

AWARD NUMBER: W81XWH-11-1-0763

TITLE: Anti-NGF Local Therapy for Autonomic Dysreflexia in Spinal Cord Injury

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REPORT DATE: December 2014

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE December 2014		2. REPORT TYPE Final report		3. DATES COVERED 30 Sep 2011 - 29 Sep 2014	
4. TITLE AND SUBTITLE Anti-NGF Local Therapy for Autonomic Dysreflexia in Spinal Cord Injury				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0763	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Naoki Yoshimura E-Mail: nyos@pitt.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pittsburgh, 3520 Fifth Ave., Pittsburgh, PA 15213-3320				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Autonomic dysreflexia (AD), which induces excessive elevation of blood pressure, is a potentially life-threatening medical emergency that occurs in persons with spinal cord injury (SCI) at or above the mid-thoracic spinal cord segment. Since the most common source of stimulation that initiates AD is the genitourinary tract including bladder distention, followed by colorectal distention, elimination of activation of bladder sensory pathways during bladder distention could significantly reduce the incidence and/or degree of AD in SCI. Because previous studies have indicated that increased levels of nerve growth factor (NGF) in sensory pathways are one of the key factors to induce increased excitability of sensory pathways after SCI, anti-NGF therapy could be an attractive treatment of AD in SCI patients. However, systemic anti-NGF treatment such as the use of NGF antibodies reportedly induces some side effects. Therefore, we hypothesize that the local therapy of NGF antisense delivery using liposomes (LPs) in the bladder could reduce the activation of bladder sensory pathways, thereby suppressing AD during bladder distention after SCI. Using adult female rats with chronic spinal cord injury induced by Th4 spinal cord transection, we will investigate: (1) the contribution of hyperexcitable bladder sensory pathways in the emergence of AD in SCI (Aim 1), and (2) the effects of intravesical delivery of NGF antisense-liposome conjugate, which reduce NGF expression in the bladder, on AD in SCI (Aim 2). If successfully completed, this study directly addresses the feasibility of local NGF antisense treatment for SCI-induced AD and provides the foundation for future clinical translation of local NGF antisense therapy in military service members, their family members, and/or the U.S. veteran population, who suffer from autonomic dysreflexia due to SCI. The local anti-NGF therapy could also be extended to a general population of people with SCI or other spinal cord lesions such as multiple sclerosis. The long-term objectives of the research program are to establish new and effective therapeutic targets and/or interventions strategies for the treatment of vascular complications of SCI.					
15. SUBJECT TERMS nothing listed					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	95	19b. TELEPHONE NUMBER (include area code)

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I. Introduction

Autonomic dysreflexia (AD), which induces excessive elevation of blood pressure, is a potentially life-threatening medical emergency that occurs in persons with spinal cord injury (SCI) at or above the mid-thoracic spinal cord segment (Khastgir et al., 2007; Krassioukov et al., 2009). The most common source of stimulation that initiates AD is the genitourinary tract including bladder distention (Krassioukov et al., 2009).

We previously showed that vascular responses (i.e., hypertension) induced by bladder distention are mediated by activation of TRPV1 (capsaicin receptor)-expressing C-fiber bladder afferent pathways in spinal intact rats (Chuang et al., 2001). In addition, the expression of TRP channels such as TRPV1 and TRPA1 are involved in sensitization of C-fiber afferent pathways (Julius, 2023). Previous studies have also indicated that increased levels of nerve growth factor (NGF) in the bladder is one of the key mediators to induce hyperexcitability of C-fiber bladder afferent pathways after SCI, resulting in detrusor overactivity (DO) (de Groat & Yoshimura, 2010; Ochodnický et al, 2012 [Appendix]; Yoshimura et al., 2014 [Appendix]), and that intrathecal application of NGF antibodies, which reduces NGF levels in bladder afferent pathways, is effective for DO as well as AD in SCI rats (Krenz et al., 1999; Marsh et al., 2002; Seki et al., 2002). However, it is not known whether a liposome-based local therapy targeting bladder NGF expression is effective to ameliorate SCI-induced AD during bladder distention.

Therefore, in this project, we aimed to investigate the feasibility of local nerve growth factor (NGF) antisense treatment for SCI-induced AD during bladder distention and provide the foundation for future clinical translation of local NGF antisense therapy in people with SCI-induced AD. For this purpose, the following two aims were proposed.

Specific Aim 1: To investigate the contribution of hyperexcitable TRPV1-expressing C-fiber bladder afferent pathways in the emergence of autonomic dysreflexia (AD) in spinal cord injured (SCI) rats.

Specific Aim 2: To examine the effects of intravesical delivery of nerve growth factor (NGF) antisense-liposome conjugate on AD and bladder afferent hyperexcitability in SCI rats.

II. Keywords

Autonomic dysreflexia (AD), spinal cord injury (SCI), nerve growth factor (NGF), liposome, antisense oligonucleotide (OND), rat, afferent pathways, resiniferatoxin (RTX), patch clamp recording

III. Overall Project Summary

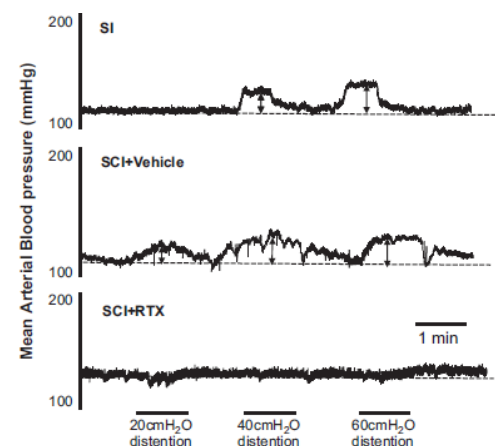
1. Comparison of blood pressure responses during bladder distention in SCI rats with or without capsaicin pretreatment

[Accomplishment]

In this project, we performed experiments to measure blood pressure responses during bladder distention using rats with Th4-5 spinal cord transection (4 weeks). In order to evaluate autonomic dysreflexia (AD), the bladder-to-vascular responses during bladder distention was examined under urethane anesthesia. For bladder distention, intravesical pressure was increased in a stepwise manner to 20, 40 and 60 cmH₂O, for 2 min by connecting the urethra cannula through a three-way stopcock to a saline-filled reservoir, the height of which was adjusted to maintain a constant pressure in the bladder (Fig. 1).

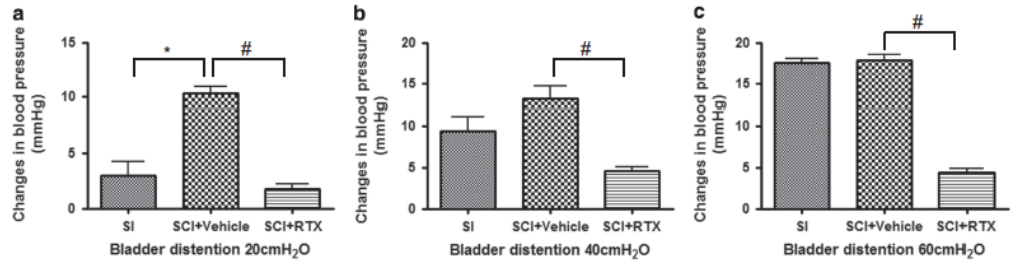
Figure 1. Recordings of mean arterial blood pressure during bladder distention in a stepwise manner to intravesical pressure at 20, 40 and 60 cm H₂O for a duration of 1 min at each pressure level with 1-min intervals in spinal intact (SI) (upper panel) and SCI rats without (SCI+ vehicle; middle panel) or with TRPV1-expressing C-fiber desensitization induced by the intravesical RTX pretreatment (SCI+ RTX; lower panel). Break lines and bidirectional arrows indicate the baseline mean arterial blood pressure and the increases of mean arterial blood pressure during bladder distention, respectively.

We have found that: (1) arterial blood pressure elevation at 20 cmH₂O intravesical pressure was significantly higher in SCI rats compared with spinal intact (SI) rats (Fig. 2), and (2) arterial blood pressure elevation induced by bladder distention was significantly reduced in SCI rats when C-fiber bladder



afferent pathways were desensitized by intravesical application of resiniferatoxin (RTX; 10 μ M) for 30 min, which was administered 24 hours prior to the experiment (Fig. 2).

Figure 2. Changes in mean arterial blood pressure responses to bladder distention at 20, 40 and 60 cm H₂O of intravesical pressure (a–c, respectively) in SI rats (n=5) and SCI rats without (SCI+vehicle, n=5) or with RTX-induced C-fiber desensitization (SCI+RTX, n=5). *P<0.05: SI vs. SCI+vehicle, #P<0.05: SCI+vehicle vs. SCI+RTX.



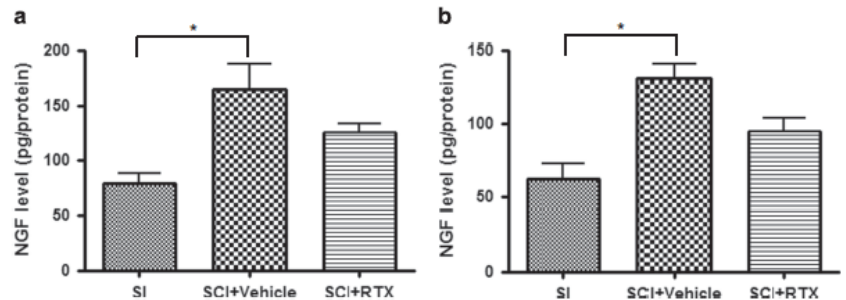
These results indicate that SCI (4 weeks) induced AD as evidenced by enhanced arterial blood pressure elevation during bladder distention to a low pressure level (20cmH₂O) in SCI rats vs. SI rats and that enhanced AD in SCI rats is dependent on C-fiber afferent activation in the bladder (Yoshizawa et al., 2015 [Appendix]).

2. Tissue analysis: Optimization and analysis of NGF expression using molecular techniques such as PCR and ELISA in spinal intact and SCI rats

[Accomplishment]

In this project, we performed ELISA experiments to measure NGF protein levels and confirmed that **the NGF levels are significantly increased in both mucosa and detrusor layers of the bladder after SCI** (Fig. 3) (Yoshizawa et al., 2015 [Appendix]).

Figure 3. Expression of NGF protein in mucosa (a) and detrusor layers (b) of the bladder in SI (n=5) rats and SCI rats without (SCI+vehicle, n=5) or with RTX-induced C-fiber desensitization (SCI+RTX, n=5). *P<0.05: SI vs SCI+vehicle.

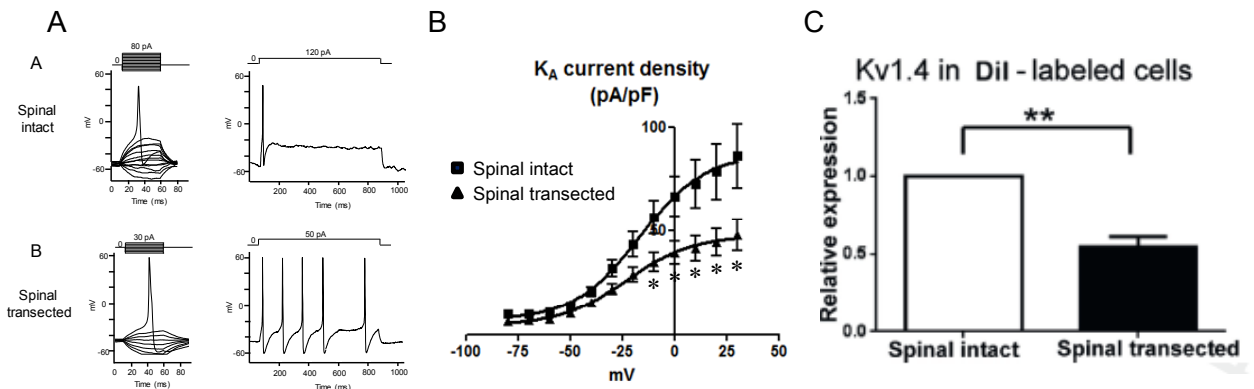


3. Electrophysiological and tissue analyses: Comparison of properties of bladder afferent neurons from spinal intact and SCI rats

[Accomplishment]

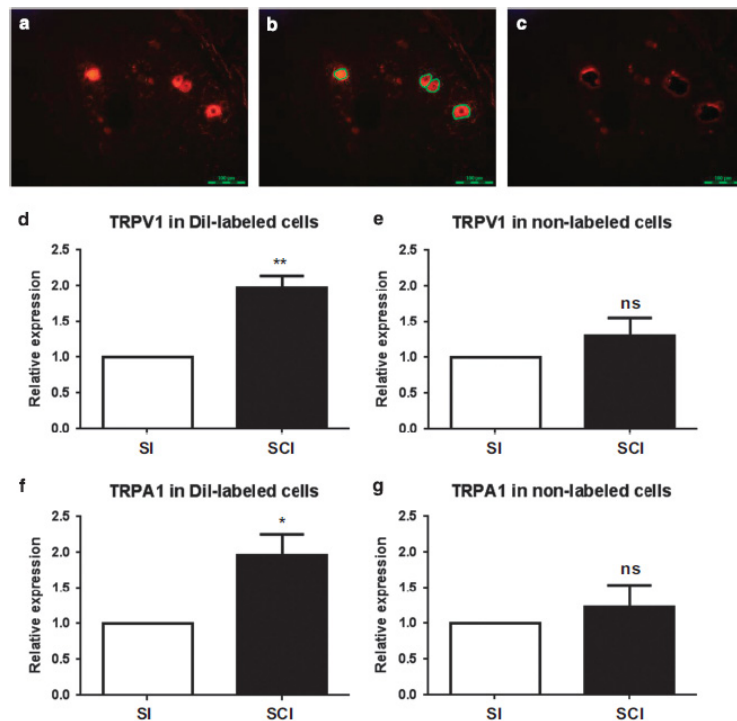
In this project, we performed the electrophysiological experiments using patch-clamp recordings and histological analyses of DRG neurons innervating the urinary bladder.

