Urethral function in overactive bladder syndrome

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CHAPTER 2

INITIAL REPORT ON DISTRIBUTION OF β3-ADRENOCEPTOR IN THE HUMAN FEMALE URETHRA

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INTRODUCTION

The micturition cycle consists of a storage phase and a voiding phase. The bladder functions as the reservoir where urine is storaged, as long as the urethral pressure withstands the pressure in the bladder[1]. When this coordinated function is disturbed, lower urinary tract symptoms (LUTS) or overactive bladder symptoms (OAB) can occur. Barrington was the first to describe seven reflexes involved in bladder storage and micturition in the cat[2, 3], of which four have their afferent origin in the urethra. Therefore, a disturbance in urethral function can probably result in OAB symptoms as well. When outlet resistance suddenly changes because of urethral pressure variations, leakage of urine into the urethra can occur, which in turn may stimulate urethral afferents inducing an involuntary voiding reflex[4]. In the past, a functional disturbance in urethral function leading to OAB was defined as urethral instability (URI)[5]. Yet, since the initial reports the clinical relevance of URI has been controversial and because lack of consensus, the condition was abandoned in terms of the International Continence Society (ICS). A recent review together with the report from the ICS Research Society meeting in 2014 concluded that urethral instability may be regarded a potentially pathophysiological entity of its own within cohorts of patients with OAB[6, 7]. In addition, a recent review of overactive bladder pathophysiology underlined the importance of identifying subgroups of patients within OAB to optimize tailor treatment[8].

Studies to urethral closure function in female patients in the past concluded that the sympathetic nerve system dominates in regard to maintaining the tonus in the urethra[9, 10]. Throughout the entire length of the urethra, administration of noradrenaline or β2-adrenoceptor agonist resulted in a contractile response. Currently, the ADRB3 agonist mirabegron has an important role in the treatment of OAB. Recently, Coelho et al described bladder structures expressing ADRB3[11]. The presence of ADRB3 within the human urethra has not been demonstrated to date. The aim of this study is to investigate the presence of ADRB3 in the human female urethra to contribute to elucidating the effect and side effects of current therapy with mirabegron.

MATERIAL AND METHODS

We performed anatomical studies in 5 female specimen. We started our experiment with three urethra specimen from the body donation program. These results were presented at the annual ICS-meeting in Tokyo in 2016. However, because of tissue decay, the quality of tissue sampling wasn't optimal. We then continued our study with tissue from two female patients with muscle invasive bladder cancer, where radical resection of bladder and urethra was performed. Pre-operatively, the patients were not treated with intravesical or systemical chemotherapy. Patients consented to use the tissue for scientific research anonymously. When bladder neck and urethra were macroscopically tumor free, the urethra up till the bladder neck was separated from the rest of the bladder and freshly obtained for this research. The urethra was transversely divided in 4 areas from meatus to bladder neck. The areas are the bladder neck, the proximal urethra, the mid-urethra and the distal urethra/meatus. Half of the tissue was embedded in optimal cutting temperature compound (OCT) (TissueTEK OCT) and stored at -80°C or directly fixed in 4% paraformaldehyde overnight and processed for paraffin-embedding. For demonstrating ADRB3 expression, we used rabbit polyclonal anti-human ADRB3 LS-A4198.

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Paraffin-embedded tissues were sectioned at 5 μm and deparaffinized. OCT-embedded tissues were cut at 5-μm using a cryomicrotome, and fixed in 4% paraformaldehyde for 10 min at room temperature. All sections were permeabilized in 0.1% Triton-100 in Tris Buffered Saline (TBS). Endogenous peroxidase was blocked by incubation in 1% H2O2/ TBS. Parafin-embedded sections, but not the cryosections, were boiled for 7 minutes in citrate buffer (0.01M) for antigen retrieval. Sections were blocked with 10% Fetal Calf Serum (FCS)/TBS for 30 minutes at room temperature, before O/N incubation with the primary antibody: a rabbit polyclonal anti-human β3AR (LS-A4198, Life Span Biosciences), directed against the N-terminus, and diluted 1:400 in 1%BSA (Sigma)/TBS, and no first antibody served as negative controls.

