Towards Conditional Stimulation of the Overactive Bladder: Electrophysiology and Neuromodulation in a Rat Model

Mahipal Choudhary

Towards Conditional Stimulation of the Overactive Bladder: Electrophysiology and neuromodulation in a rat model

Mahipal Choudhary

Colophon

Towards Conditional Stimulation of the Overactive Bladder: Electrophysiology and neuromodulation in a rat model

Doctoral dissertation, Erasmus Medical Center, the Netherlands

This research was supported by Marie Curie actions FP7 of European Union, TRUST (Training of Urology Scientists to develop new Treatments) programme (Grant 238541).

Towards Conditional Stimulation of the Overactive Bladder: Electrophysiology and neuromodulation in a rat model

Op weg naar conditionele stimulatie van de overactieve blaas: Elektrofysiologie en neuromodulatie in een rattenmodel

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the rector magnificus

Prof.dr. H.A.P. Pols

and in accordance with the decision of the Doctoral Board.

The public defense shall be held on
Wednesday May 25, 2016 at 11:30 hours

Mahipal Choudhary
born in Haryana, India

Erasmus University Rotterdam

Doctoral Committee

Promotor:	Prof.dr.ir. R. van Mastrigt
Other members:	Prof.dr. C.H. Bangma Prof.dr. J. van der Steen Prof.dr. G.A. van Koeveringe
Copromotor:	Dr. E. van Asselt

Contents

Part I

Introduction

Chapter 1

Introduction and outline of the thesis

Introduction

Neurophysiology of voiding

The functional unit of the lower urinary tract consists of a reservoir called the urinary bladder and an outlet consisting of the bladder neck, the urethra and the urethral sphincter. Coordinated activity of these components regulates the urine storage (continence) and periodic expulsion (micturition) [1]. The smooth detrusor muscle of the bladder and bladder neck is able to maintain continence for extended periods at low pressure. During this storage phase, the detrusor is relaxed while the bladder neck and the urethra are closed [1]. The continuous urine production in the kidneys results in gradual filling, which causes a distension of the bladder until a certain threshold is reached, at which the outlet opens and the detrusor contracts, relieving the bladder of its contents [1].

The seemingly simple process of micturition is regulated by a complex mechanism of afferent (sensory) and efferent (motor) neural signaling between the bladder and the central nervous system [2]. Afferents convey the sensation of bladder fullness to the neural circuits in the spinal cord and brain via pelvic and hypogastric nerves, whereas sensory information from the bladder neck and the urethra is conducted through the pudendal and hypogastric nerves [2]. The afferent components of these nerves primarily consist of two types of fiber; the Aδ and the C-fibers. The Aδ afferents have myelinated axons and are mechanosensitive in nature; activated by both low (non-nociceptive) and high (nociceptive) bladder pressure. C-fiber afferents have unmyelinated axons and do not respond to bladder distension but are activated by noxious stimuli such as cold, heat, or chemical irritation of the bladder [2]. The efferent signals are carried by different fibers in the same set of nerves to facilitate voluntary control of the micturition by the central nervous system.

The overactive bladder syndrome

The normal micturition process can be affected by pathology of the lower urinary tract. One such malfunction is the overactive bladder (OAB) syndrome. This syndrome is characterized by a multitude of symptoms [3]. It is associated with urinary urgency, defined as "*complaint of a sudden, compelling desire to pass urine which is difficult to defer*", with or without urge urinary incontinence or

"*involuntary leakage of urine, associated with a sudden compelling desire to void*". Other symptoms such as a high voiding frequency (more than seven voiding episodes during waking hours) and nocturia (three or more nighttime voiding episodes) have been frequently observed in OAB patients [4].

OAB affects approximately 12% of the European and 17% of the North American population and has a significant negative impact on the quality of life and mental health [5]. Several theories have been proposed to explain the mechanisms underlying OAB [6]. The most prevalent -the neurogenic theory -relates the OAB syndrome to a dysfunction of brain and/or spinal cord as well as to increased sensitivity of bladder afferent sensors [7]. However, despite years of research, the exact pathophysiology of the OAB syndrome remains obscure.

Current treatment methods

Treatment of overactive bladder symptoms may start with behavioral therapy including consultation on regulating the diet and the daily fluid intake [4]. Clinicians might also suggest bladder training in which the patient is asked to delay bladder emptying for a relatively short length of time in an attempt to regain the inhibitory control of the bladder. If the symptoms persist despite conservative measures, treatment might proceed with pharmacotherapy and/or neuromodulation [8].

Treatment with antimuscarinic drugs has been shown to be efficacious in ameliorating the symptoms of OAB patients, but due to adverse effects, more than half of the patients terminate this treatment [9]. Injection of botulin toxin in the bladder wall is another method which has been shown to be effective in clinical trials. It suppresses contractions by partial paralysis of the detrusor, which might lead to urine retention [10].

Neuromodulation offers an alternative for patients who are refractory to other modes of treatment [11]. Neuromodulation refers to the manipulation of electrical activity of one neural pathway that can modulate the function of others. Typical clinical neuromodulation routines involve electrical stimulation of the pudendal, sacral or tibial nerves [11]. Most of these techniques have been reported to be effective in restoring bladder function, with varying success rates. The physiological basis of neuromodulation is however not fully understood.

Aim and outline of the thesis

Aim

The aim of the research in this thesis was to study the neurophysiology of bladder afferent mechanisms in simulated detrusor overactivity in rats and to develop a conditional electrical stimulation technique based on the frequency of bladder non-voiding activity to inhibit undesired detrusor contractions.

Outline

Chapter 2 describes a mathematical model for breakdown of electrical activity from mixed nerve recordings into afferent and efferent traffic.
Chapter 3 describes a novel single compartment *in vitro* setup for recording bladder afferent nerve activity and electrical stimulation via efferent nerves.

Chapter 4 presents the results of a pattern recognition algorithm, which automatically identifies changes in the pattern of the bladder pressure and predicts an impending voiding contraction.

Chapter 5 and chapter 6 discuss the effect of continuous tibial nerve stimulation on bladder neurophysiology. The effectiveness of conditional electrical stimulation of the tibial and dorsal nerve of the penis to inhibit undesired bladder contractions is also described.

Chapter 7 describes the implementation of a closed-loop real-time pattern recognition technique to suppress bladder overactivity by event-triggered conditional stimulation of the dorsal nerve of the penis.

Chapter 8 summarizes the thesis in a general discussion.

References

1. Andersson KE, Arner A. Urinary bladder contraction and relaxation: physiology and pathophysiology. Physiol Rev. 2004;84(3):935-86.
2. Fowler CJ, Griffiths D, de Groat WC. The neural control of micturition. Nat Rev Neurosci. 2008;9(6):453-66.
3. Abrams P. Describing bladder storage function: overactive bladder syndrome and detrusor overactivity. Urology. 2003;62(5 Suppl 2):28-37; discussion 40-2.
4. Gormley EA, Lightner DJ, Faraday M, Vasavada SP, American Urological A, Society of Urodynamics FPM. Diagnosis and treatment of overactive bladder (non-neurogenic) in adults: AUA/SUFU guideline amendment. J Urol. 2015;193(5):1572-80.
5. Milsom I, Abrams P, Cardozo L, Roberts RG, Thuroff J, Wein AJ. How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. Bju Int. 2001;87(9):760-6.
6. Hashim H, Abrams P. Overactive bladder: an update. Curr Opin Urol. 2007;17(4):231-6.
7. de Groat WC. A neurologic basis for the overactive bladder. Urology. 1997;50(6A Suppl):36-52; discussion 3-6.
8. Nambiar A, Lucas M. Chapter 4: Guidelines for the diagnosis and treatment of overactive bladder (OAB) and neurogenic detrusor overactivity (NDO). Neurourol Urodyn. 2014;33 Suppl 3:S21-5.
9. Abrams P, Andersson KE. Muscarinic receptor antagonists for overactive bladder. Bju Int. 2007;100(5):987-1006.
10. Nitti VW. Botulinum toxin for the treatment of idiopathic and neurogenic overactive bladder: state of the art. Rev Urol. 2006;8(4):198-208.
11. Groen J, Bosch JL. Neuromodulation techniques in the treatment of the overactive bladder. Bju Int. 2001;87(8):723-31.

Part II

Methods in bladder neurophysiology

Chapter 2

Neurophysiological modeling of bladder afferent activity in the rat overactive bladder model

Mahipal Choudhary

Els van Asselt

Ron van Mastrigt

Francesco Clavica

J Physiol Sci. 2015 Jul;65(4):329-38

Abstract

The overactive bladder (OAB) is a syndrome based urinary dysfunction characterized by "urgency, with or without urge incontinence, usually with frequency and nocturia". Earlier we developed a mathematical model of bladder nerve activity during voiding in anaesthetized rats and found that the nerve activity in the relaxation phase of voiding contractions was all afferent.

In the present study, we applied this mathematical model to an acetic acid (AA) rat model of bladder overactivity to study the sensitivity of afferent fibers in intact nerves to bladder pressure and volume changes. The afferent activity in the filling phase and the slope i.e. the sensitivity of the afferent fibers to pressure changes in the post void relaxation phase, were found to be significantly higher in AA than in saline measurements, while the offset (nerve activity at pressure ~0) and maximum pressure were comparable.

We have thus shown, for the first time, that the sensitivity of afferent fibers in the OAB can be studied without cutting nerves or preparation of single fibers. We conclude that bladder overactivity induced by AA in rats is neurogenic in origin and is caused by increased sensitivity of afferent sensors in the bladder wall.

Introduction

The International Continence Society (ICS) defines the 'Overactive Bladder' (OAB) as a "symptom syndrome suggestive of lower urinary tract dysfunction". The OAB is characterized by "urgency, with or without urge incontinence, usually with frequency and nocturia" [1]. Despite years of research the pathophysiology of the OAB syndrome is still unknown. Several theories have been proposed to explain the mechanisms behind OAB [2]. The most prevalent – the neurogenic theory - relates the OAB syndrome to a dysfunction of brain and/or spinal cord as well as to increased sensitivity of bladder afferent sensors. Most researchers classify afferent fibers in two broad categories: the Aδ fibers which respond to bladder stretch and/or filling, pressure, stress [3] under normal conditions and the C-fibers responding to noxious chemical or thermal stimuli [4, 5]. Alterations in the sensitivity of any/both of these bladder sensors/fibers can lead to bladder overactivity.

To study the change in bladder sensation in OAB several animal models have been proposed [6, 7]. The Acetic Acid (AA) rat model of bladder overactivity i.e. evoking voiding contractions in anaesthetized rats by filling the bladder with AA has been reported to simulate, to some extent, the human OAB pathophysiology. It has shown an increase in the frequency of spontaneous (involuntary) contractions of the bladder [8, 9], an increased residual volume and a decreased inter-voiding interval [9, 10].

To measure the afferent activity in bladder nerves various techniques have been proposed [11-14]. Among the popular ones are single fiber recordings and measurements after a central/peripheral transection of bladder nerves [14]. Apart from the difficulties involved in the preparation of single fibers and transection of nerves, they pose several other complications. Firstly, a single fiber may not represent the whole nerve. Afferent measurements obtained from single fibers only show the frequency of firing and fail to take into account the interaction among the unitary spikes [15]. Measurement and modeling of these interactions and of the recruitment of new fibers when the bladder is exposed to external stimuli are necessary for the development of implantable electrical devices for neural control of micturition e.g. in spinal cord injured patients. Additionally,

transection of the pelvic nerve has been reported to affect the afferent, sympathetic and parasympathetic fibers neural activity, hence affecting the complete physiological voiding cycle [16].

Previously, in order to study the afferent nerve activity in intact, mixed (i.e. containing both afferent and efferent fibers) postganglionic bladder nerves, we developed a mathematical model which enabled the differentiation of total measured nerve activity into afferent and efferent activity without cutting the nerve or preparation of single fibers [11]. In short, lesion experiments were done in which bladder pressure and nerve activity were recorded before and after a central cut of the bladder nerve. Afferent activity was linearly related to the recorded pressure. Similar relationships were found when afferent activity was recorded from cut nerves and when it was estimated from intact nerve experiments. Additionally, peripheral cutting and pelvic afferent nerve stimulation showed that when electrical stimulation of the pelvic nerve was suddenly aborted, resulting in a sudden stop of efferent nerve activity, a decrease in bladder pressure was found that was very similar to the decrease that occurred during voiding contractions. This model was developed and validated in saline (physiologically normal) bladder filling experiments. Using this model, the slope of the linear dependence of afferent activity on bladder pressure (which is a measure for the sensitivity of the afferent fibers/sensors for pressure change) can be estimated.

In the present study, we applied this mathematical model to the AA rat model of bladder overactivity to study the changes in sensitivity of afferent nerve fibers in intact nerves i.e. without transection or separation of single fibers.

Methods

Experimental procedures

All the laboratory and animal experimental procedures described in this study were approved by the local Erasmus MC Animal Experiment Committee. 18 male Wistar rats (mean weight 420 ± 44g) were anaesthetized with urethane (1g/kg) intraperitoneally. An abdominal incision was made, the postganglionic bladder nerves close to the bladder (branches of the pelvic nerve) were carefully dissected and thin non-absorbable, sterile sutures were tied around the identified nerves. Warm saline (0.9 % NaCl) was poured into the abdomen to prevent drying out of the organs during surgery.

After the surgical procedures, the saline was removed and the pelvic cavity was kept moist by warm paraffin oil throughout the electrophysiological measurements. Bladder pressure measurement and bladder filling (0.05 ml/min) were done by inserting a 23G needle at the top of the bladder. The other end of the needle was attached to a disposable pressure transducer and an infusion pump using a 2- way connector. Pressure was measured using a Statham SPI1400 blood pressure monitor. The bladder was repeatedly filled with saline or 0.5 % acetic acid (AA) until a voiding contraction occurred or for a maximum duration of 20 min (up to ~1 ml), whichever happened first. Custom made bipolar electrodes consisting of two thin (diameter 0.1mm) platinum-iridium hook shaped wires separated by a distance of 0.5 - 1mm were used for the nerve signal recordings. The electrode was mounted on a micromanipulator and one of the identified nerves was carefully placed on the wires. A brief illustration of the experimental setup is shown in Figure 1. In 4 of the 18 rats, a left side postganglionic pelvic nerve branch was crushed between the major pelvic ganglion and the electrode, to eliminate efferent nerve signals and record afferent activity only. The pressure transducer was calibrated using a column of water before the start of each experiment. The nerve signal was calibrated using a 1 μV (500 Hz) sinusoidal test signal. The rats were euthanized at the end of the experiment with an overdose of KCl, injected into the heart.

Data acquisition and storage

The nerve activity was amplified by a DISA 15C01 EMG amplifier (amplification range: 100 - 200,000) and band-pass filtered with a Krohn-Hite 3944 filter (Bessel, 4th order, 200 - 2000 Hz). Bladder pressure and nerve activity were displayed in real-time on a computer screen using a custom written LabVIEW® (National Instruments, USA) program. In the first few rats the pressure and nerve signals were recorded for periods of 30s, which contained at least one voiding contraction. The computer program was later optimized to record intervals of 5 minutes.

Signal processing

Nerve activity and pressure signals were sampled at 25 kHz and 25 Hz respectively. Measurements with movement artefacts due to catheter displacement, air bubbles in the set-up or movement of the rat etc. were excluded from analysis. These artefacts were observed during data acquisition and were seen as random, sharp high amplitude peaks in the pressure curve. Recorded signals were processed and analysed with a custom written MATLAB® (Mathworks, USA) program. In the first step, the nerve signal was rectified and averaged [17, 18] by taking the mean of each 1000 samples, effectively reducing a 1s interval to 25 data samples.

The quality of the nerve activity recordings was assessed by the signal to noise ratio (SNR) of the averaged signal [3, 11]. Assuming that the lowest values in the averaged nerve activity recording represented baseline nerve activity and the highest values are caused by nerve action potentials, the SNR was estimated as the difference between the means of the ten highest and the ten lowest average nerve activity values divided by the mean of the ten lowest average values.

To allow for a comparison of results between animals, the averaged nerve signal was normalized by dividing it by the maximum afferent activity, calculated as the mean of the ten highest average nerve activity values. Similarly, pressure was normalized by dividing by the maximum pressure.

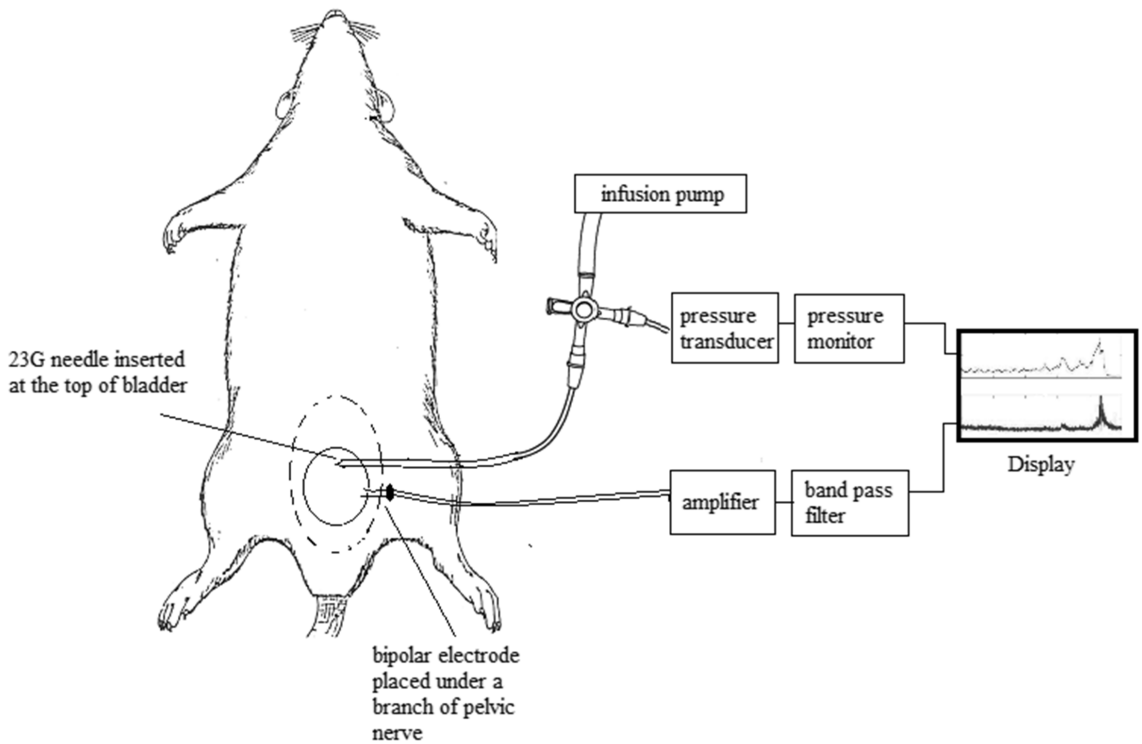

Figure 1. Experimental setup

After exposing the abdominal cavity, a 23G needle was inserted at the top of the bladder for filling and pressure measurement. The other end of the needle was attached to a disposable pressure transducer and an infusion pump using a 2-way connector. Custom made bipolar electrodes consisting of two thin platinum-iridium hook shaped wires separated by a distance of 0.5 - 1 mm were placed under one of the branches of the pelvic using a micromanipulator. The recorded nerve signal was then amplified and band pass filtered.

The whole micturition cycle shown in Figure 2 was divided into three phases:

1. *The filling phase ($t<t_1$)*

   The filling phase was further divided into two sub-phases:

- Passive filling phase: from start of filling (at t = 0) when the bladder was compliant and the pressure was relatively low.
- Active contraction: the part of the pressure curve where the bladder contracts (marked as beginning of an active contraction) and the pressure increased till t_1, which represents the start of voiding. The pressure at t_1 is the maximum

pressure immediately before the start of flow and has been termed 'pressure threshold' in the literature [7, 19].

2. *The voiding phase (t_1-t_2):* During this phase the actual voiding takes place. A typical rat voiding contraction is associated with high frequency oscillations (HFO), caused by rapid contractions of the urethral sphincter [7]. The HFO were visually identified in the pressure recording (Figure 2, t_1-t_2). In Figure 2, t_1 is the time at the start of HFO, the pressure at t_1 has been termed threshold pressure (p_{thres}) [7]. t_2 is the time at maximum pressure, after cessation of the HFO. The start of flow is characterized by the expulsion of urine droplets, which is synchronous with the initiation of HFO, both of which can be observed visually. The period t_1-t_2 was excluded from analysis because of movement artefacts in the recorded nerve and pressure signal.
3. *The relaxation phase ($t>t_2$)*: in this phase the bladder relaxed isovolumetrically and pressure decreased to baseline. The baseline pressure ($p_{baseline}$) was determined by a computer algorithm that divided the pressure signal ($t>t_2$) into successive windows of 1 second. The mean pressure in each window was compared to that in the next window. When the difference between the means of two consecutive windows was < 2 cmH_2O, the mean pressure in the first window was taken as the baseline.

Modeling

Step 1: Test of absence of efferent contribution in the relaxation phase

We have earlier shown in saline filling experiments that nerve activity after a voiding contraction is all afferent. To confirm that this is also the case in the AA measurements, we calculated the time constant of pressure decay in the interval t_2-$t_{baseline}$ and compared it between saline and acetic acid measurements.

The time constant of exponential decay was calculated by fitting:

$$p(t) = Ae^{-t/\tau}$$

Where p (t) is the pressure in the interval t_2-$t_{baseline}$, A is constant and τ is the time constant.

To provide additional support for the assumption that there is no efferent activity in the relaxation phase, only afferent nerve activity was measured (see

experimental procedures section) and the slope of afferent nerve activity and pressure in the relaxation phase in saline and AA measurements was calculated. The slope in these crushed nerve experiments was compared with that of afferent nerve activity and pressure in the relaxation phase in intact nerve experiments (see step 2).

Step 2: Derivation of afferent activity in the filling phase

The averaged nerve signal in the relaxation phase t_2-$t_{baseline}$ was smoothed with a first order Savitzky-Golay FIR smoothing filter and a linear regression model was fitted to the nerve activity-pressure data using the MATLAB® function 'polyfit' to calculate slope and offset:

$$NA(t) = m.p(t) + NA_0$$

Where *m* is the slope of the pressure-nerve activity data in the interval t_2-$t_{baseline}$, NA_0 is the offset which represents the baseline nerve activity, p(t) is the pressure during the relaxation phase t_2-$t_{baseline}$ and NA(t*)* is the afferent nerve activity in the interval t_2-$t_{baseline}$. Once the slope and offset were known, the afferent activity in the *filling phase* was calculated using the same formula.

Step 3: Modelling of afferent activity and volume in the filling phase

In addition to the afferent nerve activity-pressure modelling, we also modeled the relationship between afferent activity and bladder volume in the filling phase. A linear regression model was fitted to the afferent nerve activity-volume data to calculate slope and offset, as described in step 2. Additionally, the linear correlation coefficient (Pearson's Product-Moment Correlation), which measures the linear dependence of afferent activity on the bladder volume was also calculated using the MATLAB® function 'corrcoef'.

All data are presented as mean ± SD. Mann-Whitney U test was performed using SPSS® statistical package (version 21.0, SPSS Inc., Chicago, IL, USA) to compare groups. A $p < 0.05$ was considered significant.

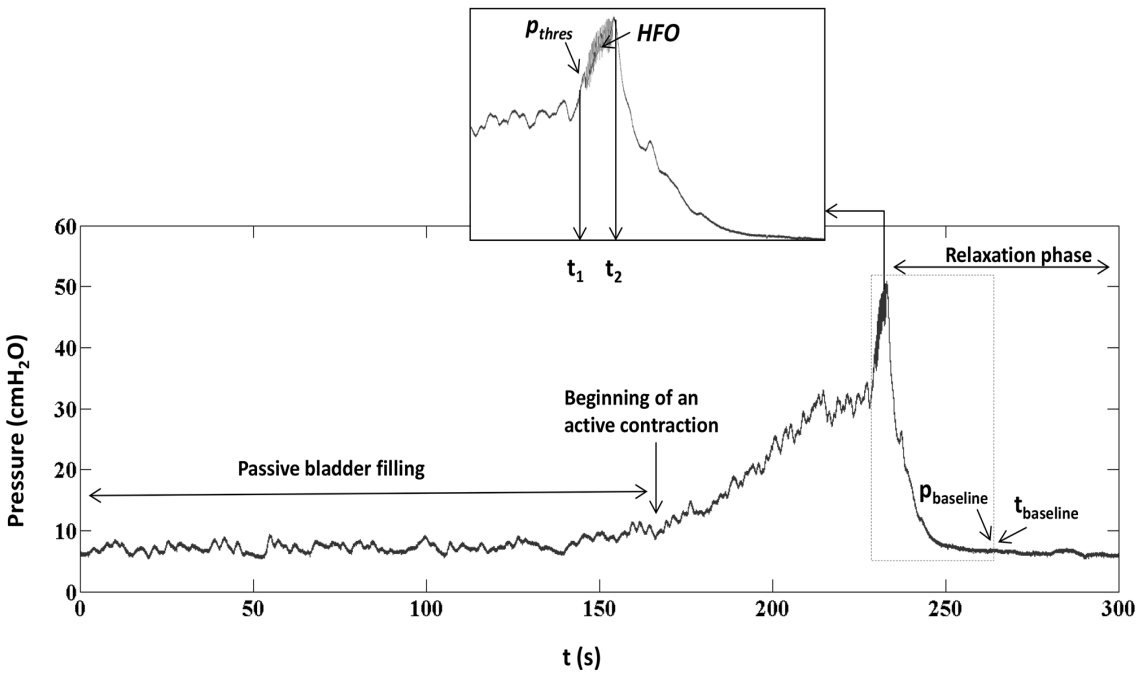

Figure 2. Pressure development during bladder filling

Bladder pressure recorded during one complete voiding cycle of a rat. t = 0 represents the start of bladder filling, at t_1-t_2 voiding takes place and $t>t_2$ represents the relaxation phase.

Results

Intact nerve experiments

Postganglionic bladder nerve activity and bladder pressure were successfully measured in 10 out of 14 rats. A total of 84 measurements were recorded from these 10 rats (Table 1), 28 measurements were excluded because of artifacts, as described in the preceding section. The remaining 56 measurements had a SNR >0.5, i.e. the nerve activity was ≥ 50 % higher than baseline activity. For the analysis of the filling phase, an additional 21 measurements were excluded because of artifacts and parameters were calculated for 35 measurements (saline = 15, AA = 20). Figure 3 shows an example of a good pressure-nerve activity recording with saline and AA filling from the same rat. The bladder filling

started at t = 0 and was stopped at the beginning of a voiding contraction. After the voiding contraction, both in saline and AA, the pressure declined smoothly to baseline in a similar fashion. It can be noted that similar to pressure the nerve activity also followed a smoothly declining pattern.

In the filling phase, the baseline afferent activity and the threshold pressure (p_{thres}) before a voiding contraction did not differ significantly between saline and acetic acid measurements. However, the afferent activity in the active contraction interval was significantly higher in acetic acid as compared to saline measurements. The bladder capacity (filled volume at which voiding occurred) was reduced by 31 % in acetic acid measurements. (Table 2). The slope of afferent nerve activity-volume was significantly higher in AA measurements than in saline measurement. The offset did not differ significantly between the two groups. The correlation coefficient of the linear dependence of afferent activity on bladder volume was also significantly higher in AA measurements (Table 2).

In the post void relaxation phase, the time constant of exponential pressure decay (τ) in the interval t_2-$t_{baseline}$ was not significantly different between AA and saline measurements. The average afferent activity, the maximum afferent activity and the mean value of the normalized slope in the interval t_2-$t_{baseline}$ were significantly higher in AA than in saline measurements, whereas the mean value of pressure (t_2-$t_{baseline}$) was found to be higher in saline measurements. The baseline afferent activity and baseline pressure ($p_{baseline}$) after the voiding contraction did not differ significantly (Table 2). The correlation coefficient (Pearson's Product-Moment Correlation), which is a measure of the linear dependence of nerve activity on bladder pressure, was significantly higher in AA (Table 2), whereas the SNR and the goodness of fit (R^2), which describes how well the model fitted the data, did not differ significantly. An example of a fitted line in a saline and an acetic acid measurement is shown in Figure 4.

Crushed nerve experiments

In the crushed nerve experiments, the slope of afferent nerve activity and pressure was higher in AA (n = 10) as compared to saline (n = 9) measurements. After removal of 2 obvious outliers from the saline measurements, the slope in the AA measurements was twice (0.007 ± 0.005) that of the saline (0.003 ± 0.002)

measurements ($p = 0.06$), similar to the change in slope in intact nerve experiments. The offset did not differ significantly between saline (2.3E-7 ± 5.0E-8) and AA (2.1E-7 ± 3.0E-8) measurements ($p = 0.5$). The threshold pressure between saline (33.0 ± 6.0 cmH_2O) and that in AA (31.0 ± 7.5 cmH_2O) was also found to be comparable ($p = 0.5$).

Table1. The number of saline and acetic acid measurements in 10 rats

Rat number	Saline		Acetic acid	
	Total	Included	Total	Included
Rat1	2	2	3	3
Rat2	7	6	6	2
Rat3	4	2	2	1
Rat4	4	2	3	3
Rat5	4	3	6	4
Rat6	2	2	3	2
Rat7	3	2	5	3
Rat8	5	1	6	4
Rat9	5	3	6	3
Rat10	4	4	4	4
Σ=	40	27	44	29

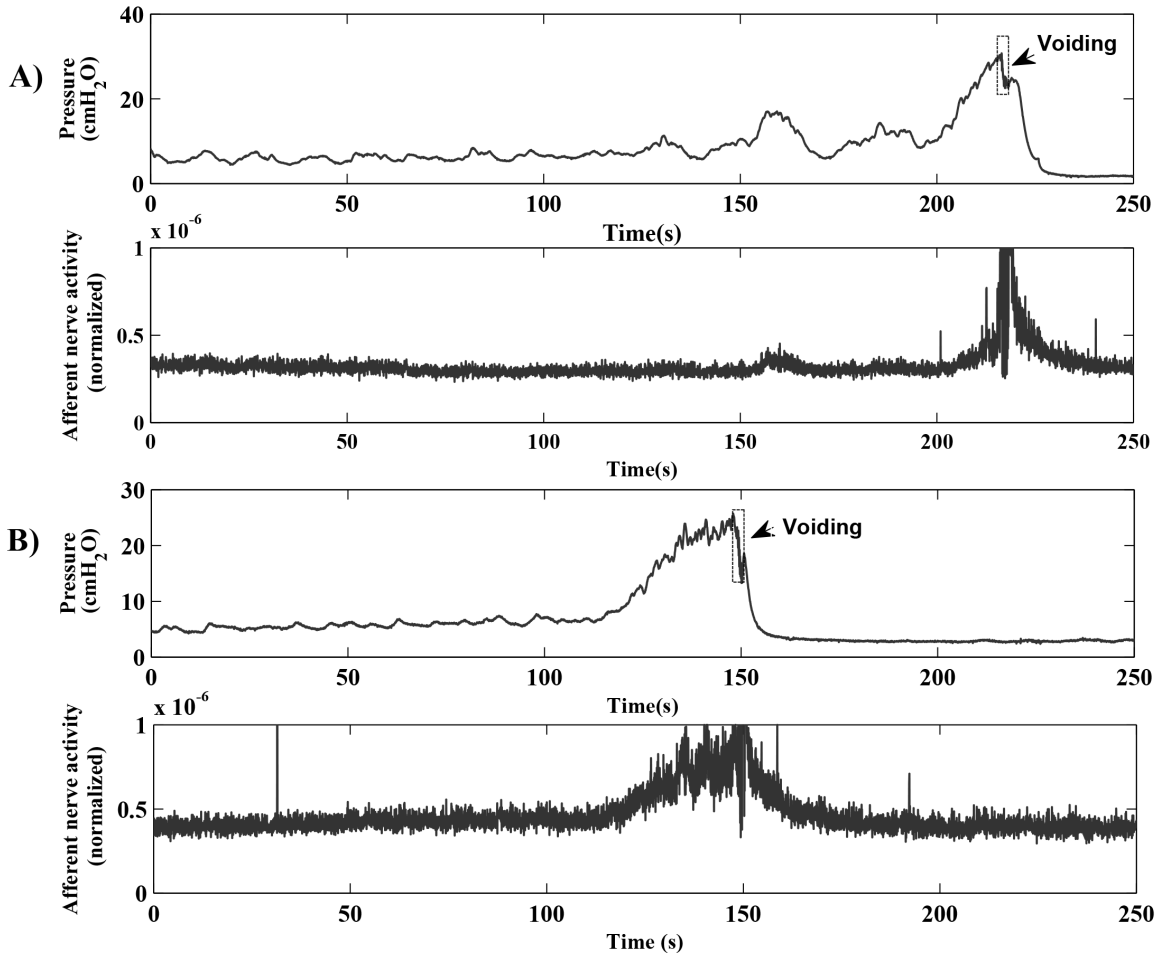

Figure 3. Nerve activity and bladder pressure during a voiding cycle

An example of a pressure-afferent nerve activity measurement with saline (A) and AA (B) filling in the same rat. The bladder filling started at t = 0. The upper panel of both A) and B) shows the pressure during the filling phase and a typical rat voiding contraction. The lower panel of A) and B) shows the measured nerve activity.

