammatory and peripheral analgesic activity while causing very little psychoactive side-effects (9), thus presenting an interesting clinical opportunity.
Conclusions

Lack of CB2R was associated with longer ICI and higher BC and Bcom than when CB2R is present (WT controls). This was unexpected since in WT, an increase in BC and ICI from baseline was observed after CB2R agonist administration, and this action was reversed by a CB2R antagonist. Since there were no differences in the in vitro responses to carbachol and EFS in bladder strips, it may be speculated that the urodynamic differences are caused by a change in the central nervous micturition control in CB2RKO animals.
References

CHAPTER VIII

Discussion and future perspectives
The studies described in the previous chapters have determined that the dopaminergic and CB system play a significant role in the CNS control of micturition. We set out as a first aim to determine the impact of CNS DA deficiency and allogeneic stem cell replacement therapy on the long term voiding function in an animal model of PD. We have demonstrated that a unilateral lesion with 6-OHDA induce prolonged urodynamic effects for up to 8 weeks, and that allogeneic rBMSC promote improvements in urodynamic pressures, possibly by preserving or stimulating a faster recovery of dopaminergic cells. These findings, limited to areas of the brain, shed some light on potential underlying pathophysiology of LUTS in patients with PD and may lead to future neuroregenerative therapeutic approaches.

In order to understand more clearly how the application of rBMSC transplantation in our animal model produced significant urodynamic changes, the underlying effect of the 6-OHDA lesion needs to be established, along with its time-course. Injection of 6-OHDA in the nigrostriatal pathway causes dopaminergic and adrenergic cells to take it up and destroys nigral cell bodies and dopaminergic striatal nerve terminals through neurotoxic oxidative stress mechanisms. It forms reactive oxygen species and free radicals and is a potent inhibitor of the mitochondrial respiratory chain complexes I and IV. It also acts via extracellular auto-oxidation and the induction of oxidative stress from the oxidative products generated. We did not determine how long 6-OHDA remains present within the area injected or the extent of the area involved, as a function of the dose given. However, we chose to use an animal model where 6-OHDA was injected in the MFB as compared to the striatum, as it has been shown to achieve a more complete
and severe lesion within the nigrostriatal pathway. This would represent a more advanced stage of PD, when LUTS are more prevalent. This model has been previously characterized for 28 days, showing increased MP, TP, SA and decreased Bcom.(6) We have therefore extended these findings to 42 days.

We chose the SNpc for injection of stem cells for several reasons. We speculated that injection of stem cells in the same area where the 6-OHDA was injected was not optimal and may present a hostile injection site. As DA neurons in the SNpc degenerate, they produce high levels of reactive oxygen species, DA and iron which renders the remaining damaged neuron even more vulnerable to oxidative injury.(7) Therefore, as the cell bodies within the SNpc are in the most precarious situation, we hypothesized that they would benefit from a direct possible neuroprotective antioxidant effect of BMSC.(8) The site of injection in cell replacement therapy is important, because certain areas of the brain are known to harbor progenitor cells capable of proliferating and remain active throughout adult life.(9) The hippocampal subgranular zone and the subventricular zone can take part in neurogenesis and cell proliferation and therefore could be a future source of cell replacement therapy. The notion of neuroprogenitor cell sites could be applied to target brain areas more likely to be involved in regenerative processes, by either directing cell migration or proliferation or by harvesting these cells and transplanting them to affected areas.

The BMSC may provide some pro-survival and neuroprotective factors by secreting different growth factors and cytokines such as glial-derived neurotrophic factor (10),
vascular endothelial growth factor (11) and nerve growth factor (12), to name a few. They may be considered analogous to drug delivery carriers, where pharmacological principles such as a dose-response effect may apply. This has been described in other models looking at functional recovery after severe skeletal muscle injury, where a logarithmic dose-response relationship of mesenchymal stem cell transplantation was observed for both maximum twitch and tetanic contraction forces.(13) Future studies could determine the most optimal concentration of cells required to obtain functional urodynamic improvements.

We describe that the identification of GFP positive cells in the brains of rats transplanted with rBMSC decrease over time. This could mean that rBMSC survival decreases with time through a process of immunological destruction, migration or apoptosis. However, we cannot rule out the possibility that GFP expression decreases without affecting the viability of the cells. Although we infected our rBMSC with a reliable EGFP tagged lentivirus, and selected GFP positive cells with flow cytometry, it is possible that the fluorescent signal is somewhat time-sensitive and impairs our detection by fluorescent microscopy.(14) An alternative to more consistently detect GFP positive cells would be to use a GFP antibody to quantitatively analyze the expression volume of GFP.(15)

As the animals implanted with rBMSC alone without microencapsulation generated more profound urodynamic changes at 42 days than animals implanted ErMBSC, we suggest that a juxtacrine effect may be required to induce functional changes. However, this finding should be carefully interpreted because other confounding factors may play a role
in our differential improvement. Indeed, the microcapsules isolate the cells from possible immunological destruction while preserving the effect of growth factor release. The microcapsules were found to create small cavities within the SNpc which may cause underlying trauma to the damaged DA neurons. The void created by microcapsule has been seen when implanted in the striatum, but not reported to cause adverse effect or hinder outcomes.(16) In order to determine whether the effect of BMSC is mediated via juxtacrine or paracrine, further in vitro studies using co-culture or conditioned media of BMSC and neurons would provide some clarification on whether cell-to-cell interaction is required in process of neuroregeneration or if cells act mainly as vehicles to deliver cytokines and growth factors to host neurons.

Regenerative pharmacology has been defined as “the application of pharmacological sciences to accelerate and optimize (either in vitro or in vivo) the maturation and function of bioengineered tissues”.(17) The endocannabinoid system has the potential to be exploited to facilitate neuroregeneration in conditions causing LUTS. However, prior to developing regenerative strategies using CB compounds, we need to understand in more details the role of cannabinoid receptors in terms of subtype and primary site of action in the control of micturition.

