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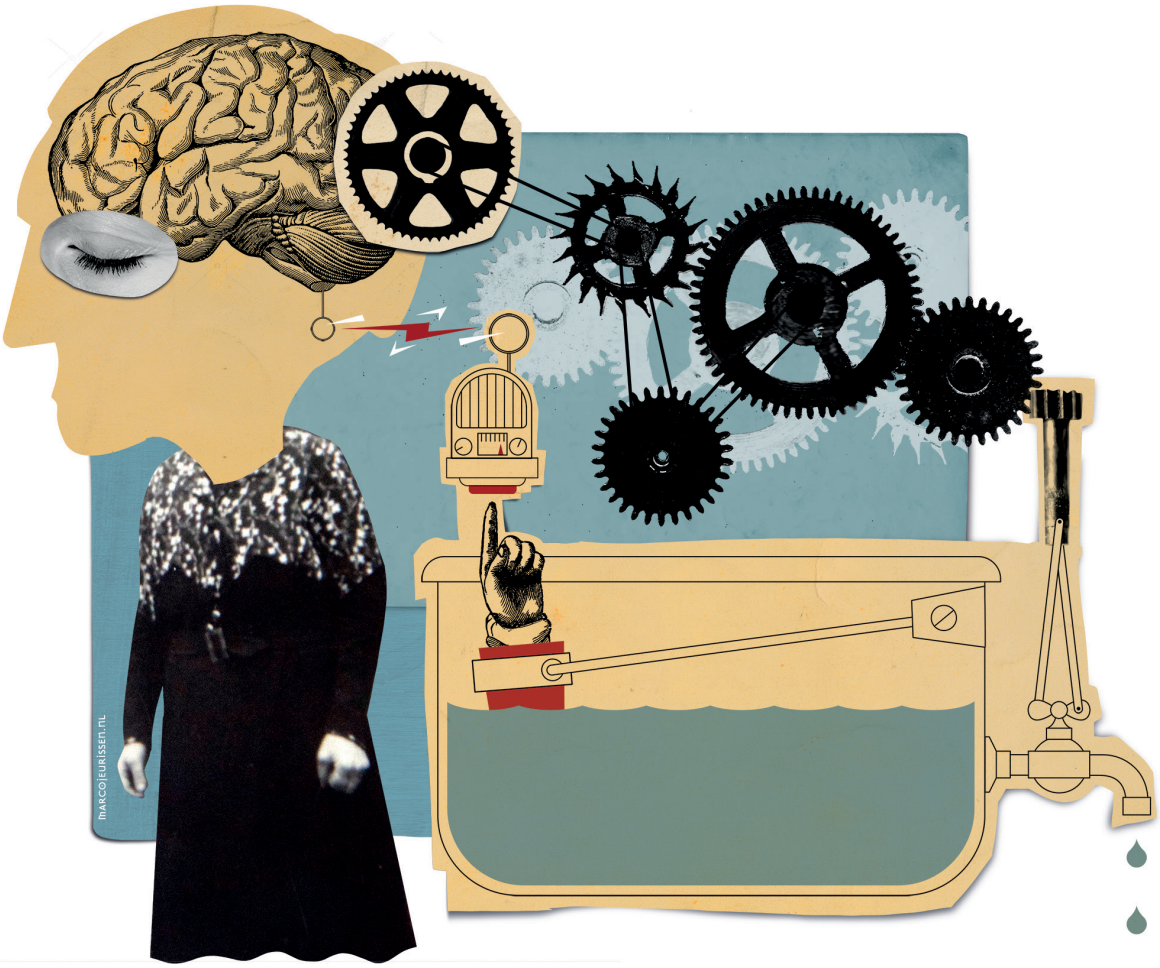
Translational models in functional and neuro-urology



Mathijs de Rijk

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Interactive control of the lower urinary tract: *Translational models in functional and neuro-urology*

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CONTENTS

CHAPTER 1

General Introduction

INTRODUCTION

Urine is a liquid biological waste product produced by humans and many other animals, which is expelled from the body via the lower urinary tract (LUT). The LUT consists out of the urinary bladder, urethral sphincter, and the urethra. The main functions of this system are storage of urine until the capacity of the bladder is reached and micturition (i.e., evacuation of urine) when an appropriate time and place is found to empty the bladder.

In most animal species, as well as in young children and individuals with lesions in the central nervous system (CNS), micturition is primarily controlled by an involuntary reflex at the level of the autonomic nervous system[1]. Exercising voluntary control over our bladder is a learned behavior that forms an integral part of our upbringing. We are taught to monitor the fullness of our bladders and estimate the time until we need to go to the bathroom. Voiding is a behavior that is highly influenced by our social environments and we continuously have to evaluate our levels of bladder fullness to prevent us from having to void in socially unacceptable circumstances. In addition to providing a system to evacuate urine from the body, in most animals evacuation of urine has numerous other functions. More primal reasons to initiate a micturition episode are territorial marking or defensive behavior and sexual behavior. In the human situation appropriateness of emptying one's bladder is mainly a learned social behavior, and the previously mentioned instinctive reasons to micturate are largely part of our evolutionary past.

Bladder morphology

The bladder is a hollow muscular organ, designed as a distensible reservoir to optimally store and excrete urine. The outlet region of the LUT consists of the bladder neck, urethral sphincter, and urethra.

The bladder wall can be subdivided into several structurally and functionally different layers (Figure 1). The innermost surface of the bladder is lined by a mucosal membrane consisting of the multi-layered urothelium and the underlying lamina propria. The urothelium can be subdivided into a superficial layer of hexagonal umbrella cells, an intermediate layer, and a basal layer[2]. The urothelium provides a barrier protecting the underlying tissue from irritative influences of urine and executes a signaling role via release of mediators that influence bladder functioning[3]. The underlying lamina propria contains afferent and efferent nerve endings, as well as many elastic fibers. The lamina propria fulfills an important integrative role in signal transduction to the central nervous system and between the urothelium and the underlying bladder layers. Additionally, the lamina propria determines bladder compliance and distention in response to increasing bladder volumes[4]. The bladder mucosa is arranged in numerous folds in the empty bladder, which disappear as the bladder fills with urine. The urothelial umbrella cells adjust their shape and become stretched and squamous. Simultaneous processes of exocytosis and endocytosis in umbrella cells provide additional cellular membrane surface area supporting the accommodation to increasing bladder volumes[5].

The detrusor muscle layer is located just below the lamina propria. The detrusor muscle is composed out of three layers of smooth muscle fibers running in longitudinal (inner and outer layers) and circular (middle layer) directions. The detrusor muscle remains relaxed and stretched, while maintaining a low constant muscle tone, during storage of urine in order to optimize the bladder capacity and contracts during micturition in order to expel urine from the bladder.

The serosa forms the outermost layer of the bladder and covers the detrusor muscle. The serosa consists out of a mesothelial membrane which secretes serous fluid and a connective tissue layer.

The structural and functional integrity of the bladder may be decreased in a number of bladder disorders, and damage to the urothelium is a likely contributor to the development or exacerbation of LUTS[6,7]. Several bladder elements may be negatively impacted by the aging process including mucosal, muscular, stromal and neural components, and can also involve surrounding structures such as the prostate in males[8,9].

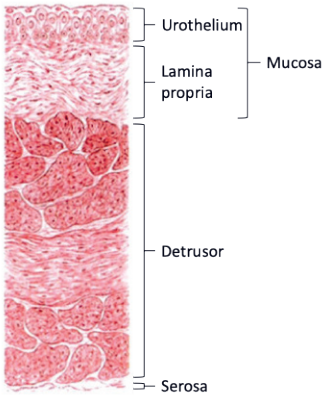

Figure 1: Overview of general bladder wall morphology. Adapted from [10].

PELVIC FLOOR CONTINENCE MECHANISMS

The urinary bladder is located in the pelvic cavity and rests on the muscular structures of the pelvic floor. The pelvic floor provides structural support for the bladder and urethra[11,12]. The main structures that provide support to the bladder and urethra are the endopelvic fascia, arcus tendineus fasciae pelvis, and the levator ani musculature[13] (Figure 2). The endopelvic fascia is a connective tissue network which surrounds the pelvic viscera. It supports the pelvic organs and fuses with the arcus tendineus fasciae pelvic, which in turn connects to the pelvic sidewalls, pubic bones, and the spine. The levator ani is a muscle group with bilateral components in the pelvic floor and are covered by the superior and inferior fascia of the levator ani. The iliococcygeus muscle portion of the levator ani is formed by a somewhat thin band of muscle fibers spanning from one pelvic sidewall to the other. The pubococcygeus portion is a hammock-like muscular

structure and stretches from the pubic bone to the coccyx[14]. The puborectalis portion forms a sling from the posterior side of the pubis on each side around the rectum and fuses with the external anal sphincter. The levator ani contains openings for the rectum, vagina (in the female), and urethra to pass through. These muscles provide essential structural support for the pelvic viscera. The levator ani muscles and their fascia together constitute the pelvic diaphragm.

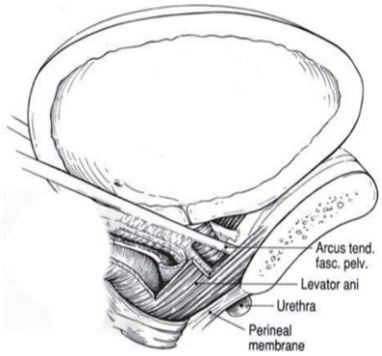

Figure 2: *Overview of the pelvic support system for the bladder and urethra. Adapted from[13].*

During daily activities the bladder is continuously exposed to fluctuations in abdominal pressure and the intravesical pressure (which is the sum of the abdominal pressure and the pressure resulting from the detrusor muscle tone) fluctuates as a result. In order to maintain continence during moments of high abdominal pressure, the pressure in the urethra has to rise to match the increase in intravesical pressure. The external urethral sphincter provides contractile activity, keeping the bladder outlet closed and preventing leakage. The urethra contains multiple layers of smooth muscle oriented in longitudinal (inner layer) and circular (outer layer) directions. The circular smooth muscle layer provides tonic activity constricting the urethral lumen during storage, while the longitudinal smooth muscle may help to open the lumen at the initiation of micturition. During episodes of high intra-abdominal pressure, the pelvic floor provides indispensable support mechanisms helping to maintain continence. The endopelvic fascia, arcus tendineus fasciae pelvis, and the levator ani musculature act as supportive structures against which the urethra is compressed and "kinks" during episodes of high abdominal pressure, resulting in an increase of urethral pressure and, subsequently preventing urine from leaking from the bladder[13,15].

If pelvic supportive structures are damaged or weakened by, for example, parturition, the endopelvic fascia and periurethral supportive tissue will not provide a stiff enough layer for optimal kinking of the urethra, and urethral closing pressures cannot overcome the increase in abdominal pressure. In this case, the pressure difference between the urethra and the bladder results in the involuntary loss of urine. This condition is termed stress urinary incontinence (SUI). Insufficient support from the supportive tissue elements is proposed to be reflected on

video urodynamic investigations in upward and downward movement of the urethra during coughing episodes[16]. This observation has been termed urethral hypermobility and has traditionally been regarded as an indicator of SUI[17,18], however some studies have indicated that the relationship between urethral mobility and SUI complaints are not as causally related to each other as has traditionally been assumed[19].

NEURAL INNERVATION OF THE LOWER URINARY TRACT

Storing urine and emptying the bladder is a multifaceted process that requires complex and coordinated activity, and integration of peripheral afferent and central efferent signals at different levels. The control of the lower urinary tract is hierarchically organized within the CNS and the peripheral nervous system (PNS)[20]. In healthy adults, the micturition reflex is controlled by dedicated areas at the level of the spinal cord (mainly Onuf's nucleus (ON)), the brain stem (e.g. pons and periaqueductal gray (PAG)), and higher cortical and subcortical areas (e.g. prefrontal cortex, anterior cingulate cortex, and insula).

The CNS exerts control over micturition by nerves connecting the bladder and the spinal cord. The pelvic nerves originate from the sacral spinal cord at the S2-S4 levels and carry parasympathetic information to the detrusor muscle of the bladder. Activation of the parasympathetic fibers of the pelvic nerves leads to contraction of the detrusor muscle, which promotes evacuation of urine. The bladder receives sympathetic innervation from the hypogastric nerves, which originate at T11-L2 levels in the spinal cord and promote relaxation of the detrusor muscle and contraction of the bladder neck and urethral sphincter muscles, in order to facilitate storage of urine. In addition to parasympathetic and sympathetic innervation, the bladder also receives somatic innervation through the pudendal nerve which originates in the spinal cord at S2-S3 and connects to the urethral sphincter muscles. The pudendal nerve fibers originate in the ON [21] and, by innervation from this nucleus, facilitate voluntary initiation and interruption of micturition.

The ON has been thought to be responsible for the contraction and relaxation of the external urethral sphincter - also called the urethral rhabdosphincter - and the pelvic floor muscles[22]. Both functions are fundamental in optimal execution of the bladder's storage and evacuation functions. The ON receives information from the pons and PAG in the brain stem regarding urine storage and the initiation of a micturition episode. Continuous activity by motor neurons in the ON facilitates the tonic muscle tonus in the external urethral sphincter that helps to maintain continence during the storage phase. The ON facilitates voluntary initiation of micturition by somatic innervation through the pudendal nerves, causing relaxation of the external urethral sphincter[23]. Generally, signals passing from the brain to the bladder and vice versa are relayed by nuclei in the spinal cord[24]. In pathophysiological conditions of the CNS, lesions to the sacral spinal cord -where the ON is located- frequently lead to dysfunctional detrusor-sphincter coordination. Particularly, an underactive bladder and or a hypotonic

sphincter can be observed. In the same manner it is likely, that peripheral pathology at the level of the bladder, through afferent signaling, influences the functioning of the ON via the PAG and pons. The relevance of the ON for both storage and evacuation of urine indicates its value as a reliable sensor on this gating mechanism. In addition to the role the CNS has in regulating bladder functioning there is also a reciprocal effect of the bladder on the CNS. This can be thought of as sensory afferent feedback, which can lead to activation in distinct areas in the central nervous system, such as the PAG[25-28].

The pons contains two areas that are primarily involved in bladder control. The first is the pontine storage center, which promotes the storage of urine, and the second is the pontine micturition center (PMC) - also known as Barrington's nucleus - which facilitates the initiation of micturition[29,30]. In SCI, the interruption of connections between the pons and ON can lead to a number of different voiding problems[31], like detrusor-sphincter-dyssynergia which is the main risk factor for vesicourethral reflux[32]. These pontine areas receive a large part of their information from the PAG[29]. The PAG is indicated to serve as a relay station projecting afferent information from the bladder to cortical and subcortical brain areas and as a gatekeeper projecting efferent information from these higher brain areas via the pontine storage and micturition centers to the ON and, ultimately, the bladder[28,33,34]. The PAG is a brain stem nucleus that is centrally located in the hierarchical system of lower urinary tract control. The PAG is organized in a symmetrical columnar fashion, and the ventrolateral PAG and dorsolateral PAG are indicated to be involved in the control of evacuation and storage of urine respectively[26,27,35-37]. The PAG is also an integral part of the emotional motor system and as such is highly involved in a wide array of processes related to nociception control, cardiovascular control, respiration, micturition, defecation, parturition, reproduction and many more[38]. Neuroimaging studies have consistently observed activity in the PAG related to bladder fullness and bladder sensations[39-42], and have underpinned the importance of the role of the PAG in the switching mechanism during the initiation and inhibition of micturition[43].

Among the subcortical structures that are involved in bladder control, the thalamus fulfills a monitoring role in processes of awareness and attention and may be involved in the control of what bladder-sensory information needs to reach the cortex for further processing. The hypothalamus is involved in emotional stress responses, and with respect to LUT control it has been proposed that direct connections from the hypothalamus to the PMC offer an extra level of control permitting micturition only when the environment is considered "safe"[44]. The limbic system is a network of nuclei involved in processing related to emotion, memory and sexual functioning. The main components of the limbic system are the amygdala and hippocampus. The limbic system has extensive connections with brain areas involved in LUT control and may be involved in unconscious monitoring of the emotional aspects and social acceptability of voiding. The insula is known as the sensory cortex of the autonomic nervous system and interoceptive information regarding the location and state of body is send via the PAG and thalamus to the insula. The insula helps to shift our attention to homeostatic processes when

needed. Regarding LUT control, the insula monitors levels of bladder fullness and brings this to our awareness once our bladder is full enough to start planning a bathroom visit. The prefrontal cortex evaluates all afferent information regarding bladder sensations and integrates this information with information regarding our environment in order to make a conscious decision about when to initiate micturition. The anterior cingulate cortex (ACC) functions as the autonomic motor cortex and the command to initiate micturition is relayed through the ACC to the PAG, PMC, and via motor neurons in ON ultimately to the LUT.

PHYSIOLOGY OF MICTURITION

The continuously ongoing process of storage and evacuation of urine is referred to as the micturition cycle. During the filling, or storage, phase of the micturition cycle the bladder slowly fills with urine while the its elastic properties allow the bladder to maintain a relatively constant low intravesical pressure. During the filling phase both the external urethral sphincter and the urethra are closed[45].

It has been proposed that reflex contractile activity of the bladder muscles, initiated by sensory stretch receptors in the bladder wall and regulated via the sacral spinal cord, start to periodically increase the intravesical pressure as the bladder volume approaches the functional capacity[46]. This reflex contractile activity has been investigated in animal models, predominantly in isolated bladders, and might be involved in motor sensory processes monitoring bladder volumes in humans as well. These periodical contractions are suggested to be very small in amplitude and current available methods lack the sufficient resolution to register these pressure changes in vivo in human participants. As the bladder continues to fill the, this reflex becomes progressively more powerful and the duration of episodes of sustained elevated pressures becomes longer[47]. More powerful reflex contractions require conscious signals from the brain to the external sphincter to inhibit urine evacuation. If the signal from the brain to the external sphincter is not sufficiently high, or even absent, reflex micturition will occur. When the prefrontal cortex makes the conscious decision to initiate a micturition episode it sends a "permission to initiate a micturition episode" signal down towards the bladder via subcortical, brain stem and spinal structures. In turn, somatic innervation via the pudendal nerve relaxes the external urethral sphincter, and parasympathetic signaling contracts the detrusor muscles and the urethra opens to facilitate the evacuation of urine from the bladder[45].

LOWER URINARY TRACT DYSFUNCTION

Lower urinary tract symptoms (LUTS) comprise complaints regarding storage and/or evacuation of urine. In an ageing society these problems are highly relevant and increasingly important. If involuntary loss of urine goes together with a decrease in mobility, as is often the case in aging, this can lead to a decrease in self-reliance. In addition, micturition disorders may be the cause of

high urinary frequency, multiple bathroom visits, nocturia, increased risk of falls and even overflow incontinence and is often accompanied by recurrent urinary tract infections, risk of urinary sepsis with hospital admissions and prolonged catheterization. A disturbed urinary control due to incontinence and/or micturition disorders are amongst the main reasons for elderly people to move to a nursing home. In a survey conducted in the Netherlands in 2017 participants were asked if they experienced any involuntary urine loss during the previous 6 months[48]. Participants' responses indicated that around 36.8% of people older than 18 years could answer this question with "yes" (for women this percentage was 49%, and for men 22.6%). This percentage increases with age and above 60 years of age the prevalence increased to 56.6% in women and 30.9% in men. These numbers provide an indication of the prevalence of involuntary urine loss in society at present, and may give a glimpse to what can be expected as our life expectancy increases. In the Netherlands in 2018, 518.000 people suffered from clinically relevant incontinence, the costs related to this amount to an expense of €256 million on an annual basis spread over incontinence materials (e.g. diapers, incontinence pads), pelvic floor physiotherapy, medications, and specialized medical care. The majority of these expenses are related to care for the symptoms (incontinence materials) rather than therapeutic interventions. The Dutch national institute for health and environment (RIVM) predicts that, by 2040, 743.000 people will suffer from incontinence. These numbers focus on the costs of patient care, but it is to be expected that costs for institutionalization to provide care for elderly people who need help with basic hygiene will be even higher.

Altered processing in the nervous system has been indicated to be related to LUTS[44,49,50], but current diagnostic and therapeutic approaches almost exclusively focus on the peripheral organ, and knowledge regarding predictors of treatment effectivity is lacking. Due to this approach, patients are often matched to a successful therapy based on a trial-and-error approach and often have to go through unsuccessful interventions before finding a therapy that alleviates their symptoms. A better understanding of the mechanisms underlying LUTS at the different levels of the nervous system, as well as at the local level in the bladder and the surrounding pelvic structures, is essential to improve effectivity of therapeutic interventions and the development of neuroimaging biomarkers of LUTS. A more complete understanding of the disease mechanisms associated with LUTS along the multiple levels of the bladder-brain axis will enable a shift towards more personalized diagnostic measurement-based treatments.

AIMS AND HYPOTHESES

LUTS can affect the structural and functional integrity of the systems responsible for healthy micturition at different, or multiple, levels along the bladder-brain axis (e.g. bladder wall, support structures, spinal cord, brain stem, higher brain areas). The interaction between these different components is currently poorly understood, and it is not clear what the effects of (mal)adaptive changes in the bladder are on central sensory and motor mechanisms. The specific aim of this

thesis is to investigate the different elements involved in LUT control, in healthy and pathological states, using both animal studies as well as in studies in human volunteers.

The investigation of changes at the local level of the bladder associated with LUTS is addressed in chapters 2, 3, and 4. In chapter 2 we have investigated the effects of aging on urothelial health in a rodent model and hypothesized that aging is associated with negative changes in urothelial health, resulting from processes of oxidative stress and an associated deterioration of mitochondrial health and bioenergetics. In chapter 3 we have used a stress model in rodents to assess the effects of chronic stress on vascular perfusion and angiogenesis in the bladder neck. We hypothesized that chronic exposure to stress increases levels of trophic factor expression in the bladder neck linked to angiogenesis. Chapter 4 focuses on the mechanisms of action underlying the alleviation of complaints related to SUI by intravesical implantation of a gas-filled balloon, both at the level of the bladder as well as at the level of the pelvic floor. We have analyzed video urodynamic data obtained from patients undergoing implantation of a gas-filled balloon. We hypothesize that the balloon will significantly reduce in size during coughing episodes, indicating that it absorbs some of the increase in intravesical pressure resulting from the coughing episode. Additionally, we hypothesized that the balloon significantly alters the shape of the bladder allowing for more optimal travel of the abdominal pressure through the pelvic floor and thereby improving the kinking capacities of the urethra.

Chapter 5 and 6 focus on the development of neuroimaging methods aiming to assess the functional organization and activity of the human PAG in unprecedented resolution. In chapter 5 we introduce a methodological approach that can be used to subdivide the PAG into distinct clusters based on resting-state functional magnetic resonance (fMRI) data. We hypothesize that clusters resulting from this approach will show consistency across different states of bladder fullness and subjectively reported bladder sensations, and will show a symmetrical lateralized organization corresponding to what has been observed in prior histochemical work in animals and post-mortem human tissue. Additionally, we hypothesize that the functional connectivity between PAG clusters will change significantly as a result of changes in bladder fullness and bladder sensations. This will enable us to develop personalized dynamic response profiles of PAG connectivity patterns related to bladder sensory processing. In chapter 6 we show that PAG clusters, as determined by the method introduced in chapter 5, show consistency at the group level.