Discussion

In this study we quantitatively compared the sensitivity of afferent nerve fibers in a rat OAB model with that in normal rats without cutting nerves or preparation of single fibers. Several models have been reported in the literature to study the afferent mechanisms of the urinary bladder. Sadananda et.al [13] proposed a decerebrated arterially perfused rat *in situ* model (DAPR) that allows the study of micturition in rats. The model offers the advantage of studying the bladder neurophysiology without the need for anesthesia and a faster recording setup. However, the authors reported a 4 hours duration of viability which imposes a limitation to experiments requiring more time. Additionally, the nerves were transected in order to record the afferent nerve activity. The cutting of a nerve has been reported to disrupt the protein supply to the nerve, leading to nervous tissue death, which would be disadvantageous for recording nerve activity for longer periods of time [20]. Zvara et. al. [12] presented an *in situ* model where afferent recordings were made by transecting bladder nerves in non-anesthetized mice. The model also has the advantage of afferent nerve activity measurements without the need for anesthesia. However, electrophysiological recordings in freely moving animals suffer from movement artefacts. Additionally once the nerve is enclosed and the abdominal cavity is sutured it is very difficult to manipulate/modify the nerve-electrode interface. The model presented in the current study offers the advantage of minimal surgical procedures and enables recordings from intact nerves with minimal damage. Although the effect of anesthesia on neurophysiology cannot be denied, it has been reported to spare the micturition [21].

The mathematical model used in the current study assumes that the nerve activity measured from an intact, multifiber rat bladder nerve in the relaxation phase of a voiding contraction is purely afferent and that it depends linearly on the bladder pressure. In an earlier study in anaesthetized rats of the same age, weight and gender, these hypotheses were verified by proximal cutting of bladder nerves and electrical stimulation of the pelvic nerve [3, 11]. In the present study the mathematical model was applied to an OAB rat model. It was found that the correlation between pressure and nerve activity was even better in AA measurements than in saline fillings, implying that the linear relationship between

pressure and afferent nerve activity holds true for the overactive bladder (Figure 4). The coefficients of that linear relationship, derived from the post void relaxation phase, can be used to estimate the afferent activity in the filling phase. Although this finding has no direct clinical implication, it could be useful in closed loop implantable devices to detect changes in afferent nerve activity based on pressure recordings and electrically inhibit bladder contractions. We also modeled the relationship between afferent nerve activity and bladder volume in the filling phase, and it was found that the afferent nerve activity (in both saline and AA) related better with pressure than with volume. This can be attributed to the compliance of the bladder during filling, while the bladder volume increases constantly, the bladder pressure and the afferent nerve activity remain relatively unchanged (or very low) till a certain threshold is reached, at which a voiding contraction begins and both pressure and afferent activity rise simultaneously.

To verify the absence of efferent activity in the relaxation phase in intact nerve measurements, we calculated the time constant of the isovolumetric pressure decay after a voiding contraction. It was assumed that in the absence of any efferent activation, the bladder relaxes passively and the time constant would be comparable in saline and AA measurements. Conversely, any efferent activity would result in a contraction or relaxation of the bladder, which in turn would result in a larger or smaller time constant. In Figure 3, it can be seen in both saline and AA filling pressure curves that, after a voiding contraction, the bladder relax passively and the pressure decline smoothly to baseline and stayed low. Conversely, in the filling phase, the bladder shows continuous non-voiding contractions (pressure transients with an amplitude 0.5 - 3 cmH_20 [22]), which have been reported to be associated with pelvic efferent nerve discharge during the continence process [23]. In addition to our earlier findings that there is no efferent activity after a voiding contraction in saline measurement [11], the slope of the relationship between pressure and afferent nerve activity in intact and crushed nerve experiments showed a similar relationship in saline and AA measurements. The similar pattern of pressure decrease after a voiding and the comparable time constants in saline and AA fillings indicate that there is no (or insignificant) efferent activity after a voiding contraction neither in saline nor in AA measurements.

Bladder overactivity on instillation of 0.5 % AA was assessed by various urodynamic parameters. To provide reassurance that in our model we have produced sensitization like that previously reported, we measured the urinary bladder capacity. We found a 31 % reduction of capacity in acetic acid instillation measurements as compared to saline filling, which is in line with other studies reporting a relevant reduction of bladder capacity with acetic acid [9, 24]. Figure 3 shows a good example of the difference between bladder capacities in saline and AA in the same rat, where it can be seen that due to reduced bladder capacity voiding occurred earlier (at lower volume) in AA. The threshold pressure just before a voiding contraction was not significantly different between acetic acid and saline filling, which is also supported by other studies [9, 25]. This implies that compared to saline fillings, there was higher afferent activity and comparable bladder pressure in acetic acid measurements at a significantly lower volume. 0.2 - 0.5 % AA has been reported to induce bladder overactivity in rats without significant morphological alterations [26]. In this study, repeated infusion with saline was followed by repeated infusion with AA in order to compare and validate our results with literature. However in a different study (unpublished), where a 'saline-AA-saline' filling routine was followed, we found that the bladder capacity was significantly reduced by AA and in consequent fillings with saline the bladder capacity did not return to its original value within the time scheme (60 - 90 minutes) in which these experiments were done. This indicated that although AA does not cause significant damage to bladder morphology, it does cause a 'carry on' effect on bladder afferent sensitization. The AA rat model of OAB in this study closely resembles the rat model of cystitis [27, 28] with overlapping symptoms such as an increased urgency, frequency and nocturia. However, there might still be a different mechanism of action behind OAB and cystitis.

Table 2. Mean ± SD of the estimated normalized parameters in saline and acetic acid measurements, n = 35 (saline = 15, AA = 20) for filling phase parameters and n = 56 (saline = 29, AA = 27) for relaxation phase parameters. Mann-Whitney test (*significance $p < 0.05$).

	Filling Fluid	*Saline*	*AA*	*Statistical significance*
Filling phase ($t<t_1$)	*Baseline afferent activity*	0.59±0.2	0.60±0.2	0.9
	Mean afferent activity	0.68±0.1	0.82±0.1	0.001*
	Pressure before void, p_{thres}	0.88±0.13	0.86±0.09	0.2
	Bladder capacity	0.75±0.16	0.58±0.31	0.02*
	Slope (volume-afferent activity)	1.6E-7±2.9E-7	8.0E-7±1.3E-7	0.0001*
	Offset (volume-afferent activity)	2.7E-7±6.0E-8	2.9E-7±6.0E-8	0.9
	Correlation coefficient (volume-afferent activity)	0.36±0.17	0.57±0.11	0.001*
Relaxation phase (t_2-$t_{baseline}$)	*Mean pressure*	0.45±0.15	0.37±0.12	0.02*
	Mean afferent activity	0.59±0.12	0.67±0.09	0.01*
	Maximum afferent activity	0.75±0.13	0.87±0.11	0.002*
	$p_{baseline}$	0.17±0.12	0.15±0.13	0.4
	Slope (pressure-afferent activity)	0.005±0.004	0.011±0.009	0.004*
	Offset (pressure-afferent activity)	0.48±0.15	0.47±0.2	0.9
	Time constant (τ)	4.0 ±2.5s	3.7 ±2.5s	0.6
	Correlation coefficient (pressure-afferent nerve activity)	0.62±0.2	0.73±0.2	0.04*
	Signal to noise ratio (SNR)	3.01±4.8	3.72±6.49	0.2
	Goodness of fit (R^2)	0.68±0.2	0.74±0.2	0.06

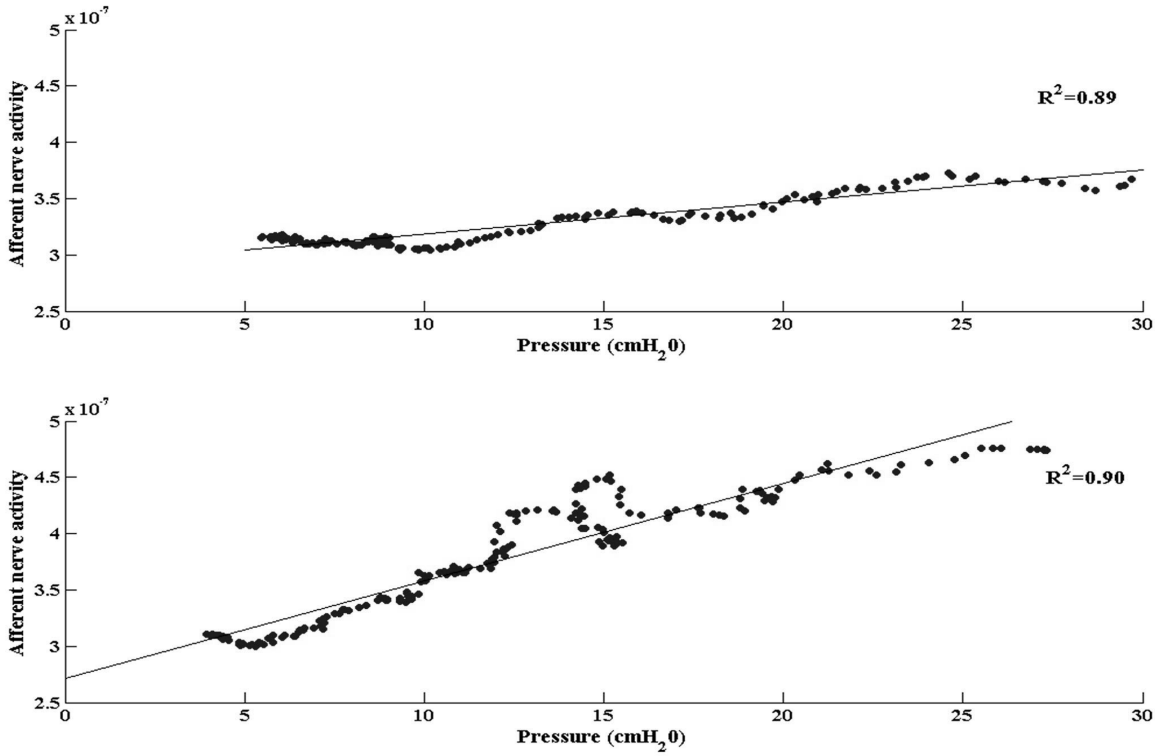

Figure 4. Linear relationship between afferent activity and bladder pressure

A linear polynomial fit of afferent nerve activity as a function of bladder pressure recorded during the relaxation phase of a voiding contraction in the same rat after saline (upper panel) and acetic acid (lower panel) filling. Dots represent the measured afferent activity and the solid line represents the fitted model.

We found higher afferent activity both in the filling phase and the relaxation phase when the bladder was filled with acetic acid than when it was filled with saline. This is in accordance with other models of bladder overactivity [12, 29]. The multifiber whole nerve activity recorded in our study does not allow the distinction of myelinated and unmyelinated fibers. However, the significant changes in afferent nerve activity in response to AA filling indicated that whole nerve recordings do reflect (pathophysiological) changes in nerve activity. The slope of the linear dependence of afferent nerve activity on bladder pressure, which defines the sensitivity of the afferent fibers/sensors for pressure changes, was significantly higher in acetic acid measurements than in saline measurements.

This implies a higher sensitivity (response) of afferent fibers under overactive bladder conditions at a given pressure. The increased afferent nerve activity indicates an underlying neurogenic mechanism, the bladder overactivity induced by acetic acid could be due to a direct sensitization of afferent nerve endings by AA. However it could also be due to an effect of acetic acid on the bladder urothelium, which in turn signals strongly to the afferents, thus causing an increased sensitivity. The relationship between afferent nerve activity and pressure was studied at a filling rate of 50 µl/min which is within the practical range of physiological filling rates [14]. In previous studies it was found that at supraphysiological (200 µl/min and above) filling rates the afferent response was significantly reduced [14]. The baseline nerve activity, both in the filling phase and the relaxation phase did not differ significantly between saline and acetic acid measurements. This implies that the sensitivity of afferent fibers at very low pressure (bladder empty) was not affected by bladder overactivity induced by acetic acid. The calculated baseline was compared with a conventional method by recording signals after euthanizing animals, where no difference was found (4 rats, $(1.01 \times 10^{-7} \pm 8 \times 10^{-10})$ vs $(1.008 \times 10^{-7} \pm 3 \times 10^{-10})$, $p = 0.15$). A plausible explanation for this could be that at low bladder pressure, the afferent fibers have a very low firing rate and the measured signal consists mainly of noise (~constant). The results on baseline nerve activity is in agreement with other studies in the rat [5, 30, 31], where it was reported that pelvic afferent fibers showed little or no nerve activity when the bladder was empty.

Conclusion

We have shown that our previously proposed mathematical model is applicable to the acetic acid rat model of bladder overactivity. Using this model the sensitivity of afferent fibers could be studied in the overactive bladder condition in intact bladder nerves without the need for cutting nerves or preparation of single fibers. To our knowledge, this is the first study comparing the afferent nerve activity measured from intact mixed nerves, in a physiologically optimal condition, in normal and overactive bladders. We found that the sensitivity of afferent fibers estimated using this model was higher in acetic acid measurements than in saline measurements while the maximum pressure before the voiding contraction did not differ significantly between both groups. This leads to the conclusion that the bladder overactivity induced by acetic acid in rats is neurogenic in origin and is caused by increased sensitivity of afferent sensors in the bladder wall.

References

1. Abrams P. Describing bladder storage function: overactive bladder syndrome and detrusor overactivity. Urology. 2003;62(5 Suppl 2):28-37; discussion 40-2.
2. Hashim H, Abrams P. Overactive bladder: an update. Curr Opin Urol. 2007;17(4):231-6.
3. le Feber J, van Asselt E, van Mastrigt R. Afferent bladder nerve activity in the rat: a mechanism for starting and stopping voiding contractions. Urol Res. 2004;32(6):395-405.
4. Wyndaele JJ. Investigating afferent nerve activity from the lower urinary tract: highlighting some basic research techniques and clinical evaluation methods. Neurourol Urodyn. 2010;29(1):56-62.
5. Moss NG, Harrington WW, Tucker MS. Pressure, volume, and chemosensitivity in afferent innervation of urinary bladder in rats. Am J Physiol. 1997;272(2 Pt 2):R695-703.
6. Parsons BA, Drake MJ. Animal models in overactive bladder research. Handb Exp Pharmacol. 2011(202):15-43.
7. Andersson KE, Soler R, Fullhase C. Rodent models for urodynamic investigation. Neurourol Urodyn. 2011;30(5):636-46.
8. Tai C, Shen B, Chen M, Wang J, Liu H, Roppolo JR, et al. Suppression of bladder overactivity by activation of somatic afferent nerves in the foot. BJU Int. 2011;107(2):303-9.
9. Kakizaki H, de Groat WC. Role of spinal nitric oxide in the facilitation of the micturition reflex by bladder irritation. J Urol. 1996;155(1):355-60.
10. Mitsui T, Kakizaki H, Matsuura S, Ameda K, Yoshioka M, Koyanagi T. Afferent fibers of the hypogastric nerves are involved in the facilitating effects of chemical bladder irritation in rats. J Neurophysiol. 2001;86(5):2276-84.
11. Le Feber J, Van Asselt E, Van Mastrigt R. Neurophysiological modeling of voiding in rats: bladder pressure and postganglionic bladder nerve activity. Am J Physiol. 1997;272(1 Pt 2):R413-21.
12. Zvara P, Wright AJ, Roach K, Ursiny M, Shapiro B, Dagrosa LM, et al. A non-anesthetized mouse model for recording sensory urinary bladder activity. Front Neurol. 2010;1:127.

13. Sadananda P, Drake MJ, Paton JF, Pickering AE. An exploration of the control of micturition using a novel *in situ* arterially perfused rat preparation. Front Neurosci. 2011;5:62.

14. De Wachter S, De Laet K, Wyndaele JJ. Does the cystometric filling rate affect the afferent bladder response pattern? A study on single fibre pelvic nerve afferents in the rat urinary bladder. Neurourol Urodyn. 2006;25(2):162-7.

15. Dick DE, Meyer JR, Weil JV. A new approach to quantitation of whole nerve bundle activity. J Appl Physiol. 1974;36(3):393-7.

16. Vaughan CW, Satchell PM. Urine storage mechanisms. Prog Neurobiol. 1995;46(2-3):215-37.

17. Andresen MC, Yang M. Interaction among unitary spike trains: implications for whole nerve measurements. Am J Physiol. 1989;256(4 Pt 2):R997-1004.

18. Hopp FA, Seagard JL, Kampine JP. Comparison of four methods of averaging nerve activity. Am J Physiol. 1986;251(4 Pt 2):R700-11.

19. Yamada A, Torimoto K, Obata K, Hirayama A, Fujimoto K, Takaki M. Persistent overexpression of SERCA2a affects bladder functions under physiological conditions, but not in bladder outlet obstruction-induced sub-acute pathological conditions. J Physiol Sci. 2014;64(1):21-30.

20. Gilley J, Coleman MP. Endogenous Nmnat2 is an essential survival factor for maintenance of healthy axons. PLoS Biol. 2010;8(1):e1000300.

21. Khadra MH, Satchell PM, Vaughan CW. Sympathetic nervous system effects on feline bladder wall compliance throughout continence. Acta Physiol Scand. 1995;155(1):31-9.

22. Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JI. Phasic non-micturition contractions in the bladder of the anaesthetized and awake rat. BJU International. 2006;97(5):1094-101.

23. Satchell P, Vaughan C. Efferent pelvic nerve activity, ganglionic filtering, and the feline bladder. Am J Physiol. 1989;256(6 Pt 2):R1269-73.

24. Matsuta Y, Mally AD, Zhang F, Shen B, Wang J, Roppolo JR, et al. Contribution of opioid and metabotropic glutamate receptor mechanisms to inhibition of bladder overactivity by tibial nerve stimulation. Am J Physiol Regul Integr Comp Physiol. 2013;305(2):R126-33.

25. Kitagawa Y, Wada M, Kanehisa T, Miyai A, Usui K, Maekawa M, et al. JTS-653 blocks afferent nerve firing and attenuates bladder overactivity without affecting normal voiding function. J Urol. 2013;189(3):1137-46.

26. Mitobe M, Inoue H, Westfall TD, Higashiyama H, Mizuyachi K, Kushida H, et al. A new method for producing urinary bladder hyperactivity using a non-invasive transient intravesical infusion of acetic acid in conscious rats. J Pharmacol Toxicol Methods. 2008;57(3):188-93.

27. Aizawa N, Igawa Y, Nishizawa O, Wyndaele JJ. Effects of Nitric Oxide on the Primary Bladder Afferent Activities of the Rat With and Without Intravesical Acrolein Treatment. Eur Urol. 2011;59(2):264-71.

28. Minagawa T, Aizawa N, Igawa Y, Wyndaele JJ. The role of transient receptor potential ankyrin 1 (TRPA1) channel in activation of single unit mechanosensitive bladder afferent activities in the rat. Neurourol Urodynam. 2014;33(5):544-9.

29. Ikeda M, Kawatani M, Maruyama T, Ishihama H. Prostaglandin facilitates afferent nerve activity via EP1 receptors during urinary bladder inflammation in rats. Biomed Res. 2006;27(2):49-54.

30. Sengupta JN, Gebhart GF. Mechanosensitive properties of pelvic nerve afferent fibers innervating the urinary bladder of the rat. J Neurophysiol. 1994;72(5):2420-30.

31. van Asselt E, le Feber J, van Mastrigt R. Threshold for efferent bladder nerve firing in the rat. Am J Physiol. 1999;276(6 Pt 2):R1819-24.

Chapter 3

A novel single compartment *in vitro* model for electrophysiological research using the Perfluorocarbon FC-770

Mahipal Choudhary

Francesco Clavica

Ron van Mastrigt

Els van Asselt

Accepted: Physiological Research 2016

Abstract

Electrophysiological studies of whole organ systems *in vitro* often require measurement of nerve activity and/or stimulation of the organ via the associated nerves. Currently two-compartment setups are used for such studies. These setups are complicated and require two fluids in two separate compartments and stretching the nerve across one chamber to the other, which may damage the nerves. We aimed at developing a simple single compartment setup by testing the electrophysiological properties of FC-770 (a perfluorocarbon) for *in vitro* recording of bladder afferent nerve activity and electrical stimulation of the bladder. Perflurocarbons are especially suitable for such a setup because of their high oxygen carrying capacity and insulating properties.

In male Wistar rats, afferent nerve activity was recorded from postganglionic branches of the pelvic nerve *in vitro*, *in situ* and *in vivo*. The bladder was stimulated electrically via the efferent nerves. Organ viability was monitored by recording spontaneous contractions of the bladder. Additionally, histological examinations were done to test the effect of FC-770 on the bladder tissue. Afferent nerve activity was successfully recorded in a total of 11 rats. The bladders were stimulated electrically and high amplitude contractions were evoked. Histological examinations and monitoring of spontaneous contractions showed that FC-770 maintained organ viability and did not cause damage to the tissue. We have shown that FC-770 enables a simple, one- compartment *in vitro* alternative for the generally used two compartment setups for whole organ electrophysiological studies.

Introduction

In electrophysiology, isolated whole organ systems are often studied for a better understanding of their function. In experiments requiring stimulation of the organs via efferent nerve fibers and/or recording of the afferent nerve activity from the organ, an *in vitro* setup is often preferred over an *in vivo* setup because: i) it offers greater control over the extracellular environment and therefore enables a more accurate measurement of the response of the organ to various stimuli (e.g. drugs, mechanical and/or electrical stimulation), without interference of the central nervous system or other functions such as circulation and breathing [1], ii) experimental variation is less leading to better reproducibility, iii) it does not require complex surgical procedures or extensive monitoring [2]. Additionally, *in vitro* experimentation is a great step forward towards reduction of experimental animal usage [3].

Two compartment *in vitro* setups have frequently been reported in the literature for the aforementioned research [2, 4-7]. In a typical two compartment setup, the first compartment (filled with an oxygenated Krebs solution), containing the organ under investigation, serves the purpose of maintaining organ viability. The second compartment acts as a nerve recording/stimulating chamber and is filled with paraffin oil to provide electrical insulation. The nerve, still attached to the organ, is pulled into the recording chamber through a small hole or gate between the two chambers. The opening is then sealed with silicon grease to prevent leakage [2, 5]. Apart from the implicit requirement of a relatively large length of nerve, guiding the nerve from the viability chamber to the recording chamber is a delicate procedure which can easily damage the nerve (e.g. by overstretching). Another popular technique used for electrophysiological studies on a nerve-organ system involves suction electrodes [6, 7]. In this setup the nerve is sucked into a micropipette filled with a conductive fluid. This setup has some drawbacks. Firstly, the diameter of the tip of the micropipette has to be adjusted to the size of the nerves. Secondly, the micropipette tip is made of thin, fragile glass which can break easily during handling and may damage the nerves. Thirdly, only monopolar recordings can be made through the fluid filled suction electrodes, which have a low signal to noise ratio as compared to bipolar differential

electrodes [8]. Lastly, high frequency signal components cannot be measured as accurately as with bipolar electrodes [8].

To overcome the complications of two compartment setups, we developed a single compartment set up using a perfluorocarbon (PFC), FC-770[9]. PFCs are basically organofluorine compounds produced by replacing all hydrogen atoms of hydrocarbons with fluorine. They do not interact with biological tissue due to the high stability and inertness of carbon-fluoride bonds [10] and have a high capacity for dissolving respiratory gases at atmospheric pressure. Additionally, PFCs are highly insulating because of their high dielectric strength (>40 kV, 0.1" gap, k = 1.9) [11]. The aim of the current study was to provide a proof-of-concept that FC-770 can be used as a new method for recording bladder afferent nerve activity and electrical stimulation of the bladder through efferent nerve fibers in a single compartment *in vitro* setup as well as for *in vivo* and *in situ* electrophysiology.

Methods

Experimental procedures

All laboratory and experimental procedures were conducted in accordance with Erasmus MC guidelines. Male Wistar rats were anaesthetized with urethane (1g/kg) intraperitoneally for the experiments. A total of 19 rats (animal weight = 390 ± 40g, 8-9 weeks old) were used in this study. Fig. 1 shows the distribution of animals used for different (combinations of) experiments. The electrophysiological suitability and functionality of FC-770 was tested in *in vivo*, *in situ* and *in vitro* conditions:

In vivo experiments

In anaesthetized rats, bladder filling (volumetric flow rate = 0.11 ml/min) and measurement of pressure were done by inserting a 23G needle at the top of the bladder. The other end of the needle was attached to a disposable pressure transducer (BD DTX Plus™) and an infusion pump using a 2-way connector. Pressure was measured using a Statham SPI 1400 blood pressure monitor. The pressure transducer was calibrated using a column of water before the start of

each experiment. Postganglionic bladder nerves, presumably branches of the pelvic nerve, were mounted on a bipolar electrode consisting of two thin (diameter 0.1 mm) platinum-iridium hook shaped wires separated by a distance of 0.5-1 mm. The bladder was repeatedly filled with saline up to a certain volume (~1ml) or until voiding occurred. The nerve activity was recorded and amplified by a DISA 15C01 EMG amplifier (gain: 100-200,000) and band-pass filtered with a Krohn-Hite 3944 filter (Bessel, 4th order, 200-2000 Hz). Bladder pressure and nerve activity were sampled at 25Hz and 25 kHz respectively and 30-900 s episodes were stored using a custom written Labview® program. The recorded signals were processed with a custom written MATLAB® program.

In situ experiments

For *in situ* experiments, the rats were euthanized after the *in vivo* measurements by injecting an overdose of KCl in the heart, while keeping the original setup intact. Care was taken not to disturb the nerve-electrode configuration. The bladder was repeatedly filled with saline and the corresponding pressure-nerve activity was recorded as described in the preceding section.

In vitro experiments

After euthanizing the rats, the bladder along with prostate, urethra, attached nerves and surrounding tissue were removed and placed in a pre-warmed (30 °C) Krebs solution. After careful removal of the surrounding tissue, the preparation was transferred to a preheated (~30 °C) FC-770 (3M™ Fluorinert™ Electronic Liquid) filled single compartment setup. Some tissue surrounding the bladder was not removed and was used to fixate the preparation to the bottom of the compartment, to avoid floating of bladder. The whole setup was placed on a heated platform which maintained the temperature of the fluid around 30 ˚C. The FC-770 was perfused continuously with carbogen throughout the experiment. Bladder filling and nerve activity recording were performed in the same way as described in the *in vivo* experiments section. After filling the bladder with up to ~1ml of saline, a mechanical stimulus was applied by pushing a cotton swab on the bladder for a period of ~4-5 seconds until there was a discernible increase in the nerve activity. The bladder pressure and nerve activity were stored, as described in the *in vivo* experiments section.

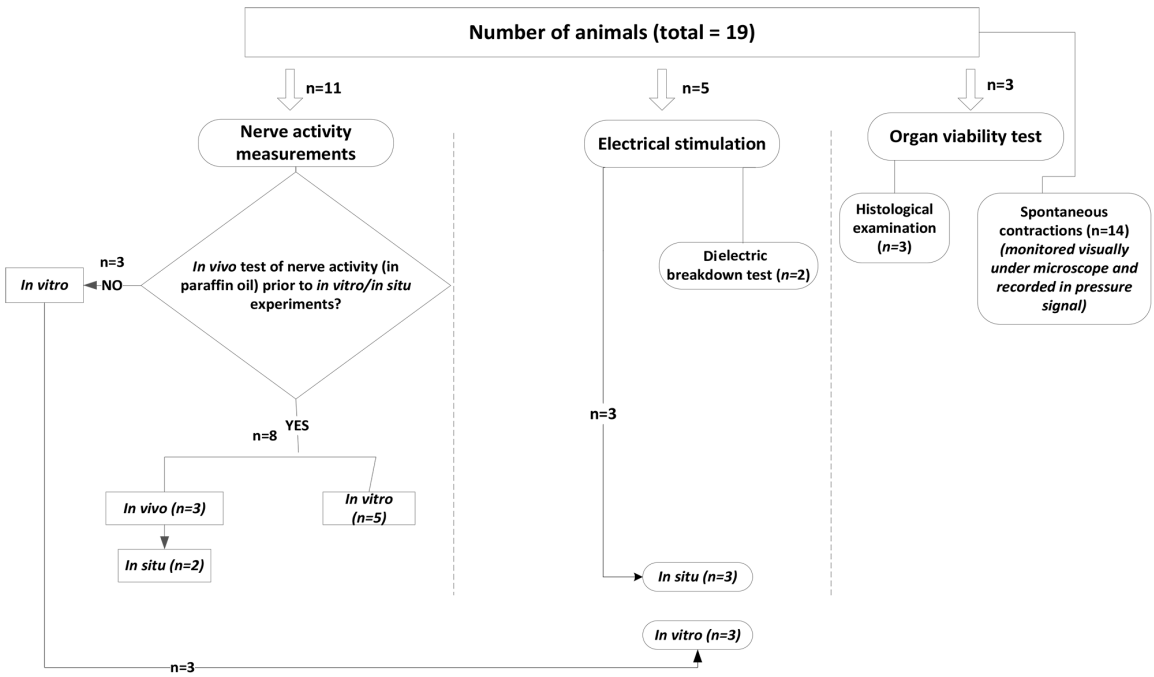

Fig. 1. Schematic overview of experiments on all animals used

All experiments were done in FC-770 except the *in vivo* test of nerve activity (shown in the diamond box).

