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Effect of Onabotulinum Toxin A on Substance P and Receptor Neurokinin 1 in the Rat Ventral Prostate

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Abstract

Introduction: The objective of this work is to examine if sensory innervation impacts lower urinary tract symptoms (LUTS). Onabotulinum toxin A (BoNTA) has been used for the treatment of overactive and neurogenic bladder and as a treatment for LUTS secondary to benign prostatic hyperplasia (BPH). The mechanism of how BoNTA impacts LUTS/BPH is unclear. In rats, BoNTA injection causes prostate denervation, apoptosis and atrophy. In clinical trials reduced prostate size and LUTS are observed inconsistently, suggesting a neurologic component. We will examine if BoNTA treatment inhibits substance P production in sensory nerve fibers in the rat prostate.

Methods: Twenty Sprague Dawley rats were divided into four groups including 1X PBS (control, n=6), 2.5 units Onabotulinum toxin A (BoNTA, n=6), 5 units BoNTA (n=6) injected into both lobes of the ventral prostate (VP) and sham surgery (n=2). Rats were Euthanized after one week. Substance P and its receptor neurokinin 1 localization and quantification were performed by counting the number of stained neurons and nerve bundles, by semi-quantitative immunohistochemical analysis and by western analysis.

Results: Substance P was localized in neuronal axons and bundles in the stroma of the VP but not in the epithelium. Receptor neurokinin 1 was identified in neuronal bundles of the stroma and in columnar epithelium of the VP ducts. Substance P decreased ~90% after BoNTA treatment (p=0.0001) while receptor neurokinin 1 did not change by IHC (p=0.213) or Western (p=0.3675).

Conclusions: BoNTA treatment decreases substance P in the rat VP.

Keywords: Prostate; LUTS/BPH; BoNTA; Substance P

Introduction

Lower urinary tract symptoms (LUTS) are commonly associated with benign prostatic hyperplasia (BPH) [1]. BPH is a histologic diagnosis that refers to smooth muscle and epithelial cell proliferation within the transition zone of the prostate [2]. Approximately 50% of men with BPH have moderate to severe LUTS symptoms [3] and BPH is age-dependent appearing in 50% of men aged 60, and 90% of men aged 85 [4]. Classically, the enlarged gland has been proposed to contribute to the overall LUTS complex via at least two mechanisms 1) direct bladder outlet obstruction (BOO) from enlarged tissue (static component) and/or 2) from increased smooth muscle tone and resistance within the enlarged gland (dynamic component). Voiding symptoms have often been attributed to the physical presence of BOO. Longstanding BOO and bladder over-distension have been proposed to cause fibrotic changes of the bladder wall, which leads to changes in detrusor function (i.e. detrusor instability). It is becoming increasingly clear that bulk or tone alterations in the bladder outlet are insufficient to explain the spectrum of male LUTS. Thus it has been proposed that LUTS may result from systemic derangements or neuropathic abnormalities of the peripheral and/or central nervous systems that regulate the lower urinary tract [5].

The lower urinary tract, including the prostate gland, is uniquely dependent on both somatic and visceral neuro-reflex activity for normal function. This supports our hypothesis that the origin of LUTS/BPH stems from neural dysregulation of the prostate and altered pelvic neuropeptides [6,7]. Onabotulinum toxin A (BoNTA), a potent neurotoxin, has been used extensively in clinical trials to treat over active bladder. However, few studies have assessed the usefulness of BoNTA for treatment of other pelvic disorders such as LUTS/BPH, and the findings are controversial with BoNTA treatment improving LUTS (measured by the AUA-SI and improved urinary flow rate (Qmax))[8-10] in some studies while in others it had only a marginal effect [11]. Thus the mechanism of how/if BoNTA impacts LUTS remains unclear. A preliminary study in rats suggests that intraprostatic injection of BoNTA may induce selective denervation, subsequent apoptosis and atrophy of the gland [7]. Data from clinical trials support this idea, with a marked reduction in prostate size and improvement in LUTS occuring after BoNTA injection in the prostate [9,10]. However controversy arises since prostatic involution is not a uniform finding and some clinical studies failed to show a reduction in size or decrease in prostate specific antigen (PSA) [8].