As our second aim, we wanted to study the contribution of cannabinoid receptors such as CB1 and CB2 in the central and peripheral nervous system control of micturition in an animal model. We first focused our attention to the CB contribution in the spinal cord role in normal voiding. Central application of CB receptor agonist localized to the spinal
cord was found to increase bladder capacity, possibly mediated by the activation of spinal TRPV1 channels, and likely through an afferent pathway. The overall involvement, both peripheral and central, of CB₁R and CB₂R in normal micturition were examined individually by characterizing the in vivo and in vitro bladder function in their respective KO mice. The absence of CB₁R was associated with a smaller BC and more SA during cystometry and a lower response to electrical stimulation of nerves. These findings demonstrate that CB₁R is involved at a peripheral site, and also possibly at the CNS. On the other hand, lack of CB₂R was linked with lower BC and higher Bcom than when CB₂R is present. Since there were no differences in the in vitro responses to carbachol and EFS in bladder strips, it may be speculated that the urodynamic differences are caused by a change in the central nervous micturition control in CB₂RKO animals.

Besides playing an active role in the neural control of micturition peripherally and centrally, the endocannabinoid system has been shown to be neuroprotective in several conditions such as neuro-inflammation, cerebral ischemia and chemical-induced neuro-injury. They have neuromodulatory, anti-excitotoxic, anti-inflammatory and vasodilatory properties through several different mechanism such as suppressing pro-inflammatory cytokine production and affecting intra-cellular signaling pathways.(18) Treatment with CB₂R agonist following spinal cord injury improved bladder recovery by modifying the inflammatory response.(19) Therefore, the endocannabinoid system could be a therapeutic target for neurodegenerative processes such as PD, and consequently also impact the voiding dysfunction involved.
As mentioned in previous chapters, the endocannabinoid system is involved in the modulation of post-synaptic transmission of norepinephrine and DA. They may also affect DA transmission indirectly via GABA-ergic and glutamatergic signaling particularly within the basal ganglia, and may therefore be involved in the control of movement. PD patients were found to have increased levels of endocannabinoids within their cerebrospinal fluid.(20) This suggests that the endocannabinoid tone might be increased in PD, and that patients may benefit from CB antagonists. Preclinical studies have demonstrated an increase in density of CB$_1$R in the substantia nigra in an animal model of PD generated by an intracerebroventricular injection of 6-hydroxydopamine. The injection of low-dose rimonabant, a CB$_1$R antagonist, attenuated the resulting hypokinesia observed in the animals.(21) However, the effect did not appear to modify directly DA transmission, and may possibly involve other targets such as TRPV1. In fact, TRPV1 channels are highly expressed in the substantia nigra and in the dorsal striatum.(22) TRPV1 antagonism in a similar animal model also elicited a decrease in hypokinesia during motor function evaluation tests.(23) It would be of particular interest to study the impact of allogeneic stem cell therapy in our animal model on the CB system by looking at CB receptor profile or levels of endocannabinoids, and how they correlate with voiding dysfunction on urodynamic assessment.

These findings present several interesting avenues to explore in the field of neurogenic voiding dysfunction for potential clinical translation. On one hand, chronic administration of CB agonist could slow down the progression of neurodegenerative or neuro-inflammator conditions through immunomodulatory and regenerative processes. While
on the other hand, acute administration and CB and TRPV1 antagonist may directly result in changes in voiding function caused by neurological conditions. As we continue to clarify the role of CB and DA in normal and pathological conditions for the CNS control of micturition, we can develop novel therapeutic strategies such as allogeneic stem cell replacement therapy, with the use of regenerative pharmacology applications of CB compounds.
References


APPENDIX I

Stem cell therapy ameliorates bladder dysfunction in an animal model of Parkinson's disease

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The following chapter has been published in Journal of Urology

Literature citations and figure formats are adherent to the guidelines set forth in Journal of Urology
Abstract

**Purpose:** Different cell-based therapies have been tested in Parkinson’s disease (PD), focusing on the motor function. The objective of this study was to evaluate the effect of human amniotic fluid stem cells and bone marrow derived mesenchymal stem cells (ALLCELLS, Emeryville, California) on bladder dysfunction in a rat model of PD.

**Material and Methods:** A nigrostriatal lesion was induced by 6-hydroxydopamine in 96 athymic nude female rats divided into 3 treatment groups. After 2 weeks, the groups were injected with human amniotic fluid stem cells, bone marrow derived mesenchymal stem cells and vehicle for sham treatment, respectively. At 3, 7, 14 and 28 days the bladder function of 8 rats per group was analyzed by conscious cystometry. Brains were extracted for immunostaining.

**Results:** The nigrostriatal lesion caused bladder dysfunction, which was consistent in sham treated animals throughout the study. Several cystometric parameters improved 14 days after human amniotic fluid stem cell or bone marrow derived mesenchymal stem cell injection, concomitant with the presence of human stem cells in the brain. At 14 days only a few cells could be observed, in a more caudal and lateral position. After 28 days, the functional improvement subsided and human stem cells were no longer seen. Human stem cell injection improved the survival of dopaminergic neurons until 14 days. Human stem cells expressed superoxide dismutase-2 and seemed to modulate the expression of interleukin-6 and glial cell-derived neurotrophic factor by host cells.

**Conclusions:** Cell therapy with human amniotic fluid stem cells and bone marrow derived mesenchymal stem cells temporarily ameliorated bladder dysfunction in a
Parkinson disease model. In contrast to integration, cells may act on the injured environment via cell signaling.
Introduction

Parkinson’s disease (PD) is a chronic neurodegenerative disease characterized by progressive loss of nigrostriatal dopaminergic neurons. PD pathogenesis remains unclear, but pathological processes such as inflammation, mitochondrial dysfunction, oxidative stress, and proapoptotic mechanisms may play a role.\textsuperscript{1, 2} Besides the motor abnormalities, lower urinary tract symptoms may also considerably affect the quality of life of PD patients.\textsuperscript{3, 4}

Current therapeutic options for PD are symptomatic therapies with numerous limitations.\textsuperscript{4} Thus, there has been an intense search for novel treatment. In the restorative/regenerative field stem cell (SC) based therapies aim at replacing lost neuroprotective factors and at being a font of neuroprotective factors to promote endogenous regeneration.\textsuperscript{5} The effect of various cell types from different sources has been investigated in animal models of PD.\textsuperscript{6-10} Clinical trials using human mesencephalic tissue provided the proof of principle for cell replacement in patients with PD but also showed limitations.\textsuperscript{5}

Bone-marrow-derived mesenchymal stem cells (BM-MSC) have the potential to differentiate into several lineages. These cells have a therapeutic potential because they can proliferate extensively in vitro while maintaining their ability to differentiate.\textsuperscript{11} Amniotic fluid stem (AFS) cells are capable of large in vitro expansion and can give rise to cells from all germ layers.\textsuperscript{12} The mechanism of tissue restoration of these SC may involve secretion of cytokines and growth factors, which can modulate inflammatory and immune response and promote endogenous regeneration.\textsuperscript{13, 14}

