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Urethral Musculature and Innervation in the Female Rat

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Abstract

Aims—The urethral sphincter and urethral muscle innervation are critically involved in maintaining continence, especially in the female. However, the urethral muscle type and distribution, as well as the urethral nerves are far from being well documented. Our aim was to clearly identify the distribution of urethral striated muscle, smooth muscle, and urethral nerves.

Methods—In a cohort analysis of 3-month-old female Sprague-Dawley rats, cross and longitudinal sections of female rat urethra were extensively investigated using morphological techniques. Urethras were harvested to the sections, in order to provide both global and detailed visions of the urethra. H&E, Masson's Trichrome, phalloidin and immunofluorescence stains were used. The cytoarchitecture, nitroergic, and cholinergic innervations were mainly investigated. Different layers of the segments of urethra were traced to draw curve graphs that represent the thickness of each muscle layer of urethral wall.

Results—The results showed that the primary peak of striated muscle is in the middle urethra. The inner layer close to mucosa was found to contain longitudinal smooth muscle. Near the bladder orifice, the circular smooth muscle dominates, which becomes thinner distally throughout the rest of urethra. In the middle urethra the vast majority of the urethral muscle are circularly oriented striated muscle cells. Typical nerve endings were present in high power images to show the different characteristic features of nerve innervation.

Conclusions—This study has illustrated the detailed morphological structure and innervations of the normal female rat urethra can serve as a basis for further study for stress urinary incontinence (SUI).

Keywords

muscle types; rat urethra; stress urinary incontinence; nerve innervations; cytoarchitecture

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Introduction

Stress urinary incontinence (SUI) affects 25–50% of women and costs 12 billion dollars annually in the United States (Chong, Khan, and Anger 2011). In SUI patients, the urethral striated muscle is significantly reduced, and this decline roughly parallels the decrease in maximal urethral closure pressure (Morgan et al. 2009). Urethral smooth muscle declines with age, but whether this contributes to SUI pathogenesis is not certain (Delancey 2010). In SUI animal models both striated and smooth muscles are significantly reduced (Lin et al. 1998, Cannon et al. 2002, Badra et al. 2013). While it is well known that the female rat urethral musculature comprises an inner longitudinal smooth muscle layer, a middle circular smooth muscle layer, and an outer striated muscle layer, the distribution of these layers along the length of the urethra remains disagreeable upon between dedicated studies. Specifically, while Kim et al (Kim et al. 2007) report highly variable distribution of the striated muscle along the length of the urethra, a recent study by Lim et al (Lim et al. 2013) report a nearly uniform distribution of the striated muscle along the entire length of the urethra. Therefore, a thorough investigation using more reliable and specific techniques is eagerly needed.

The urethra is composed of several types of tissues including: mucosa, submucosa, smooth muscle (which are oriented both circumferentially and longitudinally), striated muscle, and connective tissue (Gabella and Uvelius 1990). During bladder filling, the urethra provides a tight mucosal seal, playing an instrumental role in urinary continence. This tightly regulated process is controlled by the hypogastric and pelvic nerves, which transmit signals from a complex neural control system located in the brainstem and spinal cord (de Groat et al. 2001). The urethra is innervated by sympathetic, parasympathetic, and somatic nerve fibers. Given this close regulation, neurologic diseases that affect any of these fibers, can alter the coordination between the bladder and external urethral sphincter (EUS). In addition, injuries to the neuromuscular function of the EUS such as in childbirth or during operative interventions, may result in SUI (Hijaz et al. 2008).

SUI is the involuntary leakage of urine with increased abdominal pressure due to effort or exertion, such as with sneezing, coughing, laughing, straining, or lifting (Abrams et al. 2002). Because it occurs during daily living activities, it severely impacts quality of life and can be quite debilitating. A major risk factor for SUI is vaginal parity, which results in muscle, nerve and connective tissue injury. SUI following vaginal childbirth is relatively common, affecting up to 30% of primigravida mothers. Although severe SUI is self-limited in many patients, insidious damage to pelvic floor nerves, muscles, and connective tissues induced by pregnancy and parturition may remain after childbirth. Consequently, SUI often will develop during the perimenopausal years (Meyer et al. 1998, Gunnarsson and Mattiasson 1999). Age and parity are known to affect urethral anatomy. Age was found to be associated with a decline in striated muscle cells and circular smooth muscles of the urethra (Delancey 2010), while vaginal parity was demonstrated to damage the urethra by causing a decline in urethral musculature (both smooth and striated) in rat models of vaginal delivery (Lin et al. 1998).