The objective of this work was to examine a potential mechanism by which sensory innervation may impact LUTS. We will examine this by determining if BoNTA treatment inhibits substance P production in sensory nerve fibers in the rat prostate. Substance P is a neuropeptide (1.347 kDA) involved in inflammation and pain. It has been suggested in rabbit iris muscle and in cultured dorsal root ganglion neurons, that BoNTA may inhibit substance P release [12-14]. Identifying a peptidergic etiology to a portion of male LUTS complaints may help improve patient care and establish a clinical phenotype beyond the rudimentary risk factors (e.g. prostate volume, erectile dysfunction, age and obesity) currently used.

Materials and Methods

Animals

Thirty-one Sprague-Dawley rats postnatal day 120-137 (P120-P137) were obtained from Charles River. The weight of the rats was ~400 g. Twenty rats were divided into four groups as stated in the abstract. Eight rats were used for optimization of injection methodology and an additional three rats were required for optimization of immunohistochemical staining.

Ethics statement

All animals were cared for in accordance with institutional IACUC approval and the National Research Council publication Guide for Care and Use of Laboratory Animals.

Optimization of injection volume

Eight Sprague Dawley rats were used to optimize the injection volume into the ventral prostate. Volumes of methylene blue ranging from 10-100 µl were injected into the rat ventral prostate (n=4) to determine the optimal volume that allowed for full dispersion through out the ventral prostate without overflow or leakage. Once the optimal volume was determined, fluorogold and PBS injections (10 µl per ventral prostate lobe) were made to show complete dispersion of the fluid throughout the prostate after injection (n=4) and to determine if leakage occurred within 7 days. Frozen ventral prostate tissue was sectioned 14 µ in thickness and sections were examined under a fluorescent microscope.

BoNTA injection

Twenty rats were utilized for this portion of the study. Two concentrations of BoNTA (Allergan) were prepared including 0.125 units/µl and 0.25 units/µl. A dorsal ventral paramedian incision was made in the lower abdomen of Sprague Dawley rats to expose the prostate for injection (n=18). Rats were randomly divided into four groups including 1X PBS (n=6), 2.5 units BoNTA (n=6), and 5 units BoNTA (n=6) injected into both lobes of the ventral prostate and sham surgery (n=2) in which the urogenital organs were exposed but no injection was made. A volume of 10µl was slowly injected into each lobe of the prostate (20 µl total) using a 50 µl Hamilton syringe with 30-gauge needle. Rats were sacrificed after one week and ventral prostates were frozen in liquid nitrogen prior to analysis.

Immunohistochemical analysis

Normal Sprague Dawley rats were used for optimization of immunohistochemical analysis (n=3). Briefly, frozen VP tissue was sectioned at 14 µ. Tissue capture (Daido Sangyo Co., Ltd., Tokyo, Japan) was used with Superfrost Plus slides (Fisher, Pittsburgh, PA) to aid in section adherence. Sections were post fixed with acetone prior to analysis. OCT was removed by washing in PBS 2 × 5 minutes. Sections were blocked in 3% milk in PBS for 1 hour at room temperature prior to incubation with primary antibodies overnight at 4°. Primary antibodies were rabbit polyclonal substance P (1/100, Millipore, Billerica, MA), mouse monoclonal neuron specific enolase (1/100, Millipore, Billerica, MA) and guinea pig neurokinase 1 (substance P receptor, 1/100, Millipore, Billerica, MA). After washing in PBS 2 × 5 minutes, sections were incubated with secondary antibodies for 1 hour at room temperature in the dark. Secondary antibodies were Alexa Fluor 488 chicken anti-rabbit (1/200 Life Technologies, Grand Island, NY), Alexa Fluor 594 donkey anti-mouse (1/250, Life Technologies, Grand Island, NY) and Alexa Fluor 488 goat anti-guinea pig (1/300, Life Technologies, Grand Island, NY). Negative controls were performed with secondary only (without primary) to test for non-specific staining and auto-fluorescence. Sections were mounted using Pro-Tex Mounting Medium (Baxter Diagnostics, Inc., Pittsburgh, PA). Microscopy was performed using a fluorescent microscope (Leitz) and photographed using a Nikon digital camera. Dual staining for substance P and neuron specific enolase was performed by overnight incubation with substance P, as stated above, followed by a one-hour incubation with neuron specific enolase.