Three types of measurements were done:

1. Nerve activity measurements

In 8 rats, nerve activity was tested *in vivo* prior to the *in vitro* and *in situ* experiments. Rats in which nerve activity was recorded successfully in at least 25 % of the *in vivo* measurements were selected for further *in vitro/in situ* experiments. In 3 rats nerve activity was measured *in vitro,* without prior *in vivo* experiments. To quantify the nerve activity, first the baseline nerve activity was determined by taking the average of 30 seconds of the resting nerve activity at the beginning of filling when the bladder was empty and pressure was low. Next, the nerve signal

was divided into windows of 1s and the peaks (local maxima) exceeding the baseline nerve activity were counted with a custom written Matlab® program. A measurement was considered successful when there was a good correlation between nerve activity and bladder pressure i.e. the number of peaks in the nerve activity was maximal during the maximum pressure. We also determined the effect of FC-770 on the linear dependence of nerve activity on bladder pressure. A linear regression model was fitted to the afferent activity-pressure data in MATLAB® to calculate slope and offset.

2. Electrical stimulation

Bladders were stimulated electrically via the pelvic nerve *in situ* and *in vitro* by rectangular pulses of width 400 µs, frequency 10 Hz and an amplitude (starting with 1 V) increasing in steps of 1 V until a bladder contraction in the form of a pressure rise of approximately 10 cmH_2O above the baseline was observed and then the stimulation of switched off. Bladders were stimulated for ~2 hours, with rest periods of 10 min between successive stimulations of ~3-4 s. To verify that the bladders were stimulated via the nerves and not via the muscle, the electrical coupling between nerve and bladder was verified at the end of experiments by cutting the nerve and stimulating again.

3. Organ viability test

To test the viability of bladder and nerves in FC-770, the pressure during spontaneous contractions of the bladder in nerve activity and electrical stimulation experiments was recorded upon filling with saline. The spontaneous contractions were also observed under the microscope. The effect of FC-770 on bladder tissue was histologically compared to that of a physiological buffer (carbogenated and heated Krebs solution).

Statistical analysis

All data are presented as mean ± SD. Using the SPSS® statistical package (version 21.0, SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was used to test normality and Levene's test for homogeneity of variance. A one-way ANOVA test was used for normally distributed data and a Kruskal–Wallis test for non-normally distributed. A $p < 0.05$ was considered significant.

Results

1) Nerve activity measurements

Nerve activity measurements were done in a total of 11 rats. In 8 of these it was measured *in vivo* in paraffin oil and minimally 3 measurements per rat were obtained in which the number of peaks in the nerve activity was maximal during the maximum pressure. The effect of FC-770 on the electrical conduction of the nerves was tested in 3 rats by comparing the amplitude of the nerve activity recordings *in vivo* (7 measurements) in paraffin oil with that of the nerve activity recordings *in vivo* in FC-770 (5 measurements). No significant difference ($p = 0.8$, one-way ANOVA) in the number of peaks (175052 ± 171168 vs 156725 ± 163342) was found. An example of nerve activity recorded *in vivo* in FC-770 is shown in Fig. 2(I).

The slope and intercept of nerve activity and pressure was compared between measurements done in paraffin oil and FC-770, where no significant difference (0.20 ± 0.21 vs 0.20 ± 0.18 ± 0.10, $p > 0.05$, Kruskal-Wallis test) was found. Furthermore, in 2 of these 3 rats, afferent nerve activity was also measured *in situ,* i.e. after sacrificing the rats, where 3 episodes of nerve activity were recorded (Fig. 2 (II)). After confirming the presence of nerve activity in the *in vivo* settings, nerve activity experiments were done in 5 bladder preparations in the single compartment *in vitro* setup. 6 successful measurements were done in 3 rats (Fig. 3 (I)), whereas no nerve activity could be recorded in the other 2 rats. A linear polynomial fit of nerve activity as a function of bladder pressure is shown in Fig.3 (II). In 3 other rats, nerve activity was recorded *in vitro* without any prior *in vivo* nerve activity test. In 2 of those rats, 3 successful measurements of nerve activity were recorded *in vitro*.

2) Electrical stimulation

Electrical stimulation was performed in a total of 6 rats *in situ* and *in vitro*. In 3 of these rats nerve activity measurements had been done before. The pelvic nerve was stimulated until a rise of ~10 cmH_2O above the baseline in the bladder

pressure was observed and then stimulation was switched off, which resulted in an immediate decline in pressure. In 3 rats, the bladder nerve was electrically stimulated during a period of 2 hours *in situ* and 15 episodes of bladder contraction upon stimulation were recorded. Fig. 4a shows an example of such an *in situ* electrical stimulation. In the other 3 rats, 8 episodes of high amplitude bladder contractions were recorded upon stimulation *in vitro*. An example of an *in vitro* electrical stimulation is shown in Fig. 4b. In 2 of these 3 rats, the nerve was cut midway between the bladder and the stimulation site and no contractions could be evoked after that, verifying that the nerve was electrically stimulated, not the muscle.

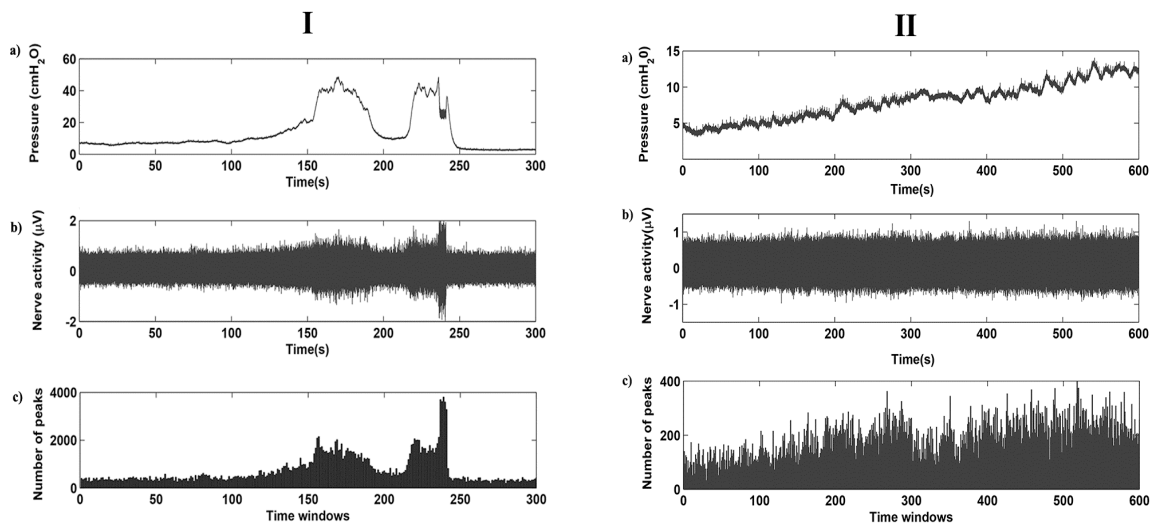

Fig. 2. An *in vivo* and *in situ* pressure-nerve activity measurement with the abdominal cavity of the anaesthetized rat filled with FC-770.

Ia) The pressure development during the filling phase and a voiding contraction. A typical rat voiding contraction is marked by high frequency oscillations (HFO) of the urethral sphincter. IIa) The pressure development during bladder filling in an *in situ* preparation. In both I & II, b) shows the corresponding nerve activity. c) The number of peaks in nerve activity overcoming the baseline.

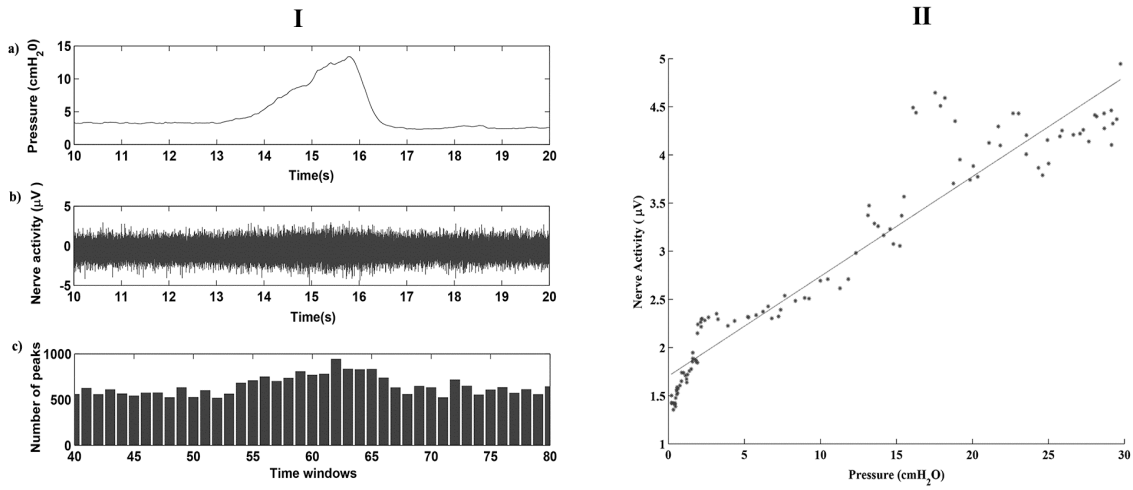

Fig. 3. An *in vitro* pressure-nerve activity measurement of a rat bladder-nerve preparation in FC-770.

I) After filling the bladder up to a certain volume (approx. 1 ml), the bladder was slightly pushed with a cotton swab and then the stimulus was removed. a) Shows the recorded pressure during the mechanical activation. b) The corresponding nerve activity. c) The number of peaks in nerve activity overcoming the baseline nerve activity. II) A linear polynomial fit of nerve activity as a function of bladder pressure recorded during the filling phase of a voiding contraction in one rat.

3) Test of organ viability

Spontaneous bladder contractions

Bladder viability was tested by continuous filling of the bladder with saline. In 14 out of the 16 animals in which nerve activity and electrical stimulation experiments (*in vitro* and *in situ)* were done, spontaneous bladder contractions were observed for ~90-120 min after sacrificing the rats.

Histological examinations

Histological examination of tissue from 3 rats showed no evidence of autolysis and necrosis. Muscle bundles were found to be intact with no visible signs of edema (fig. 5).

Discussion

In the current study, we showed that a single compartment *in vitro* setup using FC-770 can be used as a new method for recording rat bladder afferent nerve activity and electrical stimulation of the bladder via efferent bladder nerve fibers. One of the essential requirements for recording nerve activity in an *in vitro* setup is the insulation of electrodes, which is primarily the reason why two-compartment setups are used. We have shown that FC-770 provides the necessary electrical insulation in a single compartment setup by successfully recording afferent nerve activity in our *in vitro* setup. Additionally, no significant difference was found between the nerve activity recorded *in vivo* in paraffin oil and FC-770, which implies that FC-770 affect the amplitude of nerve activity no more than paraffin oil does.

We also tested the functionality of FC-770 in an *in situ* model, in which afferent nerve activity recording and electrical stimulation of the bladder were measured successfully. The *in situ* model enables a quick and easy switch from *in vivo* to *in situ* without changing the setup, hence reducing the damage to bladder and nerves. The *in situ* set up also enables the study of the rat micturition cycle without interference from the central nervous system in a whole animal model. The spontaneous contractions recorded in our *in situ* experiments have also been reported in other animal models in isolated bladders [12]. The origin of these non-voiding contractions has been shown to be autonomous in nature and their frequency and amplitude largely depends on bladder volume [13, 14]. Therefore, the recorded *in situ* nerve activity is assumed to be afferent. However, the autonomous spontaneous contractions in *in vivo* studies, have been associated with pelvic efferent nerve discharge [15, 16], indicating a regulation by the central nervous system. A similar mechanism might exist *in situ* leading to an efferent component in the recorded measurements.

Electrical stimulation of the bladder via efferent nerve fibers was favorably tested *in vitro* and *in situ* in FC-770. In both cases high amplitude bladder contractions were evoked upon stimulation. In 3 rats, bladder contractions were evoked up to 2 hours after sacrifice. Due to the high oxygen carrying capacity (approx. 25 times that of blood), FC-770 provides good organ viability, which was confirmed by the

spontaneous contractions of the bladder throughout the experiments. The viability of the bladder nerves was ascertained by evoking bladder contractions upon electrical stimulation of these nerves. In our study, histological examinations of bladders kept in FC-770 showed no signs of cell death, muscle damage or any disruption in the anatomy of the tissue, which strongly suggests that FC-770 maintained tissue viability without damaging it.

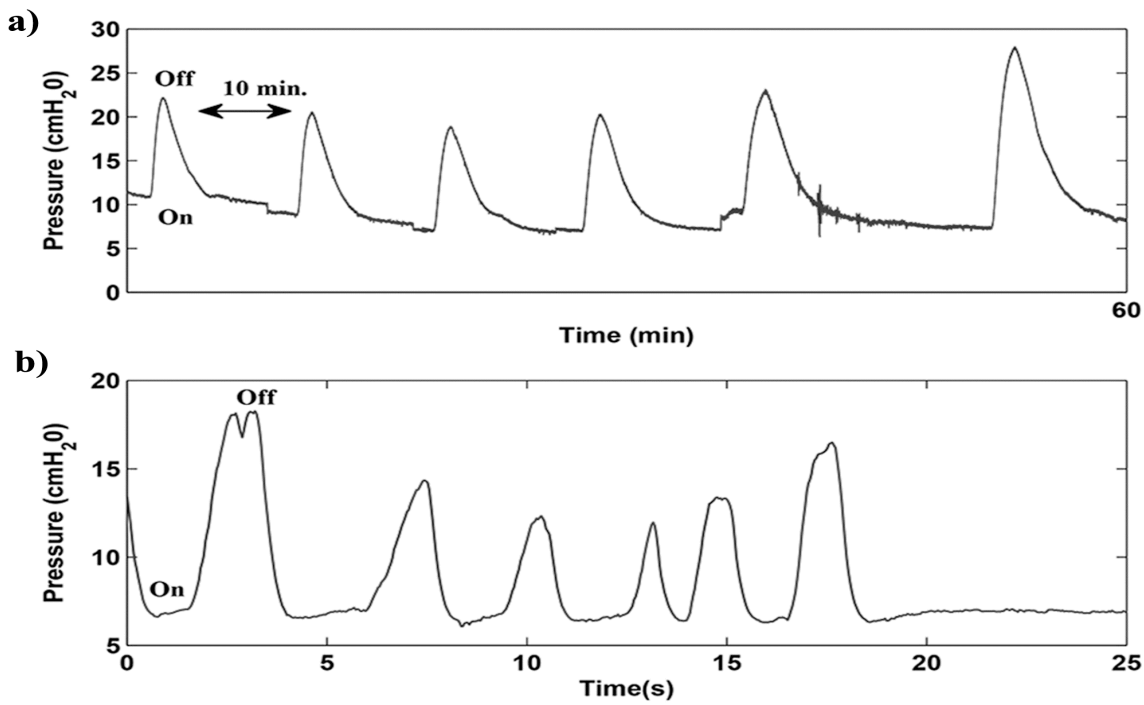

Fig. 4. Electrical nerve stimulation of a rat bladder-nerve preparation *in situ* and *in vitro* in FC-770.

The upper panel (a) shows the bladder pressure change during electrical stimulation *in situ*. In this rat the bladder was stimulated intermittently with 10 minutes rest periods between consecutive stimulations. The bladder was stimulated until a peak in pressure was observed, then the stimulation was switched off. The lower panel (b) shows evoked bladder contractions upon electrical stimulation *in vitro*. In this example multiple On/Off electrical stimulations were done in a short period of time.

FC-770 is immiscible with aqueous and hydrophobic solutions, which facilitates the removal of blood or any other unwanted liquid from the chamber. A limitation caused by this property of FC-770 is that it makes extravesicular administration of drugs difficult, which can be overcome by preparation of emulsions and nutrient solutions of PFCs [17]. Additionally, most of the drug administration tests done on bladders can also be done via intravesicular administration [18, 19] or through arterial perfusion [20]. Another minor limitation of FC-770 is that due to its high density [11], the bladder floats on the surface of the fluid. This requires fixation of the bladder to the bottom of the chamber [21].

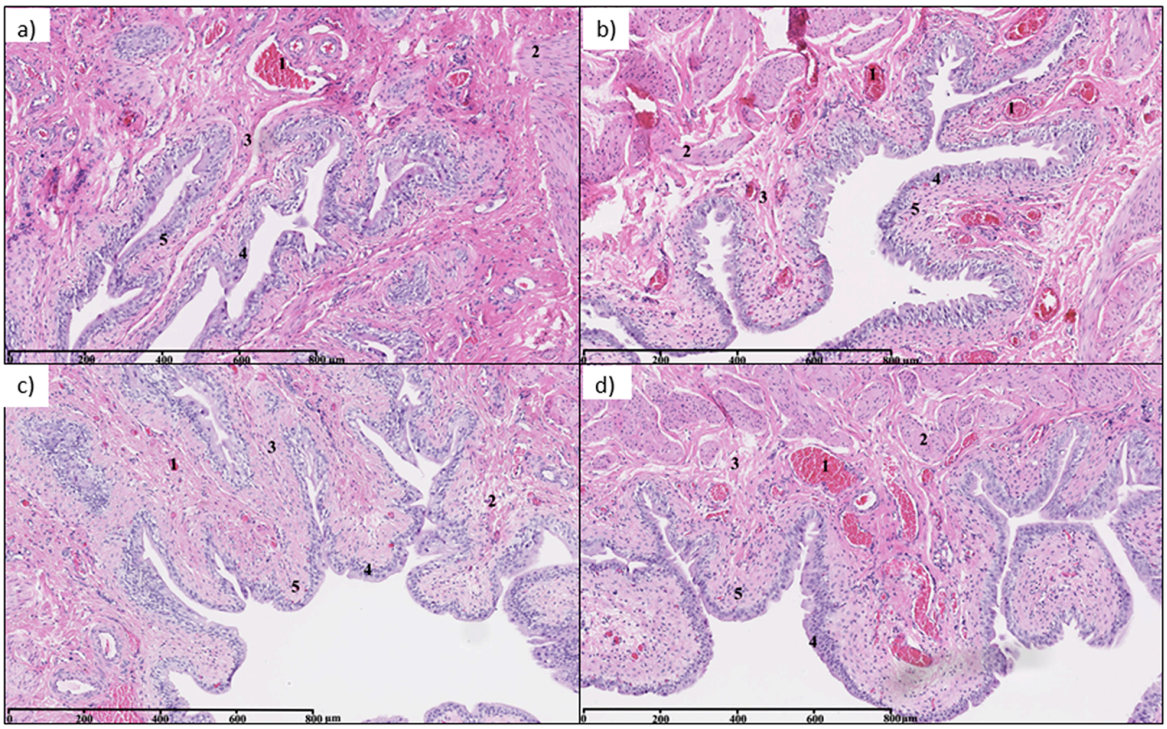

Fig. 5 (A-D). Histological examination of bladder tissue

Hematoxylin and Eosin stained tissue from two bladders, cut in half. One half was kept in Krebs solution (A, C) and the other in FC-770 (B, D) for a period of 90 minutes. Histological examination of bladder kept in FC-770 showed well preserved tissue architecture similar to the tissue kept in Krebs solution: 1-blood vessels, 2-smooth muscle, 3-connective tissue, 4-urothelium and 5-lamina propria.

Our proposed single compartment model can be applied to a broad field of electrophysiology in various other animal models in which two compartment models are generally used. For example, in a study of afferent nerve fibers in ureters [22], a two chamber setup – an oxygenating chamber and a paraffin oil filled recording chamber was used. In another study, for control of a prosthetic hand, an *in vitro* peripheral nerve preparation in a two chamber grease-gap bath setup was used for stimulation and recording [5]. A similar two compartment design was also used in the study of esophagus and stomach afferents [23]. The setup design described in preceding examples requires difficult preparation and is vulnerable to failures. These limitations could be overcome with our proposed one compartment setup.

Conclusion

We have shown that FC-770 enables a simple, one compartment *in vitro* alternative for the existing two compartment models used in various whole organ electrophysiological studies. The results of this study provide sufficient indications that PFC could be a valuable tool for electrophysiological studies. Further studies are warranted on a larger group of animals in order to definitively prove the value of this setup in a broader field of electrophysiology.

References

1. Hutcheson JC, Stein R, Chacko S, Carr M, Canning DA, Zderic SA. Murine in vitro whole bladder model: a method for assessing phenotypic responses to pharmacologic stimuli and hypoxia. Neurourol Urodyn. 2004;23(4):349-54.
2. Namasivayam S, Eardley I, Morrison JF. A novel in vitro bladder pelvic nerve afferent model in the rat. Br J Urol. 1998;82(6):902-5.
3. Russell WMS BR. The principles of humane experimental technique. London: Methuen. 1959.
4. Yu Y, de Groat WC. Effects of stimulation of muscarinic receptors on bladder afferent nerves in the in vitro bladder-pelvic afferent nerve preparation of the rat. Brain Res. 2010;1361:43-53.
5. Valderrama-Gonzalez GD, Gasson MN, Warwick K, Whalley BJ, editors. Prosthesis grasp reflex via peripheral nerve control - An in vitro study. Cybernetic Intelligent Systems (CIS), 2010 IEEE 9th International Conference on; 2010;1-6.
6. Rong W, Burnstock G. Activation of ureter nociceptors by exogenous and endogenous ATP in guinea pig. Neuropharmacology. 2004;47(7):1093-101.
7. Daly DM, Nocchi L, Liaskos M, McKay NG, Chapple C, Grundy D. Age-related changes in afferent pathways and urothelial function in the male mouse bladder. J Physiol. 2014;592(Pt 3):537-49.
8. Issa ZF, Miller JM, Zipes DP. CHAPTER 2 - Electrophysiological Testing. In: Issa ZF, Miller JM, Zipes DP, editors. Clinical Arrhythmology and Electrophysiology (1). Philadelphia: W.B. Saunders; 2009. p. 27-56.
9. Choudhary MS, van Asselt E, van Mastrigt R, Clavica F. A novel single compartment in vitro model: Perflurocarbons for electrophysiological studies of the rat urinary bladder. Conf Proc IEEE Eng Med Biol Soc. 2013;1446-9.
10. Clark LC, Jr., Gollan F. Survival of mammals breathing organic liquids equilibrated with oxygen at atmospheric pressure. Science. 1966;152(3730):1755-6.
11. Datasheet/FC-770. Data sheet Fluorinert™ Electronic Liquid FC-770: 3M Electronics, http://multimedia.3m.com/mws/mediawebserver?mwsId=66666UgxGCuNyXTtmxMtO8T_EVtQEcuZgVs6EVs6E666666--&fn=FluorinertElecLiqFC770Info.pdf; 2007.

12. Drake MJ, Harvey IJ, Gillespie JI. Autonomous activity in the isolated guinea pig bladder. Exp Physiol. 2003;88(1):19-30.

13. Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JI. Phasic non-micturition contractions in the bladder of the anaesthetized and awake rat. BJU Int. 2006;97(5):1094-101.

14. Clavica F, Choudhary M, van Asselt E, van Mastrigt R. Frequency analysis of urinary bladder pre-voiding activity in normal and overactive rat detrusor. Neurourol Urodyn. 2014;34(8):794-9.

15. Satchell P. The initiation of non-micturating contractions in the feline bladder. Acta Physiol Scand. 1991;143(3):339-44.

16. Choudhary M, van Asselt E, van Mastrigt R, Clavica F. Neurophysiological modeling of bladder afferent activity in the rat overactive bladder model. J Physiol Sci. 2015;65(4):329-38.

17. Haiss F, Jolivet R, Wyss MT, Reichold J, Braham NB, Scheffold F, et al. Improved in vivo two-photon imaging after blood replacement by perfluorocarbon. J Physiol. 2009;587(Pt 13):3153-8.

18. Fowler CJ. Intravesical treatment of overactive bladder. Urology. 2000;55(5A Suppl):60-4; discussion 6.

19. Pandita RK, Andersson KE. Intravesical adenosine triphosphate stimulates the micturition reflex in awake, freely moving rats. J Urol. 2002;168(3):1230-4.

20. Sadananda P, Drake MJ, Paton JF, Pickering AE. An exploration of the control of micturition using a novel in situ arterially perfused rat preparation. Front Neurosci. 2011;5:62.

21. Kuroda Y, Fujino Y, Kawamura T, Suzuki Y, Fujiwara H, Saitoh Y. Excellence of perfluorochemical with simple oxygen bubbling as a preservation medium for simple cold storage of canine pancreas. Transplantation. 1990;49(3):648-50.

22. Cervero F, Sann H. Mechanically evoked responses of afferent fibres innervating the guinea-pig's ureter: an in vitro study. J Physiol. 1989;412:245-66.

23. Page AJ, Blackshaw LA. An in vitro study of the properties of vagal afferent fibres innervating the ferret oesophagus and stomach. J Physiol. 1998;512 (Pt 3):907-16.

Chapter 4

Frequency Analysis of Urinary Bladder Pre-Voiding Activity in Normal and Overactive Rat Detrusor

Francesco Clavica

Mahipal Choudhary

Els van Asselt

Ron van Mastrigt

Neurourol Urodyn. 2015 Nov;34(8):794-9

Abstract

Aims

To test the hypothesis that voiding in anaesthetised rats is preceded by recurrent changes in the pattern of bladder pressure. To explore the use of frequency analysis as an analytical tool for automatically detecting these changes and to provide quantitative data on bladder pre-voiding activity.

Methods

We developed an algorithm, based on frequency analysis, to study bladder pressure during the filling phase in anaesthetised rats. Two applications of the algorithm were tested: i) as a predictor of a voiding contraction with alarms generated which would make conditional nerve stimulation to prevent incontinence possible ii) as a new index to quantify rapid pressure transients in normal and overactive detrusor conditions (i.e. induced by acetic acid instillation into the bladder).

Results

The results show that a very high percentage (~90 %) of the alarms were generated by the algorithm within 100 s before the voiding. The index of rapid transients and the bladder volume before voiding were respectively ~13 % less and ~42 % less in acetic acid.

Conclusions

We have shown that a simple algorithm, based on frequency analysis of bladder pressure, can be used to predict voiding and to provide quantitative data on non-voiding bladder activity and its changes due to pathology. Although the results refer to anesthetized rats, they are promising and warrant further urodynamic investigation to identify if similar patterns occur in non-anesthetized rats and in humans.

Introduction

For a long time the urinary bladder has been considered passive during the filling phase. However, transient rises in bladder pressure due to local contractions of the bladder wall, known as Non-Voiding Contraction (NVC) activity, have been recorded in normal individuals of many species, including humans [1-7]. Ambulatory urodynamic recordings show NVC activity in almost 70 % of healthy volunteers [8] implying that this activity is normal rather than an exception. A few studies have provided relevant information on this activity both through *in vivo* [4, 7] and *in vitro* experiments using bladder filling at physiological rates. Streng et al. [4] have shown that NVC activity, in unconscious rats, is manifested by slow transients (ST) in bladder pressure (with frequencies below 0.1 Hz) which appear soon after the start of the bladder filling. Small rapid transients (RT), in the frequency range of 0.2-0.5 Hz, are superimposed on these ST oscillations. Often, RT were recorded only shortly before a voiding contraction, which suggests that automatic pattern recognition of RT may be a promising tool to predict an impending voiding contraction. The appearance of a change in the pattern of the bladder pressure with bladder filling was also documented in *in vivo* [7] and *in vitro* [2] experiments on guinea pig bladders. A relation between an increase in the frequency of pressure transients with bladder volume was suggested in both cases [2, 7]. However, the origin of RT (and in general of NVC), their role in the initiation of micturition, the relative contributions of the central nervous system and the intrinsic properties of the bladder wall are still unclear [6, 7] with many contradictory opinions mostly based on qualitative observations. Drake et al. [3] have shown that localized bladder contractions, during bladder filling, were more sustained and at a higher frequency in women with increased bladder sensation (urinary urgency group) than in asymptomatic women (i.e. control group). In this respect, a better understanding of the NVC changes in the overactive bladder (OAB), normally associated with increased bladder sensation and detrusor overactivity, would be highly desirable. The OAB mechanism is still poorly understood because of the number of components involved i.e. epithelium lining the surface of the urinary bladder, nervous system, interstitial cells and smooth muscle cells. For a fast and a more objective diagnosis of the overactive bladder,

attention must be given to techniques which can provide quantitative data, in addition to the qualitative data supplied by the patients (OAB being a symptom syndrome). The aim of the present work is therefore to provide a tool to produce quantitative data on NVC activity of the bladder, with a particular focus on the rapid transients just before voiding (i.e. pre-voiding activity). An algorithm based on frequency analysis has been developed to automatically identify changes in the pattern of the bladder pressure and potentially predict an impending voiding contraction by generating an alarm. On the basis of this algorithm, a closed loop device may be developed to prevent incontinence by conditional stimulation. The algorithm has also been used to provide an index to quantify differences in the NVC activity in normal and pathologic conditions e.g. associated with detrusor overactivity. It has been applied to bladder pressure recordings in rats in which the bladder was filled with saline and acetic acid to induce overactivity [9, 10].

Materials and methods

Approval for the animal experiments was obtained from the local Erasmus MC Animal Experiment Committee. All laboratory and experimental procedures were conducted in accordance with institutional guidelines.

Fourteen male Wistar rats (Charles River, US) (weight: 454 ± 44 g) were anaesthetized with urethane (1 g/kg). Access to the bladder was gained through an abdominal incision. A 23G needle was used to insert a small catheter (of the same size of the needle) at the top of the bladder to allow for bladder filling and measurement of bladder pressure. A disposable pressure transducer was used to connect the catheter to a Statham SP1400 blood pressure monitor. Bladder pressure was sampled at 25 Hz and recorded using custom written software in LabVIEW (National Instrument, USA). The rats were euthanized at the end of the experiment with an overdose of KCl, injected into the heart.

Saline and Acetic acid fillings

Saline (0.9 % NaCl) and acetic acid (0.5 %) were instilled into the rat bladder using an infusion pump (model 2202, Harvard apparatus, US), set at 0.05 ml/min. Before each filling, the bladder was emptied with a syringe that was connected to the

catheter [11] through a 3-way connector, thereafter the pump was run until voiding occurred or to a maximum filling of 1ml. The bladder was repeatedly filled first with saline, then with acetic acid (0.5 %). The first filling with acetic acid was used to remove the last saline from the bladder, it was not used for analysis. A rest interval of 15 min between fillings was considered sufficient to allow for bladder relaxation.

Design of the algorithm

Two main features were commonly observed in the bladder pressure recordings (Fig.1A): i) the appearance of rapid transients (RT) and ii) the actual voiding. The actual voiding was identified manually by the start (t_1) and the end (t_2) point of high frequency voiding oscillations in the bladder pressure (Fig.1A, inset), which are caused by contractions of the external urinary sphincter and are normal in the male rat urination [12]. The algorithm, custom written in Matlab (Mathworks, USA), focuses on the detection of the RT. It uses 10 s windows of the pressure recordings: starting from the first 10 s, subsequent windows were obtained by moving one sample forward each time, similar to a moving average. An amplitude spectrum was calculated from each window and the Area Under the Curve (AUC) in the range 0.2-0.6Hz (Fig.1) was calculated as described in our earlier work [13]. This frequency range was also identified by Streng et al. [4], referring to RT. If N_s is the number of samples of each recording, N_s values of AUC were obtained. An alarm was provided by the algorithm as soon as the value of AUC_i (where AUC_i is the i^{th} value of AUC) overcame a specified threshold, see paragraph.

Test of the algorithm

In all rats, each recording of bladder pressure began with the start of the infusion pump. The minimum recording time was 300 s, only recordings including exactly one voiding event were processed. Three intervals were identified in each recording (Fig.2):

The voiding interval (X: $t_1 < t < t_2+15$ s): this region was discarded as the high frequency voiding oscillations interfered with the results of the algorithm. Since in this period the actual voiding is taking place, any alarms from the algorithm would

be too late for actions to prevent voiding and therefore not useful. We considered t_2+15 s (not t_2) as the end point of this interval in order to reset the AUC values to baseline.

