Researching Bladder Afferents—Determining the Effects of β_3 -Adrenergic Receptor Agonists and Botulinum Toxin Type-A

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ABSTRACT

A substantial portion of the current research on lower urinary tract dysfunction is focused on afferent mechanisms. The main goals are to define and modulate the signaling pathways by which afferent information is generated, enhanced and conveyed to the central nervous system. Alterations in bladder afferent mechanisms are a potential source of voiding dysfunction and an emerging source for drug targets. Established drug therapies such as muscarinic receptor antagonists, and two emerging therapies, β_3 -adrenergic receptor agonists and botulinum toxin type-A, may act partly through afferent mechanisms. This review focuses on these two new principles and new and established methods for determining their sites of action. It also provides brief information on the innervation of the bladder, afferent receptors and transmitters and how

these may communicate with the urothelium, interstitial cells and detrusor smooth muscle to regulate micturition. Peripheral and central mechanisms of afferent sensitization and myogenic mechanisms that lead to detrusor overactivity, overactive bladder symptoms and urgency sensations are also covered. This work is the result from ‘Think Tank’ presentations, and the lengthy discussions that followed, at the 2010 International Consultation on Incontinence Research Society meeting in Bristol, UK.

Key words: β 3-adrenergic receptor agonists, bladder afferents, botulinum toxin type-A, optical mapping.

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INTRODUCTION

Innervation of the urinary bladder and urethra. The coordination between the urinary bladder and the outlet (the bladder neck, urethra and urethral sphincters) is mediated by a complex neural control system located in the brain, spinal cord and peripheral ganglia as depicted in figure 1. This includes innervation through sympathetic (hypogastric), parasympathetic (pelvic) and somatic (pudendal) nerves. Sympathetic nerves in the human and mouse spinal cords originate in the T₁₁-L₂ segments¹. They run through the ganglia in the inferior mesenteric plexus and then the hypogastric nerves to enter the pelvic plexus and then the base of the bladder and urethra². Parasympathetic nerves arise from the S₂-S₄ (L₆-S₂ in mice) spinal segments and travel in the sacral roots and pelvic nerves through ganglia in the pelvic plexus and into the bladder wall. Somatic nerves innervating the striated muscle of the external urethral sphincter arise from S₂-S₄ (L₆ in mice)³ motor neurons in Onuf's nucleus and pass through the pudendal nerves.

Afferent fiber types and distribution in the bladder wall. Sympathetic, parasympathic and somatic nerves to the bladder may all contain sensory (afferent) nerves. In the urinary bladder there are both A δ - and C-fibers afferent populations, which have been demonstrated to consist of low and high threshold mechanosensitive fibers⁴. The localization and characteristics of these fibers in the bladder has been investigated in various animal models^{5, 6} and a summary of the results is described in figure 2⁷. There is also a class of 'silent' C-fibers that are not excited by known physiological stimuli even at intensities that can damage the innervated tissue⁸. These fibers are particularly numerous in the bladder and other viscera and may constitute up to 90% of the C-fibers⁹. It is suggested that these fibers are nociceptors with their orthodromic activity

summing spatially and temporally at second order neurons to contribute to different pain states⁸. This correlates with the activity of C-fibers increasing in pathological conditions including bladder outlet obstruction¹⁰, spinal cord injury¹¹, diabetes¹², cystitis¹³ and colitis¹⁴. Therefore, specific drug targeting of sensitized afferents could lead to more effective treatments.

