Association of overactive bladder and stress urinary incontinence in rats with pudendal nerve ligation injury

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OVERACTIVE BLADDER (OAB) SYNDROME is defined as a symptom complex of urgency usually associated with daytime frequency and nocturia, with or without urgency incontinence (1). The prevalence of OAB is ~17% of the general adult population (30, 48). In addition, 25% of women from 20 years of age live with urinary incontinence, of which one-half of all urinary incontinence is stress urinary incontinence (SUI), 11% urgency, and 36% mixed incontinence (14), indicating that approximately one-third of patients with SUI also suffer from urgency incontinence, one of the major symptoms of OAB.

The main risk factors for SUI, which are defined as involuntary loss of urine secondary to an increase in abdominal pressure during events such as sneezing, coughing, or laughing, include parity, age, and obesity (7). Childbirth injury to muscles, connective tissues, and nerves seems to be the most important risk factor for lifetime incontinence because damage of pudendal nerves innervating to the external urethral sphincter is often found in SUI patients (4, 43, 44, 49) and about 30% of mothers become urinary incontinent after their first vaginal delivery (28). In addition, it has been reported that patients with pudendal nerve entrapment that induces compression or stretching of the pudendal nerve at the ischial spine or in Alcock’s canal exhibit urgency, urinary incontinence (stress, urgency, and mixed types), and chronic pelvic pain such as vulvodynia, perineal pain, and proctalgia (3, 36). These findings raise the possibility that pudendal nerve injury that damages the urethral continence mechanisms to induce SUI is also involved in OAB pathogenesis.

Because the pudendal nerve carries motor and sensory fibers, injury to this nerve can affect both efferent and afferent pathways. Reflex interaction between pelvic organs, necessary for the normal regulation of sexual, bladder, and bowel function, is likely mediated by the convergence of afferent pathways in the spinal cord or innervation of multiple organs by the same primary afferent neurons (10, 16). Because a neural substrate for pelvic organ reflex interaction or cross-talk exists under normal conditions, alterations in this cross-talk by injury or disease may play a role in the development of pelvic organ dysfunction (35, 37). Thus, there is the possibility that pudendal nerve injury can induce increased activity of bladder C-fiber afferent pathways, which is reportedly involved in the development of OAB (8, 53).

Nerve growth factor (NGF) may be an important factor inducing afferent sensitization and OAB symptoms. Increased NGF levels have been found in the bladder of patients with idiopathic OAB (23), and intravesical application of NGF or injection of a viral vector encoding for NGF into the bladder wall induces OAB in rats (11, 20). Moreover, NGF may play an important role in somato-visceral cross sensitization because intravesical application of NGF induces hyperalgesia of the hind limb (an example of viscerosomatic convergence or cross talk) (15), and injection of NGF-encoding vectors into the bladder wall enhances the responses to colorectal distension (an example of viscero visceral convergence or cross talk) (5).

Therefore, the present study first examined whether PNL was able to induce OAB and SUI conditions using cystometry and leak point pressure (LPP) measurements, respectively. We also examined the effects of capsicain pretreatment that inhib...
duces C-fiber desensitization on bladder activity and the levels of NGF protein and mRNA in the bladder to clarify whether sensitization of C-fiber afferent pathways and increased bladder NGF levels were involved in PNL-induced bladder overactivity. In addition, the responses of detrusor muscle strips to muscarinic or α1-adrenoceptor (AR) stimulation were also studied to investigate changes in detrusor contractility after PNL.

MATERIALS AND METHODS

Pudendal nerve ligation injury. Eighty adult female Sprague-Dawley rats weighing 240–280 g were divided into pudendal nerve ligation injury (PNL; n = 40) and sham-operated control groups (sham, n = 40). In PNL rats, under isoflurane anesthesia (Hospira, Lake Forest, IL), pudendal nerves were exposed bilaterally near the internal iliac vessels through a lower midline abdominal incision (26) and ligated with 4–0 silk threads to compress approximately one-half of the nerve’s diameter. Sham rats underwent the same procedures without ligation. After the surgery, the animals were treated with ampicillin (100 mg/kg sc; Fort Dodge Animal Health, Fort Dodge, IA) and buprenorphine (0.5 mg/kg sc; Reckitt Benckiser Pharmaceuticals, Richmond, VA) for 3 days. All experiments were conducted in accordance with institutional guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