The neurotoxin 6-Hydroxydopamine (6-OHDA), which selectively destroys dopamine and norepinephrine containing neurons, is widely used to induce parkinsonism in
animals.² We previously reported chronological development of bladder dysfunction in a model of unilateral lesion of the medial forebrain bundle (MFB) induced by 6-OHDA.¹⁵ The primary aim of this study was to evaluate the effect of cell therapy with human BM-MSC and AFS cells on bladder dysfunction using that PD model.¹⁵ We also studied some general markers of (anti)inflammation, oxidative stress and neurogenesis to determine the direction of future studies.
Methods

1. Cell preparation and characterization. BM-MSC expressed MSC markers such as CD105, CD166, CD29, CD44 (each greater than 90%). Cells were negative for CD14, CD34 and CD45. BM-MSCs were expanded in modified α-modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin. For our purposes cells were used at passage 2 or 3.

AFS cells were obtained as previously described. Cells were grown in α-modified Eagle's medium supplemented with 10% ES-FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. AFS cells were positive for CD29, CD44, CD73, CD90, CD105 and SSEA-4 and negative for CD45, CD34 and CD133. Cells of passage 3-4 were used for the present study.

All hSC were fluorescently labeled before injection with a green fluorescent protein (GFP) lentivirus using the system of Klages et al.

2. Animals and experimental design. Female NIH rnu athymic nude rats, weighing 150 gm at the beginning of the experiment, were housed at the Wake Forest University Health Sciences animal facilities. Experiments were approved by the local animal care and use committee and done in accordance with the National Research Council Guide for Care and Use of Laboratory Animals. A total of 96 rats underwent a unilateral nigrostriatal lesion, by stereotactically injecting 8µg 6-OHDA into the right MFB (coordinates: AP: -0.4; ML: -1.6; DV: -7 from bregma). Rats were divided into 3 treatment groups of 32 each. At 2 weeks groups 1 to 3 were injected at the same site with hAFS (10^5 in 4 µL PBS), hBM-MSC (10^5 in 4 µL PBS), and sham treatment vehicle (4 µL PBS), respectively. At 3, 7, 14 and 28 days the bladder function of 8 animals per group was
analyzed by conscious cystometry, and the brain was extracted (figure 1). Two groups of 6 age-matched healthy non-lesioned controls each underwent cystometry to provide a baseline comparison data for the 3 and 7 days groups, and the 14 and 28 days groups.

3. **Cystometry.** Cystometric investigations was done three days after bladder catheterization without anesthesia as previously described.\(^{15}\) We investigated maximum pressure \((P_{\text{max}}:\) maximum bladder pressure during micturition), threshold pressure \((P_{\text{thres}}:\) bladder pressure at onset of micturition), basal pressure \((P_{\text{base}}:\) minimum bladder pressure between two micturitions), intermicturition pressure \((P_{\text{im}}:\) mean bladder pressure between two micturitions), spontaneous activity \((SA: P_{\text{min}} - P_{\text{base}})\), micturition frequency \((MF:\) number of micturitions per hour), bladder capacity \((BC: \) infused volume/MF), micturition volume \((MV)\), residual volume \((RV: BC - MV)\), and bladder compliance \((\text{Com}: BC/(P_{\text{thres}}-P_{\text{base}}))\).

4. **Brain sections staining.** Rats were perfused intracardially with 4% paraformaldehyde, before decapitation. Free floating brain sections (40µm) were incubated overnight with the specific primary antibody: 1. Anti-Tyrosine Hydroxylase (TH) (1:1000; Abcam); 2. Anti-Superoxide dismutase-2 (SOD-2) (1:1000; Abcam); 3. Anti-Interleukin-6 (IL-6)
(1:500; Abcam); and 4. Anti-Gliarial Cell-Derived Neurotrophic Factor (GDNF) (1:50; R&D Systems). The sections were incubated with either biotinylated or Alexa Fluor 488 labeled secondary antibodies (1:200) for 2 hours at room temperature. The sections were mounted on slides and counterstained with either hematoxylin (TH) or DAPI (others).

5. Image analysis. The lesion of the striatal dopaminergic terminal was evaluated by measuring the optical densities of the TH-immunoreactive fibers in the striatum, as previously described.¹⁸ TH-positive cells in the substantia nigra (SN) were counted in both hemispheres in every sixth section, as previously described.¹⁹

6. Statistical analysis. Results are shown as mean ± SEM. Comparisons among groups were made by 1-way ANOVA, followed by Student-Newman-Keuls test. Statistical significance was considered when p<0.05.
Results

1. Bladder function. Three and 7 days after treatment injections, all groups showed altered cystometric parameters compared to healthy age-matched animals. Sham treated rats still showed altered parameters, except for $P_{\text{max}}$, after 14 days and altered BC, MV, MF, SA and Com after 28 days. In contrast, hAFS cells-injected animals showed significant improvement of cystometric parameters after 14 days: BC, MF, SA and $P_{\text{thres}}$. hBM-MSC-injected animals also showed significant improvement after 14 days of: BC, MV, MF, $P_{\text{im}}$, SA, Com and $P_{\text{thres}}$. After 28 days there was no difference regarding any cystometric parameters among the groups (figure 2). Rats did not show changes in behavior during cystometry.
2. Survival and localization of transplanted hSC. At days 3 and 7 after injection hAFS and hBM-MSC remained around the site of injection (coordinates: AP: -0.4; ML: -1.6). At day 14, hSC were in a more caudal and lateral position, corresponding to the internal capsule (coordinates: AP: -3.36; ML: -3.35). The number of cells decreased with time. At
14 days only a few cells were seen, while at 28 days there were no surviving cells. At days 3 and 7 most of the hSC exhibited a normal morphology, with an elongated shape; whereas in 14 days they were mainly rounded (figures 3, 4 and 5).

![Figure 3](image)

Expression of SOD-2 – immunofluorescence. Red is SOD-2, blue is DAPI (nuclei) and green is GFP-labeled human stem cells. Orange/yellow represents co-localization (red+green). A, D and G: some expression of SOD-2 by the rat brain cells was seen in a uniform distribution at day 3, 7 and 14 in sham animals. B, E and H: Expression of SOD-2 was seen in the hAFS cells and in the surrounding rat brain cells at days 3 and 7. At day 14 the hAFS cells no longer expressed SOD-2. C, F and I: hBM-MSC expressed SOD-2 at days 3 and 7, but did not at day 14. Rat brain cells surrounding the hSC expressed SOD-2. Representative pictures of the 28 day endpoint are not presented, because no hSC were observed at this point and no changes in SOD-2 expression was seen.