Peripheral and central afferent sensitization. Alterations in bladder afferent mechanisms may contribute to voiding dysfunction in pathologies such as spinal cord injury or outlet obstruction. Accordingly, drug therapies such as muscarinic receptor antagonists, β_3 -adrenergic receptor agonists and botulinum toxin type-A (BTX-A) injections may act partly through suppression of afferent activity. One mechanism for alteration involves neuronal remodeling and sprouting. Following spinal cord injury, there can be changes in the function or architecture of afferent terminals in the periphery or the spinal cord. It has recently been demonstrated that there is increased sprouting of unmyelinated calcitonin gene-related peptide containing nerves in the lumbosacral region of the cord of spinalized rats^{15, 16}. This regeneration and remodeling of afferent nerves is believed to facilitate the emergence of reflex bladder activity as well as contribute to the development of visceral pain. The functional remodeling of afferent nerves may be driven by a variety of neurotrophic factors including nerve growth factor. An increase in the responsiveness of dorsal root neurons to noxious stimuli and changes in gene expression can cause a decrease in afferent thresholds that characterize the phenomenon of central sensitization. Stimuli that can cause central sensitization include tissue inflammation and nerve injury where there is repeatable or intense C-fiber stimulation. Primary afferents excited by a variety of strong noxious stimuli release neurotransmitters (including glutamate, tachykinins, *etc.*) antidromically from the peripheral terminals as well as in the spinal cord dorsal horn. Neurotransmitters

released in the latter case can sensitize second or third order sensory neurons leading to the transmission of noxious signals to the brain with an intact spinal cord. In the bladder, nociceptive C-fibers can become sensitive to mechanical stimulation in pathology and contribute to incontinence.

SITES OF ACTION OF β_3 -RECEPTOR AGONISTS AND BTX-A IN THE BLADDER

Role of the urothelium in afferent activation. Stretch of the mucosa (comprised of the urothelium and suburothelium) generates the release of several molecules^{17, 18} that could have a role in afferent signaling, including ATP, acetylcholine (ACh) and nitric oxide (NO•). This provides a mechanism that links bladder filling to afferent excitation. The transmitters could act directly on afferent nerve fibers or indirectly through suburothelial interstitial cells (IC). Recent studies demonstrate that NO• synthase exists in the rat urinary bladder and that its substrate, L-arginine, can inhibit both mechanosensitive A δ - and C-fiber afferents¹⁹. The role of ATP has been investigated more extensively and it has been demonstrated that P2X_{2/3} receptor knockout mice display reduced afferent firing in response to distension and have increased bladder volumes^{20, 21}. In animal studies where ATP was intravesically instilled, findings suggest that there are direct effects mainly on capsaicin-sensitive C-fibers²². These studies suggest that urothelial ATP normally plays a part in the initiation of micturition. The release of ATP has been shown to increase in various pathologies, including spinal cord injury^{23, 24} and painful bladder syndrome^{25, 26}. Moreover, agents such as BTX-A that reduce bladder overactivity normalize enhanced ATP release²⁴.

β_3 -receptor agonists and BTX-A for treating bladder overactivity. There are several drug therapies used or under investigation for ameliorating the symptoms of bladder overactivity and overactive bladder (OAB) symptoms. Anti-muscarinics are the standard drug treatment and have many sites of action in the lower urinary tract (LUT). They were initially thought to act by inhibiting contractile activation of the detrusor during voiding by blocking activation of the M_3 receptor pathway²⁷. However, the major effect of anti-muscarinics is during the filling phase, when parasympathetic nerves are silent. This suggests that anti-muscarinics may instead be affecting the sensory function of the bladder. This idea is supported by studies that demonstrated muscarinic receptor expression on the urothelium^{28, 29}, IC³⁰ and bladder afferent DRGs³¹. Direct inhibition of afferent nerves by the antimuscarinic receptor antagonists, oxybutynin (nonselective) and darifenacin (M_3 selective) have been demonstrated in the rat through single-unit measurement of A δ - and C-fiber afferents^{32, 33}. Animals chronically treated with antimuscarinics showed decreased spinal cord *c-fos* expression³⁴, also suggesting that these drugs can affect afferent nerve function.

