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Understanding the Underlying Mechanism of Age-Related Underactive Bladder and Proposing a Treatment Option to Mitigate its Symptoms

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UNDERSTANDING THE UNDERLYING MECHANISM OF AGE-RELATED UNDERACTIVE BLADDER AND PROPOSING A TREATMENT OPTION TO MITIGATE ITS SYMPTOMS

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Arezoo Gerami Pour

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To: Dean John Volakis
College of Engineering and Computing

This dissertation, written by Arezoo Gerami Pour, and entitled Understanding the Underlying Mechanism of Age-Related Underactive Bladder and Proposing a Treatment Option to Mitigate its Symptoms, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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ABSTRACT OF THE DISSERTATION

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The prevalence of underactive bladder (UAB) increases with age, suggesting a link between age-related processes and lower urinary tract (LUT) symptoms; however, the underlying mechanisms of age-related UAB are poorly understood. UAB is characterized by inefficient voiding and bladder overdistension. Due to the unknown etiology, current therapeutic options are insufficient. Thus, a detailed understanding of its mechanism will facilitate the discovery of new treatments.

In Aim 1, we investigated the relationship between age and systems-level function of the LUT reflexes in three age groups of rats, testing the hypothesis that aging causes voiding reflexes to weaken. We systematically investigated the reflex bladder contractions evoked by combinations of rates of urethral infusion and bladder fill volumes as a function of age. We found that, with the same flow rate, older animals had fewer urethral to bladder contractions (augmenting reflex) than younger animals. Furthermore, old animals needed more fluid in their bladders before augmenting reflex (AR) could be triggered, suggesting a delay in switching the LUT to “voiding mode.”

In Aim 2, we recorded electroneurography from the sensory branch of the pudendal nerve (urethral afferents) in young and old rats to map how the interaction of age and urethral flow rate evokes discharges from sensory neurons. We found that both structural changes in the urethra and degradation of the urethral afferents are responsible for attenuated urethral afferent activation. This is critical because AR is mediated by urethral sensory nerves, and this reflex is required for efficient voiding.

Aim 3 examined the effects of percutaneous pudendal nerve stimulation on UAB symptoms in aged rats. The bladder was filled, and the stimulation was applied continuously until the end of the first voiding contraction. We showed that the minimally invasive stimulation of urethral nerves could increase voiding efficiency and prevent the bladder from overdistension.

In conclusion, we found that there is functional weakening in LUT reflexes with age and reduced urethral sensation, urethral muscle and nerve degradation might be contributing factors to that. This study helps us understand the underlying mechanism of age-related UAB, identify the mechanistic targets and develop therapies to mitigate its symptoms.

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LIST OF ABBREVIATIONS

Lower urinary tract	LUT
Underactive bladder	UAB
Bladder capacity	BC
Augmenting reflex	AR
Micturition reflex	MR
Electroneurogram	ENG
External urethral sphincter	EUS
Electromyogram	EMG
Root mean square	RMS
Voiding efficiency	VE
Threshold stimulation	TS

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to lower urinary tract system

The lower urinary tract (LUT) system has two important functions: continence (holding urine in the bladder) and micturition (complete emptying of urine from the bladder at an opportune time). In these processes, the sympathetic (hypogastric nerve), parasympathetic (pelvic nerve), and somatic (pudendal nerve) nervous systems are all engaged, and neural circuits in the spinal cord, brainstem, and higher centers are responsible for regulating them (5). Pelvic afferents are activated by detrusor muscle distention and send the sensory input from the bladder to the spinal cord. Excitatory efferent signals are sent to the detrusor through the pelvic efferents (23, 43). The hypogastric nerve innervates the ureters and bladder neck, and it provides information related to bladder pressure and/or tension (87, 95), (39). The pudendal afferents carry the sensory information related to the urethra to coordinate continence and voiding, as well as transmitting local cutaneous, rectal, and genital sensations (58, 82). The pudendal afferents are activated when the urethral flow distends the urethra. Since afferent activity was equivalent in response to equal pressures imposed by static or moving fluids, it might indicate that they exclusively respond to pressure (52). However, the urothelium may be sensitive to several dynamic variables such as flow, turbulence, distention, pressure, and derivatives of any of these variables (22). Therefore, more study is needed to map the link between urethral and pudendal afferent output (20).

We require a network of intact reflexes to store and empty the urine. Pelvic afferents are responsible for continuously monitoring bladder volume and/or pressure. When the bladder contains little urine, the guarding reflex prevents unintentional leaking by contracting the urethra and relaxing the detrusor muscle. The LUT is considered to be in continence mode (2, 28) at low bladder pressures, but if the pelvic afferents become sufficiently active, the LUT switches to voiding mode. In this mode, fluid entering the urethra activates pudendal afferents, which initiates a reflex (augmenting reflex) from pudendal afferents to pelvic efferents, relaxing the urethra and causing a bladder contraction (4, 28). For effective voiding, the augmenting reflex (AR) must be activated (66, 67, 78). High levels of pelvic afferent activity activate another reflex from pelvic afferents to pelvic efferents (micturition reflex (MR)), which can elicit a bladder contraction even if there is no urethra infusion (21).

1.2 Underactive bladder

Approximately 10% of elderly people in the U.S. have underactive bladder (UAB) and this number is expected to approach 20% by 2050 (88, 90). UAB is characterized by incomplete bladder emptying, high residual volume, and high bladder capacity (60, 102). The etiology of UAB is multifactorial, leading some urologists to consider it an incurable problem (60, 102). The current treatment options are pharmacotherapy, stem cell therapy, and neuromodulation; however, none of these options are viewed by clinicians or patients as sufficient to manage UAB (60, 102). The lack of entirely effective treatment, in part, is due to the unresolved etiology of UAB. Due to the substantial burden of age-related UAB on global healthcare expenses, a thorough understanding of its mechanism is vital to facilitate the discovery of new treatments for the disease.

1.2.1 UAB diagnostic tests

There are several methods for identifying UAB including, physical examination, catheterization, imaging tests, and urodynamic tests. Physical examination tests evaluate the physical and cognitive ability as this can impact the ability to control the bladder. The catheterization is done by inserting a flexible tube into the bladder through the urethra to measure post-void residual (PVR) volume. The portable bladder scanner is used to measure PVR and determine bladder voiding efficiency. Additionally, it eliminates the danger of damage, pain, and contamination associated with the use of a catheter to determine PVR. Cystometry is a test that identifies UAB with the observation of a large bladder capacity (the bladder volume required to evoke a bladder voiding contraction), a poor sensation of bladder distention, abnormally high bladder compliance, and lack of detrusor contractility (11).

1.2.2 Pathophysiology of UAB

Given the critical role of an intact afferent system in voiding function, UAB may develop when afferent activity levels are impaired (11). Age-related loss of nerve fibers impairs the central and peripheral nervous system, and the resultant loss of function affects the LUT system’s functionality directly. Recent studies have emphasized the deficiency in either afferent signaling or the integrative control of sensory information in spinal and supraspinal centers, which results in weak efferent signaling (27, 34, 61). Degradation of the pudendal nerve with increasing age, for example, may impair the functionality of voiding reflexes because the failure of pudendal afferents can lead to UAB-like symptoms (66, 67, 78, 86). Pelvic afferents have an essential role in bladder fullness sensation. An age-related decrease in lysosomal function in the urothelium can change pelvic afferents' signaling and

alter the bladder's physiological function (84). The amount of acetylcholinesterase-positive nerve in the human bladder decreases with age, suggesting reduced parasympathetic innervation (34). With aging, structural and biochemical changes in nerve fibers, as well as a decrease in regeneration and reinnervating capabilities, degrade the central and peripheral neural systems (91), which can affect the LUT reflexes directly. Oshiro et al. demonstrated that the external urethral sphincter atrophy occurs in elderly rats (62), and urethral sphincter muscle fibers appear to be replaced by connective tissue increases throughout aging (96), both of which could result in a more compliant urethra. Histological studies also revealed an age-related decrease in the number and density of striated urethral sphincter nerve and muscle fibers (70). If the urethra becomes more compliant, the lumen will experience less pressure at the same flow rate. Because urethral afferents are thought to be driven by urethral pressure (53), this could reduce the activity of pudendal afferents, resulting in fewer AR contractions. The AR is required for effective voiding, and its disruption results in UAB-like symptoms (66, 68, 78). Therefore, the impairment of lower urinary tract (LUT) reflexes resulting from the degradation of bladder and urethral nerves may contribute to UAB symptoms.

1.2.3 Treatment options for UAB

The goal of treatment for under bladder (UAB) is to enhance bladder emptying and quality of life. Regular bladder emptying decreases intravesical bladder pressure and distension. Patients with UAB usually use Intermittent catheterization to empty their bladder at regular intervals; however, it increases the risk of infection (11).

Stimulation of bladder muscarinic receptors by agonists such as bethanechol or carbachol has shown beneficial effects in treating UAB. However, they can cause side effects, including nausea, vomiting, diarrhea, difficulty with visual accommodation, and headache. Sacral neuromodulation is a therapy option for neurogenic bladder dysfunction that is currently used in many bladder research studies (38, 72, 75). It can empty the bladder by direct activation of the parasympathetic efferent innervation of the bladder. However, to prevent dyssynergic contraction of the external urethral sphincter, this technique needs dorsal afferents transection. But, by transection of the dorsal nerve roots, remaining sensations and responses such as defecation, erection, and ejaculation are abolished (7). Therefore, there is great interest in alternative approaches that activate the detrusor and cause voiding via electrical stimulation.

Peripheral nerve stimulation is an alternative to sacral nerve stimulation for targeting restoring bladder function with more specificity. Sacral neuromodulation targets the entire sacral nerve instead of pudendal nerve stimulation, which targets a specific nerve or nerve branch. A recent animal study indicates activating sensory fibers in the pudendal nerve can cause bladder emptying (7, 40) by inducing synergic bladder activation and external urethral relaxation (6). This method would be significantly less invasive than sacral anterior root stimulation. The risk associated with surgically exposing the pudendal nerve and implanting electrodes for experimental purposes, however, limits its potential to go further.

1.3 Rational

The prevalence of underactive bladder symptoms increases with age; however, the underlying mechanism of age-related bladder dysfunction is unclear, and its multifactorial nature further complicates attempts to understand it. Currently available therapeutic

options include medication, stem cell therapy, and neuromodulation; however, none of these approaches is deemed sufficient by doctors or patients to manage underactive bladder symptoms (60, 102). Part of the reason for the absence of entirely effective treatment is the unresolved etiology. Given the significant impact of the age-related underactive bladder on global healthcare costs, detailed knowledge of its mechanism is critical for facilitating the development of innovative treatments for this disease.

Aging may lead to LUT dysfunction by compromising the state-dependent network of reflexes responsible for properly maintaining continence and efficient micturition. We require a network of intact reflexes to store and empty the urine and aging might disrupt their coordination. Augmenting reflex is a reflex from urethral afferents to bladder efferent whose activation is required for efficient voiding, and the lack of that leads to UAB-like symptoms (67, 78). Evaluating the functional changes in the LUT reflexes with age may help us understand how aging disrupts the normal function of the bladder.

Degeneration of peripheral nerves associated with age may have a role in the multifactorial pathogenesis of the underactive bladder. The degree of sensory neuropathy caused by diabetes, multiple sclerosis, and normal aging all increase with age and have a strong correlation with UAB (18, 27, 49, 74). By silencing (78, 80) or transecting the urethral afferents (68), animals' voiding efficiency is significantly reduced, indicating that these afferents play a key part in UAB symptoms. Furthermore, people with UAB usually have decreased urethral sensation (26, 47, 63), which made us speculate that reduction in the urethral sensory outflow might be one of the contributing factors to UAB. There are two likely sources from which a reduction in urethral sensory outflow might arise. First, hypotrophy of the pudendal nerve that carries urethral afferents could directly reduce

pudendal afferent activity by damaging fibers that convey urethral sensation. Second, an increase in compliance of the urethral muscle with age could reduce the afferent signaling during a void. As the bladder pushes fluid into the urethra, increases in intraurethral pressure or urethral wall stress drive afferent activation (52); therefore, the same amount of fluid entering a more compliant urethra generates less pressure and wall stress, and in turn, less afferent signaling.

Recent animal and human research show that electrical stimulation (ES) of the pudendal nerve promotes the augmenting reflex and thereby improves bladder voiding in UAB models (13, 66, 98). If age-related loss of urethral sensitivity contributes to geriatric UAB by impairing voiding reflexes, improving the signaling of the pudendal nerve by electrical stimulation might regain some lost function. Therefore, we explored the feasibility of utilizing percutaneous stimulation of the pudendal nerve to improve UAB symptoms. If percutaneous stimulation of the pudendal nerve can improve voiding efficiency in our animal study, it would open a transformative new approach for treating age-related UAB by achieving non-surgical direct access to the human pudendal nerve.

1.4 Design goals

The objectives of this study are to investigate the effect of aging on the dysfunction of LUT reflexes; quantify the contributions to pudendal afferent loss of sensitivity from mechanical factors, which change the pressure the urethra experiences in response to flow, and non-mechanical factors, which are putatively intrinsic to the nerve itself; and to determine if percutaneous stimulation of the pudendal nerve can mitigate UAB-like symptoms using *in vivo* rat models.

1.5 Specific aims

1.5.1 Specific Aim 1: Determine the sensitivity of urethral flow-evoked voiding reflexes with age in the rat

We assessed the ability of fluid entering the urethra at different rates to evoke a bladder contraction as a function of bladder volume and age in three groups of anesthetized rats (young 4-7 months, mature 11-14 months, and old 18-24 months). To understand how aging affects voiding reflexes, we evaluated the reliability of the AR and MR as a function of age, rate of urethral infusion, and bladder fullness. We controlled bladder volume (to activate the pelvic afferents) and urethra flow rate (to activate the urethral afferents) independently using nested catheters that allowed us to fill the bladder and urethra and prevent bladder contractions from expelling urine through the urethra (which is blocked by the catheter) (10, 20, 21). An increase in the urethral flow rate needed to trigger the AR would implicate the sensitivity reduction of AR with age as a contributing factor to UAB. Additionally, to evaluate the possibility of reflex dysregulation with age, we compared the bladder volume at which AR and MR occur in different age groups.

1.5.2 Specific Aim 2: Quantify the effect of age on pudendal afferents activity in response to urethral flow in rats

We evaluated the reduction in urethral sensory with age by recording pudendal afferents activity (electroneurogram (ENG)) and comparing its transient response to flow in young and old animals while we infused the urethra with fluid. The sensory branch of pudendal nerve histologically was examined, and the pressures that developed in the urethra during infusion were measured to understand the driving factors of afferent reduction. While Aim 1 investigated the functionality of LUT reflexes with age, Aim 2 focused on understanding

the age-related urethral sensory loss and helps delineate the mechanical and non-mechanical (neuropathy) factors to that.

1.5.3 Specific Aim 3: Evaluate how percutaneous electrical stimulation of pudendal nerve influences UAB symptoms

We assessed the effectiveness of percutaneous stimulation of the pudendal nerve on UAB symptoms in aged rats. We used a standard urodynamic filling protocol to fill the bladder and apply the stimulation continuously until the end of first observed voiding contraction. The stimulation was applied with a fixed excitatory frequency and different amplitudes. The performance of stimulation on voiding efficiency and bladder capacity was compared with no-stimulation control. These are outcome measures that are relevant to UAB symptoms, and our results showed that the stimulation could increase the voiding efficiency and prevent the bladder from overdistension, which would suggest that percutaneous stimulation of the pudendal nerve may be a viable future therapeutic target for new UAB therapies.

1.6 Organization of the dissertation

Chapter 1 is an introduction to the dissertation that addresses the design goals, the rational, specific aims and organization of the dissertation.

Chapter 2 evaluates the functionality of urethra to bladder reflex with age. We also evaluate the strength of bladder contractions and possibility of delay in the activation of voiding reflexes.

Chapter 3 examines the age-related loss of urethral signaling capability by measuring the afferent activity directly. The age-related changes of urethral tissue (by measuring urethral pressure) and histological changes of urethral afferents with age are evaluated.

Chapter 4 describes the potential of percutaneous stimulation of urethral afferents in improving UAB symptoms in aged rats.

Chapter 5 focuses on the significance of the dissertation, its impact, limitations and the future works that can be addressed to understand the age-related UAB.

CHAPTER 2

EVALUATE THE SENSITIVITY OF URETHRAL FLOW-EVOKED VOIDING REFLEXES DECLINE WITH AGE IN THE RAT

2.1 Introduction to age-related underactive bladder

This chapter was previously published in the American Journal of Physiology-Renal Physiology (32).

Approximately 10% of elderly people in the U.S. have underactive bladder (UAB), and this number is expected to approach 20% by 2050 (40, 42). UAB is characterized by incomplete bladder emptying and high residual volume, and its etiology is multifactorial, leading some urologists to consider UAB an incurable problem (88, 90). The current treatment options are pharmacotherapy, stem cell therapy, and neuromodulation; however, none of these options are viewed by clinicians or patients as sufficient to manage UAB (60, 102). The lack of completely effective treatment, in part, is due to the unresolved etiology of UAB. Due to the substantial burden of age-related UAB on global healthcare expenses, a thorough understanding of its mechanism is vital to facilitate the discovery of new treatments for the disease.

Age-related loss of nerve fibers impairs the central and peripheral nervous system, and the resultant loss of function affects the LUT directly. Recent studies have emphasized the deficiency in either afferent signaling or the integrative control of sensory information in spinal and supraspinal centers, which results in weak efferent signaling (27, 34, 41, 61, 91). Degradation of the pudendal nerve with increasing age, for example, may impair the functionality of voiding reflexes because the failure of pudendal afferents can lead to UAB-

like symptoms (66, 68, 78, 86). Pelvic afferents have an important role in bladder fullness sensation, and age-related decrease in lysosomal function in urothelium can change the signaling of pelvic afferents and alter the physiological function of the bladder (84). The amount of acetylcholinesterase-positive nerve in human bladder decreases with age, suggesting reduced parasympathetic innervation (34). Therefore, the impairment of lower urinary tract (LUT) reflexes resulting from degradation of bladder and urethral nerves may contribute to UAB symptoms.