 Conscious cystometry with or without capsaicin pretreatment. Conscious cystometry was performed in eight sham and eight PNL rats 4 wk after the operation. Under isoflurane anesthesia, the bladder was exposed through a lower midline abdominal incision and a polyethylene (PE)-50 catheter (Clay Adams, Parsippany, NJ) was implanted into the bladder through the bladder dome. The intravesical catheter was passed through the abdominal wound when the wound was closed with sutures, and local anesthetics (EMLA cream, AstraZeneca, Wilmington, DE) were applied to the abdominal wound. The animals were then placed in a restraining cage and allowed to recover from anesthesia, so that cystometry was performed while they were awake. The intravesical catheter was connected via three-way stopcocks to a pressure transducer (Transbridge 4M, World Precision Instruments, Sarasota, FL) and a syringe pump (Harvard Apparatus, Holliston, MA). Saline was infused at 0.04 ml/min for about 2 h until rhythmic bladder contractions became stable. Cystometric parameters were then measured during saline infusion for 1 to 2 h to evaluate bladder function. In another group of animals, conscious cystometry was performed in eight sham and eight PNL rats 4 wk after the pretreatment with capsaicin (125 mg/kg sc; Sigma, St. Louis, MO) 4 days before cystometry under isoflurane anesthesia to examine whether PNL-induced changes in bladder activity were mediated by activation of capsaicin-sensitive C-fiber afferent pathways (27). An eye wipe test was performed on each unanesthetized animal just before the experiment to evaluate the effectiveness of capsaicin pretreatment as previously described (9).

Saline voided from the urethral orifice was collected and measured to determine voided volume (VV). After constant voided volumes were collected, the infusion was stopped and postvoid residual volume (RV) was measured by clamping the catheter and withdrawing intravesical fluid through the catheter by gravity. Voiding efficiency (VE) was calculated with the formula, VV/(VV + RV) × 100. Intercontraction interval (ICI), baseline pressure (BP), voiding threshold pressure (TP), and maximal voiding pressure (MVP) were also recorded using a data acquisition software (sampling at 40 Hz, Chart, ADInstruments, Castle Hill NSW, Australia) on a computer system equipped with an analog-to-digital converter (PowerLab, ADInstruments).

LPP measurement. LPPs were measured using the vertical tilt table/intravesical pressure clamp method (21) in another group of animals (8 sham and 8 PNL rats) 4 wk after the surgery. Under isoflurane anesthesia, the animals underwent spinal cord transection at the T8-T9 level after laminectomy to eliminate spontaneous reflex voiding mediated by spino-bulbo-spinal pathways passing through a micturition center in the pons. This manipulation does not interfere with urethral reflexes induced by bladder distension, which are predominantly organized in the lumbosacral spinal cord (12, 18). The bladder was exposed through a lower midline abdominal incision, and a PE-90 catheter was implanted into the bladder through the bladder dome. Feces were removed from the distal colon through a small incision in the colon wall.

After the surgery, isoflurane anesthesia was turned off and replaced with urethane anesthesia (1.2 g/kg sc; Sigma). The animals were then mounted on a tilt table and placed in the vertical position. Intravesical pressure was clamped by connecting the bladder catheter to a saline reservoir (60-ml syringe; Becton Dickinson, Franklin Lakes, NJ) and a pressure transducer (Transbridge 4M; World Precision Instruments) via three-way stopcocks, and recorded using a Chart software on a PowerLab system (sampling at 10 Hz, ADInstruments). The reservoir was mounted on a metered vertical pole for controlled height adjustment. Intravesical pressure was increased in 2.5-cm steps from zero upward until visual identification of leakage of fluid from the urethral orifice. The pressure at leak point was regarded as LPP. The average of three consecutive LPPs was taken as a data point for each animal. In addition to the measurement of LPPs, all of the bladders were dissected and weighed to examine whether PNL induced bladder hypertrophy.