![Figure 4](image)

Expression of IL-6 – immunofluorescence. Red is IL-6, blue is DAPI (nuclei) and green is GFP-labeled human stem cells. A, D and G: Scarce expression of IL-6 by the rat brain cells was seen in sham animals at any endpoint. B, E and H: Expression of IL-6 was seen in the rat brain cells surrounding and in close contact to the hAFS cells at days 3, 7 and 14. C, F and I: Expression of IL-6 was seen in the rat brain cells surrounding and in close contact to the hBM-MSC cells at days 3, 7 and 14. Representative pictures of the 28 day endpoint are not presented, because no hSC were observed at this point and no changes in IL-6 expression was seen.
3. Dopaminergic nigrostriatal lesion. The 6-OHDA injection induced progressive loss of TH-positive cells in the ipsilateral SN and dopaminergic terminals (striatal density). The number of dead dopaminergic cells in the SN was significantly lower in the animals transplanted with either hAFS cells or hBM-MSC at days 7 and 14 compared with sham animals. The density of dopaminergic terminals in the striatum was significantly higher in the hBM-MSC-transplanted compared with sham treated rats at day 14. (figure 6)
4. Brain and stem cells staining.

4.a. SOD-2. At days 3 and 7, both hAFS cells and hBM-MSC expressed SOD-2. Rat cells surrounding the injected hSCs also expressed the enzyme. In the sham treated group some SOD-2 expression in the host cells was also noted at days 3 and 7. At day 14 hSC no longer expressed SOD-2. Rat brain cells still expressed SOD-2 in a uniform distribution until 28 days (figure 3).
4.b. **IL-6.** Rat brain cells in close contact with the hSC expressed IL-6 at days 3, 7 and 14. In sham treated rats expression of IL-6 was sparse. No IL-6 expression was seen at 28 days in any of the groups (figure 4).

4.c. **GDNF.** At day 3, all three groups showed GDNF expression in the host neurons surrounding the injection area. At day 7, few host cells expressed GDNF in the sham treated group, while in the hSC-injected animals GDNF-positive rat cells were still present close to hSC. The same scenario was seen at 14 days, while no GDNF expression was noted in sham treated animals. At 28 days no GDNF expression was seen in any group. Transplanted human cells did not express GDNF at any time point (figure 5).
**Discussion**

Injection of 6-OHDA into the nigrostriatal pathway is a well established dopaminergic lesion model. When injected into MFB, there is acute cell death, and the model is used to study the late phase of PD.\(^{20}\) We previously evaluated bladder dysfunction chronologically after 6-OHDA injection into the MFB. A defined pattern of bladder hyperactivity was established 14 days post-lesion, which persisted for 28 days.\(^{15}\) Thus, in the current study bladder dysfunction was expected to be established in the animals by the time hBM-MSC and hAFS cells were injected. The rationale for this study was to improve local conditions and/or promote tissue restoration of the initial lesion and to test the possible impact on bladder function. The model has previously been well characterized with respect to motor dysfunction, so this was not investigated this.

Since to our knowledge the effect of SC therapy on PD bladder dysfunction has not yet been reported, we followed the rats at different time-points after the SC injection. The earlier time-points allowed early detection of changes caused by the cell therapy, while the later time-points showed whether changes were sustained. Several urodynamic parameters demonstrated significant improvement at 14 days post treatment injections in rats implanted with either hMB-MSC or hAFS cells, which did not persist for 28 days. This transient improvement may be caused by cell destruction with time.

We studied human derived cells since they lead to a more direct clinical translation. By implanting xenogenic SC into nude athymic rats we tried to avoid host immune reactions. Although they lack T cells, they have normal NK and B cells,\(^ {21}\) which may have been a factor influencing the short survival of the implanted hSC.
Corresponding to the functional improvement, the hSC were seen in the rat brains only until day 14. At this point they had migrated laterally and caudally, which may represent an attempt to reach the SN area, where the dopaminergic cell bodies were progressively undergoing apoptosis. Also consistent is the fact that the survival of TH-immunoreactive cells in the SN of the rats injected with hSC was double the rate seen in the sham animals at 14 days, but reached very low levels, similar to the one seen in sham animals, after 28 days.

The concurrent functional improvement and survival of the hSC in the rat brains and the fact that no clear signs of integration were observed may suggest that the hSC exerted neuroprotection via a paracrine/juxtacrine mechanism. After transplantation, SC may act through the synthesis and secretion of chemokines, cytokines and neurotrophic factors that promote an environment favorable to neuroprotection and neuroregeneration. To test some general mechanisms of action of these cells in this animal model, and to get some directions for future, more focused studies, markers of (anti)inflammation (IL-6), oxidative stress (SOD-2), and neurogenesis (GDNF) were tested. While the host cells expressed SOD-2, the presence of hSCs seemed to modulate host cell expression of IL-6 and GDNF.

IL-6 is a multifunctional cytokine, which is produced in the brain during inflammatory processes. Its role may vary depending on the physiological context. When MSC were implanted in an animal model of traumatic brain injury there was an increase of IL-1α, IL-1β, and IL-6. In in vitro studies using co-cultures of MSC and neural SC (NSC) direct cell-to-cell contact was required for MSC to induce IL-6 production by the NSC and consequent neuroprotection. In our study, the same phenomenon seems to have
occurred in vivo since IL-6 expression was almost exclusively seen in the rat brain cells in close contact to the hSC. In this scenario, both hAFS and hBM-MSC may have modulated the inflammatory response via cell-to-cell signaling to the host cells. However, a paracrine effect cannot be excluded.

The role of oxidative stress in the pathogenesis of neurodegenerative diseases has been reported. Oxidative stress occurs when the production of free radical species overcomes the natural antioxidant mechanisms, which is followed by mitochondrial dysfunction and neuronal damage. Human MSC were shown to be highly resistant to oxidative stress-induced death. These cells constitutively expressed and exhibited activity of enzymes involved in the elimination of reactive oxygen species like SOD-1, SOD-2, catalase and glutathione peroxidase 1. In another in vitro study hBM-MSC were shown to secrete SOD-3 and protect rat cerebellar neurons against oxidative stress. This effect took place via cell-to-cell contact and/or of soluble factor secretion. In our study, we noted that hBM-MSC and hAFS cells expressed SOD-2 in vivo. This may represent an attempt to improve the local scavenging system in an environment of oxidative stress caused by the injection of the neurotoxin.