Age-related attenuation of peripheral nerves may also lead to LUT dysfunction by compromising the state-dependent network of reflexes responsible for properly maintaining continence and efficient micturition. Pelvic afferents monitor bladder volume and/or pressure continuously. When the bladder contains little fluid, the guarding reflex prevents unintended leaking by contracting the urethra and relaxing the detrusor muscle. At low bladder pressures, the LUT is said to be in continence mode (2, 28), but once the pelvic afferents become sufficiently active, the LUT transitions to voiding mode. In this mode, fluid entering the urethra activates pudendal afferents, which triggers a reflex from pudendal afferents to pelvic efferent (augmenting reflex) that relaxes the urethra and leads to a bladder contraction (2, 4, 28, 33, 65). The activation of the augmenting reflex (AR) is necessary for efficient voiding (66, 67, 78, 86). High levels of pelvic afferent activity trigger another reflex from pelvic afferents to pelvic efferents (micturition reflex (MR)) whose activation alone (regardless of urethra infusion) is sufficient to evoke a bladder contraction (21). The bladder volume required for activation of the AR (VOL_{AR}) is approximately 75% of the bladder volume required for activation of MR (VOL_{MR}) in young rats (21, 94), and the potential for fluid in the urethra to trigger AR persists for the entire

time that the LUT is in “voiding mode” (Figure 1). Therefore, the reduced sensation of pudendal and pelvic nerves with increasing age may disrupt the activation of urinary tract reflexes and lead to UAB.

In this study, we assessed the ability of fluid entering the urethra at different rates to evoke a bladder contraction as a function of bladder volume and age in anesthetized rats. To understand how aging affects voiding reflexes, we evaluated the reliability of the AR and MR as a function of age, rate of urethral infusion, and bladder fullness. We controlled the bladder volume (to activate the pelvic afferents) and urethra flow rate (to activate the urethral afferents) independently by using nested catheters that allowed us to fill the bladder and urethra and prevented bladder contractions from expelling urine through the urethra (which was blocked by the catheter) (21, 45, 46). Furthermore, infusing the urethra during continuous bladder filling allowed us to compare the volume at which AR and MR occur in different age groups.

Methods

2.2 Ethical approval

All animal care and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Florida International University. Three groups of young (n=12, 4-7 months), mature (n=12, 11-14 months), and old (n=12, 18-24 months) female Sprague-Dawley rats (Charles River, Charleston, SC, USA) were used in this study, and all animals received food and water ad libitum. All studies were acute. The young and mature rats were bought from Charles River, and rats in the “old” group were kept for 6 to 12 months in an animal care facility under a 12-hour light/dark cycle until they reached the appropriate age for the study. In total, we conducted the protocol on 13

young, 14 mature, and 23 old animals, but we excluded 13 from this cohort (1 in young, 2 in mature, and 11 in old groups) from all reported analysis because we never observed a reflex bladder contraction to urethral infusion under any condition during the study in these animals. Lluel et al. showed that 20% of aged Wistar rats had no bladder contractions, and 60% of them had unstable bladder contractions before voiding in cytometry measurements (56). We also acquired 18 additional animals on which we were unable to perform the experiment (8 died in their home cages of old age, 5 died during or did not respond to anesthesia, and 5 suffered nerve or urethral tissue damage during surgery).

Animals were anesthetized initially with isoflurane gas followed by injecting 1.2g/kg urethane (dissolved as 0.2 mg ml-1 in 0.9% saline solution) subcutaneously using a 27-gauge needle. The foot pinch reflex of animals was tested one hour after injection, and the supplemental doses (0.1 g/kg, every 30 minutes) were injected until the foot withdrawal reflex abated. We used urethane because it provides a stable depth of anesthesia and preserves the urinary tract reflexes in animal studies (57). The body temperature, heart rate (determined via ECG), and vaginal impedance (as a surrogate marker for the estrous cycle (44)) (Stoelting, Illinois) of animals were measured.

Animals were euthanized by intraperitoneal injection of Sodium Pentobarbital after the experiment.

2.3 Experimental protocol

The bladder was exposed via an abdominal incision, and we passed a double-lumen catheter (outer: PE 205 and inner: PE 90, heat-flared tips) through a small incision in the apex of the bladder dome. The outer catheter was tied in place with a suture at the incision site around the flared tip and placed in series with a pressure transducer to monitor bladder

pressure (sampled at 100Hz). The inner catheter extended through the intravesical space and was tied around the proximal urethra, preventing the bladder from voiding through the urethra (Figure 2B). The distal urethral outlet was not obstructed, allowing the fluid introduced to the urethra via the internal catheter to exit distally. We used two computer-controlled infusion pumps to infuse independently room temperature 0.9% saline solution (sodium chloride in distilled water, 154 mM) to the bladder and the urethra. Electromyogram of the external urethral sphincter (EUS) was recorded in a subset of animals by inserting two insulated stainless-steel wires (de-insulated at the tips) percutaneously into the EUS (bandpass filtered from 3 to 3000 Hz, 1000×amplification, sampled at 4000 Hz). All data were recorded on a PowerLab 16/35 system (ADInstruments, Colorado Springs, CO).

Each experiment started with three bladder fills with room temperature saline at the rate of 2-5ml/hr to measure the bladder capacity (BC). Since the urethral catheter prevented any urine expulsion, we identified BC by large, sharp increases in bladder pressure, indicating an attempted voiding bladder contraction. The initial filling rate for BC measurements was 2ml/hr; but the filling rate of the second and third BC measurements was increased in some cases to obtain a bladder contraction after approximately 10 minutes of filling. We increased filling rate since older animals typically had higher BCs (mean ± standard error: 0.43ml±0.09, 0.72ml±0.12, and 0.78ml±0.08 for young, mature, and old animals, respectively), and a larger bladder volume was needed in older animals to evoke a bladder contraction.

One experiment block in our protocol consisted of two phases, an initial bladder filling phase and a second very slow bladder filling phase. In the first phase of the block, the

bladder was filled by a computer-controlled infusion pump (Ellite 11, Harvard Apparatus, Holliston, MA) to one-third of BC over 10 minutes, where BC was determined by the average volume triggering a bladder contraction during the three fill cycles performed before the block. In the second phase, the bladder was filled at 0.5BC/hr for 100-120 minutes (Figure. 2A). In the second phase of the block, a second computer-controlled infusion pump was used to infuse the urethra with room temperature saline at two-minute intervals (the pudendal nerve sensory response to flow can recover after two minutes (19)) for 10 seconds to test the ability of the flow to trigger the AR. Flow rates (denoted with colored vertical lines in Figure 2A) were selected pseudo-randomly by drawing randomly without replacement from the set [0.1, 0.2, 0.3, 0.4, 0.5, 1] ml/min, and when this set was finished, the process was repeated. This selection procedure minimized order effects and ensured that the effect of all flow rates on activation of the AR through a wide range of bladder volumes was tested. The volume at which the AR can evoke bladder contraction (VOL_{AR}) in young rats is approximately 75% of the volume required for activation of MR (VOL_{MR}) (21); therefore, initially filling the bladder to one-third of BC prior to phase 2 saved time while still enabling us to observe the start of bladder contractions evoked by AR and MR as bladder volume increased. At the end of the block, the bladder volume was removed and measured.

To investigate the role of urethral afferents in evoking bladder contractions (AR), the urethra was anesthetized with lidocaine solution (2%) in two young and two old animals, and then the animals were prepared according to the protocol for our main experiment in Figure 2.

Before running the main experiment on two young and two old animals, the suppression of urethral afferent activity by lidocaine anesthesia of the urethra was tested in two additional animals (one young and one old animal). The sensory branch of the pudendal nerve was exposed through a posterior approach, and a bipolar nerve cuff electrode (Platinum–iridium insulated with the contact spacing of 2mm, World Precision Instruments) was used to record the neural activity before and after urethral anesthesia. A catheter was passed through the urethra and tied in place at the bladder neck, preventing backflow of the lidocaine into the bladder. Approximately 0.07ml lidocaine solution (2%) was infused into the urethra for 5-7 minutes. The urethra was washed (after 5-7 minutes) with saline and was infused with 11ml/min. Pudendal nerve activity was suppressed after lidocaine anesthesia of the urethra and continued for at least 100 minutes (which was the approximate length of our experiment). The terminal half-life of lidocaine hydrochloride 2% is 90-120 minutes (25).

2.4 Data Analysis

2.4.1 Bladder contraction detection and classification

Bladder contractions were identified using age group-specific bladder pressure threshold crossings. We computed each age-specific threshold as the average peak-to-peak amplitude of the small bladder contractions that occurred before the big bladder contraction (identified by a sharp rise in bladder pressure) during BC measurements. These small contractions are ostensibly the non-voiding contractions that typically occur prior to unobstructed voiding. The size of the pre-contractions, and therefore the thresholds we used, for young, mature, and old animals were P_{young}=15, P_{mature}=15, P_{old}=12. We used age-specific thresholds because older animals had weaker bladder contraction overall.

Bladder contractions were classified as either caused by the AR or the MR. The AR is triggered by urethral afferents, whereas the MR is triggered by bladder afferents. If the amplitude changes of bladder pressure 30 seconds following urethral infusion crossed the threshold (P_{young}=15, P_{mature}=15, P_{old}=12), the contraction was classified as AR contraction. We classified contractions occurring later than 30 seconds following infusion as MR contraction. Since pudendal afferent outflow subsides within 30 seconds of terminating urethral infusion (8), we attribute contractions occurring after this period to the MR because they are highly unlikely to be caused by the AR, which requires pudendal activation.

2.4.2 Identification of bladder volume threshold for voiding reflex occurrence

Urethral infusion activates pudendal afferents that mediate the AR; however, this reflex is only active in "voiding mode" when the bladder is full enough to generate substantial pelvic afferent activity (21). We refer to the critical bladder volume at which urethral infusion can trigger the AR as VOL_{AR}. We obtained VOL_{AR} from our data in the second phase of the experiment by determining the bladder volume at which the pressure amplitude change of the first urethra flow-evoked bladder contraction is greater than a pressure threshold: P_{young}=15, P_{mature}=15, P_{old}=12. We determined these thresholds by averaging the peak-to-peak amplitude of small bladder contractions (pre-contractions) before the big contraction in BC measurements. Similarly, the volume at which the first MR contraction occurs was named VOL_{MR}. VOL_{MR} represents the bladder volume at which the high level of bladder afferent activity directly leads to a bladder contraction via MR, regardless of the presence of flow in the urethra. Therefore, VOL_{AR} is expected to be less than VOL_{MR} in a normal LUT system (21).

2.4.3 Bladder volume computation

The ureters were not ligated, and their contribution to bladder filling was computed by subtracting the total volume in the bladder at the end of the block (which we removed and measured) from the amount infused through the bladder catheter. We could compute the volume contribution of ureters in this way because all volume remained in the bladder during each block since it was unable to flow around the urethral catheter. We assumed a constant flow rate (21) for ureters in the block and added the ureter contribution in bladder volume to the infused bladder volume. Therefore, we estimated the bladder volume throughout the block and used that information when we were computing VOL_{AR} and VOL_{MR}. We did not ligate the ureters to minimize the invasiveness of our experiment.

2.5 Results

We used independent control of bladder volume and urethra flow under quasi-isovolumetric conditions to evaluate the activation of the augmenting (AR) and micturition (MR) reflexes in young, mature, and old animals. We filled the bladder continuously (to generate a broad range of bladder afferent activation throughout the experimental block) and infused the urethra (to activate urethral afferents) with different flow rates ([0.1, 0.2, 0.3, 0.4, 0.5, 1] ml/min) to compare the ability of each urethra flow rate to evoke a bladder contraction (AR contraction) across different age groups. We also evaluated differences in bladder volumes at which the AR and MR were activated (VOL_{AR} and VOL_{MR}) across ages.

2.5.1 Age-related sensitivity reduction of AR to urethral infusion

We computed the proportion of trials (lines in figure 2A) in which applied urethral flow evoked a bladder contraction at bladder fill volumes above the AR threshold (VOL_{AR}).

Summary data in figure 3 show the proportion of evoked AR contractions averaged across blocks and separated by age group and flow rate. This metric represents the AR activation sensitivity to urethral infusion. We found that the same urethral flow rates evoked fewer AR contractions in older animals than younger ones, suggesting a reduction in urethral sensitivity. This analysis likely underestimates the effect of age-related reduction in AR activity because we excluded animals where we could not evoke any bladder contractions, and the percentage of excluded animals increased with age (7.8%, 14.2%, and 47.8%).

We found the main effects of both age and urethral flow rate ($p<0.001$) on the proportion of evoked AR contractions (bladder contractions evoked by AR), and no interaction effect between age and urethral flow rate ($p=0.224$). We used the generalized estimating equation (GEE, in SPSS) for statistical analysis, where age and urethra flow rate were considered independent variables, and the presence of a contraction for each trial (0 or 1) was considered the response which we modeled with the logit link function. Post hoc statistical tests (Bonferroni) between young and old groups showed that the same urethra flow rate evoked bladder contractions less often in old groups ($p=0.003$ for 0.1ml/min and $p=0.041$ for 1ml/min, and $p<0.001$ for all other urethra flow rates), indicating that the average proportion of bladder contractions evoked by all urethral flow rates decreases with age. The highest tested urethra flow rate (1ml/min) evoked the same proportion of bladder contractions in young and mature rats ($p=0.086$), but it evoked fewer contractions in old rats. The reduction in the number of evoked bladder contractions by the same urethra flow rate across age is consistent with the hypothesis that the sensitivity of AR to urethra flow decreased with age.

To confirm that the augmenting reflex (through activation of urethral afferents) was responsible for the bladder contractions presented in Figure 3, we repeated the experimental procedure on two young and two old animals after lidocaine anesthesia of the urethra. Figure 4 shows the electroneurogram (ENG) of the sensory branch of the pudendal nerve (gray line) and urethral pressure (dashed black line) in response to urethral infusion before (Figure. 4A) and after (Figure. 4B) lidocaine anesthesia of the urethra. The results showed that the ENG response to flow was abolished after urethral anesthesia.

Anesthetizing the urethra with lidocaine eliminated the AR but spared the MR, affirming that the AR effects we observe throughout the paper are driven by urethral afferents. To make this determination, after anesthetizing the urethra, we filled the bladder and infused the urethra with saline using the main experimental protocol (Figure 2). Urethral infusion never induced a bladder contraction, indicating AR was eliminated, but at large bladder volumes, spontaneous bladder contractions occurred that were uncorrelated with urethral infusion, indicating the MR (mediated by bladder afferents) remained intact. This is illustrated in Figure 5, showing bladder pressure from the last 30 minutes of blocks in two old and two young animals (see Data Analysis section for classification of AR and MR contractions). The bladder contractions do not immediately follow (are evoked by) urethral infusion (vertical lines), demonstrating the absence of AR. The presence of MR-evoked bladder contractions (bladder contractions that occur spontaneously at large bladder fill volumes and are not synchronized with the urethral flow) indicates that the lidocaine did not anesthetize the bladder since the MR is mediated by pelvic afferents (20). The result is compatible with previous studies, which indicated that the lidocaine anesthesia of the urethra could abolish the urethra to bladder reflex (67, 78).

2.5.2 Age-related dysregulation of AR and MR

As the lower urinary tract (LUT) system transitions from “continence mode” to “voiding mode” (29), the AR and MR become active to promote voiding; we examined when, as a function of bladder volume, these two reflexes become active and how the activation volumes are affected by age. The AR is gated by pelvic afferent discharge, such that only at high bladder fill volumes ($\geq VOL_{AR}$) the flow in the urethra will reflexively trigger a bladder contraction (21). In young rats, the bladder volume at which we could trigger the AR with urethral infusion was less than the volume at which the bladder underwent spontaneous distention-evoked MR contractions, that is $VOL_{AR} < VOL_{MR}$. However, the volume difference between VOL_{AR} and VOL_{MR} decreased with age and even reversed for the oldest group. Figure 6 shows segments from three experiments starting when we observed the first bladder contraction in the block. These examples illustrate that we could activate the AR at far lower volumes than we could activate the MR, while in old animals, the MR was triggered before we filled to VOL_{AR}.

To quantify the presence of delay in the activation of AR with respect to the activation of MR with age, we compared the proximity of VOL_{AR} to VOL_{MR} in different age groups according to equation (1):

$$Proximity = \frac{VOL_{MR} - VOL_{AR}}{VOL_{MR}} \times 100 \quad (1)$$

The proximity was computed for each animal and averaged across the corresponding animal age group. Figure 7 shows the proximity values for all blocks separated by age groups. The value of the mean and standard error of proximity was 15.9±4.1, 9.4±3.4, and -8.7±7.7 for young, mature, and old groups, respectively. The proximity of 0 indicates that VOL_{MR} is equal to VOL_{AR}, the proximity of 100 indicates that VOL_{AR} occurs even when

the bladder is completely empty, and negative proximity indicates VOL_{AR} does not occur until after VOL_{MR}. The two-sample t-test showed that the proximity of AR and MR in young animals was larger than in old animals ($p=0.006$).

To quantify the transition from continence to voiding mode and activation of voiding reflexes (AR and MR), we use the normalized bladder volume measure, computed as bladder volume/ VOL_{MR} x100. To aggregate bladder response data across animals (which all have different bladder sizes), we divided the filling cycle into bins representing 15% of each animal's normalized bladder volume (Figure 8).

Figure. 8 (top) shows the average pressure-time integral for each urethra flow rate (colored curves) at each bin (x-axis locations). This shows the work (force-time) done by the bladder in response to each urethra infusion trial, as a function of bladder fullness, to understand the transition from continence (no bladder contraction) to voiding mode. Increases in the pressure-time integral begin at lower normalized bladder volumes in younger animals, showing that the AR is activated before MR (bladder volumes $\leq$ 100% VOL_{MR}) in young animals and engaged later (bladder volumes $\geq$ 100% VOL_{MR}) in older animals.

Figure. 8 (bottom) shows the histogram of urethral flow trials within each normalized bladder volume bin, that is, the number of trials used to compute the average curves at each point in panel A. There are unequal numbers of trails across bins because the bladder filling rate (computed based on a series of pre-block fills) was a slightly different fraction of VOL_{MR} for each animal, so each 2-minute intertrial interval represented a slightly different amount of normalized bladder volume. There are no observations at low normalized bladder volumes because urethral infusion starts only after the bladder is filled to one-third capacity, and fewer observations at large volumes because the block duration was based

on a set number of trials. Lastly, the differences in the total number of urethral flow trials between age groups come from the number of blocks for each animal group; we ran two blocks in most of the young animals, but most of the mature and old animals were not stable enough under anesthesia for a second block (i.e., they died or had no bladder contractions in the second block).

2.5.3 Bladder contractions characteristics

We examined changes in peak contraction pressure and contraction duration across age groups during isovolumetric bladder contractions evoked during BC measurements that were performed prior to each block. Figure 9 shows the average peak-to-peak amplitude of bladder contractions and their duration in young, mature, and old animals. Old animals had longer bladder contractions than mature or young animals (t-test, $p<0.001$ and $p=0.01$). In addition, the peak-to-peak amplitude of bladder contractions in old animals was lower than mature ($p=0.007$) and young ($p<0.001$) groups.