Bladder muscle strip study. The bladders of eight sham and eight PNL rats 4 wk after the operation were harvested. Transverse muscle strips (2 × 8 mm) of the posterior wall of the bladder were prepared in a cold Krebs-Henseleit (K-H) solution composed of 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, and 10 mM glucose. Muscle strips were then suspended in a 30-ml organ bath filled with K-H solution at 37°C and gassed with a 95% O₂ and 5% CO₂ mixture. Contractile responses were monitored with a pressure transducer (Transbridge 4M, World Precision Instruments) and recorded using a Chart software on a PowerLab system (sampling at 40 Hz; ADInstruments). Each strip was adjusted to a resting tension of 1 g and then allowed to equilibrate for at least 60 min. After the responses to 80 mM KCl were assessed, cumulative concentration-dependent contractions induced by carbachol (10⁻⁷ to 10⁻³ M, Sigma), a muscarinic receptor agonist, and phenylephrine (10⁻⁷ to 10⁻³ M, Sigma), an α1-AR agonist, were recorded in a stepwise manner after the response to the previous concentration had reached a plateau. Contractile responses were expressed as a percentage of the response to 80 mM KCl, and contractile forces were calculated as grams of active force per cross-sectional area using the following formula, weight/length × 1.05, where 1.05 is the assumed density of muscles (32). EC₅₀ values that are the concentration required to produce 50% of the maximal contractile response were obtained from contractile response curves with an iterative nonlinear least square curve-fitting program using a Prism program (GraphPad Software, San Diego, CA). Emax values that are the maximal contractile response that can be obtained for a particular stimulus are also reported.

NGF protein measurement by ELISA. The bladders were removed following conscious cystometry without capsaicin pretreatment in eight sham and eight PNL rats 4 wk after the operation for the measurement of NGF protein by ELISA (40). The tissues were rapidly frozen and stored at −80°C until protein extraction. The bladders were homogenized with the buffer composed of 2.66% Trisma HCl (Sigma), 0.985% Trisma Base (Sigma), 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 1 µM leupeptin (Sigma), 1 µM pepstatin A (Sigma), and 0.3 µM aprotinin (Sigma) in 400 µl. The homogenate was centrifuged at 10,000 × g for 4 min, and the supernatant was diluted with four volumes of Dulbecco’s phosphate buffer saline (Invitrogen, Carlsbad, CA). The samples were acidified by 10 N HCl to pH 2.0 to 3.0 for 15 min and then neutralized by 10 N NaOH to pH 7.5 to 8.0 to activate the immunological recognition of all biologically active...
antibodies to NGF (55). After acid treatment, the samples were stored at −80°C until assayed.

The samples were assayed by ELISA Emax ImmunoAssay System (Promega, Madison, WI) according to the manufacturer’s instructions. In addition, total protein concentration in the same samples was detected with a Bradford Assay Kit (Pierce, Rockford, IL). All tissue NGF values were then standardized by tissue protein levels and expressed in picograms per microgram total protein.

**NGF mRNA measurement by real-time RT-PCR.** The bladders were harvested from eight sham and eight PNL rats 4 wk after the operation for the measurement of NGF mRNA. The tissues were rapidly frozen and stored at −80°C until RNA extraction. Total RNA was extracted from frozen tissues using TRIzol (Invitrogen) according to the manufacturer’s instructions. The concentration of RNA was estimated by measuring the absorbance at 260 nm with an ELx800 system (Bio-Tec Instruments, Winooski, VT). The 260/280 ratio was used to check for purity. RT reaction mixture contained 3 μg of total RNA, 500 ng oligo(dT)15 Primer (Promega), 0.5 mM dNTP Mix (Invitrogen), 1× first-strand buffer, 10 mM DTT, 20 units RNasin ribonuclease inhibitor (Promega), and 200 units SuperScript RT (Invitrogen) in a total volume of 20 μl. The mixture was incubated at 42°C for 50 min, heated to 70°C for 15 min to denature the RT, and then cooled to 4°C. The cDNA was stored at −20°C until assayed by real-time PCR.