Quantification of substance P

Dual staining was performed for substance P (green) and neuron specific enolase (red) as stated above. Quantification of substance P was performed by counting the number of substance P and neuron specific enolase positive neurons (axons) and nerve bundles present in ten photos per section and five sections from each rat (50 photos taken randomly in the VP per rat). Photos were taken in both the green and red channels at 100×. If staining appeared in the green channel and not the red it was not counted. The nerve bundle counting was straightforward. For the axons, each piece of axon that was visible was counted separately. This was done the same for all rats so error introduced by not knowing if each piece of axon was from one fiber or multiple fibers should cancel out across groups. Counting was performed on ventral prostates injected with PBS (n=6), 2.5 units BoNTA (n=6), 5 units BoNTA (n=6) and sham (n=2) rats. Image J could not be used for quantification because of the low area of staining relative to the size of the tissue.

Quantification of receptor neurokinin 1

Quantification of receptor neurokinin 1 was performed by Image J analysis (Image J version 1.45s, down load date 5/22/2012) measuring the integrated density with background subtraction. Total fluorescence for receptor neurokinin 1 was measured in 40 photos taken randomly in the VP per rat (10 photos per section and 4 sections per rat). Quantification was performed on ventral prostates injected with PBS (n=3), 2.5 units BoNTA (n=3), 5 units BoNTA (n=3) and sham (n=2) rats.

Hematoxylin and eosin stain

The morphology of the VP was examined in sections stained with hematoxylin and eosin (H&E) as outlined previously [15].

Western analysis

PBS (n=6), 2.5 units BoNTA (n=6), 5 units BoNTA (n=6) treated VPs and shams (n=2) were suspended in 3x lysis buffer (Cell Signaling Tech, Boston, MA) containing Sigma Protease Inhibitor Cocktail and Sigma Phosphatase Inhibitor Cocktail (Sigma-Aldrich, Saint Louis, MO), 1mM PMSF, and 0.25% Na-deoxycholate. Samples were homogenized individually with a glass homogenzer for 3 minutes on ice. After centrifugation, Laemmli buffer was added to the supernatant and the mixture was boiled for 3 minutes. Samples were frozen prior to Western analysis.

Samples were run on 10% Tris-Tricine gels and proteins were transferred to a nitrocellulose membrane (BioRAD, Hercules, CA) using a mini-Trans-Blot Electrophoretic Transfer Cell (BioRAD, Hercules, CA), for 1.5 hours at 200 mAmps. After blocking for one hour with 5% non-fat dry milk in PBS Tween at room temperature, membranes were incubated with guinea pig neurokinin 1 (1/7,500, Millipore, Billerica, MA) antibody overnight at 4°C or mouse β-ACTIN (1/60,000, Sigma-Aldrich, Saint Louis, MO) antibody for two hours at room temperature. After rinsing three times in PBS, membranes were treated with horseradish peroxidase conjugated goat anti-guinea pig (1/10,000, Santa Cruz, Santa Cruz, CA) or goat anti-mouse (1/40,000, Santa Cruz, Santa Cruz, CA) secondary for 45 minutes at 37°C. Protein bands were visualized using ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences, Piscataway, NJ) on Amersham Hyperfilm ECL (GE Healthcare Life Sciences, Piscataway, NJ). Quantification was performed by densitometry using Carestream Molecular Imaging Software (Rochester, NY). The ratio of receptor neurokinin 1/β-ACTIN was determined for individual tissues in triplicate and tissues were averaged and reported ± the standard error of the mean.

Statistical analysis

Statistical analysis was performed using ANOVA and Scheffe's posthoc test. Results were reported ± the standard error of the mean and differences were considered significant if $p \leq 0.05$.

Results

Optimization of injections

The VP was injected with 10-100 µl methylene blue in order to determine the optimal injection volume that filled the VP but did not leak into surrounding tissues. The optimal volume was found to be injection of 10 µl per VP lobe (Figure 1A). Fluorogold was used to determine if the volume injected would remain in the VP and not leak into surrounding tissues. After 7 days fluorogold was still apparent under UV light within the lobes of the VP and no leakage into surrounding organs was apparent. Sectioning of the tissue revealed that the fluorogold dispersed evenly throughout both lobes of the VP and was still apparent after 7 days (Figure 1B).

H&E staining of the VP tissue showed normal morphology with a single layer of columnar epithelium within the prostatic ducts and intact stroma (Figure 1C), indicating that the injection did not harm the ductal morphology.