GDNF is a neurotrophic factor that mediates dopaminergic neuronal survival. GDNF infused into the nigral region decreased or prevented dopaminergic degeneration in animal models of PD. Conditioned GDNF-null mice in which GDNF expression is markedly reduced in adulthood showed extensive catecholaminergic neuronal degeneration and progressive motor dysfunction. In our study, rat brain cells expressed GDNF, which seemed initially to be a consequence of the injection itself, since in both the sham treated and hSC groups expression was seen.
in this area at 3 days. In the presence of the hSC host cell GDNF expression was observed up to 14 days after the injection, while in the sham treated group expression was already sparse at 7 days and absent after 14 days. Thus, we assume that the injected hSC may have had a role in modulating GDNF expression by the host cells.

Experimental studies of cell therapy in animals with PD revealed improved motor function, which was usually associated with graft integration, dopamine production by the injected cells, neurogenesis and neurorescue, and ultimately enhanced neuronal dopaminergic survival. To our knowledge we report the first study of the effect of SC therapy on bladder dysfunction in an animal model of PD. Although there was dopaminergic neuronal preservation and indications of juxtacrine/paracrine effects, the precise mechanism by which the hSC acted to improve the bladder function is yet to be determined. Also, these results bring up discussion about the effect of long-term administration of these factors on bladder dysfunction and on SC survival. Future studies will attempt to prolong the observed urodynamic improvements and to clarify the juxtacrine/paracrine contribution.
Conclusions

The implantation of human BM-MSC and AFS cells in a PD animal model of bladder dysfunction promoted transient urodynamic improvements. The underlying mechanisms are yet to be clarified but they are likely linked to juxtacrine or paracrine effects that are initiated and sustained by the presence of hSC at the injury site.
References


APPENDIX II

Role of spinal cord fatty acid amide hydrolase (FAAH) in normal micturition control and bladder overactivity in awake rats

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The following chapter has been published in Journal of Urology

Literature citations and figure formats are adherent to the guidelines set forth in Journal of Urology
Abstract

Purpose: We assessed whether spinal inhibition of the cannabinoid degrading enzyme FAAH would have urodynamic effects in normal rats and rats with bladder overactivity induced by partial urethral obstruction or prostaglandin E2. We also determined the expression of FAAH, and the cannabinoid receptors CB1 and CB2 in the sacral spinal cord.

Materials and Methods: We used 44 rats for functional (cystometry) and Western blot experiments. The FAAH inhibitor oleoyl ethyl amide (3 to 300 nmol) was administered intrathecally (subarachnoidally) or intravenously. The expression of FAAH and CB1/CB2 receptors was determined by Western blot.

Results

Oleoyl ethyl amide given intrathecally affected micturition in normal rats and rats with bladder overactivity but effects were more pronounced in the latter. In normal rats oleoyl ethyl amide only decreased micturition frequency, while it decreased frequency and bladder pressures in rats with bladder overactivity. Intravenous oleoyl ethyl amide (3 to 300 nmol) had no urodynamic effect. FAAH and CB1/CB2 receptors were expressed in the rat sacral spinal cord. The expression of CB1/CB2 receptors but not FAAH was higher in obstructed than in normal rats.

Conclusions: FAAH inhibition in the sacral spinal cord by oleoyl ethyl amide resulted in urodynamic effects in normal rats and rats with bladder overactivity. The spinal endocannabinoid system may be involved in normal micturition control and it appears altered when there is bladder overactivity.
Introduction

The presence of cannabinoid (CB1 and CB2) receptors, mediating the effects of endogenous CBs (endocannabinoids), has been noted in bladders of various species.\textsuperscript{1-5} Cannabis extracts have improved LUT symptoms in patients with multiple sclerosis.\textsuperscript{6, 7} Systemic administration of CB receptor agonists can decrease urodynamic signs of BO in rats\textsuperscript{8-10}, and pharmacological targeting of the CB system is considered a potential new therapeutic approach to LUT dysfunction.\textsuperscript{11} Unfortunately, exogenous CB receptor agonists may induce serious CNS side effects, such as memory disorders and psychotropic effects.\textsuperscript{12} Inhibiting the breakdown of endocannabinoids, which inherently have a short half-life, and enhancing their endogenous activity might be a way to harness the CB system and decrease side effects.\textsuperscript{12} FAAH is an enzyme important for the endocannabinoid degradation.\textsuperscript{13} FAAH inhibition leads to an increase in N-arachidonoylethanolamide (anandamide) and 2-arachidonyldonoylglycerol and other fatty acid amides that act on CB1 and CB2 receptors.\textsuperscript{12, 13} FAAH is present in the bladder of various species and it may have a role in the local regulation of afferent bladder function.\textsuperscript{14, 15} In normal rats acute systemic inhibition of FAAH decreased micturition frequency\textsuperscript{14}, and FAAH inhibition reduced the urodynamic signs of BO.\textsuperscript{15} Even if the effects of systemically administered FAAH inhibitors may be explained by actions on the bladder\textsuperscript{14}, a CNS site of action cannot be excluded. FAAH has been identified in various CNS regions\textsuperscript{16, 17}, but to our knowledge not at the spinal cord levels involved in micturition control. Activity of the endocannabinoid system is up-regulated, and the effects of FAAH inhibition are more pronounced when in
inflammation and tissue damage are present.\textsuperscript{16, 18} However, it is currently unclear whether the effects of FAAH inhibition are also more pronounced on BO than on normal micturition.

Our main aim was to evaluate the urodynamic effects of a FAAH inhibitor OEtA, given intrathecally (subarachnoidally) in normal rats and rats with BO induced by either PGE2 (acute) or PUO (chronic) to determine whether the drug may have a spinal site of action. We also assessed the expression of FAAH and related CB receptors in the sacral spinal cord of normal rats and rats with BO.
Material and Methods

Study design

We used 44 Sprague-Dawley rats weighing about 200 gm. After receiving ethical committee approval, the rats were housed at the San Raffaele University animal facilities. Four rats died after the PUO procedures and 3 were not evaluated due to development of overflow incontinence.