The sequence of oligonucleotide primers for amplifying NGF mRNA were sense; 5′-AACAGGACTCAGGAGC3′, antisense; 5′-CTTCCTGCTGAGCACACA3′ (GeneMark, Atlanta, GA). Amplification of β-actin mRNA served as an internal standard. The primers used for β-actin were 5′-CTATGAGCTGCTGAGCG- GTC-3′, antisense; 5′-AGTTTCATGGATGCCACAGG-3′, giving a 115-bp fragment. Real-time PCR was performed with QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) using an Mx3000P QPCR system (Stratagene, La Jolla, CA). The real-time PCR reaction mixture contained 1× QuantiTect SYBR Green PCR Green, 0.3 μM primer pairs of NGF and 1 μl cDNA of the samples in a total volume of 25 μl. The mixture was heated at 95°C for 15 min to activate DNA polymerase, followed by 35 cycles with denaturation at 95°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. Each assay for the samples was performed in duplicate wells. After PCR products had been made, melt curve protocols designed for increment temperatures of 1°C (starting at 55°C and ending at 95°C) were performed to

| Table 1. Parameters of bladder activity during conscious cystometry in sham and PNL groups with or without capsaicin pretreatment |
|----------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                                | n  | BP, cmH2O | TP, cmH2O | MNP, cmH2O | ICI, s | VV, ml | RV, ml | VE, %   |
| Sham                            |    |           |           |            |       |       |       |        |
| without capsaicin               | 8  | 4.5±0.4   | 8.2±0.4   | 32.2±3.4   | 608.1±24.3 | 0.41±0.05 | 0.08±0.01 | 81.4±2.0 |
| with capsaicin                  | 8  | 4.7±0.1   | 9.3±0.7   | 33.9±1.1   | 693.1±56.2 | 0.47±0.06 | 0.06±0.01 | 87.6±2.1 |
| PNL                             |    |           |           |            |       |       |       |        |
| without capsaicin               | 8  | 5.1±0.5   | 8.2±0.6   | 32.8±3.1   | 482.0±23.9## | 0.30±0.03# | 0.08±0.01 | 77.5±3.8 |
| with capsaicin                  | 8  | 5.2±0.3   | 9.9±0.8   | 35.8±1.9   | 663.8±11.3** | 0.41±0.02* | 0.04±0.01* | 88.0±1.7* |

All parameters are represented as mean values ± SE. PNL, pudendal nerve ligation; BP, baseline pressure; TP, threshold pressure; MNP, maximal voiding pressure; ICI, intercontraction interval; VV, voided volume; RV, residual volume; VE, voiding efficiency. *P < 0.05 and **P < 0.01; PNL rats without capsaicin pretreatment (PNL without capsaicin) versus sham rats without capsaicin pretreatment (Sham without capsaicin). *P < 0.05 and **P < 0.01; PNL rats with capsaicin pretreatment (PNL with capsaicin) vs. PNL rats without capsaicin pretreatment (PNL without capsaicin). There are no significant differences in any parameters in sham groups (with vs. without capsaicin) or in capsaicin-treated groups (Sham vs. PNL).
Table 2. Contractile responses of bladder muscle strips of sham and PNL groups to carbachol and phenylephrine

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<th>EC50, µM</th>
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<td>Phenylephrine</td>
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<td>Sham</td>
<td>8</td>
<td>2.2 ± 1.3</td>
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All data are represented as mean values ± SE. EC50: concentration required to produce 50% of the maximal contractile response. Emax: maximal contractile response. *P < 0.05 indicates a significant difference compared with the sham group.

RESULTS

Cystometry and LPP testing. Representative conscious cystometric recordings in sham and PNL groups were shown in Fig. 1. During cystometry, ICI and VV were significantly decreased in PNL rats compared with sham rats (ICI, PNL: 482.0 ± 23.9 s vs. sham; 608.1 ± 24.3 s, VV, PNL: 0.30 ± 0.03 ml vs. sham; 0.41 ± 0.05 ml), whereas BP, TP, MVP, RV, or VE were not different between PNL and sham rats. However, the differences of ICI and VV were not seen when PNL and sham rats were pretreated with capsaicin to desensitize C-fiber afferents (ICI, PNL: 663.8 ± 11.3 s vs. sham 693.1 ± 56.2 s; VV, PNL: 0.41 ± 0.02 ml vs. sham 0.47 ± 0.06 ml).