2.5.4 Urethra flow-evoked external urethral sphincter response

We evaluated the feasibility of our experimental protocol in detecting voiding contractions in different age groups. In the current study, voiding is not possible because the urethra is occluded; however, the presence of external urethral sphincter (EUS) bursting is a strong indication that voiding would have occurred if the urethra had been unobstructed (19) because rats have bursting activity in the EUS exclusively during voiding (51, 78). We recorded an electromyogram of the EUS in a subset of 3 animals per age group. In Figure 10, the urethra was infused with 0.3ml/min and 1ml/min in three typical animals from different age groups (examples taken from trials during which the LUT system was in voiding mode). An example of bursting activity superimposed on the bladder pressure from

1 second before to 25 seconds following urethra infusion is shown. The results in Figure 10 indicated that the bursting activity is coincident with the rise in bladder pressure in all animals. Therefore, if we are interested in studying systems physiology, yet we separated bladder and urethra, we can identify voiding contractions even though no volume was expelled.

2.6 Discussion

We evaluated the role of sensory feedback from the bladder and the urethra independently on reflex bladder contractions using a split preparation method (21, 45, 46). The results showed that the same urethra flow rate evoked bladder contractions in older animals less often than in younger ones, and within all age groups, higher urethra flow rate trials evoked contractions more frequently. We also found that the AR required a fuller bladder to become active in old rats than in young rats, suggesting a functional decline in age-related activation of urinary tract reflexes associated with the "voiding mode." Old rats also exhibited longer and weaker bladder contractions than the young or mature groups. Taken together, the results are consistent with the hypothesis that the sensitivity of urinary tract reflexes declines with age, which may contribute to some age-related voiding dysfunction such as UAB.

There are a few possible explanations for less flow-evoked AR activation in old animals, including 1) degradation of the pudendal nerve, 2) degradation of the urethral musculature or, 3) the reduced number of urethral cells expressing serotonin, which we briefly discuss below. To determine which of these are fundamentally causally responsible for the reduction in AR activation we observed, we will require more detailed, targeted experiments and could be helpful in identifying new therapeutic targets.

When holding the urethra flow rate constant, the number of contractions evoked by AR decreases with age (Figure 3), suggesting that the sensitivity of AR to urethra infusion weakens with age. We found that there was not a difference in the number of bladder contractions evoked by all urethral flow rates greater than 0.1ml/min (which is likely an insufficient flow rate to recruit any urethra fibers (21)) ($p>0.05$ among all flow rates except 0.1ml/min using Bonferroni). This suggests that 0.1 ml/min is a flow rate threshold for young rats, above which higher flows can activate the AR more frequently. As it was shown in Figure 3, this number was not 100% even in young animals for the highest urethra flow rate. Repeated urethral infusion in a block might limit the effectiveness of the stimulation on evoking the bladder contractions due to the urethra and/or bladder muscle fatigue in our experimental protocol. The proportion of contractions evoked by AR for all urethra flow rates decreased from young to mature groups of animals (except 1ml/min, $p=0.086$). The same urethra flow rates evoked bladder contractions less often in old animals than young animals, indicating that the sensitivity of AR to urethra flow rate decreased with age. Since efficient voiding is dependent upon the activity of urethral afferents responding to urine flow (85), age-related degradation of the pudendal nerve might play a role in decreased activation of AR and incomplete bladder emptying and other UAB symptoms observed in older patients.

Another potential explanation for the observed decrease in AR sensitivity could be age-related degradation of the urethral musculature. If the urethra becomes more compliant, then the lumen will experience less pressure at the same flow rate. Since urethral pressure is the putative driver of urethral afferents (19, 53), this could also lead to fewer AR contractions at the same rate of urethral infusion.

Another contributing factor for less AR activation can be the reduced number of urethral cells expressing serotonin (5-hydroxytryptamine [5-HT]) with age (15). It is believed that the urethra-to-bladder reflex is partially generated by cross-talk between urethral afferents and urethral cells expressing serotonin (50), and the blockade of serotonin receptors in nerve fibers innervating the urethra can block AR (16). Because the low voiding efficiency in aged rats could be enhanced by the application of 5-HT to the urethral lumen, the lower number of 5-HT-positive urethral cells may be responsible for decreased excitation of the urethral afferents and less AR in older animals (15). Additional studies are needed to distinguish between these potential contributing causes to the loss of AR sensitivity.

The late engagement of AR with respect to MR in older animals might also be related to UAB symptoms (straining to void, difficulties with stream). We computed the bladder volumes at which AR and MR were activated and compared their proximity across age groups. The AR was activated far earlier than MR in young animals. However, the activation of these two reflexes occurred almost at the same bladder volume in old animals. Woock et al. (94) showed that there is a strong correlation between bladder volume (which is highly correlated with pelvic afferent activity) and the magnitude of pudendal nerve stimulation-evoked bladder contractions, indicating that the superposition of pelvic and pudendal afferent activity evokes the augmenting reflex bladder contraction. Therefore, a possible reason for the delay in the activation of the AR in older animals might be the reduction in the sensitivity of urethral afferents to urethra infusion. In other words, after urethral afferent sensitivity loss, the activation of AR may require so much pelvic afferent outflow that the AR occurs only after the MR becomes active (i.e., $VOL_{AR} \geq VOL_{MR}$).

Pelvic afferent outflow (bladder filling) is the putative driver by which the lower urinary tract switches between continence and voiding modes (21), a process that may be disrupted as we age and lead to UAB symptoms. Older animals' bladders needed to be filled to a larger percentage of their maximum before urethral flow could engage the augmenting reflex (Figure 6 and Figure 7), a reflex necessary for efficient bladder emptying (66, 68, 78). This suggests the switch to voiding mode happens early in the filling cycle (i.e., $VOL_{AR} < VOL_{MR}$) for young animals, allowing the augmenting reflex to aid in micturition during voiding events. However, older animals require substantially fuller bladders before the augmenting reflex engages (perhaps due to compromised peripheral sensation), effectively delaying the switch to voiding mode. If this delay also happens in humans, it will result in people attempting to void without the benefit of the augmenting reflex because the urinary tract has not fully switched to voiding mode. Voiding without the augmenting reflex leads to UAB symptoms like straining to void, difficulty initiating voiding, and weak urine streams (30, 68, 89). Together, this suggests that the functional weakening of urethra-mediated voiding reflexes during the filling cycle in old rats contributes to age-related UAB symptoms.

The International Continence Society has defined underactive bladder as low amplitude and/or inadequate duration to empty the bladder completely (1). We examined peak-to-peak amplitude and duration of bladder contractions in BC measurements. Peak-to-peak amplitude increased, and the duration of bladder contractions decreased with age. Purinergic and cholinergic components of the bladder are involved in the magnitude and maintenance (duration) of bladder contractions (81). There was a significant positive correlation between age and the purinergic component of human bladder contraction and a

significant negative correlation between age and the cholinergic component of human bladder contraction (99, 100). Therefore, the impairment of purinergic and cholinergic components of the bladder might alter the bladder contractile properties and leads to incomplete bladder emptying with increasing age.

We also measured the vaginal impedance (a biomarker for the estrous cycle (44)) to control for the potential confounds of hormone cycles on amplitude and duration of bladder contractions. We computed the correlation between vaginal impedance values and bladder contraction amplitude and duration. Neither bladder contraction amplitude ($R=-0.1$, $p=0.5$) nor bladder contraction duration ($R=0.26$, $p=0.12$) was correlated with vaginal impedance, indicating the reflex effects we observe for each animal are unlikely to depend on which phase of the estrous cycle they are in during the study. Our results are consistent with previous studies, which showed bladder contraction pressure does not differ among different stages of the estrous cycle (64).

The development of therapies for UAB requires an understanding of its pathophysiological mechanism (10). For example, Gonzalez and Grill (35) showed that the amplitude of bursting activity of the EUS (which is critical for efficient bladder emptying in rats) was reduced in obesity-prone rats. Therefore, electrical stimulation of pudendal efferent improved voiding efficiency in their study. Since our results indicated that the sensitivity of AR to urethra infusion decreased with age and voiding reflex dysregulation occurred with age, stimulation of the pudendal nerve may be a viable strategy to treat age-related UAB. Therefore, this study can help to frame our understanding of geriatric UAB and develop therapies to improve the voiding reflexes.

Figures

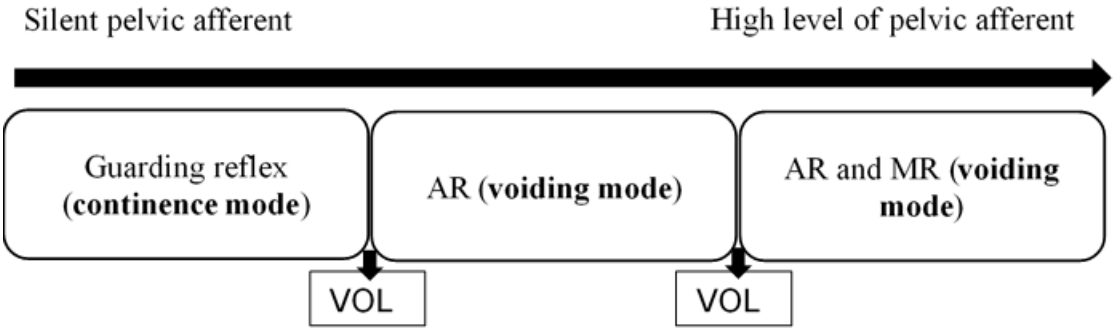

Figure 1. The activation of LUT reflexes in the nervous system. In continence mode, the guarding reflex inhibits the bladder and contracts the urethra to keep the LUT in continence mode. A sufficient level of pelvic afferent activity switches the LUT to the voiding mode. In the voiding mode, fluid entering the urethra activates the pudendal afferent leading to a bladder contraction via the augmenting reflex (AR). High level of pelvic afferent activity triggers the micturition reflex (MR) from pelvic afferents to pelvic efferents, which also evokes a bladder contraction.

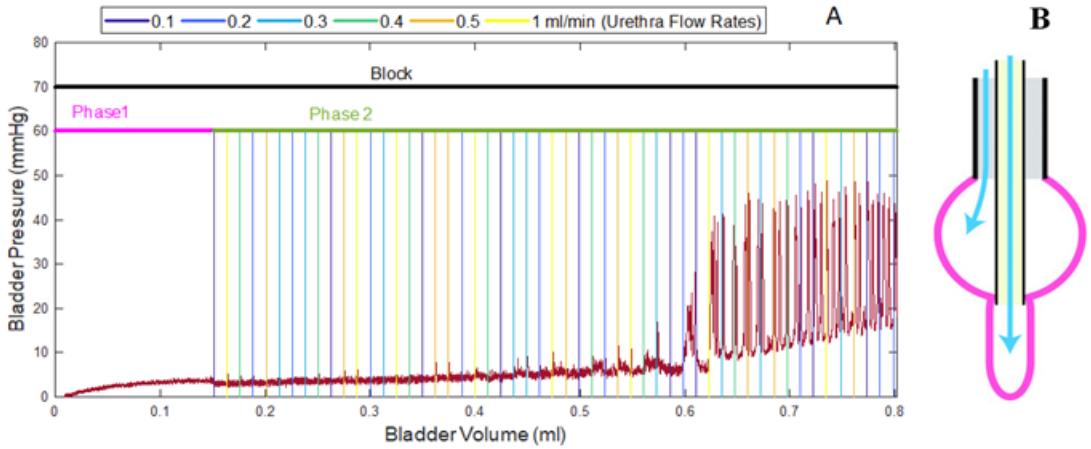

Figure 2. The bladder pressure was recorded (red trace) while the bladder was filled continuously. At two-minute intervals, the urethra was infused at a pseudo-randomly selected flow rate (vertical lines). This block was recorded from a mature rat. In this example, infusing the urethra, at any rate, did not evoke bladder contractions when the bladder volume was less than 0.59 ml, likely because the lack of sufficient pelvic afferent activity kept the LUT system in continence mode; however, above 0.59ml, the voiding reflexes were activated. B: Experimental setup, which shows the double-lumen catheter in the bladder dome and proximal urethra.

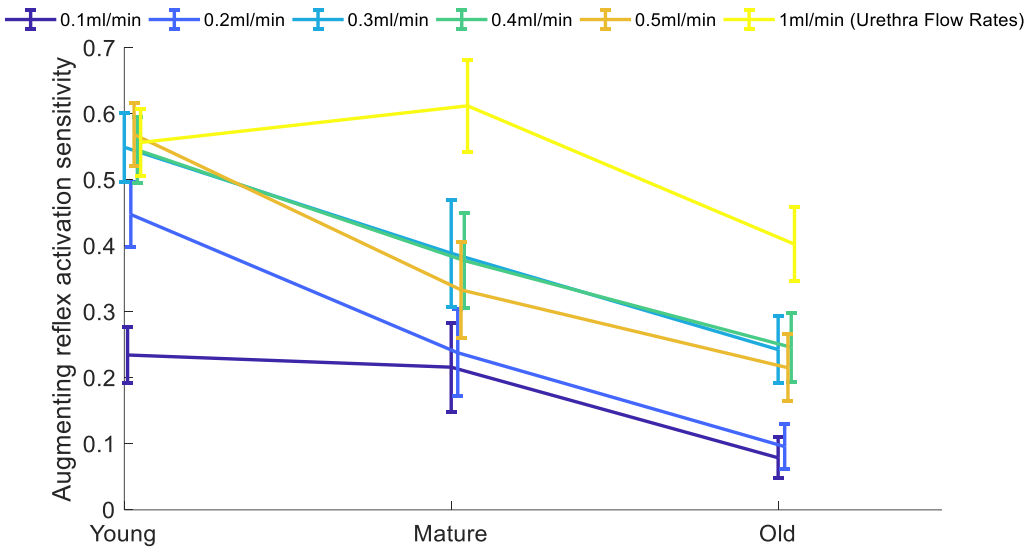

Figure 3. AR activation sensitivity to urethral flow shows fewer evoked bladder contractions in older animals than in younger ones at equal flow rates. The proportion of trials in which applied urethral flow evoked a bladder contraction for each flow rate was computed for each block, averaged across blocks, and separated by age group. Data are displayed as the mean proportion with standard error bars. Horizontal jitter is used to allow clear visualization of mean values. There are main effects of both age and urethral flow rate ($p<0.001$) on the proportion of evoked AR contractions (bladder contractions evoked by AR), and no interaction effect between age and urethral flow rate ($p=0.224$). Horizontal jitter is used to allow clear visualization of mean values.

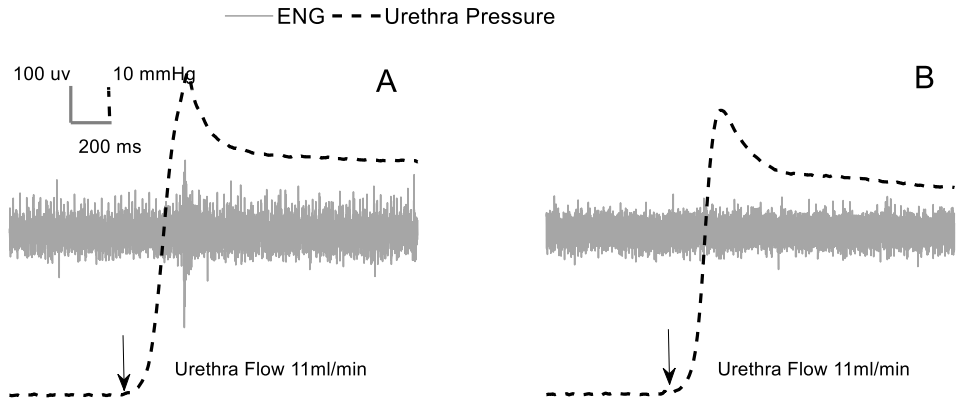

Figure 4. The pudendal nerve activity (gray line) and urethral pressure (dashed black line) before (A) and after (B) lidocaine anesthesia of the urethra to 11ml/min urethral flow (black arrow).

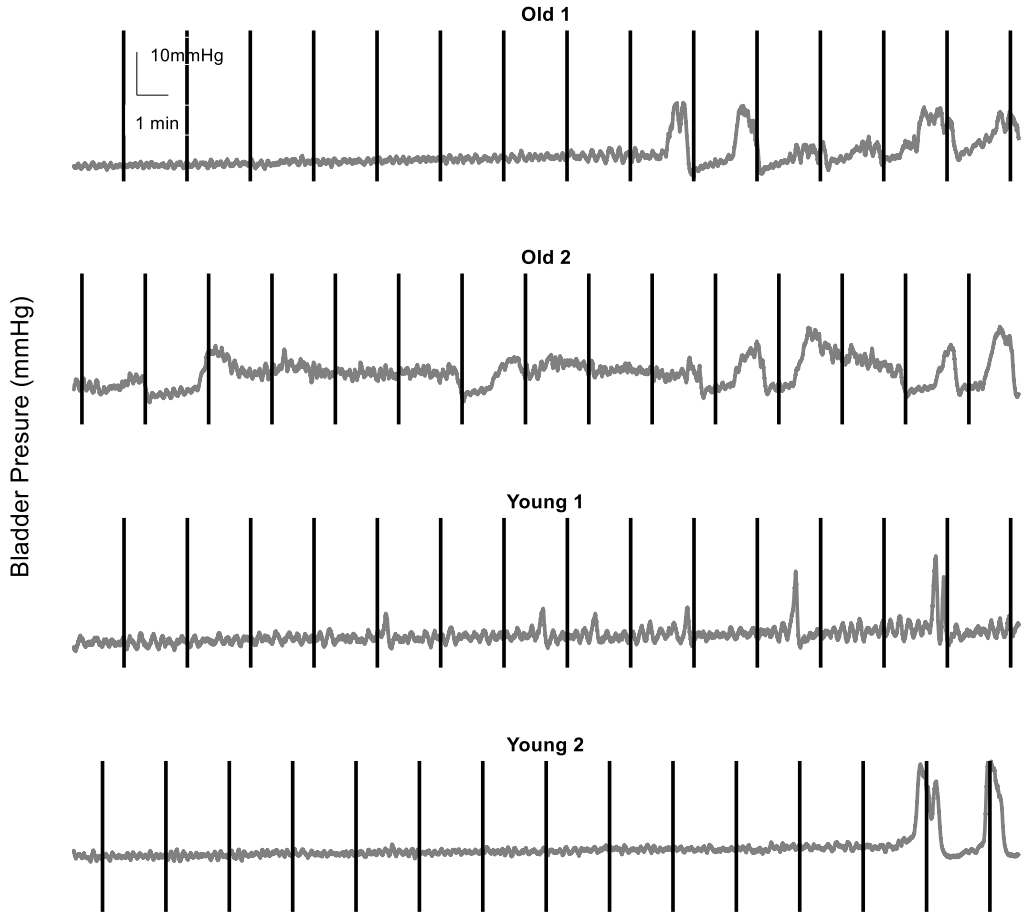

Figure 5. The bladder pressure (gray) at the last 30 minutes of the block in two old and two young animals after lidocaine anesthesia of the urethra in response to the urethral infusion (vertical black lines). The results show that the MR was spared, but the AR was abolished.