Figure 4. Electrophysiological properties and histological/molecular analysis of bladder afferent neurons from spinal intact and SCI (spinal transected) rats. A: Representative recordings of action potentials in capsaicin-sensitive bladder afferent neurons from spinal intact (A) and SCI rats (B). Left panels show the action potentials evoked by 50-ms depolarizing current pulses injected through patch pipettes during current-clamp recording. Right panels show the firing patterns during a sustained membrane depolarization (800 ms of duration). Note the



lower threshold for spike activation and repetitive firing pattern in the bladder afferent neuron from SCI rats compared to control rats. B: Current-voltage relationships of K_A currents in capsaicin-sensitive bladder afferent neurons from SI and SCI rats ($n=20$ cells from 12 rats and $n=17$ cells from 10 rats, respectively). Data are expressed as mean $\pm$ s.e.m. *; $P < 0.05$ compared with the spinal intact rats at corresponding membrane potentials. C: Kv1.4 mRNA levels in Dil-labeled bladder afferent neurons (30 cells per rat), which were laser-captured from L6 DRG sections of SI and SCI rats ($n=5$ rats each). ** $P < 0.01$ vs. spinal intact.

Figure 5. (a-c) Photomicrographs of the same L6 DRG section taken during laser capture microdissection (LCM) of Dil-labeled bladder afferent neurons before (a and b) and after LCM (c). Arrows point to neurons positively stained with Dil, which was injected into the bladder wall 1 week earlier. Green-line circles in b indicate the areas that were laser captured. Scale bar: 100 μ m. (d) TRPV1 mRNA levels in Dil-labeled neurons (30 cells per rat) from SI and transected rats (SCI; $n=5$ rats each). (e) TRPV1 mRNA levels in unlabeled neurons (30 cells per rat) from spinal intact (SI) and SCI rats ($n=5$ rats each). (f) TRPA1 mRNA levels in Dil-labeled neurons (30 cells per rat) from SI and SCI rats ($n=5$ rats each). (g) TRPA1 mRNA levels in unlabeled neurons (30 cells per rat) from SI and SCI rats ($n=5$ rats each). Data are expressed as mean $\pm$ s.e.m. * $P < 0.05$ and ** $P < 0.01$, compared with the SI group. ns: not significant.



We have found that: (1) capsaicin-sensitive bladder afferent neurons from SCI rats showed increased cell excitability, as evidenced by lower spike activation thresholds and a tonic firing pattern (Fig. 4A), (2) the peak density of transient A-type K^+ (K_A) currents in capsaicin-sensitive bladder afferent neurons from SCI rats was significantly less than that from spinal intact rats (Fig. 4B), (3) the K_A current inactivation curve was displaced to more hyperpolarized levels after spinal transection, (4) the protein and mRNA expression of Kv1.4 α -subunits, which can form transient K_A channels, was decreased in bladder afferent neurons after spinal transection (Fig. 4C) (Takahashi et al., 2013 [Appendix]) and (5) the mRNA expression of TRPA1 and TRPV1 was increased in bladder afferent neurons, which were laser-captured from L6 DRG sections of SCI rats (Fig. 5) (Yoshizawa et al., 2015 [Appendix]).

These results indicate that SCI induces hyperexcitability of capsaicin-sensitive C-fiber bladder afferent neurons due to reductions in K_A channel activity and Kv1.4 α -subunit expression as well as upregulation of TRP channels in bladder afferent pathways after SCI.

We also performed histological and molecular experiments to characterize the expression pattern and functional properties of Kv4 family Kv channel subunits (Kv4.1, Kv4.2 and Kv4.3) and their auxiliary subunits because they comprise K_A channel in DRG neurons (Matsuyoshi et al., 2012 [Appendix]). Using immunohistochemistry, in situ hybridization and RT-PCR technique, we have found that: (1) the two pore-forming subunits Kv4.1 and Kv4.3 show distinct cellular distributions; that is, Kv4.3 is predominantly in isolectin B4 (IB4)-positive, small-sized C-fiber neurons, whereas Kv4.1 is seen in DRG neurons in various sizes although Kv4.2 was not expressed in DRG neurons and (2) the two classes of Kv4 channel auxiliary subunits are also distributed in different-sized cells; that is, KChIP3 is the only significantly expressed Ca^{2+} -binding cytosolic ancillary subunit in DRGs and present in medium to large-sized neurons whereas the membrane-spanning auxiliary subunit DPP6 is seen in a large number of DRG neurons in various sizes, whereas DPP10 is restricted in small-sized neurons (Matsuyoshi et al., 2012 [Appendix]).

Furthermore, in the electrophysiological study using patch-clamp recordings, we have found that: (1) all IB4 intensely stained cells are negative for a fluorescent dye, Fast Blue (FB), injected into the bladder wall (Fig.

6), whereas a fraction of somatic neurons labeled by FB, injected to the external urethral dermis, is intensely stained with IB4, (2) in whole-cell, patch-clamp recordings, phrixotoxin 2 (PaTx2), a voltage-gated K^+ (K_v)4 channel blocker, exhibits voltage-independent inhibition of the K_A current in IB4 intensely stained cells but not the one in bladder innervating cells (Fig. 7), and (3) $Kv4.1$ and $Kv4.3$ mRNA levels were higher in laser-captured, IB4-stained neurons than in bladder afferent neurons (Fig. 8) (Yunoki et al., 2014 [Appendix]).

Figure 6. Morphological characterization of dissociated L6-S1 dorsal root ganglion (DRG) neurons from rats injected with Fast Blue (FB) into the bladder wall. A: single DRG neurons in the same field, observed by light-field and fluorescent microscope, are shown in the top and bottom, respectively. Top: an arrow indicates a FB-positive bladder afferent neuron identified under the fluorescent light. Bottom: single DRG neurons with various green-staining intensities of isolectin B4 (IB4)-FITC. The numbers (0–2) beside the cells indicate the intensity of staining: 0, no or weak (negative) staining; 1, moderate staining; 2, intense staining. B: cell-size distributions of FB-positive and -negative DRG cells with different IB4 staining intensities (grades 0–2). Note that there are no grade 2-stained cells in FB-positive bladder afferent neurons.

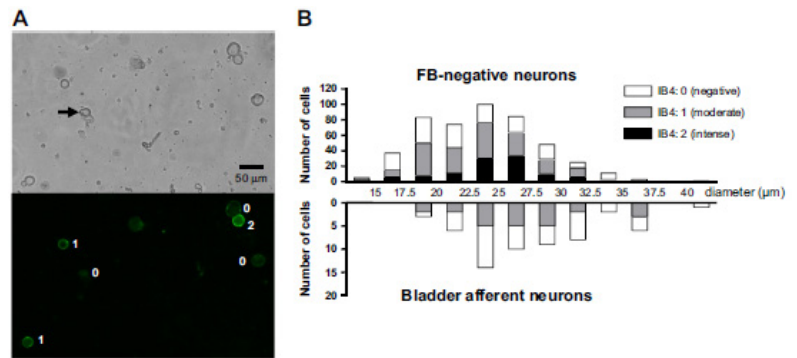
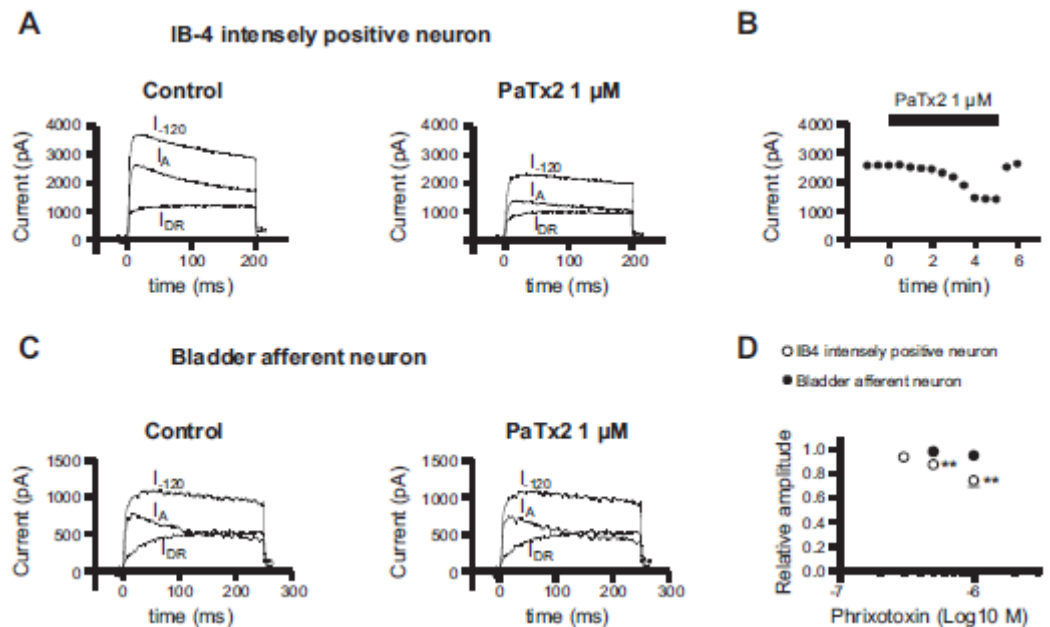


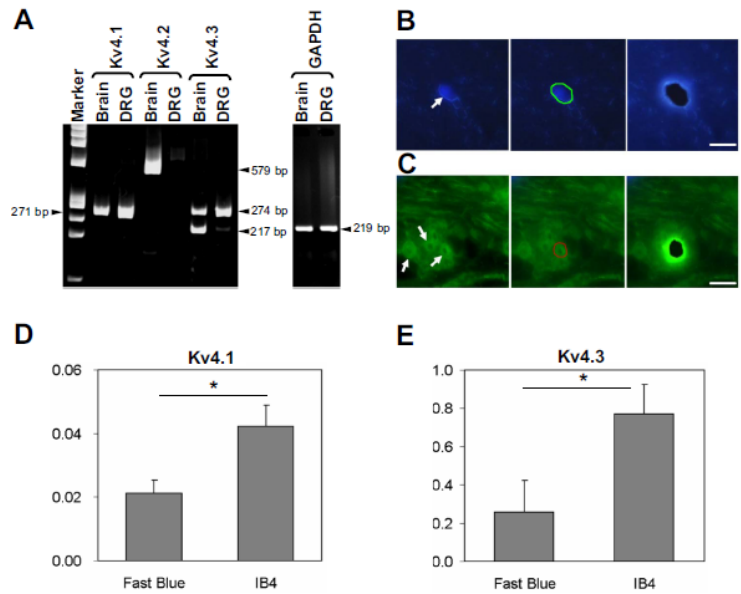
Figure 7. Effects of phrixotoxin 2 (PaTx2) on A-type transient K^+ (K_A) currents in IB4 intensely stained and bladder afferent neurons. K_A current (I_A) trace was obtained by subtracting the delayed rectifier-type K^+ (K_{DR}) current (I_{DR}) from the outward currents activated from the holding potential of -120 mV (I_{-120}).



I_{DR} and I_{-120} were evoked by a depolarizing voltage step to 0 mV from the holding potential of -40 mV and -120 mV, respectively. A and C: representative I_{-120} , I_{DR} , and I_A before (Control) and after application of $1 \mu\text{M}$ PaTx2 in an IB4 intensely positive neuron (A) and a bladder afferent neuron (C). B: the time course of PaTx2 effects on peak I_A amplitudes in an IB4 intensely positive neuron. D: the concentration-response relationship of PaTx2. Relative peak I_A amplitudes were determined using the level before application of PaTx2 (control) as 1 from the same cell. In IB4 intensely positive cells, PaTx2 decreased the K_A current in a concentration-dependent manner. Peak I_A amplitudes at 500 and 1,000 nM PaTx2 were significantly different from the control level in IB4 intensely positive cells (** $P < 0.01$ for PaTx2, 500 nM; ** $P < 0.01$ for PaTx2, 1,000 nM). In contrast, PaTx2 exhibited no significant changes in peak I_A amplitudes in bladder afferent neurons. The data are expressed as means $\pm$ s.e.m.

Figure 8. Expression of $Kv4$ mRNAs in DRG. A: RT-PCR data with tissue samples. PCR was performed with primers for $Kv4.x$ designed to detect splicing variants and the housekeeping gene, GAPDH. The 2 bands with distinct sizes in $Kv4.3$ correspond to the short and long splicing isoforms ($Kv4.3S$ and $Kv4.3L$). Note that DRGs contain abundant $Kv4.1$ and $Kv4.3L$, but not $Kv4.2$. B: photomicrographs of the same L6 DRG section taken during laser-captured microdissection (LCM) of Fast blue (FB)-labeled bladder afferent neurons before (left and middle) and after (right) LCM. An arrow indicates a laser-captured bladder afferent neuron labeled with FB

injected into the bladder wall (left). A green-line circle represents the area captured by this procedure. C: photomicrographs of the same L6 DRG section taken during LCM of IB4-stained DRG neurons before (left and middle) and after (right) LCM. Arrows point to DRG neurons positively stained with IB4 (left). A red-line circle indicates the area that was laser captured (middle). D: Kv4.1 mRNA levels in laser-captured, FB-labeled bladder afferent neurons and IB4-stained DRG neurons. E: Kv4.3 mRNA levels in laser-captured, FB-labeled bladder afferent neurons and IB4-stained DRG neurons. D and E: data are expressed as relative values normalized with GAPDH mRNA as a control. Note that Kv4.1 and Kv4.3 mRNA levels are significantly higher in IB4-stained cells than those in FB-labeled bladder afferent neurons. * $P < 0.05$ for both Kv4.1 and Kv4.3.



Taken together, these results indicate that Kv4.1/4.3 and DPP10 are predominantly expressed in IB4-positive, non-peptidergic C-fiber afferent neurons and that Kv4.1 and possibly Kv4.3 subunits functionally participate in the formation of K_A channels in a subpopulation of somatic C-fiber neurons but not in visceral C-fiber neurons innervating the bladder (Matsuyoshi et al., 2012; Yunoki et al., 2014 [Appendix]).

Overall, based on our findings in electrophysiological, histological and molecular analyses, Kv1.4 is one of the key K_A subunits, the reduced expression of which is responsible at least in part for the reduction of K_A currents to induce hyperexcitability of C-fiber bladder afferent pathways after SCI whereas Kv4.1 and possibly Kv4.3 subunits contribute to the formation of K_A channels in somatic C-fiber neurons. In addition, bladder afferent hyperexcitability after SCI is associated with upregulation of TRP receptors, which are also important excitatory channels, in bladder afferent neurons as well as increased NGF levels in the bladder.