In addition, no difference was observed in any of the other parameters (BP, TP, MVP, RV, or VE) in sham and PNL rats with capsaicin pretreatment (Table 1). Moreover, there was no significant difference between the bladder weight of PNL (0.13 ± 0.01 g) and sham (0.12 ± 0.01 g) rats.

In another group of animals, LPP was significantly decreased in PNL (31.9 ± 2.2 cmH₂O) rats compared with sham (38.2 ± 1.5 cmH₂O) rats 4 wk after PNL (P < 0.05).

Contractile responses of the bladder muscle strips. An analysis of concentration-dependent responses of the bladder muscle strips from PNL and sham rats to carbachol (10⁻⁸ to 10⁻⁴ M) and phenylephrine (10⁻⁷ to 10⁻³ M) yielded values for functional affinity constants, EC50, and maximal contractions, Emax, for the agonists (Table 2). EC50 and Emax values of the detrusor responses to carbachol were similar in PNL and sham rats (Fig. 2, A and B). However, EC50 values of the detrusor responses to phenylephrine were significantly decreased in PNL (2.2 ± 1.3 µM) rats compared with sham (6.8 ± 1.4 µM) rats (Fig. 2C), and Emax values of the detrusor responses to phenylephrine were significantly increased in PNL (1.1 ± 0.1 g/mm²) rats compared with sham (0.7 ± 0.1 g/mm²) rats (Fig. 2D), indicating increased contractile responses of the detrusor to α1-AR stimulation after PNL.

Levels of NGF protein and mRNA in the bladder. Four weeks after the operation, NGF protein levels of the bladder were also significantly increased in PNL (70.9 ± 7.9 pg/µg) rats than in sham (44.8 ± 3.2 pg/µg) rats (Fig. 3A). Similarly, NGF mRNA levels of the bladder were also significantly increased in PNL (2.6 ± 0.4) rats compared with sham (1.4 ± 0.2) rats (Fig. 3B).

DISCUSSION

The results of the present study demonstrate that 1) pudendal nerve injury induces not only SUI indicated by reduced LPPs, but also OAB conditions indicated by reduced ICI and VV; 2) OAB induced by PNL is at least in part mediated by...
activation of capsaicin-sensitive C-fiber afferents; 3) PNL increases the levels of NGF protein and mRNA in the bladder; and 4) PNL enhances α1-AR-mediated contractile responses of the detrusor.

SUI is the most common type of urinary incontinence in women, and approximately one-third of women with urinary incontinence exhibit both SUI and urgency incontinence (i.e., mixed incontinence) (14). A recent cross-sectional population-based study also has revealed that SUI is strongly associated with urgency, the primary symptom of OAB (50). The mechanisms that contribute to development of SUI and OAB symptoms in the same patients are not well understood, although a previous study has suggested that leakage of urine into the urethra (i.e., stress incontinence) may stimulate urethral afferents that induce an involuntary voiding reflex (i.e., urgency incontinence) (17). Vaginal parity has been described as one of the major risk factors inducing SUI, in addition to age and obesity. Childbirth injury can damage muscles, connective tissues, and nerves, including the pudendal nerves (7, 28). Previous clinical studies in SUI patients have provided evidence of damage of pudendal nerves (4, 43, 44, 49). The results of the present study raise the possibility that the mixed stress and urgency incontinence condition is induced by pudendal nerve injury, which sensitizes bladder afferent pathways and induces bladder overactivity by increasing the levels of bladder NGF. In addition, SUI conditions induced in PNL rats seem to be modest because the reduction in LPPs after PNL in the study was smaller (16% decrease) than that observed in our previous study using rats with pudendal nerve transection (22% decrease) (19).