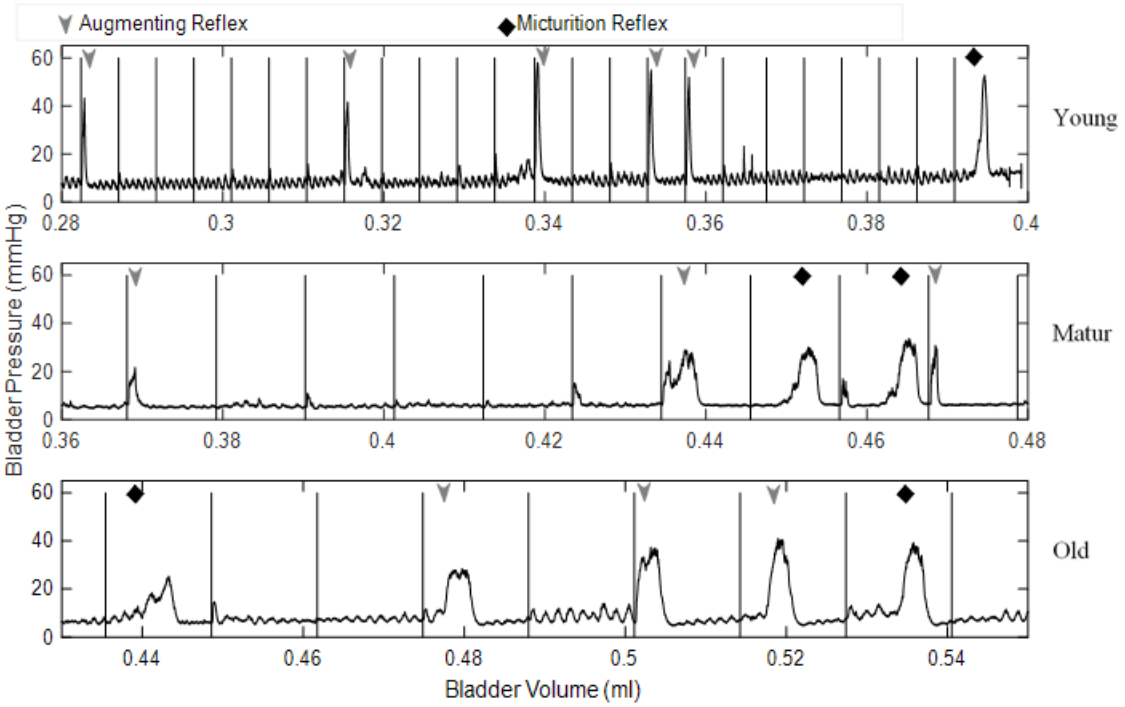

Figure 6. Examples from three experiments beginning at the first bladder contraction in the block for a young (top), mature (middle), and old (bottom) animal. The panels illustrate that the augmenting reflex responds to flow early in the filling cycle for young animals, but the augmenting reflex does not engage until very late in the filling cycle for old animals, not typically occurring until after the bladder is full enough to trigger the distention-evoked micturition reflex contraction. Vertical lines showed when the urethra was infused, and the overlaid symbols indicate augmenting (gray arrow) or micturition (black diamond) reflex contractions. Horizontal and vertical scales are the same across all panels.

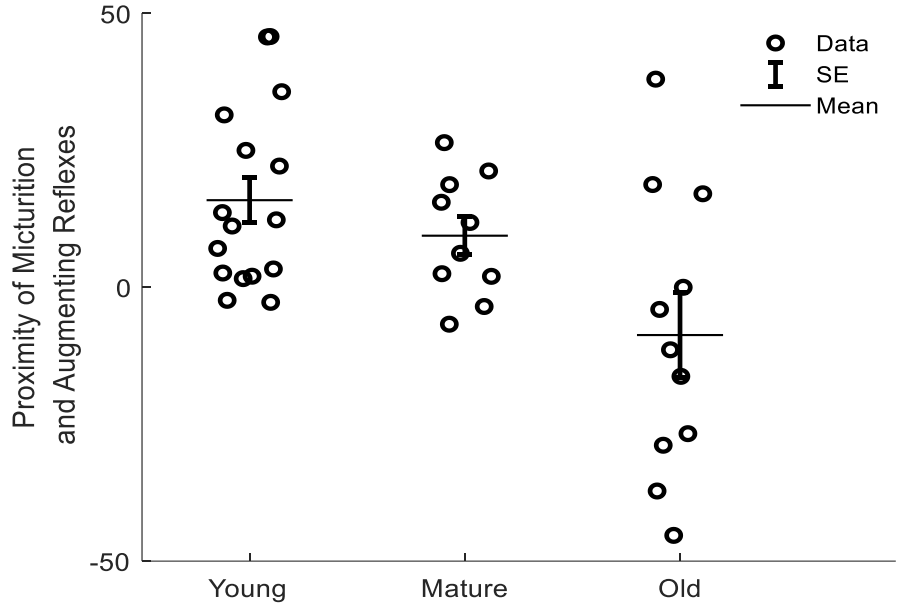

Figure 7. Delay in the activation of AR with respect to the activation of MR for each block, separated by age groups. The proximity (equation 1) was computed for each block and averaged across the corresponding animal age group. A positive proximity value demonstrates that we could activate the AR at far lower volumes than we could activate the MR, while in old animals, the negative proximity value shows that the MR was triggered before we filled to VOL_{AR}. The two-sample t-test showed that the proximity of AR and MR in young animals was larger than in old animals ($p=0.006$).

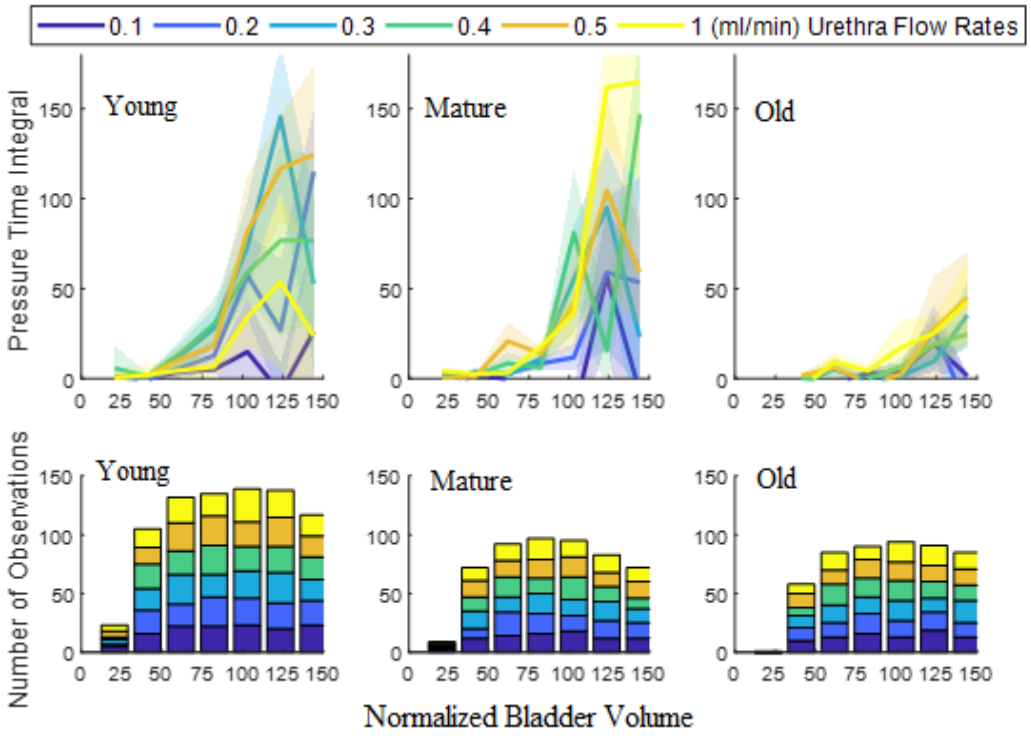

Figure 8. As rats age, the AR is active later in the bladder filling cycle. Top) Average pressure-time integral across all blocks for all animals as a function of bladder volume (percent of VOL_{MR}), separated by urethral flow rate. Bottom) Histogram of the number of trials (urethral infusion) in each bin colored by each trial's flow rate.

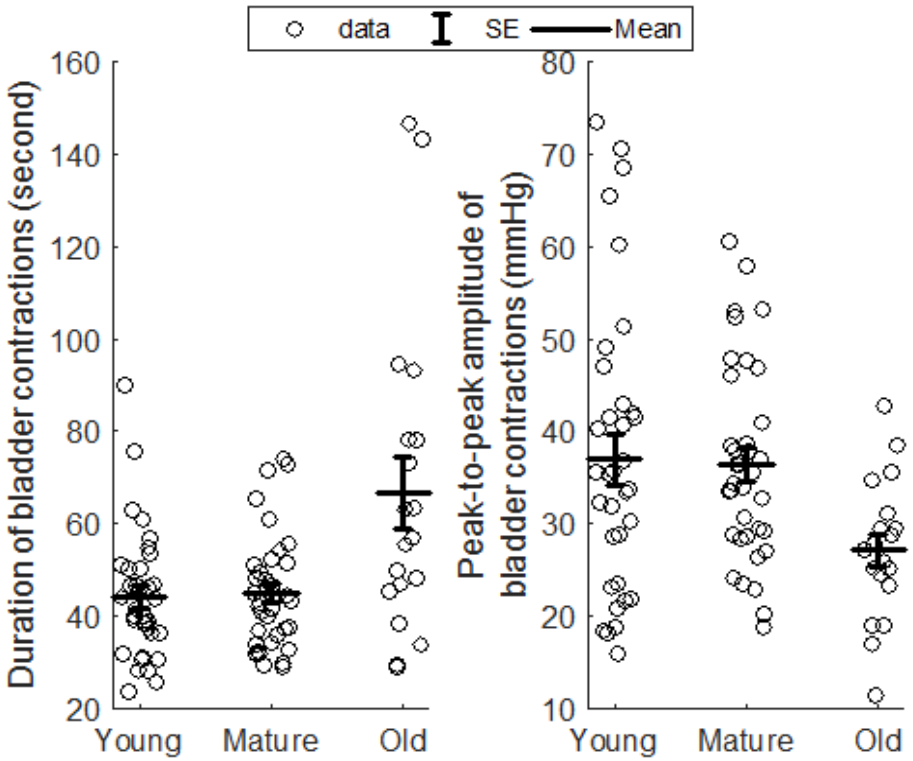

Figure 9. The peak-to-peak amplitude of bladder contractions decreases with age, and the duration of bladder contractions increases with age. Each point is one bladder contraction, and the error bars show the mean ± standard error. Old animals had longer bladder contractions than mature or young animals (t-test, $p<0.001$ and $p=0.01$). In addition, the peak-to-peak amplitude of bladder contractions in old animals was lower than mature ($p=0.007$) and young ($p<0.001$) groups.

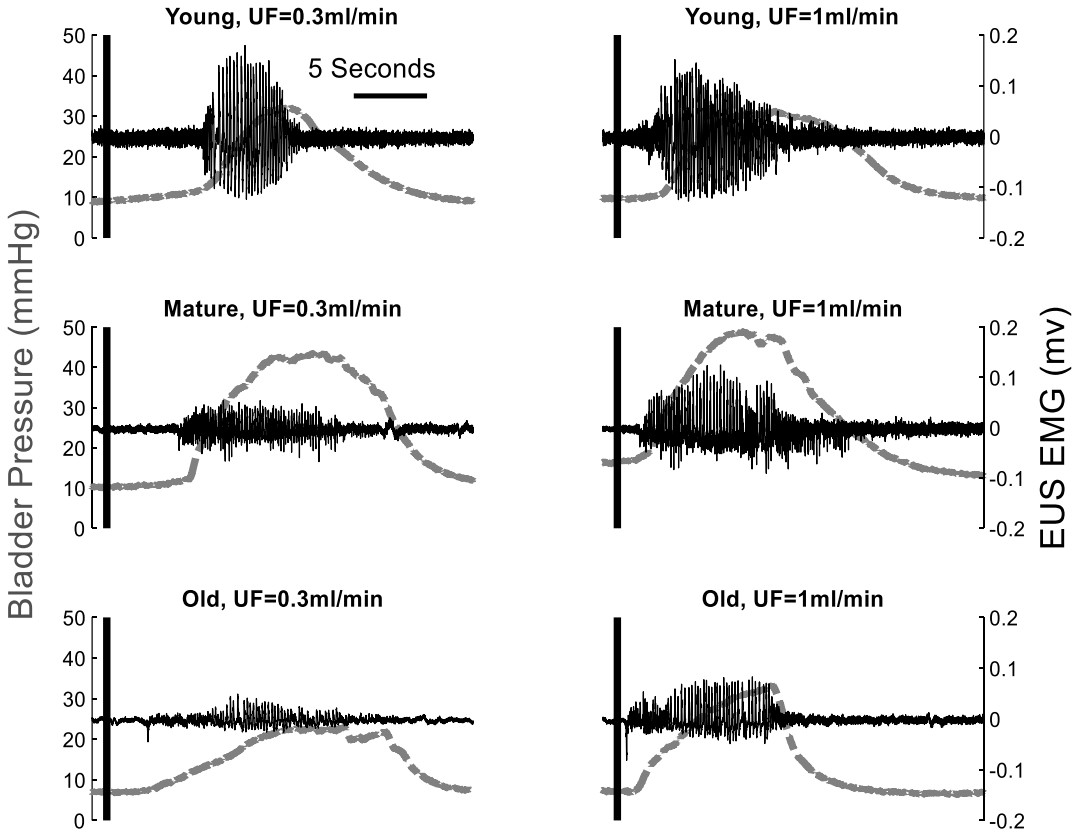

Figure 10. The bursting activity of the EUS superimposed on the bladder pressure for three animals from different age groups. The presence of bursting activity in all animal groups for two different urethra flow rates (0.3ml/min and 1ml/min) showed that we could identify the voiding contractions, even though the bladder cannot void around the inner lumen urethral catheter.

CHAPTER 3

AGE IS ASSOCIATED WITH REDUCED URETHRAL PRESSURE AND SENSITIVITY IN RAT

3.1 Introduction age-related underactive bladder

In our previous chapter, we demonstrated that the AR's (urethral afferents to bladder efferent reflex) sensitivity decreases with age; that is, older animals require higher urethral flow rates to elicit the AR than younger animals (32). The study in this chapter examines the age-related loss of urethral signaling capability by measuring the afferent activity directly.

Recent studies about the effect of aging on the LUT highlighted a lack of afferent signaling or integrative control of sensory information in the spinal and supraspinal centers, resulting in weak efferent signaling (27, 34, 61, 91) or compromised urethral sensation (47). There are two likely sources from which a reduction in urethral sensory outflow might arise. First, hypotrophy of the pudendal nerve that carries urethral afferents could directly reduce pudendal afferent activity by damaging fibers that convey urethral sensation. Second, an increase in compliance of the urethra could reduce the afferent signaling during a void. As the bladder pushes fluid into the urethra, the pressure buildup increases urethral wall stress that drives afferent activation (52); therefore, the same amount of fluid entering a more compliant urethra generates less pressure and wall stress, and in turn, less afferent signaling.

Evidence in the literature is consistent with age-related changes in the LUT. Degradation of urethral afferents and LUT denervation is present in older populations (9, 17, 27, 34, 59, 73, 96), which might cause bladder dysfunction symptoms if it compromises the LUT reflexes (68). Structural and biochemical changes of nerve fibers and the reduction of regenerative and reinnervating capabilities that come with age impair the central and peripheral nervous system (91), which can affect the LUT reflexes directly. Oshiro et al. showed external urethral sphincter atrophy in aged rats (62), and urethral sphincter muscle fibers appear to be replaced by connective tissue increases throughout aging (96); both of which could lead to a more compliant urethra. Histological studies also showed a decline in the number and density of striated urethral sphincter nerve and muscle fibers with age (69).

In this study, we hypothesized that afferent activity in the pudendal nerve, which carries urethral sensation, decreases with age, and explored how a combination of urethral afferent degradation and an increase in urethral compliance might contribute to a reduction in urethral sensory outflow. We recorded pudendal afferents (electroneurogram (ENG)) in young and old animals while we infused the urethra with fluid. We found that equivalent flow rates evoked less urethral afferent activity in the older rats. To understand what factors were driving the afferent reduction, we examined the pudendal nerves histologically and measured the pressures that developed in the urethra during infusion. We found evidence that axonal myelination decreased in old rats and lower pressures developed in the urethra of older rats during infusion.

3.2 Methods

3.2.1 Ethical approval

All animal care and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Florida International University. We used two groups of urethane-anesthetized female Sprague–Dawley rats (young (3-7mo), old (18-24mo), n=12 each group) to quantify the effect of aging on urethral afferent signaling. The age of old rats matches the human lifespan in a reasonable way (77), and our previous study showed that the AR becomes weak in rats at this age (32). All experiments were non-survival. Rats were bought from Charles River Laboratories, and rats in the "old" group were kept for 6-12 months under a low cholesterol diet (to keep them healthy) in an animal care facility under a 12:12-h light-dark cycle until they reached the appropriate age for the study. Animals were anesthetized initially with isoflurane gas followed by injection of 1.2 g/kg urethane (dissolved as 0.2 mg/mL in 0.9% saline solution) subcutaneously using a 27-gauge needle. The foot pinch reflex of animals was tested one hour after injection, and supplemental doses (0.1 g/kg, every 30 min) were injected until the foot withdrawal reflex abated. Urethane was used because it provides a stable depth of anesthesia and spares LUT reflexes (31). The body temperature and heart rate (determined via ECG) of animals were measured to monitor the vital signs and keep the depth of anesthesia. Animals were euthanized by an intraperitoneal injection of pentobarbital sodium after the experiment.