True Positive interval (TP: t_1-100 s<t<t_1): t_1- 100 s until the start of the voiding was chosen since increased RT activity was observed in this interval by Streng et al. [4]. For each rat, a threshold value for generating the alarms was set at half of the maximum value of AUC in the True Positive Interval in the first saline recording. This threshold was then used for all subsequent recordings (i.e. saline and acetic acid). Whenever the algorithm provided an alarm in this interval, the alarm was considered a True Positive Alarm since it means that a voiding event will occur within 100 s. The success rate of the algorithm was calculated from the ratio of the number of True Positive Alarms and the total number of alarms generated by the algorithm in each recording.

False Positive interval: the remaining interval. Whenever an alarm was provided by the algorithm in this period of time, it was considered a false positive alarm.

Additionally we defined:

False negative alarm: any alarm provided after the start of the voiding or no alarms provided by the algorithm although a voiding did occur.

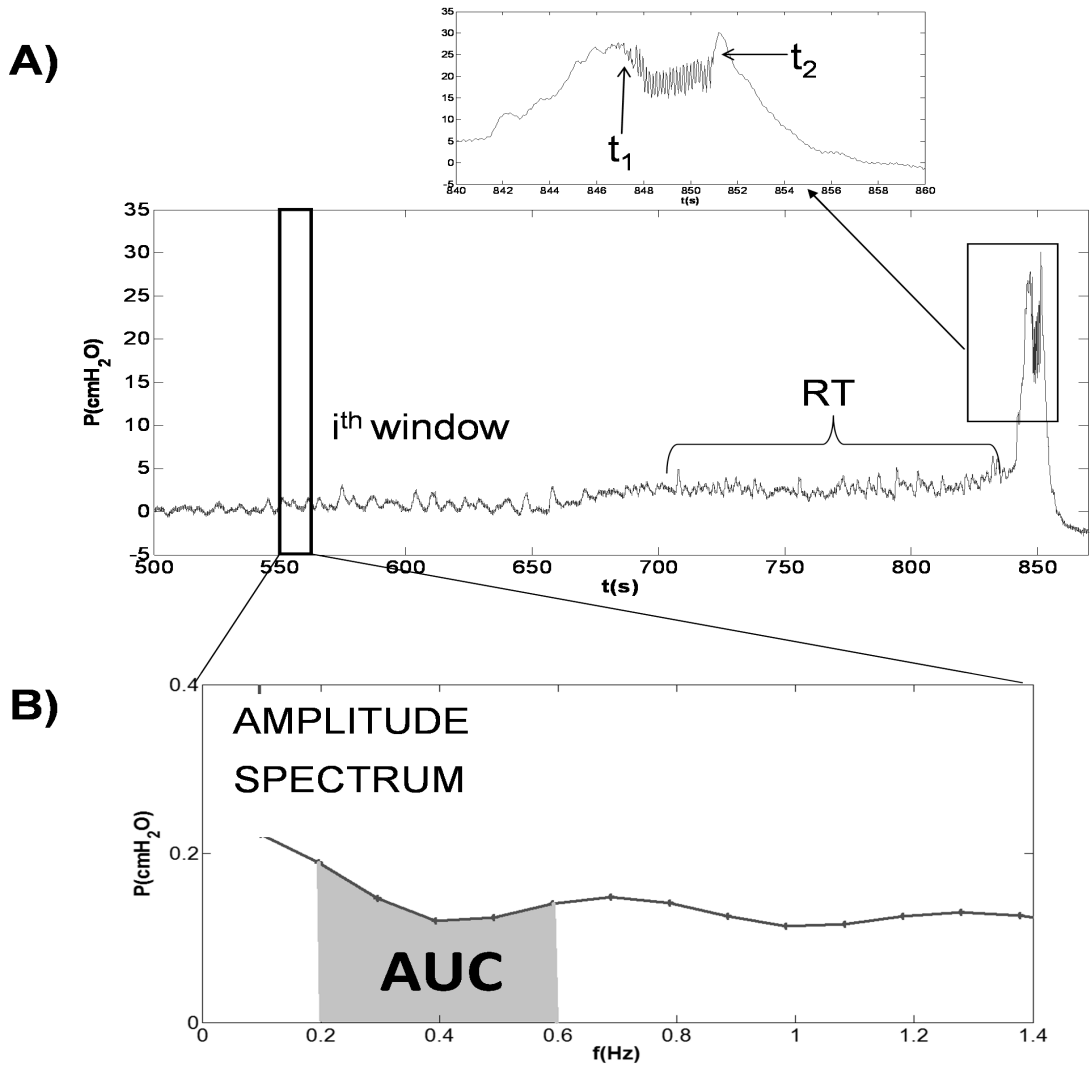

Fig. 1 A. A typical recording of bladder pressure during filling and voiding of an anesthetised rat. The inset illustrates the voiding contraction with the start (t_1) and the end (t_2) of high frequency voiding oscillations. RT refers to the rapid transients before the voiding. B. From each 10 s window an amplitude spectrum was derived and the area under the curve (AUC) was calculated in the range of frequencies: 0.2-0.6 Hz.

Index of rapid pressure transients

We defined the Index of Rapid pressure Transients as the maximum value of AUC in the True Positive interval of each recording. Additionally, since the RT have been associated with bladder volume sensation [2, 7], values of maximum pressure and filled volume at $t=t_1$ were calculated for each rat to test for possible dependencies of the rapid transients on these two variables. All data are presented as mean ± standard deviation (SD). Parameter values were compared between saline and acetic acid using the Mann-Whitney *U* test while the Kruskal-Wallis test was used for comparisons within rats (IBM SPSS Statistics, USA). Significance was set at $p < 0.05$.

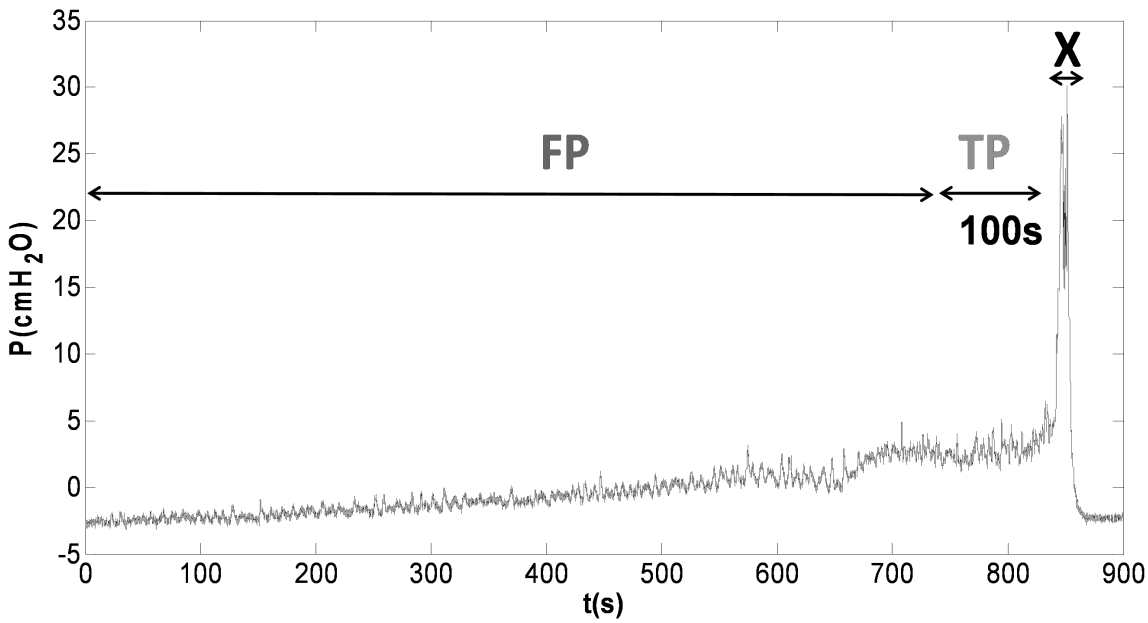

Fig. 2. The method used to test the algorithm's accuracy. In each recording three regions were defined (see text for details). TP is the true positive interval (100s before the start of the voiding), FP is the false positive interval and X is the discarded region (voiding interval: $t_1<t<t_2+15$ s).

Results

Algorithm performance

One rat was excluded from the analysis because all its pressure recordings were characterized by repetitive voiding contractions. From 115 recordings of the remaining 13 rats, 7 were excluded because of multiple voiding contractions (4 in saline and 3 in acetic acid). Eleven other recordings were excluded because of problems with the pressure catheter (air bubbles in the set-up and/or catheter not well inserted in the bladder). Table 1 shows the resulting number of selected recordings. An example of data analysis with the Matlab algorithm is shown in Fig. 3. When the voiding is approaching, a clear change is visible in the pattern of bladder pressure (Fig. 3A) with the appearance of rapid transients at t~190 s. The alarm is triggered when AUC overcomes the defined threshold (horizontal line in Fig. 3B). A summary of the algorithm performance is reported in Table 2. The average success rate was lower in saline (85 %) than in acetic acid (95 %), however the difference was not significant (Mann–Whitney U-test, $p > 0.05$). No False Negative Alarms were reported during the data analysis. Table 2 also shows the duration of the True Positive and Voiding interval as a percentage of the total recording time (total recording time: 38028 s in saline and 23333 s in acetic acid).

Table 1. Number of selected saline and acetic acid measurements.

Rat	1	2	3	4	5	6	7	8	9	10	11	12	13
Saline (n=57)	4	1	9	4	3	6	6	4	2	4	6	4	4
Acetic acid (n=40)	-	4	4	8	2	4	2	1	1	5	1	5	3

Index of rapid transients, maximum pressure and filling volumes

Three parameters were compared (Table 3) between saline and acetic acid fillings: i) maximum pressure before voiding (i.e. before t_1), ii) filled volume and iii) index of rapid transients (IRT). The three parameters were normalized, for each rat, by dividing by the maximum value of that parameter in saline fillings. The number of available values for filled volume was less than for maximum pressure and IRT (n = 31 instead of 57 for saline; n = 38 instead of n = 40 for acetic acid) because of problems encountered in emptying the bladder before the start of the filling. The mean value of normalized IRT was ~13 % higher in saline (Mann–Whitney U-test, $p < 0.05$) than in acetic acid fillings. The value of filled volume before voiding was ~42 % higher in saline (Mann–Whitney U-test, $p < 0.05$). The maximum pressure was 2 % higher in saline, however, this difference was not significant ($p > 0.05$, Mann–Whitney U-test). In saline, the values of the three parameters did not vary significantly among animals ($p > 0.05$, Kruskal-Wallis Test). In acetic acid the difference among animals was not significant for IRT ($p > 0.05$, Kruskal-Wallis Test) while it was significant for maximum pressure and filled volume ($p < 0.05$, Kruskal-Wallis Test).

Table 2. Average success percentage in saline (n = 57) and acetic acid (n = 40). In each recording: % Success = (number of True Positive Alarms / total number of alarms) ·100. The duration of the True positive interval and Voiding interval are expressed as percentage of the total recording time.

	Success (%)		True positive interval (%)		Voiding interval (%)	
	mean	SD	mean	SD	mean	SD
Saline	85	24	17	6	3	1
Acetic Acid	95	12	18	5	3	1
p-value	0.06					

Discussion

To the best knowledge of the authors, the present work represents the first attempt to use frequency analysis of bladder pressure to analyse the non-voiding activity of the bladder and to predict a voiding contraction. Preliminary results were earlier published in an abstract based on experiments in the first 5 animals [13].

The idea of using bladder associated signals to predict an impending voiding is not new, a few attempts were made using signals from the pudendal nerve [14] or EMG of the external anal sphincter [15]. However, probably due to the complexity of the recordings, none of these methods have appeared in clinical practice yet [16]. In studies on bladder neuromodulation, it has been shown that continuous or conditional electrical stimulation of the pudendal nerve inhibited bladder contractions in spinal cord injury patients and it increased the bladder capacity. Our algorithm based on frequency analysis of the bladder pressure signal together with an implantable pressure transducer, such as the one developed by Melgaard et al. [16] could potentially lead to the development of an integrated algorithm for closed loop-conditional bladder relaxation, more robust than the existing amplitude based algorithms (as the one used in Melgaard et al. [16]). The current experimental techniques for conditional stimulation rely on pressure values in the time domain [17, 18] and the stimulation of the pudendal nerve is triggered when the pressure increases above a certain threshold (e.g. 10 cmH_2O above the baseline in humans [17, 18]). The application of the Matlab algorithm, introduced in our study, may improve the current techniques by predicting a voiding contraction based on a change in the pattern, while the pressure is still low (i.e. before the voiding contraction starts). Figure 3 shows that the appearance of RT corresponds to an initial peak of AUC (i~5000) at relatively low bladder pressures (t~190 s, $P < 10cmH_2O$); subsequently the increase continued until the voiding occurred. By lowering the threshold in Fig. 3B, alarms can be generated also at low pressure, close to baseline values, in contrast to existing methods. The high success rates of the algorithm (85 % for saline and 95 % for acetic acid, Table 2) appear very promising for detecting pre-voiding activity.

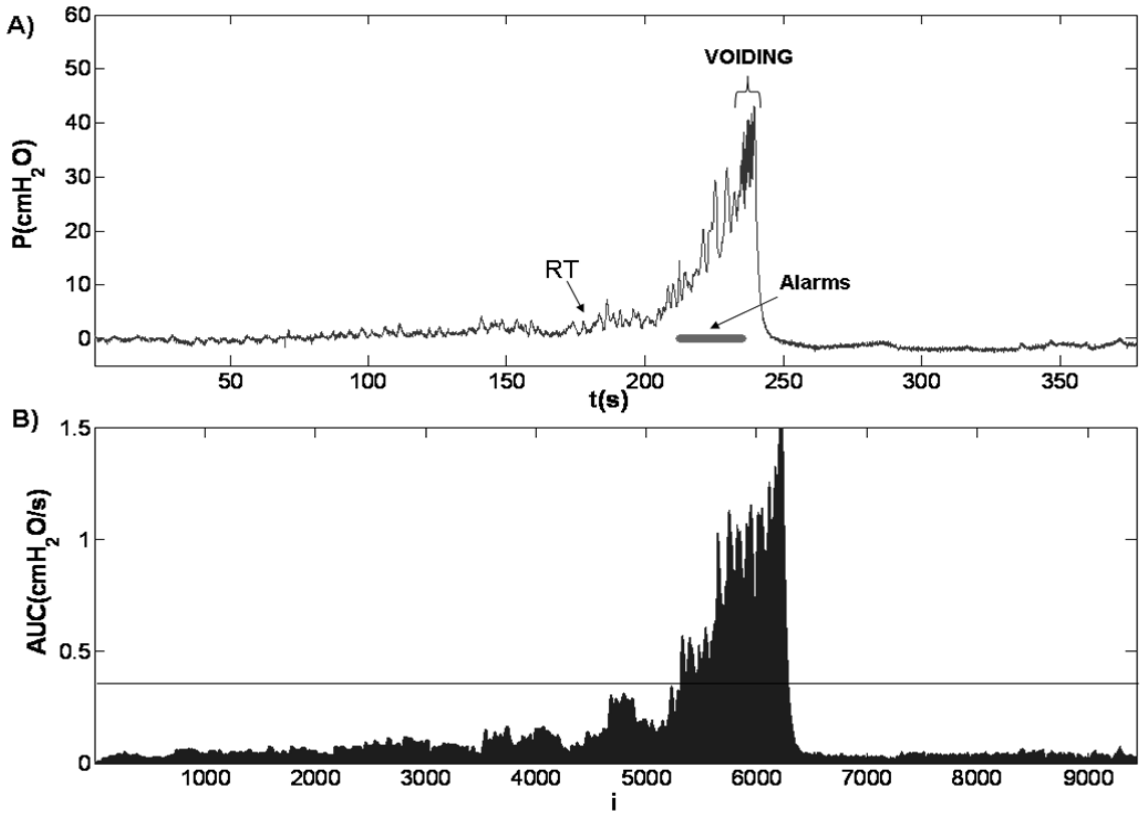

Fig. 3. Example of using the algorithm: A) measured bladder pressure and B) Area Under the Curve (AUC) of the amplitude spectrum, as defined in Fig. 1. For each value of AUC_i> threshold, an alarm is generated by the algorithm. The Threshold (in this case 0.4 cmH_2O/s) is indicated by the horizontal line. The alarms are represented by the bold line in panel A.

The threshold value for the alarm was optimized for each rat considering the first pressure recording in saline, the voiding period was identified by the high frequency voiding oscillations which are typical in rodents but not in humans. In the clinical setting, a similar optimization protocol (i.e. use of one voiding recording to set the algorithm parameters) could be applied to patients using a normal urodynamic test, where the voiding period can be identified with a flow meter.

Table 3. Maximum Pressure (p_{max}) before voiding, Filled Volume (V) at $t=t_1$ and Index of Rapid Transients (IRT) for the saline and acetic acid. Note that the data are represented as normalized values. For each parameter the mean value and standard deviation (SD) are provided. (Significance was set at $p < 0.05$, Mann–Whitney U-test).

	Maximum Pressure		Filled Volume		Index of rapid transients	
	mean	SD	mean	SD	mean	SD
Saline (n=57 for p_{max} and IRT, n=32 for V)	0.87	0.11	0.84	0.19	0.81	0.17
Acetic Acid (n=40 for p_{max} and IRT, n=28 for V)	0.85	0.13	0.59	0.29	0.72	0.17
p-value	0.310		0.0004		0.0004	

While urodynamic investigations are performed on a daily basis, too little is known yet about the role of bladder non-voiding activity and its changes due to pathologies. The present work is the first attempt to compare the non-voiding activity between normal and detrusor overactive conditions in terms of changes of bladder pressure pattern. Our choice of IRT represents a first attempt to quantify the bladder pre-voiding activity using frequency analysis. The IRT choice has shown to be successful since a significant difference was detected between saline and acid fillings (i.e. IRT index was significantly reduced during acetic acid filling). We believe that frequency analysis based parameters represent a more robust method than amplitude based algorithms since both amplitude and frequency of the transients are taken into account. Our results showed no significant difference in the maximum pressure before voiding in saline and acetic acid (Table 3), in line with the findings of other investigators using the same model of detrusor overactivity [19, 20] (i.e. acetic acid inducing detrusor overactivity). The filled

volume values also agreed with other studies reporting a significant reduction of bladder capacity when the bladder was filled with acetic acid [19, 21]. The rat model with acetic acid infusion therefore exhibited an increase in urinary frequency (i.e. voiding occurs at lower filled volumes), rather than non-voiding activity as in the human overactive bladder. Different results on non-voiding activity associated to overactive conditions were found by Gillespie et al. [22] using a different model of detrusor overactivity (i.e. partial bladder outflow obstruction).

Drake et al. [2] showed that, in *in vitro* experiments on whole guinea pig bladders, pressure transients increased in frequency as the filling volume increased. Since the *in vitro* bladder was disconnected from the central nervous system the pressure transients were locally generated in the bladder wall. Although we are still far from providing a fulfilling understanding of the mechanism behind bladder non-voiding activity, we speculate that also *in vivo* the origin of the rapid pressure transients is mainly myogenic and volume dependent. In our experiments we found fewer RT in acetic acid fillings despite the fact that these fillings are generally associated with increased afferent bladder nerve activity [23, 24]. The threshold for the alarms in the algorithm was defined referring to a filling with saline, the lower IRT in acetic acid may explain the higher success rate of the algorithm in acid (i.e. only the highest values of AUC, which are usually the closest to a voiding, overcome the threshold). In the experiments conducted by Streng et al. [4] as well as in ours, rats were anaesthetized with urethane. Although an effect of urethane cannot be denied it is known to spare the micturition reflex [25]. In our experiments, except for only a few cases, the pre-voiding activity appears 'more flat' (i.e. transients with lower amplitude) than the pressure traces of the recordings in Streng et al. [4], however, a clear change of the pressure pattern (i.e. higher frequency transients) can be observed in the 'RT' region (Figure 1). We speculate that the differences in the pre-voiding activity are associated with the rat strain and with the gender (male Wistar vs male and female Noble rats). Future developments will include data from conscious animals and will focus on identifying similar changes of bladder pressure in the human bladder. Drugs (including anaesthetics) which are known to modify

bladder physiology and mechanics before voiding can be tested using frequency analysis to quantify a phenomenon so far only qualitatively described.

Conclusions

In the present work we have shown that a simple algorithm, based on frequency analysis of bladder pressure, can provide quantitative data on non-voiding bladder activity in unconscious rats. This algorithm can potentially be used as a tool to i) predict voiding (to prevent incontinence in cases with a loss of bladder sensation by conditional neurostimulation) ii) quantify the change of bladder non-voiding activity in pathology (i.e. in overactive bladder conditions).

References

1. Van Os-Bossagh P, Kosterman LM, Hop WCJ, Westerhof BE, de Bakker JV, Drogendijk AC, et al. Micromotions of Bladder Wall in Chronic Pelvic Pain (CPP): A Pilot Study. Int Urogynecol J. 2001;12(2):89-96.
2. Drake MJ, Harvey IJ, Gillespie JI. Autonomous activity in the isolated guinea pig bladder. Experimental Physiology. 2003;88(1):19-30.
3. Drake MJ, Harvey IJ, Gillespie JI, Van Duyl WA. Localized contractions in the normal human bladder and in urinary urgency. BJU International. 2005;95(7):1002-5.
4. Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JI. Phasic non-micturition contractions in the bladder of the anaesthetized and awake rat. BJU Int. 2006;97(5):1094-101.
5. Andersson K-E, Arner A. Urinary Bladder Contraction and Relaxation: Physiology and Pathophysiology. Physiological Reviews. 2004;84(3):935-86.
6. Gillespie JI. Modulation of autonomous contractile activity in the isolated whole bladder of the guinea pig. BJU International. 2004;93(3):393-400.
7. Biallosterski BT, van Koeveringe GA, van Kerrebroeck PE, Gillespie JI, de Wachter SG. Nonvoiding Activity of the Guinea Pig Bladder. The Journal of Urology. 2011;186(2):721-7.
8. Robertson AS. Behaviour of the human bladder during natural filling: the Newcastle experience of ambulatory monitoring and conventional artificial filling cystometry. Scand J Urol Nephrol Suppl. 1999;201:19-24.
9. Mitobe M, Inoue H, Westfall TD, Higashiyama H, Mizuyachi K, Kushida H, et al. A new method for producing urinary bladder hyperactivity using a non-invasive transient intravesical infusion of acetic acid in conscious rats. Journal of Pharmacological and Toxicological Methods. 2008;57(3):188-93.
10. Yoshiyama M, Kobayashi H, Araki I, Du S, Zakoji H, Takeda M. Sex-related differences in activity of lower urinary tract in response to intravesical acid irritation in decerebrate unanesthetized mice. Am J Physiol Regul Integr Comp Physiol. 2008;295(3):R954-60.

11. Yokoyama O, Yusup A, Miwa Y, Oyama N, Aoki Y, Akino H. Effects of tolterodine on an overactive bladder depend on suppression of C-fiber bladder afferent activity in rats. J Urol. 2005;174(5):2032-6.

12. le Feber J, van Asselt E. Pudendal nerve stimulation induces urethral contraction and relaxation. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. 1999;277(5):R1368-R75.

13. Clavica F, Choudhary MS, van Asselt E, van Mastrigt R. Can an algorithm predict a voiding contraction in unconscious rats? Conf Proc IEEE Eng Med Biol Soc. 2013;2013:1334-7.

14. Wenzel BJ, Boggs JW, Gustafson KJ, Grill WM. Detecting the onset of hyper-reflexive bladder contractions from the electrical activity of the pudendal nerve. IEEE Trans Neural Syst Rehabil Eng. 2005;13(3):428-35.

15. Wenzel BJ, Boggs JW, Gustafson KJ, Creasey GH, Grill WM. Detection of neurogenic detrusor contractions from the activity of the external anal sphincter in cat and human. Neurourol Urodyn. 2006;25(2):140-7.

16. Melgaard J, Rijkhoff NJ. Detecting the onset of urinary bladder contractions using an implantable pressure sensor. IEEE Trans Neural Syst Rehabil Eng. 2011;19(6):700-8.

17. Hansen J, Media S, NØHr M, Biering-SØRensen F, SinkjÆR T, Rijkhoff NJM. Treatment of neurogenic detrusor overactivity in spinal cord injured patients by conditional electrical stimulation. J. Urol. 2005;173(6):2035-9.

18. Horvath EE, Yoo PB, Amundsen CL, Webster GD, Grill WM. Conditional and continuous electrical stimulation increase cystometric capacity in persons with spinal cord injury. Neurourology and Urodynamics. 2010;29(3):401-7.

19. Kakizaki H, de Groat WC. Role of Spinal Nitric Oxide in the Facilitation of the Micturition Reflex by Bladder Irritation. J. Urol. 1996;155(1):355-60.

20. Kitagawa Y, Wada M, Kanehisa T, Miyai A, Usui K, Maekawa M, et al. JTS-653 blocks afferent nerve firing and attenuates bladder overactivity without affecting normal voiding function. J Urol. 2013;189(3):1137-46.

21. Patra PB, Thorneloe KS. Enhanced sensitivity to afferent stimulation and impact of overactive bladder therapies in the conscious, spontaneously hypertensive rat. J Pharmacol Exp Ther. 2011;338(1):392-9.

22. Gillespie JI, Palea S, Guilloteau V, Guerard M, Lluel P, Korstanje C. Modulation of non-voiding activity by the muscarinergic antagonist tolterodine and the beta(3)-adrenoceptor agonist mirabegron in conscious rats with partial outflow obstruction. BJU Int. 2012;110(2 Pt 2):E132-42.

23. Zvara P, Wright AJ, Roach K, Ursiny M, Shapiro B, Dagrosa LM, et al. A non-anesthetized mouse model for recording sensory urinary bladder activity. Front Neurol. 2010;1:127.

24. Ikeda M, Kawatani M, Maruyama T, Ishihama H. Prostaglandin facilitates afferent nerve activity via EP1 receptors during urinary bladder inflammation in rats. Biomedical Research. 2006;27(2):49-54.

25. Matsuura S, Downie JW. Effect of anesthetics on reflex micturition in the chronic cannula-implanted rat. Neurourol Urodyn. 2000;19(1):87-99.

Part III

Electrical stimulation in overactive bladder

Chapter 5

Effect of tibial nerve stimulation on bladder afferent nerve activity in a rat detrusor overactivity model

Mahipal Choudhary

Ron van Mastrigt

Els van Asselt

Int J Urol. 2016 Mar;23(3):253-8.

Abstract

Objectives: To study the poststimulation effect of tibial nerve stimulation on rat bladder afferent activity and urodynamic parameters in normal and acetic acid induced detrusor overactivity conditions.

Methods: In urethane anaesthetized male Wistar rats, the tibial nerve was stimulated for 30 min at 5 Hz, pulse width 200 μs and amplitude approximately 3 times the threshold to induce a slight toe movement. The poststimulation effect was studied by measuring afferent nerve activity of postganglionic pelvic nerve branches and various urodynamic parameters under two different conditions: 1) In physiological saline filling experiments (simulating normal bladder condition) and, 2) in acetic acid irritated bladders (simulating detrusor overactivity).

Results: After 30 min of tibial nerve stimulation in saline filling experiments the bladder capacity, threshold pressure and afferent nerve activity were not significantly different from the prestimulation measurements. The instillation of 0.5 % AA significantly reduced the bladder capacity and increased the afferent nerve activity. Tibial nerve stimulation significantly improved the bladder capacity and suppressed the afferent nerve activity compared to prestimulation AA measurements.

Conclusions: A 30 min stimulation of tibial nerve inhibited afferent nerve activity in chemically irritated rat bladders and significantly restored the bladder capacity. This study provides important basic electrophysiological evidence to substantiate the clinical use of tibial nerve stimulation for treatment of symptoms related to detrusor overactivity.

Introduction

The Overactive Bladder (OAB) is a symptom based syndrome, detrusor overactivity being the prime indication. One of its hallmark characteristics is "urgency", often associated with involuntary bladder contractions and increased afferent sensation [1]. OAB affects approximately 12 % of the European and 17 % of the North American population and has a significant negative impact on the quality of life and mental health [2]. Antimuscarinic drugs are the first-line treatment, however, due to lack of efficacy or side effects, more than half of the patients terminate this treatment [3]. An important clinical alternative to pharmacological treatment is neuromodulation. Tibial Nerve Stimulation (TNS) is a form of peripheral neuromodulation which has been found effective in treating OAB symptoms, with lesser side effects than drugs. TNS therapy in humans is based on percutaneous stimulation of the tibial nerve for 30 minutes, performed once a week usually for 10–12 weeks followed by stimulation once per month [4]. Tibial nerve stimulation has also been reported to inhibit bladder overactivity and improve the bladder capacity in rat [5] and cat [6, 7] models.

Despite the widespread clinical use, the underlying mechanism of TNS is largely unknown [8] and animal studies to understand the basic science behind TNS have focused primarily on the effect of TNS on urodynamic parameters [5, 6], the electrophysiology has not been studied.

In an ongoing attempt to develop a method for conditional neurostimulation i.e. neurostimulation triggered by urgency [9], the aim of our present study was to investigate the poststimulation effect of tibial nerve stimulation on urodynamic parameters and bladder afferent nerve activity in normal and overactive detrusor simulated conditions.

Methods

Experimental procedures

Guidelines of the local Animal Experiment Committee for animal experiments were followed. 18 male Wistar rats (mean weight 413 ± 37g) were anaesthetized with urethane (1.2g/kg, i.p.). An abdominal incision was made and one of the postganglionic pelvic nerve branches (crushed between major pelvic ganglion and electrode, so that only afferent nerve signals were measured) was mounted on a custom made bipolar electrode consisting of two thin Platinum/Iridium hook shaped wires to record afferent nerve traffic. The electrode was placed close to the bladder, which made it possible to follow the nerves all the way to the bladder and to make sure electrical activity was recorded only from bladder nerves. Bladder pressure measurement and bladder filling (0.06 ml/min) were done by inserting a 23G needle at the top of the bladder. The other end of the needle was attached to a disposable pressure transducer and an infusion pump using a 3-way connector. Pressure was measured using a Statham SP1400 blood pressure monitor. The tibial nerve was accessed via the medial side of the right hind leg near the ankle. For stimulation a bipolar cuff electrode was placed around the nerve. The rats were euthanized at the end of the experiment with an overdose of KCl, injected into the heart.

Bladder filling and stimulation protocol

In type I measurements, the bladder was filled with physiological saline (0.9 % NaCl) until voiding occurred and 3-4 micturition cycles were recorded. The bladder was emptied before each filling and the filled volume required to evoke a voiding was considered to be the bladder capacity. Next, with an empty bladder the tibial nerve was stimulated for 30 min with monophasic rectangular pulses of frequency 5 Hz, width 200 μs and amplitude approximately 3 times the threshold to induce a slight toe movement. A range of 2-4 times the threshold is generally described in the literature [5, 6]. Poststimulation, the bladder was again infused with saline and measurements were repeated until 90 minutes after the stimulation.

In type II measurements, to determine the control bladder capacity, initially the bladder was filled 2-3 times with saline until voiding occurred. Next, the bladder was repeatedly filled with 0.5 % Acetic Acid (AA) to induce bladder overactivity and 3-4 micturition cycles were evoked. Thereafter the bladder was emptied and the tibial nerve was stimulated as described in the preceding section. Then, AA infusion was repeated to study the poststimulation effect of TNS, with the measurements done during ~90 min after the end of stimulation.