Additional evidence for a contribution of pudendal nerve injury to bladder overactivity was obtained in patients with pudendal nerve entrapment (PNE) (3, 36). The pudendal nerves, which, in humans, are derived from the sacral roots S2, S3, and S4, seem to be susceptible to compression or stretching because of their anatomical course (3, 39). Three main symptoms of PNE include urinary incontinence (both stress and urgency types) (74%), fecal incontinence (62%), and perineodynia (35%), which includes vulvodynia, perineal pain, and proctalgia (3). Patients with PNE frequently (40%) have additional symptoms such as urinary frequency and urgency (36). Thus it seems likely that pudendal nerve injury is involved in pathogenesis of OAB symptoms, such as urgency, frequency, and urgency incontinence, as well as SUI in patients with PNE.

Although the precise mechanism inducing changes in bladder activity following pudendal nerve injury is not known, cross sensitization of sensory pathways may be involved because there is extensive convergence of afferent inputs from cutaneous, muscle and visceral tissues at the level of spinal cord and/or dorsal root ganglia (31). Several examples of cross-organ sensitization have been demonstrated in the pelvic viscera, including chemically induced colitis in rats that induced urinary frequency, bladder afferent fiber hyperexcitability (35, 51), upregulation of the NGF mRNA levels and mast cell infiltration in the bladder (22, 52), and increased excitability of bladder afferent neurons, as well as spinal dorsal horn neurons receiving inputs from the bladder (25, 37). The NGF levels are reportedly elevated in the bladders of patients with benign prostatic hyperplasia (46), idiopathic OAB, and interstitial cystitis (23). Thus, it is possible that PNL-induced irritation of pudendal nerve afferents can induce sensitization of bladder-afferent pathways to increase the bladder NGF levels and elicit bladder overactivity.

It has been reported that increased NGF levels in the bladder or bladder-afferent pathways can induce OAB and C-fiber afferent hyperexcitability (5, 20, 42, 45, 47, 54). Because bladder overactivity in the PNL animals disappeared after capsaicin pretreatment that induces C-fiber desensitization in the present study, it seems reasonable to assume that NGF-induced C-fiber afferent hyperexcitability is also likely to be involved in PNL-induced bladder overactivity. Since there was no difference in bladder weight and postvoid residual volume between sham and PNL rats in the present study, it seems that PNL did not induce bladder outlet obstruction (BOO), which can lead to bladder hypertrophy. A previous study has also reported no changes in bladder weight in rats with pudendal nerve transection injury (34). Thus, upregulation of bladder NGF after PNL is likely to be induced by direct sensitization of bladder afferent pathways, rather than BOO-induced bladder hypertrophy.

In the present study, α1-AR-mediated contractility was increased in the bladder muscle strips obtained from the PNL rats, suggesting that PNL induces postjunctional changes in the bladder. α1-ARs are expressed at low levels in normal human bladder muscles (29), the highest levels being α1D-AR (66%) followed by α1A-AR (34%) and no expression of α1B-AR (24). A four-fold increase in α-ARs has been reported in the bladder muscles of OAB patients compared with normal human bladder (38). Increased contractile responses to phenylephrine have been also demonstrated in the bladder muscle strips from patients with BOO, and these responses were inhibited by tamsulosin, an α1A and α1D-AR antagonist (6). However,
other studies on human normal and obstructed bladders do not show any differences in α1-ARs mRNA expression and function (33). Hampel et al. (13) have reported that there are changes in α1-AR subtype expression from α1A to α1D subtypes in the bladder muscles of rats with BOO. Since α1D-AR has 10 to 100-fold higher affinity for the endogenous norepinephrine and epinephrine compared with other α1-AR subtypes (41), it is possible that upregulation of α1D in the bladder can increase contractility of bladder smooth muscles in response to sympathetic nerve activation, which occurs during urine storage. However, because there is already α1D-AR predominance in the normal human detrusor (24), increased α1D-AR expression may not be an important factor for generating OAB (2). More studies are needed to clarify the role of increased α1-AR-mediated contractility of the detrusor in PNL-induced bladder overactivity.

**Perspectives and Significance**

Our findings that pudendal nerve injury can induce both SUI and OAB conditions support the clinical data that approximately one-third of patients with SUI suffer from urgency and OAB conditions. Support for our findings is derived from the observation that pudendal nerve injury can induce both SUI and OAB. Our findings in chronic spinal rats can be applied to human OAB conditions and support the clinical data that approximately one-third of patients with SUI suffer from urgency and OAB conditions.