Eight rats were used for Western blot experiments and 29 were used for functional (urodynamic) experiments. We studied 4 groups, including group 1 - 6 normal rats, group 2 - 7 normal rats with intravesical PGE2 induced (acute) BO, group 3 - 6 rats with PUO induced (chronic) BO and group 4 - 4 normal rats that received vehicle intrathecally. Groups 1 to 3 received OEtA intrathecally. Another 6 rats received OEtA intravenously, of which 3 had undergone PUO creation 14 days earlier.

Partial urethral obstruction (PUO)

PUO was performed as previously described.19 The bladder was identified via a low abdominal incision. A 19 gauge needle was placed on the ventral surface of the urethra and a 3-zero polypropylene suture was tied around the urethra and needle. After suturing, the needle was removed, leaving the urethra partially obstructed. Experiments were performed 14 days after PUO creation.

Bladder catheterization

A small incision was made in the bladder dome. A polyethylene-50 catheter with a cuff was inserted into the bladder and secured with a 5-zero silk purse-string suture.20 The catheter was then tunnelled subcutaneously to the neck, and anchored. The free end was sealed thermally.
**Intrathecal (subarachnoidal) catheterization**

Immediately after bladder catheterization, the atlanto-occipital membrane was exposed and punctured using a stereotactic frame. A 32 gauge CS-1 intrathecal catheter (ReCathCo, Allison Park, Pennsylvania) was inserted and pushed forward to a line 2 fingers above the hip joints. Correct catheter positioning at L6-S2 was confirmed at necropsy after the experiments.

**Cystometry**

Cystometry without anesthesia was performed 3 days after catheterization. The rat was placed in a metabolic cage to enable the measurement of urine volume via an FT03 force displacement transducer (Grass Technologies, West Warwick, Rhode Island) and bladder pressure via a pressure transducer (BD™). Transducers were connected via a CP122 amplifier (Grass Technologies) to an MP150 data acquisition system (Biopac Systems™). AcqKnowledge™ was used for data analysis. The investigated cystometric parameters were the pressure AUC per second between micturitions as a proxy for nonvoiding contractions, BC in ml (infusion rate per minute × ICI), BP in cm H₂O (minimum bladder pressure between 2 micturitions), COM in ml/cm H₂O (MV/(TP – BP), ICI in minutes, MP in cm H₂O, MV in ml, RV in ml (BC – MV) and TP in cm H₂O (BP at micturition onset).
Drug administration

Stock solutions of OEtA (40 mg/ml) (Cayman Chemical, Ann Arbor, Michigan) and PGE2 (0.01M) (Sigma®) were made in absolute ethanol and stored at –80°C. Dilutions were made in saline on the day of the experiment. After 45 to 60 minutes of ongoing cystometry we sequentially administered 3, 30 and 300 nmol (total amounts 0.9, 9 and 90 μg, respectively) OEtA intrathecally as a bolus (10 μl). Cystometry then continued. In another group bladder infusion was changed from saline to PGE2 (100 μM) before OEtA administration. Intrathecal doses were chosen based on pilot experiments. The doses chosen (3 to 300 nmol) were tested intravenously in 3 normal and 3 obstructed rats to exclude systemic effects on urodynamic parameters.

Western blot

At 14 days after PUO 4 PUO rats and 4 nonobstructed age matched controls were sacrificed. Spinal cord segments (S1-S2) were harvested and snap frozen. Western blot was performed according to standard protocols. The primary antibodies used were rabbit anti-FAAH antibody (2 μg/ml), rabbit anti-CB2 (1:200), rabbit anti-CB1 (1:200) (Cayman Chemicals) and mouse anti-GAPDH (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, California). Proteins were visualized using relevant, species directed secondary antibodies and ECL chemiluminescent reagent (GE Healthcare, Hertfordshire, United Kingdom) for Western blots. Signals on the polyvinylidene membrane were quantified using Image Station 440 (Kodak, Rochester, new York) and SigmaScan® Pro 5.0 with GAPDH as the control Statistical analysis
SigmaPlot™ 11.0 was used for statistical analysis. One-way ANOVA for repeated measures, followed by the Student-Newman-Keuls test, was used to compare parameters before and after treatment within groups. Comparisons between 2 groups were made using the Student t test with p <0.05 considered statistically significant. Values are shown as the mean ± SEM.
Results

**Intrathecal OEtA Effects**

**In normal rats**

The table lists baseline urodynamic data on 6 rats. Figure 1, A and B shows a representative urodynamic tracing.

At an intrathecal dose of 3, 30 and 300 nmol OEtA dose dependently increased ICI by 13%, 22% and 30%, BC by 11%, 21% and 30%, and MV by 11%, 21% and 37%, respectively (each p <0.05). Intrathecal OEtA had no significant effect on RV, BP, MP, AUC or COM. At the 3 and 30 nmol doses intrathecal OEtA did not affect TP. However, at the highest intrathecal dose of 300 nmol OEtA increased TP by 45% (p <0.05, see table 1).