Signal processing

Afferent nerve activity was amplified by a DISA 15C01 EMG amplifier (amplification range: 100-200,000) and band-pass filtered with a Krohn-Hite 3944 filter (Bessel, 4th order, 200-2000 Hz). Nerve activity and pressure signals were displayed in real-time on a computer screen using a custom written LabVIEW® (National Instruments, USA) program and were sampled and stored at 25 kHz and 25 Hz respectively. Recordings with artifacts due to catheter displacement, air bubbles in the set-up or movement of the rat were excluded from analysis. These artifacts were observed during data acquisition and were seen as random, sharp high amplitude peaks in the pressure curve.

Recorded signals were processed and analysed with a custom written MATLAB® (Mathworks, USA) program. The nerve signal was rectified and averaged by taking the mean of 1000 samples, effectively reducing a 1 s interval to 25 data samples, similar to the pressure signal [10, 11]. An example of a pressure-afferent nerve activity recording is shown in fig. 1. For comparison between pre- and poststimulation measurements, mean of afferent nerve activity was calculated in the 20 s interval from t_1-20 s to t_1, where t_1 is the time at the maximum pressure immediately before the start of voiding which has been termed 'threshold pressure' [12]. This interval contains the beginning of an active contraction where the bladder contracts and the pressure increase till t_1. During t_1-t_2 the actual voiding took place. A typical male rat voiding contraction is associated with high frequency oscillations (HFO), caused by rapid contractions of the striated urethral sphincter [12]. The period t_1-t_2 was excluded from analysis because of movement artifacts in the recorded nerve and pressure signal. Urodynamic parameters such as maximum pressure, average pressure and minimum pressure were also calculated. To examine the effect of TNS on bladder non-voiding activity, we

calculated the number of non-voiding contractions occurring from the start of filling till the voiding. The non-voiding contractions have been defined as transient rises of 0.5–3 cmH_2O in bladder pressure in the literature [13]. These transients can be observed as local bladder pressure maxima (peaks) and were identified using the inbuilt MATLAB® function 'findpeaks'. A threshold (0.5–3 cmH_2O) was specified which allowed evaluation of peaks in the given range of amplitudes.

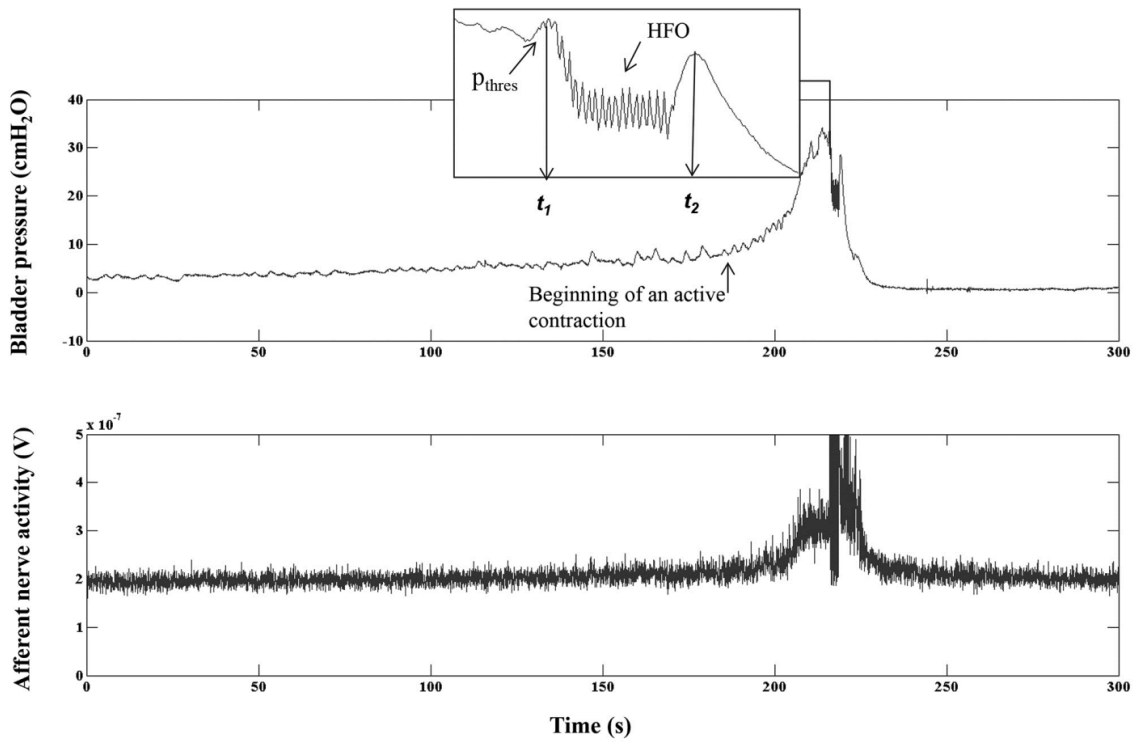

Fig. 1. Nerve activity and bladder pressure during a voiding cycle

An example of a pressure-afferent nerve activity recording with AA filling. The bladder filling started at t = 0. The upper panel shows the pressure during the filling phase with a typical male rat voiding contraction, marked by high frequency oscillations (HFO) of the urethral sphincter. The lower panel shows the corresponding afferent nerve activity.

Statistical analysis

All data are presented as mean ± SD along with median and range (maximum value minus minimum). A Shapiro-Wilk test was done for normality testing and Levene's test for homogeneity of variance. Based on these tests an ANOVA (two-way followed by Bonferroni multiple comparison) was performed on normally distributed data and the Mann-Whitney test was done on non-normal data using the SPSS® statistical package (version 21.0, SPSS Inc., Chicago, IL, USA) to compare groups. $p < 0.05$ was considered significant.

Results

Type I measurements

30 min of tibial nerve stimulation had no significant effect on the bladder capacity (1.0 ± 0.2 vs 0.9 ± 0.3 ml, $p > 0.05$) or afferent nerve activity (0.22 ± 0.02 vs 0.21 ± 0.01 μV, $p > 0.05$) in the saline filling measurements. The urodynamic parameters i.e. the threshold pressure, maximum pressure, minimum pressure and average pressure did not differ in the pre- and poststimulation measurements (Table 1, n = 8). The non-voiding activity measured as the number of spontaneous contractions was also found comparable between the two groups.

Type II measurements

Instillation of 0.5 % AA significantly reduced the bladder capacity to 40 % of the control saline experiments (0.9 ± 0.2 vs 0.5 ± 0.2, $p < 0.05$). The threshold pressure, maximum pressure, minimum pressure and average pressure were found to be comparable between the saline and AA measurements (Table 2, n = 10 rats). The sensitization caused by AA irritation caused a significant increase in the afferent nerve activity in the AA measurements (0.25 ± 0.04 vs 0.3 ± 0.01 μV, $p < 0.05$). TNS significantly increased the bladder capacity in the poststimulation AA measurements compared to the prestimulation AA measurements (0.5 ± 0.2 vs 0.7 ± 0.3 ml, $p < 0.05$) (an example is shown in fig. 2) and it significantly suppressed the afferent nerve activity (0.3 ± 0.01 vs 0.22 ± 0.04 μV, $p < 0.05$). Examples of pressure-afferent nerve activity recordings in the three groups are shown in fig. 3. The threshold pressure did not differ between the prestimulation and poststimulation AA measurements. There was no

significant change in the number of non-voiding contractions between pre- and poststimulation measurements (Table 2). An example of the identified non-voiding contractions or peaks is shown in fig. 4. The stimulation effect lasted at least until 90 min after cessation of the stimulation.

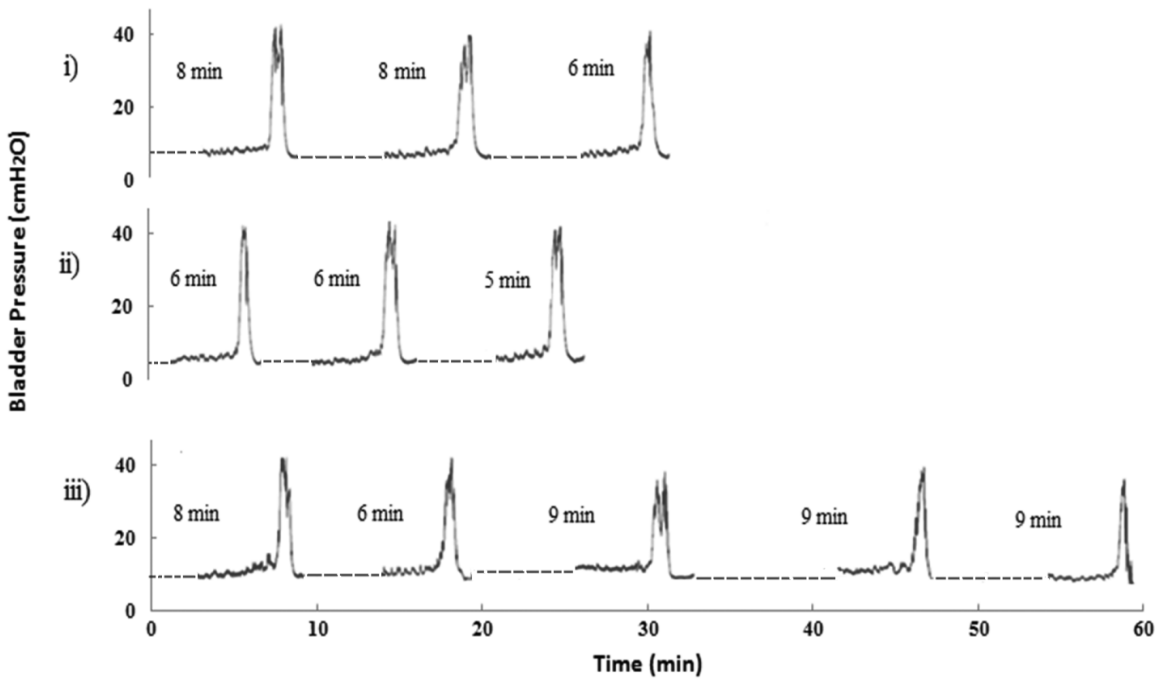

Fig. 2. Effect of tibial nerve stimulation on bladder capacity

An example of effect of TNS on filled volume (bladder capacity) in 1 rat. i) shows 3 saline voiding contractions, ii) a reduction in filling time (or filled volume) i.e. bladder capacity after instillation of AA and iii) an increase in filling time after 30 min TNS. 5 min periods of measurements, which contained at least one voiding contraction were recorded and stored. The dotted lines represent the unrecorded part of the measurement.

Table 1. Estimated parameters in type I measurements

Mean ± SD, median and range of the estimated parameters in prestimulation and poststimulation saline measurements (prestimulation, n = 19, and poststimulation, n = 19). N.S. indicates no statistical difference ($p > 0.05$) between the groups.

	Prestimulation saline mean ± SD, (median, range)	Poststimulation Saline mean ± SD, (median, range)
Afferent nerve activity (μV)	0.22 ± 0.02 (0.22, 0.07)	0.21 ± 0.01 N.S. (0.20, 0.05)
Bladder capacity (ml)	1.0 ± 0.2 (0.96, 1.0)	0.9 ± 0.3 N.S. (1.0, 0.9)
Threshold pressure, p_{thres} (cmH_2O)	29.0 ± 4.1 (29.1, 14.1)	26.1 ± 3.2 N.S. (26.6, 15.6)
Maximum pressure (cmH_2O)	32.2 ± 8.2 (31.2, 17.4)	28.9 ± 7.5 N.S. (29.2, 15.0)
Average pressure (cmH_2O)	8.4 ± 7.0 (5.0, 8.2)	8.2 ± 8.3 N.S. (3.1, 8.8)
Minimum pressure (cmH_2O)	2.6 ± 1.9 (2.0, 8.0)	2.2 ± 2.6 N.S. (0.9, 8.6)
Non-voiding activity (average number of contractions per filling)	37.8 ± 14.8 (40.5, 58)	38.8 ± 9.6 N.S. (39.0, 38)

Table 2. Estimated parameters in type II measurements

Mean ± SD, median and range of the estimated parameters in control saline, prestimulation and poststimulation AA measurements, n=81 measurements (saline = 25, prestimulation AA = 29, and poststimulation AA = 27). The symbols ϕ and * represent the statistical significance ($p < 0.05$) between group I vs II and II vs III respectively. N.S. indicates no statistical difference ($p > 0.05$) between the groups.

	Prestimulation saline (I) mean ± SD, (median, range)	Prestimulation AA (II) mean ± SD, (median, range)	Poststimulation AA (III) mean ± SD, (median, range)
Afferent nerve activity (μV)	0.25 ± 0.04 (0.24, 0.16)	0.3 ± 0.01 $^{\phi}$ (0.24, 0.64)	0.22 ± 0.04* (0.21, 0.16)
Bladder capacity (ml)	0.9 ± 0.2 (1.0, 0.9)	0.5 ± 0.2 $^{\phi}$ (0.4, 0.7)	0.7 ± 0.3* (0.6, 0.9)
Threshold pressure, p_{thres} (cmH_2O)	35.0 ± 6.3 (34.3, 30.5)	33.4 ± 7.1 $^{N.S.}$ (33.4, 29.5)	30.7 ± 5.1 $^{N.S.}$ (31.2, 19.8)
Maximum pressure, p_{max} (cmH_2O)	39.1 ± 6.7 (38.1, 26.7)	37.8 ± 7.6 $^{N.S.}$ (38.1, 27.1)	35.1 ± 5.1 $^{N.S.}$ (34.4, 24.6)
Average pressure, p_{avg} (cmH_2O)	8.3 ± 5.4 (6.9, 26.4)	6.8 ± 3.8 $^{N.S.}$ (6.2, 13.5)	7.2 ± 4.6 $^{N.S.}$ (5.8, 16.5)
Minimum pressure, p_{min} (cmH_2O)	4.5 ± 5.3 (2.5, 26.1)	3.3 ± 3.1 $^{N.S.}$ (2.0, 11.7)	4.2 ± 4.2 $^{N.S.}$ (2.9, 18.6)
Non-voiding activity (average number of contractions per filling)	43.2 ± 6.3 (44.0, 24)	42.4 ± 9.5 $^{N.S.}$ (43.5, 35)	39.1 ± 15.7 $^{N.S.}$ (41.0, 54)

Discussion

To our knowledge this study has demonstrated for the first time the poststimulation effect of tibial nerve stimulation on urodynamic parameters and bladder afferent activity in normal and simulated overactive detrusor rat bladders. TNS is known to be effective in clinical treatment of the overactive bladder syndrome in humans; the mechanism of action, however, is largely unknown [8, 14]. TNS is believed to restore the balance between excitatory and inhibitory bladder elements by modulating the signal traffic to and from the bladder via the sacral plexus [15, 16].

In our saline measurements, the 30 min stimulation did not have any effect on the measured bladder neural and mechanical parameters. The bladder capacity, threshold pressure and non-voiding contractions remained unchanged in pre- and poststimulation measurements. The unchanged bladder capacity is in contrast with a recent study by Matsuta et al. [5], where the authors reported a 40% increase in poststimulation bladder capacity. Although a definite explanation of these contrasting results is difficult, it might be due to a different nerve-electrode configuration. Matsuta et al. used a tripolar electrode which might stimulate a larger part of the nerve and/or different types of fibers compared to our bipolar electrodes. It is also noteworthy that Matsuta et al used female Sprague-Dawley rats weighing 198-200 g. Although, to the best of the author's knowledge, the effect of sex and strain on the tibial nerve stimulation has not been studied yet, some differences might exist. Several studies have highlighted the effect of sex and strain on bladder function [17-19]. Anatomical differences in spinal origin of the pelvic nerve innervation have been shown between Wistar and Sprague-Dawley rats [20]. In a comparative urodynamic study on Sprague-Dawley (SD), Wistar Kyoto (WKY) and Spontaneously Hypertensive rats (SHR), Patra et al. found significant higher voiding frequency in male as compared to female rats of all three strains [18]. In another study, a difference in effect of nerve growth factor was seen between two strains of rats, the intravesical application of nerve growth factor acutely induced bladder overactivity in WKY but not in SD rats [19]. Considering the aforementioned anatomical and functional differences, it is possible that TNS has a different mechanism of action in the two strains of rats in question.

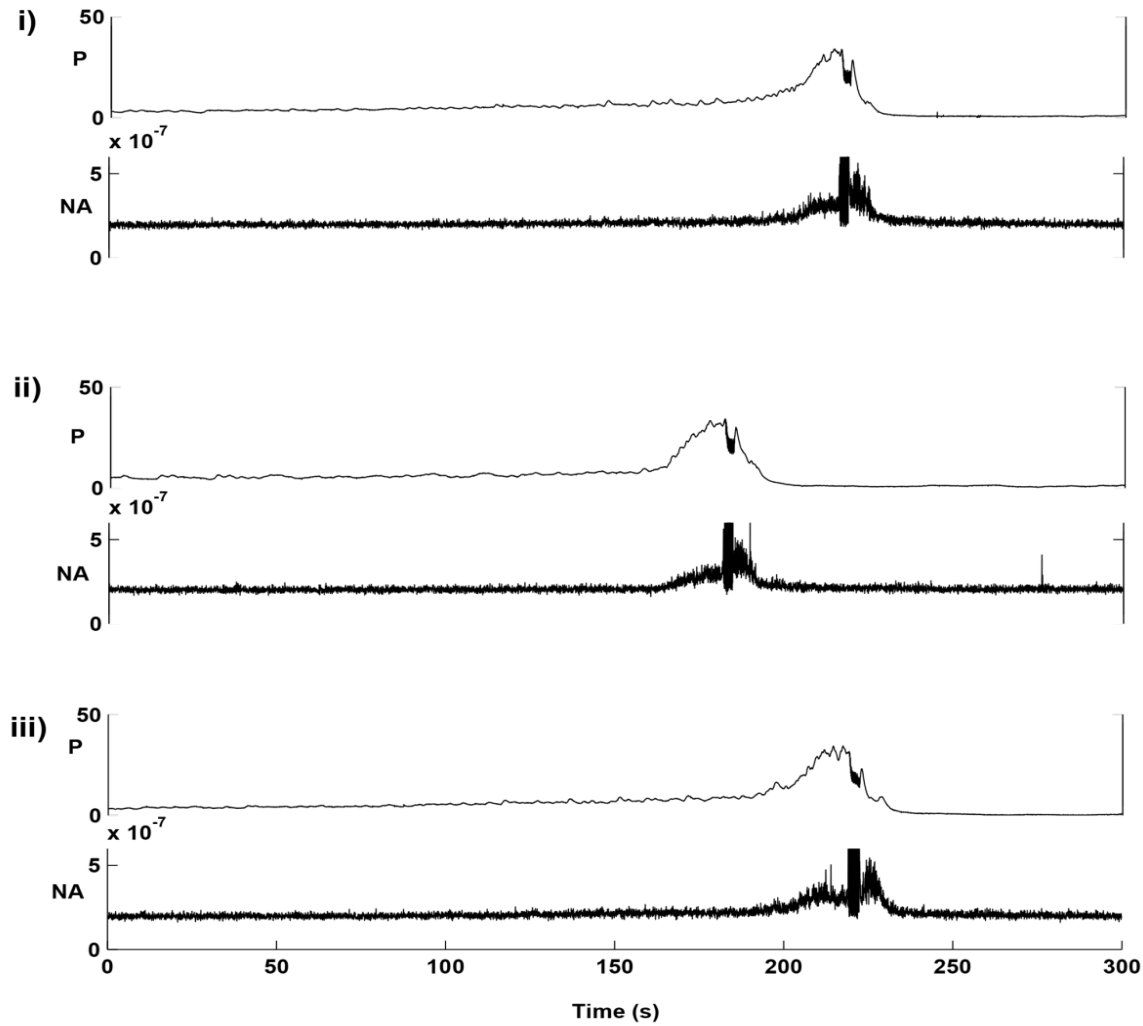

Fig. 3. Nerve activity and bladder pressure in pre- and poststimulation measurements

An example of a pressure-afferent nerve activity in the three measurement groups in one rat. i) shows a voiding contractions during a saline filling, ii) during AA filling, and iii) during AA filling after tibial nerve stimulation. For each case the upper panel (P) shows the pressure (in cmH_2O) during the filling phase and the lower panel (NA) shows the corresponding afferent nerve activity (in volts).

In the measurements with AA irritated bladders, TNS had a restoring effect on the poststimulation bladder capacity, which increased to 70 % of the control bladder capacity in saline measurements. Instillation of AA causes a sensitization of bladder C-fibers which have been reported to play a significant role in causing bladder overactivity under pathological conditions [21]. In our experiments, tibial nerve stimulation had an inhibitory effect on the bladder afferent activity. The multifiber nerve activity was recorded from a crushed postganglionic branch of the pelvic nerve innervating the bladder. We have earlier shown that crushing of nerve branch does not have a significant effect on the neurophysiology of the bladder, which is also supported by other studies [11, 22]. The recorded multifiber afferent nerve activity does not allow the distinction of myelinated and unmyelinated fibers, hence the inhibitory effect of TNS on individual fiber type could not be determined. In the literature the effect has been hinted at the suppression of C-fiber activity [6].

The role of the central nervous system in neuromodulation has been researched extensively. Chang et al. [23] showed a decreased level of C-fos expression in rat sacral spinal cord after electro-acupuncture stimulation of the hind-leg. In a different study on cats [24], it was shown that the inhibitory effect of stimulation of nerves from hind-limb muscles diminished after chronic spinal cord transection, possibly highlighting a role of the supra-spinal areas. The role of various inhibitory neurotransmitters in neuromodulation has been investigated only to a limited extent. A cat study has shown the involvement of opioid receptors (ORs) in somatic afferent inhibition of non-nociceptive bladder reflexes evoked by pudendal nerve stimulation (PNS) [25]. A recent study on the role of different subtypes of opioid receptors indicated that activation of μ and κ ORs is essential for producing TNS inhibition in irritated cat bladders [26]. The role of the spinal neurotransmitter γ-aminobutyric acid (GABA) in regulating inhibition of bladder activity in cats on PNS has also been suggested [27]. A similar study examining the role of glycine in pudendal nerve stimulation, suggested an excitatory effect in physiological conditions and a minor role in inhibition of bladder activity on PNS [28]. These studies indicate a differential role of inhibitory neurotransmitters in nociceptive and non-nociceptive bladder reflexes. It is believed that under physiological conditions, GABA and ORs inhibitory mechanism are activated to either facilitate or suppress the Aδ-fiber afferent-mediated spinobulbospinal

bladder reflex. However, in pathology, these neurotransmitters do not regulate the C-fiber mediated spinal reflex. These spinal neurotransmitter mechanisms might be used by TNS to cause an anti-nociceptive effect and suppress the bladder overactivity.

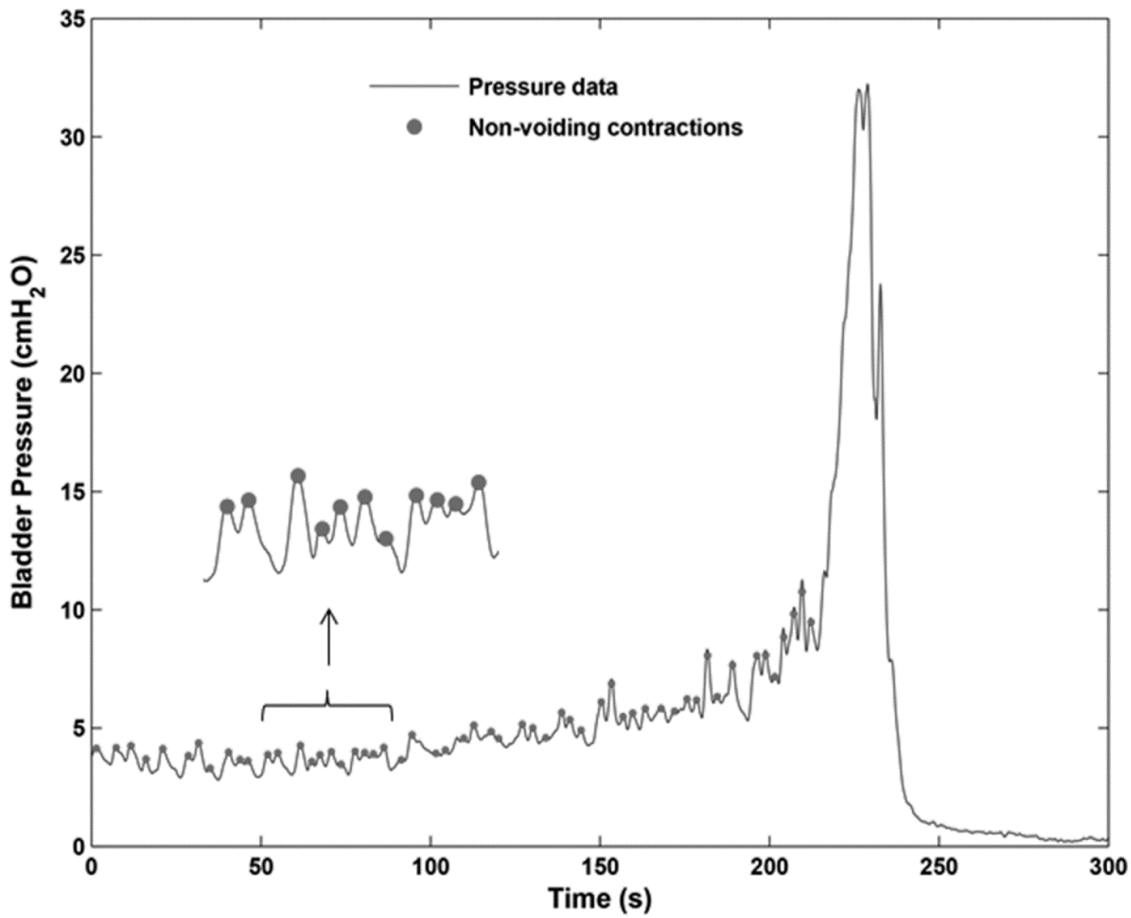

Fig. 4. Bladder non-voiding contractions

An example of non-voiding contractions occurring in the filling phase of a rat voiding cycle. The peaks (circles) were automatically identified and counted for each measurement.

The threshold pressure was not affected by the stimulation, which is in line with previous studies [5]. In a previous study, we found that the slope of pressure-afferent nerve activity was higher in acetic acid irritated bladders than in saline fillings [11]. This implies that there was higher afferent activity and comparable bladder pressure in acetic acid measurements at a significantly lower volume as compared to the saline measurements. Similarly in the current study even though the threshold pressure was comparable, the instilled volume was different. Though we did not measure the efferent nerve activity, the comparable voiding threshold pressure in pre- and poststimulation and the inhibition of afferent activity supports the hypothesis by Tai et al. [6] that TNS suppresses afferent rather than efferent pathways. The non-voiding contractions also remained unaffected by TNS, no difference was found between the pre- and poststimulation measurements. The non-voiding contractions are commonly defined as small transients of 0.5-3 cmH_2O in bladder pressure [13]. These transients are different from spontaneous contractions of bladder which are higher than 4 or 8 cmH_2O [9]. The high amplitude spontaneous contractions are a characteristic of bladder outlet obstruction and occur in animal models when the urethra is clamped [29], which was not the case in our experiments.

Although this study highlighted some important basic science behind tibial nerve stimulation, translational aspects have to be considered carefully. As the sensation of urgency cannot be simulated in animal models, the acetic acid model used in this study is only a model of detrusor overactivity and has limited direct translational impact to human OAB. There are other considerations such as the use of anaesthesia, although urethane has been reported to spare the micturition reflex [12]. The difference in stimulation technique limits the direct translation of results of animal studies to clinical application. Additional studies in unanaesthetized animals and exploration of stimulus parameters and different sites of stimulation are warranted for a better understanding of the neurophysiological mechanism behind TNS.

References

1. Abrams P. Describing bladder storage function: overactive bladder syndrome and detrusor overactivity. Urology. 2003;62(5 Suppl 2):28-37; discussion 40-2.
2. Milsom I, Abrams P, Cardozo L, Roberts RG, Thuroff J, Wein AJ. How widespread are the symptoms of an overactive bladder and how are they managed? A population-based prevalence study. Bju Int. 2001;87(9):760-6.
3. Abrams P, Andersson KE. Muscarinic receptor antagonists for overactive bladder. Bju Int. 2007;100(5):987-1006.
4. Peters KM, Carrico DJ, Wooldridge LS, Miller CJ, MacDiarmid SA. Percutaneous Tibial Nerve Stimulation for the Long-Term Treatment of Overactive Bladder: 3-Year Results of the STEP Study. J Urology. 2013;189(6):2194-201.
5. Matsuta Y, Roppolo JR, de Groat WC, Tai C. Poststimulation inhibition of the micturition reflex induced by tibial nerve stimulation in rats. Physiol Rep. 2014;2(1):e00205.
6. Tai CF, Chen M, Shen B, Wang JC, Roppolo JR, de Groat WC. Irritation Induced Bladder Overactivity is Suppressed by Tibial Nerve Stimulation in Cats. J Urology. 2011;186(1):326-30.
7. Tai CF, Shen B, Chen M, Wang JC, Roppolo JR, de Groat WC. Prolonged poststimulation inhibition of bladder activity induced by tibial nerve stimulation in cats. Am J Physiol-Renal. 2011;300(2):F385-F92.
8. Gaziev G, Topazio L, Iacovelli V, Asimakopoulos A, Di Santo A, De Nunzio C, et al. Percutaneous tibial nerve stimulation (PTNS) efficacy in the treatment of lower urinary tract dysfunctions: a systematic review. Bmc Urol. 2013;13.
9. Clavica F, Choudhary M, van Asselt E, van Mastrigt R. Frequency analysis of urinary bladder pre-voiding activity in normal and overactive rat detrusor. Neurourol Urodyn. Neurourol Urodyn. 2014;34(8):794-9.
10. le Feber J, van Asselt E, van Mastrigt R. Afferent bladder nerve activity in the rat: a mechanism for starting and stopping voiding contractions. Urol Res. 2004;32(6):395-405.
11. Choudhary M, van Asselt E, van Mastrigt R, Clavica F. Neurophysiological modeling of bladder afferent activity in the rat overactive bladder model. J Physiol Sci. 2015;65(4):329-38.

12. Andersson KE, Soler R, Fullhase C. Rodent models for urodynamic investigation. Neurourol Urodyn. 2011;30(5):636-46.

13. Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JI. Phasic non-micturition contractions in the bladder of the anaesthetized and awake rat. Bju Int. 2006;97(5):1094-101.

14. Groen J, Bosch JL. Neuromodulation techniques in the treatment of the overactive bladder. Bju Int. 2001;87(8):723-31.

15. Vandoninck V, Van Balken MR, Finazzi Agro E, Petta F, Caltagirone C, Heesakkers JP, et al. Posterior tibial nerve stimulation in the treatment of urge incontinence. Neurourol Urodyn. 2003;22(1):17-23.

16. Bemelmans BL, Mundy AR, Craggs MD. Neuromodulation by implant for treating lower urinary tract symptoms and dysfunction. European Urology. 1999;36(2):81-91.

17. Martinez-Pineiro L, Trigo-Rocha F, Hsu GL, Lue TF, Schmidt RA, Tanagho EA. Response of bladder, urethral and intracavernous pressure to ventral lumbosacral root stimulation in Sprague-Dawley and Wistar rats. J Urol. 1992;148(3):925-9.