A general discussion of our findings is presented in chapter 7 along with consideration for future scientific endeavors, and chapter 8 provides a concise summary of the main findings presented in this thesis.

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CHAPTER 2

Aging-associated changes in oxidative stress negatively impacts the urinary bladder urothelium

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ABSTRACT

Aims

Lower urinary tract symptoms are known to significantly increase with age, negatively impacting quality of life and self-reliance. The urothelium fulfills crucial tasks in serving as a barrier protecting the underlying bladder tissue from the harsh chemical composition of urine, and exhibits signaling properties via release of mediators within the bladder wall that affect bladder functioning. Aging is associated with detrimental changes to cellular health, in part by increasing oxidative stress in the bladder mucosa, and more specifically the urothelium. This, in turn, may impact urothelial mitochondrial health and bioenergetics.

Methods

We collected mucosal tissue samples from both young (3–4 months old) and aged (25–30 months old) rats. Tissue was evaluated for p21-Arc, nitrotyrosine and cytochrome C expression by western immunoblotting. Urothelial cells were cultured for single cell imaging to analyze basal levels of reactive oxygen species and mitochondrial membrane potential. Mitochondrial bioenergetics/cellular respiration were investigated by Seahorse assay and measurement of ATP release using the luciferin-luciferase assay.

Results

Aging is associated with a significant increase in biomarkers of cellular senescence, oxidative stress and basal levels of reactive oxygen species. Mitochondrial membrane potential was significantly decreased in urothelial cell cultures from aged animals, and cultures from aged animals showed a significant decrease in mitochondrial bioenergetics.

Conclusions

Aging-related increases in oxidative stress and excessive reactive oxygen species may be contributing factors underlying lower urinary tract symptoms in the older adult. The mechanisms outlined in this study could be utilized to identify novel pharmaceutical targets to improve aging-associated bladder dysfunctions.

INTRODUCTION

The prevalence of lower urinary tract symptoms (LUTS), characterized by problems regarding storage and/or voiding of urine, is known to significantly increase with age[1,2]. Multiple bladder components may become dysfunctional with age including mucosal, muscular, stromal and neural elements and can also involve surrounding structures (e.g. prostate)[3,4]. In an aging society LUTS are, therefore, highly relevant and increasingly important[5].

The inner surface of the bladder wall is covered with a mucosal membrane. This bladder mucosa consists of a multi-layered urothelium and underlying lamina propria. The urothelium is highly specialized and has important functions, including acting as a barrier to protect the underlying tissue from the harsh chemical composition of urine[6]. The urothelium furthermore fulfills essential roles in the execution of proper signaling processes in the lower urinary tract (LUT). Stress on the bladder wall induces the release of signaling molecules (e.g. adenosine triphosphate [ATP], acetylcholine, or nitric oxide) from the urothelium which influences activity of underlying detrusor muscle layers or afferent nerve fibers located in the lamina propria in close proximity to the urothelium[7-10]. The structural and functional integrity of the urothelium may be decreased in a number of bladder disorders, and damage to the urothelium is a likely contributor to the development or exacerbation of LUTS[11,12].

Healthy mitochondria are essential for the maintenance of proper urothelial function. Mitochondria, which are the primary source of oxidative stress, are particularly susceptible to age-associated abnormalities[13]. The increase in concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is assumed to be one of the underlying causes of the aging process[14]. Oxidative phosphorylation, which is the main mechanism underlying ATP synthesis, is negatively affected by the increase of ROS and RNS. Therefore, increased oxidative stress caused by the gradual accumulation of ROS and RNS is proposed to significantly impact healthy functioning of the LUT[5,16]. Previous research has indicated that there is a positive relationship between the expression of ROS in urothelial cells and aging in mice[17].

Although aging-related LUT dysfunction in rodents has been previously reported[18], the underlying mechanisms by which aging-related processes influence urothelial health and bladder functioning are not yet fully understood. In the current study we aimed to further elucidate aging-related changes in urothelial and mucosal biochemistry. We investigated biomarkers for cellular senescence and oxidative stress in mucosal tissue samples collected from young-mature and aged rodents, and assessed cellular respiration, mitochondrial function and basal ROS in urothelial cell (UTC) cultures. We hypothesized that aging is associated with augmented oxidative stress which, over time, may damage multiple components of the LUT system, leading to LUTS in the older adult.

METHODS

Animals and tissue collection

This study utilized female young, though mature (3–4 months old) and aged (25–30 months old) Fischer 344 rats ([F344] Charles River, Wilmington, MA, and the National Institute on Aging rodent colony). Animals were kept in standard housing with 12-hour light/dark cycles and ad libitum access to food and water. Mucosal tissue (separated from the detrusor muscle by blunt dissection) was obtained after sacrificing animals by exsanguination during isoflurane anesthesia.

Western immunoblotting

After surgically separating the urinary mucosa (defined as urothelium and lamina propria) from the smooth muscle, tissue was homogenized using Lysing Matrix D in a FastPrep 24 instrument (MP Biomedicals, Solon, OH) in HBSS (5 mM KCl, 0.3 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HCO_3, 0.3 mM Na_2HPO_4, 5.6 mM glucose, 10 mM Hepes, 1mM DL-Dithiothreitol, and 1mM EDTA, pH 7.4 containing complete protease inhibitor cocktail (1 tablet/10 ml, Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, 1:100). After centrifugation (16200g; 15 min at 4°C), we suspended the membrane pellets in lysis buffer containing 0.3 M NaCl, 50 mM Tris-HCl (pH 7.6) and 0.5% Triton X-100 and the same concentration of protease inhibitors as above in order to prepare the membrane protein fraction. We then incubated the suspensions on ice followed by centrifugation (16200g; 15 min at 4°C). The supernatants were combined and total protein concentrations were assessed using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). After denaturation (100°C for 5 min) with Laemmli sample buffer, lysate from each sample was separated on a 4-15% TGX Stain-Free SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA). Total protein for each sample was measured using Bio-Rad Stain Free SDS-PAGE gel technology as a loading control. UV-activated protein fluorescence was imaged on a ChemiDoc MP (Bio-Rad). Proteins were transferred to polyvinylidene fluoride membranes. We then incubated membranes in 5% (w/v) dried milk dissolved in TBS-T (20 mM Trizma, 137 mM NaCl, 0.1% Tween-20, pH 7.6). Membranes were washed with TBS-T, and left to incubate overnight at 4°C with primary antibodies (Synaptic Systems mouse monoclonal anti-p21-Arc antibody; 1:1000; 303 011; Germany [N = 14 young, 16 aged], Abcam Mouse anti-cytochrome C AB13575; 1:1000; RRID AB_300470; Cambridge, MA [N = 6 young, 7 aged] and Cell Signaling Rabbit anti-nitrotyrosine 9691; 1:1000; RRID AB_331634; Danvers, MA [N = 8 young, 11 aged]) diluted in TBS-T containing 5% (w/v) milk (p21-Arc, cytochrome C) or 5% (w/v) bovine serum albumin (BSA) (nitrotyrosine). Membranes were washed in in TBS-T, and incubated with secondary antibodies (sheep anti-mouse HRP (1:5000;

GE Amersham, Pittsburgh, PA) or donkey anti-rabbit HRP (1:10,000; Advansta, San Jose, CA) for 1 hour in 5% (w/v) milk. Membranes were then washed, incubated in WesternBright Quantum (Advansta, Menlo Park, CA) and imaged on a ChemiDoc MP (BioRad). The volume of each protein species was measured and normalized to total protein using Image Lab software (Bio-Rad).

Primary bladder UTC cultures

Bladders from both young and aged F344 rats were incubated in dispase (2.5 mg/mL; Worthington Biochemical, Lakewood, NJ) overnight at 4°C. UTCs were collected by gentle scraping, placed in trypsin (0.25%; 10-15 min, 37°C), dissociated and plated on collagen-coated glass coverslips at 0.3 × 106 cells/mL density. Preparations were used within 48-96 hours. Previous research has indicated that UTC cultures retain their structural and functional properties in short term primary cultures[8,19]. We verified epithelial nature of cultures using the epithelial cell marker cytokeratin 17, which stained >95% of cultured UTCs, comparable to previous results[20]. In all experiments reported here, each culture contained UTCs obtained from a single animal.

Reagents

We dissolved carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), oligomycin, and rotenone in DMSO at 100 mM stock solutions. ATP was dissolved in water at 10- or 100-mM stock solution. Reagents were diluted to working concentrations using HBSS or cell culture media.

Seahorse assay

UTCs were seeded (0.24×10^6 cells/mL) into the wells of an SF cell culture microplate and became ~90% confluent following 2-3 days in culture. Cells obtained from all animals ($n = 4$ rats per age group) were assayed simultaneously in 2 separate experiments using the Seahorse XF96 Extracellular Flux analyzer, which simultaneously measures two energy producing pathways in real time: mitochondrial respiration (oxygen consumption rate [OCR]) and glycolysis (extracellular acidification) in living cells. Once basal levels of energy production were established, cells were stimulated by exposure to three compounds that shift cellular bioenergetics: oligomycin, FCCP, and rotenone. Following the addition of 200 µL of protein lysis buffer, the BCA assay was performed to measure protein (results normalized to total protein content).

ATP release

Cultured urothelial cells (N = 3 rats per age group) were placed into a bath perfusion system set at a flow rate of 0.4ml/min and perfused with HBSS (5mM KCl, 0.3mM KH_2PO_4, 138mM NaCl, 4mM $NaHCO_3$, 0.3mM Na_2HPO_4, 5.6mM Glucose, 2mM $CaCl_2$, 1mM $MgCl_2$, and 10mM HEPES, pH 7.4, 300mOsm). A 100ul sample of perfusate was collected every 60secs and ATP measurements were calculated based on the luciferin-luciferase reaction using a standard curve (Adenosine Triphosphate Assay Kit, Millipore Sigma; Glomax 20/20 Luminometer, Promega, Madison, WI). Background release of ATP from the cultures was measured for 15-20 minutes. Cell swelling, used as a surrogate for cell stretch, was tested using a hypotonic stimulus (HBSS containing 69mM NaCl, 180mOsm) applied through the perfusion system for another 15-20 minutes. ATP Data were analyzed by calculating the area under the curve during each portion of the experiment and normalized by protein content in the cell culture.

Single cell imaging

Cultured UTCs (N = 4 rats per age group) were loaded with tetramethylrhodamine methyl ester (TMRM; 25 nM, 10 min, 37°C) (Thermo Fisher, Pittsburgh, PA) in order to estimate mitochondria membrane potential (Ψm), or dihydrorhodamine 123 (DHR123; 5 μM, 30 min, 37°C) (Thermo Fisher) to measure ROS. TMRM is a cell-permeant fluorescent dye sequestered by active mitochondria and intensity of the fluorescence is proportional to Ψm (i.e., lower fluorescence suggests depolarized Ψm; Ex/Em 544/574 nm). DHR123 is a cell-permeant ROS indicator localizing in mitochondria where it is oxidized to cationic rhodamine 123 and exhibits green fluorescence (Ex/Em 488/520 nm). Cells were imaged using an epifluorescence microscope (Olympus IX70; 40X oil objective) and HCImaging software (Hamamatsu Photonics, Bridgewater, NJ) with compounds applied using a gravity driven application pipette. For analysis, an area of interest was drawn around single cells and the average fluorescent intensity of pixels included in this area was taken as one measurement. Fluorescent intensity measured in an area without cells was subtracted to control for background. For TMRM and DHR123 imaging, pictures of 10-12 random areas per coverslip were taken using the same exposure settings for all coverslips. Data were quantified as fluorescence arbitrary units. For baseline ROS, cells were washed with HBSS and imaged as described above.

Statistics

Data were analyzed in GraphPad Prism 6 (GraphPad, La Jolla, CA). Statistical analysis was performed using Student's t test or a Welch's t test as applicable to compare young and aged animals. Asterisks in the figures indicate $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***).

RESULTS

We first characterized the presence of urothelial cellular senescence by use of a well-established senescence marker, p21-Arc, in mucosal samples. p21 acts as an inhibitor of the cell cycle and is significantly higher in mucosal tissue from aged rodents (Fig. 1A). The findings illustrated in Fig. 1B-C are consistent with biomarkers associated with apoptosis and risk for disease. For example, we found significant increases of cytochrome C in aged mucosal tissue (Fig 1B). Cytochrome C is present in the mitochondrial inner membrane and associated with cell death. We also found increased expression of nitrotyrosine in aged mucosa (Fig. 1C). Nitrotyrosine is considered an indicator of ROS, oxidative damage and apoptotic cell death. As further support for increased ROS and oxidative stress within the UT, we used a cell permeant reagent, DHR123, which is used to detect ROS, in isolated UTCs. DHR123 itself is nonfluorescent until oxidized to rhodamine 123 in mitochondria. Our findings revealed that basal production of ROS was significantly higher in UTC cultures from aged bladders compared to young animals ($p = \leq 0.001$, Fig. 1D).

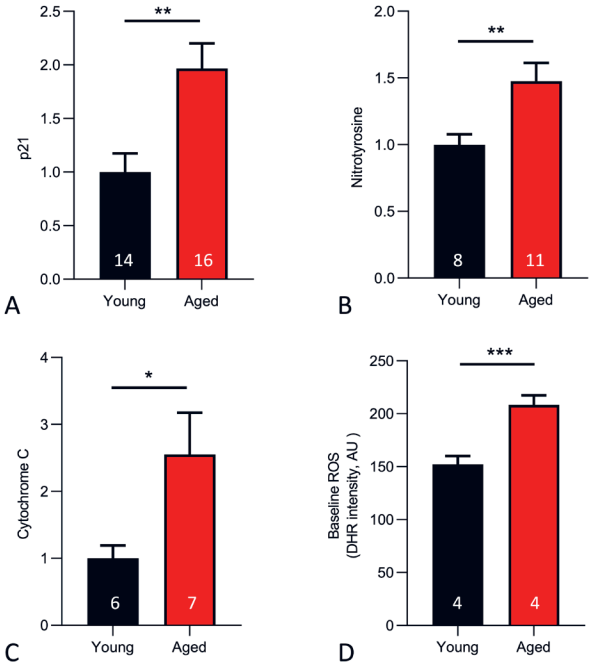


*Figure 1: Bar graphs depicting age associated changes in levels of **a)** p21-Arc $p = 0.003$ **b)** Nitrotyrosine $p = 0.006$ **c)** Cytochrome C $p = 0.04$ **d)** Baseline ROS $p = \leq 0.001$. Asterisks indicate $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***). N-numbers, indicating numbers of young versus aged rats per group, are shown in the respective bar graph.*

We then aimed to investigate the detrimental effects of increased oxidative stress and ROS production in UTCs from aged animals on mitochondrial health. Ψm is an essential component of energy storage and key indicator of mitochondrial activity. We used a cell-permeant dye, TMRM which accumulates in healthy, active mitochondrial with intact membrane potentials. Our findings revealed that TMRM signal intensity was significantly decreased in UTCs from aged bladders (indicating loss of membrane potential) compared to UTCs isolated from young bladders (Fig. 2).

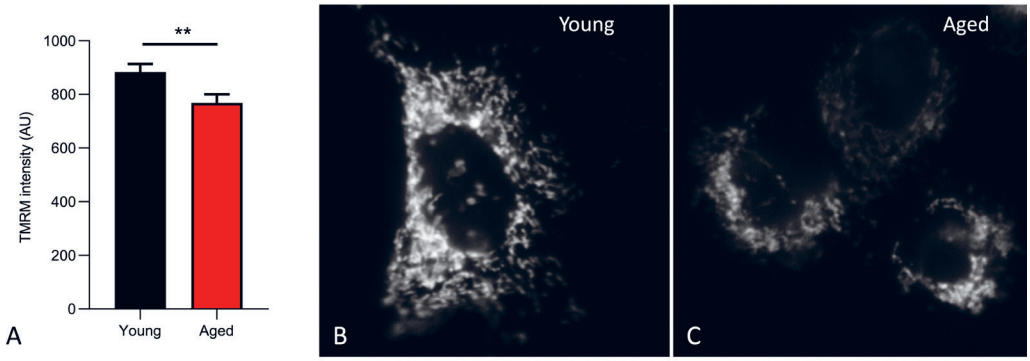
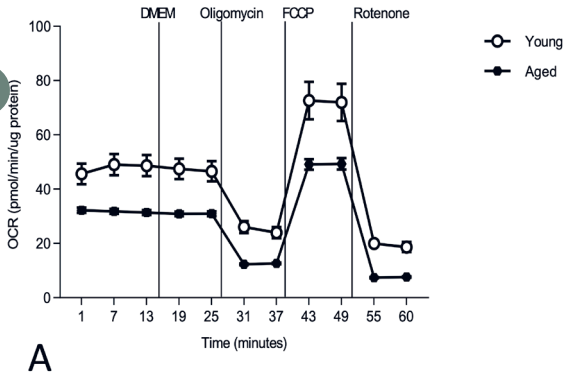
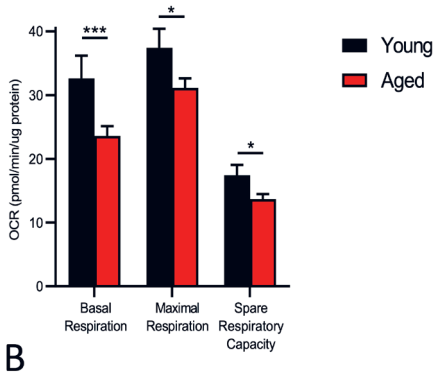


Figure 2: ***a)*** *Bar graphs depicting the TMRM intensity in cultured UTCs from young and aged rat bladders (N = 4 each). Ψm was found to differ significantly (p = 0.01) between age groups. Asterisks indicate p ≤ 0.05 (*), p ≤ 0.01 (**) or p ≤ 0.001 (***).* ***b & c)*** *Representative images of TMRM staining in cultured UTCs from young and aged animals.*

The Seahorse test is the gold standard for measuring mitochondrial function in a number of cell types and we used this method to assess the bioenergetic profile of young versus aged UTC mitochondria (Fig. 3A). After basal OCR was determined, oligomycin (2.5 μM), a complex V inhibitor, was administered to quantify OCR unrelated to ATP synthesis. The resulting OCR represents proton leak across the inner mitochondrial membrane. Next, UTCs were exposed to the uncoupler FCCP (0.7 μM) to stimulate maximal respiratory rate by separating oxygen consumption from ATP production. The resulting collapse of the Ψm triggers rapid consumption of energy and oxygen and was utilized to calculate the spare respiratory capacity of the cell, defined as the difference between maximal and basal respiration. Finally, the complex I inhibitor, rotenone (10 μM), was added to establish the non-mitochondrial OCR. UTCs from young-mature and aged rat bladders reacted to all pharmacologic compounds, indicating fully functional mitochondria and generating a measurable bioenergetic profile. The OCR measured at baseline (p = 0.001), maximal respiratory rate (p = 0.013), and the spare respiratory capacity (p = 0.017) all proved to be significantly decreased in aged animals when compared to young animals (Fig. 3B). These results are further supported by the significant decrease in hypotonic-evoked ATP release that was observed in aged UTCs compared to young-mature UTCs (p = 0.006 [Fig. 3C]).





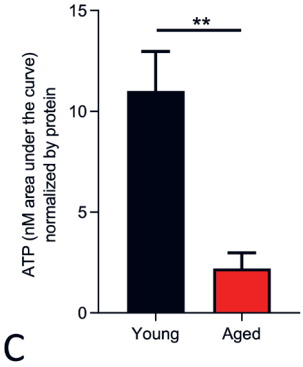


Figure 3: UTCs from aged rats showed altered mitochondria bioenergetics ***a)*** *Representative Seahorse trace measuring oxygen consumption in UTC from young (open circles) and aged (squares) rats. Trace shows basal respiration, the addition of DMEM as a vehicle control, oligomycin (2.5 μM), FCCP (0.7 μM), and rotenone (10 μM) (as described in the methods)* ***b)*** *Quantitation of data from two individual experiments (N = 4 rats/group) basal respiration p = 0.001, maximal respiratory rate p = 0.013, spare respiratory capacity p = 0.017* ***c)*** *Hypotonic evoked ATP release was significantly decreased in UTC from aged versus young rat bladders by 80%; N = 3 each, p = 0.006. Asterisks indicate p ≤ 0.05 (*), p ≤ 0.01 (**) or p ≤ 0.001 (***).*

DISCUSSION

Here, we examined how increased age in F344 rats alters urothelial health. Our findings revealed that aging negatively affects a number of biomarkers associated with cellular senescence, increased oxidative stress and mitochondrial bioenergetics. These types of detrimental changes over time could lead to defects in both the barrier and sensory aspects of urothelial functioning, culminating in abnormal bladder functioning and micturition behavior.

Mucosal samples from aged rats show a significant increase in p21-Arc, nitrotyrosine and cytochrome C when compared to those obtained from younger rats. P21-Arc has been proposed to be a useful biomarker for cellular senescence in rodent urothelial tissue[21,22]. As such, elevated levels of p21-Arc indicate aging-related phenotypical cellular changes such as: decreased responsiveness to growth-promoting stimuli, chromatin reorganization, and the secretion of a number of pro-inflammatory cytokines. Nitrotyrosine is a product of tyrosine nitration and its production is highly influenced by the accumulation of RNS. Thus, it is an indicator of oxidative stress, and often used as a marker of cell damage. As the terminal enzyme in the respiratory chain in mitochondria, cytochrome C is responsible for the storage of released energy and is thereby essential for the production of ATP. Outside the mitochondria cytochrome C is one of the main regulators of caspase activated apoptosis. Accumulation of ROS and RNS in the mitochondrion induces the release of cytochrome C into the cytosol where it binds with apoptotic protease activating factor-1 (Apaf-1), activating caspase-9 and thereby initiating the apoptotic pathway. Elevated levels of cytochrome C indicate high oxidative stress in mitochondria and are linked to a decrease in cell health and ATP production. The increase in both nitrotyrosine and cytochrome C in our study supports the view that the urothelium is significantly negatively impacted by chronic accumulation of ROS and RNS. Future studies are needed to assess the extent of apoptosis in aged animals, in part by investigating levels of caspase-9 or caspase-3 (caspase-9 is related to the initiation of the apoptotic pathway, whereas caspase-3 is involved in the execution phase of the apoptotic pathway). Previous research has reported significantly increased levels of caspase-3 in urothelial samples obtained from interstitial cystitis/painful bladder syndrome patients[23]. Apoptosis was present in samples from these patients and indicated to be mediated by the inflammatory pathway. The elevated levels of p21-Arc and cytochrome C reported here suggest that the secretion of pro-inflammatory cytokines might be elevated and apoptosis of urothelial cells might be occurring at an elevated rate. One could hypothesize that levels of caspase-9 and caspase-3 will be elevated in urothelial samples from aged animals. However, analysis of caspase-3 levels in samples from studies done in aged mice have reported no increase in apoptotic activity[17]. Thus, it is possible that cytochrome C levels increase in aged urothelial cells without execution of apoptosis. This would lead to an accumulation of damaged and unhealthy cells in the urothelium, negatively impacting bladder functioning. A better understanding of underlying mechanisms could improve bladder health and therapeutic options for the high prevalence of LUTS in older adults.

