Bladder outlet obstruction triggers neural plasticity in sensory pathways and contributes to impaired sensitivity in erectile dysfunction

Anna P. Malykhina¹, Qi Lei¹, Shaohua Chang², Xiao-Qing Pan¹, Antonio N.Villamor¹, Ariana L. Smith¹ and Allen D. Seftel³

¹ Division of Urology, Department of Surgery, University of Pennsylvania, Glenolden, PA
² Department of Surgery, Cooper University, Camden, NJ, USA
³ Division of Urology, Cooper University Hospital, Camden, NJ

Running Title: Bladder obstruction impairs penile sensory nerves

*Corresponding Author:

Anna P. Malykhina, Ph.D.
Division of Urology, Department of Surgery
University of Pennsylvania School of Medicine
500 S. Ridgeway Ave, #158
Glenolden, PA, 19036-2307
Tel. (office): (267) 350-9606
Fax: (267) 350-9609
E-mail: Anna.Malykhina@uphs.upenn.edu

Keywords: sensory neurons, corpus cavernosum, smooth muscle contractility, afferent innervation

Copyright © 2013 by the American Physiological Society.
ABSTRACT

Lower urinary tract symptoms (LUTS) and erectile dysfunction (ED) are common problems in aging males worldwide. The objective of this work was to evaluate the effects of bladder neck nerve damage induced by partial bladder outlet obstruction (PBOO) on sensory innervation of the corpus cavernosum (CC) and CC smooth muscle (CCSM) using a rat model of PBOO induced by a partial ligation of the bladder neck. Retrograde labeling technique was used to label dorsal root ganglion (DRG) neurons which innervate the urinary bladder and CC. Contractility and relaxation of the CCSM was studied in vitro and expression of nitric oxide synthase (NOS) was evaluated by Western blotting. Concentration of the sensory neuropeptides Substance P (SP) and calcitonin gene-related peptide was measured by ELISA. Partial obstruction of the bladder neck caused a significant hypertrophy of the urinary bladders (2.5-fold increase at 2 weeks). Analysis of L6-S2 DRG sections determined that sensory ganglia received input from both the urinary bladder and CC with 5-7% of all neurons double labeled from both organs. The contractile responses of CC muscle strips to KCl and phenylephrine were decreased after PBOO, followed by a reduced relaxation response to nitroprusside. A significant decrease in nNOS expression, but not in eNOS or protein kinase G (PKG-1), was detected in the CCSM of the obstructed animals. Additionally, PBOO caused some impairment to sensory nerves as evidenced by a 5-fold down-regulation of SP in the CC (p<0.001). Our results provide evidence that PBOO leads to the impairment of bladder neck afferent innervation followed by a decrease in CCSM relaxation, down-regulation of nNOS expression and reduced content of sensory neuropeptides in the CC smooth muscle. These results suggest that nerve damage in PBOO may contribute to LUTS-ED comorbidity and trigger secondary changes in the contraction/relaxation mechanisms of CCSM.
INTRODUCTION

Lower urinary tract symptoms (LUTS) associated with benign prostatic hyperplasia (BPH), and erectile dysfunction (ED) are common problems in aging males worldwide (5; 50; 53). Large-scale epidemiologic studies established the link between LUTS and ED with more severe LUTS associated with a greater prevalence of ED (6; 24; 35; 49; 56). Despite the strong epidemiological evidence of correlation between the LUTS and ED, the underlying biological mechanisms of LUTS-ED co-morbidity are not fully understood. Some of the suggested pathophysiological pathways include vascular changes, altered tone and contractility of the corpus cavernosum (CC) smooth muscle (CCSM), nitric oxide (NO) and phosphodiesterase dependent dysfunctions, and a decrease in circulating sex hormones (36; 42; 50; 59).

Physiological transition between flaccid and erect states is supported by coordinated activity of the penile blood vessels and smooth muscle, which occurs in parallel with a complex interaction between afferent and efferent nerves (1; 15). However, neural mechanisms of LUTS-ED co-morbidity are less studied in comparison to vascular and muscle components. Epidemiological studies suggested a link between BPH and increased autonomic tone (17). Likewise, autonomic hyperactivity was shown to be involved in LUTS whereas increased sympathetic activity may have a role in ED (37). Electrophysiological recordings from pelvic nerves in patients with ED identified the changes in bulbocavernosus reflex (14; 44), pudendo-urethral reflex (13), and dorsal penile nerve cerebral-evoked responses (14; 45). This clinical evidence of nerve dysfunction in ED, especially when it is co-morbid with LUTS, supports the occurrence of neural plasticity and participation of neurogenic mechanisms in the development of ED.