4. Development and optimization of liposome (LP) conjugated with NGF antisense for the local treatment in the bladder

[Accomplishment]

In this project, we first optimized and manufactured liposomes (LPs) conjugated with NGF antisense as follows. The 18mer phosphorothioate oligonucleotide (OND) with the sequence 5'-GCCCGAGACGCCTCCCGA-3' for the experiments were made, and cationic liposomes composed of DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N trimethylammonium methylsulfate) were made by thin film hydration method and hydrated with nuclease free water with the final lipid concentration of 7mM. The OND were dissolved in nuclease free water at the concentration of 2mM and were complexed with liposomes in the proportion of 6μl OND solution to 1 ml liposome lipid by incubation at room temperature for 30min (Kashyap et al., 2013 [Appendix]; Tyagi et al., 2014 [Appendix]).

Secondly, we performed in-vivo experiments to test the efficacy of LPs conjugated with NGF antisense OND using a rat mode of bladder overactivity. Rats were anesthetized with 2% isoflurane, and catheterized by a 24-gauge angiocatheter through the urethra into the bladder. After urine was drained from the bladder, 12μM of NGF antisense or scramble OND complexed with liposome or saline in a volume of 0.5ml was infused. The bladder outlet was tied with a running suture thread for 30 minutes. The efficacy of LP-antisense treatments was assessed 24h after infusion by saline and subsequent acetic acid (AA) cystometry under urethane anesthesia.

We have found that; (1) cationic liposomes were needed for bladder uptake of OND (Fig. 9), (2) at 24 hours after liposome-oligonucleotide treatment baseline bladder activity during saline infusion was indistinct in the sham and antisense treated groups (Fig. 10B), (3) acetic acid induced bladder overactivity was shown by a

decrease in the intercontraction interval to a mean of 33.2% of baseline in sham treated rats while the reduction was blunted to a mean of 75.8% of baseline in rats treated with liposomal antisense OND ($p<0.05$), (4) acetic acid induced increased NGF in the urothelium of sham treated rats, which was decreased by antisense treatment (Fig. 10A) and (5) increased NGF in bladder tissue was associated with sICAM-1, sE-selectin, CXCL-10 and 1, leptin, MCP-1 and vascular endothelial growth factor over expression, which was significantly decreased by NGF antisense treatment ($p<0.01$) (Kashyap et al., 2013 [Appendix]; Tyagi et al., 2014 [Appendix]).

Figure 9. Confocal images of harvested rat bladders at 8 (panel A) and at 24 hours (panel B) after instillation of cationic liposomal antisense with 5' tag of TYETM 563. The red fluorescence in panels A represents successful bladder distribution of OND at 8h and the fluorescence seems to be more concentrated due to bladder folds. The bright red fluorescence was more homogenous in the urothelium at 24h with diffusion to cells in deeper layers (panel B). Localization of fluorescence in urothelium demonstrates successful uptake and retention in target cells due to binding with target mRNA. Lumen side of the section is marked by white arrow. Magnification is 40x in all sections.

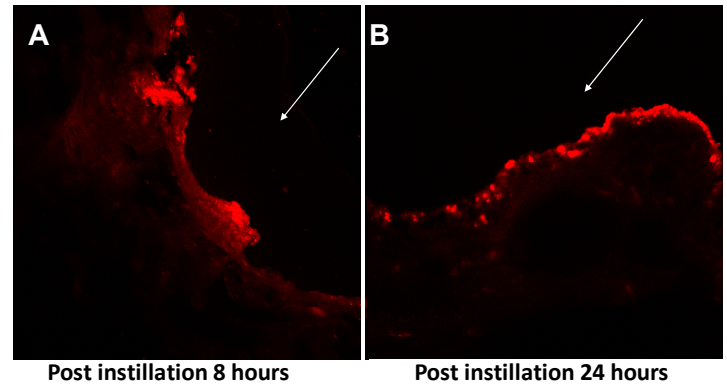
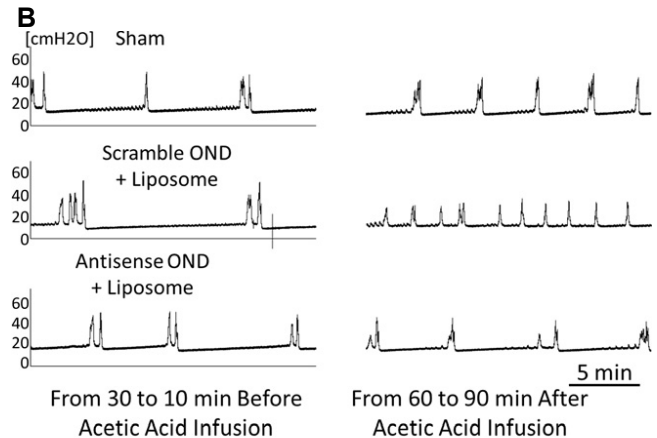
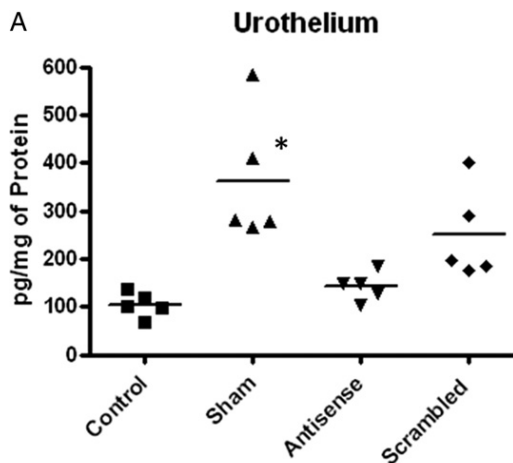


Figure 10. A: Antisense OND mediated suppression of acetic acid (AA) induced NGF protein expression in urothelium. AA exposure caused significant increase in NGF in sham treated group (Sham) vs. untreated controls.

Liposome (LP)+NGF antisense OND significantly blunted NGF increase vs. sham treated group. Data points represent NGF values of individual rats around mean (horizontal line) of each group.



B: Cystometric analysis of the effects of LP-NGF antisense treatment on bladder overactivity induced by intravesical application of AA (0.25%) in rats. Representative cystometrograms show the effects of intravesical application of AA in sham (saline treatment) (upper traces), LP-scramble OND-treated (middle traces) and LP-NGF antisense OND treated rats (lower traces). The cystometrograms before and after AA are shown in left and right traces, respectively. Note that the AA-induced reduction in intercontraction intervals (ICI) was seen in sham and scramble OND-treated rats, but not in the rat treated with LP-NGF antisense OND conjugates.

These results indicate that the cationic LPs-NGF antisense OND conjugate is suitable and effective to suppress the urothelial NGF expression and inhibit bladder overactivity induced by bladder afferent sensitization.

5. Physiological and tissue analyses: Comparison of blood pressure responses during bladder distention in SCI rats with or without NGF antisense treatment

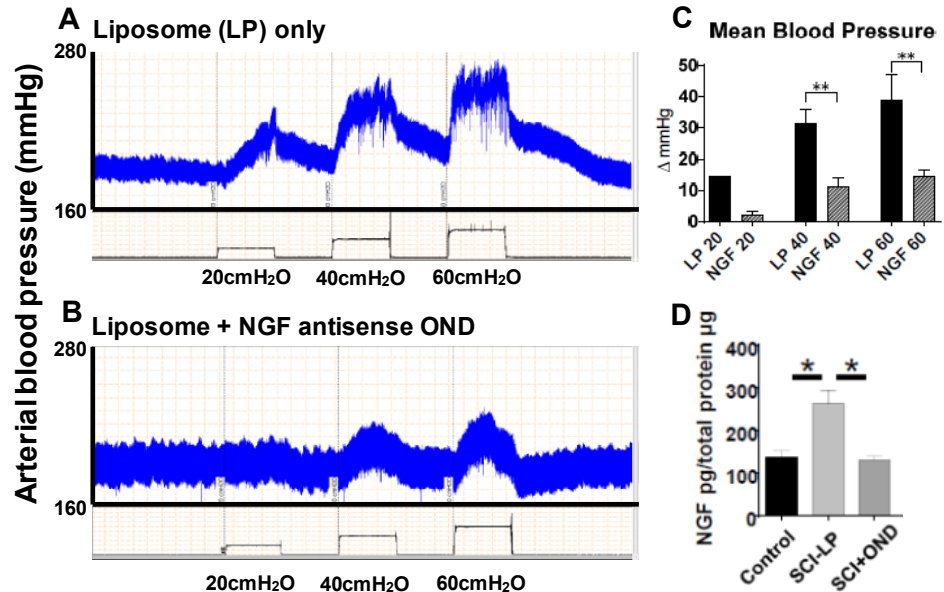
[Accomplishment]

Based on the results described in Figs. 3 & 10, which respectively described the NGF overexpression in the bladder after SCI and the successful down regulation of NGF in the bladder using liposomes (LP) conjugated with NGF antisense, we performed experiments to examine the effects of liposome-NGF antisense conjugates

on AD by measuring blood pressure responses during bladder distention using rats with Th4-5 level spinal cord transection (4 weeks). For bladder distention, intravesical pressure was increased in a stepwise manner to 20, 40 and 60 cmH₂O, for 2 min by connecting the urethra cannula through a three-way stopcock to a saline-filled reservoir, the height of which was adjusted to maintain a constant pressure in the bladder (Fig. 11A-B). LP-NGF antisense OND conjugate or empty LP was instilled into the bladder transurethrally for 30 min one week prior to experiments (i.e., 3 weeks after spinal cord transection).

We have found that: (1) the liposome-NGF antisense treatment suppressed the increases of mean blood pressures (2.3 ± 0.8 mmHg; 83% decrease at 20 cmH₂O, 11.3 ± 2.5 mmHg; 64% decrease at 40 cmH₂O, 14.7 ± 1.7 mmHg; 62% decrease at 60 cmH₂O) compared to the liposome only-treated group (LP) (Fig. 11A-C) and (2) NGF levels in the bladder mucosa is elevated in the SCI+ LP group; however it was reduced in SCI rats treated with LP-NGF antisense OND conjugate (SCI-OND) (Fig. 11D).

Figure 11. A-B: Arterial blood pressure responses during passive bladder distention to 20, 40 and 60 cmH₂O for a duration of 2 min each in spinal cord injured (SCI) rats without (A) or with liposomes-NGF antisense OND treatment (B). C: Comparison of mean blood pressure responses (Δ mmHg) during bladder distention to 20, 40 and 60 cmH₂O in SCI rats treated with liposomes (LP20, LP40 and LP60, respectively) or liposomes conjugated with NGF antisense OND (OND20, OND40 and OND60, respectively). Note that the significant (* $P < 0.05$) reduction in mean blood pressure elevation during bladder distention to 40 and 60 cmH₂O. N=5 per each group. * $P < 0.05$. D: ELISA measurements of NGF proteins in the mucosa of spinal intact (control), SCI with intravesical liposome (SCI+LP) and SCI with liposomes-NGF antisense conjugate (SCI+OND). N=5 per each group. * $P < 0.05$. Note that the NGF upregulation in the mucosa of SCI rats (SCI+ LP) was reduced by liposomes-NGF antisense OND treatment (SCI+OND).



Note that the significant (* $P < 0.05$) reduction in mean blood pressure elevation during bladder distention to 40 and 60 cmH₂O. N=5 per each group. * $P < 0.05$. D: ELISA measurements of NGF proteins in the mucosa of spinal intact (control), SCI with intravesical liposome (SCI+LP) and SCI with liposomes-NGF antisense conjugate (SCI+OND). N=5 per each group. * $P < 0.05$. Note that the NGF upregulation in the mucosa of SCI rats (SCI+ LP) was reduced by liposomes-NGF antisense OND treatment (SCI+OND).

These results indicate that AD (i.e, enhanced arterial blood pressure elevation during bladder distention) in SCI rats was significantly suppressed by the intravesical treatment with liposome-NGF antisense conjugates, which reduces the expression of mucosal NGF. These findings will be presented at the 2015 annual meeting of American Urological Association (AUA) (Kadekawa et al., 2015 AUA abstract [Appendix]).

IV. Key research accomplishments

- Confirmation of autonomic dysreflexia (AD) during bladder distention which is associated with hyper excitability of TRPV1-expressing C-fiber afferent pathways and NGF upregulation in the bladder urothelium, in a rat model of SCI
- Confirmation of hyperexcitability of bladder afferent neurons in SCI rats, which is induced by the reduction of A-type K⁺ channel activity that is associated with the decrease of Kv1.4 K⁺ channel subunit expression
- Identification and optimization of cationic liposomes-NGF antisense conjugates, which effectively suppress the expression of NGF in the bladder mucosa when administered intravesically
- Confirmation of effectiveness of the intravesical administration with liposomes-NGF antisense conjugates for the treatment of AD during bladder distention and NGF upregulation in the bladder using SCI rats

V. Conclusions

In this 3-year project, we successfully completed the works listed in the SOW. Based on the results obtained in the funding period, we observed two major implications for the SCI research. First, our results indicate that SCI induces autonomic dysreflexia (AD) during bladder distention as evidenced by enhanced arterial pressure responses during low-pressure bladder distention, which is dependent on hyperexcitability of TRPV1-expressing C-fiber bladder afferent pathways, in a rat model of SCI. Secondly, our formulation of cationic liposomes conjugated with NGF antisense OND successfully suppresses SCI-induced AD in association with the reduction in NGF expression in the bladder. We believe that these findings would provide the foundation for the future development of new therapies using local, liposome-based NGF antisense delivery targeting NGF upregulation in the bladder.