Our findings in aged bladder UTC cultures indicate a significant accumulation of basal ROS, confirming that, in agreement with our observations in mucosal tissue samples, urothelial cell

health is negatively impacted by aging. To assess the effects of oxidative stress on urothelial mitochondrial functioning we investigated the effects of age on the following: 1) Ψm using TMRM intensity measurements, 2) cellular respiration and mitochondrial bioenergetics using a seahorse assay, and 3) ATP release using a luciferin-luciferase assay. Since TMRM is a cell-permeant dye that accumulates in active mitochondria with intact membrane potentials it follows that in healthy cells with functioning mitochondria the signal intensity is bright. This is in contrast to aged UTC which show a decreased staining intensity, indicating depolarization or loss of resting mitochondrial membrane potential (Figure 2C) and suggests decreased cellular viability.

The Ψm drives a number of fundamental processes thus the observed age-associated Ψm decrease also implies a deterioration of cellular functions including cellular respiration and, thereby, ATP production. The bioenergetic profile of aged rats (studied using the seahorse method) suggests that the OCR (monitored in real time) is significantly decreased, suggesting that these cells have decreased their activity. It has been reported that a decrease of Ψm activates the Pink1/Parkin system[24]. This system is responsible for initiating signaling pathways related to the removal of damaged mitochondria. Work by our group has observed a significant aging-related increase in the expression of Parkin in the bladder wall, indicating an activation of this pathway in aged rats[25]. Decreases in Ψm are related to the induction of apoptotic processes[26]. Augmented expression of Dynamin-Related Protein 1 (DRP-1) in bladder tissue from aged rats[25] is an indicator of decreased Ψm and increased release of cytochrome C[27], which were both confirmed in the present study.

Based on the findings reported, we argue that the aging process has a substantial impact on urothelial health and that the mechanisms outlined may contribute to the deterioration of bladder morphology and bladder functioning, which may play a role in the high prevalence of LUTS in older adults[3-5]. A healthy urothelium is crucial for the maintenance of optimal barrier and signaling properties. We postulate that the observed decline of urothelial health and bioenergetic profile may alter the capacities of the urothelium to optimally execute both barrier and signaling functions. Previous work by our group has indicated that the administration of a purine nucleoside phosphorylase inhibitor, which can increase levels of 'uro-protective' precursors (e.g., inosine and guanosine) that reduce oxidative stress while simultaneously decreasing levels of 'uro-toxic' products (e.g., hypoxanthine) that are a source of ROS, significantly improves bladder functioning in aged rodents[25,28].

To conclude, in the present work we show that aging is associated with a significant increase in cellular senescence and oxidative stress in bladder mucosa samples, and that mitochondrial Ψm, mitochondrial OCR, and ATP release are significantly decreased in UTC cultures from aged bladders compared to that obtained from younger animals. We propose that these changes might significantly impact the barrier and signaling properties of the urothelium and potentially play an important role in the increased prevalence of LUTS in older adults. Future research should aim to further investigate the nature of the relationship between oxidative stress and bladder functioning with the potential to identify novel therapeutic targets which might improve the quality of life and self-reliance of many older adults suffering from LUTS.

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CHAPTER 3

Stress-induced changes in trophic factor expression in the rodent urinary bladder: possible links with angiogenesis

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ABSTRACT

Aims

There is substantive evidence supporting a role for chronic stress in the development, maintenance and even enhancement of functional bladder disorders such as interstitial cystitis/ bladder pain syndrome (IC/BPS). Increased urinary frequency and bladder hyperalgesia is reported in rodents exposed to a chronic stress paradigm. Here, we utilized a water avoidance stress (WAS) model in rodents to investigate the effect of chronic stress on vascular perfusion and angiogenesis.

Methods

Female Wistar-Kyoto rats were exposed to WAS for 10 consecutive days. Bladder neck tissues were analyzed by western immunoblot for vascular endothelial growth factor (VEGF) and nerve growth factor precursor (proNGF). Vascular perfusion was assessed by fluorescent microangiography followed by Hypoxyprobe to identify regions of tissue hypoxia.

Results

The expression of VEGF and proNGF was significantly increased in bladder neck mucosa from WAS rats. There was a trend toward increased vascular perfusion however this was not significantly different from control. WAS rats displayed a 1.6-fold increase in perfusion. Additionally, a greater abundance of vessels is observed in WAS, most notably in the microvasculature.

Conclusions

These findings show that chronic, psychological stress induces factors that can lead to increased microvasculature formation, in particular around the bladder neck, a region that contains most nociceptive bladder afferents. These findings may indicate a link between angiogenesis and other inflammatory factors that contribute to structural changes and pain in IC/BPS.

INTRODUCTION

The lower urinary tract has two main functions, namely, storing urine until the capacity of the bladder is reached and micturition once an appropriate time and place are found to empty the bladder. Proper execution of these functions is dependent on a delicate interplay between local and central processes which convey sensory information to our awareness and allow us to voluntarily initiate micturition. Lower urinary tract symptoms (LUTS) comprise bothersome problems experienced during storage and/or voiding of urine. LUTS are highly prevalent in the general population and have a major impact on patients' quality of life[1]. Clinical studies and population-based surveys have indicated that there is a strong relationship between LUTS and symptoms related to anxiety and depression, often manifesting as comorbid conditions[2,3]. This relationship appears to be of a bidirectional nature, and has been established for a variety of LUTS and syndromes including urinary frequency, overactive bladder and interstitial cystitis/bladder pain syndrome (IC/BPS)[4,5]. This paper aims to further elucidate the relationship between chronic psychological stress and IC/BPS.

IC/BPS is a characterized as a chronic condition presenting with chronic pelvic pain which is perceived to be localized to the bladder and is accompanied by storage dysfunction. The mechanisms underlying this condition are still largely unknown. However, chronic psychological stress and anxiety may initiate maladaptive changes in nociceptive pathways, ultimately resulting in stress-induced hyperalgesia and tactile allodynia[6]. Studies have indicated that increased stress is highly prevalent in IC/BPS patients and is associated with exacerbation of symptoms[7-11]. Therapeutic interventions aimed at improving psychological wellbeing are reported to additionally reduce IC/BPS symptoms[12,13]. It is likely that the stress-related exacerbation of IC/BPS is associated with maladaptive changes in nociceptive pathways.

Experimental models in which anxiety prone rodents are exposed to a chronic stress paradigm induce symptoms associated with IC/BPS, such as severe anxiety-related behavior, tactile allodynia in the suprapubic region, and a significant increase in voiding frequency[14-19]. A frequently used model to induce chronic psychological stress in studies investigating the relationship with lower urinary tract functioning is the water avoidance stress (WAS) paradigm. Previous studies utilizing WAS in rats to investigate IC/BPS associated changes in lower urinary tract functioning have indicated that exposure to WAS leads to visceral hypersensitivity during bladder filling and alterations in the central nervous system response[18].

IC/BPS is associated with increases in the expression of vascular endothelial growth factor (VEGF), an essential regulator of angiogenesis and vascular permeability crucial for maintenance of healthy vascular functioning[20]. The high expression of VEGF that can be observed in IC/BPS is associated with immature angiogenesis, leading to fragile and in some cases hemorrhagic-prone vessels, and levels of VEGF show a positive relationship with pain severity in patients suffering from IC/BPS[21,22]. VEGF is recognized as a survival factor for endothelial and other cell types[23], such as renal tubular epithelial cells[24]. It has been suggested that the increase in VEGF initially

acts a survival mechanism in response to IC/BPS, but can lead to detrimental effects, such as edema, and inflammation[25,26].

In addition to its role in the maintenance of vascular functioning research suggests that VEGF has an important role in neuroprotective processes, and reduced levels of VEGF may promote neuronal degeneration[27]. VEGF is proposed to exert its neuroplastic effects by playing a modulating role in nerve growth factor (NGF) signaling pathways, and VEGF inhibition has been shown to induce a decrease in the expression of proNGF, the precursor for NGF[28]. Urinary NGF levels have been indicated to show a direct association with IC/BPS symptom severity and treatment effectivity[29,30]. Next to its neuroplastic and neuroprotective effects proNGF, in turn, has a mediating role in the induction of angiogenic processes[31].

In the current study, we utilized WAS induced chronic stress in rats as a model for IC/BPS and have investigated levels of VEGF and proNGF along with assessment of vascular perfusion and angiogenesis in the region of the bladder neck. We hypothesized that exposing rats to a WAS paradigm induces an elevation of VEGF and proNGF levels in the bladder neck and will have an impact on vascular perfusion and angiogenesis.

METHODS

Animals

This study utilized young though adult female Wistar-Kyoto (WKY) rats (180-200 g) purchased from a commercial vendor (Charles River Laboratories International, Inc. Wilmington, MA, USA). This particular strain is genetically predisposed to elevated levels of anxiety[32] and has successfully been used to study stress-induced visceral hyperalgesia in earlier studie[17]. Rats were assigned to either WAS or control conditions at random. Animals were kept in standard housing with 12-hour light/dark cycles and ad libitum access to standard chow and water. The Institutional Animal Care and Use Committee (IACUC) approved all procedures which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Water avoidance stress paradigm

Rats were divided into WAS and control groups at random. The WAS paradigm utilized in this study was established[17] and hence used by the Multidisciplinary Approach to the Study Chronic Pelvic Pain (MAPP) research network[18]. In short, rats were placed on a pedestal (8 cm×8 cm×11.5 cm) in a water-filled container (1 hr/day for 10 consecutive days) and sacrificed on day 11. Controls were handled in the same way as the WAS rats but placed in a clean cage (1 hr/day for 10 consecutive days). All procedures for both groups were conducted in the morning to control for circadian effects. The animals were sacrificed by exsanguination during isoflurane anesthesia.

Western immunoblotting

Following removal, bladder tissue was placed in a sylgard-lined dissecting dish and cut open longitudinally in order to isolate the region around the bladder neck for further processing. After surgically separating the urinary mucosa (consisting of the urothelium and lamina propria) from the smooth muscle, tissue was homogenized using Lysing Matrix D in a FastPrep 24 instrument (MP Biomedicals, Solon, OH) in HBSS (5 mM KCl, 0.3 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HCO_3, 0.3 mM Na_2HPO_4, 5.6 mM glucose, 10 mM Hepes, 1mM DL-Dithiothreitol, and 1mM EDTA, pH 7.4) containing complete protease inhibitor cocktail (1 tablet/10 ml, Roche, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma, 1:100). After centrifugation (16200g; 15 min at 4°C), we suspended the membrane pellets in lysis buffer containing 0.3 M NaCl, 50 mM Tris-HCl (pH 7.6), 0.5% Triton X-100 and the same concentration of protease inhibitors as above in order to prepare the membrane protein fraction. We then incubated the suspensions on ice followed by centrifugation (16200g; 15 min at 4°C). The supernatants were combined, and total protein concentrations were assessed using the Pierce BCA protein assay (Thermo Scientific,

Rockford, IL). After denaturation (100°C for 5 min) in the presence of Laemmli sample buffer, lysate from each sample was separated on an Any kD TGX Stain-Free SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA). As a loading control, total protein per sample was determined using Bio-Rad Stain Free SDS-PAGE gel technology. UV-activated protein fluorescence was imaged on a ChemiDoc MP (Bio-Rad). After proteins were transferred to polyvinylidene fluoride membranes, the membranes were incubated in 5% (w/v) dried milk dissolved in tris buffered saline with tween (TBS-T) (20 mM Trizma, 137 mM NaCl, 0.1% Tween-20, pH 7.6), rinsed with TBS-T, and incubated overnight at 4°C with primary antibody VEGFA (AB46154, 1:1000 in 5% Milk, Abcam, Cambridge, MA [WAS N=10, control N=11]), proNGF (OSN00007G, 1:1000 in 5% Milk, Life Technologies, Carlsbad, CA [WAS N=6, control N=6]). After washing in TBS-T, the membranes were incubated with secondary antibody (Donkey anti-rabbit HRP, GE Healthcare, Marlborough, MA or goat anti-rabbit IgG HRP (Santa Cruz) for 1 hour in 5% (w/v) Milk TBS-T, washed, incubated in WesternBright Quantum (Advansta, Menlo Park, CA) and then imaged on a ChemiDoc MP (BioRad). A single immunoreactive band was observed for both VEGF (45kDa) and proNGF (54kDa). Optical density of each protein species was determined and normalized to total protein using Image Lab software (Bio-Rad).

Fluorescent microangiography and quantification of vessel diameter

Control and WAS rats were subjected to fluorescent microangiography (N=3 per group) as previously described[33,34]. In brief, deeply anesthetized rats were intra-cardiac injected with low temperature wax (1% low melting agarose gel; Sigma) containing fluorescent beads (5 ml of 1% FluoSpheres Carboxylate-Modified Microspheres 0.02 µm, Life Technologies). Following the infusion with low melting temperature wax and beads, the bladders were excised and placed on ice for 10 minutes prior to fixation with 4% paraformaldehyde.

Two hours prior to tissue harvest, injection of the gold standard immunohistochemical hypoxia marker-Hypoxyprobe (Pimonidazole HCl; Hypoxyprobe, Burlington, MA) was done in order visualize areas of tissue hypoxia. The bladders were then processed into frozen blocks, exhaustively sectioned and then imaged along with the fluorescent microbeads. These images were then quantitated using MATLAB as previously described[33,34] and high throughput analysis performed to assess regional changes in blood flow and vascular networks (e.g., micro vessel abundance and area).

Statistical analysis

Results are presented as mean ± SEM. Values for control vs. WAS were compared using two-tailed unpaired Student's t-Test for parametric distributed data or the Mann-Whitney test for non-parametric data sets, using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). P-values < 0.05 was considered statistically significant.

RESULTS

We first assessed whether VEGF protein expression was increased in bladder neck mucosa obtained from rats exposed to WAS compared to control using western immunoblotting. VEGF expression is known to be increased in IC/BPS and is involved in angiogenic processes. Bladder neck mucosa VEGF protein expression was significantly increased 1.8-fold in the WAS mucosa compared to control (80.81±10.6, N=11 vs 44.14 ± 7.96, N=10, p=0.0156) (Fig. 1.A).

Elevated levels of VEGF are associated with an increased expression of proNGF, which has been indicated to be involved in neuroplastic changes in cystitis and mediates proangiogenic processes. Western blot analysis indicated that levels of proNGF were significantly increased 2.6-fold in bladder neck mucosa from WAS animals compared to control (3.38±0.47, N=6 vs 1.28±0.16, N=6, p=0.0017) (Fig 1.B).

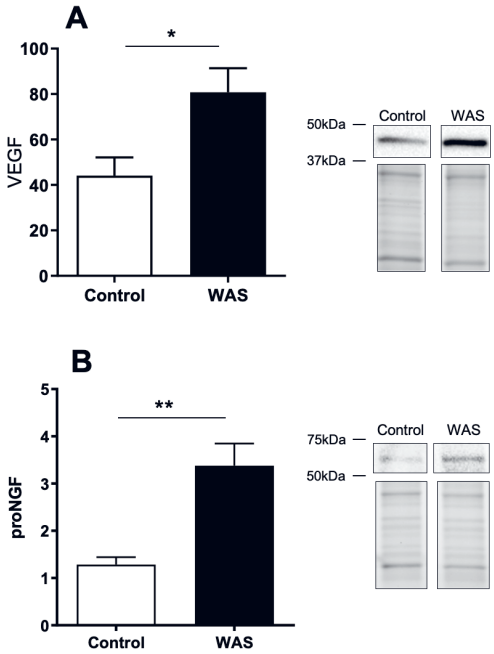

*Figure 1: Bar graphs depicting WAS associated changes in levels of **A)** VEGF p = 0.016 (WAS: 80.81±10.6, N=11, control: 44.14 ± 7.96, N=10) **B)** proNGF p = 0.002 (WAS: 3.38±0.47, N=6, control: 1.28±0.16, N=6). Western blot expression bands and representative total protein images are included. Asterisks indicate p ≤ 0.05 (*) or p ≤ 0.01 (**).*

We then perfused the urinary bladder of control and WAS rats with low melting temperature agar containing fluorescent beads. There was a trend toward increased vasculature perfusion in WAS rat bladders (Fig. 2.A, green depicts regions of perfusion throughout the bladder neck mucosa/submucosa, N=3 per group). Perfusion efficiency was calculated by measuring total blood vessel area, defined as the area containing fluorescent beads. This region was then expressed as a percentage of total tissue area. WAS rats showed a 1.6 fold increase in vascular perfusion. Representative micrographs from control and WAS bladder are shown in fig. 2.A. Quantification analysis showed a variable distribution in WAS bladder compared to a narrow distribution in control bladder. The calculated perfusion ratio in bladders from WAS (4.42±1.12, N=3) and control (2.78±0.16, N=3) animals did not significantly differ (p=0.22) (Fig. 2.B). Though genes implicated in the regulation of angiogenesis such as VEGF are known to be regulated by hypoxia, we find no obvious signs of tissue hypoxia in either control or WAS bladders, as the intensity of staining of pimonidazole adducts was similar in both WAS and control bladder tissue. We also observe a greater abundance of vessels in WAS (in particular the microvasculature).

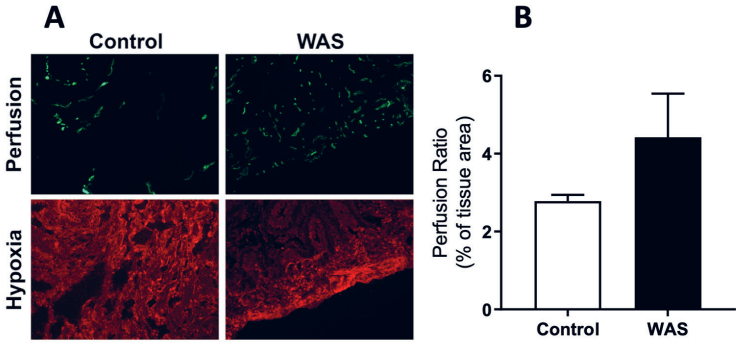

Figure 2: A) *Representative micrographs from control and WAS bladder regarding tissue perfusion and hypoxia are shown. Green depicts regions of perfusion throughout the bladder neck mucosa/submucosa, N=3 per group. An increase in perfusion. In WAS rats can be noticed. No obvious signs of tissue hypoxia in either control or WAS bladders can be observed, as the intensity of staining of pimonidazole adducts was similar in both WAS and control bladder tissue.* ***B)*** *WAS rats showed a 1.6 fold increase in vascular perfusion. The calculated perfusion ratio in bladders from WAS (4.42±1.12, N=3) and control (2.78±0.16, N=3) animals did not significantly differ (p=0.22).*

DISCUSSION

Here, we examined the effects of a chronic psychological stress paradigm to induce symptoms related to IC/BPS on mucosal biochemistry in the bladder neck region. We provide support to the notion that exposure to a chronic psychological stress changes bladder health at a structural level. Markers related to angiogenesis, inflammatory, and neuroprotective processes were significantly increased in WAS rats. The reported exacerbated expression of these factors, over time, could lead to dysfunctional alterations in nociceptive pathways and contribute to the clinical phenotype of IC/BPS.

Mucosal samples obtained from the bladder neck region of rats exposed to a WAS paradigm show a significantly increased expression of VEGF. An overexpression of VEGF in WAS rats indicates initiation of angiogenic processes in the bladder neck. This culminates in immature blood vessels which are vulnerable to become leaky and potential regions of ischemia, and is an important factor involved in the development of edema and inflammation regularly seen in IC/BPS[25,35]. VEGF has been shown to be involved in bladder wall permeability and a deterioration of the barrier functions of the urothelium[36,37]. A deterioration of the barrier functions of the urothelium will allow urea and toxins present in the harsh chemical composition of urine to pass into underlying layers of the bladder and has been linked to sensations of urgency and pain during the filling phase as well as urinary frequency[38]. VEGF is involved in neuroplastic changes in IC/BPS related to hypersensitivity in response to noxious stimuli and, interestingly, evidence indicates that cystitis induced neuroplasticity in the bladder can be prevented by administration of VEGF neutralizing antibodies[39], showing a direct modulatory effect of VEGF on the peripheral nervous system. Targeting VEGF expression by administration of anti-VEGF-neutralizing antibodies has, additionally, been shown to significantly reduce the nociceptive response to a model of acute cystitis[40]. Because of its established expression in IC/BPS and correlation to symptom severity[21], the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) Research Network suggests that VEGF might serve as a useful biomarker to diagnose presence and potentially severity of IC/BPS in bladder biopsies[22].

An important role for neurotrophins, such as NGF, as versatile signaling molecules in allostatic adaptations to exposure to stressors has been established[41]. In the central nervous system expression of neurotrophins is significantly altered after exposure to stressors. This initiates neurobiological changes that are associated with the pathophysiology of affective disorders. In particular in the hypothalamic-pituitary-adrenal axis maladaptive changes can be observed after exposure to stress inducing stimuli. Furthermore, NGF has been indicated to play an essential role in nociceptive processing[42]. Bladder neck mucosa in WAS rats shows a significant increase in the expression of proNGF as measured by western blot analysis. These results are in agreement with previously reported increases in NGF (the mature form of proNGF) in patients with IC/BPS[30,43]. The promotion of neuronal survival and differentiation by NGF is carried out by binding to Tropomyosin receptor kinase A (TrkA) and p75 neurotrophin receptor

(p75ntr). A delicate balance of NGF binding to both TrkA and p75ntr receptors is required for neuroprotective and differentiation related activity. Binding to p75ntr receptors in the absence of binding to TrkA induces processes of apoptosis and axonal pruning[44]. ProNGF can, by selective binding to p75ntr but not TrkA, evoke apoptotic processes[45,46]. Given the deterioration of urothelial health in IC/BPS[47] the accumulation of proNGF, and associated onset of apoptotic processes, is likely to negatively impact the restorative capacities of the urothelium resulting in a further decline of lower urinary tract health and potentially an exacerbation of IC/BPS related symptoms.

NGF is a notable mast cell activator and accumulation of NGF could play a key role in reported mast cell activation in IC/BPS[48]. IC/BPS is known as a chronic inflammatory disease and increased mast cell activation has been postulated to play a role in the onset and progression of the disease[49,50]. Mediators secreted by mast cells may explain symptoms of IC/BPS related to inflammatory processes, angiogenesis and neuronal hyperexcitability in the bladder wall. Mast cell activation in the bladder has been reported in rodents exposed to a 10 day WAS protocol[17], and the increased release of VEGF in an acute stress paradigm has, interestingly, been indicated to be regulated through a mast cell dependent process[37].