Among published animal data related to the changes in neural pathways underlying LUTS-ED dysfunction, the major findings included the changes in the autonomic nervous system. Animal models of neurogenic ED caused by peripheral nerve injury, detected axonal deterioration of the nerve fibers and reduced nerve density in the CCSM, as well as altered peripheral release of neuropeptides and cytokines (8; 39; 46). These models were characterized by long-lasting changes in neural pathways,
extended recovery time and partial re-innervation of the affected area (40). They mostly mimic acute conditions such as nerve injury during radical prostatectomy in humans. The animal model of partial bladder outlet obstruction (PBOO) uses a different approach of continuous chronic compression of the bladder neck nerves which mimics BPH development in patients with significantly enlarged prostate. Previous studies detected decreased innervation of the CCSM and diminished relaxation of CCSM strips \textit{in vitro} in a rabbit model of PBOO (10). Surgical obstruction of the bladder neck was also shown to trigger increased sympathetic activity contributing to the occurrence of both LUTS and ED (8).

Recent investigations provide accumulating evidence for an important role of genitourinary sensory innervation for both erectile function and voiding reflex. Transmission of sensory inputs from the periphery to the central nervous system (CNS) is a critical step for psychological/physiological limbs of both erectogenic stimulation and control of micturition. Very limited information is currently available about how BPH-associated compression of the bladder neck affects sensory nerves during the progression of prostate enlargement. Our previous studies characterized a phenomenon of afferent fiber convergence in the pelvis which is involved in neural cross-sensitization (“cross-talk”) among pelvic organs (32; 33). One of the underlying mechanisms suggests the existence of peripheral axons which split at some point and, therefore, can co-innervate either different parts of the same organ or distinct pelvic organs (9; 11; 33). No studies have been performed to date to test if the same mechanism could occur with urinary bladder and penile sensory nerves, potentially contributing to LUTS-ED co-morbidity. The objective of this work was to evaluate the effects of bladder neck nerve compression on sensory innervation of the CC and CCSM function using a rat model of PBOO. Specifically, we aimed to determine if: a) the urinary bladder and CCSM are innervated by the branches of the same sensory fibers; b) PBOO, a primary cause of LUTS in males, can trigger temporary or permanent damage to sensory nerves innervating not only the urinary bladder but also the CC correlating with impaired sensation in ED; c) PBOO affects the basal release of sensory neuropeptides in the pelvic organs; and d) to identify potential molecular players involved in PBOO associated neural plasticity in the pelvis.
MATERIALS AND METHODS

Animals and experimental groups

Adult male Sprague-Dawley rats (N=50, 250–300 g, Charles River Laboratories, Wilmington, MA) were used in this study. Animals were housed two per cage, maintained on a 12 hour light/dark cycle with ad libitum access to water and food. Animals were divided into two experimental groups: 1 - control group (sham surgery), and 2 – experimental group with surgically induced partial bladder outlet obstruction (PBOO group). Animals from each group were used for in vivo and in vitro experiments 2 weeks after the surgical procedures. All protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Animal model of partial bladder outlet obstruction

Surgical procedure to induce PBOO was performed as previously described (38). Briefly, rats were anesthetized with 2% isoflurane and held on a warming pad inside the designated hood to minimize an investigator’s exposure to the anesthetic. A low midline suprapubic laparotomy (1.5-2 cm in length) was performed under sterile conditions to gain access to the urethra. The urethra was carefully isolated from the surrounding prostate lobes. A sterile 21g needle (0.81 outer diameter) was placed on the urethral surface, and a 3-0 silk suture was tied around both the urethra and the needle. As soon as the suture was secured, the needle was carefully removed, leaving the urethra partially obstructed. Sham surgery was also performed as described above except that suture around the urethra was left untied. Incisions were sutured in layers using a 4-0 size chromic gut suture for the muscle layer and a 4-0 nylon suture for the skin. Animals were allowed to recover on a warm blanket until they gained full consciousness and then were returned to their cages.

Surgical procedure for retrograde labeling of DRG neurons

In a separate set of rats, we performed double labeling with fluorescent retrograde tracers to test the hypothesis that some of lumbosacral DRG neurons may receive convergent afferent input from
the penis and urinary bladder, and PBOO may affect the number of these cells. The sham or PBOO surgical procedures were combined with retrograde labeling of dorsal root ganglion (DRG) neurons innervating the urinary bladder and penis. First, the surgical procedure to induce PBOO was completed, then Dil (1,1'-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR, USA; 1.5% w/v in methanol, red fluorescence) was injected close to the base of the penis at 6-8 sites using a Hamilton syringe with 26 gauge needle. Injections were placed in such a way to target predominantly the CCSM. This approach allowed the dye to stay within the organ and not being flushed away with the blood flow. Likewise, Fast Blue dye (FB, Polysciences Inc., Warrington, PA, USA; 1.5% w/v in water, blue fluorescence) was injected into the urinary bladder wall using the same approach. The total volume of each dye injected into organs was 10–15 µl. Adjacent pelvic organs were isolated with gauze to soak up any spills and prevent the labeling of adjacent structures during dye injections. Additionally, the needle was kept in place for 30s after each injection and any leaked dye was immediately removed with a cotton swab. Incisions were sutured in layers under sterile conditions. Animals were allowed to recover on a warm blanket until they gained full consciousness and then were returned to their cages.