VI. Publications, Abstracts and Presentations

Refereed articles:

1. Matsuyoshi, H., Takimoto, K., Yunoki, T., Erickson, V.L., Tyagi, P., Hirao, Y., Wanaka, A., Yoshimura, N.: Distinct cellular distributions of Kv4 pore-forming and auxiliary subunits in rat dorsal root ganglion neurons. *Life Sciences*, 91: 258–263, 2012.
2. Ochodnický, P., Cruz, C.D., Yoshimura, N., Cruz, F.: Neurotrophins as regulators of urinary bladder function. *Nature Reviews Urology*, 9, 628–637, 2012.
3. Kashyap, M., Kawamorita, N., Tyagi, V., Sugino, S., Chancellor, M.B., Yoshimura, N., Tyagi, P.: Downregulation of NGF expression in the bladder by antisense oligonucleotides as new treatment for overactive bladder. *Journal of Urology*, 190: 757-764, 2013.
4. Takahashi, R., Yoshizawa, T., Yunoki, T., Tyagi, P., Naito, S., de Groat, W.C., Yoshimura, N.: Hyperexcitability of bladder afferent neurons associated with reduction of Kv 1.4 α -subunit in rats with spinal cord injury. *Journal of Urology*, 190: 2296-304, 2013.
5. Tyagi, P., Kashyap, M.P., Kawamorita, N., Yoshizawa, T., Chancellor, M., Yoshimura, N.: Intravesical liposome and antisense treatment for detrusor overactivity and interstitial cystitis/painful bladder syndrome. *ISRN Pharmacology*, 2014, Article ID 601653, 12 pages (open access at <http://dx.doi.org/10.1155/2014/601653>)
6. Yoshimura, N., Ogawa, T., Miyazato, M., Kitta, T., Furuta, A., Chancellor, M.B., Tyagi, P.: Neural mechanisms underlying lower urinary tract dysfunction, *Korean Journal of Urology*, 55: 81-90, 2014.
7. Yunoki, T., Takimoto, K., Kita, K., Funahashi, Y., Takahashi, R., Matsuyoshi, H., Naito, S., Yoshimura, N.: Differential contribution of Kv4-containing channels to A-type, voltage gated potassium currents in somatic and visceral dorsal root ganglion neurons. *Journal of Neurophysiology*, 112: 2492-2504, 2014.
8. Yoshizawa, T., Kadekawa, K., Tyagi, P., Yoshikawa, S., Takahashi, R., Takahashi, S., Yoshimura, N.: Mechanisms inducing autonomic dysreflexia during urinary bladder distention in rats with spinal cord injury. *Spinal Cord*, in press, 2015 (published online at <http://www.nature.com/doi/10.1038/sc.2014.233>).

Published abstracts:

1. Takahashi, R., Yunoki, T., Naito, S., Yoshimura, N.: Increased excitability of bladder afferent neurons in rats with spinal cord injury: a role of A-type voltage-gated potassium channels. 110th Annual Meeting AUA, Abstract No. 38, Atlanta, GA, May 19-23, 2012.
2. Yoshizawa, T., Yoshikawa, S., Takahashi, R., Takahashi, S., Yoshimura, N.: Mechanisms inducing autonomic dysreflexia during bladder distention in rats with spinal cord injury. 111th Annual Meeting AUA, Abstract No. 34, San Diego, May 4-8, 2013.
3. Takahashi, R., Yunoki, T., Naito, S., Yoshimura, N.: Increased excitability of bladder afferent neurons associated with reduced expression of Kv1.4 α -subunit in rats with spinal cord injury. 111th Annual Meeting AUA, Abstract No. 1707, San Diego, May 4-8, 2013. [NOTE] This abstract was selected as the Best Abstract and presented at the plenary lecture session during the 2013 AUA meeting.
4. Kawamorita, N., Kashyap, M., Tyagi, V., Sugino, Y., Chancellor, M., Yoshimura, N.: Downregulation of NGF expression in the bladder by antisense oligonucleotides as new treatment of overactive bladder. 111th Annual Meeting AUA, Abstract No. 1947, San Diego, May 4-8, 2013.
5. Takahashi R, Yunoki T, Naito S, Yoshimura N: Hyperexcitability of bladder afferent neurons associated with reduced expression of potassium channel Kv1.4 α -subunit in rats with spinal cord injury. 43rd Annual meeting of the International Continence Society, Abstract No. 31, Barcelona, Spain, August 26-30, 2013.

6. Tsuyoshi Yoshizawa, Pradeep Tyagi, Michael B. Chancellor, Satoru Takahashi, Naoki Yoshimura: Therapeutic effects of intravesical application of liposome conjugated with antisense oligonucleotide targeting nerve growth factor on neurogenic detrusor overactivity in rats with spinal cord injury. 112th Annual Meeting AUA, MP1-17, Orlando, FL, May 16-22, 2014.
7. Kadekawa, K, Tsuyoshi Yoshizawa, Pradeep Tyagi, Kimio Sugaya, Naoki Yoshimura: Effects of liposome-based local suppression of nerve growth factor in the bladder on autonomic dysreflexia during urinary bladder distention in rats with spinal cord injury. 113th Annual Meeting AUA, Abst #15-3882, New Orleans, LA, May 15-19, 2015.

VII. Inventions, Patents and Licenses

Not applicable

VIII. Reportable Outcomes & Other Achievements

Not applicable

IX. References

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ISRN Pharmacology, 2014, Article ID 601653, 12 pages (open access at <http://dx.doi.org/10.1155/2014/601653>).

17. Kadekawa, K, Tsuyoshi Yoshizawa, Pradeep Yyagi, Kimio Sugaya, Naoki Yoshimura: Effects of liposome-based local suppression of nerve growth factor in the bladder on autonomic dysreflexia during urinary bladder distention in rats with spinal cord injury. 113th Annual Meeting AUA, Abstract #15-3882, New Orleans, LA, May 15-19, 2015.

X. Appendices

PDF files of the following publications are appended.

1. Matsuyoshi, H., Takimoto, K., Yunoki, T., Erickson, V.L., Tyagi, P., Hirao, Y., Wanaka, A., Yoshimura, N.: Distinct cellular distributions of Kv4 pore-forming and auxiliary subunits in rat dorsal root ganglion neurons. *Life Sciences*, 91: 258–263, 2012.
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6. Yoshimura, N., Ogawa, T., Miyazato, M., Kitta, T., Furuta, A., Chancellor, M.B., Tyagi, P.: Neural mechanisms underlying lower urinary tract dysfunction, *Korean Journal of Urology*, 55: 81-90, 2014.
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Distinct cellular distributions of Kv4 pore-forming and auxiliary subunits in rat dorsal root ganglion neurons

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ARTICLE INFO

Article history:

Received 18 March 2012

Accepted 7 July 2012

Keywords:

Dorsal root ganglion

Voltage-gated potassium channel

Kv4.1

KChIP

DPP

ABSTRACT

Aims: Dorsal root ganglia contain heterogeneous populations of primary afferent neurons that transmit various sensory stimuli. This functional diversity may be correlated with differential expression of voltage-gated K⁺ (Kv) channels. Here, we examine cellular distributions of Kv4 pore-forming and ancillary subunits that are responsible for fast-inactivating A-type K⁺ current.

Main methods: Expression pattern of Kv α -subunit, β -subunit and auxiliary subunit was investigated using immunohistochemistry, *in situ* hybridization and RT-PCR technique.

Key findings: The two pore-forming subunits Kv4.1 and Kv4.3 show distinct cellular distributions: Kv4.3 is predominantly in small-sized C-fiber neurons, whereas Kv4.1 is seen in DRG neurons in various sizes. Furthermore, the two classes of Kv4 channel auxiliary subunits are also distributed in different-sized cells. KChIP3 is the only significantly expressed Ca²⁺-binding cytosolic ancillary subunit in DRGs and present in medium to large-sized neurons. The membrane-spanning auxiliary subunit DPP6 is seen in a large number of DRG neurons in various sizes, whereas DPP10 is restricted in small-sized neurons.

Significance: Distinct combinations of Kv4 pore-forming and auxiliary subunits may constitute A-type channels in DRG neurons with different physiological roles. Kv4.1 subunit, in combination with KChIP3 and/or DPP6, form A-type K⁺ channels in medium to large-sized A-fiber DRG neurons. In contrast, Kv4.3 and DPP10 may contribute to A-type K⁺ current in non-peptidergic, C-fiber somatic afferent neurons.

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Introduction

Voltage-gated K⁺ (Kv) currents in sensory neurons are divided into two major categories; sustained delayed rectifier (K_{DR}) and transient A-type K⁺ (K_A) currents (Kostyuk et al., 1981; Hall et al., 1994; Gold et al., 1996; Yoshimura et al., 1996). K_A current is activated at subthreshold of action potential and rapidly inactivates. Thus, this current is important to determine the initiation and interval of action potentials. K_A current in sensory neurons may be carried by a number of Kv pore-forming subunits including Kv1.4 and any of Kv4 subunits (Kv4.1, Kv4.2, and Kv4.3). It has been shown that Kv1.4 is localized in small-sized C-fiber DRG neurons (Rasband et al., 2001). Furthermore, K_A current in small-sized C-fiber neurons exhibits slower inactivation and sensitivity to α -dendrotoxin, a blocker of Kv1-family channels. In addition, reduced K_A current and Kv1.4 proteins are associated with

hyperexcitability of DRG neurons in animal models of bladder pain (Hayashi et al., 2009). Therefore, Kv1.4 significantly contributes to the formation of A-type channels in a subset of C-fiber neurons. In contrast to Kv1.4 subunits, relatively less is known about cellular distributions of Kv4 channel subunits in DRGs. Previous studies showed that Kv4.3 protein is predominantly expressed in non-peptidergic, small-sized DRG neurons (Chien et al., 2007). PCR analysis also detected Kv4.1 mRNA in DRG tissue and a large number of isolated, small to medium-sized DRG neurons (Phuket and Covarrubias, 2009). These findings support differential expression of the two Kv4 pore-forming subunits in distinct DRG neurons. Yet, the cell-size distribution of Kv4.1 in the entire DRG neuronal population remains unclear.

Kv4 pore-forming proteins are known to form complexes with two distinct types of auxiliary subunits that markedly alter channel expression and gating. The first type of Kv4 auxiliary subunits are small cytosolic Ca²⁺-binding proteins, namely Kv channel interacting proteins (KChIPs) (An et al., 2000), whereas the other type contains one transmembrane domain with a large extracellular portion similar to dipeptidyl peptidase (DPP6/10) (Jerng et al., 2004; Nadal et al., 2003; Ren et al., 2005). Diverse KChIPs are generated by the presence

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of four genes (An et al., 2000; Morohashi et al., 2002) and alternative splicing of transcripts (Rosati et al., 2001; Takimoto et al., 2002; Holmqvist et al., 2002; Patel et al., 2002; Boland et al., 2003). However, less is known about the distribution of KChIPs and DPPs in DRG neurons.

We wished to determine cellular distributions and subunit compositions of Kv4 channel complexes in distinct DRG neurons. We utilized PCR analysis, in-situ hybridization and immunohistochemistry to examine the expression and cellular distributions of Kv4 pore-forming and auxiliary subunits in rat DRG neurons.

Materials and methods

Experiments were performed using female Sprague–Dawley rats (220–250 g). Care and handling of animals were in accordance with institutional guidelines and were approved by the Animal Care and Use Committees of the Nara Medical University and University of Pittsburgh Institutional Animal Care and Use Committees.

PCR analysis

Total RNAs were prepared from L6–S1 DRGs and total brain using a column-based isolation method (Qiagen, Valencia CA). Synthesis of cDNA was performed as described previously (Takimoto et al., 2002). These primers were designed to detect splicing variants in different sizes (Table 1). PCR was done under the following conditions: denature at 94 °C for 5 seconds, annealing at 64 °C for 5 seconds and extension at 72 °C for 60 seconds for 28 cycles (22 cycles for GAPDH), and final extension at 72 °C for 4 minutes. PCR products were separated on a 5% polyacrylamide gel and stained with ethidium bromide for visualization. Control PCRs using cDNA made without reverse transcriptase generated no visible products.

Immunohistochemistry

Rats were perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, and L4 and L5 DRGs were then removed. Tissues were post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight, and then cryoprotected in 10, 20, and 30% series of sucrose in 0.01 M phosphate buffered saline. Tissue was cut in 5 or 10 µm thick sections for subsequent histological examination. Immunohistochemical analyses were performed on tissue sections obtained from different DRGs.

The general expression pattern of Kv4 subunits were examined in L4 or L5 DRG sections. After quenching of endogenous peroxidase activity by using 3% hydrodioxyde, the tissue sections were incubated with 5% of bovine serum albumin in 0.01 M phosphate-buffered saline at room temperature for 30 minutes. The sections were then probed with antibody against Kv pore-forming subunits anti-Kv 4.3 antibody at 1: 200; NeuroMab Facility, Davis, California) in 5% of bovine serum albumin and 0.3% Triton X-100 in 0.01 M phosphate-buffered saline overnight at 4 °C. Kv immunoreactive proteins were detected with 5 mg L^{−1} (1:200) biotin-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) at room temperature for 1 hour and then reacted with avidin–biotin peroxidase complex

(VECTSTAIN Elite ABC Kit, Vector Laboratories, Inc., Burlingame, California) at room temperature for 30 minutes. The probed sections were washed with 0.02 M Tris–HCl pH 7.4 and incubated with diaminobenzidine (DAB Substrate kit, Vector Laboratories, Inc.). For immunofluorescence staining, anti-Kv4.3 (1:200) or anti-KChIP3 (1: 200) antibody (NeuroMab Facility) in 5% of bovine serum albumin and 0.3% Triton X-100 in 0.01 M phosphate-buffered saline were applied overnight at 4 °C. The sections were incubated with 5 mg L^{−1} (1:200) biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 2 hours and then reacted with 2.7 mg L^{−1} Alexa Fluor 488-streptavidin conjugate (1:750) (Molecular probes Inc., Eugene, Oregon) at room temperature for 2 hours.

In situ hybridization

A portion of rat Kv4.1, Kv4.3, DPP6 and DPP10 cDNAs was obtained by RT-PCR and cloned into a topoisomerase-based vector (pCR2.1-TOPO or pCRII-TOPO, Invitrogen) for RNA probe synthesis. Primers used for the cloning were as follows: Kv4.1 5′-cacagacgagtaactttcag-3′ and 5′-tcacagggagagatcttgac-3′ (GenBank ID: 116695); Kv4.3 5′-tggttatctctctgtgga-3′ and 5′-ttacaagacagacaccttgac-3′ (GenBank ID: 65195); DPP6, 5′-agcaatgacaacatccagtc-3′ and 5′-agtaccatccaccacgagc-3′ (GenBank ID: 29272); DPP10 5′-gagcaaattacgggtgcgcgact-3′ and 5′-cttcattctattattagtagaagagc-3′ (GenBank accession # AY557199). Digoxigenin-labeled RNA probes were synthesized using linearized plasmids with T7 and SP6 RNA polymerases.