The development of blood vessels and nerves share many common signaling processes and pathways[51] and studies on retinal tissue suggest that proNGF is likely to be involved in angiogenic and inflammatory processes[31,52]. Studies indicate that proNGF and NGF initiate angiogenic processes via TrkA binding and downstream signaling pathways like PI3K and ERK[53]. Inflammatory processes in IC/BPS may culminate in increased vascular perfusion and permeability which is associated with peripheral edema[54].

Though not statistically significant we report a trend towards an increase in vascular perfusion in WAS rats, and the microvasculature appears to be increased in WAS rats. These changes are likely due to the direct and indirect effects of the increase in expression of VEGF and proNGF on angiogenic processes and vascular permeability. Quantification of vascular perfusion in the bladder neck is methodologically challenging and larger N-numbers might be required to accurately reflect potential changes in perfusion related to IC/BPS in this animal model. The observed vascular increases, in particular in the microvasculature, could indicate presence of immature blood vessels. Immature angiogenesis, leading to fragile vessels and potential areas of ischemia, has previously been reported in IC/BPS and is linked to an increase of VEGF expression[21,39]. Previous research has suggested that the increase of VEGF expression in IC/BPS might be caused by hypoxia in the bladder[55,56], but in our data we did not find evidence of hypoxia in WAS rats. This might indicate that NGF induced mast cell activation plays a more prominent role in the increased release of VEGF[37].

To conclude, in the present work we show that exposing rats to a 10 day WAS protocol is associated with a significant increase in the expression of VEGF and proNGF in the bladder neck mucosa, induces a trend towards an increase in vascular perfusion, and shows an increase in the abundance of vessels (particularly in the microvasculature). We propose that these detrimental

structural changes in the lower urinary tract negatively impact overall lower urinary tract health, as well as urothelial signaling and barrier functions, and play an important role in the clinical phenotype seen in IC/BPS. The current study has solely focused on changes in the bladder neck associated with IC/BPS. More work is needed to determine whether the observed changes occur in the complete bladder or are isolated to the bladder neck. Future research should aim to assess the effects of duration of WAS exposure on these outcome measure, integrate assessment of tissue markers associated with psychological stress, and to further investigate the relationships between VEGF and proNGF expression and processes related to angiogenesis, neuroplasticity and apoptosis with the potential of identifying and improving biomarkers of IC/BPS and further uncovering mechanisms that can be utilized to improve therapeutic options for IC/BPS patients.

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CHAPTER 4

Mechanisms of action of an intravesical balloon as a therapy for stress urinary incontinence

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Under review

ABSTRACT

Aims

Previous studies have indicated that the intravesical implantation of an air-filled balloon alleviates much of the symptoms caused by stress urinary incontinence (SUI) in women. However, the exact working mechanisms behind this therapy are not yet fully understood. The current study aims to elucidate the potential physiological mechanisms underlying this minimally invasive intervention.

Methods

We have evaluated video urodynamic data in women undergoing this therapy ($n = 5$), during which participants were asked to cough with increasing intensity. For each participant, we have videos before insertion of the balloon and one week following insertion. We identified a frame in a resting situation in which the maximum horizontal and vertical bladder dimensions were measured. We expressed the maximum vertical diameter as a ratio of the maximum horizontal diameter. We then used custom-written scripts to identify the bladder and balloon in each frame of the video urodynamic investigation and subtracted information regarding the location of the bladder neck and diameters of the balloon. We then used this information to plot the displacement of the bladder neck and size of the balloon during coughing.

Results

The diameters of the balloon were significantly decreased during coughing ($p \leq 0.05$). We found a significant increase of the maximum vertical diameter expressed as a ratio of the maximum horizontal diameter before and after insertion of the intravesical balloon ($p \leq 0.05$). The maximum displacement of the caudal bladder limit increased significantly after implantation of the intravesical balloon was ($p \leq 0.05$).

Conclusions

Our results imply that the balloon compresses in response to increases in abdominal pressure, and the bladder obtains a significantly more vertically oriented shape after implantation of the balloon. Moreover, it appears that implantation of the balloon significantly increases the mobility of the bladder neck. The balloon is indicated to absorb some of the increases in intravesical pressure during episodes of high abdominal pressure. We propose that the balloon inwardly pushes the bladder wall upwards, causing the organ to acquire a more vertically oriented shape. Additionally, we postulate that this change in bladder shape will increase the mobility of the bladder neck, thereby increasing the kinking capability of the urethra.

INTRODUCTION

Stress urinary incontinence (SUI) is a highly prevalent condition in women and considerably impacts the patients' quality of life[1]. SUI is characterized by involuntary urine loss during episodes of high abdominal pressure such as coughing, sneezing, or physical exertion[2]. In healthy individuals, continence is maintained by synergic coordination of low intravesical pressure, achieved by relaxation of the detrusor smooth muscle, and simultaneous sufficiently high resting pressure in the urethra, compared to the intravesical pressure, to prevent leakage of urine from the bladder. During episodes of high abdominal pressure, the urethral pressure has to rise to match the increase in intravesical pressure resulting from the abdomen's force on the bladder. Several mechanisms are proposed to contribute to maintaining continence during these episodes. A widely accepted hypothesis suggests that the endopelvic fascia and the urethro-pubic ligaments provide a supportive structure against which the urethra rests[3-5]. During increases in abdominal pressure, the urethra compresses and 'kinks' on this supportive layer, closing the urethral lumen and increasing the urethral pressure equivalent to abdominal pressure increment. Together with the muscular activity of the external urethral sphincter, this mechanism prevents the involuntary loss of urine. If pelvic supportive structures are damaged or weakened by, for example, parturition, the endopelvic fascia will not provide a stiff enough layer for optimal kinking of the urethra, and urethral closing pressures cannot overcome the increase in abdominal pressure. In this case, the pressure difference between the urethra and the bladder results in the involuntary loss of urine. Insufficient support from the endopelvic fascia is proposed to be reflected on video urodynamic investigations in upward and downward movement of the urethra during coughing episodes[6]. This observation has been termed urethral hypermobility and has traditionally been regarded as an indicator of SUI[7,8], however some studies have indicated that the relationship between urethral mobility and SUI complaints are not as causally related to each other as has traditionally been assumed[9].

We propose that the kinking capabilities of the urethra are, to some extent, dependent on the mobility of the bladder neck. In order for the urethra to successfully kink on the endopelvic fascia, displacement of the bladder neck and proximal urethra is essential. The kinking mechanism is assumed to serve as a supplementary closure mechanism supporting the urethral sphincter in compensating for increases in abdominal pressure. If the shape of the bladder limits movement of the bladder neck and urethra, as might be the case for a bladder that lies flat on the pelvic floor, kinking capacities of the urethra may be decreased, and the efficiency of indirect transfer of abdominal pressure to the urethra may be limited. Therefore, we expect that displacement of the bladder neck during coughing episodes could indicate successful kinking of the urethra, and increased bladder neck mobility in SUI patients with limited bladder neck mobility might consequently be beneficial in alleviating complaints.

A novel therapeutic option for SUI patients utilizes the insertion of a small air-filled balloon into the bladder transurethrally. The air-filled balloon is proposed to serve as an efficient and

minimally invasive treatment option for patients. Since gasses compress more easily than liquids, the balloon has been proposed to act as an absorber for increases in intravesical pressure in response to an increase in intra-abdominal pressure. It is fabricated as a free-floating biocompatible medical-grade polyurethane balloon and is implanted through the urethra without the need for anesthesia. After the balloon is inserted into the bladder and filled with 30 mL of air and 0.5 mL of a perfluorocarbon liquid that prevents the balloon from deflating, buoyant forces cause the balloon to float to the top of the bladder dome. The device successfully prevents the loss of urine during episodes of high intra-abdominal pressure. Previous clinical studies have investigated the use of this intravesical balloon to alleviate symptoms of SUI and have confirmed its therapeutic effectiveness[10-13].

We hypothesize that during coughing episodes the size and volume of the balloon will decrease as a result of successful transfer of abdominal pressure to the air-filled balloon. Additionally, we investigated the hypothesis that the buoyancy of the intravesical balloon will inwardly push the bladder wall upwards, causing the organ to acquire a more vertically oriented shape. This may result in a decreased exposure of the bladder dome to downward pressures and a more efficient exertion of intra-abdominal pressure to the bladder neck region. Additionally, we propose that this change in bladder shape may increase bladder neck mobility. We suggest that these mechanisms will aid the region around the bladder neck and proximal urethra in successfully kinking and, thereby, preventing the loss of urine during episodes of high intra-abdominal pressure. Cumulatively, these processes may cause a reduction in incontinence episodes or volume due to absorbance of the increase in intra-vesical pressure by the balloon and improved kinking capacities of the urethra resulting in a more successful closing of the bladder.

METHODS

Participants

The current study was approved by the local ethics committee and was designed and conducted in line with the agreements stated in the Declaration of Helsinki. 10 female patients were included in the study after obtaining written informed consent (mean age: 55.6, SD 7.8). In data of 6 patients, we assessed bladder shape before and after implantation of the balloon as well as measurement of changes in size of the balloon during coughing (4 patients had missing data due to technical difficulties). Data of 5 patients could be evaluated for bladder neck displacement before and after placement of the balloon (one more patient had to be excluded because of missing data for this analysis). Patients experienced predominant SUI, as confirmed by visual assessment during Valsalva maneuvers, for at least 12 months before inclusion and failed prior noninvasive treatment (e.g. behavioral modification, bladder training exercises, pelvic muscle rehabilitation, biofeedback, electrical stimulation, or drug therapy).

Study design

After obtaining informed consent, participants were asked to fill out a 3-day micturition diary prior to insertion of the balloon and following placement of the balloon. The enrollment visit consisted of a video urodynamic investigation at a sampling rate of 20 frames per second before placement of the balloon while participants were exposed to episodes of high intra-abdominal pressure using Valsalva maneuvers and coughing episodes during bladder filling with 100ml of saline with a contrast medium. The intravesical balloon was then placed according to a previously described standardized procedure[13] and filled with 30 mL of air and 0.5 mL of a perfluorocarbon liquid that prevents the balloon from deflating. Participants were invited for a follow-up visit approximately 7-10 days after balloon placement and underwent a video urodynamic investigation using the same protocol as during the enrollment visit. None of the participants experienced involuntary loss of urine during the analyzed coughing episodes.

Data analysis

We designed a custom MATLAB (MathWorks, R2018b) script, which enabled us to subtract information regarding the size of the balloon in an automated fashion. Due to the air present in the balloon it can be distinguished from the surrounding structures and tissues based on its bright appearance in the grayscale images. After optimizing the contrast of each frame of the video urodynamic assessment, we manually defined a cut-off gray value that would allow for the most optimal differentiation between the balloon and the rest of the image. Images were then converted to binary images where each pixel above our threshold was assigned the value 1

(white) and each other pixel 0 (black). The largest continuous object consisting of white pixels was considered to correspond to the balloon and was selected for further processing. The coordinates of the edge extremities (Fig. 1.A) of this object were computed and plotted over the original image grayscale image. Correspondence of the computed dimensions of the balloon with the original image were then visually verified by the researchers. The diameters of the balloon along the mediolateral axis (horizontal, or x direction) and cranio-caudal axis (vertical, or y direction) were then used for frame-by-frame computing of the size of an ellipse corresponding to the size of the balloon. The difference between the maximal (rest) and minimal (high intra-abdominal pressure) size of the computed ellipse during the video urodynamic assessment was statistically assessed using an SPSS 26 (IBM, 2019) implementation of the Wilcoxon Signed-Rank non-parametric test. Since the abdominal pressure forces emerging from the coughing episodes travel in a cranio-caudal fashion we expect the dimensions of the balloon to mainly be affected in the vertical (y) direction. We tested this by statistically assessing the difference between the maximal and minimal diameters of the balloon along the y direction using a Wilcoxon Signed-Rank test.

Next, for each video urodynamic study, the maximum diameters of the bladder along the mediolateral (horizontal) and cranio-caudal (vertical) directions were measured manually in a selected frame during resting (i.e. no active exertion of stress on the abdomen by coughing or Valsalva). The maximum measured vertical diameter was expressed as a ratio of the maximum horizontal diameter. Before and after implantation ratios were subsequently statistically compared using a Wilcoxon Signed-Rank test.

We then used a custom-written ImageJ macro script[14] to identify the bladder outline in each frame of the video urodynamic investigation in an automated computerized approach based on the recognition of contrast differences between the bladder and surrounding tissue induced by the infusion of the contrast medium. We extracted information regarding the location of the caudal limit of the bladder and computed frame-by-frame displacement of this location during intra-abdominal pressure increases. The location identified in this approach as the lower most caudal bladder limit, which contained contrast medium, is assumed to represent the bladder neck. For each urodynamic investigation, we used RStudio[15] to normalize the displacement values by subtracting the mean location. The normalized displacement of the bladder neck during coughing episodes at 100 ml infused volume was plotted to identify the maximum displacement. Before and after balloon placement, displacement values of the bladder neck were then statistically compared using a Wilcoxon Signed-Rank Test.

In addition, using the micturition diaries, we evaluated participants' daily number of episodes of involuntary urine loss and daily pad use before and after balloon placement using a Wilcoxon Signed-Rank non-parametric test.

RESULTS

We first assessed whether the implanted air-filled intravesical balloon decreased in size in response to increases in abdominal pressure emerging from coughing. The surface area of an ellipse based on the dimensions of the balloon in the frontal plane was found to significantly decrease in size during coughing episodes (p = 0.028, mean decrease = 9.26%, SD: 2.44) (Fig. 1.B). Because abdominal pressure forces due to coughing mainly travel in cranio-caudal (vertical) direction we, additionally, assessed changes in the diameter of the balloon along the vertical axis and found a significant decrease of the diameter of the balloon during coughing (p = 0.028, mean decrease = 11.5%, SD: 3.61, n = 6) (Fig. 1.B).

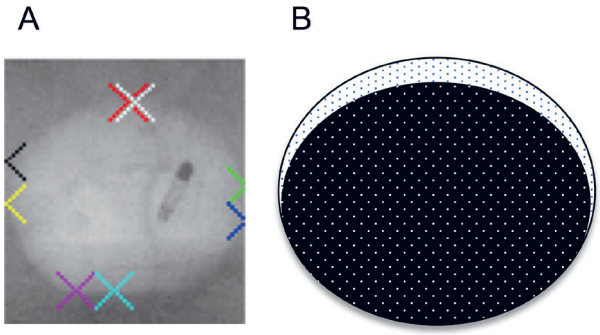

Figure 1: A) *Example of a projection of the extremities of the balloon as determined by the script projected over the original grayscale image.* ***B)*** *Illustration of the change in size and cranio-caudal diameter of the intravesical balloon as seen in the frontal plane. Maximal size is indicated by the dotted pattern and minimal size is indicated by the dark patterned area. The surface area of the ellipse based on diameters of the balloon along the mediolateral and cranio-caudal axis significantly decreased during coughing (p = 0.028). Mean decrease = 9.26%, SD = 2.44, mean minimal ellipse = 3485.34 pixels, SD = 1558.53, mean maximal ellipse = 3945.58 pixels, SD = 1804.63. Additionally, the decrease of the cranio-caudal diameter of the balloon also proved to be significant (p = 0.028). Mean decrease = 11.5%, SD = 3.61, mean minimal vertical diameter = 59 pixels, SD = 12.92, mean maximal vertical diameter = 65 pixels, SD = 13.93.*

The computed ratios of the maximum vertical bladder diameter expressed as a ratio of the maximum horizontal diameter before implantation of the intravesical balloon had a mean of 92.637 (SD: 30.24) and ratios one week following implantation had a mean of 136.605 (SD: 39.784). The difference between the maximum vertical diameter expressed as a ratio of the maximum horizontal diameter before and after implantation after the intravesical balloon proved to be statistically significant (p = 0.028, n = 6). Showing that the bladder acquired a significantly more vertically oriented shape after implantation of the intravesical balloon (Fig. 2.A, B).

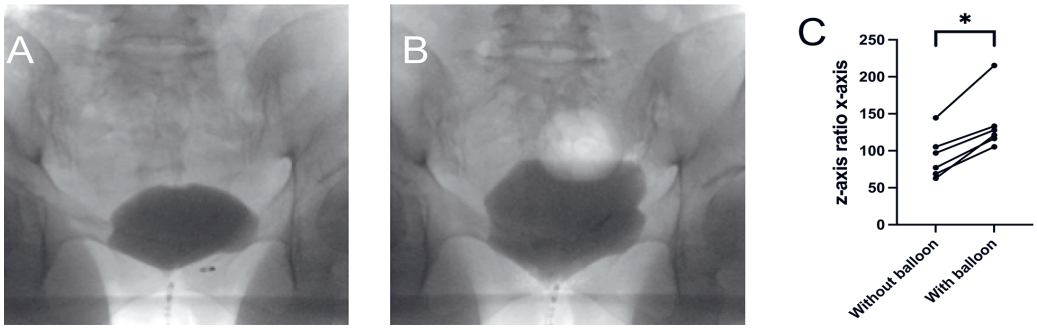

Figure 2: A) *Representative example of bladder shape in frontal view after infusion of 100ml of saline before implantation and* ***B)*** *after implantation of the intravesical balloon. Both images originate from the same patient.* ***C)*** *Vertical bladder diameter expressed as a ratio of the maximum horizontal diameter before implantation of the intravesical balloon had a mean of 92.637 (SD = 30.24) and was significantly lower than ratios one week following implantation (mean = 136.605, SD = 39.784) (p = 0.028).*

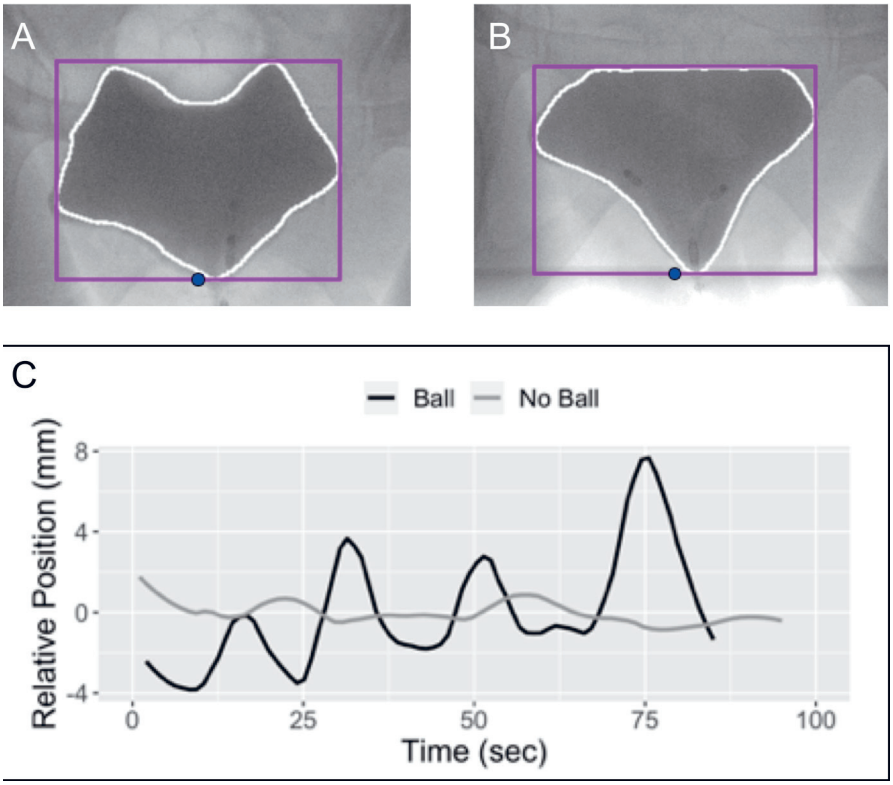

Figure 3: A) Representative examples of bladder neck landmarks identified by the script before implantation of the intravesical balloon. The bladder was automatically traced (white) and the 'bounding box' of the bladder region was identified (magenta). The location of the caudal bladder limit was taken to be the lower boundary position of the bounding box, indicated with the blue dot. ***B)*** *Representative sample of bladder dimensions measured after implantation of the intravesical balloon.* ***C)*** *Displacement of the caudal bladder limit during coughs, at a bladder volume of 100ml, was significantly increased after implantation of the intravesical balloon (p = 0.034). Mean displacement without balloon = 7.56 mm, SD = 7.18, mean displacement with balloon = 14.48 mm, SD = 11.48. Representative trace illustrating the normalized frame-by-frame displacement of the bladder neck region during coughing episodes with (black) and without (grey) the intravesical balloon.*

The mean normalized maximum downward displacement of the caudal bladder limit during coughing was 7.56 mm before placement of the intravesical balloon and 14.48 mm one week following balloon placement (n = 5). The difference between the maximum displacement of the caudal bladder limit before and after implantation of the intravesical balloon was statistically significant (p = 0.034) (Fig. 3.C).

The micturition diaries showed a significant decrease in participants' SUI symptoms. The daily number of episodes of involuntary urine loss decreased from an average of 4.4 episodes before intervention to 1.8 episodes after balloon placement (p = 0.020). Average daily pad use decreased from 3 pads per day to 1.3 after placement of the intravesical balloon (p = 0.017).

DISCUSSION

Here, we have investigated the therapeutic effectivity and potential working mechanisms of the implantation of an intravesical air-filled balloon were assessed. The reported effects of implantation of an intravesical balloon in SUI patients are proposed to contribute to the observed clinical effect of this therapy.

The therapeutic effectivity of this minimally invasive treatment options has been evaluated in previous studies using larger sample sizes[10-13], however for the current study we confirmed that the therapy had a positive effect on SUI complaints in our sample as well. The dimensions of the balloon in the frontal view show a significant decrease during episodes of high abdominal pressure, mainly along the cranio-caudal axis. We argue that the decrease in size of the balloon is a direct result of compression of the air inside the balloon. This indicates that the balloon is successful in absorbing at least part of the intra-abdominal pressure which is transferred to the bladder. Thereby decreasing the extent to which the intravesical pressure increases in response to episodes of high intra-abdominal pressure. Previous in-vitro tests have measured increases of pressures in a bench top pressure chamber while pressure pulses of physiological intensities were applied with and without the balloon present in the pressure chamber. The balloon was observed to reduce the amplitude of the pressure pulse by 65%[16]. An in-vitro test of the extent to which the balloon absorbs pressure increases in a pressure chamber provides a very controlled environment to test the hypothesis that increases in intravesical pressure during episodes of high intra-abdominal pressure can be absorbed by the balloon. In-vivo tests have shown to be limited because the separation of the intra-abdominal and intravesical spaces requires accurate pressure monitoring in both compartments which is, especially concerning abdominal pressure, highly prone to disturbances of accurate pressure transmission to the catheter[17]. Additionally, the pressure amplitudes emerging from coughing episodes will be highly variable across repetitions compared to pressure pulses generated in a pressure chamber which will be the same for every repetition.