**Isolation and analysis of L6-S2 dorsal root ganglia**

Animals were sacrificed at 2 weeks after the surgeries and L6, S1 and S2 DRG ganglia were isolated bilaterally along with the bladder and penis. Isolated structures were placed in HistoChoice tissue fixative solution (Amresco, Solon, OH) for 1-2 days. Dorsal root ganglia were then transferred to 30% sucrose for cryoprotection whereas bladder and penile tissues were embedded in paraffin for histological analysis of the injection cites. Dorsal root ganglia were placed in optimal cutting temperature (OCT) compound (Sakura Tissue Teck, Torrance, CA) and rapidly frozen on dry ice. Frozen ganglia were sectioned on a cryotome at 10 µm increments and every fifth section was mounted onto slides. This approach allowed minimization of double counting of labeled neurons in adjacent sections as an average diameter of a DRG neuron lies within 20-50 µm. Sections were mounted in
Vectashild (Vector Laboratories, Inc., Burlingame, CA, USA) and visualized using a Nikon Eclipse Ti inverted microscope (Nikon, Lewisville, TX, USA) connected to the Nikon Digital Sight Ri1 camera (Nikon, Lewisville, TX, USA). Images were acquired with MetaMorph® vs 6.2 software (Universal Imaging Corp., Downingtown, PA, USA). Quantitative analysis of the photomicrographic images was carried out by using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA). Due to the large total number of neurons (labeled and unlabeled), only Dil labeled (CC afferent input), FB labeled (urinary bladder afferents) and dual labeled (convergent input) neurons were counted. Double labeled neurons were identified by the presence of both red and blue fluorescence. The percentage of dual labeled neurons was determined as a ratio of the sum of Dil and FB single labeled neurons. Only cells with a clear nucleus and specific neuronal morphology were considered to be neurons.

**Isolation of rat corpus cavernosum smooth muscle**

Two weeks after the surgeries, rats were euthanized with overdose of sodium pentobarbital (120 mg/kg). The grossly dissected bladder-penis organ preparation was placed in cold oxygenated Tyrode’s buffer composed of (in mM): 124.9 NaCl, 2.5 KCl, 23.8 NaHCO₃, 0.5 MgCl₂·6H₂O, 0.4 NaH₂PO₄·H₂O, 1.8 CaCl₂ and 5.5 glucose. Care was taken to remove overlying skeletal muscle without damaging the underlying tunica albuginea containing the corpus cavernosum. The corpus cavernosum muscle strips were dissected free of the tunica bilaterally and placed either directly in an organ bath containing warm (37 C) Tyrode’s buffer equilibrated with 95% O₂/5% CO₂ for physiological studies (one set of animals) or immediately snap frozen in liquid nitrogen for subsequent mRNA and protein analyses (another set of animals).

**In vitro measurements of CCSM contractility**

For *in vitro* recordings of CCSM contractility, isolated CCSM strips were suspended from L-shaped hooks in 15-ml organ bath chambers. The chambers were filled with Tyrode buffer (in mM): 125 NaCl, 2.7 KCl, 23.8 NaHCO₃, 0.5 MgCl₂·6H₂O, 0.4 NaH₂PO₄·H₂O, 1.8 CaCl₂, and 5.5 dextrose,
maintained at 37°C, and perfused continuously with a mixture of 95% O$_2$ and 5% CO$_2$. After a 1-hour equilibration, the length of optimal force development (Lo) was determined by increasing the length of each strip by 1-mm increments until a maximal contractile force to 125 mM KCl stimulation were achieved. The muscle was kept at Lo in Tyrode's buffer for 30 minutes to allow stabilization at the resting level. The tissues were washed three times (10 min each) with Tyrode buffer before the application of α-adrenoreceptor agonist L-phenylephrine (PHE, 10 µM) to trigger CCSM contractions followed by application of sodium nitroprusside (SNP, 10 µM) to induce muscle relaxation. Contraction parameters were measured using PowerLab Lab-Chart version 7.1.2 software (ADinstruments, Colorado Springs, CO). The raw traces were analyzed manually and then exported into SigmaPlot 11.2 Software (Systat Software, San Jose, CA).

**Protein extraction from CCSM and bladder tissues**

Frozen bladder and CCSM samples isolated from sham and PBOO rats were homogenized using PowerGen 500 homogenizer (Fisher Scientific, Rockford, IL) in ice-cold lysis buffer containing 25% glycerol, 62.5 mMTris-HCl, 1xprotease inhibitors (Roche, Complete mini) and phosphotase inhibitors (Roche, PhosSTOP). 10% SDS was added to the samples, vortexed and boiled for 4 min. The extracts were centrifuged at 10,000 rpm for 15 min at 4°C, and supernatants with the total protein were collected. Protein concentration in each sample was detected using BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Bovine serum albumin (BSA) was used to generate the standard curve. Each protein sample was diluted 1:20 with 1% SDS. All standards and samples were run in duplicate. The absorbance was measured at 562 nm on the Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT) and data analysis was performed using Gen5 Microplate Data Collection & Analysis Software (BioTek Instruments, Winooski, VT).