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The polyclonal anti-human β3AR (LS-A4198, Life Span Biosciences, 1:200) and the mouse monoclonal α-beta III Tubulin (SC-80005, Santa Cruz, 1:1000) in PBS-T with 1% BSA (PBST/BSA) were incubated overnight. Next morning slides were rinsed in 2 times PBS and PBS-Tween. Then the sections were incubated with a mixture of donkey anti-rabbit-Alexa-555 (A-31572, Life technologies) and donkey anti-mouse-Alexa-488.(A-21202, Life technologies) for 60 minutes. Both antibodies were diluted 1:200 in PBST/BSA. After rinsing again the sections were incubated with a 0,3 μM DAPI (D3571, Molecular probes) for 5 minutes. The slides were rinsed with 2 times PBS and mounted in ProLong Gold antifading Reagent (P36930, Molecular probes). The imaging was performed with the panoramic 250 Flash (3DHISTECH)

RESULTS

In the adult female, the urethra has a length of 3-4 cm average and consists of three layers[12]. From inside to outside the first layer is the mucosal layer. In the region of the bladder neck and proximal urethra this is lined by urothelium (figure 2.1B, TE). In the middle and distal part, the urethra is lined by stratified squamous epithelium (figure 2.1D). The second layer is the submucosal layer (figure 2.1D), consisting of connective tissue, elastic tissue, a vascular plexus and periurethral glands (figure 2.1F). The third layer is the muscular layer, consisting of an inner longitudinal and circular smooth muscle layer (figure 2.1E) and an outer circular striated muscle layer, also referred to as EUS. The EUS has its thickest part in the middle third section of the urethra, where striated

Figure 2.1 HE-staining of transverse cross-sections of the urethra and bladder neck. **A:** bladder neck with transition to the proximal urethra. L=lumen. Square box indicates transitional epithelium. **B:** Detailed image of the square box in A. L=lumen bladder, SM=submucosal layer, TE=transitional epithelium. **C:** transverse cross-section of mid-urethra. L=lumen. Square box D demonstrates stratified squamous epithelium and is in detail in image 1D. Square box F refers to figure 1F, an example of a peri-urethral gland. **D:** Detailed image of the square box in C. L=lumen. SE= squamous stratified epithelium. SM=submucosal layer **E:** able- fluorescence image of C. L=lumen. LSMC=longitudinal smooth muscle layer. CSMC=circular smooth muscle layer. **F:** detailed image of the peri-urethral gland. Scale bar A,C,E=1000μm. B,D,F=50μm