The bladder obtained a significantly more cranio-caudally (vertically) oriented shape after implantation of the intravesical balloon. Proper support of the urethra by pelvic musculo-fascial structures and sufficient mobility of the bladder neck is essential for the maintenance of continence[18]. The loss of integrity of the supportive structures, or any limitation in bladder neck mobility, may affect an efficient transfer of intra-abdominal pressure to the bladder neck, and thereby successful kinking of the urethra[18]. We propose that the change in bladder shape after implantation of the intravesical balloon may compensate for a relative immobilization of the bladder neck, possibly due to an altered orientation of the bladder on the pelvic floor muscles such as the levator ani. A more sideways expansion of the bladder on the pelvic floor may lead to a reduced craniocaudal mobility. The more vertically oriented bladder will likely improve kinking capacities and lead to higher urethral pressures during episodes of elevated intra-abdominal pressure. The observed change in bladder shape may be related to the buoyancy of the balloon,

and alternatively it can be hypothesized that the continuous contact of the balloon with the bladder dome induces reflex activity in the detrusor muscle, which results in the observed change in bladder shape. Another possible mechanism may be that a low-grade irritation or sensory stimulation of the bladder dome causes a reflex contraction of the bladder neck leading to improved continence.

Research on colorectal functioning has shown that the passive tone and reflex activity of the internal and external anal sphincters and the endopelvic fascia show coordinated activity to maintain continence during episodes of high intra-abdominal pressure[19]. A reflex contraction of the external anal sphincter complex in response to increases in intra-abdominal pressure prevents fecal incontinence by kinking of the anal canal in opposite directions[20]. Although the external urethral sphincter is structurally and fundamentally different from the external anal sphincter complex the functional task of preventing leakage during episodes of high intra-abdominal pressure is comparable. Our results indicate that the downward displacement of the caudal limit of the bladder, assessed in frontal view during coughing episodes with a bladder volume of 100ml, increases significantly after implantation of an intravesical balloon. The observed increase in cranio-caudal displacement of the bladder neck seen in frontal view is expected to occur with a simultaneous increase in anterio-posterior movement leading to more optimal kinking of the urethra on the musculo-fascial structures of the pelvic floor. Urethral kinking on the stiff pelvic musculo-fascial structures of the pelvic floor results in an indirect transfer of force from the abdomen to the kinking point of the urethra. In combination with possible reflex activity of the bladder neck this will increase the urethral pressure equivalent to the increase in intra-abdominal pressure. The reported increase in bladder neck mobility is proposed to create a longer urethral lever for kinking on endopelvic musculo-fascial structures. Thereby, this mechanism will help prevent involuntary urine loss during high intra-abdominal pressure episodes.

Lastly, the results of the current study confirm the therapeutic effectivity of the intravesical placement of an air-filled balloon 7 days post-placement on SUI by their significantly positive effect on patients' daily involuntary urine loss and pad use. We propose that the therapeutic effect is related to absorption of the increase in intravesical pressure by the balloon, along with an improvement of urethral kinking efficacy.

CONCLUSIONS

In the present study, we show that implantation of an intravesical balloon in patients with SUI complaints significantly reduces patients' daily episodes of involuntary urine loss and pads used. We have looked at several mechanisms which could underlie this therapeutic effect. The balloon significantly decreased in size indicating that it successfully absorbed intra-abdominal pressure increases. Additionally, we report that the implantation of an air-filled balloon causes the bladder to obtain a more vertically oriented shape and increases mobility of the bladder neck. These changes are proposed to facilitate an improved kinking of the bladder neck and urethra area, which will aid the absorbing function of the balloon in preventing involuntary loss of urine during episodes of high intra-abdominal pressure.

The observations made in this study can be utilized to further maximize the potential benefits of this therapy and help us understand which patients can be expected to obtain the largest advantage of the implantation of an intravesical balloon.

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CHAPTER 5

Parcellation of human periaqueductal gray at 7T fMRI in full and empty bladder state: the foundation to study dynamic connectivity changes related to lower urinary tract functioning

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ABSTRACT

Aims

The periaqueductal gray (PAG) is a brain stem area involved in processing signals related to urine storage and voiding. The PAG is proposed to be responsible for projecting afferent information from the bladder to cortical and subcortical brain areas and acts as a relay station projecting efferent information from cortical and subcortical areas to the pons and spinal cord. Here, we use 7 Tesla functional magnetic resonance imaging to parcellate the PAG into functionally distinct clusters during a bladder filling protocol.

Methods

We assess the similarity between parcellation results in empty and full bladder states and show how these parcellations can be used to create dynamic response profiles of connectivity changes between clusters as a function of bladder sensations.

Results

For each of our 6 healthy female participants we found that the agreement between at least one of the clusters in both states resulting from the parcellation procedure was higher than could be expected based on chance ($p = \leq 0.05$), and observed that these clusters are significantly organized in a symmetrical lateralized fashion ($p = \leq 0.05$). Correlations between clusters change significantly as a function of experienced sensations during bladder filling ($p = \leq 0.05$).

Conclusions

This opens new possibilities to investigate the effects of treatments of lower urinary tract symptoms on signal processing in the PAG, as well as, the investigation of disease-specific bladder filling related dynamic signal processing in this small brain structure.

INTRODUCTION

The control of micturition is hierarchically organized within the central nervous system (CNS) and the peripheral nervous system[1]. In healthy adults, storage and voiding of urine is controlled by dedicated areas on the level of the spinal cord (SC) (e.g. Onuf's nucleus (ON)), the brain stem (e.g. pons and periaqueductal gray (PAG)), and higher cortical and subcortical areas (e.g. prefrontal cortex, anterior cingulate cortex, insula, thalamus). The main purpose of this system is to facilitate storage of urine and to initiate the micturition episode once a time and place is found where it is safe and socially acceptable to void.

The PAG is centrally located in the hierarchical system of micturition control, and is assumed to serve as a relay station projecting afferent information from the bladder to cortical and subcortical brain areas and as a gatekeeper projecting efferent information from these higher areas to the pontine micturition center (PMC) and ON[2-4]. The PAG is organized in a symmetrical columnar fashion, and the ventrolateral PAG and dorsolateral PAG are indicated to be involved in the control of voiding and storage of urine respectively[5-8]. Patients with selective lesions of the PAG are known to present with micturitional disturbances[9-11]. This indicates the relevance of gaining a better understanding of the role of the PAG, both as a whole and at the level of individual columns, on the control of micturition.

Neuroimaging studies have consistently observed activity in the PAG related to bladder fullness and bladder sensations[12-15], and have underpinned the importance of the role of the PAG in the switching mechanism during the initiation and interruption of voiding[16]. Altered CNS processing has been indicated to be related to lower urinary tract symptoms (LUTS) like urge incontinence[17-19].

The utilization of ultra-high-field functional magnetic resonance imaging (fMRI) at 7 Tesla will enable us to investigate PAG activity in human subjects at a sub-millimeter resolution. We aim to parcellate the PAG into clusters based on their fMRI blood-oxygen-level-dependent (BOLD) signal using connectivity-based parcellation methods. This approach allows the identification of anatomical regions that differ from one another based on correlations in BOLD fluctuations during resting-state[20]. Resting-state based parcellations reflect an underlying anatomical construct[21], and this approach enables us to parcellate the PAG into anatomically distinct clusters. Since the underlying anatomy remains constant we hypothesize that PAG parcellations based on resting-state fMRI data during an empty and a full bladder state show a high overlap, and show a symmetrical organization that corresponds to what has been observed in prior histochemical work.

To demonstrate the clinical potential of a robust functional parcellation of the PAG, we propose an exploratory application which can be used to study connectivity changes between different subregions of the PAG related to changes in bladder fullness and bladder sensations. To this end, we introduce a novel methodological approach to identify dynamic response profiles of PAG activity that, when established in healthy controls, could serve as a benchmark to which LUTS patients' response profiles can be compared.

METHODS

Participants

This study was designed and conducted in line with the Declaration of Helsinki and was approved by our local ethics committee. Written informed consent was obtained from each of our participants. We enrolled 9 female participants in our study (mean age: 47, SD: 20) without any clinically significant history neurological disease or dysfunction as judged by the medical investigator. We chose to include only female participants in this project to control for gender as a confounding factor. Future studies should assess sex-related differences in PAG activity related to bladder fullness and bladder sensations. We had to exclude data obtained from 3 participants due to technical difficulties. All participants were able to complete all steps of the bladder filling and scanning protocol. For the development of the exploratory application of PAG parcellations we demonstrate our novel methodological approach on data obtained from a single subject (age: 24).

Study design

After obtaining informed consent, participants were asked to fill out a 3-day micturition diary in order to gain baseline values of their voiding- and drinking patterns and to familiarize the participants with scoring their bladder fullness sensations on a visual analogue scale (VAS) and their perception of urgency on the 4-point Indevus Urgency Severity Scale (IUSS)[22].

Participants were then invited to visit our clinic for the urodynamic and fMRI studies. Upon arrival at our institute participants were instructed to void until empty in private after which a nurse inserted a filling catheter (FR: 8) transurethrally. Participants were instructed to lie down in a supine position on the MRI bed while an MRI-compatible syringe pump was connected to the filling catheter. Head motion was restricted using foam cushioning. Scanning was conducted on a 7T MRI scanner (Siemens, MAGNETOM) using a 32-channel head coil (NOVA Medical, Wilmington, MA, USA).

For each participant; first we ran an empty bladder resting-state fMRI scan in which we collected 420 T2*-weighted multiband echo planar imaging volumes (mb-EPI sequence, acceleration factor = 2, MB-factor = 2, TR = 1400ms, TE = 22ms, resolution = 1.1 x 1.1 x 1.1mm) we scanned 40 slices covering the supramedullary portion of the brain stem, next we ran a T1-weighted whole brain anatomical scan using an MP2RAGE sequence. After the anatomical scan, participants were instructed to report their experienced bladder sensations, using a joystick they could control from the scanner, on a VAS for their perceived bladder fullness and the IUSS for their perceived levels of urgency. Reported bladder sensations were visually presented to the researcher in the operating room in real-time and were logged every second using a custom written MATLAB script. We manually prefilled the bladder with a syringe at a rate

of approximately 30ml/min to 50% of the volume of the micturition episode with the smallest volume reported on the micturition diary with a score of 1 on the 4-point IUSS (range 0-3) in order to decrease individual differences in total filling time. Next, a filling resting-state fMRI scan was started simultaneously with an automatic syringe pump to fill the bladder at a rate of 30ml/min (mb-EPI sequence, acceleration factor = 2, MB-factor = 2, TR = 1700ms, TE = 22ms, resolution = 0.9 x 0.9 x 0.9mm). The bladder was filled until participants indicated urgency levels of 2 on the 4-point IUSS. This is defined by Nixon et al.[22] as "enough urgency discomfort that it interferes with or shortens your usual activity or tasks" (p. 607). The length of this scan was variable and depended on each participant's individual bladder capacity. After the participants indicated a strong desire to void the syringe pump and filling scan were stopped and a full bladder resting-state fMRI scan was started using the same scanning parameters as the empty bladder scan. The scanning protocol is visualized in figure 1. After the full bladder scan finished participants were assisted out of the scanner and instructed to void until empty in private while recording their voided volume.

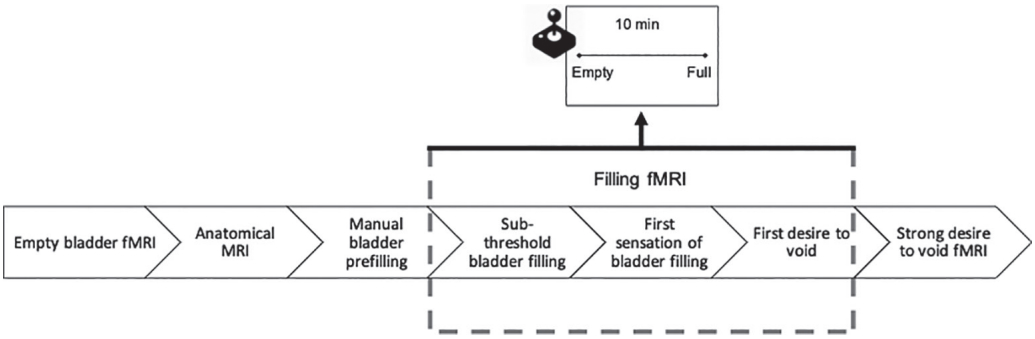

Figure 1: *The scan paradigm consisted of an empty bladder resting state fMRI scan which was followed by an anatomical scan. Next, we manually filled the bladder to a predetermined participant-specific level. A filling fMRI scan was then started, which could later be subdivided into different sensory states based on participant reported data acquired using a joystick script. Lastly, a full bladder or strong desire to void fMRI scan was started.*

Data processing

Functional data were preprocessed using BrainVoyager 20.6 (Brain Innovation, Maastricht, The Netherlands). Functional volumes were first corrected for slice scan-time differences and 3D head motion using 3 translation and 3 rotation parameters. Subsequently, linear trends and low frequency temporal drifts were removed from the data using a high-pass filter, removing temporal frequencies below 0.1 Hz, which is suggested to be an appropriate threshold for preservation of functional connectivity information in the data[23,24]. Functional data were subsequently coregistered to the anatomical images and rotated to ACPC space.

Periaqueductal gray parcellation

For each participant we identified and segmented out the PAG in the anatomical data using BrainVoyager 20.6. For both the empty bladder and the full bladder resting-state fMRI scans, the time course of each voxel within the PAG mask was correlated in a pair-wise fashion, to obtain a voxel-by-voxel square connectivity matrix for both datasets. We removed the lowest 5% correlations that are assumed to represent spurious connections from our matrix[25].

To generate parcellation maps of the PAG, we partitioned the connectivity matrices of both the empty bladder and full bladder data into clusters using a MATLAB implementation of the Louvain module detection algorithm[26]. This algorithm outputs clusters with stronger within-cluster connectivity than between-cluster connectivity[27]. The assignment of voxels to modules by the Louvain module detection algorithm has a stochastic nature, and in order to compensate for this we ran the algorithm for 500 iterations and selected the parcellation with the largest modularity statistic (Q-value) for further analysis. The Q-value quantifies the extent to which a network has a modular underlying data structure[28]. Our parcellation algorithm then assigned a label to each voxel in the PAG assigning it to a module. These labels were then used to create a module map for each participant for, both, empty and full bladder datasets.

Quantification of parcellations

We propose that if the clusters resulting from the parcellation procedure correspond to an underlying anatomical construct, these should not be influenced by subjective bladder sensations or objective bladder filling. In order to ascribe meaningful interpretations to our PAG parcellations and quantify our results we, therefore, aimed to assess the agreement between parcellations based on the empty and full bladder datasets.

Using the empty bladder parcellation, we computed random parcellations using a custom MATLAB script that randomly places N anchor points in the 3D space of the PAG mask, with N representing the number of clusters in the original dataset. From each of these anchor points, a cluster was grown until it reached a similar voxel count as the original cluster. We iterated this script to obtain 1000 different random parcellations. These random parcellations were subsequently used to assess the agreement between our parcellations based on fMRI data acquired during an empty and full bladder state compared to what could be expected based on chance. The Sørensen–Dice coefficient is a useful measure of spatial overlap which can be applied to studies of reproducibility and accuracy in image segmentation, it is based on the percentage of spatial overlap between two images[29]. For each participant, we computed the Sørensen–Dice coefficient between each cluster in the empty bladder parcellation and each cluster in the full bladder parcellation. Next, we computed the Sørensen–Dice coefficient between each cluster from the empty and full bladder parcellations and each cluster from the 1000 iterations of the random parcellation, obtaining a distribution of Dice coefficients under

the null hypothesis. This allowed us to statistically test the extent to which the similarity between empty and full bladder parcellations was higher than could be expected based on chance on a single subject level using a non-parametric permutation test.

Second, to evaluate the anatomical characteristic of a lateralized organization of the human PAG, we assessed the lateralization of each of the identified clusters during the full bladder scan. We identified the midline of the PAG mask for each participant and divided all clusters in the full bladder parcellation into a left and right homologue. We then computed the center of mass (CoM) of the whole PAG and used this as a reference point to plot the CoM of the left and right homologue of each cluster (figure 2). This enabled the assessment of the difference in distance of the CoM from each left cluster to the CoM of the PAG versus the distance of the CoM from each right cluster to the CoM of the PAG. We expected the difference in distance of the CoM of the matching clusters in each hemisphere to the CoM of the PAG to be significantly lower than for non-matching clusters. This effect was statistically tested using a Wilcoxon signed-rank test.

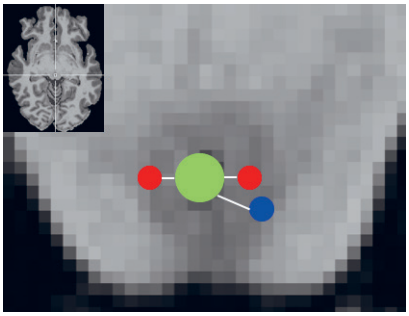

Figure 2: *The relative location of the PAG in transversal view is indicated by the crosshairs in the top left corner of the figure. The center of mass (CoM) of the whole PAG is indicated in green. The red dots represent the left and right CoM of homologues of the same cluster. The blue dot represents the CoM of a non-matching cluster. We compared the distance of the CoM of the cluster in left/right PAG to the CoM of the whole PAG for matching and non-matching clusters.*

Dynamic response profiles

From the filling resting-state fMRI scan we could delineate 3 different states of bladder sensation using the data regarding participants' perceived levels of bladder fullness and urgency. The period before participants indicated any experienced bladder fullness was classified as "sub-threshold bladder fullness", once participants moved the cursor along the VAS the sensory state was classified as "first sensation of bladder filling", and when participants switched the IUSS indicator from 0 to 1 this was classified as "first desire to void". Together with the empty and full bladder resting-state scans we had 5 different states of interest: 1) empty bladder, 2) partially filled bladder without sensation of bladder filling, 3) first sensation of bladder filling, 4) first desire to void, 5) strong desire to void. In order to obtain a dynamic response profile of PAG connectivity changes related to bladder sensations we computed the correlations between the different clusters identified in the parcellation procedure of the full bladder dataset for each of the 5 different sensory states. To investigate how connectivity between PAG clusters changes during bladder filling we fitted a second-degree polynomial to the data points and statistically tested the amplitude of the polynomial using a non-parametric permutation test.

RESULTS

After completion of the scanning protocol all participants voided a volume that was higher than the average volume of voids with an urgency level of 2 reported on the micturition diary, indicating that the interoceptive nature of the study did not significantly influence the tolerated infused volume.

Parcellation analysis

We found that for each of our participants the agreement between at least one of the clusters resulting from the empty and full bladder resting-state fMRI parcellation procedures was higher than could be expected based on chance ($p = \leq 0.05$, corrected for multiple comparisons using a false discovery rate ($q=0.05$)) (table 1). The mean similarity for modules identified in empty and full bladder parcellations, when compared to random iterations, was 69.4%, and for two of our participants this percentage was as high as 100% (figure 3). This confirms our hypothesis that resting-state fMRI parcellations of the PAG are stable despite changes in subjective bladder sensations and bladder fullness.

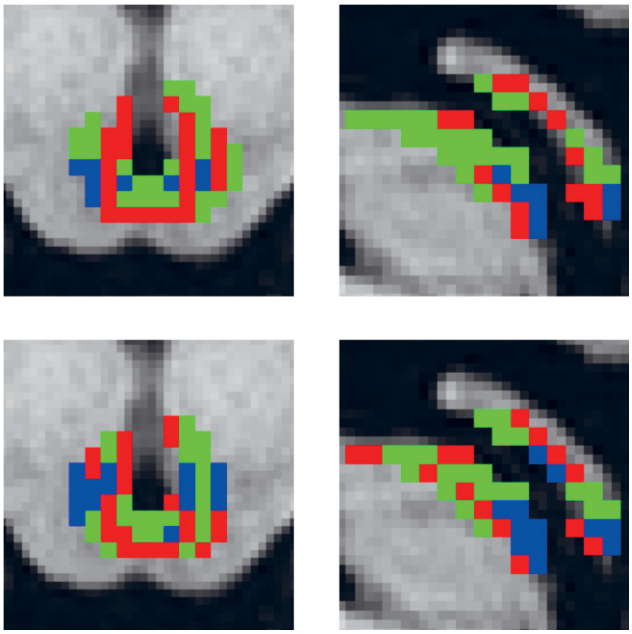

Figure 3: *Visualization of the spatial correspondence between modules that show a significantly larger spatial overlap than could be expected on chance for a representative participant.*
Top row: transversal and sagittal view of parcellations based on data acquired during an empty bladder state.
Bottom row: transversal and sagittal view of parcellations based on data acquired during a full bladder state. Colors indicate corresponding modules.

Table 1. *Number of modules per participant for each parcellation and significant similarity ratio*

Participant	Number of modules empty	Number of modules full	Significant similarity ratio
1	3	3	2/3
2	3	4	2/3
3	3	3	3/3
4	3	3	1/3
5	3	3	3/3
6	3	2	1/2

The distance of the center of mass of clusters in each side of the PAG to the center of mass of the whole PAG mask was significantly lower for matching clusters than for non-matching clusters over our participants ($p = 0.016$). These results indicate that the clusters resulting from our parcellation procedures are organized in a lateralized fashion.

Dynamic response profiles

The modularity Q-value resulting from our parcellation of the full bladder scan using the Louvain module detection algorithm was above 0.4, which is an indicator of a modular underlying data structure. The parcellation procedure in the representative subject used in the figures yields a subdivision of the PAG into three distinct clusters in the full bladder resting-state scan. The change in functional connectivity as a function of experienced bladder sensations between PAG clusters 2 and 3 was significant ($p = 0.017$) after correcting for multiple comparisons using a false discovery rate ($q = 0.05$). The patterns of connectivity between clusters 1 & 2, and 1 & 3 did not reach statistical significance (figure 4). This suggests that connectivity between clusters within the PAG changes significantly as a function of experienced sensations during bladder filling, and gives an indication of the potential implications of these dynamic response profiles in studying PAG activity related to bladder fullness and participants' experienced bladder sensations.