In spite of its prevalence and significant impact on quality of life, there are few effective non-surgical and pharmacological therapies. Part of this may stem from the complex anatomy and tight neuromuscular control of micturition. Therefore, a better understanding of the neurologic innervations, muscles, and connective tissues of the female urethra is required. We consequently sought to better characterize these elements in the female rat urethra, which will be instrumental and beneficial for future SUI research and development of SUI treatment options.

Accurate and representative animal models are essential to test hypotheses regarding mechanisms of urinary continence, SUI pathophysiology, and potential therapeutic interventions. Although urethral and sphincter anatomy and function have been extensively investigated (Praud et al. 2003, Brown 2011), only a few studies have described the morphology of the pudendal nerve (Kane et al. 2002, Kim et al. 2007). Additionally, although the striated muscle fibers of the rhabdosphincter have been previously described microscopically (Russell et al. 1996, Praud et al. 2003), these studies have not provided a clear view of the different types of muscles in the urethra. Furthermore, the standardized location of EUS and its innervations remains controversial.

A clear understanding of the female rat urethral anatomy is essential to fully utilize this as an appropriate animal model for the study of SUI. The aim of our study is to further characterize gross and microscopic urethral anatomy, define the location and different types of muscle fibers in the urethra, and delineate urethral nerve innervations utilizing immunohistochemical techniques in adult female rats.

Materials and Methods

Animals

Twelve adult female Sprague-Dawley rats at age 12 weeks and weighing 250 to 300 grams were used. Rats were housed and cared for according to guidelines of the Institutional Animal Care and Use Committee (IACUC) of University of California, San Francisco. All efforts were taken to minimize animal suffering and minimize the number of animals used.

Urethral tissue preparation

Rats were anesthetized with isoflurane and sacrificed by thoracotomy. After euthanasia, the rat urethras were harvested en bloc with anterior vaginal wall and fixed in cold 2% formaldehyde and 0.002% saturated picric acid in 0.1M PBS, pH 8.0 for four hours followed by overnight immersion in 0.1M PBS containing 30% sucrose. The specimens were then embedded in OCT Compound (Sakura Finetic USA, Torrance, CA, USA). Urethral specimens from six animals were fixed with catheters in place and six animals without catheters. In brief, after the entire urethra was dissected from the rat, a polyethylene-90 tube was inserted into the lumen to provide rigidity during fixation, and the fixed urethra was then sectioned without the tubing. These were then embedded transversely and sectioned longitudinally, or embedded longitudinally and sectioned transversely respectively.

The urethra was divided into four parts. These were defined as the proximal, middle, distal, and meatus segments. Transverse sections (10 microns) were mounted onto SuperFrost-Plus

charged slides (Fisher Scientific, Pittsburgh, PA), and then divided into 18 sets (60 sections per series).

H&E staining and Masson's trichrome staining

Each set of sections was processed with H&E staining to delineate overall cytoarchitecture and Masson's trichrome staining for urethral muscle fiber and collagen components. Urethral tissues were immersed in warm (58°C) Bouin solution for 20 minutes, rinsed, then stained with Weigert Hematoxylin for 10 minutes, and then rinsed until only nuclei remained stained. The sections were then stained with Biebrich Scarlet-Acid Fuchsin for 3 minutes, rinsed, and immersed in phosphomolybdic acid for 45 minutes. Next, the sections were stained with Aniline Blue for 3 minutes, rinsed in distilled water for 2 minutes, immersed in 1% acetic acid for 2 minutes, and rinsed twice in distilled water for 2 minutes. Finally, the sections were dehydrated through increasing concentrations of ethanol, air dried, and mounted.

Phalloidin staining

Tissue sections were incubated with Alexa-488/594-conjugated phalloidin (1:500; Invitrogen, Carlsbad, CA, USA), which was diluted 1:200 in 1% bovine serum albumin, for 20 minutes at room temperature. After rinsing with PBS, the tissues were stained with 4',6-diamidino-2-phenylindole (DAPI), for nuclear staining, 1 µg/ml (Sigma-Aldrich, St. Louis, MO, USA).