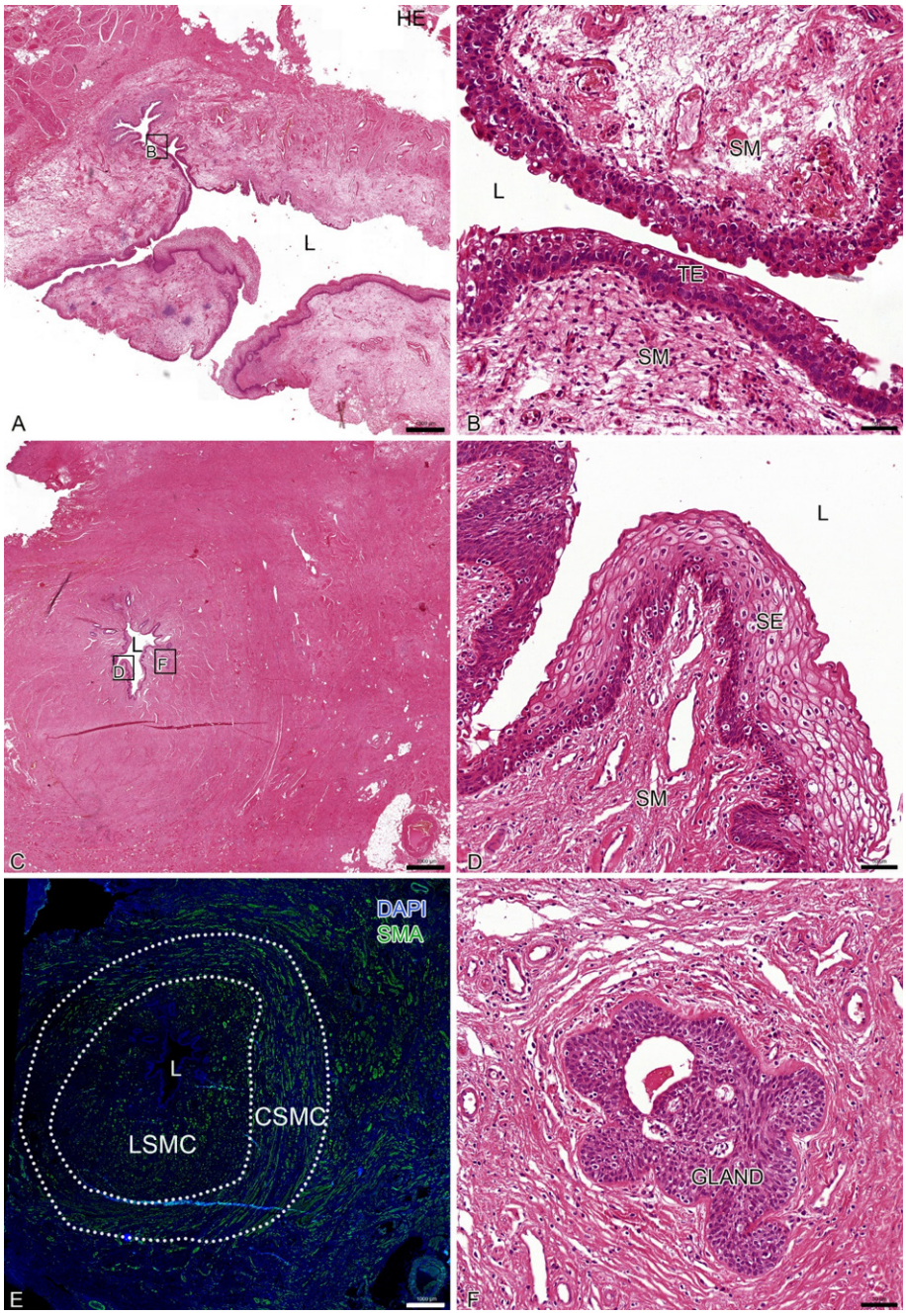

Figure 2.1 For caption see left page

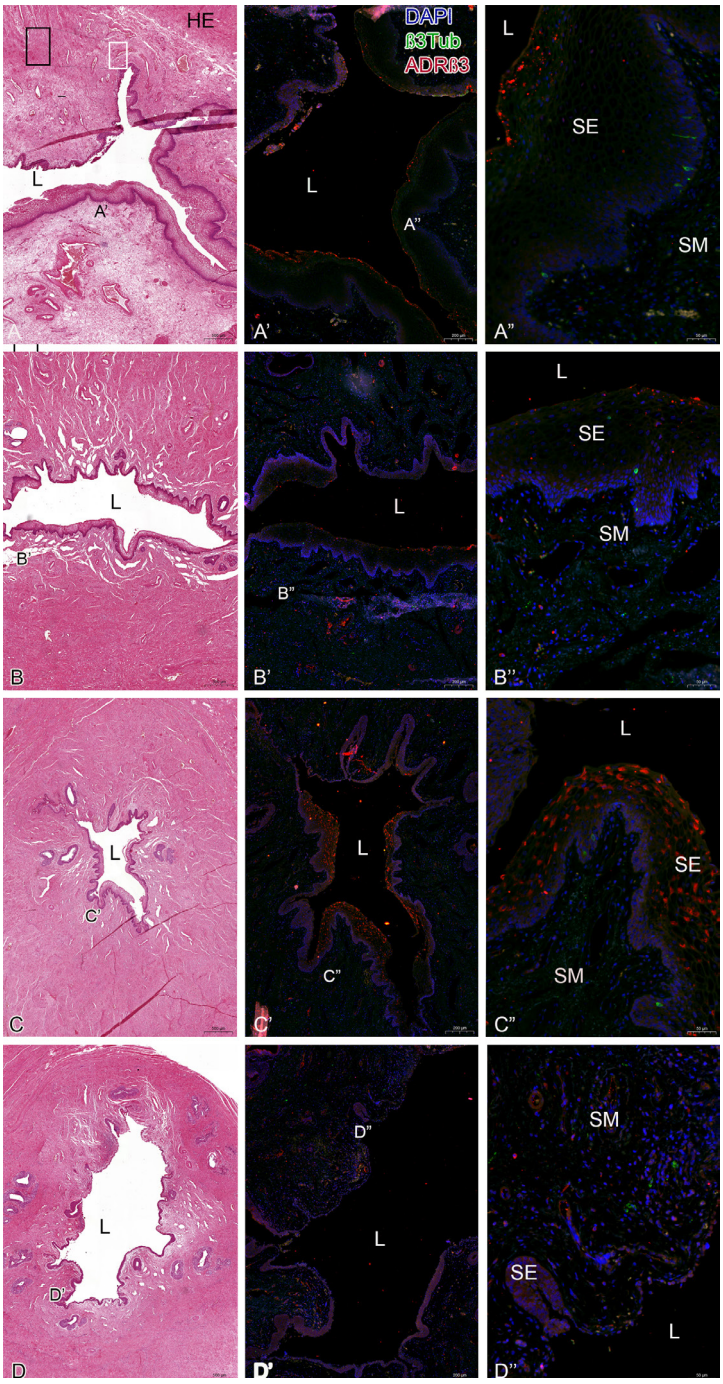

Figure 2.2 For caption see right page

muscle completely surrounds the urethra, although the posterior part is relatively thin. In the proximal and distal part of the urethra, no striated muscle is present in the posterior part.

ADRB3-expression

Expression of ADRB3 was demonstrated in the urothelium and squamous stratified epithelium in all urethral parts, except at the meatus. The pattern and degree of ADRB3 expression differs between urethral regions. At the bladder neck, ADBR3 is expressed in the superficial layer of the transitional epithelium only (fig 2A'). In the proximal urethra, there is a lower level of ADRB3 expression in the superficial layer of the transitional epithelium and the expression is more scattered than at the bladder neck (fig 2B'). The highest level of ADRB3 expression is present in the mid-urethra (fig 2C'). In the distal urethra, at the meatus and the pelvic floor, there is no intraluminal expression of ADRB3 (fig 2D'). ADRB3-expression is also demonstrated in the stratified muscular layer (fig 3). This stratified muscular layer represents the EUS and is the only urethral part with stratified muscles. The other urethral parts contain smooth muscle cells only, where ADRB3-expression is absent. Within the periurethral glands no ADRB3 expression was demonstrated.