3.2.2 Experimental Protocol

A catheter (PE90) was passed through the intravesical space into the urethra from the bladder dome to allow infusion of fluid through the urethra. The abdomen was sutured

closed, but we left the bladder incision open so that the bladder remained empty during the experiment. A Millar pressure catheter (Millar, SPR-1000, Houston, TX) was passed through the urethral catheter and into the urethra just proximal to the trigon to measure the pressure in the proximal urethra. This low-profile, solid-state transducer lets us record pressure while minimally interfering with the flow. A computer-controlled infusion pump (Ellite 11, Harvard Apparatus, Holliston, MA) was connected to the catheter and used to pass a solution of room temperature saline through the urethra at controlled flow rates. The pudendal nerve was exposed using the posterior approach, and the sensory branch was isolated from the compound nerve and connective tissue. The sensory branch is almost completely of sensory fibers and can be isolated through careful dissection (19, 82). A bipolar nerve cuff electrode (Platinum–iridium insulated with the contact spacing of 2mm, World Precision Instruments) was placed on the sensory branch of the pudendal nerve to record the electroneurogram (ENG). At 2-minute intervals (the pudendal nerve sensory response to flow can recover after two minutes (19)), the urethra was infused at pseudo-randomly selected flow rates ([0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 5, 11] ml/min) for 15 seconds (this time is sufficient for the flow rates to develop required pressure in the urethra). In a physiological condition, the average flow rate is calculated as average volume voided over the average duration of the voiding period, which is shown to be 6.4 ml/min) (19). However, if we assume that urethral flow is only permitted during the silent phase (not the bursting phase), the average flow rate will be 10.4 ml/min (19). If instead we assume that urethral flow is slowed during sphincter contractions, rather than stopped, then a typical average flow rate during voiding lies between 6.4 and 10.4 ml/min. We expect old rats to have lower flow rates on average during voiding and may also experience slow leaking

from stress incontinence; therefore, the flow rates we used were chosen to represent a range from barely detectable to the maximum physiologically relevant flow the urethra might experience. Electrical activity of the pudendal sensory nerve was sampled at 20 kHz (PowerLab 8/35, ADInstruments), amplified at a gain of 104, and bandpass filtered from 100 to 10000 Hz (SR560, Stanford Research Systems). Pressure signals were amplified at a gain of 10, sampled at 100 Hz, and recorded unfiltered. The experimental setup is displayed in Figure. 11

The sensory branch of the pudendal nerve along with the cuff electrode was completely removed after the experiment, fixed in 10% buffered formalin, and embedded in paraffin. The middle segment of the nerve tissue was cut into slices with 2 μm thickness. We used H&E staining (42), the light microscope (Leica DM 4500b), and ImageJ to measure axonal density and myelin thickness of the afferents in the sensory branch of the pudendal nerve.

3.3 Data Analysis

Before data analysis, ENG signals were rectified and filtered (100–3000 Hz). To better visualize the initial transient neural response to infusion, a moving average filter with a 200 ms sliding window was applied. We computed the initial transient peak in response to the urethral infusion (19) to quantify trends of the pudendal afferent activity and urethral pressure across flow rates and trials. To capture the transient response of urethral pressure to flow, we subtracted the root mean square (RMS) of baseline urethral pressure before urethral opening (caused by flow) from the RMS activity after urethral opening in a 2-second window (Figure. 12). The urethral opening was defined as the time after flow onset when the urethral pressure first exceeded the 75% of RMS value of the pressure across the

entire duration of the flow. We subtracted the root mean square (RMS) of baseline neural activity before urethral opening (caused by flow) from the RMS activity after urethral opening in a 2-second window to quantify change from baseline. We did not start the RMS calculation immediately (or at a fixed latency) after turning on the infusion pump because pudendal afferents are not activated until the urethra opens, and depending on the flow rate, different amounts of time were necessary to overcome urethral opening pressure. (Figure. 13).

3.4 Results

Pudendal sensory nerve activity was recorded in response to different urethral flow rates. Figure. 13 shows the rectified and moving averaged filtered ENG response to flow in a young and old animal. These data show a large transient initial response to the onset of flow followed by a persistent, steady-state activity. The ENG decayed back to baseline once the flow was terminated. The onset of neural activity of urethral afferents with respect to the start of the urethral infusion occurred earlier for high flow rates because higher flow rates require less time to develop enough catheter pressure to overcome the urethral opening resistance (36). All flow rates except 0.1ml/min could activate the pudendal afferents of young animals, probably because this flow rate is not strong enough to stretch the urethral wall sufficiently to activate the urethral afferents.

To capture the transient ENG response to flow onset, we subtracted the integrated RMS of baseline activity before urethral opening by flow from the RMS activity after urethral opening in a 2-second window (see Figure. 12). The same urethral flow rates evoked less

nerve activity in older animals [($F(1,33)=10.5$, $p=0.003$), mixed ANOVA], which shows there is a deficit in neural response to flow in older animals.

Flow in the urethra increases the urethral pressure and distension, which leads to activation of pudendal afferents (19, 53). However, the pressure generated in the urethra caused by a fixed flow rate may be different across animals and across ages, which may affect the evoked neural activity in pudendal afferents. Urethral muscle diameter and elasticity are key factors that determine what pressure will be generated in the urethra in response to fluid infusion. Therefore, maintaining constant infusion rates across animals and age groups does not guarantee we generate equal urethral pressures. Previous studies on mice (55), rats (73), and adult women (12) showed that the urethral diameter does not change with age, indicating that pressure differences caused by equivalent flow rates are likely due to differences in compliance.

The precise transduction mechanism of mechanical stimuli into urethral afferent activity is not fully known, and flow, volume, pressure, distention, and wall stress are all plausible candidates (20). However, the afferent response to a given pressure is identical whether the urethral fluid causing that pressure is flowing or static (53), suggesting that pressure is the primary sensed stimuli or that flow sensors represent a negligible portion of the response. Danziger et al. also showed that pressure (and its derivative) was sufficient to capture urethral afferent responses across a wide range of flow rates and flow profiles (19). Tension in the urethra wall will also be highly correlated with pressure in many physiological conditions. Therefore, even if pressure itself is not the primary physical quantity being sensed by urethral afferents, it is highly likely to be a good approximation.

We computed the urethral pressure evoked by each applied flow rate to understand how differences in intraurethral pressures between ages could drive observed differences in evoked ENG. Figure. 15 shows that the same flow rates evoked less pressure in the urethra in older animals than younger ones ($F(1,33)=8.7$, $p=0.006$, mixed ANOVA). The change in urethral pressure was computed by subtracting the integrated RMS of baseline pressure before urethral opening by flow from the RMS activity after urethral opening in a 2-second window (see data analysis). The likely explanation for this difference in pressure between age groups is that the urethral lumen in old rats is more compliant and thus experiences less pressure at equivalent flow rates.

The lower pressures in older animals may partially explain their lower flow evoked ENG response (Figure. 14). To understand the relationship between urethral pressure and evoked afferent activity, we averaged the resulting urethral pressures and averaged the evoked afferent activity for each applied flowrate across each age group. This lets us visualize the relationship between pressure (dependent variable) and evoked ENG (dependent variable) across each flow rate (color–independent variable) for both young (circles) and old (diamonds) rats. Figure. 16 shows that evoked ENG in old rats is strictly less than for young rats at equivalent urethral pressures, which controls for the effects of differential tissue-related responses to a fluid infusion between old and young rats. For example, the pressure evoked by the 5ml/min infusion in young animals is approximately equal to the pressure evoked by 11ml/min in old animals (approximately 19 mmHg in both cases); however, the ENG response to 11ml/min in the old group is less than the ENG response to 5ml/min in the young group. Therefore, an increase in passive urethral compliance with age, which

results in less intraurethral pressure compared to young rats, is not sufficient to explain the entirety of the reduction in urethral afferent signaling we observe in old rats.

To examine the nerve-related factors that could lead to reduced afferent signaling, we measured the axonal density (3 young and 3 old animals) and myelin thickness (6 young and 6 old animals) of the sensory branch of the pudendal nerve after the experiments. The myelin thickness (young group: 1.53±0.03um; elderly group: 1.03±0.01 um; $p<0.001$, t-test) of the elderly group was smaller than those of the young group. The axonal density of the young and old groups was 0.013±0.004 and 0.01±0.006 fiber/μm2, respectively. Figure. 17 shows the boxplot of pooled myelin thickness from all samples in young and old animals. Figure. 18 (A, B) shows the examples of axons in young (A) and old (B) animals with 20x magnification and Figure. 18 (C, D) shows the examples of axons in young (C) and old (D) animals with 100x magnification.

3.5 Discussion

We measured age-related loss of urethral signaling capability by measuring the afferent activity directly. We found that urethral afferents (measured by electroneurogram) are less responsive to fluid flow in old rats and that there is both a mechanical component that contributes to this (a more compliant urethra) and a non-mechanical component (presumably neuropathy). Overall, the same urethral flow rates evoked less activity in pudendal afferents of older animals. We also found that pressure in the urethra decreases with age, meaning the urethra in older rats is more compliant, and their lumen experiences less pressure than for the same flow rate in the urethra of young rats. We showed that even after controlling for age-related pressure differences, less activity is generated in pudendal

afferents of old animals at equivalent pressures, suggesting both mechanical and non-mechanical causes. Histological results of the pudendal nerve showed significant myelin thickness reduction with age, suggesting the non-mechanical factors may have a neuropathic origin. Our results suggest that this age-related reduction in urethral afferent outflow is mediated by a mechanical component, presumably a more compliant urethra, and a non-mechanical component, presumably neuropathy.

3.5.1 Two Contributing Factors to Age-Related Loss of Urethral Sensitivity: Mechanical and Non-Mechanical

There are several possible explanations for less flow-evoked pudendal afferents activation in old animals. The horizontal axis in Figure. 16 indicates that with the same urethral flow rate, less pressure is generated in the urethra of older animals, suggesting that mechanical changes in the urethral tissue attenuate the physical stimulus that urethral afferents respond to. Interestingly, even at the same urethral pressure, less neural activity is generated in the pudendal afferents of old animals. Therefore, an increase in passive urethral compliance with age, which results in less intraurethral pressure compared to young rats, is not sufficient to explain the entirety of the reduction in urethral afferent signaling we observe in old rats.

3.5.2 Computing the Contributions of Mechanical and Non-Mechanical Factors to Total Sensory Loss

Here we quantify the contributions to pudendal afferent loss of sensitivity from mechanical factors, which change the pressure the urethra experiences in response to flow, and non-mechanical factors, which are putatively intrinsic to the nerve itself. We find that old rats

lose over 50% of their urethral sensitivity overall when compared to young rats and that 20% of that loss is due to mechanical factors of the urethra, with the remaining 80% from intrinsic neural factors (Figure. 19D). The remainder of this subsection describes the procedure to obtain this result.

The total loss of urethral sensitivity is the difference in evoked afferent responses between old and young rats to the same flow rate (quantified in Figure. 14). To visualize how this total decomposes into the two factors, in Figure. 19A, we create a schematic representation of the afferent response to flow in the urethra as a function of pressure (actual data are in Figure. 16). Formally,

$$\boldsymbol{L_t(Q) = L_n(Q) + L_m(Q) = N_y(Q) - N_o(Q)} \quad (1)$$

where $\boldsymbol{L_t(Q)}$ is the total loss as a function of flow rate, $\boldsymbol{Q}$, and $\boldsymbol{N_y(Q)}$ and $\boldsymbol{N_o(Q)}$ are the neural responses of young and old rats as a function of flow rate. The first equality in equation (1) states that the total sensory loss is the sum of the sensory loss due to non-mechanical (or neural) factors, $\boldsymbol{L_n(Q)}$, and mechanical factors, $\boldsymbol{L_m(Q)}$, which is shown in Figure. 19A. The second equality is visualized as the gray patch in Figure. 19B, which is the difference between nerve responses of young and old animals as a function of flow rate.

At a given urethral pressure, the difference between the neural afferents of young and old rats must be the non-mechanical contribution to sensory loss since the mechanical contribution only has its effect through changes in the pressures the urethra experiences. This is shown as the difference between the young and old curves in Figure. 19A. We can write this as

$$\boldsymbol{L_n = N_y(Q_k) - N_o(Q'_k)} \quad (2)$$

where $\boldsymbol{N_y(Q_k)}$ is the neural response of the young rats to flow rate $\boldsymbol{Q_k}$, and $\boldsymbol{N_o(Q'_k)}$ is the neural response of the old rats at flow rate $\boldsymbol{Q'_k}$, which is the flow rate required to generate urethral pressure in old rats that $\boldsymbol{Q_k}$ generates for young rats. The remaining mechanical contribution to sensory loss is then the difference between the neural response of old rats at the given pressure minus the sensory response of the old rats to the flow rate that produced the given pressure in young rats (shown geometrically in Figure. 19A):

$$\boldsymbol{L_m = N_o(Q'_k) - N_o(Q_k)} \quad (3)$$

To compute $\boldsymbol{L_n}$ and $\boldsymbol{L_m}$ we need expressions for $\boldsymbol{N_y(Q)}$, $\boldsymbol{N_o(Q)}$, and $\boldsymbol{Q'_k}$. $\boldsymbol{N_y(Q)}$ and $\boldsymbol{N_o(Q)}$ can be computed by direct curve fits to data in Figure. 14 using the form $\boldsymbol{N(Q) = c_1 + c_2 log_{10}(Q)}$, yielding the fits shown in Figure. 19B. The constants $\boldsymbol{c_1}$ and $\boldsymbol{c_2}$ were fit separately for each age group ($\boldsymbol{c_{1y} = 2.00}$, $\boldsymbol{c_{2y} = 1.79}$, $\boldsymbol{c_{1o} = 0.94}$, $\boldsymbol{c_{2o} = 1.05}$), and yield R^2=0.98 and R^2=0.97 for young and old groups. To compute $\boldsymbol{Q'_k}$ (the flow rate in old rats that causes the same pressure as the flow rate $\boldsymbol{Q_k}$ causes in young rats) we require an expression relating the pressure to flow for young rats, $\boldsymbol{P_y(Q)}$, and old rats $\boldsymbol{P_o(Q)}$. We can obtain these expressions through direct curve fits to data in Figure. 15 using the form $\boldsymbol{P(Q) = b_1 Q^{b_2}}$, yielding the fits shown in Figure. 19C. The constants $\boldsymbol{b_1}$ and $\boldsymbol{b_2}$ were fit separately for each age group ($\boldsymbol{b_{1y} = 7.90}$, $\boldsymbol{c_{2y} = 0.54}$, $\boldsymbol{c_{1o} = 6.08}$, $\boldsymbol{c_{2o} = 0.49}$), and yield R^2=0.99 and R^2=0.99 for young and old groups. To compute $\boldsymbol{Q'}$ in general, we set $\boldsymbol{P_y(Q) = P_o(Q')}$, which yields $\boldsymbol{Q' = \left(\frac{b_{1y}}{b_{1o}} Q^{b_{2y}}\right)^{1/b_{2o}}}$. Plugging Q' into equation (3)

and using the expressions for $N_y(Q)$ and $N_o(Q)$, we can decompose the loss into mechanical and non-mechanical factors. The mechanical loss as a function of flow rate is then

$$L_m(Q) = c_{2o} log_{10}\left(\left(\frac{b_{1y}}{b_{1o}}\right)^{1/b_{2o}} Q^{\frac{b_{2y}}{b_{2o}}-1}\right) \quad (4)$$

The red trace in Figure. 19D shows the percentage of the total loss, $L_t(Q)$, due to mechanical factors, $L_m(Q)$, expressed as $L_m(Q)/L_t(Q) \cdot 100$. For the larger flow rates that occur during voiding, mechanical factors account for only 20% of the total sensory loss, with the balance coming from non-mechanical factors. Since the mechanical loss is proportional to log of $Q^{\frac{b_{2y}}{b_{2o}}-1}$, and the exponent is less than 1, we expect a very weak dependence of $L_m(Q)$ on flow rate for flows above 1 ml/min. Mechanical factors may represent a larger share of the impact at low flow rates, as voiding is beginning, although at low flow rates the total sensory loss is much lower overall. The black trace in Figure. 19D shows the overall percentage of neural activation that old rats lose, $N_o(Q)/N_y(Q) \cdot 100$. This indicates that most of the functional loss comes at high flow rates. Taken together, most of the age-related urethral sensitivity loss occurs at higher flow rates, and at higher flow rates over 80% of the loss originates with non-mechanical factors, which suggests that urethral afferents may be a promising therapeutic target for restoring urethral sensation and mitigating age-related UAB symptoms.

3.5.3 Drivers of Urethral Sensation Loss

One potential explanation for the observed decrease in pudendal afferents activity could be age-related degradation of the urethral musculature (i.e., a mechanical factor). The increase in the urethral resistance generally makes the urine stream difficult in the urethra, which

does not let the flow develop required urethral pressure to activate pudendal afferents. On the other hand, if the urethra is more compliant (very loose), the flow cannot distend the urethra to develop enough pressure and activate pudendal afferents. Since urethral pressure is the putative driver of urethral afferents rather than flow (53), this might also decrease the activity of pudendal afferents and lead to fewer AR contractions. Our results showed that the same flow rates evoked less pressure in the urethra in older animals. The increased EUS connective tissue, reduced number of EUS fibers (96), and EUS muscle atrophy (62) with age might explain the reduced pressure generated by urethral infusion in older animals.

Functional deficits in flow-evoked pudendal nerve activity may be the consequence of structural and biochemical changes that result in a gradual loss of neurons and myelin (i.e., a neural factor). Therefore, age-related degradation of the pudendal nerve might be another contributing factor for less flow evoked ENG response in older animals. The myelin thickness of the old group was significantly smaller than those of the young group. Previous research has revealed a significant reduction in mRNA and protein levels for myelin sheath components in the sciatic nerves of older rats (59). A decrease in myelin of the myelinated fibers in the aged subjects is the main morphologic change responsible for the nerve conduction changes, and this delay might change the normal timing of the neural circuit in the spinal cord and disrupt AR activation (32).

Reduced number of urethral cells expressing serotonin (5-hydroxytryptamine [5-HT]) with age can be another contributing factor for less pudendal afferents activity in older animals (15). It is believed that cross-talk between urethral afferents and urethral cells expressing serotonin involved in transmission of sensory information from the urethra to the central

nervous system (50), and the blockade of serotonin receptors in nerve fibers innervating the urethra can block AR (16). Previous research showed that the application of 5-HT to the urethral lumen could increase the voiding efficiency. Therefore, the lower number of 5-HT-positive urethral cells may be responsible for decreased excitation of the urethral afferents (15).