An emerging treatment for bladder overactivity is β_3 -receptor agonists. It has been shown that β_3 -receptors are highly expressed in the bladder, particularly in the detrusor and the urothelium³⁵. Activation of these receptors is believed to reduce the symptoms of bladder overactivity by relaxing the detrusor smooth muscle. However, whether this is the sole mechanism of action is not known. Studies have suggested β_3 -receptor agonists may inhibit the firing of mechanosensitive afferent nerves in the bladder³⁶. In addition, β_3 -receptors may influence transmitter release from the urothelium as β -receptor stimulation can induce NO• release³⁷.

BTX-A has also shown promise as a therapy for the symptoms of bladder dysfunction in patients with neurogenic detrusor overactivity and possibly other conditions such as idiopathic overactivity and painful bladder syndrome³⁸. Although the procedure for BTX-A administration is invasive, the benefit of this treatment is that it is long-acting, with effects lasting several months, and may be repeated several times³⁹. BTX-A was thought to act by inhibiting muscle contractions through block of neurotransmitter release. However, it has been found that BTX-A does not affect the spontaneous intrinsic contractions of the detrusor⁴⁰, suggesting therapeutic effects are mediated from a different site. There is evidence that BTX-A inhibits bladder sensory pathways that could contribute to storage symptoms. Animal studies have shown BTX-A can decrease transmitter release, such as ATP and NO•, from the urothelium²³ and neuropeptide release from bladder afferents⁴¹. Thus, the effects on afferent nerves may contribute to the beneficial aspects of BTX-A treatment, even in neurogenic bladder overactivity, where the main goal is to reduce the contractility of the bladder. Overall, there are multiple sites and mechanisms of action for the mentioned agents. The effectiveness of these treatments depends upon selectivity for the urinary bladder as well as minimizing undesirable side effects.

NEW APPROACHES FOR STUDYING DRUG EFFECTS ON AFFERENT ACTIVITY

Neurogenic and myogenic bladder overactivity. Animal models exhibiting neurogenic and myogenic bladder overactivity include new bladder and colon irradiation-induced overactivity models developed by the corresponding author's laboratory (figure 3A and B) and the established spinal cord transected (SCT) mouse (figure 3C). While all three of these models

exhibit bladder overactivity when studied using cystometry, when the bladders are excised and studied as whole sheets, only those from SCT mice exhibit intrinsic detrusor contractions (figure 3Eiv). This suggests that irradiation-induced overactivity involves only central sensitization (figure 3D-E/ii-iii). Urinary diversion prior to transection prevents the development of overactivity (not shown), demonstrating the importance of detrusor-sphincter dyssynergia, urinary retention and bladder overdistension in the development of myogenic overactivity.

Simultaneous measurements of afferent nerve firing and spontaneous bladder contractions. In order to investigate the multiple components contributing to bladder overactivity, we have developed an *in vitro* method which involves isolating the bladder with the associated spinal roots that make up the pelvic and hypogastric nerves. This preparation allowed us to record single-unit afferent nerve firing simultaneously with tension measurements of intrinsic bladder contractions and optical maps of their initiation sites (figure 4). Using this method, we have demonstrated that there is direct correlation between spontaneous contractions and afferent nerve firing in the bladders of SCT mice (figure 4D)⁴². Accordingly, this approach allows us to evaluate the sites of action and therapeutic benefits of agents such as β_3 -receptor agonists and BTX-A in decreasing afferent firing and intrinsic bladder contractions.

Effects of β_3 -receptor agonists and BTX-A on afferent sensitization, neuropeptide release and detrusor overactivity. The β_3 -receptor is highly expressed in the bladder, particularly on smooth muscle⁴³ and activation of this subtype leads to relaxation and decreased intrinsic contractile activity. β_3 -receptors are also expressed on other cell types including urothelium⁴⁴, IC³⁵ and afferent nerve terminals³⁵. However, a direct effect on decreasing afferent sensitization and