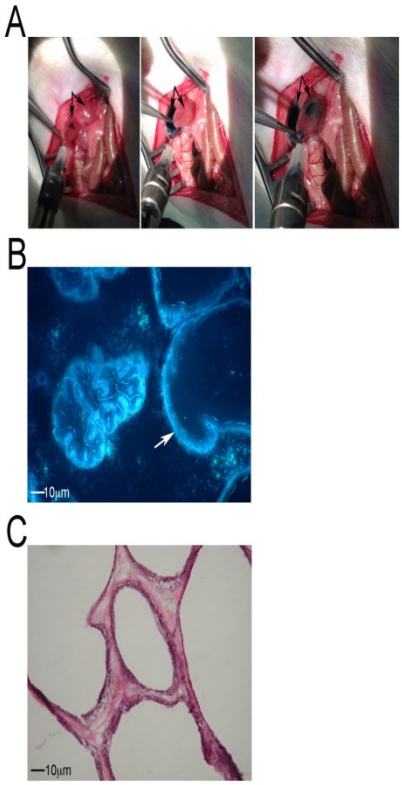

Figure 1: Optimization of injections into the Sprague Dawley VP. (A) Methylene blue (100-10µl) was used to determine the optimal volume for injection that filled both lobes of the VP but did not leak into other tissues. Two injections of 10µl into each VP lobe were optimal. Arrows indicate the VP lobes. (B) Fluorogold injected into both lobes of the VP was used to determine dispersion of fluid within the VP. Fluorogold was observed evenly dispersed in sectioned VP 7 days after injection. (C) H&E staining of VP injected with 10 µl PBS shows that the injection did not damage normal VP morphology since normal columnar epithelium and stroma were observed.

No change in VP size with BoNTA treatment

VP weights were determined for both the right and left prostatic lobes from sham (n=2), PBS (n=6), 2.5 units BoNTA (n=6), and 5 units BoNTA (n=6) treated VPs. No difference in weight was observed in any of the groups (p=0.321).

Localization of substance P and receptor neurokinin 1

Normal, adult ventral prostate was assayed for substance P and its receptor neurokinin 1. Substance P protein was identified in neuronal axons and bundles in the stroma of the VP (Figure 2A). Staining for substance P was not present in the epithelium. Receptor neurokinin 1 was localized in neuronal bundles of the stroma and in the columnar epithelium of the VP ducts (Figure 2B).

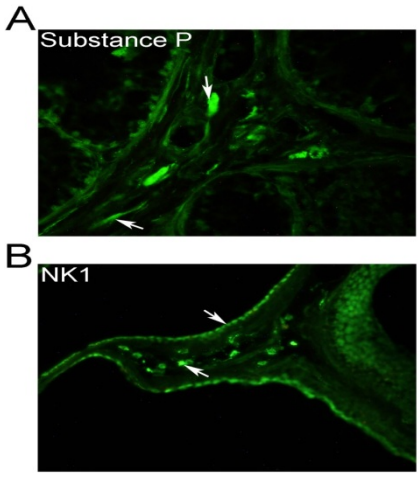

Figure 2: Localization of substance P and its receptor neurokinin 1 in normal Sprague Dawley VP. (A) Substance P is localized in neurons and nerve bundles in the stroma but was not identified in the epithelium. 400× magnification. (B) Receptor neurokinin 1 was identified in neurons and nerve bundles in the stroma and in columnar epithelium of the prostatic ducts. 250X magnification. Arrows indicate substance P and receptor neurokinin 1 proteins.

Quantification of substance P

Substance P was quantified by counting the number of substance P and neuron specific enolase positive neuronal bundles and axons in the stroma of PBS (n=6), 2.5 units BoNTA (n=6), 5 units BoNTA (n=6) and sham (n=2) treated VPs.

Dual immunohistochemical analysis was performed for substance P (green) and neuron specific enolase (red) and photos of both were merged for counting (Figure 3A). Western analysis for substance P was not possible due to the extremely small size of the mature form of substance P (1.347 kDa), which transfers through nitrocellulose membranes too quickly to visualize.

While the staining quality was good, it was also not feasible to use Image J due to the low percentage of the tissue that stained (nerve bundles and axons) relative to the entire tissue. There was no difference in the number of axons and neuronal bundles in the sham and PBS treated VPs ($p>0.05$, Figure 3B).