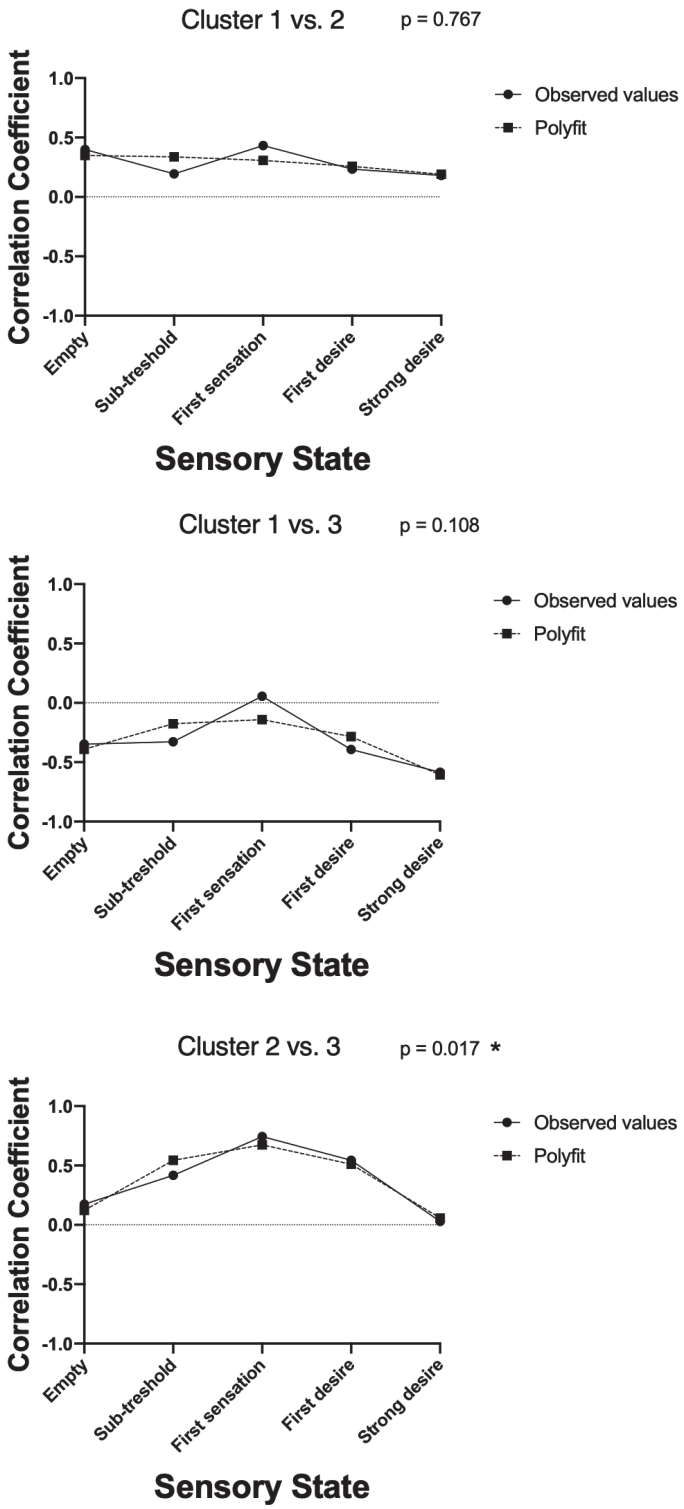

Figure 4: *The observed correlations between periaqueductal gray clusters at different sensory states. The correlation between clusters 2 and 3 was found to change significantly as a function of bladder sensations.*

DISCUSSION

Parcellation analysis

Our results indicate that PAG activity in an empty and full bladder state can reliably be subdivided in clusters that are largely independent of bladder state, and show a higher similarity with each other than could be expected based on chance. Thereby supporting the idea that voxels from clusters resulting from resting-state fMRI based parcellation of the PAG have a high correlation in their BOLD time courses related to an underlying anatomical construct.

As an additional measure of the extent to which the clusters from the parcellation procedures have an underlying anatomical construct we observed that clusters are organized in a significantly more lateralized fashion than can be expected by chance. This implies that our parcellation correctly identified the symmetrical organization of the PAG.

These results imply that this method can be used to reliably parcellate the PAG using ultra-high-field resting-state fMRI data. This opens opportunities to use parcellation methods to study changes in dynamic connectivity between PAG clusters related to bladder fullness and participants' experienced bladder sensations.

The current study is the first to parcellate the human PAG using 7 Tesla resting-state fMRI data utilizing a largely data driven approach. We validated our methodological approach by running parcellation procedures on two datasets from the same subject in different physiological and sensory states, namely with an empty bladder and no desire to void versus a full bladder and a strong desire to void. Using ultra-high-field MRI methods we are nearing spatial resolutions that allow for direct translations of invasive electrophysiological animal work to the human situation in non-invasive research designs. In view of our limited samples size, for the scope of this study we have investigated the agreement between PAG parcellations at the within-subject level. In order to advance this line of research future studies should assess whether an agreement can be found between PAG parcellations at the between-subjects level. Additionally, since the current study only included female participants, more research is needed to investigate the agreement between PAG parcellations across genders. This will allow for further validation of underlying (functional) anatomical organizations. Furthermore, the observation of such an agreement will enable the assignment of potential changes in PAG organization due to pathophysiological conditions to specific anatomical areas within this region.

Dynamic connectivity profiles

Our results suggest that connectivity between PAG clusters identified in parcellations of full bladder resting-state fMRI data, changes significantly between different sensory states during a bladder filling protocol. The observed changes in connectivity between PAG clusters agrees with changes PAG activity in animal studies[8], and future studies should assess changes in connectivity

between PAG clusters during the voiding phase. These distinct connectivity patterns can be seen as dynamic response profiles of PAG activity. These profiles, when established in healthy controls, can serve as a benchmark to which LUTS patients' response profiles can be compared. The separation of resting-state fMRI data by participants' reported bladder sensations is a completely new approach to study CNS activity related to bladder functioning. This approach will help to better understand CNS activity related to subjective bladder sensations and bladder filling. This technique will thus allow us to monitor bladder filling dependent activity in the reciprocal communication between the lower urinary tract and the CNS. Therefore, it can be utilized to develop a new and necessary imaging biomarker framework for translational research into new therapeutic targets, and new diagnostic strategies combined with treatment effectivity predictors and evaluators.

CONCLUSIONS

In the present study, we aimed to assess whether the PAG can reliably be parcellated using a resting-state fMRI scanning protocol. Our results support that these parcellations are independent of participants' reported changes in bladder sensations or bladder filling. Additionally, we found that these parcellations show a symmetrical lateralized organization that could be expected based on previous post-mortem neuro-anatomical studies[30] and work in rodents[31]. Correlations between the identified clusters are indicated to change significantly as a function of experienced sensations during bladder filling. The current study lays the foundation to take an interdisciplinary and translational approach to find new and more optimally targeted treatment and diagnostic options for LUTS patients.

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CHAPTER 6

Between-subject similarity of functional connectivity-based organization of the human periaqueductal gray related to autonomic processing

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ABSTRACT

Aims

The periaqueductal gray (PAG) is a brain stem area designated to play an essential role in lower urinary tract (LUT) control. Post-mortem human and animal studies have indicated that the PAG is symmetrically organized in functionally and anatomically distinct columns which are involved in sympathetic or parasympathetic autonomic control of the LUT. The current study aims to find consistency across subjects and identify homologous clusters between subjects.

Methods

Here, we evaluated data from 10 female participants. During a bladder filling protocol, we ran a resting-state fMRI scan while participants experienced a strong desire to void. A voxel-by-voxel correlation matrix of the PAG was created and parcellated using the Louvain module detection algorithm. Resulting in a map of the PAG in which each voxel is assigned to a cluster as determined by the Louvain module detection algorithm. The spatial similarity of resulting clusters between participants was assessed by computing the Dice similarity coefficient for all cluster comparisons. Next, we designed a permutation test to create randomized parcellation maps which enabled us to statistically test the similarity values observed across participants.

Results

We observed several significantly similar clusters between subjects compared to permutations ($p \leq 0.05$). These results show that the PAG can be parcellated into distinct clusters which show a similar spatial distribution at the group level.

Conclusions

This analysis is a crucial step to determine the agreement between in vivo PAG parcellations and the functional and anatomical columnar organization of the PAG which is known from previous research. These advancements may enable us to identify the relationship between LUT symptoms, such as urgency, and activity patterns in the PAG in normal and pathological states.

INTRODUCTION

There is a continuous exchange of information between the peripheral nervous system (PNS) and central nervous system (CNS) regarding the internal state of the body. Most of the time these processes do not reach our conscious awareness, but our attention will be shifted to visceral functioning when needed in order to maintain a proper homeostatic balance[1]. The spinal cord and brain stem have key functions in relaying interoceptive information from peripheral organs to higher brain areas. That way, these structures serve a crucial role in the bidirectional transfer of visceral information to our awareness and to the autonomic nervous system for subconscious regulatory purposes[2]. Proper exchange of information between the CNS and PNS is essential for the regulation of autonomic processes and dysfunction of this system can present in disturbances in nociceptive, cardiovascular, respiratory, urinary, gastrointestinal, and thermoregulatory functions.

Storing and voiding of urine is regulated by a delicate interplay between visceral sensations regarding bladder fullness and socially learned behavior concerning appropriateness of emptying one's bladder at a certain place and time. Therefore, the control of the bladder and urinary sphincter is arguably one of the most evident visceral functions that is highly dependent on multifaceted processes that require complex and coordinated activity, and integration of peripheral afferent and central efferent signals at different levels[3]. The control of the lower urinary tract (LUT) is hierarchically organized within the CNS and the PNS. In healthy adults, bladder sensory processing and the initiation of micturition is controlled by dedicated areas at the level of the spinal cord (mainly Onuf's nucleus (ON)), the brain stem (e.g. pons and periaqueductal gray (PAG)), and higher cortical and subcortical areas (e.g. prefrontal cortex, anterior cingulate cortex, and insula)[4].

The PAG is a brain stem structure surrounding the cerebral aqueduct and is approximately 14mm in length. The PAG is an integral part of the emotional motor system and as such is highly involved in a wide array of processes related to nociceptive control, cardiovascular control, respiration, micturition, defecation, parturition, and many more[5]. The PAG is centrally located in the hierarchical system of micturition control, and is assumed to serve as a relay station projecting afferent information from the bladder to cortical and subcortical brain areas and as a gatekeeper projecting efferent information from these higher areas to the pontine micturition center (PMC) and ON[6-9]. Animal research has indicated that the PAG is organized in a symmetrical columnar fashion, and the ventrolateral and dorsolateral areas of the PAG are indicated to be involved in the control of voiding and storage of urine respectively[10-14].

Previous functional neuroimaging research has identified some of the pathways and has indicated that the insula and anterior cingulate cortex, known as the sensory and autonomic areas for the autonomic nervous system, are involved in processing bladder sensory information and LUT control[15,16]. The insula monitors bladder sensory information and, when necessary, shifts our attention to our bladder so that we can look for an appropriate time and place to initiate

voiding. These higher level processes and the ultimate decision to void require frontal cortex activity[17-19]. These cortical and subcortical structures are likely to exercise their control over the LUT via the PAG.

Optimal functioning of these central systems enables healthy adults to accurately assess their experienced levels of bladder fullness at any given time and to reliably predict for how long they will be able to postpone micturition. In conditions of LUT dysfunction, such as overactive bladder (OAB), participants might struggle with these tasks. The International Continence Society defines OAB as urgency, with or without urge incontinence, and often with frequency and nocturia. Over the past years, it has become more apparent that OAB is associated with altered bladder sensations. Mechanisms of central sensitization might underlie bladder hypersensitivity and sensory disturbances leading to alarm falsification[20]. A disturbance in the neural processing of visceral sensory information might be related to this type of LUT dysfunction. Recent neuroimaging work has reported the first indications that PAG activity reflects subjective reported bladder sensations[21]. PAG activity might, therefore, offer insights into processing of visceral sensory information and could help identify alterations in interoceptive processes which might cause "false alarms" in patients with OAB.

Previous research has indicated that the PAG can reliably be subdivided into distinct clusters of functional regions that can be differentiated based on resting-state functional magnetic resonance imaging (fMRI) data at 7 Tesla (T). At the within-subject level, these clusters show a symmetrical organization and high level of similarity between empty and full bladder states[21], which is in line with what would be expected based on animal work.

In the current study we aimed to assess the similarity between PAG organization at the group level using resting-state fMRI at 7T. We expected to observe a significantly higher spatial overlap between clusters from different participants than would be expected based on chance.

METHODS

Participants

This study was designed and conducted in line with the Declaration of Helsinki and was approved by our local ethics committee. Written informed consent was obtained from each of our participants. Participants provided consent for untraceable use of their functional and structural MRI data. We enrolled 13 female participants in our study (mean age: 46.31, range: 21-73) without any clinically significant history of lower urinary tract dysfunction, or neurological disease or dysfunction, as judged by the medical investigator. We chose to include only female participants in this project to control for gender as a confounding factor. We had to exclude data obtained from 3 participants due to technical difficulties. All participants were able to complete all steps of the bladder filling and scanning protocol.

Study Design

After obtaining informed consent, participants were asked to fill out a 3-day micturition diary in order to gain baseline values of their voiding- and drinking patterns and to familiarize the participants with scoring their perception of urgency on the 4-point Indevus Urgency Severity Scale (IUSS)[22]. Participants were then invited to visit our clinic for the fMRI study. Upon arrival at our institute participants were instructed to void until empty in private after which a nurse inserted a filling catheter (FR: 8) transurethrally. Any residual urine was emptied from the bladder via this catheter. Participants were then instructed to lie down in a supine position on the MRI bed while an MRI-compatible syringe pump was connected to the filling catheter. Head motion was restricted using foam cushioning. Scanning was conducted on a 7T MRI scanner (Siemens, MAGNETOM) using a 32-channel head coil (NOVA Medical, Wilmington, MA, USA).

First, we ran a T1-weighted whole brain anatomical scan using an MP2RAGE sequence. We then manually prefilled the bladder with a syringe at a rate of 30ml/min to 50% of the volume of the micturition episode with the smallest volume reported on the micturition diary with a score of 1 on the 4-point IUSS (range 0-3) in order to decrease individual differences in total filling time. The bladder was then further filled using an automatic syringe pump at a speed of 30ml/min. Participants were instructed to report their experienced levels of urgency, using a joystick they could control from the scanner, on the IUSS for their perceived levels of urgency. Reported bladder sensations were visually presented to the researcher in the operating room in real-time and were logged every second using a custom written MATLAB script. Once participants indicated urgency levels of 2 on the 4-point IUSS, defined by Nixon et al.[22] as "enough urgency discomfort that it interferes with or shortens your usual activity or tasks" (p. 607), the bladder filling protocol was stopped and a full bladder resting-state scan was started.

During this resting-state scan we collected 420 T2*-weighted multiband echo planar imaging

volumes (mb-EPI sequence, acceleration factor = 2, MB-factor = 2, TR = 1400ms, TE = 22ms, resolution = 1.1 x 1.1 x 1.1mm). Participants were instructed to keep their eyes open during this scan, and try not to think of anything in particular. We scanned 40 slices covering the supramedullary portion of the brain stem. After the full bladder scan was finished, the participants were assisted out of the scanner and instructed to void until empty in private while recording their voided volume.

Data Processing

Functional data were preprocessed using BrainVoyager 22.2 (Brain Innovation, Maastricht, The Netherlands). Functional volumes were first corrected for slice scan-time differences and 3D head motion using 3 translation and 3 rotation parameters. Subsequently, linear trends and low frequency temporal drifts were removed from the data using a high-pass filter, removing temporal frequencies below 0.1 Hz, which is suggested to be an appropriate threshold for preservation of functional connectivity information in the data[23,24]. Functional data were then co-registered to the anatomical images. The whole brain anatomical images were transformed to MNI space, after which the functional data was transformed to MNI space using the same parameters as the structural data.

Periaqueductal Gray Parcellation

We identified and segmented out the PAG in the MNI template using BrainVoyager 22.2. The mask file resulting from this approach contained 1169 voxels within the region of interest. For the full bladder resting-state fMRI scans of each participant, the time course of each voxel within the PAG mask in MNI space was selected and correlated in a pair-wise fashion, to obtain a voxel-by-voxel symmetric square connectivity matrix. We removed the lowest 5% correlations that are assumed to represent spurious connections from our matrix[25]. To generate parcellation maps of the PAG, we partitioned the connectivity matrices of the full bladder resting-state data into clusters using a MATLAB implementation of the Louvain module detection algorithm[26]. This algorithm outputs modules with stronger within-module connectivity than between-cluster connectivity[27] without the need for a predetermined number of clusters. The assignment of voxels to modules by the Louvain module detection algorithm has a stochastic nature, and in order to compensate for this we ran the algorithm for 500 iterations and selected the parcellation with the largest modularity value (Q-value) for further analysis. The Q-value quantifies the extent to which a network has a modular underlying data structure[28]. Our parcellation algorithm then assigned a label (a number between 1 and the total number of clusters) to each voxel in the PAG allocating it to a module. These labels were then used to create a module map for each participant for full bladder datasets.

Cluster matching across Participants

We expect that if our parcellations reflect an underlying physiological construct present in the general population we should be able to observe spatially highly similar clusters across participants. We assessed the similarity between PAG parcellations across participants and statistically tested our observations by designing a permutation test.

The similarity of clusters resulting from the parcellation procedure between participants was assessed by computing the Sørensen–Dice similarity coefficient for all cluster comparisons across participants. The Sørensen–Dice coefficient is a metric that assesses spatial overlap by multiplying the area shared by both datasets by 2 and subsequently dividing this number by the total area of both datasets combined. In this way the Sørensen–Dice coefficient is a useful measure to assess the similarity between two images.

Next, we designed a custom MATLAB script in which, for each iteration, we randomly picked a subject's correlation matrix and shuffled the observed correlation values across the matrix. Subsequently, this code created a randomized symmetric correlation matrix reflecting the same range of correlation values as the original data. We iterated this script in order to generate 1000 randomized correlation matrices which were subsequently parcellated using the Louvain module detection algorithm in the same approach as the original data. From these 1000 randomized PAG parcellation maps we computed the Sørensen–Dice coefficient for 100,000 randomly selected cluster combinations between maps in order to obtain a distribution of Sørensen–Dice coefficients under the null hypothesis.

We then statistically assessed the significance of the original Sørensen–Dice coefficients between cluster pairs from different participants by ranking them to the distribution of Sørensen–Dice values observed in the permutations after correcting the false discovery rate for multiple comparisons.

RESULTS

In order to visualize the functional organization of the human PAG we determined the consistency of resting state functional connectivity based parcellations across participants. Data from each participant was transformed to the MNI template in order to be able to include the same voxels for each participant in our analyses. After successful parcellation of PAG connectivity matrices for all participants, we assessed the similarity between PAG parcellation maps by comparing Sørensen–Dice coefficients observed between cluster combinations across participants to values expected under the null hypothesis as determined by our permutation test. In this permutation test, the similarity of blood-oxygen-level-dependent signal fluctuations of voxels based on spatial proximity and functional connectivity was randomized across the matrix in order to generate parcellation maps based on data without topographical data regarding PAG organization, but with maintenance of correlation values corresponding to the original data. We found that for each of our participants the agreement between at least one of the clusters, resulting from the resting-state fMRI parcellation procedure, with a cluster from another participant was significantly higher than could be expected based on the empirical distribution under the null hypothesis resulting from our permutation test ($p \leq 0.05$, corrected for multiple comparisons using a false discovery rate ($q=0.05$)). We observed a significantly higher similarity between cluster pairs across subjects compared to permutations. For 23 cluster combinations across participants we observed a significantly higher similarity than could be expected based on the distribution under the null hypothesis (Fig. 1). Between two of our participants (participant 5 and 7, Fig. 1) we found a significant match for all clusters (Fig. 2). Additionally, there seems to be a higher order consistency between clusters across participants. For example: There is a significant similarity between participant 1 – cluster 1 and participant 2 – cluster 2 (Fig. 1), as well as between participant 1 – cluster 1 and participant 5 – cluster 1. Moreover, the similarity between participant 2 – cluster 2 and participant 5 – cluster 1 is significantly higher than would be expected based on the distribution under the null hypothesis as well (Fig. 1). This suggests that the functional organization of the PAG, as determined by parcellating this structure using the Louvain module detection algorithm in MNI space, shows measurable consistency at the group level.

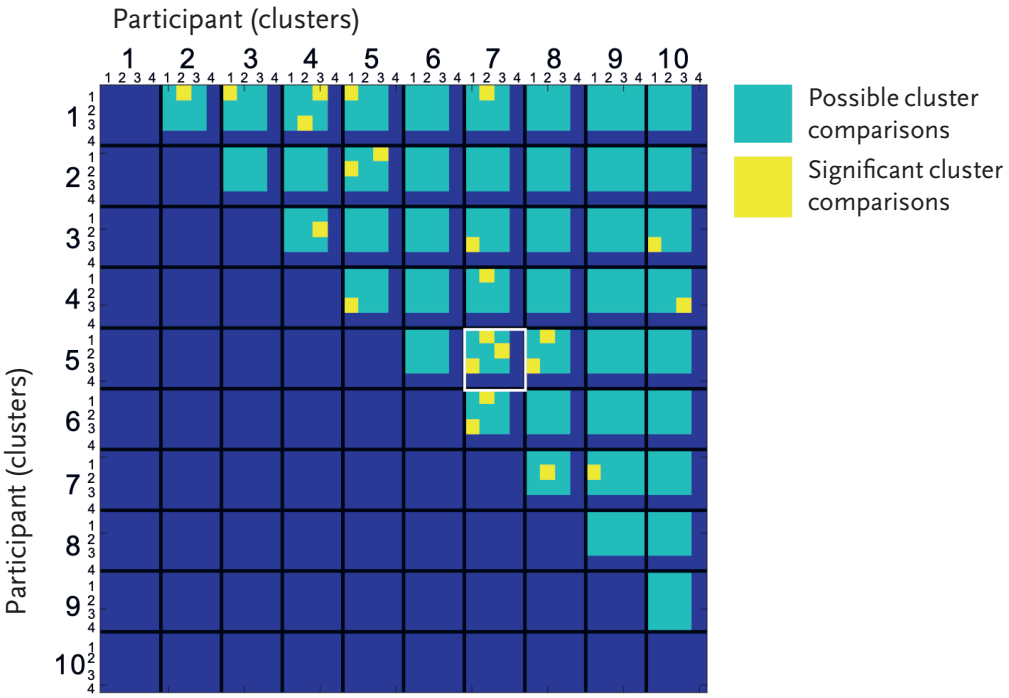

Figure 1: Group level assessment of spatial similarity of PAG clusters across participants. Data from each participant are represented in a single cell. Along the x and y direction comparisons between subjects and clusters are plotted. Subjects are not compared with themselves and only in one direction. Each yellow block marks a statistically significant similarity between clusters ($p \leq 0.05$, after FDR corrections ($q = 0.05$)). The green area indicates the comparisons made (the Louvain detection algorithm parcellated some PAGs into 4 clusters and others into 3 clusters). The comparison between participants 5 and 7 has been highlighted in white since all clusters from these two participants showed significantly high similarity.