| Table 1: Urodynamic parameters in healthy nonPUO rats after increased OEtA and with ongoing intravesical PGE2 before and after 30 nmol OEtA, and in PUO rats after increased OEtA |
|---|---|---|---|---|
| | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM | Mean ± SEM |
| | ICI (ms) | BC (ml) | MV (ml) | RV (ml) | BP (cm H₂O) | TP (cm H₂O) | MP (cm H₂O) | AUC (m/cm H₂O) |
| Baseline OEtA (nmol): | 3 | 7.7 ± 0.4* | 1.27 ± 0.07* | 1.19 ± 0.05* | 0.09 ± 0.03 | 6.0 ± 1.6 | 25.1 ± 4.5 | 75.8 ± 6.3 | 9 ± 1 | 0.068 ± 0.008 |
| | 30 | 8.3 ± 0.5*,† | 1.38 ± 0.08*,† | 1.30 ± 0.10*,† | 0.09 ± 0.02 | 6.2 ± 1.6 | 25.4 ± 2.5 | 70.4 ± 9.0 | 9 ± 1 | 0.069 ± 0.004 |
| | 300 | 8.9 ± 0.5*,†,‡ | 1.49 ± 0.08*,†,‡ | 1.43 ± 0.09*,†,‡ | 0.08 ± 0.05 | 5.1 ± 1.5 | 32.2 ± 4.5* | 66.2 ± 7.1 | 8 ± 1 | 0.058 ± 0.007 |
| Healthy, no PUO, ongoing intravesical PGE2 before + after 30 nmol OEtA (7 rats) | | | | | | | | |
| PGE2 | 3.0 ± 0.3* | 0.49 ± 0.04* | 0.49 ± 0.06* | 0.03 ± 0.01 | 11.6 ± 1.5* | 46.7 ± 6.5* | 110.2 ± 11.1* | 23 ± 5* | 0.016 ± 0.003* |
| 30 nmol OEtA | 3.6 ± 0.35 | 0.60 ± 0.05* | 0.61 ± 0.05* | 0.06 ± 0.02 | 8.4 ± 1.05 | 34.1 ± 5.55 | 97.9 ± 9.05 | 16 ± 45 | 0.029 ± 0.0065 |
| PUG after increased OEtA (6 rats) | | | | | | | | |
| Baseline OEtA (nmol): | 3 | 4.2 ± 0.5 | 1.40 ± 0.17 | 1.37 ± 0.11 | 0.15 ± 0.11 | 16.0 ± 4.3 | 83.1 ± 6.8 | 164.7 ± 24.4 | 24 ± 2 | 0.001 ± 0.003 |
| | 30 | 5.2 ± 0.7* | 1.73 ± 0.23* | 1.68 ± 0.10* | 0.19 ± 0.18 | 12.7 ± 3.5* | 52.2 ± 9.1* | 117.0 ± 24.7* | 17 ± 2* | 0.052 ± 0.011* |
| | 300 | 5.7 ± 0.7* | 1.90 ± 0.24* | 1.83 ± 0.12* | 0.16 ± 0.15 | 11.3 ± 2.7* | 52.4 ± 9.7* | 113.1 ± 25.6* | 14 ± 1* | 0.055 ± 0.012* |

* p <0.05 vs baseline.
† One-way ANOVA, p <0.05 vs 3 nmol OEtA.
‡ One-way ANOVA, p <0.05 vs 30 nmol OEtA.
§ One-way ANOVA, p <0.05 vs PGE2.
Intrathecal administration of only vehicle in 4 rats had no effect on any parameter assessed (data not shown). OEtA (3 to 300 nmol) had no urodynamic effect in 3 rats when administered systemically (intravenously) (data not shown). Neither intrathecal nor intravenous OEtA resulted in an obvious change in behavior in normal or PUO rats.

**In acute BO model using PGE2**

In 7 normal rats acute intravesical instillation of PGE2 (100 μM) decreased ICI by 44%, BC by 45% and MV by 57% compared to baseline values before PGE2. RV remained unaffected by PGE2 but PGE2 increased BP by 132%, TP by 95%, MP by 49% and AUC by 130%, while COM was decreased by 70%. Except for RV, all changes were significantly different from values before PGE2 instillation (p <0.05, see table and fig. 1, C and D).

In rats with PGE2 induced BO the 30 nmol intrathecal dose of OEtA significantly increased ICI by 20%, BC by 22%, MV by 24% and COM by 81%, while it decreased BP by 28%, TP by 27%, MP by 12% and AUC by 31% (each p <0.05, see table and fig. 1, E and F). There was no effect on RV.

![Figure 1](image_url)  
Figure 1: Representative 10-minute urodynamic recordings in healthy rats. A and B, before drug. C and D, during PGE2 instillation. E and F, after 30 nmol intrathecal OEtA. A, C and E, intravesical pressure in cm H2O. B, D and F, voided volume in ml.
In chronic BO model (PUO)

Mean bladder weight in PUO vs nonPUO rats was 413 ± 44 vs 144 ± 8 mg (p <0.05). There was no difference in body weight.

The table lists baseline urodynamic data on 6 PUO rats. Figure 2, A and B shows a representative urodynamic tracing. Compared to healthy controls, PUO rats had higher intravesical BP, increased micturition frequency and decreased bladder COM (see table and fig. 2). In obstructed rats 30 nmol intrathecal OEtA significantly increased ICI by 23%, BC by 23%, MV by 22% and COM by 67%, and decreased BP by 21%, TP by 18%, MP by 13% and AUC by 30% (each p <0.05). There was no effect on RV. Compared to baseline, intrathecal OEtA (300 nmol) decreased ICI by 35%, BC by 35%, MV by 33%, COM by 80%, BP by 30%, TP by 18%, MP by 16% and AUC by 42%. These changes were significant compared to baseline but not to the changes induced by the 30 nmol dose of OEtA (see table and fig. 2, C and D). OEtA intravenously (3 to 300 nmol) had no urodynamic effect in 3 rats (data not shown).

Figure 2 Representative 10-minute urodynamic recordings in PUO rats. A and B, before drug. C and D, after 30 nmol intrathecal OEtA. A and C, intravesical pressure in cm H2O. B and D, voided volume in ml.
Western Blot

The spinal cord of 4 controls and 4 PUO rats showed bands at the expected weight for CB1, CB2 and FAAH. When quantifying CB1, CB2 and FAAH expression in relation to GAPDH, no difference in the amount of FAAH was noted between the groups. In contrast, CB1 and CB2 were significantly increased in the sacral spinal cord of PUO rats (fig. 3).

Figure 3 A and B, Western blot bands of FAAH, CB1, CB2 and GAPDH in 4 normal and 4 PUO rats. C to E, quantified signals in relation to GAPDH in healthy (open bars) and PUO (black bars) rats. C, CB1. Triple asterisks indicate Student t test p < 0.001. D, CB2. Single asterisk indicates Student t test p < 0.05. E, FAAH.
Discussion

The current study shows that FAAH, and CB1 and CB2 receptors are expressed in the rat sacral spinal cord. Intrathecal administration of the FAAH inhibitor OEtA in normal rats decreased micturition frequency with little or no effect on intravesical bladder pressure. It was previously noted that FAAH is expressed in the bladder mucosa and intravesical CB receptor antagonism effectively inhibits the effects of OEtA, suggesting a role for the bladder endocannabinoid system in micturition regulation. Effects on urodynamic parameters after spinal inhibition of FAAH are similar to previous data on intravenous administration of OEtA.

However, since OEtA can penetrate into the CNS, we could not exclude that the drug also has a site of action in the CNS after systemic administration. Assuming an equal distribution of OEtA in the rat body, intravenous injection of 0.3 mg/kg would result in a CSF concentration of around 0.3 μg/ml. Considering the volume of the CSF in the rat, the amounts given intrathecally in our study would result in a CSF concentration of 3 to 300 μg/ml. When corresponding amounts were administered intravenously we noted no effect on urodynamics, suggesting that the effects of intrathecally OEtA were mediated exclusively via a CNS site of action. Also, according to the intrathecal catheter location, as confirmed by necropsy, the site of drug administration was the subarachnoidal space at the sacral spinal cord level.