The number of stained axons and neuronal bundles decreased 85% in the 2.5 units BoNTA injected VP relative to the PBS treated VP ($p<0.0001$, Figure 3B). The number of axons decreased 89% and neuronal bundles decreased 91% in the 5 units BoNTA injected VP relative to the PBS treated VP ($p<0.0001$, Figure 3B). There was no difference in the number of axons and neuronal bundles in the 2.5 and 5 units BoNTA treated VPs ($p>0.05$, Figure 3B).

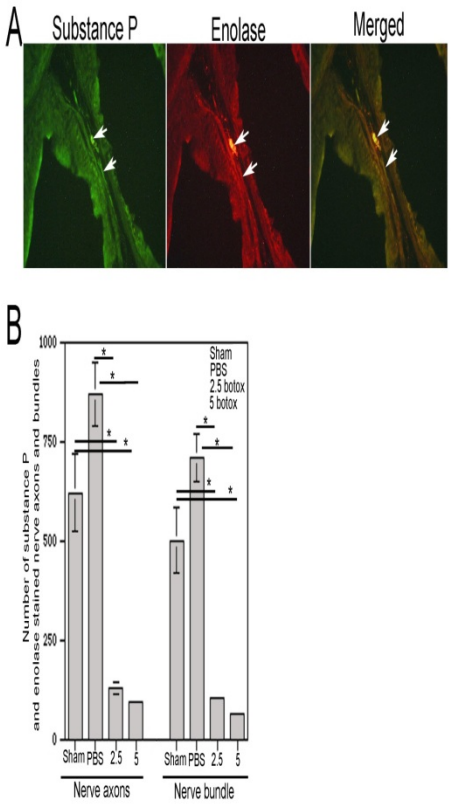

Figure 3: Quantification of substance P was performed by counting the number of substance P and neuron specific enolase stained axons and nerve bundles at 100X magnification in sham, PBS, 2.5 units BoNTA and 5 units BoNTA injected Sprague Dawley VP. (A) Immunohistochemical analysis was performed for substance P (green) and neuron specific enolase (red) and the photos were merged prior to quantification by counting. (B) Substance P stained nerve axons and bundles were significantly decreased with 2.5 and 5 units BoNTA treatment relative to sham and PBS controls ($p<0.0001$).

Quantification of receptor neurokinin 1

Receptor neurokinin 1 was quantified by Image J analysis of the total fluorescence (integrated density minus background) in PBS (n=3), 2.5 units BoNTA (n=3), 5 units BoNTA (n=3) and sham (n=2) treated VPs. Representative photos for all groups used for quantification are shown in Figure 4A. There was no difference in receptor neurokinin 1 observed between any of the groups when quantifying by Image J ($p=0.213$, Figure 4B). This result was verified by Western analysis of PBS (n=6), 2.5 units BoNTA (n=6), 5 units BoNTA (n=6) and sham (n=2) VP, which showed no difference between groups ($p=0.3675$, Figure 4C).

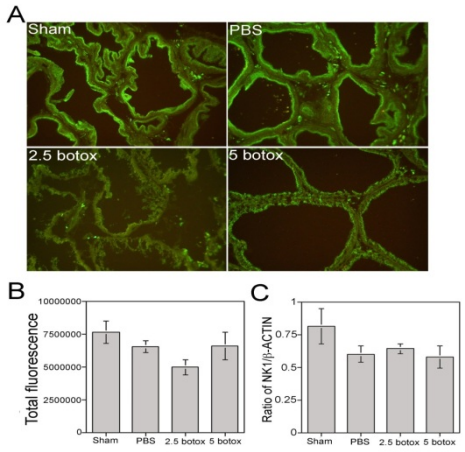

Figure 4: (A) Representative photos of receptor neurokinin 1 immunohistochemical analysis used for quantification by Image J. No difference in receptor neurokinin 1 was observed between groups by Image J (B, p=0.213) and by Western (C, p=0.3675) analysis.

Discussion

The purpose of this study was to examine the impact of BoNTA on the sensory innervation of the prostate, specifically as impacted on the nosioception pathways mediated by substance P. We show that at higher concentrations of BoNTA, there was a profound decrease (~90%) in substance P staining. However there was no change in protein abundance of the substance P receptor, receptor neurokinin 1. We did not observe a change in prostate weight, as was reported previously in a rat model and in a small number of human studies [7,9,10].