The methodology of our study has a number of limitations that we note here to help clarify the interpretation of our results. 1) We tied a catheter around the bladder neck to infuse the urethra. This might prevent mechanical distension of the proximal urethra and influence flow-evoked pressure (or tension) and afferent activity results. 2) It is possible that urethane anesthesia dosed according to body weight (the gold-standard approach) does not result in equal responses in young and old animals. If urethane suppresses afferent activity or urethral muscle tone to a different extent in old rats than young rats, the anesthesia itself could contribute to the differences we observe between ages. 3) Our study isolated and quantified the fundamental response of urethral afferents and urethral pressure to applied urethra flow. To perform this characterization, we needed to remove many potentially confounding aspects present in natural voiding, which included enforcing a constant flow rate throughout urethral infusion and maintaining an empty bladder, neither of which is typical of physiological voiding. Therefore, our results specifically highlight changes in fundamental afferent activity related to aging and cannot be used to directly infer functional age-related differences. This work only discovered that age-related differences in afferent responses and pressures exist, but their explanatory role in functional age-related LUT decline remains to be determined. 4) We measured urethral pressure rather than tension, which is very likely the primary sensed urethral stimuli. If these two quantities diverge

substantially during infusion, our estimate of the mechanical effects on age-related afferent loss may also change. We expect any effects of using pressure as a surrogate for tension to be small, since these variables will be highly correlated with each other in many physiological conditions, and multiple models have shown success predicting urethral afferent discharge from pressure information.

3.5.4 Future Directions

Underactive bladder (UAB) is very common in elderly, and its etiology is under studied (14); here we propose a mechanism that could explain age-related UAB symptoms that is consistent with the literature and the results of this work. In prior work we showed that there is a deficit in the urethra-to-bladder (augmenting) reflex that worsens with age (32). Disrupting the augmenting reflex by administration of intraurethral lidocaine (32, 45, 78), or transecting the pudendal nerve (67) significantly decreases voiding efficiency, which is a hallmark UAB symptom. Here we report that the urethral afferents, the fibers directly responsible for activating the augmenting reflex, weaken with age. The afferent weakening we observed is consistent with the reported decline in urethral sensation and neuropathy in age-related UAB (26, 63). We hypothesize that age-related neuropathy and increases in urethral compliance lead to less urethral sensory outflow that, in turn, leads to a less sensitive and robust augmenting reflex. Since the augmenting reflex is necessary for efficient voiding, a neuropathic weakening of this reflex could contribute to the poor voiding efficiency we observe in age-related UAB patients. To fully verify this hypothesis, further work is needed to demonstrate 1) that the reduction in urethral afferent outflow is causally responsible for the weakened augmenting reflex in old rats, and 2) that a weakened

augmenting reflex materially contributes to the loss of voiding efficiency or hesitancy observed in UAB.

Figures

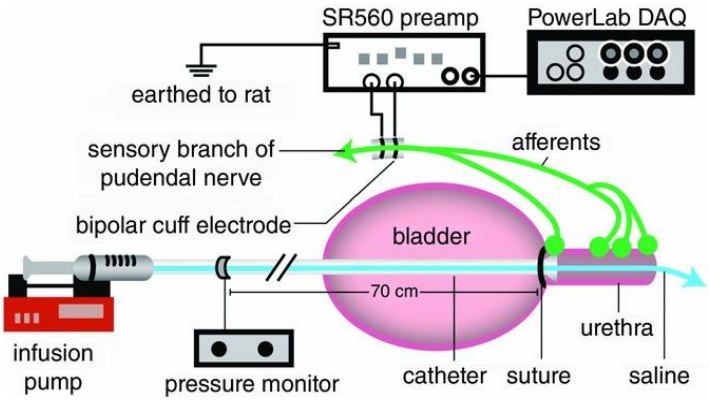

Figure 11. Experimental setup for nerve recording (19). The catheter is inserted to the urethra through bladder dome and is tied with suture around bladder neck. The urethra is infused with selected flow rates and the neural activity of the pudendal afferents is recorded.

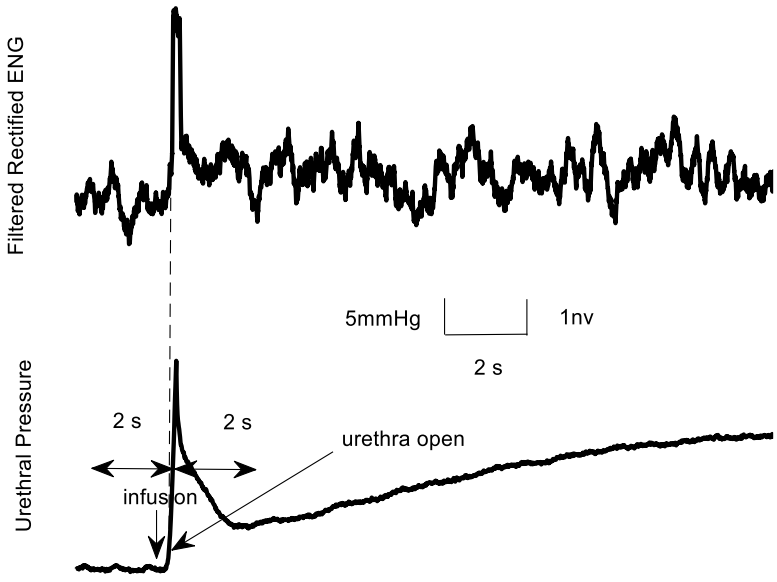

Figure 12. The initial transient response of pudendal nerve activity (ENG) and urethral pressure to flow. The urethral opening was defined as the time after flow onset (infusion) when the urethral pressure first exceeded the 75% of RMS value of the pressure across the entire duration of the flow.

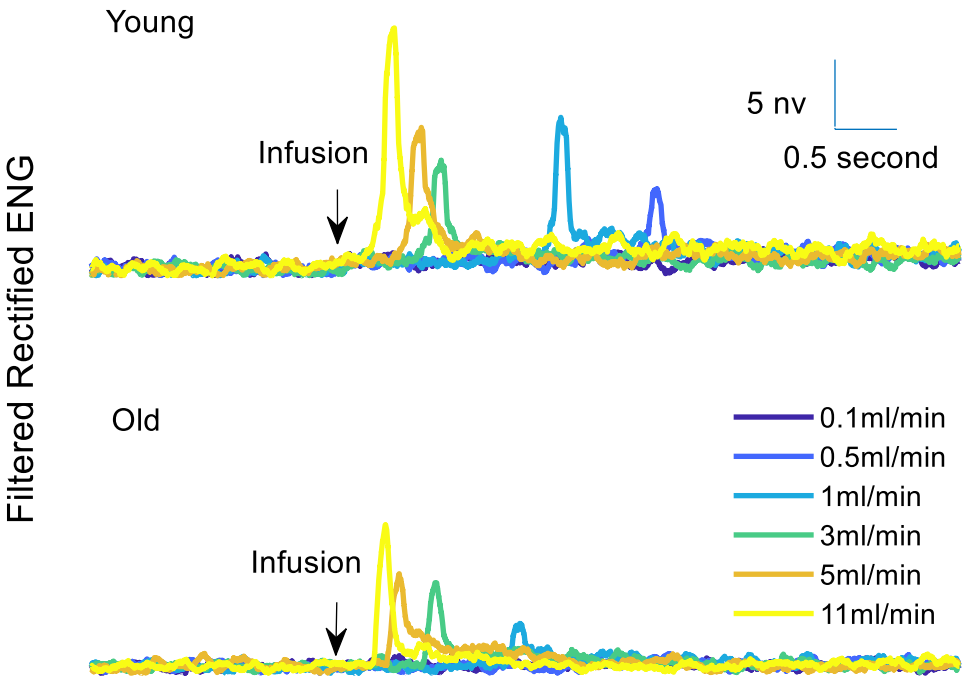

Figure 13. The transient response of pudendal afferent activity to a subset of the different applied flow rates in a typical young and old animal. Higher flow rates initiate more neural activity in both groups of animals. 0.5ml/min urethral infusion evoked detectable levels of ENG in the young animal but could not generate activity in the old animal.

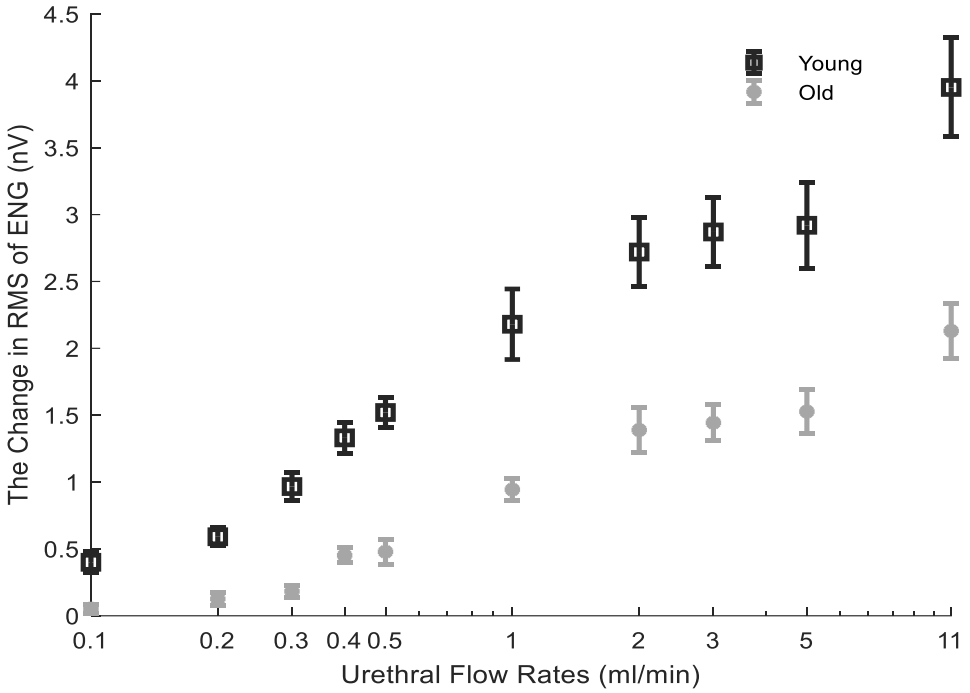

Figure 14. The initial transient peak response of pudendal nerve activity to urethral infusion in young (black) and old (grey) animals. The change in RMS of ENG response to all flow rates in a 2-second window was computed and averaged across the corresponding age group. The same flow rates evoked less pudendal afferent activity in older animals. Each animal's average ENG response to multiple applications of each flow rate was computed, then that number was averaged across all animals in each age group for each flow rate. Bars are standard error across animals (n=12 per age group), plotted on a log-scale horizontal axis.

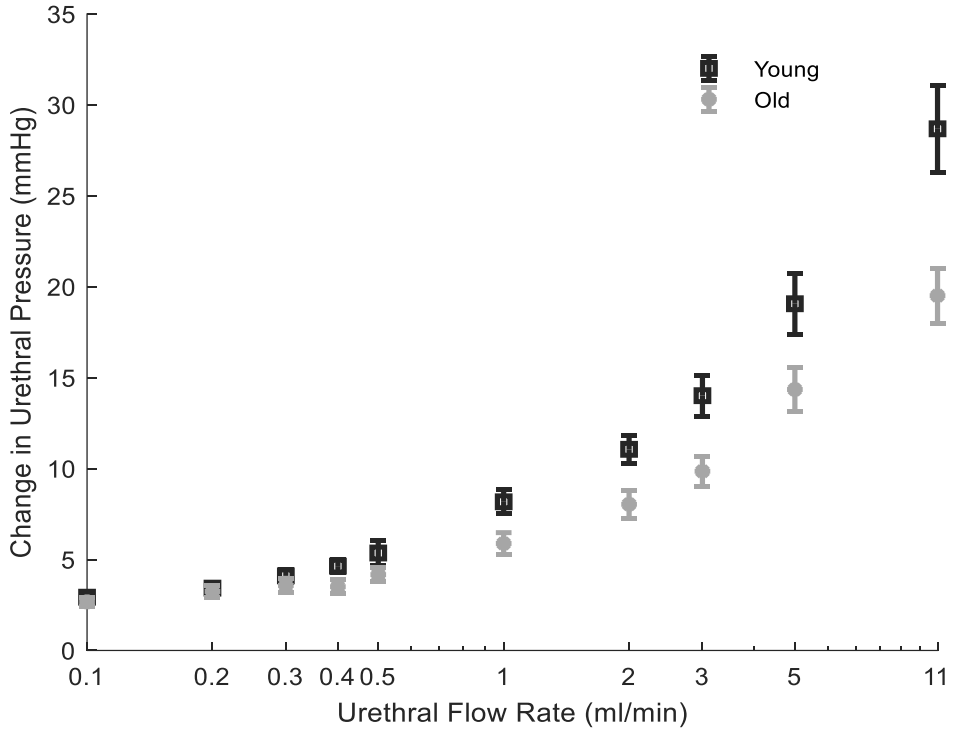

Figure 15. The flow-evoked urethral pressure response in young and old animals. The change in RMS of urethral pressure was computed in a 2-second window for each flow rate and averaged across the corresponding animal age groups. The same urethral flow rates created less urethral pressure in older animals. The change in urethral pressure is displayed as the mean with standard error, plotted on a log-scale horizontal axis.

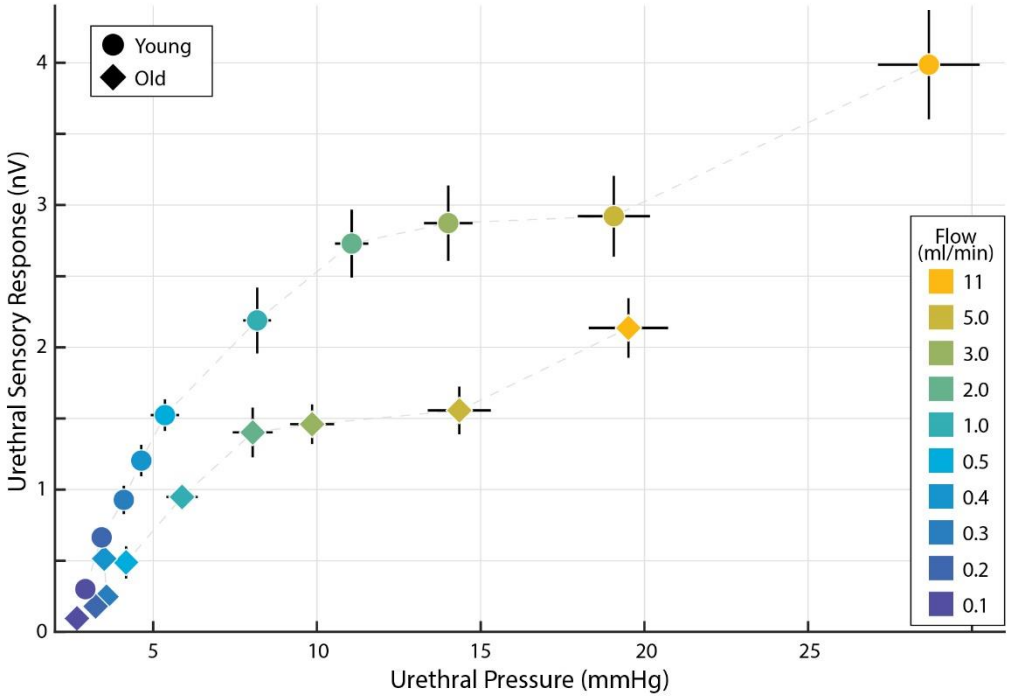

Figure 16. Old rats (diamonds) have smaller urethral sensory responses than young rats (circles) to equivalent pressures generated by experimentally applied flow rates (color). Larger flow rates were required in old rats to generate the same urethral pressures (see also Figure. 15), but even when comparing old and young rats at equivalent pressures (the same extent along the abscissa), old rats showed a less sensory response. Ordinate data were pooled according to Figure. 14 and abscissa data were pooled according to Figure. 15, error bars for both are standard errors.

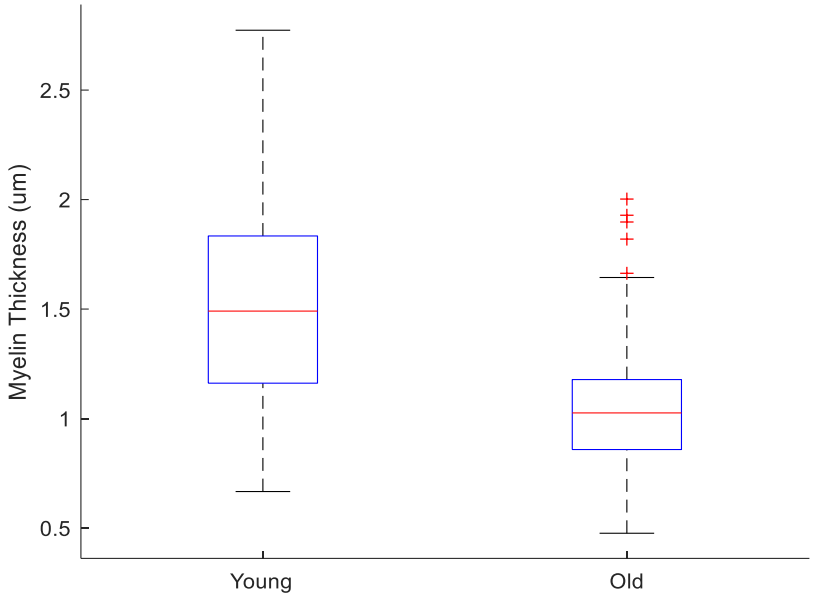

Figure 17. Axonal myelin in the sensory branch of the pudendal nerve is thicker in younger animals.