In situ hybridization was performed according to the procedure described previously (Tatsumi et al., 2005) and the signal was detected by dig-NBT/BCIP system. Briefly, after rehydration with 0.1 M phosphate buffer, the sections of L4 DRG were treated with 0.2 M HCl. The sections were then treated with 10 µg mL^{−1} proteinase K in 50 mM Tris–HCl and 5 mM EDTA and then fixed with 4% formaldehyde in 0.1 M phosphate buffer. The sections were acetylated by 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated by ethanol series (70, 95 and 100%), defatted in chloroform, rinsed with ethanol, and dried. Denatured labeled RNA probes were applied and hybridized at 50 °C. Remaining probes were eliminated with RNase A, followed by washing with 50% formamide in sodium chloride sodium citrate. Digoxigenin-labeled probes were processed by anti-digoxigenin antibody-conjugated alkaline phosphatase, and visualized using nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolylphosphate p-toluidine salt.

Histological analysis

Sections stained by *in situ* hybridization technique and immunohistochemistry with DAB were viewed under an Olympus BX51 Microscope (OLYMPUS Corp., Tokyo, Japan) in bright field. Fluorescent images were captured on an Olympus FluoView 1000 confocal microscope (OLYMPUS Corp., Tokyo, Japan). Randomly selected two sections from each DRG at more than 50 µm intervals were used for counting of positively stained cells to avoid double counting of cells. Cross-sectional areas of all neuronal profiles, in which nuclei were identified, were measured by using Scion Image (Scion Corp., Frederick, Maryland). Neuronal profiles were then divided into small-, medium- and large-sized neuronal populations based on the

Table 1
Primers used in the RT-PCR study.

Gene	GenBank accession no.	Sequence 5′–3′	Position	Product sizes
KChIP1	AY082657	atgggggccgtcatgggac accacaccactggggcactcg	1–20 239–219	KChIP1a 239 KChIP1b 206
KChIP2	XM_342059	gctcctatgaccagcttacgg cctcgttgacaatcccactgg	275–295 598–578	KChIP2a 324 KChIP2b 270 KChIP2c 174
KChIP3	NM_032462	aggctcagacagcagtgaca gggggaagaactgggaataa	197–216 393–374	197
KChIP4	AF345444	agatgaacgtgagaagggtgga gtgcataatgtggtggagcttc	376–397 753–733	KChIP4a 378 KChIP4b 276
GAPDH	NM_017008	gccatcactgccactcag gtgagcttcccgttcagc	608–625 739–756	149

area size (area < 600 μm^2 , 600 μm^2 < area < 1200 μm^2 and area > 1200 μm^2 , respectively). The staining intensity was rated on a four point scale, from completely negative (grade 0) to intense staining (grade 3), and the neurons that exhibited grades 2 or 3 were regarded as positively stained cells. The number of positively stained cells as well as the total DRG neurons was counted, and the percent ratio of positively stained cells against the total DRG cells was calculated.

Results

Cellular distributions of Kv4 pore-forming subunits in DRGs

Previous PCR analysis suggested significant expression of Kv4.1 and Kv4.3 mRNAs in DRG neurons (Phuket and Covarrubias, 2009). We also observed abundant Kv4.1 and long-isoform of Kv4.3 transcripts, but not Kv4.2 mRNA, in L6-S1 DRGs (unpublished observation). Thus, we first examined the cellular distributions of the two pore-forming subunits, Kv4.1 and Kv4.3. Since commercial anti-Kv4.1 antibodies appeared less suitable for immunohistochemistry, we used *in situ* hybridization to test for differential distributions of Kv4.1 and Kv4.3 in DRG neurons (Fig. 1). Antisense Kv4.3 probe preferentially stained small-sized neuronal cell bodies (Fig. 1B). Kv4.3 transcript-positive cells represented approximately 32.8% of DRG neurons (total 4 sections), equivalent to that of the corresponding channel proteins (33.8%). Most Kv4.3 mRNA-positive cells were less than 900 μm^2 in cell area size. Consistent with mRNA distribution, anti-Kv4.3 antibody stained small-sized neuronal cell bodies (data not shown). Kv4.3 protein-positive cells represented approximately 33.8% of DRG neurons.

In contrast, antisense Kv4.1 probe detected DRG neurons in various sizes (Fig. 1A). Kv4.1 mRNA-positive cells represented 59.5% of DRG neurons (total 4 sections) and were distributed in all sizes. Thus, the two Kv4 pore-forming subunits are differentially distributed in DRG neurons.

Cellular distributions of Kv4 channel auxiliary subunits

Kv4 pore-forming subunits may be associated with the two distinct types of auxiliary subunits that significantly alter channel expression and gating. We first tested expression of KChIP1–4 mRNAs by RT-PCR analysis (Fig. 2A). All four gene transcripts were abundant in the brain, whereas only KChIP3 mRNA was significant in L6-S1 DRGs. Immunostaining with anti-KChIP3 antibody showed that KChIP3 proteins were prominent in a subset of medium to large-sized neuronal cell bodies (Fig. 2B). Staining with anti-Kv4.3 antibody of adjunct sections suggested that Kv4.3 and KChIP3 are not colocalized in the same cells (Fig. 2B).

We next examined cellular distribution of the other type of Kv4 channel auxiliary subunits, DPP6 and DPP10. Our previous study demonstrated abundant mRNA expression of these two auxiliary subunits in DRGs (Takimoto et al., 2006). Since several available antibodies against these proteins failed to provide reliable staining, we performed *in situ* hybridization analyses (Fig. 3). DPP6 mRNA was widely distributed in DRG neuronal cell bodies in all sizes (Fig. 3A), whereas DPP10 transcript was expressed mostly in small to medium-sized neurons ranging 400–1600 μm^2 of cell area size (Fig. 3B). The proportions of DPP6 and DPP10 mRNA-positive cells among DRG neurons per section were 72.8% and 27.2%, respectively (the mean of $n = 2$ sections). Thus,

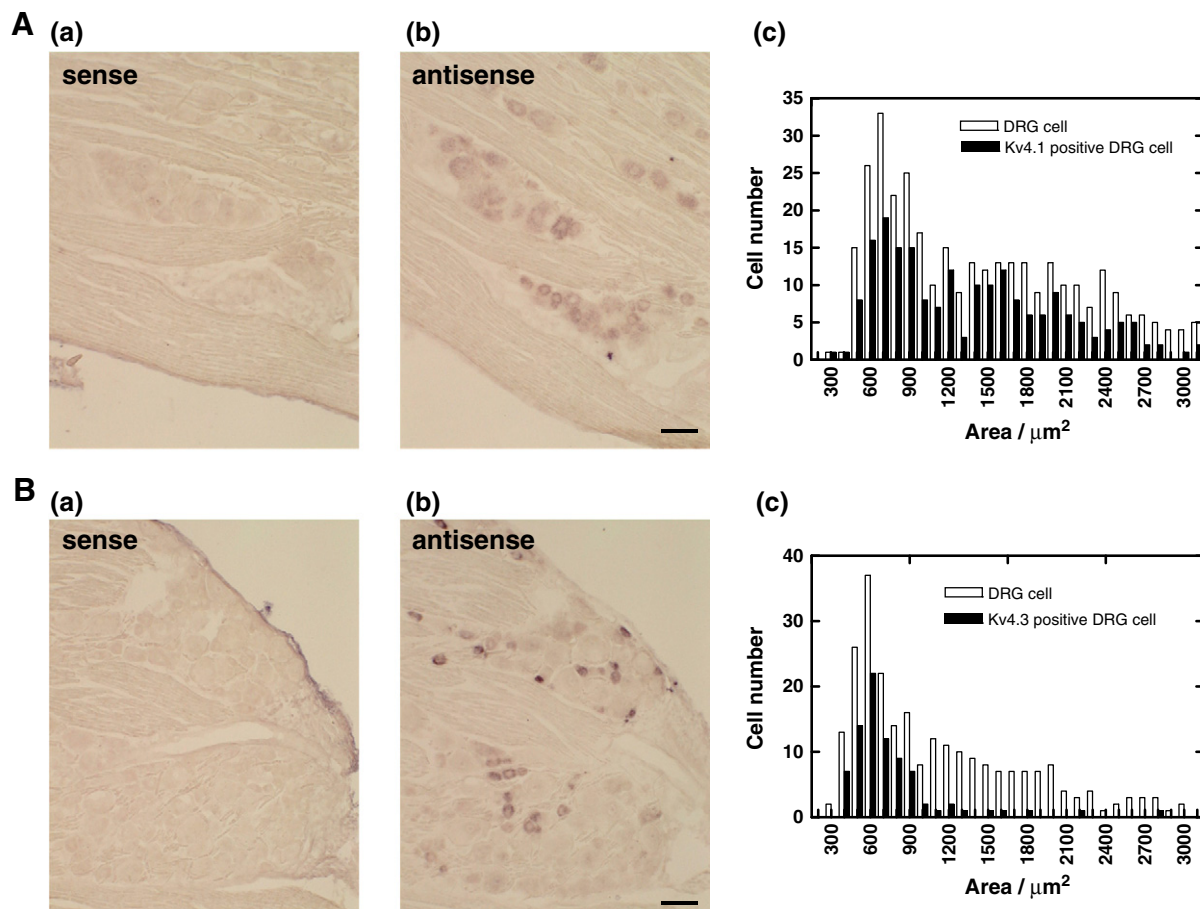


Fig. 1. Cellular distributions of Kv4.1 and Kv4.3 mRNA in rat DRGs. A. Rat DRG sections stained with Kv4.1-sense RNA probe (a) or Kv4.1-antisense RNA probe (b), are shown. Cell-size distributions are shown with filled and open columns indicating Kv4.1 mRNA-positive and total DRG neuronal cell bodies, respectively (c). B. *In situ* hybridization picture (a, b) and cell-size distributions (c) for Kv4.3 are shown. Scale bar, 50 μm . Bin width: 100 μm^2 .

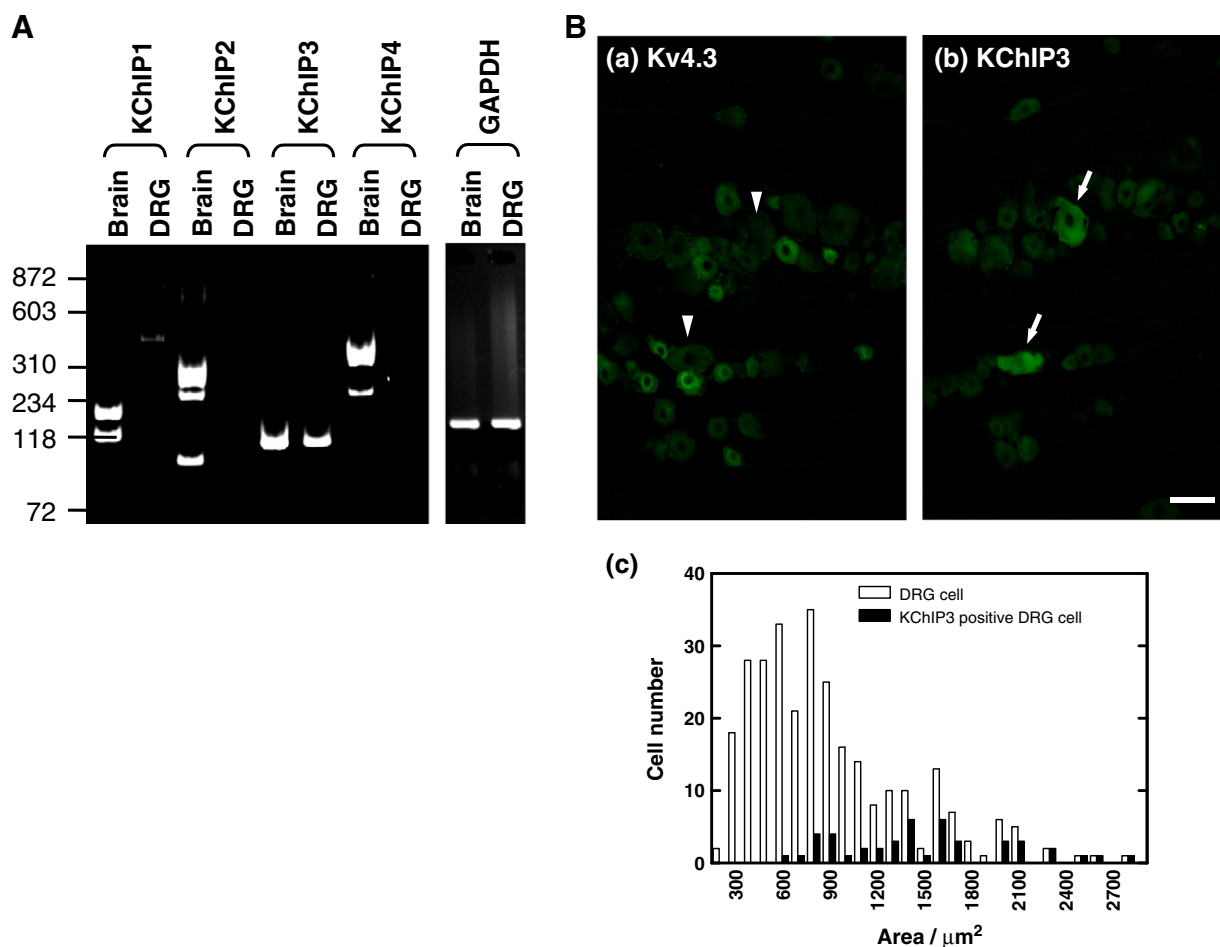


Fig. 2. Expression of KChIPs in rat DRGs. A. RT-PCR data with primers for KChIP1–4 in the brain and L6–S1 DRG are shown. Different sizes of bands represent splicing variants: KChIP1a and KChIP1b (Boland, et al., 2003); KChIP2a, KChIP2b and KChIP2c (Takimoto, et al., 2002); KChIP4 (Takimoto, et al., 2002). Note that DRGs contain only KChIP3. B. Adjacent L5 DRG sections at a 10 μm interval stained with anti-Kv4.3 antibody (a) or anti-KChIP3 antibody (b) are shown. Scale bar, 50 μm . Arrow heads (a) and arrows (b) indicate the same cells that are negative for Kv4.3 and positive for KChIP3, respectively. Cell-size distributions are shown with filled and open columns indicating KChIP3 protein-positive and total DRG neuronal cell bodies, respectively (c). Bin width: 100 μm^2 .

the two auxiliary subunits differently contribute to the production of Kv4 channel complexes in DRG neurons with different sizes.