**Western blotting**
Equal amounts of protein (20 µg per lane, N=4 per group) were separated on 4-15% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with ODYSSEY blocking buffer for 45 min at room temperature, the membranes were incubated overnight at 4°C with primary antibodies, including rabbit anti-nNOS (Cat.#4236, Cell Signaling Technology, Danvers, MA, 1:1000), anti-eNOS (Cat.#9586, Cell Signaling Technology, Danvers, MA, 1:1000), anti-PKG-1 (Cat.#3248, Cell Signaling Technology, Danvers, MA, 1:1000), and mouse anti-α-actin (sc-17829, Santa Cruz Biotechnology, Santa Cruz, CA, 1:200). The membranes were washed and incubated with secondary horseradish peroxidase–linked anti-rabbit antibody (Cat.#16284, Abcam, 1:5000) or goat anti-mouse IgG-Cy3 (GE Healthcare, PA43009, 1:2500) for 1 hour at room temperature. Target proteins were detected using an enhanced chemiluminescence kit (Cat.#RPN2108, Amersham Life Sciences, Arlington Heights, IL), followed by scanning on a Fujifilm Image Reader LAS-3000 (Fujifilm, Valhalla, NY). The amount of protein in a band was determined by Optical Density scanning using Multi Gauge V 3.0 software (Fujifilm).

Quantification of Substance P and calcitonin gene related peptide content in the CCSM and urinary bladder

The levels of Substance P (SP) in CCSM and urinary bladder tissues were measured using a rat Substance P ELISA kit (MD Biosciences Inc., St. Paul, MN) according to the manufacturer’s instructions. Each sample and standards were run in duplicate. The concentration of total protein was 400 µg/ml for SP assay. Briefly, a 96-well microplate was loaded with 25 µl of primary antibody specific for rat SP. 50 µl of each sample and 50 µl of the standard SP dilutions (as a control) were mixed in the assigned wells followed by the addition of biotinylated SP into each well except the Blank. The plate was incubated for 2 h at RT, and then washed 6 times with provided in the kit wash buffer. Subsequently, 100 µl of biotinylated anti-SP antibody solution was added, incubated for 1 h, and then washed four times. 100 µl of streptavidin-horseradish peroxidase conjugate solution was added to each well except chromogen blank, incubated for 1 hour, and washed again. After that, 100 µl of substrate
solution provided in the kit was added to each well and the plate was incubated for 1 hour at room
temperature. The reaction was stopped with 2N HCl and the optical density values were read at 450 nm
using a Biotek Synergy 2 plate reader (BioTek Instruments Inc., Winooski, VT).

Calcitonin gene-related peptide (CGRP) levels were measured using CGRP Enzyme
Immunoassay (EIA) kit for rats (ALPCO Diagnostic, Salem, NH) according to the manufacturer’s
instructions. The total protein concentration from each sample for CGRP assay was 200 μg/ml. Briefly,
a 96-well microplate was coated with 100 μl of antibody specific for rat CGRP mixed with 100 μl of EIA
buffer provided in the kit plus 100 μl of standard and sample aliquots. Plate was incubated for 16–20 h
at room temperature followed by the aspiration of the samples and washing them 3 times with wash
buffer. Subsequently, 200 μl of Ellman’s reagent was added to each well, incubated for 30–60 min in
the dark and then read between 405 and 414 nm using a Biotek Synergy 2 plate reader (BioTek
Instruments Inc., Winooski, VT). The protein concentrations of both SP and CGRP were statistically
analyzed using two-way repeated measures ANOVA followed by comparisons between control and
experimental groups using Bonferroni’s method (Systat Software Inc., San Jose, CA).

**Statistical analysis**

All data are expressed as the mean±standard error of the mean (SEM). Statistical significance
between the groups was assessed by two-way repeated measures ANOVA followed by
Bonferroni’s post test when appropriate (Systat Software Inc., San Jose, CA). Difference between the
groups and treatments was considered statistically significant at p≤0.05.
RESULTS

Histological and morphological evaluation of the CC and urinary bladder in a rat model of PBOO. Animals from both sham and PBOO groups were sacrificed 2 weeks after the surgery. After perfusion with paraformaldehyde, each bladder-penis organ preparation was carefully dissected from overlying skeletal muscle and connective tissues. First, specimens were screened for dye injection sites to confirm that the dyes did not cross-leak after the surgery was completed. Fig. 1 A shows one of the bladder-penis organ preparations isolated from an animal in PBOO group. The penis presents with the red coloring predominantly in the lower half of the organ, and the urinary bladder has visible yellow spots of injected FB which has blue color under fluorescent microscope. As seen in the figure, the silk suture at the base of the urinary bladder and large volume of residual urine confirm that the bladder neck was partially obstructed in this animal. After the initial evaluation was completed, the bladder was cut off, weighed and a section with the most abundant dye spots was embedded in paraffin for histological evaluation. A section of the penis with the dye in the wall was also saved the same way as described for the urinary bladder. Histological evaluation of longitudinal sections of the penis showed that most of the dye was located within the CC muscle layer (Fig.1 B). The FB spots were predominantly detected in the muscle bundles of the detrusor in rats from both sham and PBOO groups (Fig.1C). No overlap in dye labeling was observed in bladder-penis organ preparations isolated from rats in control and experimental groups. Additionally, a significant increase in bladder mass was detected in PBOO group when compared to sham control (N=7 for both groups): the average bladder weight was 123±7.8 mg in sham operated rats and 286.5±17.1 mg in animals with PBOO (132 % increase, p≤0.01, Fig. 1D).