Figure 2.2 Distribution of ADRB3 and beta III tubulin in the female urethra with LS-A4198. **A** transverse cross- section of the bladder neck with HE-staining. L=lumen. **A'** able-fluorescence staining for ADRB3 of A. Red spots represent ADRB3 expression in the superficial layer of transitional epithelium. L=lumen. **A''** able-fluorescence staining with beta III tubulin. Arrows indicate the course of neurons in the lamina propria. L=lumen SM=submucosal layer. SE=stratified squamous epithelium **B** transverse cross-section of the proximal urethra with HE-staining. L=lumen **B'** immunofluorescence staining for ADRB3 of B. SM=submucosal layer. SE=stratified squamous epithelium **B''** immunofluorescence staining of B' with beta III tubulin. Arrows indicate expression of beta III tubulin positive neurons. **C** transverse cross-section of the mid-urethra with HE-staining. L=lumen. **C'** able-fluorescence staining of C for ADRB3. L=lumen. **C''** able-fluorescence staining of C' with beta III tubulin. Arrows indicate expression of neurons. L=lumen SM=submucosal layer. SE=stratified squamous epithelium **D** transverse cross-section of the distal urethra with HE-staining **D'** immunofluorescence staining of D for ADRB3 is negative. **D''** immunofluorescence staining of D' with beta III tubulin. Arrows indicate beta III tubulin positive neurons in the submucosal layer. L=lumen SM=submucosal layer. SE=stratified squamous epithelium. Scale bar A,B,C,D=500 μm A'BÇ'D'=200 μm A"B"C"D"= 50 μm