neuropeptide release has not yet been reported. In part, this is because intrinsic bladder contractions, as shown in figure 4D, can directly stimulate afferent nerves⁴². This makes it difficult to determine if the effects of β_3 -receptor agonists on afferent firing are direct, or an indirect consequence of smooth muscle relaxation. Accordingly, we have developed another approach to address this issue that was utilized to obtain the unpublished findings in figure 5. These data suggest that the β_3 -receptor agonist, BRL37344, can reduce afferent firing in bladder sheets from SCT mice through a direct action on these nerves. After obtaining single unit activity, in response to stepper motor-controlled stretches, addition of the β_3 -receptor agonist decreased afferent firing without changing the smooth muscle tension profile (figure 5B). As indicated by the red arrow pointing to the tension profile in figure 5A, the effects of spontaneous bladder contractions on afferent firing can be discerned during controlled stretches. The length, duration and rate of these stretches typically range from 10-20% of resting length, 1-30 sec and 1-100 $\mu\text{m}/\text{sec}$, respectively. However, these parameters can be set to mimic the slow rates of normal bladder filling. The addition of a β_3 -selective receptor antagonist, L-748,377, enhanced afferent firing over controls (figure 5C), unmasking activity that was most likely inhibited by endogenous norepinephrine released from sympathetic nerves.

BTX-A has been utilized in numerous clinical trials as a therapeutic agent to reduce neurogenic bladder overactivity. BTX-A is classically thought to act upon parasympathetic cholinergic nerve terminals. The toxin selectively cleaves SNAP-25 which inhibits ACh release thereby reducing muscle contractility³⁹. However, by using the mouse bladder-pelvic nerve approach in figure 4 with BTX-A, it was determined that this agent can also directly inhibit afferent nerve firing (not shown). Since BTX-A has an onset of action of days, bladders were injected with the

toxin *in vivo*, and then excised and studied 48-72 hours later. BTX-A was injected at 2 to 3 sites from the serosal side along with a blue dextran dye of comparable molecular weight (150 kDa) to track its distribution. It was determined that half the bladder wall could be filled with BTX-A (figure 6A) permitting changes in activity to be studied in treated and untreated sides of the same bladder (figure 6B) offering a built-in control. It was determined that BTX-A decreased the firing frequency of bladder afferent nerves in response to stretch and spontaneous contractions (not shown).

Neuropeptide release from afferent nerves could also be studied in the bladder-pelvic nerve sheet preparations. This was accomplished by using the detrusor as a sensor and optically imaging neuropeptide evoked action potentials (figure 7A) while measuring tension generation in response to capsaicin (figure 7B). The bladder in this figure was injected on the left side (inscribed by the blue box) with BTX-A (1 Unit). Low dose (10-150 nM) capsaicin must be used to permit multiple applications before desensitization. Electrical field or bipolar stimulation cannot be used because they evoke much larger efferent mediated responses which mask neuropeptide release. The response could be blocked with a cocktail of NK₁/NK₂ receptor antagonists (not shown). In rodents, NK₁ and NK₂ receptors are located on detrusor smooth muscle, and NK₂ receptors are located on afferent nerve terminals⁴⁵. In humans, NK₁ receptors are on afferent terminals and NK₂ receptors are on muscle^{45, 46}. This same approach can be used to test the effect of β_3 -receptor agonists on neuropeptide release, only the drug must be bath applied and will affect the whole sheet preparation (not shown).

Potential mechanism for the actions of β_3 -receptor agonists and BTX-A in the bladder wall.

We hypothesize that antimuscarinic agents exert an effect on signaling pathways linking bladder wall stretch and the release of transmitters and afferent nerve activation, as shown in figure 8. Urothelial ACh may act in an autocrine fashion to promote ATP release or act on downstream signaling pathways, and antimuscarinic agents disrupt these processes. Furthermore, in afferent nerves, blockade of muscarinic receptors could decrease neuropeptide release reducing irritative symptoms as well as afferent sensitization. Decreasing IP_3 levels may inhibit the opening of voltage-gated Ca^{2+} channels and peptide release. This, in turn, could decrease positive feedback on neurokinin 2 (NK_2) receptors in rodents and NK_1 receptors in humans. This would remove inhibition on K^+ channels causing hyperpolarization and decreased afferent firing^{47, 48}.