Partial obstruction of the bladder neck affects sensory innervation of the corpus cavernosum. Single DiI or FB labeled neurons were identified in L6-S2 sectioned ganglia. Fig.2 shows an example of S1 ganglion cross-section isolated from control (top panels) and PBOO (lower panels) animals. Sensory neurons receiving input from the urinary bladder were identified by blue fluorescence
and neurons innervating the CC were labeled in red. Merged images show a few neurons in pink color (depicted by arrows) which contain both blue and red dyes suggesting that they received the tracers from both organs. Double labeled (from here on referred to as convergent) cells were identified in all analyzed ganglia. Due to a large total number of neurons (labeled and unlabeled), only Dil labeled (CC projecting), FB labeled (bladder projecting) and dual labeled (convergent input) neurons were counted and statistically analyzed. The percentage of dual labeled neurons was determined as a ratio of the sum of Dil and FB single labeled neurons. Analysis of retrograde labeled sensory neurons is shown in Fig. 3 for both sham operated (A, N=5) and PBOO (B, N=5) animals. The majority of labeled neurons in L6 ganglion received input from the urinary bladder (92.4%). The ratio of bladder/CC labeled cells decreased in the sacral DRG with bladder projecting neurons comprising 36.8% and CC innervating cells 63.1% in S2 DRG. Convergent neurons were present at all L6-S2 levels ranging from 5.7±0.9% (L6 DRG) to 10.5±1.3% (S2 level, Fig.3 C). Partial bladder outlet obstruction eliminated the level-dependent distribution of single labeled neurons with the number of bladder projecting cells reaching 74-82% among the ganglia (Fig.3 B). Additionally, the number of convergent sensory neurons was reduced by PBOO down to 2-3% in sacral DRG (Fig.3 C). These results suggest that PBOO triggers neural plasticity in sensory afferents supplying the CC, thereby, affecting the sensory component of ED development. Reduced number of convergent sensory neurons innervating the urinary bladder and CC may contribute to a neurogenic component of co-morbidity between the LUTS and ED.

Changes in CCSM contractility and relaxation induced by PBOO. Strips of CCSM isolated from sham and PBOO rats (~25 mg, Fig.4 A) were longitudinally suspended in organ baths for contraction/relaxation experiments. The mean maximum force generated CCSM strips isolated from sham rats in response to 125 mM KCl was 4.9±0.9 g/g (N=4, n=7) whereas strips from PBOO group had a mean of 2.6±0.3 g/g, reflecting an approximately 47% decrease in maximum force (N=4, n=7, p≤0.02, Fig.4 B). Diminished contractile response of CCSM to stimulation with phenylephrine (PHE) was also observed in the PBOO group reaching 2.8±0.3 g/g (n=7) in comparison to 4.3±0.5 g/g in sham
In addition, pre-contracted with PHE strips of CCSM from PBOO rats were more difficult to relax by sodium nitroprusside (SNP) in comparison to sham operated animals (Fig.4 C).

**Modulation of NOS and PKG expression by PBOO in CCSM.** To identify molecular candidates involved in altered contractility and relaxation of the CCSM we evaluated protein expression of both neuronal and endothelial NOS, as well as PKG-1, an established modulator of NOS activity, in the CCSM (30). Figure 5 A shows the gels with protein bands from control (S1-S4) and PBOO (P1-P4) groups. Analysis of relative protein expression (Fig. 5 B) determined that obstruction of the bladder neck caused a significant decrease in n-NOS expression in the PBOO group (by 50%, p≤0.05) when compared to sham CCSM. In contrast, the expression of e-NOS showed a trend of increased expression without reaching statistical significance. There was no change in PKG-1 expression between control and PBOO groups of rats (Fig.5 B).