Discussion

DRGs contain cell bodies for heterogeneous populations of primary afferent neurons. These neurons may be categorized by cell body sizes and innervating tissues. A α / β -fiber neurons with large-sized cell bodies generally carry mechanical information, whereas small-sized cell bodies correspond to C and A δ -fiber neurons that are responsible for pain sensation. The latter small-sized neurons are also implicated in the development of chronic pain. In this study, we determined the cellular distribution of Kv4 pore-forming subunits and their associating auxiliary subunits in DRG neurons. We found that Kv4.1 mRNA is widely expressed in DRG neurons with various cell body sizes. Similarly, mRNA for the auxiliary subunit DPP6 is ubiquitous in neurons with various cell body sizes, whereas DPP10 transcript is more concentrated in small-sized neurons. In addition, KChIP3 protein seems more abundant in medium to large-sized neurons. These new findings suggest that Kv4.1 channel complexes containing DPP6 and KChIP3 contribute to K_A currents in medium to large-sized A-fiber neurons, whereas Kv4.3 channel complexes containing DPP10 may be responsible for K_A currents in small-sized C-fiber somatic neurons.

DRG neurons are known to contain two types of K_A currents with distinct kinetics (fast vs. slow-inactivating) and toxin sensitivities.

Fast-inactivating K_A current is sensitive to heteropodotoxins and phrixotoxins that influence the gating of Kv4 channels, but not Kv1 or Kv2 channels (Sanguinetti et al., 1997; Diochot et al., 1999; Escoubas et al., 2002). Thus, it is assumed that Kv4 channel complexes are responsible for the fast K_A current. While fast-inactivating K_A current is prominent in medium to large-cell sized A-fiber neurons (Gold et al., 1996; Yoshimura and de Groat, 1996), it may also be present in a subset of small-sized C-fiber neurons. Our immunostaining and *in situ* hybridization clearly showed the presence of Kv4.3 pore-forming subunit and DPP10 auxiliary subunit in small-sized DRG neurons. We have recently observed that phrixotoxin-sensitive K_A current is prominent in somatic sensory neurons, but not bladder afferent cells (unpublished observation). Thus, Kv4.3 pore-forming and DPP10 ancillary subunits may contribute to the formation of fast K_A channels in somatic C-fiber neurons. In addition, Kv4.1 mRNA has been detected in dissociated, small to medium-cell sized DRG neurons (Phuket and Covarrubias, 2009). Our *in situ* hybridization study further demonstrated that Kv4.1 mRNA is expressed not only in small to medium-sized DRG neurons, but in DRG neurons with various cell sizes. Therefore, it is likely that this Kv4.1 pore-forming subunit may also participates in forming fast K_A channels in small-sized DRG neurons.

Kv4 channels may simultaneously contain the two distinct auxiliary subunits, KChIPs and DPP6/10. In the brain, immunoprecipitation studies indicated ternary channel complexes containing the two types of auxiliary subunits (Jerng et al., 2005; Amarillo et al., 2008).

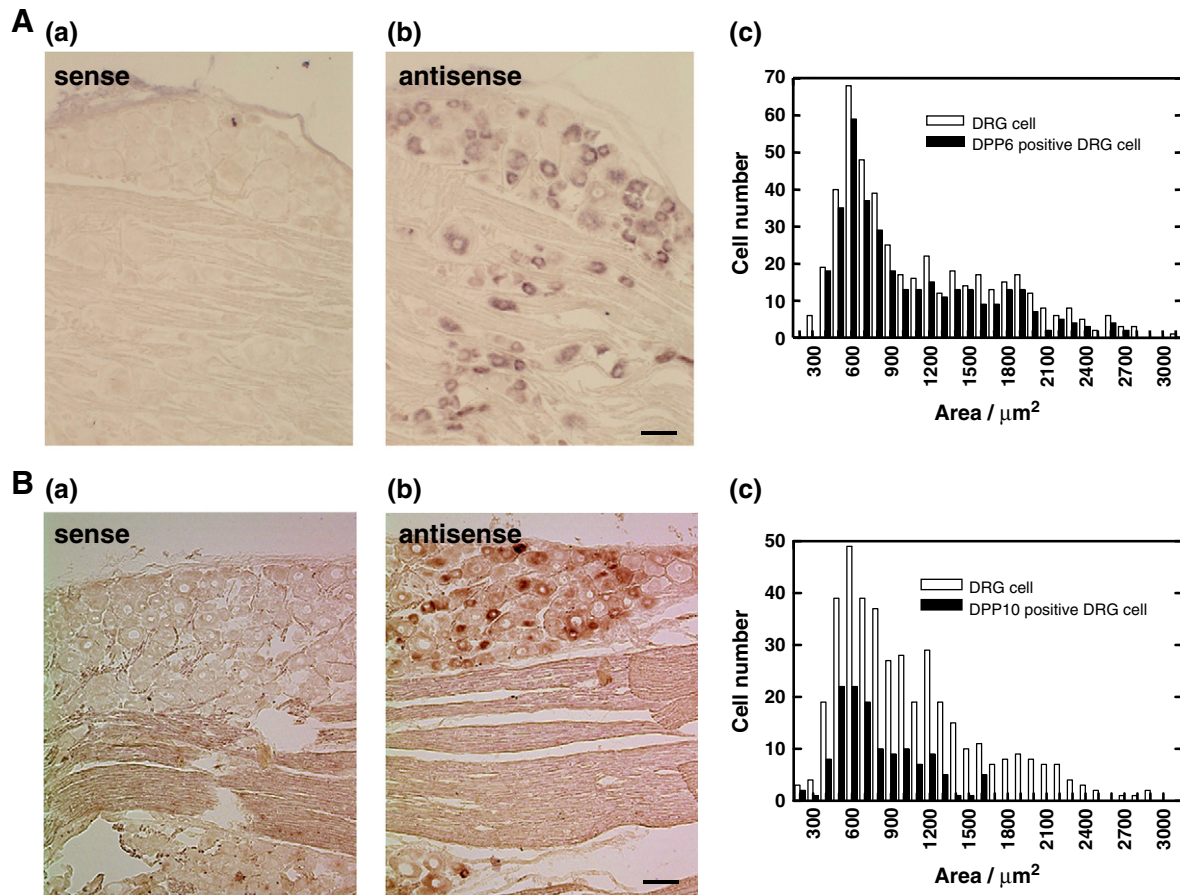


Fig. 3. Cellular distributions of DPP6 or DPP10 mRNA in rat DRGs. A. Rat DRG sections stained with DPP6-sense (a) or antisense RNA probe (b), are shown. Cell-size distributions are shown with filled and open columns indicating DPP6 mRNA-positive and total DRG neuronal cell bodies, respectively (c). B. In situ hybridization picture (a, b) and cell-size distributions (c) for DPP10 are shown. Scale bar, 50 μm . Bin width: 100 μm^2 .

However, our RT-PCR analysis and *in situ* hybridization suggest that some Kv4 channel complexes may not contain KChIPs. RT-PCR analysis detected a high level of KChIP3 without apparent expression of other three KChIPs in DRGs, whereas all four auxiliary subunit mRNAs were abundant in the brain. Moreover, immunohistochemistry indicated that KChIP3 protein is present in medium to large-sized neurons, but not in small-sized cells. A simple explanation for these observations is that Kv4 channel complexes in small-sized DRG neurons consist of Kv4.1/Kv4.3 and DPP6/10, but not any KChIPs, whereas Kv4 channel complexes in medium to large-sized DRG neurons consist of Kv4.1, DPP6 and KChIP3. Heterologous expression studies suggest that KChIPs and DPP6/10 somewhat play redundant roles in raising expression of the associated pore-forming subunits and inducing faster recovery from inactivation. Therefore, it is possible that a subset of small-sized C-fiber neurons contain Kv4 channel complexes without any KChIPs.

Sensory neuron-type selective expression of different channel subunits may provide the basis for the development of new therapeutic strategy or drugs for chronic pain and other disorders. We have previously showed that reduced expression of Kv1.4 subunits is associated with hyperexcitability of DRG neurons in an animal model of bladder inflammation (Hayashi et al., 2009). In contrast to visceral pain, less attention is focused on molecular correlates for Kv channel plasticity in primary afferents that transmit somatic pain, such as arthritis and chronic back pain. Further studies on alterations in the expression of Kv4 pore-forming and auxiliary subunits and functional properties of Kv4-mediated K_A currents could identify the molecular correlates that contribute to somatic pain conditions.

Conclusion

Kv4 channel complexes in small-sized, somatic DRG neurons consist of Kv4.1/Kv4.3 and DPP6/10, but not any KChIPs, whereas Kv4 channel complexes in medium to large-sized DRG neurons consist of Kv4.1, DPP6 and KChIP3.

Conflict of interest statement

None.

Acknowledgements

This work was supported by grants from the National Institute of Health (DK057267 and DK088836), the Department of Defense (SC100134 and PR110326) and the Ministry of Education, Science, Sports and Culture of Japan (22591798).

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Neurotrophins as regulators of urinary bladder function

Peter Ochodnický, Célia D. Cruz, Naoki Yoshimura and Francisco Cruz

Abstract | Increased voiding frequency and urgency are among the most prevalent storage lower urinary tract symptoms (LUTS), often diagnosed as part of overactive bladder syndrome (OAB). It has been suggested that these symptoms are caused by excessive sensory activation of the neural micturition circuit. It seems likely that sensory pathway remodelling is also responsible for pain perception upon bladder filling in patients with bladder pain syndrome (BPS). Neurotrophins—including nerve growth factor (NGF), brain-derived nerve factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4)—represent master modulators of neural plasticity, both in peripheral and central nervous systems. Accumulating evidence points towards a role for neurotrophins in the control of neural sensory function during micturition and indicates their involvement in the emergence of OAB-related and BPS-related LUTS. Neurotrophins could potentially be used as urinary biomarkers to improve diagnostic accuracy for OAB and BPS and monitor therapy effectiveness. Proof-of-principle clinical evidence has confirmed that NGF is a potential target for treating human bladder overactivity.

Ochodnický, P. et al. *Nat. Rev. Urol.* 9, 628–637 (2012); published online 9 October 2012; doi:10.1038/nrurol.2012.178

Introduction

Function of the urinary bladder (the storage and voiding of urine) is controlled by highly complex neural circuits involving supraspinal, spinal and peripheral mechanisms.¹ Increased daytime voiding frequency and urgency to void—resulting from excessive sensory activation of the neural micturition circuit—are among the most prevalent storage lower urinary tract symptoms (LUTS) and are often diagnosed as part of overactive bladder syndrome (OAB).² Urgency and frequency—representing storage or ‘irritative’ LUTS (distinguishable from voiding LUTS)³—can arise as a consequence of neurological conditions, such as multiple sclerosis or spinal cord injury (SCI). Storage LUTS can also occur as a result of peripheral disorders, such as BPH and bladder pain syndrome/interstitial cystitis (BPS). However, in many cases, the underlying pathology of OAB is poorly defined (referred to as idiopathic OAB).

Frequently, but not always, storage LUTS are associated with detrusor overactivity during the bladder-filling phase (observed upon urodynamic examination). Detrusor overactivity is thought to contribute towards incontinence episodes and, for this reason, early treatments for OAB—for example, antimuscarinic agents and β_3 -adrenergic agonists—were designed to target excessive detrusor contractility. However, the focus has since shifted towards therapies that target neuronal remodelling, which is responsible for OAB-related storage

LUTS.^{4,5} Urgency is the defining (and most bothersome) symptom of OAB and the suppression of urgency implies the inhibition of detrusor overactivity in most patients. Thus, interference with neural plasticity could represent an important therapeutic strategy for tackling both storage LUTS and detrusor overactivity in patients with OAB. In addition to urgency and increased frequency, sensory pathway remodelling is probably also responsible for the perception of pain upon bladder filling in patients with BPS.^{6,7} Analogously to OAB-related LUTS, modulators of sensory neural plasticity could also interfere with bladder pain in BPS.

Neurotrophins—including nerve growth factor (NGF), brain-derived nerve factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4)—represent master modulators of neural plasticity, both in peripheral and central nervous systems. In this Review, we discuss the clinical and experimental evidence that supports a role for neurotrophins in the neural control of bladder function and in the emergence of OAB-related and BPS-related LUTS. We also consider the potential utility of neurotrophins as urinary biomarkers for improving the accuracy of OAB diagnosis and monitoring therapy efficacy, and review proof-of-principle clinical evidence that confirms NGF as a potential target in the treatment of bladder disorders.

Neuronal remodelling in bladder overactivity

Coordinated storage and voiding of urine is achieved by autonomic efferent control of bladder detrusor muscles via sympathetic and parasympathetic innervation; the latter representing the major excitatory mechanism of bladder smooth muscle.⁴ The sensory nature of urgency

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Competing interests

P. Ochodnický, C. D. Cruz and N. Yoshimura declare no competing interests. F. Cruz declares associations with the following companies: Allergan, Astellas, Recordati, AMS and Pfizer. See the article online for full details of the relationships.

indicates functional alterations of bladder afferents. Normal sensation of bladder filling is communicated to the lumbosacral spinal cord by fast-conducting A δ -myelinated afferent axons, responding to normal bladder distention and contraction. In addition, unmyelinated C fibres mediate responses to noxious stimuli—such as chemical irritation or inflammation—and do not respond to bladder filling under normal conditions (also known as ‘silent’ C fibres). In humans, the primary cell bodies of A δ and C fibres are localized in the lower lumbar (T11–L2) and sacral (S2–S4) dorsal root ganglia (DRG) and project to spinal interneurons and tract neurons, relaying sensory information to central micturition control pathways.¹

Although the A δ fibre endings are located in the vicinity of the smooth muscle layers, many of the unmyelinated C fibres terminate in the urothelial and immediate suburothelial layers (Figure 1).⁸ It has been suggested that urothelial cells form an important part of the bladder ‘sensory web’, responding to stretch or chemical stimuli by producing mediators—such as acetylcholine, ATP and, ultimately, neurotrophins—that modulate the function of underlying urothelial or suburothelial afferent fibres.^{9,10} Intensity of afferent firing is primarily determined by the activity of mechanosensitive, acid-sensitive, ligand-gated and voltage-gated ion channels, including transient receptor potential cation channel vanilloid subfamily (TRPV) members 1 (TRPV1) and 4 (TRPV4) and P2X purinoceptor 2 and P2X purinoceptor 3 (P₂X₃) receptors.^{5,11} Opening of the sensory ion channels can be enhanced or suppressed by a wide variety of urothelial and inflammatory-cell-derived regulators, including neurotrophins.