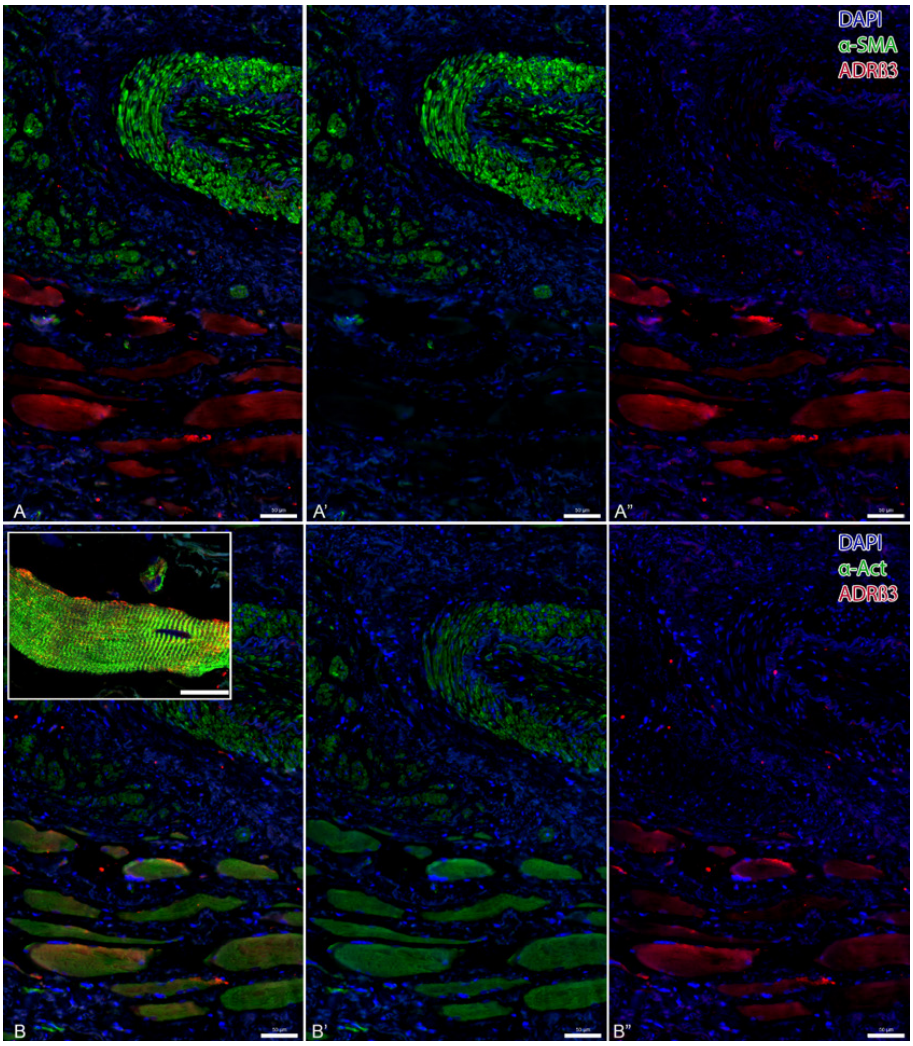

Figure 2.3 Transverse images of the muscular layers of the urethra.
A: fluorescent image of smooth muscle cells, **A'** staining αsmooth muscle actin(αSMA = green) **A''** no ADRB3-expression in smooth muscle cells could be found.
B: Fluorescent image of stratified muscular cells. In the upper left corner detailed image of a stratified muscular cell with scale bar 20μm **B'** demonstrates positive actin staining, **B''** demonstrates ADRB3-expression within the stratified muscular cells. Scale bar all images 50μm

Beta III tubulin staining

Staining with beta III tubulin is positive in the lamina propria of the bladder neck (fig 2A"). There is no direct contact between the cells that express the ADRB3 receptor and the neurons. In the proximal urethra, there is a scattered presence of neurons within the distal part of transitional epithelium and in the submucosal layer (fig 2B"). In the mid-urethra, there is expression of few neurons both in the distal epithelial layer as in the submucosal layer (fig 2C"). The distance between ADRB3 in the squamous stratified epithelial layer and beta III tubulin positive neurons in the submucosal layer is smallest in this section. At the distal urethra, there are few neurons in the submucosal layer(fig 2D").

DISCUSSION

In this study, we performed an anatomical mapping of ADRB3 in five human female urethras. This resulted in two main findings. First, we demonstrated the presence of ADRB3 in the superficial epithelial layer of the urethra. Second, we found that there was no direct contact between ADRB3 receptor and nerve endings.