**Obstruction of the bladder neck affects the release of sensory neuropeptides in the CC and urinary bladder.** Since PBOO can affect both afferent and efferent nerve fibers, we sought to distinguish the effects on sensory nerves from the effects on efferent fibers. It is well established that sensory afferents release specific sensory neuropeptides in the viscera upon peripheral stimulation (23; 34). We ran SP and CGRP ELISA assays for the proteins isolated from the urinary bladder and CCSM in both control and PBOO samples. These experiments established that the CC had higher level of SP content (2.01±0.23 ng/ml, N=5, n=10) in comparison to the urinary bladder (0.3±0.05 ng/ml, N=5, n=8, p≤0.001, Fig.6 A) under normal physiological conditions. However, physiological concentration of CGRP was significantly higher in the urinary bladder reaching 120.6±37.2 pg/ml in comparison to 14.5±0.9 pg/ml in the CC (p=0.003, Fig.6 B). PBOO diminished SP release from sensory terminals as evidenced by a 5-fold down-regulation of SP concentration in the CC (N=5, n=10, p≤0.001, Fig.6 A) without significant changes in the obstructed bladder. Interestingly, concentration of CGRP was down-regulated in the urinary bladder by PBOO without parallel changes in the CC, however, the decrease
did not reach the level of statistical significance (Fig. 6 B). These results provide evidence that PBOO causes neural plasticity in afferent pathways associated with down-regulation of neuropeptide content in the affected organs.
DISCUSSION

This study investigated the neural mechanisms of LUTS-ED co-morbidity with focus on sensory innervation of the CC and urinary bladder using a rat model of PBOO. We provided direct evidence for the presence of convergent bladder-penis DRG neurons in lumbosacral sensory ganglia and established that the number of these neurons is decreased after partial obstruction of the bladder neck. Additional important findings included reduced relaxation of CCSM strips in response to NO donor, down-regulation of neuronal NOS expression and a decreased content of sensory neuropeptides in the CCSM of rats with PBOO. The results of our work provide additional support for a neurogenic component in ED development and suggest that PBOO induces neural plasticity in afferent pathways of the penis and urinary bladder, thereby, contributing to LUTS-ED co-morbidity and impaired sensation during ED development.

Previous clinical studies suggested that neurogenic ED often results from surgical management of invasive bladder cancers and other low pelvic malignancies including colon and rectal cancers (3). The peripheral nervous system demonstrates limited intrinsic ability to recover after axonal damage due to pelvic surgery or trauma. While peripheral axons can regenerate and partially re-innervate a damaged organ, these changes do not guarantee the full recovery of a neurogenic function (39). In this study, we tested the hypothesis that one of the mechanisms linking LUTS and ED involves impairment of sensory nerves supplying the urinary bladder and penis. Our group has previously shown that there is a network of sensory nerves which have dichotomized axons supplying adjacent pelvic structures (33). These convergent afferents play a role in the development of cross-sensitization among pelvic organs via neural pathways (4; 7; 32; 54). The presence of sensory neurons receiving convergent afferent input from the pelvic viscera in animal models was previously shown for the lower gastrointestinal, urinary and reproductive organs (9; 11; 33). The percentage of DRG neurons with multiple or dichotomizing axons usually lies within 3-10% (9; 11; 12; 33) which correlates with the number of bladder-penis convergent cells observed in this study. Our results from retrograde tracing experiments in rats with PBOO also supported our suggestion that mild obstruction of the bladder neck
nerves can be associated with impairment of sensory axons supplying both organs. Therefore, we provide additional evidence that PBOO, a primary cause of LUTS in males, induces damage to the nerve fibers innervating not only the bladder but also the CC contributing to a neurogenic development of ED. We would like to emphasize that alterations in neural pathways triggered by PBOO are not suggested to be the primary cause for LUTS-ED co-morbidity but rather play a modulatory role in addition to main mechanisms such as vascular changes, altered tone/contractility of CCSM, phosphodiesterase dependent pathway (36; 42; 50; 59).

Impairment of nerve and blood supply by obstruction of the bladder are associated with a number of changes in the contractility of visceral smooth muscle. The majority of the available reports investigated the effects of PBOO on the function of bladder detrusor and only few studies focused on the contractility of the CCSM. The exact mechanisms by which PBOO can trigger changes in CCSM contractility and relaxation remain unknown. In our rat model of PBOO, we observed a ~2.5-fold increase in bladder weight after 2 weeks of obstruction. This correlates with the previous studies which established that denervation of the pelvic organs was associated with smooth muscle proliferation and thickening (16; 60). Partial bladder outlet obstruction can result in either mild or severe obstruction depending upon several factors such as the duration of obstruction (58) and diameter of the surgical ligation (51). The inclusion of sham operated animals in our study ruled out direct injury by trauma due to surgical intervention since a suture was also placed around the urethra of sham operated rats, although without ligation. Thus, constant compression of the nerves that travel through the bladder neck region to supply the CCSM was not present in sham operated controls, and may have contributed to the decreased sensory innervation in the CCSM in rats with PBOO. Despite the benefits of the surgical approach used in our study in a recapitulation of the events that take place in clinical PBOO, the acute nature of surgical intervention does not fully mimic the slow progression of BPH and/or development of ED observed in ageing men. The major difficulty in studying the correlation between LUTS/BPH and ED is a limited number of experimental animal models combining prostate enlargement, bladder obstruction and ED all at once. In order to test whether the impairment of
sensory nerves depends on the method of PBOO induction, our findings should also be tested in additional animal models such as estrogen/testosterone-induced BOO (41) or in testosterone-supplemented spontaneously hypertensive rats (43).