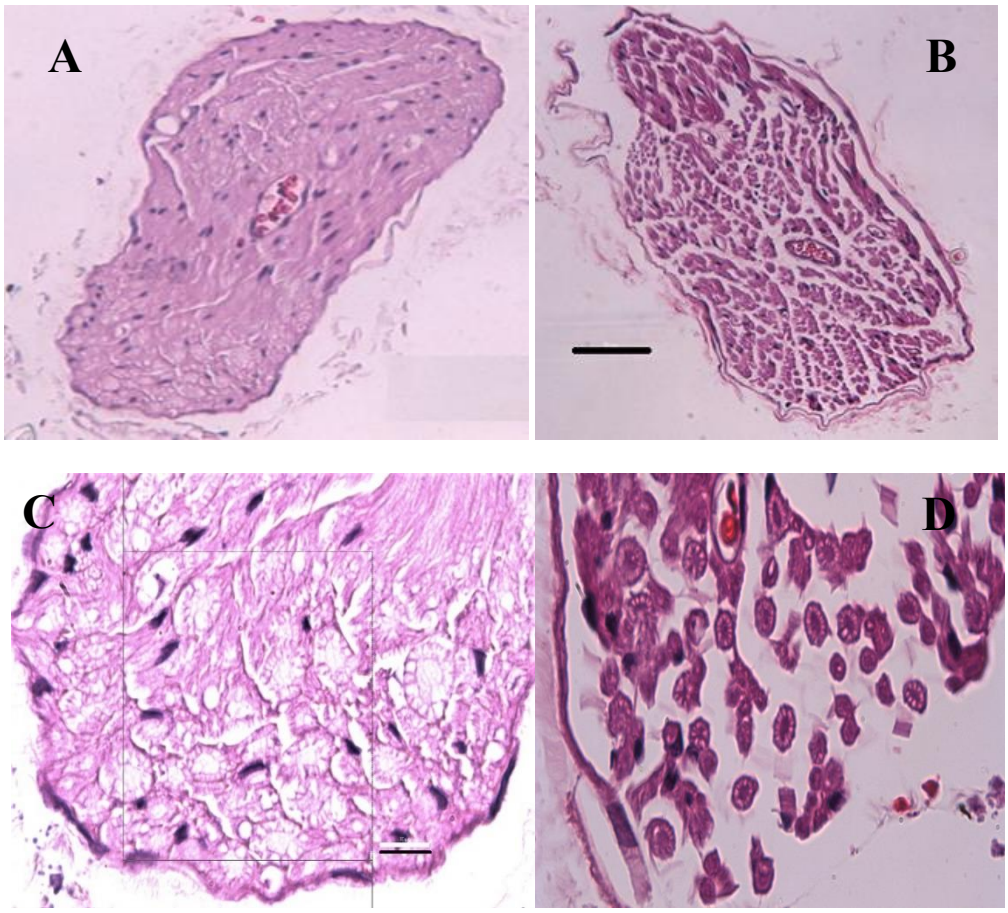

Figure 18. The examples of axons in the sensory branch of the pudendal nerve in young and old animals. A (young), B (old) are with 20x magnification and C (young), D (old) are with 100x magnification. The black bar in B, shows 50um in A and B; and the black bar in C, shows 10 um in C, D.

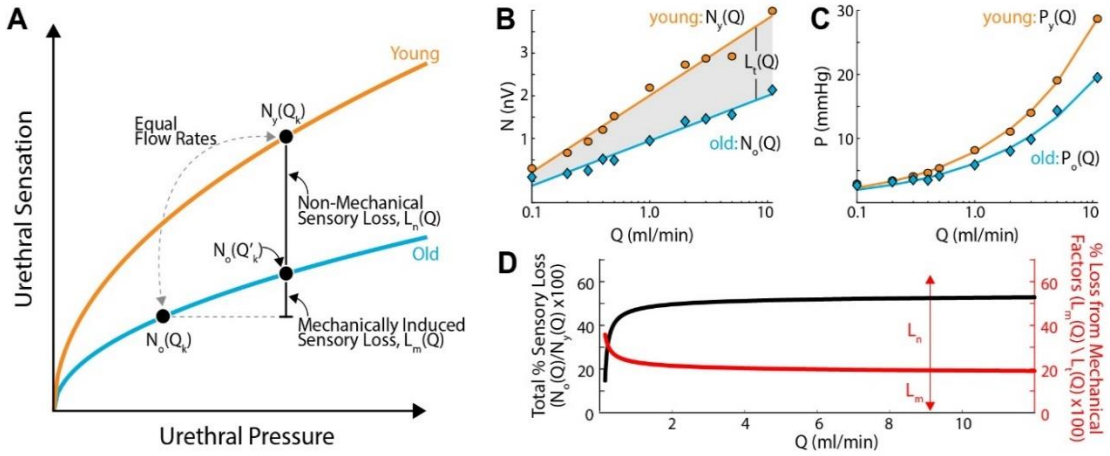

Figure 19. Decomposition of age-related urethral sensory loss into its mechanical and non-mechanical factors. A) Schematic (not actual data) illustrating how the total sensory loss in old rats breaks down into its non-mechanical and mechanical components. The first equality in equation (1) expresses this depiction of the total loss. B) Curve fits to data show in Figure. 14 for neural response as a function of applied flow rate. The gray patch depicts the total sensory loss and is expressed in the second equality in equation (1). C) Curve fits from data in Figure. 15 expressing urethral pressure as a function of applied flow rate. D) The ratio of evoked neural activity in old to young rats as a function of applied flow rate (black). The percentage of sensory loss in old rats due to mechanical factors as a function of applied flow rate.

CHAPTER 4

POTENTIAL OF PERCUTANEOUS ELECTRICAL STIMULATION OF PUDENDAL NERVE IN MITIGATING UNDRACTIVE BLADDER SYMPTOMS IN AGED RATS

4.1 Introduction to UAB and treatment options

The sensitivity of the AR decreases with age (chapter 2); that is, higher urethral flow rates are required in older animals compared to younger ones to evoke the AR (32). In chapter 3, we found evidence that axonal myelination decreased in old rats and that lower pressures developed in the urethra in older animals during infusion, possible explanations for weaker afferent signaling. If age-related loss of urethral sensitivity contributes to geriatric UAB by impairing voiding reflexes (possibly AR), improving the signaling of urethral afferents by electrical stimulation might regain some lost function.

It has been suggested that pudendal nerve stimulation may be an effective therapy for neurogenic bladder dysfunction (76, 92). Recent animal studies indicate that activating sensory fibers in the pudendal nerve can cause bladder emptying (7, 40) by inducing synergic bladder activation and external urethral relaxation (6). The risk associated with surgically exposing the pudendal nerve and implanting electrodes for experimental purposes, however, limits its potential.

Needle-based percutaneous electrodes have been widely utilized in clinical settings for many years (37). In the lower urinary tract system, Spinelli et al. investigated the effect of percutaneous stimulation of the pudendal nerve on patients with overactive bladder (79). Because the pudendal nerve innervates the pelvic muscles, external urethral and anal

sphincters, and pelvic organs, there have been several attempts to stimulate it, all with the goal of improving multiple impaired LUT functions (48, 76, 92).

In this chapter, we evaluated the effectiveness of percutaneous electrical stimulation of a pudendal nerve in aged rats on voiding efficiency (VE) and bladder capacity (BC). Low VE and high BC (bladder overdistension) are common symptoms observed in UAB patients. BC is the volume at which the first voiding contraction is observed. VE indicates how well the bladder can expel the urine and is defined according to equation 1, where V_v is the volume voided and V_r is the residual volume.

$$VE = \left(\frac{V_v}{V_v + V_r}\right) \times 100 \quad (1)$$

4.2 Method

All animal care and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Florida International University. Fifteen old (18-24 months) female Sprague-Dawley rats (Charles River, Charleston, SC, USA) were used in this study, and all animals received food and water ad libitum. All studies were acute. Animals were anesthetized initially with isoflurane gas followed by injecting 1.2g/kg urethane (dissolved as 0.2 mg ml-1 in 0.9% saline solution) subcutaneously using a 27-gauge needle. The foot pinch reflex of animals was tested one hour after injection, and the supplemental doses (0.1 g/kg, every 30 minutes) were injected until the foot withdrawal reflex abated. We used urethane because it provides a stable depth of anesthesia and preserves the urinary tract reflexes in animal studies (57).

Animals were euthanized by intraperitoneal injection of Sodium Pentobarbital after the experiment.

4.3 Experimental protocol

The bladder was exposed via an abdominal incision, and we passed a catheter (PE 50, heat-flared tips) through a small incision in the apex of the bladder dome and placed in series with a pressure transducer to monitor bladder pressure (sampled at 100Hz). The distal urethral outlet was not obstructed, allowing the fluid introduced to the urethra via the internal catheter to exit distally. We used a computer-controlled infusion pump to infuse room temperature 0.9% saline solution (sodium chloride in distilled water, 154 mM) to the bladder and the urethra. Electromyogram (EMG) of the external urethral sphincter (EUS) was recorded by inserting two insulated stainless-steel wires (de-insulated at the tips) percutaneously into the EUS (bandpass filtered from 3 to 3000 Hz, 1000×amplification, sampled at 4000 Hz). All data were recorded on a PowerLab 16/35 system (ADInstruments, Colorado Springs, CO). A pair of needle electrodes (stainless steel, BIOPAC System) was placed close to the sensory branch of the pudendal nerve. We applied the stimulation starting low amplitude (and 1Hz frequency) until we get a motor response in EUS and called that motor threshold stimulation (TS). Then, the bladder was filled continuously with saline, the bladder pressure was recorded, and the effect of different percentages of motor threshold stimulation ([0, 0.25-0.4, 0.6-0.75, 1, 1.5-3] × TS, 20Hz, 0.1 ms) on VE and BC was evaluated. Each stimulation condition was called a “trial” and tested once in most animals, and twice in a couple of them. Initially, the set of ([0, 0.25, 0.5, 0.75, 1, 1.5, 3] × TS) was used to test the effect of stimulation on VE and BC. However, after the experiment was finished and during data analysis, we realized that in two animals in the bladder filling phase, TS-level stimulation did not evoke motor response in EMG data (it did initially when the bladder was empty). Therefore, we replaced the 1.5 × TS stimulation amplitude

with TS-level stimulation, adjusted the other stimulations correspondingly and categorized our stimulation values in different bins instead of a single value. Since the bladder capacity is defined as the bladder volume at which the first voiding contraction occurs, we computed the BC as the bladder filling rate multiplied by the time duration when the first voiding contraction was observed. To minimize the carryover effect, we randomized the trials for each animal and introduced a delay of 20 minutes between them.

Figure. 20 shows EUS EMG in response to different stimulation amplitudes. The figure shows that starting from Figure. 20C, the stimulation amplitude can generate the motor response in EUS EMG. The amplitude of the response becomes larger as the stimulation amplitude increases (Figure. 20C to Figure. 20E), meaning more fibers are recruited. The increase in the amplitude of response continues until the point at which all the fibers are recruited (Figure. 20E and Figure. 20F have almost the same EMG response).

One-way ANOVA was used for statistical analysis, and the post-hoc analysis was computed by Turkey HSD.

4.4 Results

The bladder was filled, the bladder pressure was recorded and the effect of different trials on VE and BC was tested. Figure. 21, Figure. 22 and Figure. 23 show the bladder pressure and their corresponding VE in 3 typical age animals. In these figures, panel “A” shows the bladder pressure and VE at control condition (no stimulation), panel “B” shows the bladder pressure and VE when stimulation amplitude= (0.25-4) × TS, panel “C” shows the bladder pressure and VE when stimulation amplitude= (0.6-0.75) × TS and panel “D” shows the bladder pressure and VE when stimulation amplitude= TS. Stimulation amplitudes less than TS (panels B and C) can evoke a bladder contraction and increase VE.

To show the effect of stimulation on VE and BC in old animals, we computed the change of VE and BC by subtracting the VE and BC values at no stimulation trial (control) from their value in response to stimulation at each trial (Figure. 24A and Figure. 24B). VE and BC values at each trial were shown as solid circles and different colors represent different animals. Each trial was tested once in most animals, and twice in a couple of them. When more than one trial was performed for an animal, the average value of VE or BC was considered. Figure. 24C and Figure. 24D show VE and BC in old animals and Figure. 24E and Figure. 24F show the average VE and BC at each trial across animal ages. The results in Figure. 24 show that when the stimulation amplitude is [0.6-0.75] × TS, voiding efficiency increases ($p=0.031$, one-way ANOVA), and bladder capacity decrease ($p=0.004$, one-way ANOVA). However, if the stimulation amplitude equals TS, the voiding efficiency and bladder capacity will not change ($p=0.69$, $p=0.86$).

4.5 Discussion

In this chapter, we evaluated the effectiveness of percutaneous stimulation of the pudendal nerve on voiding efficiency and bladder capacity in aged animals. The low stimulation amplitudes (≤TS) could increase VE and decrease BC. The largest increase in VE was at ([0.6-0.75]) × TS), at which the bladder capacity decreased significantly. However, higher stimulation amplitudes (≥TS) could not improve VE and BC.

The results in Figure. 24 showed that the stimulation amplitudes less than TS could improve UAB symptoms but the high amplitude stimulation did not have any effect. The differences in responses elicited by low and high stimulation intensities might be due to the activation of distinct classes of pudendal afferents based on their diameters. Pudendal afferents include myelinated Aβ, Aδ and unmyelinated C fibers (101). Larger diameter

nerve fibers like Aα or Aβ have low threshold for extracellular stimulation, whereas unmyelinated C fibers have high stimulation threshold (24, 54). These findings are consistent with bladder excitation caused by activation of larger myelinated A-type fibers at low stimulation amplitudes and inhibition caused by activation of the inhibitory urethro-vesical reflex mediated by urethral c-fibers at higher stimulation amplitudes (83).

The underlying mechanism by which sacral or pudendal nerve stimulation can improve bladder dysfunction symptoms remains unclear (31). Our results showed that percutaneous stimulation of the pudendal nerve could increase voiding efficiency and decrease the volume threshold for evoking bladder contraction (BC). This is consistent with a previous study which showed that electrical stimulation of pudendal afferents could evoke the bladder contraction at lower bladder volumes than distension-evoked activity (94). The fact that the superposition of pelvic and pudendal afferent activity is responsible for bladder excitation evoked by pudendal afferents (94), and Figure. 6 and Figure. 7 showed the engagement of AR (urethral to bladder reflex) is delayed with age (possibly due to reduced pudendal afferent activity), indicate that the stimulation might have improved the voiding efficiency by increasing the signaling of pudendal afferents and prevent the bladder from overdistension. In fact, percutaneous stimulation of the pudendal nerve might have facilitated the engagement of AR to lower bladder volumes by improving the signaling of urethral afferents. However, additional studies are required to evaluate the causal link.

AR is a reflex from pudendal afferents to bladder efferent whose activation is required for efficient voiding. Figure. 3 showed that the sensitivity of AR decreases with age which can have mechanical or non-mechanical (neuropathy of pudendal nerve) origins (chapter 3). In this chapter, we tested the effect of stimulation of pudendal nerve on VE of all animals in

aging group. Figure. 24C shows that the VE in aged animals (at no stimulation) has a big variability ranging from 20% to 80% and the stimulation is more effective in increasing VE in animals which have low VE. That means, in animals with low VE, there might be severe neuropathy in pudendal nerve and electrical stimulation could have improved it by increasing the signaling of pudendal afferents. In fact, the effectiveness of electrical stimulation on improving VE depends on the extent of neuropathy in the pudendal nerve. Therefore, in animals with large VE, the stimulation may not be very effective due to the low level of neuropathy in the pudendal nerve. However, since the pudendal afferents of aged animals after the stimulation study were not histologically tested, additional studies are required to evaluate the casual link.

Figure. 21, Figure. 22 and Figure. 23 showed that each stimulation condition (trial) was tested once in most animals, and twice in a couple of them because based on our age-reflex study (chapter 2), most of the old animals are not stable enough under anesthesia for a long time (they die or have no bladder contraction in the second run) and we wanted to test the effect of all trials on each individual animal's VE and BC. However, based on our previous experiments (3, 32) and other studies (97), the within animal variation of voiding efficiency in a same trial is very small.

While stimulation of pudendal afferents with low frequency (<10Hz) can inhibit the bladder contractions, high-frequency stimulation (20-40Hz) produces bladder excitation (8, 66, 67). Boggs et al. (6) found that higher frequency stimulation (20–40 Hz) is more effective than 10 Hz stimulation in triggering bladder contractions, which is consistent with the firing rates of urethral afferents in response to urethral fluid flow (43). The results of Figure. 24 are based on using a fixed frequency (20Hz) with varying stimulation

amplitudes. Our results showed that the low stimulation amplitudes (less than TS) could increase voiding efficiency, and high stimulation amplitudes could prevent the bladder from expelling urine at 20Hz frequency. However, more research is needed to determine the appropriate stimulation parameters and to confirm the long-term results.

Figure. 21, Figure. 22 and Figure. 23 showed that electrical stimulation was applied continuously until the end of first observed voiding contraction. It has been shown that continuous stimulation may limit the effectiveness of stimulation for a long time due to nerve habituation. However, conditional stimulation might be more effective and save power by reducing the stimulation time by 67% (93).

Figures

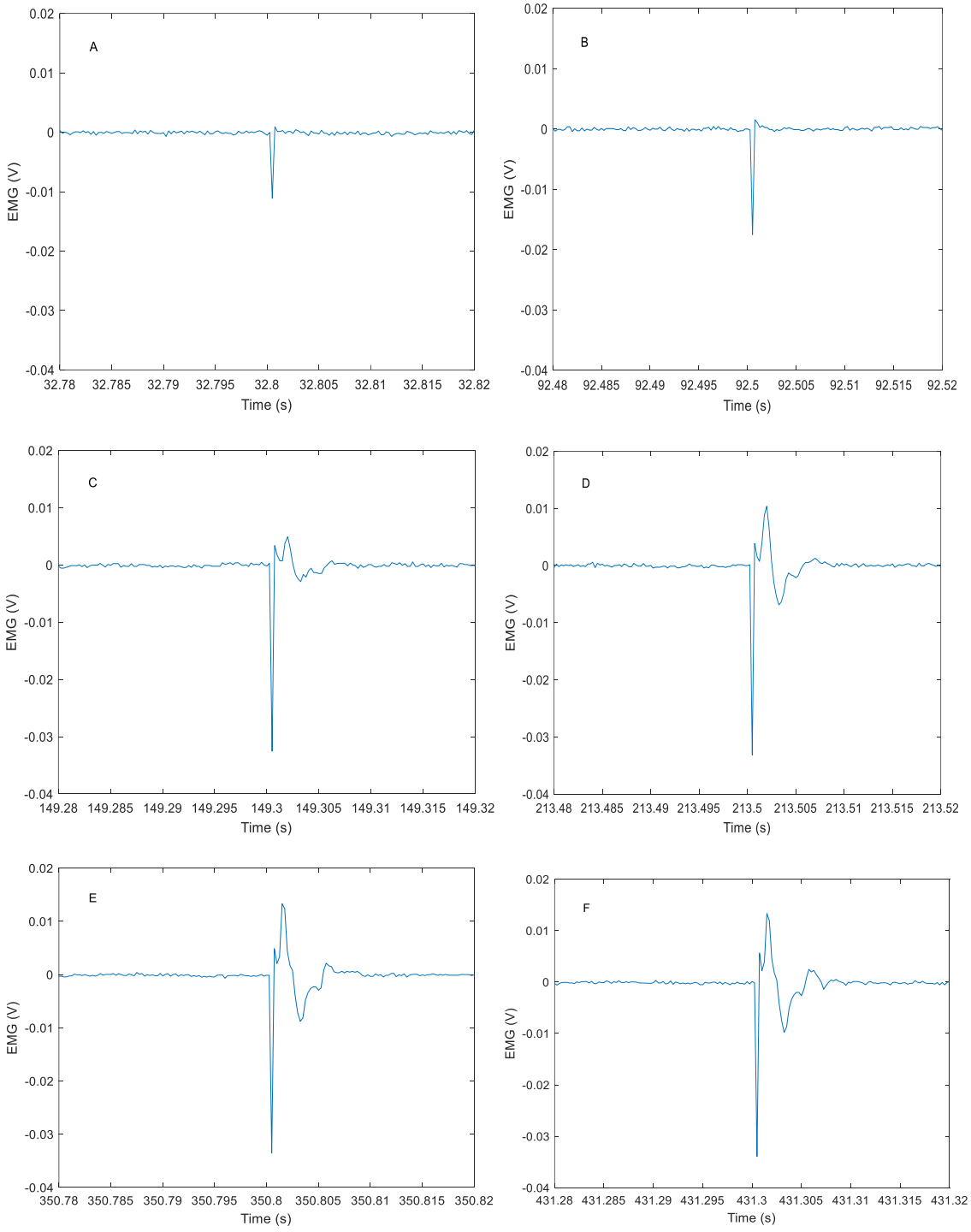

Figure 20. The EUS EMG in response to different stimulation amplitudes. A: 0.15ma, B: 0.25ma, C: 0.5ma, D: 0.7ma, E: 0.9ma, F: 1.2ma. The figure shows that starting from C, the stimulation amplitude can generate the motor response in EUS EMG. The amplitude of

the response becomes larger as the stimulation amplitude increases (C to E), meaning more fibers are recruited. The increase in the amplitude of response continues until the point at which all the fibers are recruited (E and F have the same EMG response).