Immunofluorescence staining

Tissues were rehydrated with PBS for 5 minutes, and treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity. After rinsing with water, sections were washed twice in PBS for 5 minutes, followed by 30 minutes of incubation with 3% horse serum/PBS/0.3% triton X-100. After excess fluid was drained, sections were incubated overnight at 4°C with primary antibodies including anti-neuronal nitric oxide synthase (nNOS; 1:200; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-tyrosine hydroxylase (TH; 1:200; Millipore Corp, Bedford, MA, USA), anti-choline acetyltransferase (ChAT; 1:200; Millipore, , Bedford, MA, USA) and anti-myosin heavy chain (MHC; 1:500; Abcam, Cambridge, MA, USA) . Secondary antibodies used included Alexa-488- and Alexa-594- conjugated antibodies (1:500; Invitrogen). Nuclei were stained with DAPI. For image analysis, five randomly selected fields per tissue were photographed and recorded using Retiga Q Image digital still camera and ACT-1 software (Nikon Instruments Inc., Melville, NY).

Results

Anatomy of female rat urethra

In adult female rats, the urethra is 15.26 ± 0.21 mm (n=6) in length, extending from the internal urethral orifice at the bladder neck to the external urethral meatus (Fig. 1A). Additionally, urethral smooth muscle layers are not as regularly oriented as in the gut. Rather, there are two layers of smooth muscle fibers, an inner layer oriented longitudinally and an outer layer surrounding it oriented in a circular circumferential manner (Fig. 1B).

The external urethral sphincter (EUS) is located in the middle part of the urethra (Fig. 2A). At this level, the urethral epithelium is surrounded by a submucosal layer containing well-developed venous plexus and smooth muscle. Surrounding this are fibers of the striated muscle. Striated muscle fibers cover part of the proximal and middle urethra (30–40%). The striated urethral sphincter is circular in its proximal part with only a fibrous raphe connecting both sides on its dorsal aspect. The distal part differs from the proximal part in that it has dorsolateral wings that cover the vagina laterally (Fig. 2C).

The distal urethra becomes the external urethral meatus, which is located between the clitoris and the vagina in the vulvar vestibule of the female genitalia. At this level, we do not find significant amounts of smooth muscle or striated muscle bundles, rather more clitoral gland tissue.

Urethral muscular layers

As mentioned previously, there are longitudinal and circular smooth muscle layers that span from the bladder neck proximally along the entire length of the urethra. However, the striated muscle fibers that comprise the EUS are located in the middle portion of the urethra. The internal and external urethral sphincters cannot be distinguished by H&E staining or Phalloidin staining (sFig1).

In order to further quantify and classify our morphological observations of rat urethral muscle fibers, myosin heavy chain/smooth muscle antigen (MHC/SMA) double staining was implemented to distinguish the striated muscle layer from the smooth muscle layers. We noted that the maximal thickness of the sphincter with striated muscle fibers (MHC staining) was located in the middle third of the urethra (Fig. 2B).

Representative transverse sections from 18 sets were chosen for measurement. Different layers of the segments of urethra were traced in the transverse plane through the 18 sections. We plotted the thickness of each type of urethral muscle as a function of distance from the bladder neck using tissue from 3 female rat urethras (Fig. 2D). This clearly demonstrates a peak in striated muscle fibers in the middle urethra representing the EUS.

Additionally, the composition of the smooth muscle also differs drastically based on the distance from the bladder neck. Circular smooth is more prominent proximally at the bladder neck then becomes extremely thin at middle section and again increases distally with some expansion at the level of the urethral meatus. On the other hand, longitudinal smooth muscle fibers remain thin but more consistent throughout the length of the urethra. As mentioned previously, most of the striated muscle fibers are found in the middle part of the urethra. Interestingly, the smooth muscles are partly interlaced with the striated muscles (sFig2).

Nitroergic, adrenergic and cholinergic innervations

Urethral nerve distribution was detected by phalloidin and nNOS/TH/ChAT double staining (Fig. 3, 4, 5, sFig3). In longitudinal sections, nNOS staining fibers are expressed in greater quantities at the middle urethra, whereas ChAT is expressed evenly throughout the urethral.

TH is expressed relatively more in the smooth muscle layers in the middle part of the urethra.