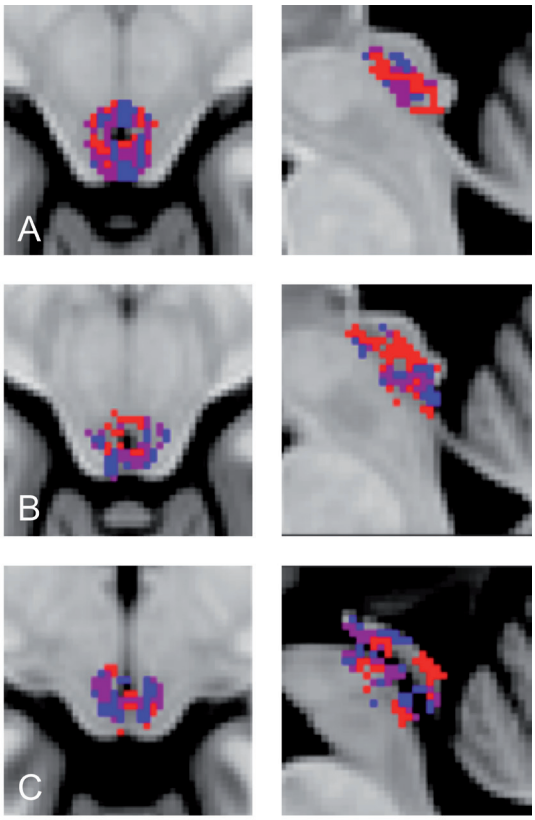

Figure 2: *Similarity between the different clusters from participants 5 and 7. The purple area indicates the region of overlap between participants.* ***A)*** *Transversal and sagittal view of the overlap between participant 5, cluster 1 (red) and participant 7, cluster 2 (blue)* ***B)*** *Transversal and sagittal view of the overlap between participant 5, cluster 2 (red) and participant 7, cluster 3 (blue)* ***C)*** *Transversal and sagittal view of the overlap between participant 5, cluster 3 (red) and participant 7, cluster 1 (blue).*

DISCUSSION

Here, we aimed to use 7T fMRI data acquired during a full bladder state to functionally subdivide the human PAG into clusters which show consistency at the group level. Our findings show that parcellating the PAG using the Louvain module detection algorithm resulted in clusters that show significant agreement across subjects. The reported results improve our understanding of the functional organization of this small brain stem structure and enable assessment of the role specific topographical PAG regions have in the control of the LUT.

We have previously assessed some of the known anatomical characteristics regarding anatomical organization of the PAG and have shown that resting-state fMRI based parcellations result in clustering which show symmetrical organization as well as consistency between empty and full bladder states in healthy subjects[21]. The results we report here, additionally, show that the PAG can be subdivided into distinct functional clusters with a similar spatial distribution across participants in full bladder states. These findings provide additional support that full-bladder resting-state fMRI based PAG parcellations show characteristics which are in line with organizational characteristics known from earlier neuroanatomical studies.

Since the 1990's there is a general consensus that functionally distinct areas within the PAG are arranged in symmetrical longitudinal columns extending along the rostrocaudal axis of the PAG[29,30]. The PAG is assumed to be organized in three functionally and histologically distinct pairs of columns: the ventrolateral, lateral, and dorsolateral columns, and a single dorsomedial column. Using diffusion MRI, researchers have previously segmented the PAG into columnar modules using tractography-based segmentation methods[31]. This method uses the diffusion direction of water molecules to group together voxels in a region with similar diffusion properties. Along the cerebral aqueduct this resulted into clusters showing a columnar organization along the rostrocaudal axis of the PAG, with spatial similarity to animal models. Investigation of connectivity patterns for the different columns with predefined cortical and subcortical target regions, based on previous animal and human studies, showed unique connectivity patterns for the different columns[31].

On visual inspection, the functional clusters observed in our parcellation maps do not evidently show the columnar organization that one might expect to see based on animal research and post-mortem human studies. We argue that the nature of the resting-state fMRI signal during an awake full bladder state in humans reflects a fundamentally different signal from animal and post-mortem work. It is known that, to some extent, there are regional differences in somatotopic and viscerotopic organization along the rostrocaudal axis within individual PAG columns. Additionally, neuroanatomically distinct columns appear to have partially overlapping functional areas[30]. The awake and alert full bladder state in which our participants were scanned, with continuously ongoing autonomic processes, might result in PAG parcellation maps consisting of functional clusters that are in an actively recruited state to execute these processes and show high correlations in their activity which might reflect communication across columns or within restricted areas along the rostrocaudal axis. The exact nature of the relationship between clusters resulting from resting-state fMRI based parcellations and the columnar neuroanatomical organization of the PAG requires further research to fully be elucidated. Analysis of brain stem functional connectivity patterns investigating different characteristics of resting-state fMRI, such as regional homogeneity and amplitudes of low-frequency fluctuations, may be useful future approaches.

By the assessment of functional connectivity patterns between voxels within the PAG we have been able to group together voxels that show a high similarity in their blood-oxygen-level-dependent (BOLD) signal during a full bladder state. This provides a new way to functionally map this small brain stem structure without the need for a priori assumptions regarding functional anatomy of this small structure. The advantage of the method presented in the current paper is the largely data driven approach of parcellating the PAG, without the need for a predetermined number of clusters or hand drawn constraints for the shape and orientation of clusters. Once PAG parcellation maps based on resting-state functional connectivity patterns are established, future research should investigate dynamic changes in functional connectivity patterns within this region, as well as with cortical and subcortical areas involved in LUT control, during manipulation of bladder sensory processing.

Neuroimaging data inherently contains some level of signal noise. We argue that the permutation test we designed enables us to assess whether the observed similarity between cluster combinations can be explained solely by signal noise. The cluster combinations that were significantly more similar than would be expected under the null hypothesis, resulting from the permutation test, give an indication of the reliability and reproducibility of the reported results and suggest that the significantly high dice similarity coefficients between clusters across participants cannot be explained by signal noise alone. The small sample size, focus on female participants, and relatively large age distribution of participants puts limits to the results of the current study However, the analysis shown here is a crucial step to determine the agreement between in vivo fMRI based PAG organizational maps and the functional and neuroanatomical organization of the PAG which is known from previous research. These advancements are essential in order to enable us to identify the relationship between LUT symptoms, like urgency, and activity patterns in the PAG in normal and pathological states. Establishing this relationship will allow determination of interindividual conformity or diversity.

Further investigation of how CNS activity patterns relate to subjective bladder fullness and urgency sensations can lead to identification of fMRI imaging biomarkers regarding OAB. A further unraveling of mechanisms such as alarm falsification in OAB could potentially lead to new, non-invasive therapies like interoceptive bladder awareness training via bio-feedback. Moreover, this research may help us understand the underlying mechanisms of current therapies, including sacral neuromodulation, and improve patient selection strategies.

To conclude, the PAG is an important brain stem nucleus involved in sensation and control of the LUT and various other visceroceptive processes. At the within-subject level, resting-state fMRI data of the PAG can be used to subdivide this nucleus into clusters that show some of the anatomical characteristics known from animal and post-mortem studies. Here, we show that PAG clusters additionally show high spatial organizational similarity at the group level. This paves the way for analysis of PAG activity related to bladder sensation and control at the group level, as well as studies aiming to unravel the interaction between the PAG and the rest of the brain. Utilizing these approaches to study CNS changes in response to successful therapeutic interventions will not only help to improve current therapies and patient selection strategies, but also may lead to the development of new therapies.

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CHAPTER 7

General discussion and conclusion

Efficient control of visceral organs, such as the lower urinary tract (LUT), is dependent on optimal exchange of information between the periphery and the central nervous system (CNS). Additionally, adequate integrity and functioning of support from pelvic and organ confined structures play an important role[1]. However, the interactions between the CNS and the periphery regarding LUT control, and the influence of the musculoskeletal system (i.e. pelvic supportive structures) on this interaction, is still poorly understood.

In this thesis, changes at the local level in the bladder wall associated with LUT dysfunction are investigated. Moreover, methodological developments that enable the assessment of (mal) adaptive change in the pelvic support system and CNS are introduced.

In the first part of this thesis maladaptive changes at the local level (in the bladder wall), in response to LUT dysfunction, are studied in rat models. It is likely that the CNS will undergo neuroplastic adaptive changes aiming to compensate for LUT dysfunction. Besides this, it is expected that in some cases the CNS will be the origin of dysfunction and compensatory adaptions are made at the level of the bladder wall. Furthermore, pelvic floor dysfunction (associated with stress urinary incontinence [SUI], but also urinary retention) may also be (partially) compensated by adaptive changes at the level of the CNS or LUT. Due to the complex nature of the interplay between the different components of the systems responsible for LUT control, most studies have only focused on parts of this system and methodological innovations are essential to gain a better understanding of the bidirectional relationships between the different levels of this control system. In conditions of LUT dysfunction different, or multiple, components of this system may be affected.

Examples of LUT dysfunction in which multiple levels along the bladder-brain axis may be involved are overactive bladder (OAB) or SUI. The prevalence of OAB is known to increase with age and aging-related changes at the local level of the bladder, such as inflammatory processes and oxidative stress, are associated with OAB[2-4]. On the other hand, aging-related changes in CNS activity measured using functional magnetic resonance imaging (fMRI) during a infusion and withdrawal protocol, are also suggested to be involved in the development of urge incontinence[5]. Most predominantly a decrease of activity in the insula and dorsal anterior cingulate cortex was observed (known as the sensory and motor areas of the autonomic nervous system respectively). In SUI associated with weakened pelvic support structures, the CNS might compensate by increasing the resting tonus of the external urethral sphincter or more conscious recruitment and "tightening" of pelvic musculature. In the second part of this thesis, novel methodological approaches are proposed which could help to further elucidate the interaction between the different levels of LUT control in both physiological and pathophysiological states.

In chapter two the impact of aging on urothelial health in a rat model was investigated. It was shown that the bladder mucosa of aged rats expresses a significant elevation of p21-Arc, which is a well-known marker of cellular senescence. Additionally, it was reported that markers

associated with cell death and oxidative damage (cytochrome C and nitrotyrosine) are significantly higher in aged animals. This is in agreement with previous research that has shown that there is a positive relationship between oxidative damage and aging in the mouse urothelium[6]. In cultured urothelial cells it was observed that aged rats show significantly decreased mitochondrial membrane potentials and mitochondrial bioenergetic profiles. Oxidative damage can severely impact mitochondrial health, and as a result the overall functioning of the urothelium[7]. The detrimental effects of aging on mitochondrial bioenergetics reported in this chapter furthermore confirm this. A significantly decreased oxygen consumption rate in mitochondria has a large and highly amplified effect on the production of energy through adenosine triphosphate (ATP) by oxidative phosphorylation. The production of ATP by glycolysis alone has an output of around 2 molecules of ATP for every molecule of glucose. The complete oxidation of glucose by the process of oxidative phosphorylation, on the other hand, is a tremendously efficient process with an output of around 30 ATP molecules for each glucose molecule. This implies that the decrease in oxygen consumption rate observed in aged animals may cause a cascade that has an exacerbated effect on the decrease of ATP production in aged animals. In line with this hypothesis, the results showed that ATP release in cultured urothelial cells from aged animals is significantly lower compared to young animals. These mechanisms have a substantial impact on the capacity of the urothelium to optimally execute its barrier and signaling properties and may contribute to the deterioration of bladder morphology and bladder functioning, which is associated with the increased prevalence of LUTS in older adults[3,8,9]. It is still unclear what the downstream effects of a decrease in urothelial mitochondrial health are, but it can be postulated that efficiency of transmission of sensory information from the urothelium to the underlying afferent nerve fibers in the lamina propria will be negatively impacted. This could be reflected in aging-associated changes in the distribution, number, thickness and density of afferent nerve fibers in the lamina propria.

In the third chapter changes in vascular perfusion and angiogenesis in the bladder neck in a rodent water avoidance stress (WAS) model associated with interstitial cystitis/bladder pain syndrome (IC/BPS) were studied. A significant increase in vascular endothelial growth factor (VEGF) and nerve growth factor precursor (proNGF) was observed in rats exposed to the WAS model, as well as a trend towards increased vascular perfusion. Besides this, a greater abundance of vessels in the microvasculature was noticed.

Chronic psychological stress and anxiety are associated with the initiation of dysfunctional adaptations in nociceptive pathways, which lead to stress-induced hyperalgesia and tactile allodynia[10]. Previous research has shown that increased stress is highly prevalent in IC/BPS patients and can be linked to an exacerbation of symptoms[11-15]. The elevated expression of VEGF that is seen in IC/BPS is associated with immature angiogenesis, leading to fragile and in some cases hemorrhagic-prone vessels. Furthermore, high levels of VEGF show a positive association with symptom intensity in patients diagnosed with IC/BPS[16]. The elevation of VEGF may initially

act as a protective mechanism in response to IC/BPS, but research suggests that these elevations can progress to harmful adaptations, such as edema, and inflammation[17,18]. Additionally, VEGF increases permeability of the bladder wall and negatively impacts the barrier functions of the urothelium[19,20]. This may lead to exposure of the underlying tissues to the harsh chemical composition of urine and is linked to sensations of urgency and pain during the filling phase as well as urinary frequency[21]. Downstream this may be associated with maladaptive changes in the lamina propria and detrusor, or a suboptimal communication between the urothelium and the nervous system.

It was also shown that bladder neck mucosa in WAS rats expresses a significant increase in proNGF, which can be linked to previously reported increases in nerve growth factor (NGF - the mature form of proNGF) in IC/BPS patients[22,23]. ProNGF can, binding selectively to p75ntr in the absence of binding to TrkA, initiate apoptotic processes[24,25]. Considering the decay of urothelial health in IC/BPS[26], the elevated expression of proNGF, and associated initiation of apoptotic processes, may adversely affect restorative capacities of the urothelium leading to a further deterioration of LUT health and potentially worsening of IC/BPS complaints.

In chapter four the working mechanisms that may underlie de established therapeutic effect of the implantation of an intravesical balloon for the management of SUI were evaluated. In SUI, the outlet region of the LUT is unsuccessful in maintaining continence during episodes of high intra-abdominal pressure. When the pressure in the abdomen increases the pressure in the bladder increases with the same magnitude and the urethral pressure needs to increase as well in order to keep the bladder outlet closed. In SUI the urethral pressure does not rise sufficiently and urine leaks from the bladder. Therapies for stress incontinence aim to decrease the pressure difference between the bladder and the urethra and most often do this through mechanisms that help to elevate the urethral pressure (such as pelvic floor physiotherapy or the implantation of a tension-free vaginal tape). In contrast, by implanting an intravesical balloon that absorbs the increase in intravesical pressure in response to an increase in intra-abdominal pressure the pressure difference between the bladder and urethra decreases because the magnitude with which the intravesical pressure increases is lowered.

Video urodynamic data were collected from patients before and one week following placement of the balloon system. It was shown that the balloon significantly decreased in size during episodes of high intra-abdominal pressure (coughing), and that this change in shape is seen mainly along the cranio-caudal axis. The decrease in size of the balloon is suggested to reflect partial absorption of the increase in intravesical pressure resulting from the increase in intra-abdominal pressure. This effect may decrease the pressure difference between the bladder and the urethra and may help to maintain continence in SUI patients.

The pelvic floor provides essential support to the LUT and fulfills a crucial task in the maintenance of continence[27-29]. The data showed that the bladder changes in shape after implantation of the balloon, becoming significantly more vertically oriented. This may change

the supportive contact with pelvic floor muscles and may improve kinking capabilities of the urethra, leading to higher urethral pressures during episodes of elevated intra-abdominal pressure. To provide additional support for this proposed mechanism, displacement of the bladder neck before and after implantation of the balloon was investigated. An R script that identifies the most caudal limit of the bladder and extracted the maximal displacement of the bladder neck from the data was designed. The difference between maximal displacement before and after implantation of the balloon was statistically tested and proved to be significantly increased after balloon implantation. This indicates that optimal bladder neck mobility, and hence urethral kinking capacities, are improved by implantation of the balloon.

Frame-by-frame computerized analysis of video urodynamic data offers an approach to study biomechanics in the pelvic floor related to LUT control that has largely been underutilized. The analyses presented here show how working mechanisms and effectivity of therapeutic developments can be evaluated using this approach. Future developments can employ video urodynamic data to extract parameters that can be fed into computational models of the bladder and pelvic floor. Computational modelling of the bladder and pelvic floor would, for example, enable investigation of the effects of pelvic floor stiffness on bladder shape, and the effects of bladder shape on pressure transfer to the urethra under conditions in which all other parameters can be kept constant. These developments are indispensable to develop more specific curative concepts for patients suffering from SUI.

The brain stem fulfills crucial tasks in the control of a number of autonomic processes, but until now has not been studied in detail in the awake and conscious human situation. Although spinal cord and brain stem activity related to efferent bladder control have been well studied in rodents[30,31], due to a lack of good models, not much research has focused on human brain stem activity related to these processes. In addition to studying efferent bladder control, human models are also indispensable to assess CNS activity related to subjective bladder sensations. Recent neuroimaging developments have enabled the investigation of cortical activity related to bladder sensation and bladder control[32-34]. Moreover, ultra-high-field neuroimaging approaches can now be utilized to investigate not only structure but also function of the human brain stem at unprecedented resolution in order to translate rodent studies to the human situation and gain a more integrated understanding of the CNS control of the LUT.

In chapter five a novel methodological approach to study functional organization of the human PAG was proposed. The PAG was parcellated into clusters based on their fMRI blood-oxygen-level-dependent (BOLD) signal using connectivity-based parcellation methods on 7 Tesla resting-state functional magnetic resonance imaging (fMRI) data. This approach allows for the identification of anatomical regions that differ from one another based on correlations in BOLD fluctuations during resting-state[35]. These resting-state based parcellations reflect an underlying anatomical construct[36] and this enabled us to parcellate the PAG into anatomically distinct clusters. Since the underlying anatomy remains constant, it was shown that PAG

parcellations based on resting-state fMRI data during an empty and a full bladder state express a high overlap with one another, and are organized in a symmetrical lateralized fashion that corresponds to earlier neuroanatomical findings[37,38]. To demonstrate the clinical potential of a robust functional parcellation of the PAG, an exploratory application which can be used to study connectivity changes between different subregions of the PAG related to changes in bladder fullness and bladder sensations was introduced. To this end, a novel methodological approach was developed to identify "dynamic response profiles" of PAG activity related to subjective bladder sensations during a bladder filling and emptying protocol that, when established in healthy controls, could serve as a benchmark to which LUTS patients' response profiles can be compared.

These dynamic response profiles have, so far, been established by our group at the level of the PAG, but several other brain stem regions involved in the control of the LUT, like the pontine micturition center and pontine storage center, can be analyzed in the near future according to the same principle. These connectivity profiles can be established within a given brain stem nucleus, but can also be utilized to investigate the connectivity patterns between different nuclei.

Chapter six further investigates this data-driven approach to functionally subdividing the human PAG based on ultra-high-field fMRI data. The similarity of PAG parcellations at the group level in was assessed order to pave the way for the development of population maps of PAG functional connectivity patterns. The establishment of robust PAG maps in both healthy and patient populations is a crucial step in the movement of this methodological approach towards clinical applications. For this group level analysis, all data was transformed to a standardized space (MNI space) and a PAG mask was drawn on the MNI template. This corrected for individual differences in orientation of the brain and enabled selection of the same voxels for each participant. The data was parcellated using the approach developed in chapter five and random parcellations were created by randomly shuffling the correlation matrix that holds information regarding the correlations between all voxel combinations within the PAG mask. In this way information regarding spatial proximity and functional connectivity was removed from the matrix, but a range of correlation values that corresponds to the original data was maintained. These randomized matrices were used to create parcellation maps against which the similarity between clusters resulting from the data from study participants could be statistically tested. The results showed that in many cases the similarity between clusters from different participants was significantly higher than could be expected based on chance.

These analyses enable functional mapping of the human PAG at the group level. In this study there was a focus on fMRI data obtained during full bladder states in healthy subjects, but this approach opens the door to compare consistency of empty and full bladder parcellations in patients suffering from LUT dysfunction, to group based parcellations obtained from healthy volunteers. Since the PAG fulfills an important role relaying bladder sensory information from the bladder to higher subcortical and cortical areas, as well as

relaying information from these higher brain areas towards the pontine micturition center and spinal cord[39], PAG functional connectivity patterns and activity patterns in the different clusters may reflect the PAG's current task in the central control system of the LUT. As such these maps of functional organization of the PAG will enable future assessment of dynamic connectivity changes within the PAG at the group level during bladder sensory tasks or micturition paradigms. Although the columnar organization of the PAG is not directly apparent when visually assessing the parcellation maps the resting-state fMRI signal in the awake and alert full bladder state in which participants were scanned is fundamentally different from the animal and post-mortem human studies that have been conducted in the past. Future research is needed to further elucidate the exact nature of the relationship between resting-state fMRI based parcellations and the neuroanatomical columnar organization that can be observed using immunohistochemical methods. A step in this direction could be to manually segment PAG fMRI data into columnar modules and assessing the modularity by determining the Q-value, this enables assessment of the extent to which the underlying data has a modular data structure[40]. Q-values from this "forced" segmentation approach could then be compared to data-driven parcellations. Once functional maps of the PAG have been established at the group level, the hypothesis that activity of different functional clusters in the PAG changes as a function of changes in bladder sensations can be tested. Pursuing scientific endeavors in this direction can help to identify PAG response profiles of functional connectivity patterns associated with physiological and pathophysiological voiding behavior. If successful, these response profiles could be beneficial as predictive factors of therapeutic effectivity and may, for example, even help to optimize stimulation parameters for patients treated with sacral neuromodulation. Even though ultra-high-field fMRI research may be a relatively costly procedure to conduct for each patient, the establishment of reliable predictive factors for sacral neuromodulation and objective metrics quantifying the therapeutic effect could prevent patients from undergoing unnecessary invasive procedures and the implantation of costly devices in patients that will not benefit from them. Additionally, these advancements may help to readjust and optimize stimulation parameters in patients that have lost therapeutic effect after implantation of a neuromodulation device.

Voiding is a behavior that is highly influenced by our social environment and we continuously have to evaluate our levels of bladder fullness to prevent us from having to void in socially unacceptable circumstances. The learned and social aspects of voiding behavior are difficult to translate to animal models, since the sensations related to bladder fullness and desire to void are often dealt with at a lower (reflex) level in the nervous system. Some animals can be taught to void and defaecate in a designated location and that will allow to evaluate models of incontinence and therapy effectivity. Up until now this offered the only model to investigate CNS activity related to LUT control in detail. In addition, in animals voiding may also have a territorial, defensive or sexual purpose. In the human situation appropriateness of emptying one's bladder

is largely a learned social behavior, and the previously mentioned instinctive reasons to void, apart from an occasional return to these rudimentary reflexes in dysfunctional conditions, are largely part of our evolutionary past. A great deal of our current understanding of the central control of the LUT is based on animal work, and new models such as the one described in the second part of this thesis (chapters four, five, and six) are needed to evaluate and validate these findings in a human population. The development of models to measure activity in the human situation, have a high preference over alternative models like "organ-on-a-chip" or organoids. The described measurement model will provide a better understanding of the control of the LUT leading to more optimized treatment possibilities and the identification of new treatment targets compared to those used in animal studies. Moreover, this tool will even help to integrate previous findings in animal models and put these in a human perspective.