The predominating β-adrenoceptor in the human bladder is the ADRB3-receptor[13, 14]. Although presence of adrenergic nerves and of ADRB3-receptor have been demonstrated in the striated human urethral sphincter in the past[15], this is the first report on epithelial presence of ADRB3 in the human female urethra.

In animal studies, attempts to identify the subtype beta-adrenoceptor (ADRB) mediating urethral relaxation were based upon the response of urethral pressure to a non-selective agonist, and to selective ADRB1, ADRB2 and ADRB3 agonists and their inhibition by subtype selective antagonists[16]. In rats, bladder pressure was reduced by ADRB2 and ADRBR3 agonist, but urethral pressure reduction was different between ADRB2 and ADRBR3 agonist. The pressure reduction was highest in response to ADRB2 agonist. The effect of ADRB3 agonist on urethral pressure was comparable to the effect of saline. Michel and Vrydag conclude in their review that relaxation of the urethra of rat, dog and pig appear to involve a strong ADRB2-component[17]. The lack of response of urethral pressure to ADRB3 agonist could be due to dominance of ADRB2 in the urethra in rats. We want to address that many adrenoceptor antibodies lack specificity[18], but that the LS-A4198 antibody used in

the current study has been tested and described as most promising[19] to best validated ADRB3 antibody available[14].

Previous studies mainly focused on the role of alfa-adrenoceptor subtypes and the effect of α-adrenoceptor agonists in urethral pressure. However, a randomized placebo-controlled pilot study in female patients with LUTS demonstrated that the alfa blocking agent terazosine was not effective in treating symptoms[20]. The use of α-adrenoceptor agonists in the treatment of LUTS is limited because of adverse effects on the cardiovascular system. Nowadays, with the selective β3 agonist mirabegron available in treatment with patients suffering from OAB, the question rises if and how urethral pressure is influenced by this agent.

Our second finding, the lack of contact between ADRB3 and neurons in the submucosal layer, could suggest the presence of an extra afferent signaling network originating from the urethral epithelial layer. From previous studies is learned that in the bladder, the urothelium itself has neuron-like properties, contributing to sensory transduction mechanisms[21]. The release of the neurotransmitters nitric oxide (NO) and ATP by the urothelium in response to chemical and mechanical stimuli has been demonstrated [22-24]. Activation of ADRB3 receptor on urothelial cells triggers production and release of nitric oxide (NO). For the urethra, no similar findings have been reported. Although urothelium only is present in the proximal urethra, maybe the presence of ADRB3 in other parts of the urethral epithelial layer could result in NO release as well when stimulated. Past studies suggested that alterations in NO-levels may play a role in urothelial signaling in the bladder[25, 26]. The female urethra has a rich vascular plexus and vascular smooth muscle cells are recognized as targets for NO. Activity of the enzyme cyclic nucleotide phosphodiesterase 5 (PDE5) rapidly inactivates the degradation of cGMP and thus increases the effect of NO stimulation. If a similar afferent signaling pathway from the urethra exists, this could be of interest for future research to the role of pharmacotherapy in regard to urethral function. Previous research demonstrated that PDE5 inhibitors promote potent relaxation of animal and human urethral smooth muscle [27]. Male patients with erectile dysfunction and LUTS treated with PDE5 inhibitors may experience a beneficial effect both on erectile function as on their LUTS [28]. Extrapolation of these data suggest that PDE5 inhibitors could have an effect on female urethral function as well, an interesting hypothesis for future research. Given the hypothesis of an additional local mechanism in afferent signaling, future research could

also focus on therapy with a local mechanism of action, as we recently did in a pilot study with paraurethral injections of botulinum toxin-A in patients with URI [29].

The major limitations of this study are the lack of a quantitative analysis, of positive controls, of a functional evaluation and of course the small sample size. Future research should elucidate the optimal protocol for demonstrating the presence of functional ADRB3, combined with functional research, as well as exploring the role of NO pathway in the urethra.

Conclusion

In this study, for the first time expression of ADRB3 is demonstrated in the epithelial layer of the human female urethra. The expression of ADBR3 is present in almost the entire length of the urethra, with the highest level in the mid-urethra. No direct connection between ADRB3 and nerve endings was observed. These findings contribute to better a better understanding of action mechanisms of ADRB3 agonists. Future research should elucidate the local effect of pharmacotherapy.

Acknowledgements

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