18. Patra PB, Patra S. Sex differences in the physiology and pharmacology of the lower urinary tract. Curr Urol. 2013;6(4):179-88.

19. Chuang YC, Fraser MO, Yu Y, Chancellor MB, de Groat WC, Yoshimura N. The role of bladder afferent pathways in bladder hyperactivity induced by the intravesical administration of nerve growth factor. J Urol. 2001;165(3):975-9.

20. Pascual JI, Insausti R, Gonzalo LM. The pelvic innervation in the rat: different spinal origin and projections in Sprague-Dawley and Wistar rats. Brain Res. 1989;480(1-2):397-402.

21. Fowler CJ, Griffiths D, de Groat WC. The neural control of micturition. Nat Rev Neurosci. 2008;9(6):453-66.

22. Zvara P, Wright AJ, Roach K, Ursiny M, Shapiro B, Dagrosa LM, et al. A non-anesthetized mouse model for recording sensory urinary bladder activity. Front Neurol. 2010;1:127.

23. Chang CJ, Huang ST, Hsu K, Lin A, Stoller ML, Lue TF. Electroacupuncture decreases c-fos expression in the spinal cord induced by noxious stimulation of the rat bladder. J Urol. 1998;160(6 Pt 1):2274-9.

24. McPherson A. The effects of somatic stimuli on the bladder in the cat. J Physiol. 1966;185(1):185-96.

25. Chen ML, Shen B, Wang J, Liu H, Roppolo JR, de Groat WC, et al. Influence of naloxone on inhibitory pudendal-to-bladder reflex in cats. Exp Neurol. 2010;224(1):282-91.

26. Tai C, Larson JA, Ogagan PD, Chen G, Shen B, Wang J, et al. Differential role of opioid receptors in tibial nerve inhibition of nociceptive and nonnociceptive bladder reflexes in cats. Am J Physiol Renal Physiol. 2012;302(9):F1090-7.

27. Xiao ZY, Reese J, Schwen Z, Shen B, Wang JC, Roppolo JR, et al. Role of spinal GABA(A) receptors in pudendal inhibition of nociceptive and nonnociceptive bladder reflexes in cats. Am J Physiol-Renal. 2014;306(7):F781-F9.

28. Rogers MJ, Shen B, Reese JN, Xiao Z, Wang J, Lee A, et al. Role of glycine in nociceptive and non-nociceptive bladder reflexes and pudendal afferent inhibition of these reflexes in cats. Neurourol Urodyn. 2015; doi: 10.1002/nau.22821.

29. Kwak TI, Lee JG. Inhibitory effects of propiverine, atropine and oxybutynin on bladder instability in rats with infravesical outlet obstruction. Br J Urol. 1998;82(2):272-7.

Chapter 6

Inhibitory Effects of Tibial Nerve Stimulation on Bladder Neurophysiology in Rats

Mahipal Choudhary

Ron van Mastrigt

Els van Asselt

SpringerPlus 2016, 5:35

Abstract

Tibial Nerve Stimulation (TNS) is a form of peripheral neuromodulation which has been found effective in treating overactive bladder symptoms, with lesser side effects than first line pharmacotherapy. Despite its widespread clinical use, the underlying mechanism of action is not fully understood. Our aim was to study its effect on the bladder neurophysiology and the trigger mechanism of voiding in the overactive detrusor, simulated by Acetic Acid (AA) instillation. In urethane anaesthetized male Wistar rats, the tibial nerve was stimulated for 30 min at 5 Hz, pulse width 200 μs and amplitude approximately 3 times the threshold to induce a slight toe movement.

The pressure at which a voiding contraction was triggered (p_{thres}) did not change significantly between the pre- and post- TNS measurements in acetic acid (AA) induced detrusor overactivity. It was found that TNS significantly reversed the effects of AA irritation by increasing the bladder compliance and the bladder volume at p_{thres}, as well as suppressed the threshold afferent nerve activity. The slope of the linear relationship between pressure and the afferent activity increased after AA instillation and decreased significantly after stimulation.

In addition to its well-known central inhibitory mechanisms, this study has demonstrated that TNS improves bladder storage capacity by delaying the onset of voiding, via an inhibitory effect on the bladder afferent signaling at the peripheral level.

Introduction

A voiding reflex is initiated by bladder afferent nerve fibers which run through the major pelvic ganglion via the pelvic nerve to the L4-S3 level of the spinal cord. Bladder efferent fibers originate at the same level, travel via the spinal nerve, synapse in the major pelvic ganglion and innervate the bladder smooth muscle [1, 2]. At the same spinal level the sciatic nerve originates which at the mid-thigh level splits into tibial, peroneal and sural nerves [3]. It is generally believed that superseding a threshold of afferent nerve activity triggers efferent firing to the bladder wall i.e. initiates a voiding contraction [4]. In pathological conditions such as detrusor overactivity this afferent-efferent mechanism is disturbed [5]. Evidence of this anomaly is the increased sensitivity of afferent fibers, particularly C-fibers, which has been reported to be one of the underlying causes of detrusor overactivity [6]. The increased sensitivity leads to symptoms like urgency and frequency. One treatment technique is based on stimulation of posterior tibial nerves for 30 min a few times a week for several months [7, 8]. Although this technique has been reported to be promising, the underlying mechanism of action is only partially understood [9].

To explain the inhibitory effect of tibial nerve stimulation (TNS) on the bladder, most studies have focused on urodynamic parameters. As neuromodulation takes place via the afferent and/or efferent pathways, our aim was to study its effect on the bladder neurophysiology and the trigger mechanism of voiding in a detrusor overactivity rat model.

Methods

Ethics, consent and permissions

All laboratory and experimental procedures were conducted in accordance with institutional guidelines of the local Erasmus MC Animal Experiment Committee (Dier Experimenten commissie (DEC)), DEC number: EMC 2092 and 3164, Protocol number: 102-10-06.

Experimental Procedures

A total of 14 male Wistar rats (432 ± 45 g) were used in this study. The animals were anesthetized with urethane (1.2 g/kg body weight, intraperitoneally) and placed on a heated undercover. The abdomen was surgically opened and a branch of the postganglionic pelvic nerve (crushed between the major pelvic ganglion and the electrode, so that only afferent nerve signals were recorded) was mounted on a custom made bipolar electrode consisting of two thin Platinum/Iridium hook shaped wires to record afferent nerve traffic. Since we only crushed one branch of the postganglionic pelvic nerve, leaving intact all other branches, this did not have a significant effect on the bladder function [10]. A catheter connected to a disposable pressure transducer and infusion pump was inserted through the apex of the bladder dome. It was used to fill the bladder with saline (0.06 ml/min) and to record pressure (Statham SP1400 pressure monitor). The tibial nerve was accessed via the medial side of the right hind leg near the ankle. For stimulation a bipolar cuff electrode was placed around the nerve. The abdominal cavity was filled with paraffin oil to prevent the tissue from drying out. At the end of the experiments, the animals were euthanized using intracardiac KCl.

Initially the bladder was filled with physiological saline (0.9 % NaCl) until voiding occurred and 3-4 micturition cycles were recorded. Next, the bladder was repeatedly filled with 0.5 % Acetic Acid (AA) to induce bladder overactivity. Thereafter the bladder was emptied and the tibial nerve was stimulated for 30 min with monophasic rectangular pulses of frequency 5 Hz, width 200 μs and an amplitude approximately 3 times the threshold to induce a slight toe

movement. Then, AA infusion was repeated to study the poststimulation effect of TNS.

Signal processing

Afferent nerve activity was amplified by a DISA 15C01 EMG amplifier (amplification range: 100-200,000) and band-pass filtered with a Krohn-Hite 3944 filter (Bessel, 4th order, 200-2000 Hz). Nerve activity and pressure signals were displayed in real-time on a computer screen using a custom written LabVIEW® (National Instruments, USA) program and were sampled and stored at 25 kHz and 25 Hz respectively. A custom written MATLAB® (Mathworks, USA) program was used to process and analyse the recorded signals. The nerve signal was rectified and averaged by taking the mean of 1000 samples, effectively reducing a 1 s interval to 25 data samples, similar to the pressure.

The pressure threshold at which a voiding contraction began was determined by another custom written MATLAB® program. The program first smoothed the signal using a Savitzky-Golay filter and then calculated the second derivative of the pressure signal. The pressure at which the second derivative superseded a certain threshold was defined as the pressure threshold (p_{thres}) and the afferent nerve activity at this threshold was calculated. Additionally, we also calculated the filled volume (V_{thres}) and the bladder compliance, $\frac{dV_{thres}}{dp_{thres}}$ at the pressure threshold.

The automatically derived pressure threshold and afferent activity were compared to values derived manually at the point where the pressure started to increase rapidly (t_0, Figure 1). In rats, voiding is marked by rapid contractions of the urethral sphincter, seen as high frequency oscillations (HFO) in bladder pressure recording. In Figure 1, t_1 is the time at the start of HFO and p_{max} is the maximum pressure just before the start of HFO.

To compare the relationship between bladder pressure and afferent activity pre- and post-tibial nerve stimulation, a linear regression model was fitted to the afferent activity-pressure data in the interval t_0-t_1 to calculate slope and offset.

All data are presented as mean ± SD. To compare groups a two-way ANOVA followed by Bonferroni multiple comparison was done using the SPSS®

statistical package (version 21.0, SPSS Inc., Chicago, IL, USA). A $p < 0.05$ was considered significant.

Results

No measurements could be done in 4 of the 14 rats due to equipment failure and the absence of bladder contractions. In the remaining 10 rats a few measurements were excluded from the analysis due to movement artifacts, or a high noise level caused by electrodes touching the surrounding tissue. These artefacts were manifested by irregular peaks in the pressure signal. The p_{thres} which marks the beginning of a voiding contraction did not vary among the saline, AA and poststimulation measurements (Table 1).

Irritation of the bladder with AA induced an increase in the threshold afferent activity, which was significantly reversed in the poststimulation AA measurements. As expected, the threshold volume and bladder compliance were significantly reduced after AA irritation, and were also restored in the poststimulation AA measurements (Table 1). The maximum pressure (p_{max}) just before the HFOs was found comparable between the three groups, whereas the corresponding afferent nerve activity increased on AA instillation and decreased poststimulation. The pressure rise time (t_0-t_1, Figure 1) from the p_{thres} to the actual voiding seemed higher in saline measurements, however the difference between the three groups was not statistically significant. The slope of the pressure-afferent nerve activity increased on AA instillation and decreased strongly after tibial nerve stimulation. An example of afferent nerve activity as a function of bladder pressure represented by a linear polynomial fit is shown in Figure 2. The offset which represents nerve activity at pressure ~ 0 did not differ between the groups (Table 1).

To validate the automatic calculation of pressure threshold and afferent activity by the custom written program, we also calculated these parameters by manual identification of the pressure threshold and the corresponding afferent threshold (Table 1). No statistical difference in results between the two methods was observed.

Discussion

Tibial nerve stimulation is a clinical alternative to pharmacotherapy and has been found effective in restoration of bladder capacity by inhibiting undesired detrusor contractions [7, 8, 11]. In addition, it has been shown to suppress simulated bladder overactivity in anaesthetized animal models [12, 13]. To further the understanding of the working mechanism of TNS, we studied its effect on the pressure-afferent nerve activity relationship and the trigger mechanism of voiding.

A voiding reflex is initiated by bladder afferent firing superseding a certain threshold necessary to trigger efferent firing of the bladder nerves [4]. This threshold has been defined as the point where pressure rises above the baseline and increases rapidly until voiding occurs. Although a change was expected during an inhibitory response, in our study, the pressure threshold did not change significantly between the pre- and post- tibial nerve stimulation measurements. However, the volume threshold was different between both groups after stimulation, implying that the stimulation delayed the onset of voiding to a higher filled volume, without affecting the pressure at this point.

The threshold afferent nerve activity and the slope of the linear pressure-afferent activity relation increased when the bladder was irritated by AA and decreased significantly poststimulation (Figure 2). The increase in afferent activity can be ascribed to chemical irritation of bladder [14], which causes hypersensitization of nociceptive C-fiber afferents [12]. Whether the poststimulation decrease is a reversal of the C-fiber sensitization, cannot be ascertained due to the mixed, multifiber nature of our afferent recordings. Though urethane is believed to spare the micturition reflex [15], a suppressing effect of anaesthesia on pressure development and/or afferent signaling cannot be ruled out, which however may be expected to affect the pre- and post- stimulation measurements to the same degree.

A peripheral effect of TNS on afferent nerve terminals, to the best of author's knowledge, has not been reported elsewhere. It is possible that this effect is caused by a change in bladder compliance. Poststimulation the volume threshold increased while the pressure threshold remained unchanged, implicating an increase in detrusor compliance. It has been shown that pudendal nerve

stimulation, via sympathetic efferent pathways, can activate β3- adrenoceptors in the detrusor muscle and/or α-adrenergic receptors at the vesical ganglia to inhibit detrusor contractions and regulate bladder compliance [16-19]. Similar hypotheses have been proposed for the working mechanism of tibial stimulation. According to Matsuta et al.: "TNS might activate a reflex output to the bladder through the sympathetic hypogastric nerves and relax the detrusor via β-adrenergic mechanisms" [20]. Further studies on the role of different nerve types in TNS inhibition are warranted for a better understanding.

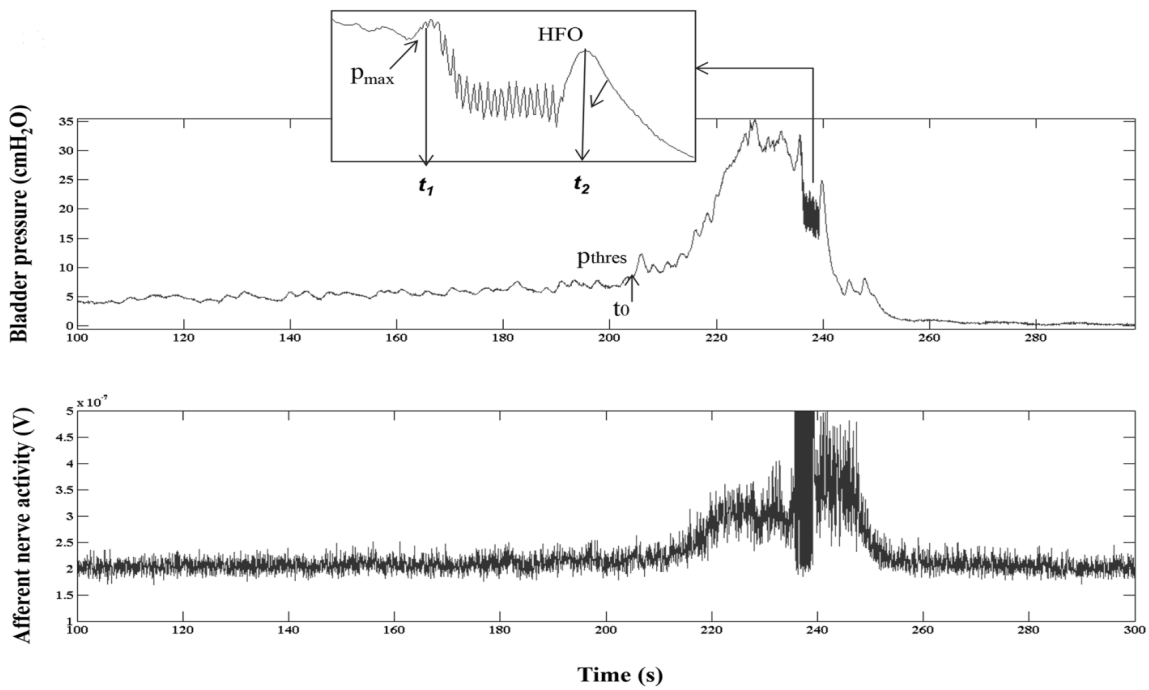

Figure 1. Nerve activity and bladder pressure development during a typical rat voiding cycle

This figure shows a part of a bladder filling cycle. The upper panel shows the pressure during the filling phase with a voiding contraction (t_1-t_2), marked by high frequency oscillations (HFO) of the urethral sphincter. At p_{thres} the pressure rises above the baseline and increases rapidly until a voiding occurs. p_{max} represents the maximum pressure just before the start of urine flow. The lower panel shows the corresponding afferent nerve activity.

Table 1. Statistical comparison of estimated parameters in pre- and poststimulation measurements

Mean ± SD of the estimated parameters in control saline, prestimulation and poststimulation AA measurements, n = 72 measurements (saline = 18, prestimulation AA = 27, and poststimulation AA = 27). The symbols ϕ and * represent the statistical significance ($p < 0.05$) between group I vs II and II vs III respectively. N.S. indicates no statistical difference ($p > 0.05$) between the groups.

		Prestimulation saline (I)	Prestimulation AA (II)	Poststimulation AA (III)
Automatically calculated	Threshold afferent activity (μV)	0.21 ± 0.02	0.23 ± 0.04$^{\phi}$	0.19 ± 0.01*
	Maximum afferent (μV)	0.25 ± 0.04	0.41 ± 0.07$^{\phi}$	0.22 ± 0.04*
	Threshold pressure, p_{thres} (cmH_2O)	5.2 ± 2.4	4.4 ± 2.7 N.S.	5.4 ± 2.8 N.S.
	Maximum pressure, p_{max} (cmH_2O)	34.4 ± 7.0	32.5 ± 7.8 N.S.	30.5 ± 5.2 N.S.
	Pressure rise time (s)	59.2 ± 55.9	54.6 ± 55.1 N.S.	54.6 ± 47.9 N.S.
	Slope (μV/ cmH_2O)	1.6 ± 1.4	1.9 ± 1.5$^{\phi}$	0.7 ± 0.6*
	Offset (μV)	0.21 ± 0.03	0.22 ± 0.04 N.S.	0.19 ± 0.02 N.S.
Manually calculated	Volume, V_{thres} (mL)	0.84 ± 0.2	0.53 ± 0.2$^{\phi}$	0.76 ± 0.3*
	Compliance (mL/ cmH_2O)	0.17 ± ± 0.03	0.10 ± 0.07$^{\phi}$	0.17 ± 0.06*
	Threshold afferent (μV)	0.21 ± 0.02	0.23 ± 0.04$^{\phi}$	0.20 ± 0.21*
	Threshold pressure (cmH_2O)	6.5 ± 2.9	5.3 ± 2.3 N.S.	6.7 ± 2.5 N.S.
	Rise time (s)	53.2 ± 55.9	48.6 ± 55.1 N.S.	45.5 ± 33.9 N.S.

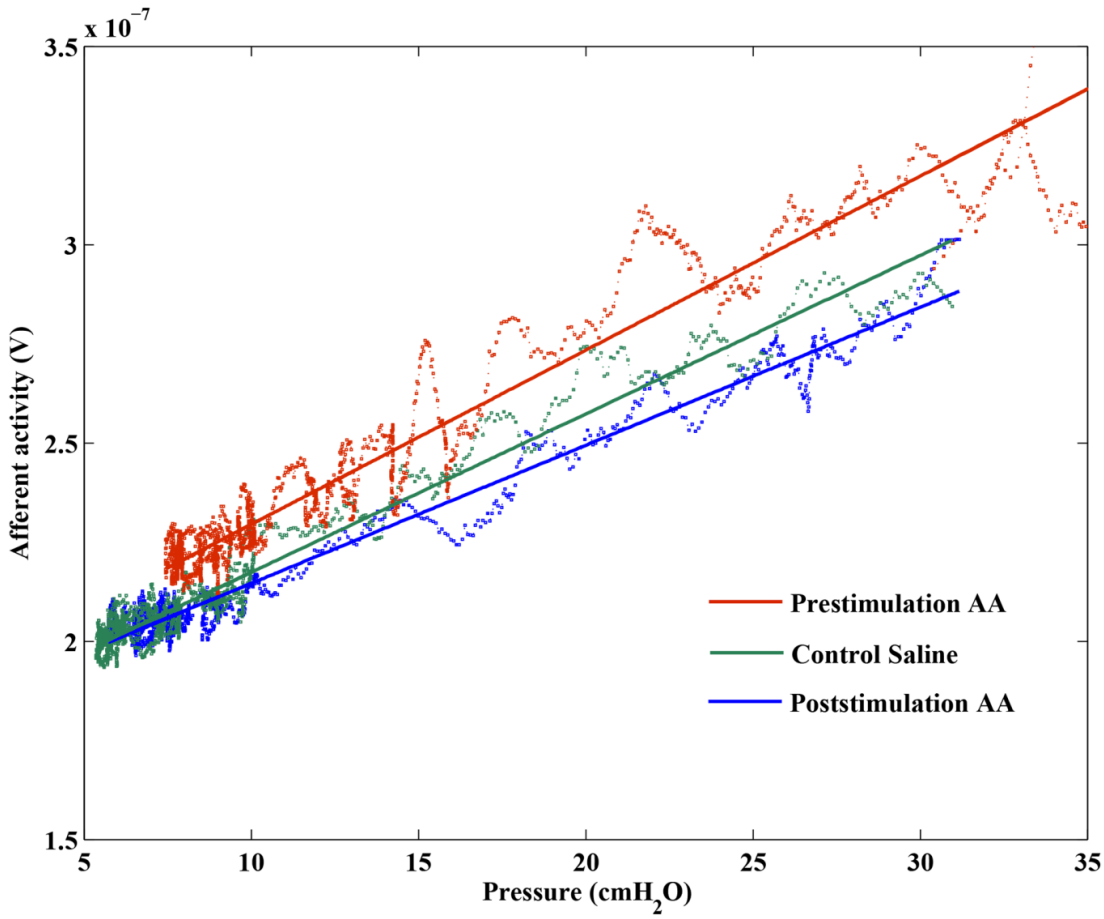

Figure 2. Example of the effect of tibial nerve stimulation on the slope of the pressure-afferent nerve activity

The slope of afferent nerve activity as a function of bladder pressure was fitted with a straight line representing a linear polynomial fit. Instillation of 0.5 % AA significantly increased the slope of this line in the prestimulation AA measurement (topmost line) as compared to the control saline measurement (middle) and decreased significantly after tibial nerve stimulation (bottom) (3 measurements within 1 animal).

The role of the central nervous system in neuromodulation has been well established in various studies [16, 21-23]. As the posterior tibial nerve projects to the sacral micturition center and the nucleus of Onuf, the same area where bladder projections are found, it is generally accepted that TNS evokes a central inhibition of micturition pathways and the therapeutic effect of TNS takes place via these areas [24, 25]. Recent studies have also suggested the involvement of inhibitory neurotransmitter mechanisms for TNS inhibition of bladder overactivity modulated by activation of μ, κ and δ opioid receptors [21]. As evident from the multitude of suggested modes of action, the working mechanism of tibial nerve stimulation is highly complex and occurs at several levels, incorporating a combination of central inhibitory mechanisms as well as an inhibition at the peripheral level, as suggested by our present study.

Although the results presented in this study underline some important basic neurophysiology behind tibial nerve stimulation, the large differences between rats and humans require a careful consideration of the translational aspects. Sensory urgency in humans is a subjective urodynamic diagnosis and cannot be simulated in animal models; therefore the acetic acid irritation model used in this study only mimics one symptom of the human overactive bladder syndrome, the detrusor overactivity.

Conclusions

We studied the effect of tibial nerve stimulation on the neurophysiology of voiding in terms of the linear relationship between bladder pressure and afferent nerve activity. It was found that 30 min of stimulation favorably affected the slope of this linear relationship, by inhibiting bladder afferent activity and increasing bladder compliance in an anaesthetized rat model of detrusor overactivity. In addition to the established central inhibitory mechanism of TNS, our study provides evidence for an inhibitory effect on afferent signaling at the peripheral level. Additional studies in unanaesthetized animals and the effect of TNS on different neural pathways are warranted for a comprehensive understanding of the neurophysiological mechanism behind TNS.

References

1. Pascual JI, Insausti R, Gonzalo LM. The pelvic innervation in the rat: different spinal origin and projections in Sprague-Dawley and Wistar rats. Brain Res. 1989;480(1-2):397-402.
2. Keast JR, Booth AM, de Groat WC. Distribution of neurons in the major pelvic ganglion of the rat which supply the bladder, colon or penis. Cell Tissue Res. 1989;256(1):105-12.
3. Brunner R, Zimmermann P, Klussmann FW. Localization and Neurophysiological Properties of Moto-Neurons of the M-Triceps Surae of the Rat after Retrograde Labeling with Evans Blue. Cell and Tissue Research. 1980;212(1):73-81.
4. van Asselt E, le Feber J, van Mastrigt R. Threshold for efferent bladder nerve firing in the rat. Am J Physiol. 1999;276(6 Pt 2):R1819-24.
5. de Groat WC. A neurologic basis for the overactive bladder. Urology. 1997;50(6A Suppl):36-52; discussion 3-6.
6. Steers WD. Pathophysiology of overactive bladder and urge urinary incontinence. Rev Urol. 2002;4 Suppl 4:S7-S18.
7. Peters KM, Carrico DJ, Wooldridge LS, Miller CJ, MacDiarmid SA. Percutaneous Tibial Nerve Stimulation for the Long-Term Treatment of Overactive Bladder: 3-Year Results of the STEP Study. J Urology. 2013;189(6):2194-201.
8. Govier FE, Litwiller S, Nitti V, Kreder KJ, Jr., Rosenblatt P. Percutaneous afferent neuromodulation for the refractory overactive bladder: results of a multicenter study. J Urol. 2001;165(4):1193-8.
9. Gaziev G, Topazio L, Iacovelli V, Asimakopoulos A, Di Santo A, De Nunzio C, et al. Percutaneous tibial nerve stimulation (PTNS) efficacy in the treatment of lower urinary tract dysfunctions: a systematic review. Bmc Urol. 2013;13.
10. Choudhary M, van Asselt E, van Mastrigt R, Clavica F. Neurophysiological modeling of bladder afferent activity in the rat overactive bladder model. J Physiol Sci. 2015;65(4):329-38.
11. van Balken MR, Vandoninck V, Gisolf KWH, Vergunst H, Kiemeney LALM, Debruyne FMJ, et al. Posterior tibial nerve stimulation as neuromodulative treatment of lower urinary tract dysfunction. J Urology. 2001;166(3):914-8.

12. Tai CF, Chen M, Shen B, Wang JC, Roppolo JR, de Groat WC. Irritation Induced Bladder Overactivity is Suppressed by Tibial Nerve Stimulation in Cats. J Urology. 2011;186(1):326-30.

13. Su X, Nickles A, Nelson DE. Differentiation and interaction of tibial versus spinal nerve stimulation for micturition control in the rat. Neurourol Urodyn. 2015;34(1):92-7.

14. Zvara P, Wright AJ, Roach K, Ursiny M, Shapiro B, Dagrosa LM, et al. A non-anesthetized mouse model for recording sensory urinary bladder activity. Front Neurol. 2010;1:127.

15. Matsuura S, Downie JW. Effect of anesthetics on reflex micturition in the chronic cannula-implanted rat. Neurourol Urodyn. 2000;19(1):87-99.

16. McGee MJ, Danziger ZC, Bamford JA, Grill WM. A spinal GABAergic mechanism is necessary for bladder inhibition by pudendal afferent stimulation. Am J Physiol Renal Physiol. 2014;307(8):F921-30.

17. Craggs M, McFarlane J. Neuromodulation of the lower urinary tract. Exp Physiol. 1999;84(1):149-60.

18. Lindstrom S, Fall M, Carlsson CA, Erlandson BE. The neurophysiological basis of bladder inhibition in response to intravaginal electrical stimulation. J Urol. 1983;129(2):405-10.

19. De Groat WC, Saum WR. Sympathetic inhibition of the urinary bladder and of pelvic ganglionic transmission in the cat. J Physiol. 1972;220(2):297-314.

20. Matsuta Y, Roppolo JR, de Groat WC, Tai C. Poststimulation inhibition of the micturition reflex induced by tibial nerve stimulation in rats. Physiol Rep. 2014;2(1):e00205.

21. Zhang Z, Slater RC, Ferroni MC, Kadow BT, Lyon TD, Shen B, et al. Role of micro, kappa, and delta opioid receptors in tibial inhibition of bladder overactivity in cats. J Pharmacol Exp Ther. 2015;355(2):228-34.

22. Xiao ZY, Reese J, Schwen Z, Shen B, Wang JC, Roppolo JR, et al. Role of spinal GABA(A) receptors in pudendal inhibition of nociceptive and nonnociceptive bladder reflexes in cats. Am J Physiol-Renal. 2014;306(7):F781-F9.

23. van der Pal F, Heesakkers JP, Bemelmans BL. Current opinion on the working mechanisms of neuromodulation in the treatment of lower urinary tract dysfunction. Curr Opin Urol. 2006;16(4):261-7.

24. Bemelmans BL, Mundy AR, Craggs MD. Neuromodulation by implant for treating lower urinary tract symptoms and dysfunction. European Urology. 1999;36(2):81-91.

25. Vandoninck V, Van Balken MR, Finazzi Agro E, Petta F, Caltagirone C, Heesakkers JP, et al. Posterior tibial nerve stimulation in the treatment of urge incontinence. Neurourol Urodyn. 2003;22(1):17-23.

Chapter 7

The frequency spectrum of bladder non-voiding contractions as a trigger-event for conditional stimulation: closed-loop inhibition of bladder overactivity in rats

Mahipal Choudhary

Ron van Mastrigt

Els van Asselt

Submitted

Abstract

Aim: To test the hypothesis that the frequency of bladder non-voiding contractions (NVCs) can be used as a trigger event for closed-loop conditional inhibition of detrusor contractions via tibial nerve (TN) or dorsal penile nerve (DPN) stimulation.

Methods: In urethane anaesthetized male Wistar rats, the bladder was filled continuously with saline to evoke contractions. To test the plausibility of conditional inhibition via the tibial nerve, electrical stimulation was switched on manually when the pressure increased 10 cmH_20 above the baseline. For testing conditional stimulation via the DPN, the incoming pressure signal was continuously stored and the area under the curve (AUC) of the amplitude spectrum in the frequency range 0.2-20 Hz of a 5 s window at the beginning of filling was calculated. When the AUC of subsequent pressure windows superseded the predefined threshold, the penile nerve was automatically stimulated.

Results: Stimulation of the tibial nerve failed to inhibit filling evoked voiding contractions in all of the 8 tested rats. The NVC frequency spectrum based conditional stimulation of DPN successfully inhibited 70 % of the filling evoked contractions and resulted in a 45 % increase in the bladder capacity.

Conclusions: While conditional TNS failed to suppress bladder contractions, stimulation of the DPN, automatically triggered by an increased frequency of bladder non-voiding activity, resulted in bladder inhibition and a consequential increase in bladder capacity. This study for the first time has shown the plausibility of using frequency of non-voiding contractions as a trigger event for conditional inhibition of detrusor contractions.

Introduction

Spinal cord injury (SCI) may cause disruption of the coordinated neural signaling between the central nervous system and the bladder. The lack of regulatory input from higher centers often results in involuntary contractions of the bladder muscle in the storage phase [1]. This condition, neurogenic detrusor overactivity (NDO), is symptomized by urinary incontinence, urgency and frequency [2]. Neuromodulation is an important treatment alternative for NDO patients who are refractory to pharmacologic interventions [3].