β_3 -receptor agonists may produce similar results in IC and afferent nerves, working through increased protein kinase A (PKA), which inhibits elevations in intracellular Ca^{2+} (figure 8B). In UC, β_3 -receptor agonists also increase $NO\bullet$ production which may diffuse to and disrupt IC coupling and the drive on intrinsic spontaneous contractions. In the urethra, $NO\bullet$ produces smooth muscle relaxation. Its action in the detrusor is questionable because the levels of cyclic guanosine monophosphate (cGMP) generated in response to exogenously applied $NO\bullet$ are extremely low⁴⁹. The action of BTX-A in UC may decrease ATP release (figure 8C) which is vesicular, but not ACh release which is non-vesicular⁵⁰. BTX-A may also decrease ACh and prostaglandin (PG) release from IC. In afferent nerves, it may decrease neuropeptide release and, therefore, afferent sensitization.

CONCLUSION

The discussed topics indicate there are still many unknown factors related to afferent mechanisms and methods for treating sensory disorders of the bladder. Much of the emphasis was on new therapeutic options, namely β_3 -receptor agonists and BTX-A, and their potential sites and mechanisms of action. In summary, it is necessary to use new approaches and techniques, some of which were discussed in this review, in order to further our understanding of the sensory function of the LUT and improve upon treatment methods.

FIGURE LEGENDS

Figure 1. Innervation of the LUT.

Figure 2. Classes and distribution of afferent nerves in the LUT. A) The distribution of the different classes of afferent fibers in the bladder wall and urethra. B) Four types of mechanosensitive fibers were identified in the pelvic nerve by stretch, stroke and probing of the bladder. C) Proportions of afferent fiber types recorded in the pelvic nerve. D) Distribution of low- and high-threshold receptive fields of pelvic nerve muscle fibers based on responses to stretch. E) Distribution of receptive fields of the four classes of pelvic nerve fibers. The data and illustrations in B to E are adapted from Xu and Gebhart ⁵.

Figure 3. Animal models of neurogenic and myogenic bladder overactivity. Bladder irradiation-induced (A) and colon irradiation-induced (B) neurogenic overactivity models. C) Spinal cord transection (T8-T9), a mouse model for neurogenic and myogenic bladder overactivity. D) *In vivo* cystometry data. E) *In vitro* bladder sheet tension measurements. *Live animal photos by permission of the IACUC, University of Pittsburgh.*

Figure 4. Simultaneous recordings of afferent nerve activity and spontaneous bladder contractions. A) Optical mapping system showing the light source, filters and photodiode array cameras above a whole bladder sheet-nerve preparation. B) Spontaneous intracellular Ca^{2+} transients recorded from the bladder sheet. C) Isochronal map derived from the data in B, where the white area corresponds to the initiation site for spontaneous contractions. D) Large spontaneous contractions in a SCT mouse bladder stimulate afferent nerve firing. E) Small spontaneous contractions in a control bladder do not.

Figure 5. Effects of β_3 -adrenergic agonists on afferent activity. Single unit activity in response to stretch is decreased by addition of a β_3 agonist (BRL37344) to a SCT mouse bladder sheet (B). The β_3 -antagonist, L-748,337, abolished the effect of BRL37344 (C).

Figure 6. BTX-A injected mouse bladder. A) A mouse bladder injected over half its wall surface with BTX-A (1 Unit) and blue dextran dye of comparable weight (150 kDa) to track its distribution. B) The bladder after excision with the associated pelvic nerves mounted in a recording chamber.

Figure 7. Measurement of neuropeptide release from afferent nerves. A) Optical action potentials and B) tension generation in response to capsaicin-evoked neuropeptide release in a SCT mouse bladder sheet.

Figure 8. Hypotheses for the pathways mediating the effects of M₃-muscarinic receptor antagonists, β_3 -adrenergic receptor agonists and BTX-A in the LUT.

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