Increased pain perception upon bladder filling in patients with BPS and the occurrence of urgency in patients with OAB are associated with sensitization of afferents, resulting from long-term changes in the expression and biological properties of the ion channels and receptors that modulate channel function. Remodelling of the central micturition pathways—either primary or secondary to peripheral sensitization—could eventually lead to excessive efferent stimulation of detrusor muscle and, ultimately, to detrusor overactivity (Figure 2). It is important to note that alternative hypotheses have been proposed to explain the emergence of detrusor overactivity. These implicate the primary myogenic, rather than neurogenic, alterations or localized exaggerated sensory responses that lead to the propagation of spontaneous myogenic bladder activity, without major involvement of central pathways.^{4,12,13}

Neurotrophins and bladder dysfunction

Neurotrophins and their receptors

All neurotrophins are structurally related peptides generated from homologous genes that encode pre-peptides. These 240–260 amino acid sequences lose their signalling peptide via proteolytic cleavage to produce propeptides, which are then modified to form mature proteins of 118–129 amino acids. Two identical mature peptides combine to form an active neurotrophin

Key points

- Urgency, frequency and bladder pain are all associated with hypersensitization and remodelling of bladder peripheral afferents
- Exogenous administration of nerve growth factor (NGF) to the bladder or spinal cord induces bladder overactivity in experimental animal models
- NGF—and possibly brain-derived neurotrophic factor (BDNF)—is produced in the bladder by urothelium and smooth muscle cells upon stretch and inflammation to sensitize underlying bladder afferent C fibres
- Peripheral or central NGF sequestration reduces bladder overactivity in experimental models of spinal cord injury, bladder inflammation and outlet obstruction; local NGF delivery improves bladder underactivity in experimental diabetic cystopathy
- NGF and BDNF represent potential disease biomarkers for bladder pain syndrome/interstitial cystitis (BPS/IC) and overactive bladder syndrome; increased urinary excretion correlates with symptom severity and can be modulated by therapy
- Neurotrophin system intervention using the monoclonal NGF antibody tanezumab has been shown to improve self-reported bladder pain scores and urgency episode frequency in patients with BPS/IC

homodimer that is able to recruit, phosphorylate and activate two tropomyosin-related kinase (Trk) receptors with high-affinity neurotrophin-binding sites. Neurotrophins have varying specificities for Trk receptor subtypes; NGF binds to TrkA, BDNF and NT-4 bind to TrkB and NT-3 binds to TrkC. Trk receptors display the structure of classical tyrosine kinase receptors, with two extracellular immunoglobulin (Ig)-like domains that are responsible for interaction with neurotrophins. Modified Ig-like domains are able to sequester specific neurotrophins when injected *in vivo*, suggesting that this interaction can be exploited for therapeutic purposes. The intracellular tyrosine kinase domain of Trk receptors serves as a cytoplasmic docking site for signal transduction adaptors and enzymes. Transphosphorylated Trk receptors mediate the survival and differentiation effects of neurotrophins via the activation of phospholipase C, mitogen-activated protein kinase and phosphatidylinositol-3-kinase pathways. In addition to Trks, all neurotrophins bind with lower affinity to the pan-neurotrophin receptor p75^{NTR}—a member of the death-domain tumour necrosis factor superfamily of receptors. These receptor types are known to promote apoptosis (by activating c-Jun-N-terminal kinase and nuclear factor κ -light-chain-enhancer of activated B cells transcription factor) when activated by pro-neurotrophins. Upon binding of mature neurotrophins, on the other hand, p75^{NTR} serves as a regulator and interacting partner of Trk receptors that supports Trk-induced signalling, modifies Trk-neurotrophin binding affinities and regulates endocytosis and retrograde transport of Trks to downstream regulators.

Neurotrophins modulate neuronal plasticity

NGF—a principal neurotrophic peptide—is produced by peripheral tissues to ensure their innervation during organ development. Changes in bladder levels of various neurotrophins have been described during prenatal and postnatal development in rodents.^{14–17} In the bladder, the urothelium and densely innervated detrusor smooth

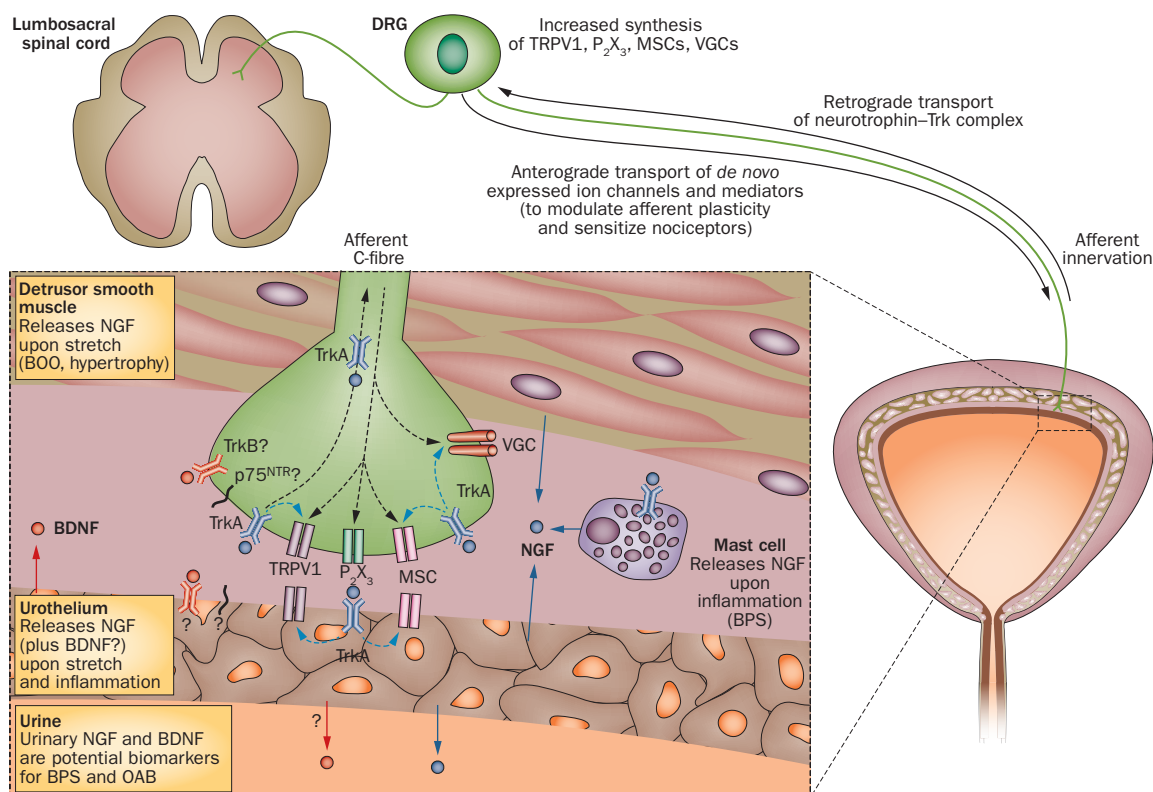


Figure 1 | Peripheral mechanisms involved in the neurotrophin-mediated development of bladder overactivity. In urinary bladder, NGF (shown in blue) is produced by several cell types—including urothelium, mast cells and detrusor smooth muscle cells—upon stretch or inflammation. The urothelium also potentially produces BDNF (shown in red). NGF binding to TrkA receptors on the urothelium might directly activate urothelial sensory ion channels, such as TRPV1 (shown in purple), or increase expression of TRPV1 and MSC (shown in pink). Increased TRPV1 and MSC activity stimulate the release of urothelial mediators, such as ATP, which sensitize the underlying afferents. In addition, NGF activates TrkA receptors expressed on suburothelial afferent C-fibre terminals, directly sensitizing neuronal TRPV1, MSCs and voltage-gated ion channels (shown in orange). The TrkA-NGF complex is internalized (dashed lines) and retrogradely transported to cell bodies in lumbosacral DRG, where *de novo* transcription of TRPV1, VGCs, MSCs and additional sensory ion channels (including purinergic P_2X_3 receptor for ATP; shown in green) is initiated. These newly synthesized ion channels are anterogradely transported back to afferent terminals to contribute to peripheral hypersensitivity. Neurotrophin receptors TrkB (shown in red) and p75^{NTR} (shown in black) are also expressed on both urothelium and afferent terminals, although their role has not yet been defined. Abbreviations: ATP, adenosine triphosphate; BDNF, brain-derived nerve factor; BOO, bladder outlet obstruction; BPS, bladder pain syndrome; DRG, dorsal root ganglia; MSC, mechanosensory channel; NGF, nerve growth factor; OAB, overactive bladder syndrome; P_2X_3 , P2X purinoceptor 3; TrkA, tropomyosin-related kinase A; TrkB, tropomyosin-related kinase B; TRPV1, transient receptor potential cation channel vanilloid subfamily member 1; VGC, voltage-gated ion channel.

muscle are thought to be the major sources of NGF (Figure 1). In culture, NGF controls the survival and outgrowth (axon and dendrite) of sensory neurons from the DRG, as well as the sympathetic neurons of the major pelvic ganglia (MPG; projecting postganglionic efferent axons to the bladder).^{18,19} This essential facet of neurotrophin action has been demonstrated in the bladders of transgenic mice that overexpress NGF in the urothelium, which are characterized by extensive hyperinnervation caused by nerve fibre expansion of sympathetic neurons, unmyelinated C fibres and A δ -myelinated afferents.²⁰

The survival-promoting role of neurotrophins is not restricted to neuronal development; these peptides also protect neurons against various types of injury, including hypoxic, excitotoxic and hypoglycaemic damage. Thus, it is not surprising that infiltrating inflammatory cells such as mast cells can produce NGF, which modulates

neuronal function under inflammatory conditions.^{21,22} Elevated bladder NGF levels have been reported in animal models of chemical, neurogenic and immune-mediated inflammation,^{23–25} suggesting that an increase in NGF release represents a generalized response to bladder inflammation.²⁶ During the development of adult organisms, neurotrophins switch roles from pro-survival factors to general regulators of differentiation and functional neuronal phenotype. This neuromodulation is achieved via the regulation of neuronal expression profiles, including those of neuropeptides, neurotransmitters and membrane ion channels.^{11,27} Peripheral sensory neurons are specifically sensitive to the neuromodulatory effects of NGF, as highlighted by the role of this peptide in the regulation of pain perception.^{11,27} In clinical trials of patients with neuropathy and neurodegeneration, NGF has been shown to induce acute

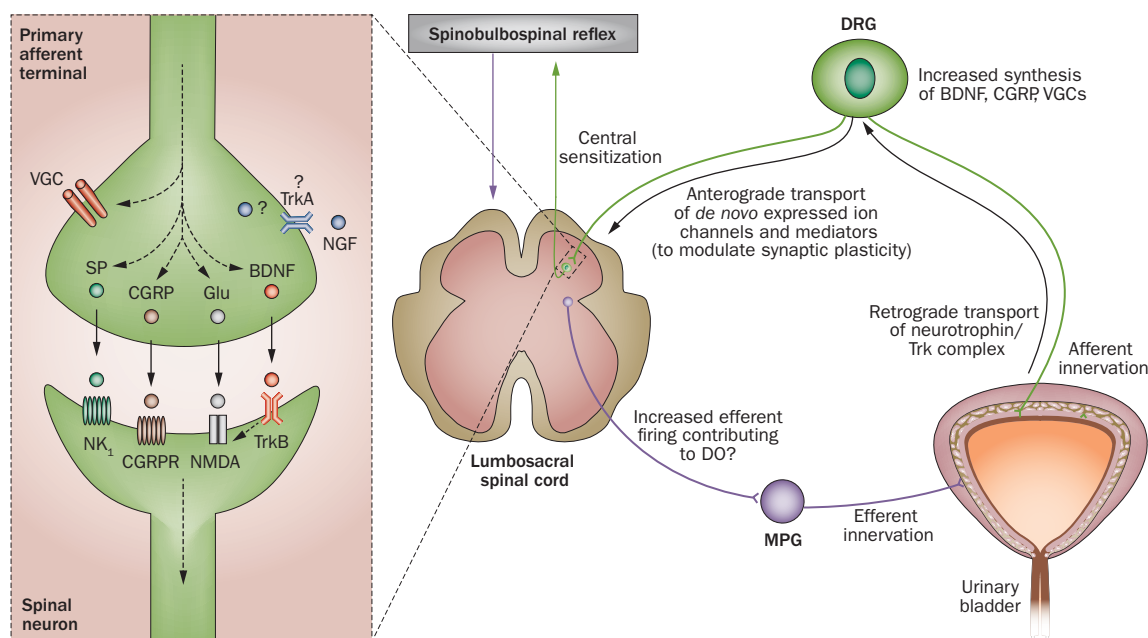


Figure 2 | Central mechanisms involved in the neurotrophin-mediated development of bladder overactivity. Upon retrograde transport of TrkA-NGF complexes along the afferent fibres from the bladder, DRG neurons increase synthesis of excitatory neuromediators, such as substance P, CGRP, BDNF and voltage-gated ion channels, which are transported anterogradely to primary afferent terminals in the lumbosacral spinal cord. Following the synaptic release, substance P (shown in green), CGRP (shown in brown) and BDNF (shown in red) activate their corresponding receptors (NK₁, CGRP receptor and TrkB, respectively) to induce central sensitization of nociceptive, and possibly also micturition, pathways. One of the mechanisms of central sensitization involves BDNF-induced activation of the NMDA receptor for excitatory mediator glutamate (shown in grey). Enhanced voltage-gated ion channel activity could contribute towards increased firing of bladder afferents. NGF and TrkA are also detected in the spinal cord, but their origin and function remain unknown. Following sensitization and activation of the central (spinobulbospinal) micturition reflex, excessive efferent stimulation could eventually contribute to DO. Abbreviations: BDNF, brain-derived nerve factor; CGRP, calcitonin gene-related peptide; CGRPR, calcitonin gene-related peptide receptor; DO, detrusor overactivity; DRG, dorsal root ganglia; Glu, Glutamate; NGF, nerve growth factor; NK₁, neurokinin-1 receptor; NMDA, N-methyl-D-aspartate; MPG, major pelvic ganglia; SP, substance P; Trk, tropomyosin-related kinase; VGC, voltage-gated ion channel.