The increase in ICI (and of TP at the highest dose) as indicator parameter(s) of sensory bladder function suggests that OEtA at the spinal level affects neural pathways that regulate afferent signals for bladder control. This is supported by studies of FAAH in pain models, which showed that FAAH inhibition decreased neuronal sensitivity to
noxious stimuli. When given systemically, the nonsubtype specific CB receptor agonist CP55940 also increased ICI and TP. Based on functional data and staining experiments, in which CB2 immunoreactivity colocalized with TRPV1 in sensory nerves, it was suggested that CB2 receptor agonists may have a role in modulating bladder afferent signals. Similarly, the synthetic tetrahydrocannabinol analogue systemic IP-751 increased ICI and TP.

FAAH inhibition had effects on micturition in normal rats and rats with BO. In contrast, α1-adrenoceptor antagonists and muscarinic receptor antagonists, which are types of drugs commonly used to treat LUT disorders and may also act at spinal sites, have no urodynamic effects on normal animals but affected animals with experimentally induced BO. The investigators concluded that pathways containing α1 and muscarinic receptors had little importance for normal micturition but may be activated in pathological conditions. Current data suggest that the spinal endocannabinoid system may also be relevant for micturition control under normal conditions.

In line with previously published urodynamic data, intravesical instillation of PGE2 as a model of acute BO increased bladder pressure and decreased bladder volume and ICI. Intrathecal OEtA counteracted these effects, decreasing bladder pressure and increasing bladder volume and ICI. In various models of acute BO systemic (intravenous) administration of other compounds that target the CB system had effects similar to those of intrathecal OEtA. In turpentine inflamed rat bladders anandamide attenuated urodynamic signs of BO. Similarly, the nonselective CB receptor agonist WIN-55,212-2 decreased urodynamic signs of BO in the same model; In cyclophosphamide induced
cystitis increased bladder afferent firing on electrophysiological recording was suppressed by intravesical administration of the nonselective CB receptor agonist AZ12646915. Overactivity due to intravesical PGE2 is caused by local effects in the bladder and increased afferent input generated by sensory nerve activation. Intrathecal OEtA reduced the effect of PGE2, implying that in the spinal cord the drug affected the afferent input. Since intrathecal OEtA also decreased gall bladder pressure, including maximum pressure, which reflects motor functions of the detrusor, the drug may also have other effects, eg on interneurons that modify the spinal cord efferent output.

As a chronic model of BO, PUO resulted in increased bladder pressure and AUC, and decreased ICI and micturition volume. These findings are in accord with previous studies. Intrathecal administration of OEtA changed most urodynamic parameters 20% to 30% toward normal, including 60% to 80% for COM. Gratzke at al reported that chronic administration of the selective CB2 agonist cannabinor prevented nonvoiding contractions after PUO in rats. They reported no difference in bladder pressure or ICI. However, since cannabinor was given daily for 2 weeks after surgical PUO to prevent BO and in our series OEtA was administered acutely in rats with already established BO, it is difficult to compare the results of these studies.

Analogous to PGE2/BO, the effects of intrathecal OEtA were more pronounced in rats with PUO/BO than in normal rats, including a decrease in all increased bladder pressures. Furthermore, in contrast to normal rats, in which a dose dependent effect was noted, there was no further effect of an intrathecal OEtA dose of 300 nmol compared to 30 nmol in PUO rats.
In agreement with these findings, in spinal cord segments from PUO rats compared to nonobstructed controls we noted up-regulation of CB1 and CB2 receptors. Similar up-regulation was found in models of chronic pain, inflammation or nerve injury.\textsuperscript{29, 30} In contrast to nerve injury models, in which spinal FAAH decreased, we observed no difference in FAAH expression in PUO vs control rats. Still, our data suggest that the endocannabinoid system in the sacral spinal cord is altered due to PUO, which may be linked to differences in the effects on urodynamic parameters caused by intrathecal FAAH inhibition between normal rats and rats with PUO.
Conclusions

The current study provides evidence that the spinal endocannabinoid system is involved in the regulation of normal and pathological bladder function. Sacral intrathecal administration of the FAAH inhibitor OEtA counteracted urodynamic changes due to acute and chronic BO. Outflow obstruction may be linked to the plasticity of sacral spinal CB receptors, which are targets for FAAH substrates.

Acknowledgements

Giorgio Gandaglia, Urological Research Institute, San Raffaele University, provided technical assistance.
References


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<tr>
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INVITED PRESENTATIONS:

March 21 2013: Exploring new avenues for the treatment of OAB - Cannabinoid Contribution to Micturition. Urology Grand Rounds, Department of Urology, SUNY, Syracuse, USA

November 21 2012: Exploring new avenues for the treatment of OAB - Cannabinoid and Dopaminergic Contribution to Micturition. Urology Grand Rounds, Division of Urology, McGill University, Montreal, Canada

November 24 2011: Stem cell therapy in neurogenic detrusor overactivity - Applications in a Parkinsonian animal model. Urology Research Seminar, Urology Research Institute, Vita-Salute San Raffaele Hospital, Milan, Italy

July 18 2012: Dopaminergic and cannabinoid contribution to central nervous system control of micturition. Urology Grand Rounds, Department of Urology, Ludwig Maximilian University, Munich, Germany
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PUBLICATIONS

a) PEER-REVIEWED ARTICLES

2013
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3) Fullhase C*, Campeau L*, Sibaev A, Storr M, Hennenberg M, Gratzke C, Stief C, Hedlund P, Andersson KE. Cannabinoid receptor type 1 (CB1) is important for normal micturition – results from in vitro and in vivo bladder evaluation of a CB1 knock-out mouse model (accepted to British Journal of Urology International)
*both authors contributed equally to the work

4) Hicks AN, Campeau L, Burmeister D, Bishop CE, Andersson KE. Lack of Nicotinamide mononucleotide adenyltransferase 2 (Nmnat2) - consequences for mouse bladder development and function. Neurourology and Urodynamics. 2013 Jan 31


2012


2011


2007-2009


b) INVITED EDITORIALS


c) OTHER ARTICLES


c) BOOK CHAPTERS


References and other additional information available upon request.

Date of last revision: 7-31-2013