In the wide connective tissue spaces between the smooth muscle bundles, there were many nerve fibers. High power imaging revealed that nNOS fibers were longer and thicker compared to TH/ChAT, and they were densely distributed around both smooth muscle layers and striated muscle layer. While around the EUS in the middle urethra, there were more nitrergic and cholinergic nerve innervations compared to other areas of the urethra.

Smooth muscle cells in the wall of the bladder and urethra were densely innervated by autonomic nerve fibers. Although striated muscle fibers were mainly innervated by somatic motor nerves, we did find these were also innervated by autonomic nerves as these fibers were partly interlaced with urethral circular smooth muscles (Fig. 6A).

Typical nerve endings were present in high power images to show the different characteristic features of nerve innervations. The nNOS positive fibers were noted frequently in the striated muscle layer of the EUS. These nerve endings, unlike those innervating smooth muscle cells, did not form any terminal swellings, but instead, gradually diminished the diameter to a tapering end (Fig. 6B). A small number of spherical TH positive ganglion cell bodies were present in the outermost striated muscle layer (Fig. 6C). A ChAT positive motor endplate was shown in the EUS as well (Fig. 6D).

Discussion

Although the structure of female rat urethra and its nerve innervations have previously been described, urethral muscle types and distribution and the details about the EUS are still controversial. Additionally, a more precise understanding and description of urethral nerve fiber distribution and type is still required, particularly nerve fiber association with specific muscle types. These findings are clinically relevant as neuromuscular injuries play a significant role in urinary incontinence.

Previous studies have provided evidence that the rat urethra contains smooth and striated muscle fibers. Additionally, smooth fibers are oriented both longitudinally and circularly (Andersson, Malmgren, and Uvelius 1990). It has also been shown that smooth muscle fibers surround the urothelial epithelium, and these fibers increase in thickness and numbers of bundles with increasing distance from the renal pelvis (Klutke et al. 1990). In the urethra, longitudinal smooth muscle may be a continuation of the bladder smooth muscle and, and if activated simultaneously with the bladder during micturition, it may function to shorten the urethra, thereby decreasing urethral resistance during voiding (Andersson, Malmgren, and Uvelius 1990).

In the present study, we provide detailed gross and immunohistochemical images of the female rat urethra. Because the majority of previous studies have only focused on urethral muscle tissue, we aimed to provide a more complete picture of rat urethral anatomy, particularly neuromuscular structures. Particularly, we characterized and quantified the different types of muscle fibers in the urethra. In addition, we characterized and described the specific nerve fibers and their distribution in the urethra. This detailed neuromuscular

description provides a more complete view of the EUS and its function in urine storage and micturition.

Recent histological studies have described the percentage of muscle in the urethra (Lim et al. 2013, Kim et al. 2007). In their microscopic study, Lim et al. (2013) stated that EUS was primarily distributed in the proximal urethra. Our results, however, indicate that there is an increase in striated muscle fibers that actually starts at the end of the proximal urethra and peaks in the middle part of the urethra. These findings are more consistent with Kim et al [7]. However, they described twin peaks of both striated muscle and nerve innervations, which was not observed in our study. This may be the result of their method of tissue preparation (12 sets) in comparison to our technique of sampling (18 sets).

Unlike in male rats, we did not find significant striated muscle intrusion into smooth muscle as was reported in previous studies (Watanabe and Yamamoto 1979). There was only a minor interlace of fibers observed at the outer part of the circular smooth muscle. Transdifferentiation of a smooth muscle stem cell to a striated phenotype has been previously postulated to explain the presence of striated muscle (Patapoutian, Wold, and Wagner 1995). The striated component of the urethral sphincter is the result of migration of lateral somite mesoderm cells into the periurethral smooth muscle mesenchyma. These stem cells may then differentiate into striated myotubules that are anatomically distinct from other striated muscle groups in the region (Sarig et al. 2006). According to the present study, we illustrated the “smooth muscle-striated muscle cross zone”, indicating the transdifferentiation progress. We also found the EUS had specific structural features, identified as the striated muscle wings. This structure was only observed in the distal part of EUS. In contrast, the proximal part of EUS is basically circular. Studies showed that the striated urethral sphincter of rabbit present a similar feature we observed in rat urethra (Taneli et al. 2004, Genc et al. 2002).