The signaling cascade initiated by BoNTA treatment at the motor nerve terminals has been previously described in other systems [16]. It is assumed that the prostatic signaling cascade is similar to that described in other organ systems such that BoNTA binds to ecto-receptors on the cholinergic nerve terminal and is then internalized and translocated to the cytosol where it inhibits Ca2+ dependent neurotransmitter release. It is believed that BoNTA prevents the N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent release of neurotransmitters. The SNARE target for BoNTA is SNAP-25 (synaptosomal-associated protein of 25 kDa), one of the SNARE proteins in presynaptic nerve endings. BoNTA cleaves SNAP-25 in the presynaptic nerve endings. Proteolytic cleavage of SNAP-25 disables the exocytotic machinery via inhibiting the fusion of the neuronal membrane with the neurotransmitter-containing synaptic vesicles [17,18]. Thus exocytosis of acetylcholine is inhibited. When the target tissue is muscle, this chemical denervation results in paresis and organ involution. BoNTA causes an extended neuroparalysis that lasts for months in humans [19]. Because prostate function is under the influence of acetylcholine as well as norepinephrine and testosterone, additional mechanisms are likely involved in BoNTA effects on the prostate [10,20].

Less well appreciated, BoNTA impacts noncholinergic synapses and neuropeptides and thus may have a role in nociceptive pathways by its interaction with substance P. Based on this anti-nociceptive hypothesis, BoNTA is expected to prevent peripheral nerve sensitization induced by local neurotransmitter (neuromodulator) release [21]. The nerve targets for pain are the C-fibers, which are afferent unmyelinated nerves, 0.4-1.2 mm in diameter with nerve conduction velocities of approximately 0.7-2.3 m s-1. Substance P is a neuropeptide (1.347 kDa) involved in neurogenic inflammation and the genesis of pain disorders. The release of substance P requires the SNARE protein activity that is inhibited by BoNTA [14]. In the iris muscles of rabbits and in cultured dorsal root ganglion neurons, BoNTA inhibited the release of substance P [12,13]. This is consistent with the hypothesis that sensory neuropeptides are implicated as significant regulators of prostate growth. Neurokinin 1, the receptor for substance P, has known effects on canine prostate epithelial cell proliferation, stromal differentiation, and contraction and neurokinin 1, neurokinin 2 and neurokinin 3 subtypes have been localized in canine prostate tissue by immunohistochemical analysis and ligand binding studies [22]. We have proposed that one component of bothersome LUTS is the activation of prostatic nociceptive pathways such that men complain of the bother regardless of the degree they are obstructed and independent of prostate size [23].

The current paradigm for understanding the pathophysiologic mechanisms for LUTS secondary to BPH are incomplete, and likely simplistic. We recently reported on a NIDDK sponsored trial (MIST2 Trial) composed of a Phase II RCT of BoNTA in men with LUTS and BPH [8]. In this phase II feasibility trial we noted several unexpected findings besides demonstrating the inherent safety of the treatment. We noted clinically significant improvements in symptoms (AUA-SI), urinary flow rates (Qmax) and ejaculation scores (MSHQ). Surprisingly, these clinical improvements were not accompanied by the expected decrease in prostate size and/or PSA (a proxy for prostate size). These findings closely parallel the findings in the current study in which substance P was markedly reduced without an expected decrease in prostate weight. This lack of change in prostate size noted in the clinical study and in our present rat study, suggest that the mechanism of action for BoNTA is not related to the expected efferent nerve denervation [20,24]. These findings suggest that mechanism of action of intraprostatic BoNTA is also not via the classic cholinergic-motor nerve terminal pathway as prostate volume, serum PSA and ejaculatory pathways should have been markedly altered. Given this, we propose that the mechanism of action for intraprostatic BoNTA improvement in male LUTS/BPH is via modulation of peptidergic presynaptic input on the prostatic sensory nerves.

Conclusions

In conclusion, these results show that BoNTA treatment decreases substance P in the rat VP. These results provide a possible mechanism of how BoNTA treatment improves LUTS in treated patients and suggests that interference with prostatic nosiception may be an important factor in the LUTS/BPH patient.

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