We determined that partial obstruction of the bladder neck led to a decreased contractility of CCSM upon stimulation with KCl or PHE. Other groups reported similar observations including diminished CCSM contractility under ischemic conditions resulted from occlusion of abdominal aorta in vivo (52). Lin at al (29) observed a decrease in the contractile response of the CCSM to PHE in a rabbit model of PBOO whereas another group detected the opposite effect (10). Variability in contractility responses of the CCSM among the studies is likely associated with phasic molecular changes underlying adaptation of the CC muscle to new conditions induced by PBOO development. Other important contributing factors may include species differences, severity and duration of obstruction, degree of smooth muscle hypertrophy, and variability in methodological approaches. Despite the reported differences in contractile responses of the CCSM, all groups, including ours, detected a significant decrease in relaxation of CCSM strips isolated from PBOO animals (10; 22; 29). Impairment of CCSM relaxation was suggested to be associated primarily with up-regulation of Rho-kinase expression/activity and changes in NO/NOS signaling (59). NO is a vasodilator regulating multiple physiological and pathophysiological processes, including host-defense response, neuronal communication, and vascular tone (18). NO is released from non-adrenergic non-cholinergic nerves and endothelial cells (26; 48) and relaxes CCSM through the activation of the sGC/cGMP/PKG signaling cascade (2). PKG pathway is also involved in regulation of cardiac contractility, axon guidance, bone growth, smooth muscle relaxation and erectile dysfunction (21; 30). In Rosen (50) and McVary’s (35) review papers, NO and nitric oxide synthase (NOS) related dysfunctions are listed as one of the mechanisms underlying the link between LUTS and ED. However, the information about the effects of PBOO on NO/NOS pathway is still limited.

Since the focus of our study was on neural mechanisms, we aimed to further establish if a decrease in CCSM relaxation was associated with the changes in expression of nNOS and/or PKG
related pathways. Our results confirmed a down-regulation of nNOS in CCSM of animals with PBOO, whereas other signaling molecules such as eNOS and PKG-1 did not change significantly between sham operated and PBOO rats. This data correlates with a report by Klotz et al (1997) which determined a significant decrease in nitrinergic innervation and neuronal NOS (nNOS) in hyperplastic obstructive human prostates compared to normal non-obstructive ones (27). An experimental model of rat PBOO showed a similar decrease of nNOS in the CCSM isolated from obstructed animals which may contribute to ED during PBOO development (20).

Partial obstruction of the bladder neck affects both afferent and efferent fibers coursing through the urethra. Therefore, we attempted to distinguish the effects of PBOO on sensory nerves from the effects on efferent fibers. It is well established that sensory afferents contain vasodilating neuropeptides, such as SP and CGRP, which are released from peripheral terminals upon sensory stimulation (23; 34). Calcitonin gene-related peptide and SP are the main neuropeptides released from the sensory fibers innervating pelvic organs (47; 55; 57). Both of them are highly expressed in primary afferent neurons projecting to the urinary bladder, distal colon, and reproductive organs (11; 25). Release of SP and CGRP from peripheral nerves is usually associated with development of arteriolar vasodilation. A number of important factors underlie the effects of neuropeptide release including content, transport to nerve terminals (peripheral), rate of peptide metabolism, positive/negative feedback on synthesis and/or release (31). At the subcellular level, CGRP is often co-stored with tachykinins in large dense cored vesicles in trigeminal and DRG neurons (19). However, SP does not have the storage capacity in peripheral terminals and the continual activation may be evident as a partially depleted terminal. Unlike SP, CGRP was found to be more metabolically stable than the co-released tachykinins (28). Our results showed that PBOO caused some impairment to sensory nerves associated with 5-fold down-regulation of SP in the CC without significant changes in the obstructed bladder. Interestingly, concentration of CGRP was down-regulated by PBOO in the urinary bladder without parallel changes in the CC, however, without reaching the level of statistical significance. Differential effects of PBOO on SP and CGRP basal release could be explained, in part, by various
basal concentrations of these neuropeptides in the pelvic organs under normal physiological conditions. Thus, basal level of SP in the CC was 6-fold higher in comparison to the urinary bladder, whereas the concentration of CGRP was 8-fold higher in the urinary bladder than in the CC. Together, these data suggest that PBOOO causes neural plasticity in afferent pathways associated with down-regulation of neuropeptide release from peripheral sensory terminals.

**Conclusions and Significance**

We present the novel findings of molecular and functional changes in the nerve supply of the CCSM associated with PBOO. PBOO leads to alterations in bladder neck sensory innervation followed by decreased CCSM relaxation, down-regulation of nNOS expression in the CC and reduced basal release of vasodilatory neuropeptides from peripheral sensory terminals. Our results provide evidence that altered neural plasticity induced by PBOO may contribute to the development of ED causing subsequent changes in the contraction/relaxation mechanisms of CCSM. In light of the expansive epidemiologic presence and significant LUTS-ED co-morbidity, further basic and translational studies to clarify the mechanistic link between these two pathologies are warranted.