According to the present study the inner longitudinal muscle layer of the proximal urethra received an equal distribution of adrenergic and cholinergic fibers, in agreement with Gosling and Dixon (1975) (Gosling and Dixon 1975). But, in the circular layer, as well as in the outer longitudinal layer, most of the nerve endings were adrenergic. In other words, cholinergic fibers are mainly located in longitudinal and circular smooth muscle layer in the middle and distal urethra. These findings suggest that the detrusor muscles of the bladder and the circular muscles of the proximal urethra (sphincter muscles) are under different neuronal control (Long et al. 2009). The sympathetic nerve plays a more important role than the parasympathetic nerve in elevating urethral pressure (Yoshiyama and de Groat 2002). The present morphological observations are in good agreement with these investigations.

The striated EUS is innervated by somatic motor neurons via the pudendal nerve providing voluntary and involuntary control (Radziszewski et al. 1996). There has been a recent and lively interest in EUS innervations (Johansson et al. 2002, Garcia-Pascual et al. 2010), but nitrergic nerves were found only at the most inner portion of the striated muscle layers and no relationship between nitrergic nerves and muscle endplates have been observed (Gonzalez-Soriano et al. 2003, Ho et al. 1998). Thus, our results, which give more detail and

higher accuracy, provide strongest evidence of nNOS expression in the sarcolemma and the cytoplasm of some striated muscle fibers in the EUS.

Conclusions

The present study provides a more complete and detailed description of the neuromuscular and morphological structure of the normal female rat urethra. It demonstrates the more prevalent adrenergic control on the circular and outer longitudinal muscle layers, thereby proving its more important role than cholinergic control in elevating urethral pressure. In addition, we showed that the thickest EUS layer was present in the mid-urethra, and is under strong autonomic innervation. A thorough knowledge of the cytoarchitecture, nitrergic, adrenergic and cholinergic innervations of the rat urethra is crucial for future experimental studies, development of therapeutic options, and a greater understanding of urinary incontinence. Because the muscle and nerve distribution in the rat urethra is similar to that in human, this supports the use of the rat as an appropriate animal model for both experimental studies and clinical investigations relevant to SUI caused by aging or childbirth injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Anatomy of female rat urethra. Representative morphology shows the corresponding location of the longitudinal section of female rat urethra using H&E staining (A) and Masson's trichrome staining (B). Arrows in the longitudinal sections point to levels along the urethra where proximal, middle, and distal segments were obtained. An inner layer of smooth muscle fibers is oriented longitudinally and an outer layer surrounding this is oriented in a circular manner circumferentially.

Fig. 2.

Immunohistochemical staining with MHC and SMA reveals the normal distribution of striated and smooth muscles in the proximal, middle and distal urethra. The external urethral sphincter is located in the middle part of the urethra (A), the maximal thickness of the sphincter with striated muscle fibers (MHC staining) is located in the middle third of the urethra (B). The distal part of the EUS has dorsolateral wings that cover the vagina laterally (C). The thickness of each type of urethral muscle from the bladder neck is shown in line graph (D).

Fig. 3.

Immunohistochemical double-staining with Phalloidin and nNOS in the urethra. Arrows in the longitudinal section point to the areas of the urethra where the proximal, middle, and distal segments were obtained. In the longitudinal sections, nNOS staining fibers are expressed in greater quantities at the middle urethra. High power images show nNOS fibers are long and thickly distributed around smooth muscle and striated muscle layers.

Fig. 4.

Immunohistochemical double-staining with Phalloidin and TH in the urethra. Arrows in the longitudinal sections point to the areas of the urethra where the proximal, middle, and distal segments were obtained. TH is expressed relatively more in the smooth muscle layers in the middle and distal part of the urethra.

Fig. 5.

Immunohistochemical double-staining with Phalloidin and ChAT in the urethra. Arrows in the longitudinal sections point to the areas of the urethra where the proximal, middle, and distal segments were obtained. Morphology images show that ChAT is expressed less in the proximal urethra compared to the middle or the distal part of the urethra.

Fig. 6.

Representative typical nerve endings are shown in high power images to delineate the different characteristic features of nerve innervations. The smooth muscles are partly interlaced with the striated muscles from the EUS (A). The nNOS positive fibers are noted frequently in the striated muscle layer of the EUS, which gradually tapers off and diminishes (B). A small number of spherical TH positive ganglion cell bodies are present in the outermost striated muscle layer (C). A ChAT positive motor endplate is shown in the EUS (D).