Stimulation can be applied either continuously [4, 5] or conditionally [6]. Continuous stimulation for a certain period has been found effective in suppressing detrusor contractions and restoration of bladder capacity [7]. Longer stimulation periods might result in ineffectiveness due to adaptation, increased risk of tissue damage and high battery consumption [8, 9]. In contrast, conditional stimulation is triggered only when an impending bladder contraction is detected [6, 9-11] and may therefore considerably reduce the stimulation duration and thus the above-stated complications. A number of contraction detection methods such as electromyography of the bladder [12], the urethral [13, 14] and the anal sphincter [15, 16] as well as electroneurography of the sacral root [17], the pelvic nerve [18] and the pudendal nerve have been described [19, 20]. However, due to difficulties in signal acquisition and low signal-to-noise ratio, none of these methods have been used in clinical practice. The most commonly used method triggers stimulation when a preset bladder pressure (10-15 cmH_2O) is exceeded [9-11, 21]. Although these methods have been found useful in suppressing bladder contractions, they might fail to relieve a patient of the feeling of 'urgency', which has been reported by patients about 5 seconds before a bladder pressure rise of 10 cmH_2O [10].

In pursuit of developing a method for conditional neurostimulation triggered by urgency, we recently reported that voiding in rats is preceded by recurrent changes in frequency of bladder non-voiding activity [22]. To detect these changes a moving average, Fast-Fourier Transform algorithm was developed and validated offline on pre-recorded pressure measurements.

In our current study we aimed at expanding the developed algorithm to real-time, to show that the frequency of non-voiding contractions can be used as a trigger event for closed-loop conditional inhibition of detrusor contractions. To this end we tested the plausibility of conditional stimulation of the tibial nerve, which has mostly been used for continuous stimulation [4, 23]. We also tested our technique with conditional stimulation of the dorsal penile nerve (DPN).

Methods

Animals

The local Animal Care and Use Committee approved all the animal experiments described in this study. Male Wistar rats (n = 18, 440 ± 24g) were used.

Bladder filling and pressure measurements

The animals were anesthetized by intraperitoneal administration of urethane (50% g/v, 1.2 g/kg body weight). A midline incision was made to expose the abdominal cavity. Bladder pressure measurement and bladder filling (0.06 ml/min) were done through a 23G needle inserted at the top of the bladder. The needle was connected to a disposable pressure transducer and an infusion pump using a 3-way connector. Pressure was measured using a Statham SP1400 blood pressure monitor and was displayed in real-time on a computer screen using a custom written LabVIEW® program. The pressure was sampled at 25 Hz and was analysed with a custom written MATLAB® program.

Types of experiments

i) Conditional tibial nerve stimulation

The tibial nerve was accessed via the medial side of the right hind leg near the ankle. For stimulation a bipolar cuff electrode was placed around the nerve. In each rat, the bladder was filled continuously and initially 2-3 control cystometrograms (CMGs) were recorded. After the control measurements, the tibial nerve was stimulated with monophasic rectangular pulses of frequency 5 Hz, width 200 or 400 μs and with an initial amplitude approximately 3 times the motor threshold (T) to induce a slight toe movement. The amplitude was increased in steps of 1 V until an inhibition of the voiding reflex or a maximum amplitude of 5T

was reached. The stimulation was manually switched on/off when the pressure increased 10 cmH_20 above the baseline, or decreased below it.

Urodynamic parameters

Voiding in rats is marked by rapid contractions of the urethral sphincter, seen as high frequency oscillations (HFO) in bladder pressure recording. We calculated the maximum pressure (p_{max}) immediately before the start of HFO, the baseline pressure (p_{basal}) calculated as the mean of a 5 s pressure window at the beginning of filling and the bladder capacity determined as the volume required to evoke a voiding. We also calculated the rise time of pressure from p_{basal} to p_{max} and the decay constant which gives a measure of the rate of exponential pressure decay after a voiding (Figure 1).

ii) Conditional dorsal penile nerve stimulation

A bipolar platinum-iridium electrode was mounted on a branch of the DPN. According to the described anatomy of the sensory branch of the pudendal nerve in the male rat (dorsal nerve of the penis) [24] we stimulated the so called middle branch. Plastic tubing was wrapped around the nerve-electrode system to isolate it from the surrounding tissue. The bladder was filled continuously and initially 2-3 control CMGs were recorded. After the control measurements, the DPN was stimulated as described below.

A computer program for real-time frequency domain analysis based stimulation was written in LabVIEW®. The program continuously stored the incoming pressure signal in a circular buffer. Simultaneously, it read a window of user defined length from the stored pressure signal and calculated the area under the curve (AUC) of the amplitude spectrum in the frequency range 0.2-20 Hz. From the control measurements a baseline threshold, based on the AUC of the pressure signal at the beginning of filling where the pressure was relatively low (~baseline), and a stimulation threshold, which was the AUC of a 5s pressure window just before a voiding contraction were calculated. When, during the subsequent filling cycles, the value of a 5 s pressure window superseded the predefined AUC threshold, a pulse train of frequency 10 Hz, width 200 μs and amplitude 3-5 times the threshold to induce a pudendo-anal reflex was automatically switched on to

stimulate the penile nerve. The stimulation was stopped again automatically by the software if the value of AUC fell to the threshold baseline.

Urodynamic parameters

As the filling progressed during a CMG, bladder contractions were evoked and subsequently inhibited until voiding (or leakage) occurred. For each of these stimulation CMGs, we calculated the p_{max} at the first and the last inhibited contraction (Figure 2). We also calculated the maximum pressure (p_{max}) at the loss of continence in control and stimulation CMGs. Bladder capacity in the controls was calculated as the filled volume which evoked a voiding. For stimulation measurements, the change in the bladder capacity was defined as the extension of the filling time from the first inhibited contraction up to the occurrence of voiding. The rationale behind this is that if the bladder was not stimulated, voiding would have occurred at the first inhibited contraction [9]. We also calculated to which degree the bladder pressure returned to its original level (p_{basal} before stimulation) in terms of the ratio $p_{basal\ (after\ stimulation/before\ stimulation)}$. The automatic stimulation was considered 'on time' if the change in AUC threshold (hence the start of stimulation) occurred at the onset of a visual progressive rise in pressure above the baseline. If the stimulation did not start at the onset of pressure rise it was considered 'late'.

Statistical analysis

All data are presented as mean ± SD. A Shapiro–Wilk test was done for normality testing, and Levene's test for homogeneity of variance. To compare groups, an ANOVA followed by Bonferroni test for multiple comparisons was carried out on normally distributed data, and Kruskal–Wallis test on non-normal data using the SPSS® statistical package (version 21.0, SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered significant.

Results

i) Conditional tibial nerve stimulation

The average motor threshold T to induce a slight toe movement was 1.42 ± 0.4 V. Conditional stimulation of the tibial nerve with amplitude 3-5T failed to inhibit any of the 50 voiding contractions evoked in 8 rats. Switching on the stimulation when the bladder pressure increased 10 cmH_2O above the baseline had no effect on the contraction and the pressure continued rising progressively culminating in a voiding (Figure 1). Comparison of urodynamic parameters in control CMGs and the ones in which stimulation was applied showed no significant differences in maximum or basal pressure (Table 1). The filled volume (bladder capacity) at which a voiding contraction started was comparable between the two types of measurements. The rise time of pressure from baseline to the point of incontinence and the exponential decay of pressure after a voiding also remained unaffected by stimulation (Table 1).

ii) Conditional dorsal penile nerve stimulation

Three out of 10 rats were used for optimization of the detection algorithm. The stimulation was switched on manually in these rats and the detection software was updated dynamically based on the results. Urodynamic parameters were not calculated. In the other 7 rats, stimulation was switched on automatically based on the AUC threshold determined from the control CMGs in these rats. In a total of 37 CMGs, 106 contraction inhibitions were attempted, out of which 74 (70%) were inhibited 'on-time'. The bladder pressure at which the automatic stimulation started was found to be 8 ± 5 cmH_20. In the other 30 measurements, the automatic stimulation was 'late' and the AUC threshold triggered the stimulation when the pressure had reached 20 cmH_2O or above. Only in 13 of these measurements leakage occurred, while in the other 17 voiding was still inhibited. Bladder capacity in conditional stimulation CMGs increased 45 % compared to that of controls (Table 2).

Table 1. Mean ± SD of the estimated parameters in filling evoked contractions in control (n = 28) and conditional tibial nerve stimulation (n = 50) cystometrograms.

	Control	Conditional tibial Stimulation	p-value
Rise time (s)	6.6 ± 4.3	5.5 ± 2.6	0.2
Maximum pressure (cmH_2O)	24.3 ± 7.0	23.4 ± 5.8	0.6
Baseline pressure (cmH_2O)	2.6 ± 1.6	2.5 ± 1.3	0.7
Pressure decay constant (s)	7.7 ± 4.4	8.4 ± 4.0	0.5
Bladder capacity (mL)	0.9 ± 0.3	1.0 ± 0.3	0.2

The p_{max} at the loss of continence did not differ between the control and stimulation measurements (Table 2). However p_{max} in control CMGs was found to be significantly higher when compared to that of the first inhibited contractions (Table 3, Figure 2). As a result of the continuous increase in volume, the p_{max} at the last inhibited contraction was higher than that at the first inhibited contraction (Figure 2). The ratio $p_{basal\ (after\ stimulation/before\ stimulation)}$ was higher in the first inhibited contractions than in the last inhibited ones (Table 3). In 80 % of the first inhibited contractions the bladder pressure returned to 80 % of the $p_{basal\ before\ stimulation}$ value at the start of filling, whereas only 60 % of the last inhibited contractions returned to 80 %.

Table 2. Mean ± SD of the estimated parameters in control and conditional DPN stimulation cystometrograms.

	Control	Conditional stimulation	p-value
p_{max} (cmH_2O)	35.0 ± 11.3	37.4 ± 16.6	0.2
Bladder capacity (mL)	1.1 ± 0.7	1.6 ± 0.3	0.01*

Table 3. Mean ± SD of the estimated parameters in control and conditional DPN stimulation CMGs. As the filling progressed, bladder contractions occurred and were subsequently inhibited until voiding occurred. For each cystometrogram in which stimulation was done, parameters were calculated for the first inhibited contraction and the last inhibited contraction. Statistical difference shows the difference between shown values.

	Control	First Inhibited contraction	Last inhibited contraction	p-value
p_{max} (cmH_2O)	35.0 ± 11.3	31.1 ± 17.0	-	0.04*
p_{max} (cmH_2O)	-	31.1 ± 17.0	36.5 ± 15.2	0.2
Ratio, p_{basal} (after stimulation/before stimulation)	-	1.06 ± 1.04	0.53 ± 0.4	0.01*

Discussion

Conditional tibial nerve stimulation

A weekly 30 min percutaneous stimulation of the tibial nerve for several weeks is a well-established treatment for neurogenic detrusor overactivity [4, 23]. In anaesthetized animal models too, stimulation applied to this nerve during filling cystometry inhibited bladder contractions, resulting in an increase in bladder capacity [25, 26]. We hypothesized that, similar to acute continuous tibial stimulation, acute conditional stimulation might also recruit a lumbo-sacral spinal cord route to acutely produce an inhibitory effect. Unfortunately, conditional TNS failed to suppress bladder contractions in all of the animals in our study. Only limited research has been done to test conditional tibial stimulation [27, 28]. In a study on chronic spinal cord injured cats, the authors not only reported the ineffectiveness of acute TNS, but also found that stimulation was in fact followed by increased bladder activity [28]. A study in multiple sclerosis NDO patients reported similar negative results [27]. A potential hindrance of this patient study was the maximum tolerable stimulation threshold of only 1.5 times the motor

threshold. In our animal study, in order to maximize the number of depolarized afferents, higher stimulation thresholds of up to 5 times the motor threshold and pulse widths of 200 and 400 μs were tested. However even such manipulation of the stimulation parameters, did not produce any beneficial effects. To test if stimulation would affect pressure dynamics during a voiding contraction, we examined the pressure rise time from the baseline to its culmination into a voiding and the following exponential decay to baseline (Figure 1). There was no appreciable change in these parameters between the stimulation and control CMGs. Evidently, continuous stimulation for longer periods remains the only viable option to achieve bladder inhibition via the tibial nerve.

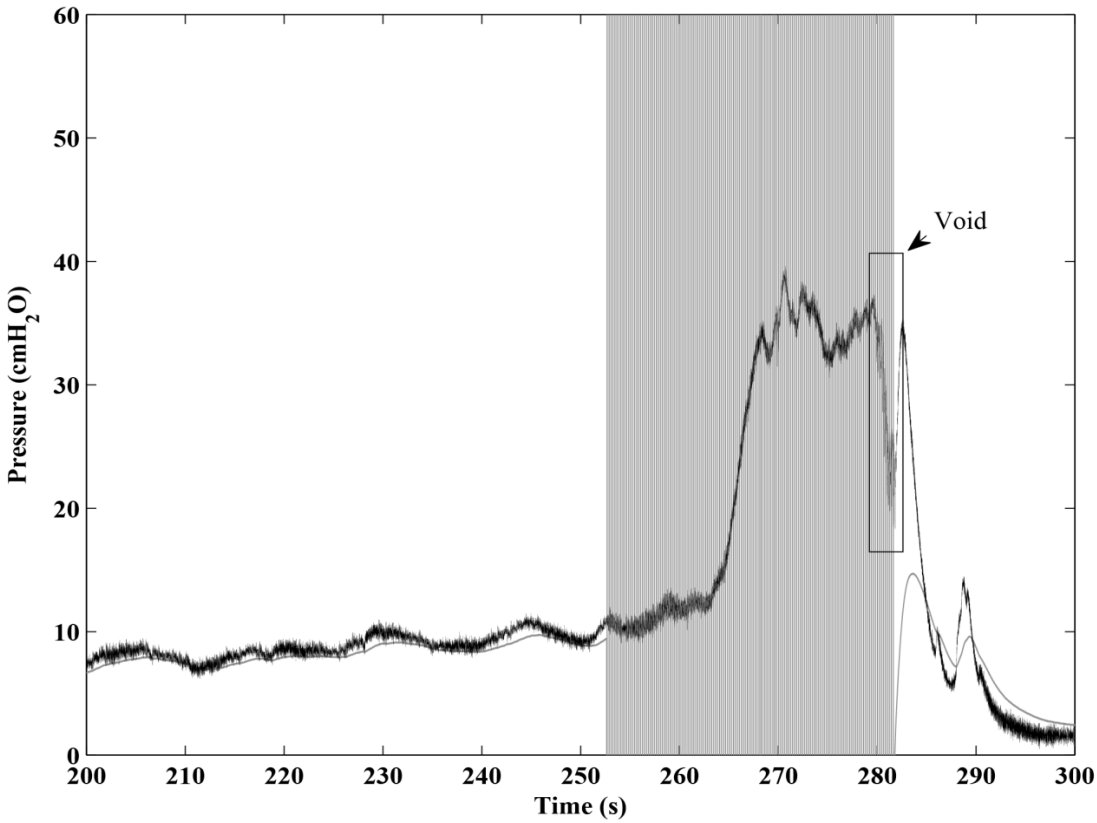

Figure 1. An example of unsuccessful conditional stimulation of the tibial nerve

The stimulation was manually switched on when the bladder pressure increased 10 cmH_20 above the baseline. The shaded region shows the stimulation period, where it is noticeable that regardless the stimulation, the pressure increased rapidly, ultimately culminating to a voiding.

Conditional dorsal penile nerve stimulation

In a recent study [22], we reported that voiding in rats is preceded by recurrent changes in the frequency of bladder non-voiding activity. These changes were detected using a moving average Fast-Fourier Transform algorithm. The algorithm was tested offline on pre-recorded pressure data and we were able to demonstrate a high success rate of 90 % in detecting frequency changes occurring from 100 s before a voiding contraction. In our current study we aimed at expanding the developed algorithm to real-time, and to show that the frequency of non-voiding contractions can be used as a trigger event for closed-loop conditional inhibition of bladder contractions via the dorsal penile nerve.

Conditional stimulation successfully suppressed 70 % of the contractions evoked by bladder filling. The resulting 45 % increase in bladder capacity is well supported by other conditional stimulation studies [9]. A common observation in conditional stimulation studies is that bladder contractions can only be inhibited up to a certain bladder volume [9, 11]. In our study too, as the bladder volume increased with continuous filling, a significant increase in p_{max} was seen from the first inhibited contractions to the last inhibited contraction, which was followed by voiding (Figure 2). An impact of increasing bladder volume was also seen in the efficiency of stimulation in bringing down the pressure to baseline. In 80 % of the first inhibited contractions the baseline pressure returned to 80 % of the pre-stimulation level, as compared to 60 % in the last inhibited contractions. Presumably when the volume threshold for voiding is reached, the bladder afferent activity overcomes the inhibitory influence of stimulation. One possible way to further inhibit afferent activity (and increase bladder capacity) would be to increase the stimulation amplitude to maximize afferent fiber recruitment, however, due to the limitation of the maximum tolerable threshold in patients, the window for stimulation parameter negotiation remains narrow [29].

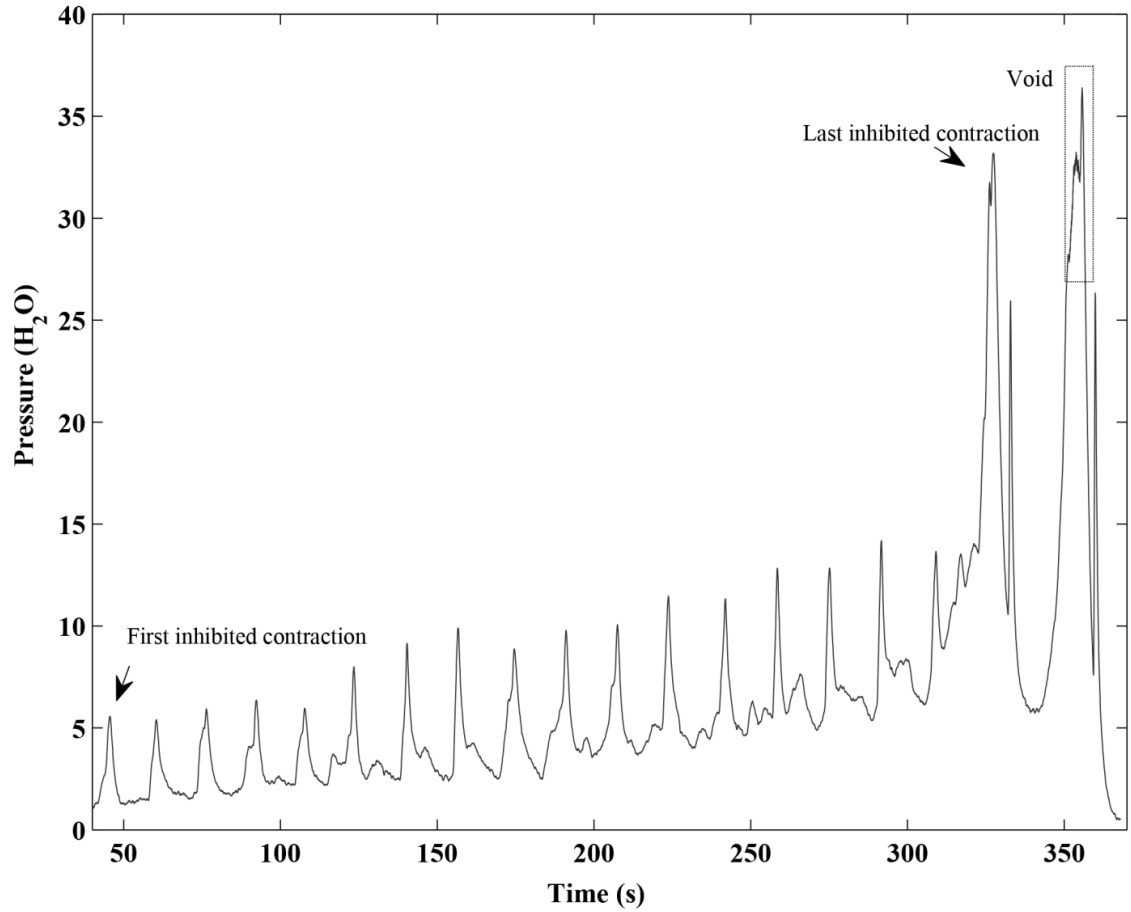

Figure 2. An example of conditional stimulation of the DPN

The stimulation was automatically switched on when the area under the curve of the frequency spectrum of pressure signal superseded the predefined threshold. As seen in the figure, a series of contractions were evoked and subsequently suppressed. The contractions could only be inhibited up to a certain volume, thereafter a leakage was observed.

To realize an implantable neural prosthesis, various bio-signals originating from the lower urinary tract have been proposed to detect the early onset of bladder contractions. Research has been done to identify the electrical activity of the bladder [12], the anal [15] and the urethral sphincter muscles [13] as a trigger event for conditional stimulation. These methods are hindered by the difficulties in recognizing whether the recorded bladder EMG is a true signal or just an electromechanical artifact and by the high rate of false positive detections in anal

and urethral EMG [13, 14, 30]. Electric activity of pudendal, pelvic and sacral nerves has also been proposed for conditional stimulation [17-19]. The method, however, suffers from low signal amplitude and poor signal-to-noise ratio. A viable solution, that has been studied extensively, would be to use a certain bladder pressure (e.g. 10-15 cmH_2O) as a trigger level, which is resilient to the above mentioned complications due to its relatively strong signal strength. However, switching on stimulation when the pressure has already reached a higher level might cause failure in inhibition as the rapid rise in pressure would probably result in an extensive increase in afferent firing, resulting in voiding [31]. The key feature of our AUC threshold detection method is that stimulation started at bladder pressure levels as low as 3 cmH_2O, long before the commonly used threshold of 10-15 cmH_2O is reached. In a conditional stimulation study [27], urgency was reported by patients about ~ 5 s before the pressure rose to 10 cmH_2O. Although the sensation of urgency cannot be simulated in animal models, a detection method such as ours might be beneficial in suppressing the feeling of urgency in patients by starting stimulation before the bladder pressure has increased significantly.

Translational impact and future prospective

The use of an anesthetized animal model restricts the drawing of parallels to humans. Nonetheless, non-voiding activity has now been accepted as an intrinsic bladder property rather than an anomaly and has been reported in anesthetized and unanesthetized animals as well as in humans [32, 33]. To utilize this property of the bladder as a trigger event for conditional stimulation in detrusor overactivity patients, further research is warranted on the pathological changes in non-voiding activity. In a recently initiated study, as yet on a small dataset of 4 patients [34], a significant power difference in a specific frequency range was found in intravesical pressure recordings from normal and detrusor overactivity patients. The results of our present study combined with this pilot study could contribute to the realization of an implantable automatic neurostimulator in a closed-loop system.

Conclusions

Conditional tibial nerve stimulation in anaesthetized rats did not inhibit bladder contractions evoked by bladder filling. Automatic stimulation of the dorsal penile nerve triggered by an increased frequency of bladder non-voiding activity resulted in successful bladder inhibition at a low bladder pressure level and a consequential increase in bladder capacity. The low pressure at this inhibition might prevent patients from experiencing urgency sensations. The presented technique could provide a robust detection system, contributing to the realization of an automatic closed-loop electrical stimulator.

References

1. Weld KJ, Dmochowski RR. Association of level of injury and bladder behavior in patients with post-traumatic spinal cord injury. Urology. 2000;55(4):490-4.
2. Abrams P. Describing bladder storage function: overactive bladder syndrome and detrusor overactivity. Urology. 2003;62(5 Suppl 2):28-37; discussion 40-2.
3. Grill WM, Craggs MD, Foreman RD, Ludlow CL, Buller JL. Emerging clinical applications of electrical stimulation: opportunities for restoration of function. J Rehabil Res Dev. 2001;38(6):641-53.
4. Peters KM, Carrico DJ, Wooldridge LS, Miller CJ, MacDiarmid SA. Percutaneous Tibial Nerve Stimulation for the Long-Term Treatment of Overactive Bladder: 3-Year Results of the STEP Study. J Urology. 2013;189(6):2194-201.
5. Chartier-Kastler EJ, Ruud Bosch JL, Perrigot M, Chancellor MB, Richard F, Denys P. Long-term results of sacral nerve stimulation (S3) for the treatment of neurogenic refractory urge incontinence related to detrusor hyperreflexia. J Urol. 2000;164(5):1476-80.
6. Kirkham AP, Shah NC, Knight SL, Shah PJ, Craggs MD. The acute effects of continuous and conditional neuromodulation on the bladder in spinal cord injury. Spinal Cord. 2001;39(8):420-8.
7. Wheeler JS, Jr., Walter JS, Zaszczurynski PJ. Bladder inhibition by penile nerve stimulation in spinal cord injury patients. J Urol. 1992;147(1):100-3.
8. Hasan ST, Robson WA, Pridie AK, Neal DE. Transcutaneous electrical nerve stimulation and temporary S3 neuromodulation in idiopathic detrusor instability. J Urol. 1996;155(6):2005-11.
9. Hansen J, Media S, Nohr M, Biering-Sorensen F, Sinkjaer T, Rijkhoff NJ. Treatment of neurogenic detrusor overactivity in spinal cord injured patients by conditional electrical stimulation. J Urol. 2005;173(6):2035-9.
10. Fjorback MV, Rijkhoff N, Petersen T, Nohr M, Sinkjaer T. Event driven electrical stimulation of the dorsal penile/clitoral nerve for management of neurogenic detrusor overactivity in multiple sclerosis. Neurourol Urodynam. 2006;25(4):349-55.
11. Dalmose AL, Rijkhoff NJM, Kirkeby HJ, Nohr M, Sinkjaer T, Djurhuus JC. Conditional stimulatzion of the dorsal penile/clitoral nerve may increase

cystometric capacity in patients with spinal cord injury. Neurourol Urodynam. 2003;22(2):130-7.

12. Ballaro A, Mundy AR, Fry CH, Craggs MD. A new approach to recording the electromyographic activity of detrusor smooth muscle. J Urology. 2001;166(5):1957-61.

13. Hansen J, Borau A, Rodriguez A, Vidal J, Sinkjaer T, Rijkhoff NJM. Urethral sphincter EMG as event detector for neurogenic detrusor overactivity. Ieee T Bio-Med Eng. 2007;54(7):1212-9.

14. Opisso E, Borau A, Rijkhoff NJM. Urethral sphincter EMG-controlled dorsal penile/clitoral nerve stimulation to treat neurogenic detrusor overactivity. J Neural Eng. 2011;8(3).

15. Wenzel BJ, Boggs JW, Gustafson KJ, Creasey GH, Grill WM. Detection of neurogenic detrusor contractions from the activity of the external anal sphincter in cat and human. Neurourol Urodyn. 2006;25(2):140-7.

16. Craggs M, Edirisinghe N, Leaker B, Susser J, Al-Mukhtar M, Donaldson N. Conditional Neuromodulation Using Trans-Rectal Stimulation in Spinal Cord Injury. Neurourol Urodynam. 2009;28(7):836-7.

17. Jezernik S, Grill WM, Sinkjaer T. Detection and inhibition of hyperreflexia-like bladder contractions in the cat by sacral nerve root recording and electrical stimulation. Neurourol Urodyn. 2001;20(2):215-30.

18. Jezernik S, Wen JG, Rijkhoff NJ, Djurhuus JC, Sinkjaer T. Analysis of bladder related nerve cuff electrode recordings from preganglionic pelvic nerve and sacral roots in pigs. J Urol. 2000;163(4):1309-14.

19. Wenzel BJ, Grill WM, Boggs JW, Gustafson KJ. Detecting the onset of hyper-reflexive bladder contractions from pudendal nerve electrical activity. Conf Proc IEEE Eng Med Biol Soc. 2004;6:4213-6.

20. Wenzel BJ, Boggs JW, Gustafson KJ, Grill WM. Detecting the onset of hyper-reflexive bladder contractions from the electrical activity of the pudendal nerve. IEEE Trans Neural Syst Rehabil Eng. 2005;13(3):428-35.

21. Melgaard J, Rijkhoff NJM. Detecting the Onset of Urinary Bladder Contractions Using an Implantable Pressure Sensor. Ieee T Neur Sys Reh. 2011;19(6):700-8.

22. Clavica F, Choudhary M, van Asselt E, van Mastrigt R. Frequency analysis of urinary bladder pre-voiding activity in normal and overactive rat detrusor. Neurourol Urodyn. 2015;34(8):794-9.

23. Vandoninck V, Van Balken MR, Finazzi Agro E, Petta F, Caltagirone C, Heesakkers JP, et al. Posterior tibial nerve stimulation in the treatment of urge incontinence. Neurourol Urodyn. 2003;22(1):17-23.

24. Peng CW, Chen JJ, Cheng CL, Grill WM. Role of pudendal afferents in voiding efficiency in the rat. Am J Physiol Regul Integr Comp Physiol. 2008;294(2):R660-72.

25. Tai CF, Chen M, Shen B, Wang JC, Roppolo JR, de Groat WC. Irritation Induced Bladder Overactivity is Suppressed by Tibial Nerve Stimulation in Cats. J Urology. 2011;186(1):326-30.

26. Choudhary M, van Mastrigt R, van Asselt E. Effect of tibial nerve stimulation on bladder afferent nerve activity in a rat detrusor overactivity model. Int J Urol. 2015 Dec 21. doi: 10.1111/iju.13033.

27. Fjorback MV, van Rey FS, van der Pal F, Rijkhoff NJ, Petersen T, Heesakkers JP. Acute urodynamic effects of posterior tibial nerve stimulation on neurogenic detrusor overactivity in patients with MS. Eur Urol. 2007;51(2):464-70; discussion 71-2.

28. Walter JS, Wheeler JS, Robinson CJ, Wurster RD. Inhibiting the hyperreflexic bladder with electrical stimulation in a spinal animal model. Neurourol Urodyn. 1993;12(3):241-52; discussion 53.

29. Martens FM, Heesakkers JP, Rijkhoff NJ. Minimal invasive electrode implantation for conditional stimulation of the dorsal genital nerve in neurogenic detrusor overactivity. Spinal Cord. 2011;49(4):566-72.

30. Brunsting CD. An interpretation of the urinary bladder electrocystogram as artefact. J Urol. 1958;79(1):165-70.

31. van Asselt E, le Feber J, van Mastrigt R. Threshold for efferent bladder nerve firing in the rat. Am J Physiol. 1999;276(6 Pt 2):R1819-24.

32. Drake MJ, Harvey IJ, Gillespie JI, Van Duyl WA. Localized contractions in the normal human bladder and in urinary urgency. BJU International. 2005;95(7):1002-5.

33. Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JI. Phasic non-micturition contractions in the bladder of the anaesthetized and awake rat. BJU Int. 2006;97(5):1094-101.

34. Zolliker D, Niederhauser T, Grämiger M, van Mastrigt R, Burkhard F, Obrist D, Clavica, F. Frequency Analysis of Urinary Bladder Pre-Voiding Activity in Normal and Overactive Human Detrusor: A Pilot Study. 37th Annual IEEE EMB Conference Proceedings 2015.

Part IV

General discussion and summary

Chapter 8

General discussion and summary

Discussion

The simple process of storage and periodic expulsion of urine is regulated by a complex mechanism of afferent and efferent neural signaling between the bladder and the central nervous system [1]. In pathological conditions such as the overactive bladder (OAB) syndrome, this afferent-efferent mechanism is disturbed, resulting in involuntary contractions of detrusor muscle, and symptoms such as urgency, urge incontinence, frequency and nocturia [2].

Study of OAB in animal models

Research in humans is restricted by law and ethical considerations. *In vivo* urodynamics (bladder pressure and flow-rate measurements) and brain mapping in consenting patients, in addition to *in vitro* studies of muscle strips and post-mortem materials are the only viable investigative tools for human research [3]. Animal modeling provides a logical, hypothesis testing alternative to human experimentation and often acts as a precursor to human clinical treatment.