The research conducted in the first part of this thesis (chapters two and three) provides insights in local changes in the bladder in conditions of LUT dysfunction. The methodological developments presented in the second part of this thesis (chapters four, five, and six) will enable future research to integrate changes at the local level with assessment with evaluation of functioning of pelvic support structures and central control of the LUT. Combining research conducted in animals with ultra-high-field fMRI studies using human participants enables an unprecedented translational approach to studying LUT control, and will even increase the translational value of animal research that has been conducted in the past. This is essential to gain a better understanding of the course of events leading to dysfunction in different patient populations as well as to gain insight in compensatory adaptations at these different levels. This will enable identification of the exact working mechanisms of medications and therapies in humans so that we can improve the care we can offer to patients, and decrease the dependence on animal research to make progress in a more effective manner.

FUTURE DIRECTIONS

Future studies aiming to elucidate mechanisms underlying LUT dysfunction in humans should aim to improve the translational value of scientific endeavors by including markers which are known to be related to LUT dysfunction from animal research. For example, in aging study populations urinary markers of oxidative stress should be analyzed and the relationship between these markers and LUT functioning should be assessed. In IC/BPS patients the urinary excretion of trophic factors can be studied alongside symptom severity. Including urinary markers associated with urothelial health and functioning in studies investigating central control of the LUT will, furthermore, enable assessment of central coping mechanisms and will elucidate the relationship between (mal)adaptive changes at the level of the periphery and CNS.

In addition to investigating PAG activity using ultra-high-field resting-state fMRI, activity and functional organization of other nuclei associated with LUT control need to be studied as well. The PAG is known to communicate with the pontine micturition and storage centers and future

studies are needed to assess changes in functional connectivity between PAG clusters and these regions in the pons during bladder filling and micturition in health and disease.

Until now, only a rudimentary understanding of the role that different CNS nuclei fulfill in LUT control was possible. The assessment of interactions between subregions of the involved nuclei will help to refine working models of central control of the LUT that will also serve to develop and assess new and existing treatments such as neuromodulation. For example, since the PAG is known to function as a bi-directional relay station, involved in both storage and evacuation of urine, whole brain connectivity analyses should be conducted for the different functional areas within the PAG.

Furthermore, these neuroimaging developments pave the way for high-resolution assessment of activity at other levels of the central nervous system. At the level of the spinal cord, the Onuf's nucleus is associated with contraction and voluntary relaxation of the external urethral sphincter and knowledge regarding activity of this nucleus in physiological and pathophysiological states will help to elucidate spinal cord mechanisms related to voluntary initiation of micturition. Using the same study protocols at different central nervous system levels will enable integration of changes in activity and functional connectivity between the cortical areas, PAG and pons with activity at the level of Onuf's nucleus.

CONCLUSION

In summary, the results presented in this thesis show that LUT dysfunction induces local maladaptive changes in the urothelium and bladder neck in rodent models. Targeting processes associated with oxidative stress and inflammation are indicated to be useful targets for therapeutic developments for LUT dysfunction associated with aging and IC/BPS respectively. Video urodynamic data was analyzed using an automated approach to assess biomechanics related to LUT function and evaluate working mechanisms underlying therapeutic approaches. Implantation of an intravesical balloon was shown to absorb increases of intravesical pressure during episodes of high intra-abdominal pressure, and enables manipulation of urethral kinking capacities. Lastly, a novel methodology was proposed, using ultra-high-field fMRI, to evaluate brain stem activity related to LUT control. The PAG was reliably subdivided into distinct clusters which show high spatial agreement within and between subjects, and functional connectivity between clusters is indicated to change significantly as a function of bladder sensations. The developments made in this thesis can be used to develop a more integrated systems physiological approach in order to gain a holistic understanding of LUT functioning. The integrated approach that combines central and peripheral measurements in both animal models and human volunteers opens a completely new avenue to investigate the bladder-brain axis. In additional to advancing our understanding of LUT control, these developments will offer novel insights and improved understanding of the regulation of visceral functions in general.

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CHAPTER 8

Summary
Samenvatting

SUMMARY

Lower urinary tract control is dependent on a delicate interplay between systems organized at the local level (bladder, sphincter, and urethra), pelvic support structures, and the nervous system. Lower urinary tract symptoms are associated with dysfunctional changes at one or multiple levels in this control system. In the first part of this thesis, maladaptive changes in the urothelium and bladder neck related to lower urinary tract symptoms are described. Subsequently, methodological approaches, that can be utilized to investigate pelvic and central nervous system control mechanisms of the lower urinary tract, are introduced.

In chapter two a study investigating changes in urothelial health in a rat aging model is presented. It is shown that aging increases expression of markers associated with cellular senescence, oxidative stress and basal levels of reactive oxygen species. Analysis of urothelial cell cultures, furthermore revealed that mitochondrial membrane potentials were significantly decreased in aged animals, and mitochondrial bioenergetics were negatively affected by the aging process. Aging-related increases in oxidative stress and excessive accumulation of reactive oxygen species could significantly impair the extent to which the urothelium can adequately fulfill its barrier and signaling functions, and may be contributing factors underlying lower urinary tract symptoms in the older adult. The mechanisms outlined in this study may be utilized to identify novel pharmaceutical targets to improve aging-associated bladder dysfunctions.

In chapter three it is shown that chronic, psychological stress in rats induces expression of trophic factors that can lead to increased microvasculature formation, in particular around the bladder neck, a region that contains most nociceptive bladder afferents. These findings may indicate a link between angiogenesis and inflammatory factors that contribute to structural changes and pain in interstitial cystitis/bladder pain syndrome.

Chapter four introduces a, largely data-driven, approach to analyze video urodynamic data and investigates potential working mechanisms related to therapeutic effectivity of implantation of an intravesical balloon to manage complaints of stress urinary incontinence. It is shown that the balloon absorbs some of the increase in intravesical pressure resulting from increases in intra-abdominal pressure. Additionally, it is shown that the bladder significantly changes in shape after implantation of the balloon system and bladder neck mobility is increased. These mechanisms may improve urethral kinking capacities and may ultimately decrease the difference in urethral pressure compared to intravesical pressure and, hence, help to maintain continence during episodes of high intra-abdominal pressure.

Chapter five focuses on the parcellation of the human periaqueductal gray using ultra-high-field functional magnetic resonance imaging data acquired during a bladder filling protocol with

simultaneous assessment of subjectively reported urgency and bladder fullness sensations. It is shown that the periaqueductal gray can reliably be parcellated into distinct clusters that show high similarity at the within-subject level between empty and full bladder states. The data, furthermore indicates that the resulting clusters show a symmetrical organization. This is in line with what can be expected based on previous animal and post-mortem studies. The connectivity between different periaqueductal gray clusters is observed to show significant variations associated with changes in perceived urgency and bladder fullness.

The sixth chapter further elaborates on these developments and here the spatial similarity of periaqueductal gray clusters across participants was assessed. It is shown that for many cluster combinations across participants the similarity of spatial organization of clusters in the periaqueductal gray is significantly higher than would be expected based on chance alone. These results indicate that this method of parcellating the periaqueductal gray into distinct clusters based on functional connectivity patterns yields parcellation maps that show high similarity at the group level, and enables further development of functional maps of the human periaqueductal gray. The functional organization and connectivity patterns of the periaqueductal gray in patients diagnosed with lower urinary tract dysfunction can be compared to patterns observed in healthy volunteers, and can be utilized to assess therapeutic effectivity and improve success rates by enabling assessment of predictive factors.

Overall, the research conducted in this thesis shows that rodent models of lower urinary tract dysfunction are associated with detrimental changes in bladder tissue. In particular, oxidative stress and mitochondrial bioenergetics are impaired in aged rodents, and trophic factors associated with maladaptive changes in the bladder neck are increased in a rodent model of interstitial cystitis/bladder pain syndrome. It is shown that implantation of an intravesical balloon in stress urinary incontinence patients initiates changes in bladder shape and mobility, which might contribute to alleviation of symptoms. Next, methodological approaches are introduced that enable the assessment of central nervous system activity related to lower urinary tract control. The periaqueductal gray matter was reliably parcellated into clusters exhibiting unique functional connectivity patterns. These clusters showed a high level of similarity across subjects, and connectivity between different clusters was observed to significantly change during a bladder filling protocol.
These advancements will help to integrate changes at the level of the lower urinary tract and the pelvic support system with adaptive changes in the central nervous system. These developments are crucial to evaluate the effects of therapeutic interventions on the complex interaction between peripheral organ and central nervous system structure and function and will help to develop new, and necessary, diagnostic markers and predictive factors for therapeutic effectivity.

SAMENVATTING

De controle van de lagere urinewegen is afhankelijk van een fijngevoelig samenspel tussen op lokaal niveau georganiseerde systemen (blaas, sluitspier en urethra), bekkenondersteunende structuren en het zenuwstelsel. Lagere urinewegsymptomen worden geassocieerd met disfunctionele veranderingen op één of meerdere niveaus in dit controlesysteem. In het eerste deel van dit proefschrift worden negatieve veranderingen in het urotheel en de blaashals beschreven die een verband vertonen met lagere urinewegsymptomen. Vervolgens worden methodologische benaderingen geïntroduceerd die kunnen worden gebruikt om controlemechanismen van de bekkenbodem en het centrale zenuwstelsel over de lagere urinewegen te onderzoeken.

In hoofdstuk twee wordt een studie gepresenteerd waarin veranderingen in de gezondheid van het urotheel in een verouderingsmodel van ratten wordt onderzocht. Hier werd aangetoond dat veroudering is geassocieerd met een verhoogde expressie van markers gerelateerd aan cellulaire senescentie, oxidatieve stress en reactieve zuurstofverbindingen. Analyse van urotheliale celculturen onthulde bovendien dat mitochondriale membraanpotentialen significant waren afgenomen bij oudere dieren en mitochondriale bio-energetica negatief werden beïnvloed door het verouderingsproces. Verouderings-gerelateerde toename van oxidatieve stress en overmatige accumulatie van reactieve zuurstofverbindingen kunnen de mate waarin het urotheel zijn barrière- en signaleringsfuncties adequaat kan vervullen aanzienlijk verminderen. Daarnaast kunnen deze factoren ten grondslag liggen aan symptomen van de lagere urinewegen bij oudere volwassenen. De mechanismen die in deze studie worden geschetst, kunnen worden gebruikt om nieuwe farmaceutische targets te identificeren om verouderings-geassocieerde blaasdisfuncties te behandelen.

In hoofdstuk drie wordt aangetoond dat chronische, psychologische stress bij ratten expressie van trofische factoren induceert die geassocieerd zijn met verhoogde vorming van microvasculatuur, in het bijzonder rond de blaashals, een gebied dat de meeste nociceptieve blaasafferenten bevat. Deze bevindingen kunnen wijzen op een verband tussen angiogenese en ontstekingsfactoren die bijdragen aan structurele veranderingen en pijn geassocieerd met interstitiële cystitis/blaaspijnsyndroom.

Hoofdstuk vier introduceert een grotendeels datagedreven benadering om video-urodynamische gegevens te analyseren. Deze methode wordt vervolgens gebruikt om mogelijke werkingsmechanismen die geassocieerd zijn met de implantatie van een intravesicale ballon, om klachten van stress-urine-incontinentie te behandelen, te onderzoeken. Het is aangetoond dat de ballon een deel van de toename van de intravesicale druk die het gevolg is van de toename van de intra-abdominale druk absorbeert. Bovendien is aangetoond dat de blaas aanzienlijk van vorm verandert na implantatie van het ballonsysteem en dat de mobiliteit van de blaashals wordt

vergroot. Deze mechanismen kunnen het knikvermogen van de urethra verhogen en zodoende helpen het verschil in urethrale druk in ten opzichte van de intravesicale druk te verkleinen. Dit mechanisme zal bijdragen continentie te behouden tijdens episodes van hoge intra-abdominale druk.

Hoofdstuk vijf richt zich op het in kaart brengen (ofwel parcellatie) van het menselijke periaqueductale grijs in de hersenstam met behulp van ultrahoogveld functionele beeldvorming door magnetische resonantie (fMRI). Dit gebied in de herstenstam speelt een belangrijke rol bij de controle over de lagere urinewegen. Er werd gebruik gemaakt van fMRI data verkregen tijdens een blaasvullingsprotocol met gelijktijdige registratie van subjectief gerapporteerde aandrang en blaasvullingsgevoel. In dit onderzoek werd aangetoond dat het periaqueductale grijs op betrouwbare wijze kan worden opgedeeld in afzonderlijke clusters die op het niveau van de proefpersoon een hoge mate van overeenkomst vertonen tussen lege en volle blaas. De data liet verder zien dat de resulterende clusters een symmetrische organisatie vertonen. Dit komt overeen met wat kan worden verwacht op basis van eerdere proefdier- en postmortemstudies. Daarnaast wordt in dit hoofdstuk aangetoond dat de connectiviteit tussen verschillende clusters in het periaqueductale grijs significante variaties vertoont die verband houden met veranderingen in ervaren aandrang en blaasvullingsgevoel.

Het zesde hoofdstuk gaat verder in op de ontwikkelingen gemaakt in hoofdstuk vijf en in dit hoofdstuk wordt de ruimtelijke gelijkenis van functionele clusters in het periaqueductale grijs tussen deelnemers beoordeeld. Het werd aangetoond dat voor veel clustercombinaties tussen deelnemers de gelijkenis van ruimtelijke organisatie van clusters in het periaqueductale grijs aanzienlijk groter is dan zou worden verwacht op basis van kans. Deze resultaten geven aan dat deze methode om het periaqueductale grijs in afzonderlijke clusters te verdelen, op basis van functionele connectiviteitspatronen, topografische kaarten van het periaqueductaal grijs oplevert die op groepsniveau een hoge mate van overeenkomst vertonen, en verdere ontwikkeling van deze functionele kaarten mogelijk maakt. Verder kunnen op basis van deze ontwikkelingen functionele organisatie- en connectiviteitspatronen van het periaqueductale grijs bij patiënten met lagere urinewegdisfunctie worden vergeleken met patronen die worden waargenomen bij gezonde vrijwilligers. Deze kunnen vervolgens mogelijk worden gebruikt om therapeutische effectiviteit te beoordelen en slagingspercentages te verhogen door de identificatie van voorspellende factoren mogelijk te maken.

Samengevat toont het onderzoek in dit proefschrift aan dat knaagdiermodellen van lagere urinewegdisfunctie geassocieerd zijn met negatieve veranderingen in het blaasweefsel. Met name oxidatieve stress en mitochondriale bio-energetica bleken getroffen bij oude knaagdieren, en trofische factoren geassocieerd met maladaptieve veranderingen in de blaashals waren verhoogd in een model van interstitiële cystitis/blaaspijnsyndroom. Verder is aangetoond dat de

implantatie van een intravesicale ballon bij patiënten met stress-urine-incontinentie, naast het absorberen van een gedeelte van de verhoging van de blaasdruk veroorzaakt door toename in intra-abdominale druk, veranderingen in blaasvorm en mobiliteit veroorzaakt. Deze processen zouden kunnen bijdragen aan de geobserveerde vermindering van symptomen als gevolg van deze therapie.

Vervolgens zijn methodologische benaderingen geïntroduceerd die de beoordeling van de activiteit van het centrale zenuwstelsel met betrekking tot de controle van de lagere urinewegen mogelijk maken. Het periaqueductale grijs is op betrouwbare wijze verdeeld in clusters die unieke functionele connectiviteitspatronen vertoonden. Deze clusters vertoonden een hoge mate van overeenkomst tussen proefpersonen, en de connectiviteit tussen verschillende clusters bleek aanzienlijk te veranderen tijdens een blaasvullingsprotocol.

Deze vorderingen zullen helpen om veranderingen op het niveau van de lagere urinewegen en het bekkenondersteuningssysteem te integreren met adaptieve veranderingen in het centrale zenuwstelsel. Deze ontwikkelingen zijn cruciaal om de effecten van therapeutische interventies op de complexe interactie tussen de structuur en functie van perifere organen en het centrale zenuwstelsel te evalueren en zullen helpen bij het ontwikkelen van nieuwe, noodzakelijke, diagnostische markers en voorspellende factoren voor therapeutische effectiviteit.

CHAPTER 9

Impact

Involuntary urine loss and lower urinary tract dysfunction are major health problems and a burden to patients and their caregivers. The impact urinary incontinence has on the quality of life of patients and their self-reliance is so severe that it is one of the main reasons to move to institutionalized care for elderly people. The lack of a complete understanding regarding the central control of micturition limits our knowledge of the dysfunctional mechanisms associated with different types urinary incontinence, possible central compensatory mechanisms, and the extent to which we understand how certain interventions lead to their therapeutic effect. This severely hampers the care that can be offered to patients because it is not known what the most optimal therapeutic targets are and for many therapies predictive factors regarding effectivity are missing. The work discussed in this thesis will improve our understanding of the control of the lower urinary tract and will, thereby, help to fill this knowledge gap.

Some of the most significant shortcomings of current diagnostic and therapeutic approaches are the almost exclusive focus on the peripheral organs, the non-specific patient characteristics related to effectivity prediction, and a broad definition of the disease. This leads to treatment selections based on trial-and-error principles and step-up therapies. In some cases, up to 6 iterations of therapy selection are needed before finding an effective treatment. Most compounds that were evaluated for pharmacological treatments showed effectivity in animal research, however, numerous of these compound candidates have entered very costly clinical trials and showed no benefit in the human situation, wasting animals' lives, money, and above all impose unnecessary risks on human trial participants.

Currently existing therapies are not only insufficient in treating lower urinary tract symptoms, but also often cause severe side-effects which are frequently the reason for therapy discontinuation. Therefore, there is a high need to better optimize existing therapies and develop novel therapeutic approaches. The developments emerging from this project contribute to unravelling the mechanisms related to interaction between neuronal control systems and lower urinary tract functioning. As such, this work is expected to contribute to the development of novel, innovative therapeutic approaches. It will enable the discovery of predictive factors, and more optimally targeting of mechanisms involved in certain types of urinary incontinence and. This will help decrease the burden on patients by more efficient matching with effective therapies. During the past decades, modifying sensory information by pharmaceutical therapies or neuromodulation has more often become the target of therapies for lower urinary tract symptoms.

In the current project the structural and functional integrity of the bladder wall in rat models associated with lower urinary tract dysfunction was assessed. Furthermore, approaches to evaluate biomechanics of the pelvic floor associated with stress urinary incontinence and successful therapeutic intervention were developed. In addition, novel neuroimaging methods that help progress our understanding of central nervous system control of the lower urinary tract in human participants at the level of the brain stem were introduced. These developments pave the way towards a unified theory of visceral lower urinary tract sensation and control. In the near

future, these developments will be integrated with more widely used diagnostic tools (e.g. (mobile) urodynamic assessment, micturition diaries, patient reported bladder sensations, LUTS questionnaires, and momentary assessment tools) to investigate correlates between the different biometric modalities. The integration of different measurement models offers new insight into the diagnostic value of each tool on its own and will enable further optimization of the differentiation capacities of diagnostic procedures.

CHAPTER 10

Appendix

CURRICULUM VITAE

Mathijs de Rijk was born in Heerlen, the Netherlands on the 25th of June 1991. He attended secondary education at the Broekland college in Hoensbroek where he obtained his VMBO-T+ degree in 2007. After following the vocational education program in Water management at Citaverde College for one year he decided to pursue secondary education at a higher level and obtained his HAVO degree from Arcus College in 2011. After turning 21 he decided to apply for the Colloquium Doctum exams that enable enrollment into university and started the bachelor program in Psychology at Maastricht University in 2012. During his bachelors he followed a minor in Biological Psychology. His bachelor thesis focused on how mechanisms of neural plasticity may influence the subjective experience of musical timbre. He finished his bachelor in 2015 after which he started the master in Neuropsychology at Maastricht University. For his master internship he worked with Dr. Peter Stiers (Department of Neuropsychology and Psychopharmacology, Faculty of Psychology and Neuroscience, Maastricht University) to assess consistency of cortical parcellations based on functional and structural connectivity measured by functional magnetic resonance imaging and diffusion tensor imaging. During the final months of his master studies he additionally worked as a research assistant with Prof. Frits Prinzen (Department of Physiology, Faculty of Health, Medicine and Life Sciences, Maastricht University) where he digitized and analyzed electrocardiograms of left bundle branch block patients for a study aiming to optimize patient selection for cardiac resynchronization therapy. While finishing his master program, Mathijs was introduced to the neuroimaging studies being developed in the urology group by Dr. Job van den Hurk (Scannexus), Dr. Sajjad Rahnama'I (Department of Urology, Faculty of Health, Medicine and Life Sciences, Maastricht University) and Prof. Dr. Gommert van Koeveringe (Department of Urology, Faculty of Health, Medicine and Life Sciences, Maastricht University) and became enthusiastic about the possibility to continue his academic adventure as a PhD-candidate in their group. During the past 5 years, Mathijs has studied the control of the lower urinary tract using a variety of methodological approaches. He presented his work at multiple international conferences and won several awards. During the last year he obtained a Prins Bernhard Cultuurfondsbeurs which enabled him to move to Pittsburgh, United States for a research visit with Prof. Dr. Lori Birder. Under Dr. Birder's supervision he investigated changes in bladder structure and functioning associated with lower urinary tract dysfunction. The results of his studies are presented in this thesis. For his postdoc, Mathijs will continue his research on interactive control of the lower urinary tract in the group of Prof. Dr. van Koeveringe at the Department of Urology, Faculty of Health, Medicine and Life Sciences, Maastricht University.

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Fernandez Chadily, S.†, **de Rijk, M.†,** Janssen, J., van den Hurk, J.‡ & van Koeveringe, G.‡ (2023). Assessment of brainstem functional organization in healthy adults and overactive bladder patients using ultra-high field fMRI. *Biomedicines, 11, 403.*

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ORAL PRESENTATIONS

de Rijk, M., Peter, S., Wolf-Johnston, A., van Koeveringe, G., Birder, L. (2022) Aging results in reduced sensory innervation of the bladder trigone in rats. *Podium short oral presentation at the annual meeting of the International Continence Society: ICS 2022, Vienna, Austria.*

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de Rijk, M., Wolf-Johnston, A., Kullmann. A., Taiclet, S., Shiva, S., & Birder, L. (2021) Increased oxidative stress with age negatively impacts the urinary bladder urothelium. *Abstract presented at the annual meeting of the International Continence Society: ICSOnline.*

de Rijk, M., Wieringa, P., & van Koeveringe, G. (2020) Implantation of an intravesical balloon increases bladder mobility and reduces complaints of stress urinary incontinence, a video urodynamic study. *Abstract presented at the annual meeting of the International Continence Society: ICSOnline.*

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de Rijk, M., Janssen, J., Fernandez Chadily, S., Birder, L., Rahnama'i, M., van den Hurk, J., & van Koeveringe, G. (2022) Unraveling functional organization related to visceroceptive processing in the periaqueductal gray**.** *ePoster presentation at the annual meeting of the International Continence Society: ICS 2022, Vienna, Austria (selected best abstract in category).*

de Rijk, M., Pinckaers, S., Freeman, J., & van Koeveringe, G. (2022) Implantation of an intravesical balloon absorbs increases in intra-abdominal pressure and reduces complaints of stress urinary incontinence, a video urodynamic study**.** *ePoster presentation at the annual meeting of the International Continence Society: ICS 2022, Vienna, Austria.*

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