**Acknowledgements**

We would like to thank Joseph Hypolite, Robert H. Seftel, and Katherine Florio for excellent technical assistance.

**Competing interests**

The authors have declared that no competing interests exist.

**Author Contributions**

Conceived and designed the experiments: APM, SC, ADS. Performed the experiments: QL, SC, XQP, ANV, ALS. Analyzed the data: APM, QL, SC, XQP, ANV. Contributed reagents/materials/analysis tools: APM, ALS, ADS. Wrote the paper: APM, ALS, ADS.
Figure legends

Fig. 1 Morphological and histological evaluation of the urinary bladder and CC during PBOO. A, A representative image of an isolated bladder-penis organ preparation with visible sites of retrograde tracer injections. CC was injected with Dil (red color) and the urinary bladder with FB (yellow color in the figure which becomes blue under the fluorescent microscope). An arrow points to the silk suture at the bladder neck suggesting that this preparation was isolated from a rat with PBOO surgery. B, Fluorescent image of the longitudinal section of the CC shows the presence of Dil in the CC wall. C, Fluorescent image of the longitudinal section of the urinary bladder using a filter for FB. Scale bar is 1 mm. D, Bladder weight in sham operated (N=7) and PBOO (N=7, p≤0.01) rats.

Fig.2 Cross section of S2 ganglion after in vivo labeling of the CC (Dil) and urinary bladder (FB). Top panels include sections from a control rat and the bottom panels represent the sections of S2 ganglion isolated from an animal with PBOO. FB labeled neurons on the left side represent urinary bladder afferent neurons, and CC projecting cells are labeled in red on the middle panels. Overlap image of both color channels reveals convergent penis-bladder DRG neurons (arrows). Scale bar is 100 μm.

Fig. 3 Percentage of retrogradely labeled sensory neurons receiving input from the CC and urinary bladder in L6-S2 DRG. A, Proportion of single labeled CC and urinary bladder neurons per each ganglion in the sham group of rats (N=5). B, Ratio of single labeled CC and urinary bladder neurons per each ganglion in rats with induced PBOO (N=5). C, Percentage of double labeled neurons in L6-S2 DRG in sham operated and PBOO animals. The percentage of convergent neurons was calculated from the total sum of single Dil and FB labeled cells per each section (taken together as 100%, p≤0.05 to sham group).
Fig. 4 Contractility and relaxation of CCSM strips in sham operated and PBOO rats. A, Weight of the muscle strips isolated from the CC (N=4 per group). B, Normalized amplitude of CCSM contractions in response to KCl and PHE (N=4, n=7, p=0.04 to sham group). C, Percentage of CCSM relaxation after pre-contraction of the muscle strips with PHE (n=7 per group, p ≤ 0.05). Maximal response to PHE before the addition of SNP was taken as 100%. The relaxant effect of SNP was evaluated as a percentage of this response. Corpus cavernosum smooth muscle from rats with PBOO relaxed less than that from sham operated rats.

Fig. 5 Expression of n-NOS, e-NOS and PKG-1 protein in sham operated and PBOO rats. Total protein was extracted from the CC of four pairs of sham-operated and PBOO rats, and equal amounts of total extractable protein were then loaded onto a mini 12% SDS-polyacrylamide gel and analyzed as described in Materials and Methods. A, Images of the Western blot membranes probed with antibodies against eNOS, nNOS and PKG-1. S1-S4 - Sham operated rats, P1-P4 – rats with PBOO. B, Relative protein expression normalized to the level of α-actin. Please note that the expression of nNOS was decreased in the CC from PBOO rats in comparison to the CC from sham group (N=4, p ≤ 0.05), while the expression of eNOS and PKG-1 was not significantly altered. Alpha actin was run as an internal control and was not significantly different between the groups.

Fig. 6 Substance P and CGRP peptide content is modulated by PBOO in the CC and urinary bladder. A, Concentration of SP in the urinary bladder and CCSM from sham (N=5, n=10) and PBOO animals (N=5, n=8, p ≤ 0.001 to sham). B, Calcitonin gene related peptide content in the penis and urinary bladder of rats without (N=5) and with bladder outlet obstruction (N=5, p=0.003 to sham).
References


12. **Christianson JA, McIlwrath SL, Koerber HR and Davis BM.** Transient receptor potential vanilloid 1-immunopositive neurons in the mouse are more prevalent within colon afferents compared to skin and muscle afferents. *Neuroscience* 140: 247-257, 2006.


57. **Wu ZM, Chen YF, Qiu PN and Ling SC.** Correlation between the distribution of SP and CGRP immunopositive neurons in dorsal root ganglia and the afferent sensation of preputial frenulum. *Anat Rec (Hoboken)* 294: 479-486, 2011.


Corpus Cavernosum

Fig. 1

Urinary bladder
Fig. 2
Fig. 5
Fig. 6

A, Substance P

B, CGRP