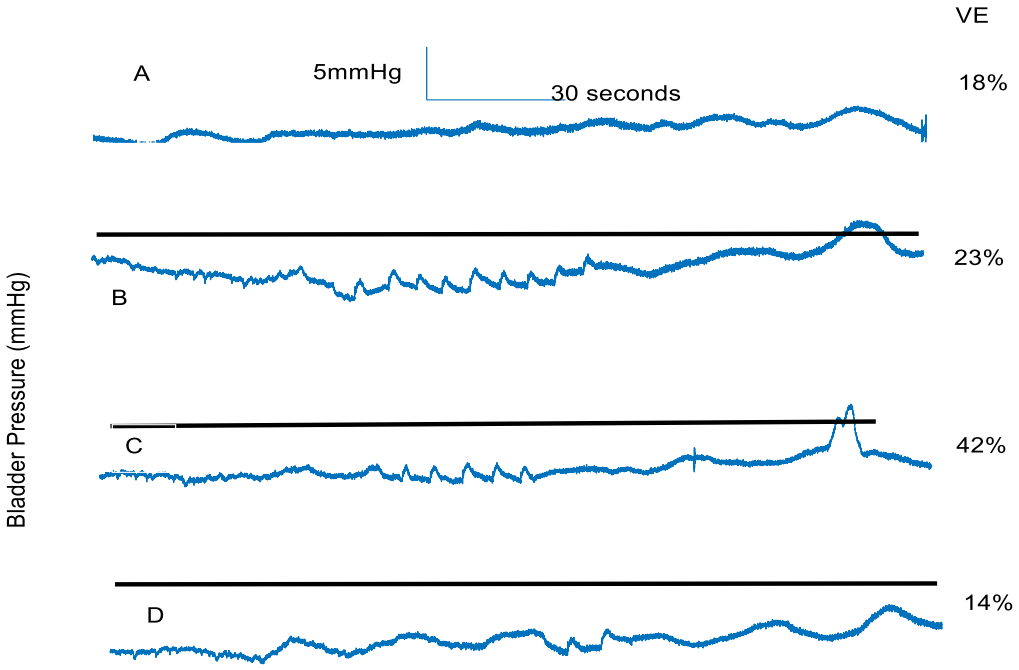

Figure 21. Bladder pressure (blue) and voiding efficiency (VE) in response to different stimulation amplitudes in an aging animal. A: no stimulation, B: stimulation amplitude= (0.25-4) × TS, C: stimulation amplitude= (0.6-0.75) × TS, D: stimulation amplitude= TS. The black bar shows the stimulation period.

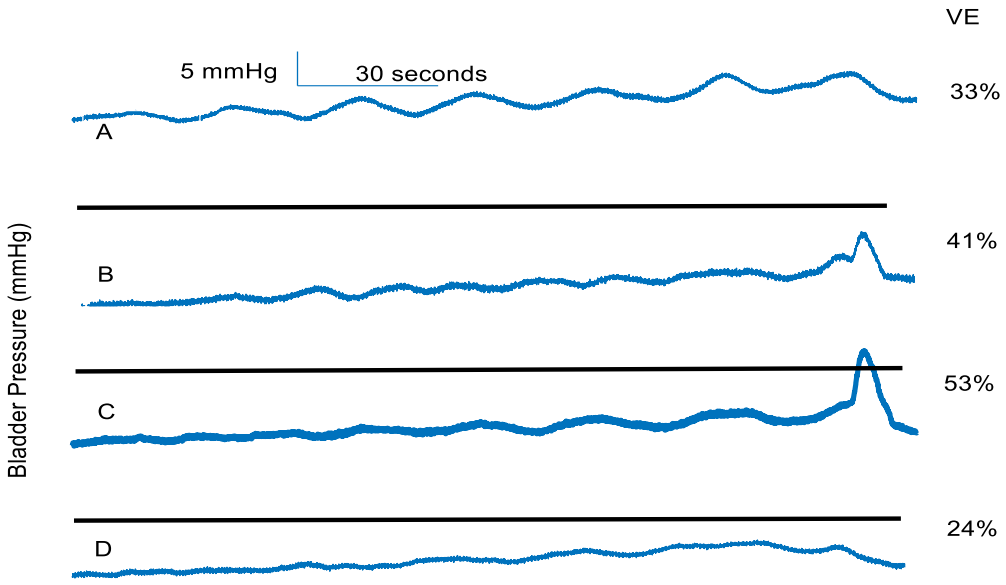

Figure 22. Bladder pressure (blue) and voiding efficiency (VE) in response to different stimulation amplitudes in an aging animal. A: no stimulation, B: stimulation amplitude=

(0.25-4) × TS, C: stimulation amplitude= (0.6-0.75) × TS, D: stimulation amplitude= TS. The black bar shows the stimulation period.

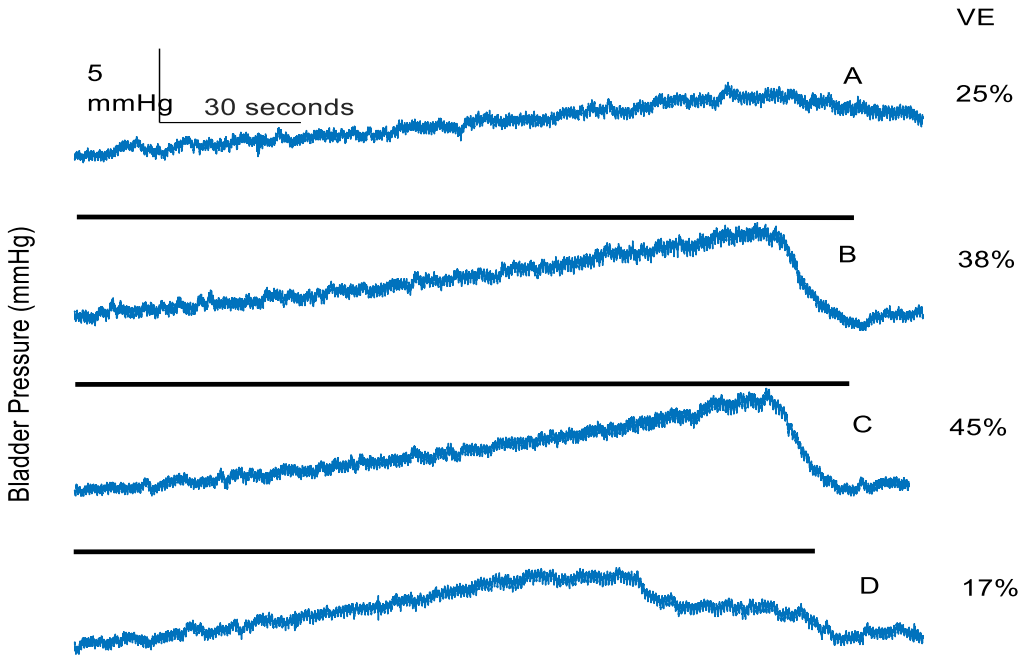

Figure 23. Bladder pressure (blue) and voiding efficiency (VE) in response to different stimulation amplitudes in an aging animal. A: no stimulation, B: stimulation amplitude= (0.25-4) × TS, C: stimulation amplitude= (0.6-0.75) × TS, D: stimulation amplitude= TS. The black bar shows the stimulation period.

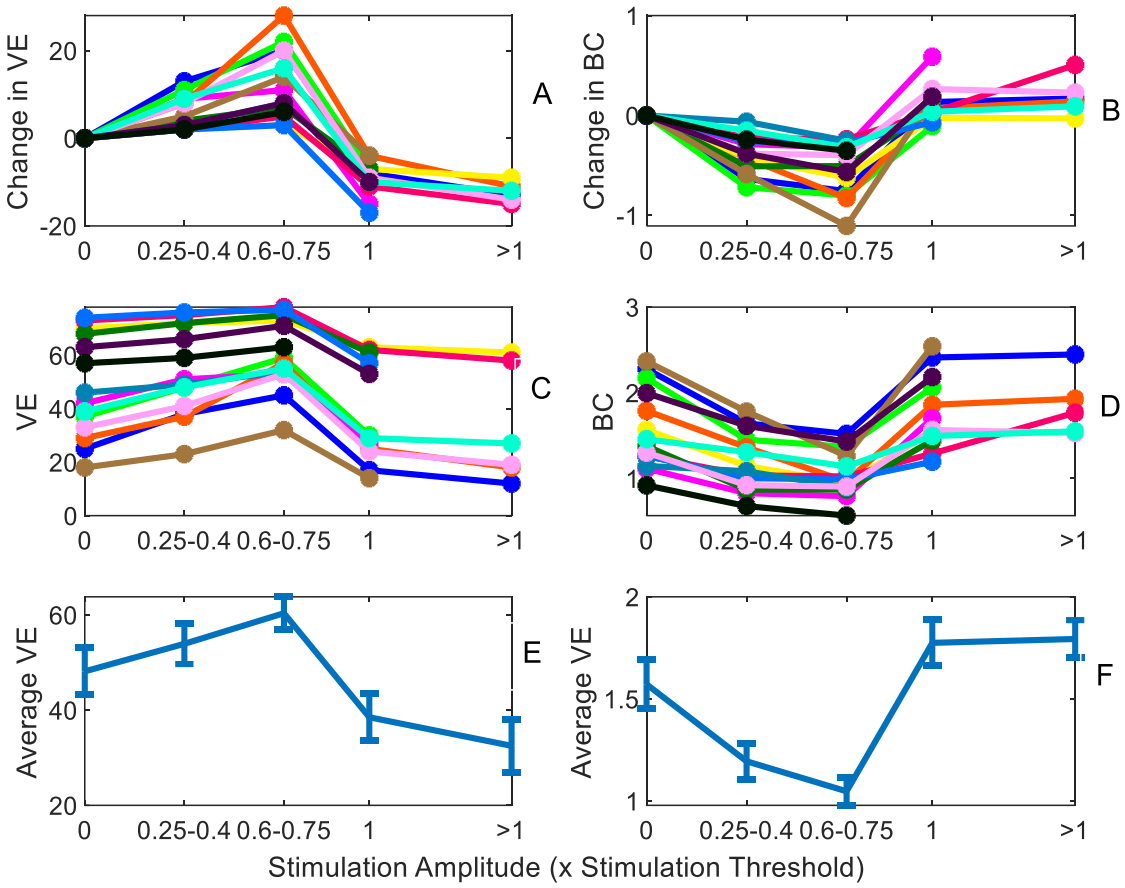

Figure 24. A: the change in voiding efficiency (VE) in old animals (each color corresponds to one animal) in response to different stimulation amplitudes. B: the corresponding change in bladder capacity (BC) in old animals in response to different stimulation amplitudes. C: the VE in old animals in response to different stimulation amplitudes. D: the BC in old animals in response to different stimulation amplitudes. E: voiding efficiency in response to different stimulation amplitudes averaged across animals. F: bladder capacity in response to different stimulation amplitudes averaged across animals. Error bars in E and F are standard errors.

CHAPTER 5

DISSERTATION CONCLUSIONS

5.1 Summary

In this study, the functionality of voiding reflexes with age was evaluated, the contributing factors that can lead to weakness of voiding reflexes were identified and the efficacy of percutaneous stimulation of urethral nerve on improving the symptoms of UAB in aged rats was tested.

We evaluated the role of sensory feedback from the bladder and the urethra independently on reflex bladder contractions using a split preparation method. We showed that the same urethra flow rate evoked bladder contractions in older animals less often than in younger ones, and within all age groups higher urethra flow rate trials evoked contractions more frequently. We also found that the AR required a fuller bladder to become active in old rats than in young rats, suggesting a functional decline age-related activation of urinary tract reflexes associated with the "voiding mode". Old rats also exhibited longer and weaker bladder contractions than the young or mature groups. Taken together, the results of our first experiment (chapter 2) are consistent with the hypothesis that the sensitivity of urinary tract reflexes declines with age, which may contribute to some age-related voiding dysfunction such as UAB.

The sensitivity reduction of AR (urethral to bladder reflex) with age encouraged us to evaluate the afferent signaling in the pudendal nerve, which carries urethral sensation. We evaluated the signaling of urethral afferents by directly measuring its activity in response to urethral infusion and found that equivalent flow rates evoked less urethral afferent activity in the older rats. To understand what factors were driving the afferent reduction,

we examined the pudendal nerves histologically and measured the pressures that developed in the urethra during infusion. We found evidence that axonal myelination decreased in old rats and lower pressures developed in the urethra of older rats during infusion. Our results suggest that this age-related reduction in urethral afferent outflow is mediated by a mechanical component, presumably a more compliant urethra, and a non-mechanical component, presumably neuropathy.

The effectiveness of percutaneous electrical stimulation of a pudendal nerve in aged rats on voiding efficiency (VE) and bladder capacity (BC) was evaluated. We showed that the minimally invasive stimulation of urethral nerves could increase voiding efficiency and prevent the bladder from overdistension in animals with UAB symptoms.

5.2 Conclusions

The following conclusions have been drawn based on the results of our experiments in previous chapters.

- There is an age-related functional weakening, dysregulation and loss of sensitivity in LUT reflexes (urethral afferent to bladder efferent reflex and bladder afferent to bladder efferent reflex), which may contribute to age-related UAB symptoms.
- Afferent activity in the pudendal nerve, which carries urethral sensation and initiates augmenting reflex, decreases with age, and a combination of urethral afferent degradation and an increase in urethral compliance might contribute to a reduction in urethral sensory outflow.
- Percutaneous stimulation of pudendal nerve could increase voiding efficiency and prevent the bladder from overdistension in animals with UAB symptoms.

5.3 Limitations

The methodology of our study has a number of limitations that we note here to help clarify the interpretation of our results. 1) We tied a catheter around the bladder neck to infuse the urethra. This might prevent mechanical distension of the proximal urethra and influence flow-evoked pressure (or tension) and afferent activity results. 2) It is possible that urethane anesthesia dosed according to body weight (the gold-standard approach) does not result in equal responses in young and old animals. If urethane suppresses afferent activity or urethral muscle tone to a different extent in old rats than young rats, the anesthesia itself could contribute to the differences we observe between ages. 3) Our study isolated and quantified the fundamental response of urethral afferents and urethral pressure to applied urethra flow. To perform this characterization, we needed to remove many potentially confounding aspects present in natural voiding, which included enforcing a constant flow rate throughout urethral infusion and maintaining an empty bladder, neither of which is typical of physiological voiding. Therefore, our results specifically highlight changes in fundamental afferent activity related to aging and cannot be used to directly infer functional age-related differences. This work only discovered that age-related differences in afferent responses and pressures exist, but their explanatory role in functional age-related LUT decline remains to be determined. 4) We measured urethral pressure rather than tension, which is very likely the primary sensed urethral stimuli. If these two quantities diverge substantially during infusion, our estimate of the mechanical effects on age-related afferent loss may also change. We expect any effects of using pressure as a surrogate for tension to be small, since these variables will be highly correlated with each other in many physiological conditions, and multiple models have shown success predicting urethral

afferent discharge from pressure information. 5) most of the stimulation trials tested only once in most of the animals. This was mainly because aged rats are not stable under anesthesia and their bladder gets non-responsive or they die. However, our previous experiments and previous research studies have shown that the within animal variation of VE and BC is small.

5.4 Future work

In this study we showed that percutaneous stimulation of the pudendal nerve could increase voiding efficiency and decrease the volume threshold for evoking bladder contraction (BC). The fact that the superposition of pelvic and pudendal afferent activity is responsible for bladder excitation evoked by pudendal afferents (94), and our results in chapter 2 that showed the engagement of AR (urethral to bladder reflex) is delayed with age (possibly due to reduced pudendal afferent activity), indicate that the stimulation might have improved the voiding efficiency by increasing the signaling of pudendal afferents and prevent the bladder from overdistension. However, the signaling of pudendal afferents needs to be recorded and evaluated in the future studies to understand the causal link.

The VE in aged animals (at no stimulation) had a big variability ranging from 20% to 80% and the percutaneous stimulation of pudendal nerve was more effective on increasing VE in animals which had low VE. That means, in animals with low VE, there might be severe neuropathy in pudendal nerve and electrical stimulation could have improved it by increasing the signaling of pudendal afferents. In fact, the effectiveness of electrical stimulation on improving VE depends on the extent of neuropathy in the pudendal nerve. Therefore, in animals with large VE, the stimulation may not be very effective due to the

low level of neuropathy in the pudendal nerve. However, since the pudendal afferents of aged animals after the stimulation study were not histologically tested, additional studies are required to evaluate the casual link.

The significant reduction in the bladder blood flow observed in elderly patients might indicate that vascular risk factors for atherosclerosis, which eventually lead to bladder ischemia, may have important role in the development of lower urinary tract dysfunctions with age. Therefore, understanding the time course of the progression of vascular dysfunction might prevent the bladder nerve and muscle degradation and bladder dysfunctions in elderly (71).

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