In vivo animal studies

To simulate the human OAB syndrome in animal models, the bladder is generally irritated by infusing chemicals. We used an Acetic Acid (AA) rat model of detrusor overactivity, which mimics the human symptoms such as frequency and involuntary contractions of the detrusor. The chemical irritation of the bladder wall causes hypersensitization of bladder C-fibers: which have been reported to play a significant role in causing bladder overactivity under pathological conditions [4].

While urodynamic investigations involving pressure and volume measurements are relatively simple, electrophysiological techniques to measure the electrical activity of the bladder nerves are rather complicated. Among the popular techniques are single fiber recordings and measurements after a central/peripheral transection of bladder nerves [5-7]. Apart from the mechanical difficulties involved in the preparation of single fibers and the transection of nerves, they pose several other complications. Firstly, a single fiber may not represent the whole nerve. Afferent measurements obtained from single fibers only show the frequency of firing and fail to take into account the interaction

among the unitary spikes. Measurement and modeling of these interactions and of the recruitment of new fibers when the bladder is exposed to external stimuli are necessary for the development of implantable electrical devices for neural control of micturition e.g. in spinal cord injured patients.

To provide an alternative to the aforementioned techniques, we previously developed a mathematical model to study the changes in sensitivity of afferent nerve fibers in intact nerves i.e. without transection or separation of single fibers [8]. The model assumes that the nerve activity measured from an intact, multifiber rat bladder nerve in the relaxation phase of a voiding contraction is purely afferent and that it depends linearly on the bladder pressure. The coefficients of that linear relationship, derived from the post void relaxation phase, can be used to estimate the afferent activity in the filling phase.

In the current study in anaesthetized rats of the same age, weight and gender as those in which the model was developed, we applied this mathematical model to an AA induced detrusor overactivity model. To verify the absence of efferent activity in the relaxation phase in intact nerve measurements, we calculated the time constant of the isovolumetric pressure decay after a voiding contraction. It was assumed that in the absence of any efferent activation, the bladder relaxes passively and the time constant would be comparable in control (saline) and AA measurements. Conversely, any (pelvic) efferent activity would result in a contraction of the bladder, which would result in a different time constant. To provide additional support for this assumption, one of the postganglionic pelvic nerve branches was crushed between major pelvic ganglion and electrode, so that only afferent nerve signals were measured. The slope of the linear relationship between pressure and afferent nerve activity in intact and crushed nerve experiments was found to be comparable, confirming our hypothesis. Although this finding has no direct clinical implication, it could be useful in closed loop implantable devices to detect changes in afferent nerve activity based on pressure recordings to electrically inhibit bladder contractions.

In vitro animal studies

Apart from *in vivo* investigations in animal models, study of isolated whole organ systems is often necessary for a better understanding of their function. In experiments requiring stimulation of the organs via efferent nerve fibers and/or

recording of the afferent nerve activity from the organ, an *in vitro* setup is often preferred over an *in vivo* setup because: i) it offers greater control over the extracellular environment and therefore enables a more accurate measurement of the response of the organ to various stimuli (e.g. drugs, mechanical and/or electrical stimulation), without interference of the central nervous system or other functions such as circulation and breathing, ii) experimental variation is less, leading to better reproducibility, iii) it does not require complex surgical procedures or extensive monitoring [9]. Additionally, *in vitro* experimentation is a great step forward towards reduction of experimental animal usage [10].

Currently two-compartment setups are used for *in vitro* measurement of nerve activity and/or stimulation of the organ via the associated nerves [11-14]. These setups are complicated because they require two fluids in two separate compartments (one to maintain organ viability and another to provide electrical insulation) and stretching the nerve across one chamber to the other, which may damage it. We developed a simple single compartment setup by testing the electrophysiological and viability preserving properties of FC-770 (a perfluorocarbon) for *in vitro* recording of bladder afferent nerve activity and electrical stimulation of the bladder. Perfluorocarbons are especially suitable for such a setup because of their high oxygen carrying capacity combined with good insulating properties. In our setup, afferent nerve activity was successfully recorded in preparations from 11 rats. The bladders were stimulated electrically and high amplitude contractions were evoked. Histological examinations and monitoring of spontaneous contractions showed that FC-770 maintained organ viability and did not cause damage to the tissue.

Understanding the mechanisms of bladder overactivity

In broad terms, three main theories have been proposed to explain the mechanism underlying bladder overactivity [15]. The myogenic theory ascribes involuntary contractions of the bladder to changes in the smooth detrusor muscle. The neurogenic theory suggests that damage to inhibitory pathways in the central nervous system or hypersensitivity of bladder afferent terminals causes detrusor overactivity [2]. A relatively new theory: the autonomous theory proposes a modular configuration of the detrusor [16]. This theory suggests that, during normal bladder filling, there is autonomous activity of detrusor (modules) i.e.

independent contractions of areas of the detrusor muscle, globally observed as non-voiding contractions (NVCs). It is substantiated by the fact that NVCs have been recorded in normal individuals of various species, including humans [17-19]. Ambulatory urodynamic recordings show NVC activity in almost 70 % of healthy volunteers implying that this activity is normal rather than an exception [17]. A few studies have shown evidence of this activity both through *in vivo* and *in vitro* animal experiments using bladder filling at physiological rates [18, 20].

The autonomous theory attributes detrusor overactivity to a disturbance in this autonomous system that can amplify the sensory information and/or cause failure of inhibiting inputs, leading to detrusor overactivity. To substantiate this theory, a better understanding of the NVC changes in the overactive bladder, normally associated with increased bladder sensation and detrusor overactivity, is necessary. To this end we developed a technique to acquire quantitative data on the NVC activity of the bladder, with a particular focus on rapid pressure transients just before voiding (i.e. pre-voiding activity) [21]. An algorithm based on frequency analysis was developed to automatically identify changes in the pattern of the bladder pressure and predict an impending voiding contraction by generating an alarm. On the basis of this algorithm, a closed loop device may be developed to prevent incontinence by conditional stimulation. The algorithm has also been used to provide an index to quantify differences in the NVC activity in normal and pathologic conditions e.g. associated with detrusor overactivity.

Treatment of bladder Overactivity

The sensation of desire and urge in OAB patients is a key symptom, often associated with increased sensitivity of afferent fibers [2]. Pharmacological compounds such antimuscarinic drugs are the first-line treatment, however, due to lack of efficacy or side effects, more than half of the patients terminate this treatment [22]. An important clinical alternative to pharmacological treatment is neuromodulation, which presumably works via afferent nerve modulation. Commonly used clinical neuromodulation routines involve electrical stimulation of sacral, pudendal or tibial nerve fibers either by percutaneous stimulation or an implanted device [23]. Despite the widespread clinical use, knowledge on the working mechanism of neuromodulation is scarce and remains incomplete.

Tibial nerve stimulation

Tibial Nerve Stimulation (TNS) therapy in humans is based on half-hourly percutaneous stimulation, performed once a week usually for 10–12 weeks followed by stimulation once per month [24]. It has been reported to inhibit bladder overactivity and improve the bladder capacity in animal models [4]. Although it has been found effective in clinical treatment of the overactive bladder syndrome in humans, the mechanism of action is largely unknown. Stimulation of somatic afferents in the tibial nerve is believed to restore the balance between excitatory and inhibitory bladder elements by modulating the signal traffic to and from the bladder via the sacral plexus [25]. To understand the inhibitory effect of tibial nerve stimulation on the bladder, most studies have focused on urodynamic parameters [24]. We studied its effect on the bladder neurophysiology and the trigger mechanism of voiding in a detrusor overactivity rat model [26]. We found that a 30 min stimulation of tibial nerve poststimulation inhibited afferent nerve activity in acetic acid irritated bladders and significantly increased the bladder capacity. In addition, the afferent threshold to initiate a voiding contraction and the slope of the linear pressure-afferent activity relation were reduced, whereas the volume threshold increased after stimulation, implying that the stimulation delayed the onset of voiding to a higher filled volume. Whether the poststimulation decrease is a reversal of C-fiber sensitization cannot be ascertained due to the mixed, multifiber nature of our afferent recordings. A peripheral effect of TNS on afferent nerve terminals, to the best of the authors' knowledge, has not been reported elsewhere. It is possible that this reduction in afferent activity is an indirect effect of a change in bladder compliance. Poststimulation the volume threshold increased while the pressure threshold remained unchanged, implicating an increase in detrusor compliance. It has been shown that pudendal nerve stimulation, via sympathetic efferent pathways, can activate β3-adrenoceptors in the detrusor muscle and/or α-adrenergic receptors at the vesical ganglia to inhibit detrusor contractions and regulate bladder compliance [27-29].

In addition to its well-known central inhibitory mechanisms, we found that TNS poststimulation improves bladder storage capacity by delaying the onset of voiding, via an inhibitory effect on the bladder afferent signaling at the peripheral

level. As evident from the multitude of modes of action suggested in the literature, the working mechanism of tibial nerve stimulation is highly complex and occurs at several levels, incorporating a combination of central inhibitory mechanisms as well as an inhibition at the peripheral level, as we found in our present study.

Conditional electrical stimulation

The long stimulation periods discussed in the preceding section might result in ineffectiveness due to adaptation and an increased risk of tissue damage [30, 31]. Furthermore, they require battery replacement in implanted devices, posing a medical risk. A possible alternative is conditional stimulation, triggered only when an impending bladder contraction is detected [32], hence considerably reducing the stimulation time and with it, the associated complications and risks. We hypothesized that, similar to continuous tibial stimulation, acute stimulation might also recruit a lumbo-sacral spinal cord route to acutely produce an inhibitory effect. Unfortunately, acute TNS failed to suppress bladder contractions in all of the 8 animals in our study. In order to maximize the recruitment of afferents, higher stimulation thresholds of up to 5 times the motor threshold and pulse widths of 200 and 400 μs were tested. Nevertheless we could not produce any suppression of bladder contractions. To test if the tibial stimulation would affect pressure dynamics during a voiding contraction, we examined the pressure rise time from the baseline until its culmination into a voiding and the following exponential decay to baseline. There was no appreciable change in these parameters between the stimulation and control cystometrograms. Evidently, continuous stimulation for longer periods remains the only viable option to achieve bladder inhibition via the tibial nerve.

Continuing the pursuit of developing a method for conditional neurostimulation triggered by urgency, we expanded our non-voiding activity detection algorithm to real-time, and to testing if the frequency of non-voiding contractions could be used as a trigger event for closed-loop conditional inhibition of bladder contractions via the Dorsal Nerve of the Penis (DNP).

Conditional stimulation, triggered by changes in the amplitude spectrum of the bladder pressure in the frequency range 0.2-20 Hz, successfully suppressed 70 % of the bladder contractions evoked by filling and resulted in a 45 % increase in

bladder capacity. A noticeable feature of this frequency based trigger-event detection method is that changes in frequency were detected at bladder pressure value as low as 3 cmH_2O. This is an advantage over the commonly used pressure amplitude based detection methods for conditional stimulation, where stimulation is triggered at a pressure level of 10-15 cmH_2O. Switching on stimulation when the pressure has already reached a higher level (e.g. 15 cmH_2O) might cause failure in inhibition as the rapid rise in pressure would result in a large increase in afferent firing, resulting in voiding. In an earlier published conditional stimulation study [33], based on a pressure value threshold, urgency was reported by patients about ~5 sec before a pressure rise of 10 cmH_2O. Although the sensation of urgency cannot be simulated in animal models, a frequency based detection method such as ours might be beneficial in suppressing the feeling of urgency in patients by starting stimulation earlier at a lower pressure.

Limitations

Animal models are essential to simulate the overactive bladder syndrome and further the understanding of its pathophysiology. The large differences between rats and humans require a careful consideration of the translational aspects. Sensory urgency in humans is a subjective clinical diagnosis and cannot be simulated in animal models. The acetic acid irritation model used in our study only mimics one symptom of the human overactive bladder syndrome, the detrusor overactivity. The chemical irritation of the bladder also has side effects such as inflammation of the bladder wall, a symptom typically associated with bladder cystitis. The use of anaesthesia further restricts the drawing of parallels to humans. Although urethane is believed to spare the micturition reflex, a suppressing effect of the anaesthesia on pressure development and/or neural signaling cannot be ruled out. The mixed, multifiber nature of our afferent recordings also made the characterization of the individual responses of the mechanosensitive Aδ and the chemosensitive C-fiber afferents impossible. Nevertheless we find the limited invasiveness of our experimental approach an asset that made important observations and conclusions possible.

References

1. Fowler CJ, Griffiths D, de Groat WC. The neural control of micturition. Nat Rev Neurosci. 2008;9(6):453-66.
2. de Groat WC. A neurologic basis for the overactive bladder. Urology. 1997;50(6A Suppl):36-52; discussion 3-6.
3. Parsons BA, Drake MJ. Animal models in overactive bladder research. Handb Exp Pharmacol. 2011(202):15-43.
4. Tai CF, Chen M, Shen B, Wang JC, Roppolo JR, de Groat WC. Irritation Induced Bladder Overactivity is Suppressed by Tibial Nerve Stimulation in Cats. J Urology. 2011;186(1):326-30.
5. Zvara P, Wright AJ, Roach K, Ursiny M, Shapiro B, Dagrosa LM, et al. A non-anesthetized mouse model for recording sensory urinary bladder activity. Front Neurol. 2010;1:127.
6. De Wachter S, De Laet K, Wyndaele JJ. Does the cystometric filling rate affect the afferent bladder response pattern? A study on single fibre pelvic nerve afferents in the rat urinary bladder. Neurourol Urodyn. 2006;25(2):162-7.
7. Wyndaele JJ. Investigating afferent nerve activity from the lower urinary tract: highlighting some basic research techniques and clinical evaluation methods. Neurourol Urodyn. 2010;29(1):56-62.
8. Le Feber J, Van Asselt E, Van Mastrigt R. Neurophysiological modeling of voiding in rats: bladder pressure and postganglionic bladder nerve activity. Am J Physiol. 1997;272(1 Pt 2):R413-21.
9. Namasivayam S, Eardley I, Morrison JF. A novel in vitro bladder pelvic nerve afferent model in the rat. Br J Urol. 1998;82(6):902-5.
10. Russell WMS BR. The principles of humane experimental technique. London: Methuen. 1959.
11. Yu Y, de Groat WC. Effects of stimulation of muscarinic receptors on bladder afferent nerves in the in vitro bladder-pelvic afferent nerve preparation of the rat. Brain Res. 2010;1361:43-53.
12. Valderrama-Gonzalez GD, Gasson MN, Warwick K, Whalley BJ, editors. Prosthesis grasp reflex via peripheral nerve control - An in vitro study. Cybernetic Intelligent Systems (CIS), 2010 IEEE 9th International Conference on; 2010 1-2 Sept. 2010.

13. Rong W, Burnstock G. Activation of ureter nociceptors by exogenous and endogenous ATP in guinea pig. Neuropharmacology. 2004;47(7):1093-101.

14. Daly DM, Nocchi L, Liaskos M, McKay NG, Chapple C, Grundy D. Age-related changes in afferent pathways and urothelial function in the male mouse bladder. J Physiol. 2014;592(Pt 3):537-49.

15. Hashim H, Abrams P. Overactive bladder: an update. Curr Opin Urol. 2007;17(4):231-6.

16. Drake MJ, Mills IW, Gillespie JI. Model of peripheral autonomous modules and a myovesical plexus in normal and overactive bladder function. Lancet. 2001;358(9279):401-3.

17. Drake MJ, Harvey IJ, Gillespie JI, Van Duyl WA. Localized contractions in the normal human bladder and in urinary urgency. BJU International. 2005;95(7):1002-5.

18. Streng T, Hedlund P, Talo A, Andersson KE, Gillespie JI. Phasic non-micturition contractions in the bladder of the anaesthetized and awake rat. BJU Int. 2006;97(5):1094-101.

19. Biallosterski BT, van Koeveringe GA, van Kerrebroeck PE, Gillespie JI, de Wachter SG. Nonvoiding Activity of the Guinea Pig Bladder. The Journal of Urology. 2011;186(2):721-7.

20. Drake MJ, Harvey IJ, Gillespie JI. Autonomous activity in the isolated guinea pig bladder. Experimental Physiology. 2003;88(1):19-30.

21. Clavica F, Choudhary M, van Asselt E, van Mastrigt R. Frequency analysis of urinary bladder pre-voiding activity in normal and overactive rat detrusor. Neurourol Urodyn. 2015;34(8):794-9.

22. Abrams P, Andersson KE. Muscarinic receptor antagonists for overactive bladder. Bju Int. 2007;100(5):987-1006.

23. Groen J, Bosch JL. Neuromodulation techniques in the treatment of the overactive bladder. Bju Int. 2001;87(8):723-31.

24. Peters KM, Carrico DJ, Wooldridge LS, Miller CJ, MacDiarmid SA. Percutaneous Tibial Nerve Stimulation for the Long-Term Treatment of Overactive Bladder: 3-Year Results of the STEP Study. J Urology. 2013;189(6):2194-201.

25. Gaziev G, Topazio L, Iacovelli V, Asimakopoulos A, Di Santo A, De Nunzio C, et al. Percutaneous tibial nerve stimulation (PTNS) efficacy in the treatment of lower urinary tract dysfunctions: a systematic review. Bmc Urol. 2013;13.

26. Choudhary M, van Mastrigt R, van Asselt E. Effect of tibial nerve stimulation on bladder afferent nerve activity in a rat detrusor overactivity model. Int J Urol. 2015 Dec 21. doi: 10.1111/iju.13033.

27. Craggs M, McFarlane J. Neuromodulation of the lower urinary tract. Exp Physiol. 1999;84(1):149-60.

28. De Groat WC, Saum WR. Sympathetic inhibition of the urinary bladder and of pelvic ganglionic transmission in the cat. J Physiol. 1972;220(2):297-314.

29. Lindstrom S, Fall M, Carlsson CA, Erlandson BE. The neurophysiological basis of bladder inhibition in response to intravaginal electrical stimulation. J Urol. 1983;129(2):405-10.

30. Hasan ST, Robson WA, Pridie AK, Neal DE. Transcutaneous electrical nerve stimulation and temporary S3 neuromodulation in idiopathic detrusor instability. J Urol. 1996;155(6):2005-11.

31. Hansen J, Media S, Nohr M, Biering-Sorensen F, Sinkjaer T, Rijkhoff NJ. Treatment of neurogenic detrusor overactivity in spinal cord injured patients by conditional electrical stimulation. J Urol. 2005;173(6):2035-9.

32. Dalmose AL, Rijkhoff NJM, Kirkeby HJ, Nohr M, Sinkjaer T, Djurhuus JC. Conditional stimulatzion of the dorsal penile/clitoral nerve may increase cystometric capacity in patients with spinal cord injury. Neurourol Urodynam. 2003;22(2):130-7.

33. Fjorback MV, Rijkhoff N, Petersen T, Nohr M, Sinkjaer T. Event driven electrical stimulation of the dorsal penile/clitoral nerve for management of neurogenic detrusor overactivity in multiple sclerosis. Neurourol Urodynam. 2006;25(4):349-55.

Summary

The Overactive Bladder (OAB) is a symptom based syndrome, detrusor overactivity being the prime indication. One of its hallmark characteristics is the sensation of "urgency", often associated with involuntary contractions of the detrusor muscle. OAB affects approximately 12% of the European and 17% of the North American population and has a significant negative impact on the quality of life and mental health. Despite years of research, a comprehensive understanding of the pathophysiology of the OAB syndrome has not yet been reached. Antimuscarinic drugs, botulinum toxin injections and neuromodulation are the most important treatment methods used in the clinical treatment of OAB patients. The aim of this research was to study the neurophysiology of bladder afferents in simulated detrusor overactivity in rats and to develop a conditional electrical stimulation technique to inhibit undesired detrusor contractions before they are sensed.

To study the electrophysiology of bladder nerves, a novel single compartment *in vitro* setup was developed which could be used to measure nerve activity. This setup was an improvement over the commonly used two-compartment models that require two fluids (kept in two separate compartments) and the need of stretching the nerve across one compartment to the other, which may damage the nerve.

Furthermore, a mathematical model was developed which allows the distinction of the electrical activity of mixed nerve recordings into afferent (sensory) and efferent (motor) traffic. Using this model, the sensitivity of afferent fibers could be studied in intact bladder nerves without the need for cutting nerves or preparing single fibers, offering the advantage of minimal surgical procedures and minimal nerve damage.

Next, a pattern recognition algorithm was developed to automatically identify changes in the frequency spectrum of the bladder pressure and predict an impending voiding contraction by generating an alarm. The algorithm provided a tool to produce quantitative data on spontaneous activity of the bladder as well as to detect a pattern which could be used to prevent incontinence (urine leakage) through event driven (conditional) stimulation in patients with a loss of bladder sensation.

To implement the developed algorithm in real time, different nerves associated with the bladder were studied. Experiments were done to investigate the effect of tibial

nerve stimulation on afferent nerve activity and bladder pressure. Furthermore, the effectiveness of acute electrical stimulation of the tibial and dorsal nerve of the penis to inhibit premature bladder contractions was studied. The dorsal nerve of the penis was found to be the most suitable for conditional stimulation.

Finally, a closed loop real-time pattern recognition based stimulation technique was developed to suppress bladder overactivity. On the basis of this technique, an implantable closed loop device could be developed to prevent incontinence in spinal cord injury or other OAB patients providing automatic control of the bladder function.

Samenvatting

De overactieve blaas (OAB) is een op symptomen gebaseerd syndroom, waarbij frequente en hevige aandrang om te plassen het belangrijkste kenmerk is. Deze aandrang hangt doorgaans samen met detrusoroveractiviteit, het optreden van onwillekeurige contracties van de blaasspier (musculus detrusor). OAB treft ongeveer 12% van de Europese en 17% van de Noord-Amerikaanse bevolking en heeft een vergaande invloed op de kwaliteit van leven en de geestelijke gezondheid. Ondanks jarenlang onderzoek is er nog geen volledig begrip van de pathofysiologie van het OAB-syndroom. Anticholinergica, botulinetoxine-injecties en neuromodulatie zijn de belangrijkste behandelingsmethoden voor OAB-patiënten. Het doel van ons onderzoek was om de neurofysiologie van blaasafferenten (d.w.z. van de blaas afkomstige gevoelszenuwen) in ratten met gesimuleerde detrusoroveractiviteit te bestuderen en om een conditionele (voorwaardelijke) stimulatietechniek te ontwikkelen om ongewenste detrusorcontracties te onderdrukken.

Om de elektrofysiologie van blaaszenuwen te bestuderen, werd een 1-compartiment *in vitro* opstelling ontwikkeld die gebruikt kon worden om zenuwactiviteit te meten. Deze opstelling is een verbetering ten opzichte van de vaak gebruikte 2-compartimentenopstelling met 2 verschillende vloeistoffen in 2 verschillende compartimenten. In een dergelijke opstelling wordt de zenuw van het ene naar het andere compartiment geleid, hetgeen tot beschadiging kan leiden.

Daarnaast is er een mathematisch model ontwikkeld dat in elektrofysiologische metingen aan gemengde zenuwen onderscheid kan maken tussen de activiteit van afferente (sensorische) en efferente (motorische) vezels. Met behulp van dit model kon de sensitiviteit van afferente vezels worden bestudeerd zonder de noodzaak om zenuwen door te nemen of enkelvoudige vezels te prepareren, met als voordeel minimale dissectie en minimale zenuwschade.

Vervolgens is er een algoritme ontwikkeld om veranderingen in het frequentiespectrum van de blaasdruk te identificeren met als doel een opkomende blaascontractie te voorspellen en een alarm te genereren. Het

algoritme maakt het ook mogelijk de spontane activiteit van de blaas te kwantificeren en hierin een patroon te herkennen. Deze patroonherkenning zou gebruikt kunnen worden om incontinentie (urineverlies) te voorkomen door middel van een conditionele (voorwaardelijke) zenuwstimulatie bij patiënten met een verminderd gevoel in de blaas.

Voor een *real time* implementatie van het algoritme werden verschillende zenuwen die betrokken zijn bij de innervatie van de blaas bestudeerd. Er werden experimenten gedaan om het effect van stimulatie van de nervus tibialis (scheenbeenzenuw) op de activiteit van afferente blaaszenuwen en de blaasdruk te onderzoeken. Verder werd er gekeken naar de effectiviteit van acute elektrische stimulatie van de n. tibialis en de dorsale zenuw van de penis om voortijdige blaascontracties te onderdrukken. De dorsale zenuw van de penis bleek de meeste geschikte kandidaat voor conditionele stimulatie.

Ten slotte werd er een *real time* op patroonherkenning gebaseerde *closed loop* stimulatietechniek ontwikkeld om detrusoroveractiviteit te onderdrukken. Op basis van deze techniek zou een implanteerbaar apparaat kunnen worden ontworpen dat incontinentie bij dwarslaesie- en andere OAB-patiënten kan voorkomen.

Addendum

Publications

Journal papers

- Choudhary M.S., van Asselt E., van Mastrigt R., Clavica F., (2015), "Neurophysiological modeling of bladder afferent activity in the rat overactive bladder model". J Physiol Sci. 2015. doi 10.1007/s12576-015-0370-y.
- Clavica F, Choudhary M.S., van Asselt E., van Mastrigt R., (2014), "Frequency analysis of urinary bladder pre-voiding activity in normal and overactive rat detrusor". Neurourol Urodyn. doi:10.1002/nau.22664.
- Choudhary M.S., van Mastrigt R., van Asselt E., (2015), "Effect of tibial nerve stimulation on bladder afferent nerve activity in a rat detrusor overactivity model". Int J Urol. 2015 Dec 21. doi: 10.1111/iju.13033.
- Choudhary M.S., van Mastrigt R., van Asselt E., (2015), "A novel single compartment *in vitro* model for electrophysiological research using Perfluorocarbons". Accepted, Physiol. Res.
- Choudhary M.S., van Mastrigt R., van Asselt E., (2016), "Inhibitory effects of tibial nerve stimulation on bladder neurophysiology in rats". SpringerPlus (2016) 5:35, doi 10.1186/s40064-016-1687-6.
- Choudhary M.S., van Mastrigt R., van Asselt E., (2016), "The frequency spectrum of bladder non-voiding contractions as a trigger-event for conditional stimulation: closed-loop inhibition of bladder overactivity in rats". Submitted.
- van Asselt E., Choudhary M.S., Clavica F., van Mastrigt R., (2015), "Urethane anesthesia in acute lower urinary tract studies in the male rat". Submitted.

Conference proceedings, posters and abstracts

- Choudhary M. S., van Asselt E., van Mastrigt R., "A rat model for the study of the effect of tibial nerve stimulation on afferent nerve activity", International Continence Society Conference (ICS), Rio de Janeiro, Brazil, October 2014.

- Choudhary M. S., van Asselt E., van Mastrigt R., Clavica F., "A Novel Single Compartment In Vitro model: Perflurocarbons for Electrophysiological studies of the Rat Urinary Bladder", Proceedings of the 35th Annual International IEEE EMBS Conference, Osaka, Japan, July 2013.
- Clavica F., Choudhary M. S., van Asselt E., van Mastrigt R., "Can an Algorithm Predict a Voiding Contraction in Unconscious Rats?" Proceedings of the 35th Annual International IEEE EMBS Conference, Osaka, Japan, July 2013.
- Choudhary M. S., van Asselt E., Clavica F., van Mastrigt R., "Validation of rat overactive bladder model by bladder wall stress estimation", International Continence Society Conference (ICS), Barcelona, Spain, August 2013.
- Choudhary, M.S., Clavica, F., van Asselt E., van Mastrigt R., "Frequency analysis of afferent nerve activity in the rat overactive urinary bladder model", Proceedings of the 34th Annual International IEEE EMBS Conference, San Diego, USA, July 2012.

Acknowledgements

"It is good to have an end to journey toward; but it is the journey that matters, in the end." — Ernest Hemingway

This journey would not have been worthwhile if I hadn't had the opportunity to share it with the amazing people around me. And for that, I sincerely thank my family, friends and colleagues for being there.

It would never have been possible to reach this point without the unconditional support of my parents. The lessons of persistence and patience I learned from my father have brought me a long way.

The freedom to explore and work on your own ideas, however ridiculous they might be, is the one of the greatest thing a supervisor could offer. Thank you Ron for giving me that freedom. The countless manuscript revisions tested your patience and your red lines mine. In the end the 'red'ing process taught me a great deal about structured writing.

Els you have been so encouraging and supporting in all aspects of my PhD life. I could never thank you enough for doing the extremely hard and demanding animal experiments.

Lab atmosphere was always fun with Arif, Jan, Geert and Tim, especially the interesting lunchtime discussions. Thank you Francesco for your help during the initial period of my PhD.

The Dutch adventure would never have been complete without the great company of my friends outside the lab. Thomas, Deep, Jeremy, Muthu, apart from the PhD journey, we took on our journey of squash and what a great fun that was! The time spent with 'The 300' - Michael, Daan, Sven, Jatin, Rony will always be in my fond memories.

Finally, Nish you have been so amazing all these years. Although you had very little clue of what I was doing, you were always there to keep me going. I thank you very much for everything.

Curriculum Vitae

Mahipal Choudhary was born in Haryana, India on 16th July, 1985. He attended high school in New Delhi and thereafter, obtained an undergraduate degree in Biomedical Engineering from GJ University of Science and Technology. His interest in medical technology prompted him to move to Germany, where he obtained his Master degree in Biomedical Engineering at Hochschule Furtwangen University.

In 2011, he joined the Department of Urology, Sector Furore at Erasmus Medical Center in Rotterdam, the Netherlands as a PhD candidate under the supervision of Prof. dr. ir. Ron van Mastrigt and dr. Els van Asselt. His project was a part of the Marie Curie Actions – an interdisciplinary research network of European universities and enterprises. During his PhD he focused on the neurophysiology of bladder afferent mechanisms and electrical stimulation of the bladder.

PhD Portfolio

Name PhD student	Mahipal Choudhary
PhD period	Nov 2011 - Sept 2015
Erasmus MC department	Urology, Sector Furore
Promotor	Prof.dr.ir. Ron van Mastrigt
Supervisor	Dr. Els van Asselt

PhD training	Year	ECTS
General and specific courses		
• Biomedical English Writing	2012	4.0
• Laboratory Animal Science, Article 9	2012	3.0
• Biostatistical Methods I: Basic Principles (CC02)	2014	5.7
Workshops		
• Basic Urodynamics - an interactive workshop, Bristol, UK	2012	1.0
• Drug Development Course: lectures & workshops (Astellas, Leiden)	2013	1.0
Presentations		
• Marie Curie Actions, TRUST meeting, Leuven, Belgium	2011	1.0
• Marie Curie Actions, TRUST meeting, Bristol, UK	2012	1.0
• Marie Curie Actions, TRUST meeting, Leiden, the Netherlands	2013	1.0
• Sector Furore meeting	2012	1.0
• Sector Furore meeting	2013	1.0
• Sector Furore meeting	2014	1.0
International conferences		
• IEEE EMBS Conference, San Diego, USA (poster presentation)	2012	1.4

• Marie Curie Actions Conference at EuroScience Open Forum (ESOF), Dublin, Ireland, (poster presentation)	2012	1.4
• IEEE EMBS Conference, Osaka, Japan, (Oral presentation)	2013	2.0
• International Continence Society Conference (ICS), Barcelona, Spain (Poster and oral presentation)	2013	3.0
• International Continence Society Conference (ICS), Rio de Janeiro, Brazil (Poster and oral presentation)	2014	3.0

Total ECTS	31.5