pain and hyperalgesia.^{28,29} Given that altered sensations in the bladder underlie the symptoms of urgency and frequency, it is not surprising that neurotrophins might be involved in the development of OAB and BPS.³⁰

Although no data have been published on the effects of exogenous neurotrophin administration on human bladder function, experimental evidence clearly shows that NGF modulates micturition pathways when administered to the bladder or spinal cord. Acute intravesical administration of NGF in rats^{31,32} and transgenic urothelium-specific NGF overexpression in mice²⁰ result in sensitization of bladder afferents, increased frequency of micturition contractions and reduced micturition threshold (as measured by cystometry). Infusion of NGF into the bladder wall increases the expression of excitatory neurotransmitters, such as calcitonin gene-related peptide in the lumbosacral spinal cord (Figure 2).³³ This process, which is thought to involve an uptake of peripheral NGF by TrkA and retrograde transport of the NGF-TrkA complex to DRG cell bodies and their spinal projections, induces synaptic plasticity in the spinal cord (Figure 2). Thus, it is not surprising that not only peripheral, but also intrathecal, delivery of NGF to the lumbosacral spinal cord induces bladder overactivity and modulates the activity of several voltage-gated ion

channels—for example, tetrodotoxin-resistant sodium channels and transient A-type potassium channels—in rat DRG afferents.³⁴ It has been shown that bladder overactivity following the systemic injection of NGF is virtually absent in TRPV1-knockout mice.³⁵ This implies that modulation of TRPV1—an acid-sensitive, heat-sensitive and capsaicin-sensitive cationic channel—represents the primary mechanism of NGF-induced hypersensitization of bladder afferents^{36,37} and modulation of urothelial function (Figure 1).³⁸ Although several other NGF-induced sensitizing mechanisms have been described in nociceptors, their importance for bladder sensory pathways has not yet been characterized in detail (Figure 2).

Several populations of mammalian sensory neurons seem to be dependent on members of the neurotrophin family other than NGF, such as BDNF, NT-3 and NT-4.^{18,39,40} Similarly to NGF, BDNF has been identified in the peripheral tissues of the bladder, including the urothelium and detrusor muscles.^{41,42} NT-3 and NT-4 might also be expressed in the bladder, although their exact role and location are unclear.⁴³ Upon TrkA-mediated stimulation with NGF, several subpopulations of DRG neurons release BDNF (Figure 2).^{44,45} BDNF released into the spinal cord might control central synaptic plasticity, possibly by potentiating activation of

postsynaptic ionotropic glutamate N-methyl-D-aspartate receptors, which have a key role in central sensitization.¹¹ Thus, it is feasible that central BDNF might be involved in the regulation of micturition pathways.⁴²

Neurotrophins in bladder pain syndrome

Given that NGF acts as a ubiquitous hyperalgesic mediator, it seems intuitive to study the role of neurotrophins in bladder pain syndromes. BPS—previously known as interstitial cystitis—is characterized by chronic pelvic pain or discomfort that is related to the bladder, accompanied by urgency to void, increased voiding frequency and nocturia, usually without detrusor overactivity.^{6,7,46,47} It is a relatively prevalent condition and current therapeutic approaches are often inadequate.⁴⁸ However, some patients seem to respond to local botulinum neurotoxin A (BoNT/A) administration.^{49,50} Although the exact nature of BPS remains enigmatic, the presence of both hypersensitivity and urgency symptoms indicates that the neural plasticity of bladder sensory afferents is involved in the development of the disease. Hyperinnervation of suburothelial and detrusor nerve fibres can be detected in bladder biopsies taken from patients with BPS, along with inflammatory infiltration (including a prominent presence of mast cells).^{51,52} Structural and functional damage to the urothelium is thought to represent the key step in BPS pathogenesis.^{53,54} Interestingly, suburothelial hyperinnervation and tissue mast cell infiltrate have also been observed in transgenic mice that exhibit urothelial NGF overexpression.²⁰ It is unknown whether the urothelium or mast cells are the primary source of NGF upon bladder inflammation (Figure 1). Excessive NGF immunoreactivity has also been observed in the urothelium and suburothelium of biopsies from patients with BPS.^{49,55} In the latter study, elevated bladder NGF mRNA expression was reduced by effective treatment with pain-reducing trigonal injections of BoNT/A. This neurotoxin is thought to modulate bladder sensations via chemical denervation, by interfering with vesicular transport of neuromediators and membrane ion channels in afferent nerve fibres and urothelium.⁵⁶ It is possible that BoNT/A also directly affects NGF release. Patients with BPS excrete large amounts of urinary NGF; levels of this neurotrophin correlate with symptom severity (including self-reported pain-severity scores).⁵⁷ Excessive urinary NGF is transiently reduced upon BoNT/A treatment, coinciding with symptomatic improvement.⁵⁸

Interestingly, urinary concentration of another neurotrophin, BDNF, is also elevated in patients with BPS and is similarly suppressed following BoNT/A treatment.⁵⁸ Several neurotrophin-system interventions alleviate cystometric bladder overactivity in experimental rat models of bladder inflammation (induced by administration of cyclophosphamide, which is converted to the bladder irritant acrolein). Effective interventions include intravesical instillation of modified NGF-antisense oligodeoxynucleotide construct⁵⁹ or sequestration of NGF protein using a recombinant fusion protein that consists of an extracellular TrkA domain (directly responsible for NGF binding with the receptor) and the Fc portion

of IgG. Studies have shown that NGF sequestration is effective, regardless of whether a systemic intravenous or intrathecal administration route is used, suggesting that both peripheral and central modes of action exist.^{60,61} Intravenous injection of recombinant TrkB-Ig₂ domain—designed to neutralize BDNF, rather than NGF—reduces the frequency of reflex contractions in animals treated with cyclophosphamide.⁴² As TrkB-Ig₂ is unlikely to cross the blood–brain barrier, peripheral uptake of BDNF is probably involved in the modulation of micturition pathway plasticity upon cyclophosphamide-induced inflammation.

Based on these interventional experimental data, the monoclonal NGF-neutralizing antibody tanezumab has been subject to clinical evaluation; phase two trials of drug efficacy have provided encouraging results. In a study of 68 patients with BPS, a single intravenous injection of tanezumab (200 µg/kg) improved self-reported pain score and urgency episode frequency within 6 weeks.⁶² Tanezumab had no significant effect on micturition frequency or mean voided volume per micturition. Among the associated adverse effects were abnormal peripheral sensation symptoms, such as paraesthesia and hyperaesthesia.⁶² Although clinical studies of NGF monoclonal antibody therapy were put on hold following reports of bone necrosis (requiring total joint replacement in some patients with osteoarthritis),^{63,64} proof-of-concept evidence exists regarding the potential efficacy of systemic intervention in the NGF system for treating BPS.

Neurotrophins in OAB

NGF in neurogenic bladder dysfunction

In humans and animals, suprasacral spinal cord lesions eliminate voluntary control of voiding, leading to an areflexic bladder and urinary retention. However, in the long-term, an automatic spinal reflex develops, resembling primitive neonatal automatic micturition control. Emergence of this reflex is associated with profound neuromodulation of afferent bladder pathways, including sensitization of ‘silent’ C fibres and increased responsiveness to mechanical stimuli. As a result of this neuronal remodelling, neurogenic detrusor overactivity (NDO) can be detected on cystometry, accompanied by storage LUTS such as increased frequency and urgency. Furthermore, inefficient bladder emptying—resulting from simultaneous bladder and external urethral sphincter contractions (bladder–sphincter dyssynergia)—has been associated with bladder hypertrophy.^{1,65} Similar bladder symptoms are observed in patients with primary neurodegenerative disease, such as multiple sclerosis.⁶⁶

Neurotrophins, especially NGF, have been implicated in the neuromodulation of micturition pathway plasticity associated with neurogenic bladder overactivity. In patients with NDO, BoNT/A therapy could improve urodynamic parameters and reduce the occurrence of storage LUTS.^{67,68} NGF production is elevated in the bladder of patients with NDO and can be reduced upon therapy with BoNT/A.⁶⁹ Urinary NGF excretion is also increased in patients with NDO, and symptomatic

improvement following treatment with antimuscarinics or BoNT/A has been associated with reductions in NGF urinary levels.⁶⁸

In experimental animal models of SCI, levels of bladder NGF and BDNF are increased;^{43,70} however, the primary stimulus for NGF release remains unknown. Excessive bladder distension caused by bladder–sphincter dyssynergia might be a contributing factor as stretch induces NGF release from bladder smooth muscle cells (BSMCs).^{71,72} Peripheral NGF could directly modulate afferent plasticity, including expression of TRPV1 and P₂X₃ receptors.^{36,73} Patients with NDO display abnormally high immunoreactivity towards these receptors in the urothelium and suburothelium (Figure 1).⁶⁷ Increased transport of NGF to DRG cell bodies or central NGF production in the injured spinal cord could modulate the micturition pathway at the spinal level; intrathecal delivery of an NGF monoclonal antibody has been shown to diminish bladder contractions, maximal voiding pressure and ameliorated detrusor-sphincter dyssynergia in rats with SCI.^{74,75} A combination of peripheral and central NGF action is probably involved in the emergence of neurogenic bladder overactivity (although interventional experimental evidence exists only for the latter). Roles for the other neurotrophins have not yet been defined.

Bladder-hypertrophy-associated OAB

Neuronal growth in the bladder is inherently associated with bladder hypertrophy. Hypertrophy of the bladder is most frequently the consequence of bladder outlet obstruction (BOO) caused by benign prostatic hyperplasia (BPH). In this clinical setting, hypertrophy is thought to occur as a compensatory response to restrained emptying of the obstructed bladder. Structurally, hypertrophy of BSMCs and excessive extracellular matrix deposition result in increased bladder wall thickness and bladder weight, both of which are related to the severity of obstruction.⁷⁶ Patients with BPH often present with storage LUTS (including frequency and urgency), which are diagnosed as part of OAB. Eventually, detrusor overactivity might be evident upon urodynamic examination. Microscopically, hypertrophy of both DRG and MPG neurons is observed in the hypertrophied bladders of rat models of BOO, implying a role for neurotrophic factors in the development of these conditions.^{77,78} Indeed, men with OAB symptoms and BOO caused by BPH display excessive levels of NGF in bladder tissue⁷⁷ and increased levels of urinary NGF.⁷⁹ Interestingly, NGF elevation in the urine is reduced following successful BPH treatment with an α -blocker or 5 α -reductase inhibitor.⁷⁹ Several studies have shown no correlation between urinary NGF levels and urodynamic parameters, possibly indicating that the extent of NGF urinary release reflects the severity of OAB-related storage LUTS, rather than detrusor overactivity severity.^{79–81} In experimental animal models, urinary NGF levels have been shown to correlate with tissue NGF protein levels, which are closely related to rates of symptom clearance following bladder obstruction relief.⁸² Most importantly, immunization of rats against NGF not only prevents the rise of NGF

in obstructed bladders, but also limits neuronal hypertrophy and associated voiding dysfunction, confirming a causal role for NGF in the emergence of OAB symptoms following BOO.⁷⁸

Steers *et al.*⁷⁷ originally proposed that NGF in the hypertrophied bladder is primarily produced by stretched BSMCs;^{72,83} however, more recent evidence suggests that the urothelium is also an important source of NGF. Transgenic mice that overexpress NGF in the urothelium display significant bladder enlargement, along with bladder overactivity and both suburothelial and BSMC hyperinnervation.²⁰ Urothelial mechanosensory function might be markedly altered in patients with BOO-related OAB.⁸⁴ Collectively, these findings suggest that NGF is crucial for the development of storage LUTS in hypertrophied bladders. However, no data are available on the role of the other neurotrophins.

NGF in idiopathic OAB

Urgency and frequency are often reported by patients with no apparent underlying pathology. Detrusor instability observed upon urodynamic examination of such patients is described as idiopathic detrusor overactivity. Several hypotheses have been proposed to explain the emergence of idiopathic detrusor overactivity, implicating either neural plasticity (neurogenic activity) or altered smooth muscle contractility (myogenic activity) as the primary underlying cause. According to one unifying theory, local spontaneous myogenic activity is normally generated via sensory activation of the urothelium, interstitial cells or afferent nerves, and exaggeration of these local responses might contribute to the emergence of urgency and detrusor overactivity.¹² Regardless, the presence of storage LUTS and afferent hypersensitization might suggest a role for neurotrophic factors in the development of idiopathic OAB.

Detrusor biopsies from patients with idiopathic detrusor instability have been shown to contain twice as much NGF as controls.⁷² There have been several reports of markedly increased urinary NGF release in patients with idiopathic OAB;^{81,85–87} one report suggested that urinary NGF release is associated with the presence and severity of urgency.⁸⁷ In a study of women with OAB (with or without detrusor overactivity), no relationship was established between levels of NGF in superficial biopsies (largely composed of urothelium and suburothelium) and urodynamic parameters,⁸⁸ suggesting that the urinary NGF that accumulates in patients with OAB does not originate from the urothelium. Similarly, elevated levels of serum NGF correlate with urinary NGF excretion in patients with OAB, reinforcing the idea that NGF has a systemic origin in bladder overactivity.⁸⁹ Nevertheless, urinary NGF is decreased in response to antimuscarinic and BoNT/A therapies, which improve storage LUTS. Upon treatment withdrawal, abnormal NGF excretion is observed once again.^{68,90} The presence of detrusor overactivity has been associated with further NGF urinary increases in some studies,⁹¹ but not others.⁸¹ As a significant proportion of the available data relate to small cohorts, additional studies are required to

better define a role for urinary NGF as a biomarker of idiopathic OAB.

Altered NGF balance in diabetic cystopathy

Patients with diabetes mellitus present with a wide variety of LUTS and diabetic bladder dysfunction is one of the most common diabetic complications, reported in about 50–70% of patients.^{92,93} Increased bladder volumes at first sensation to void have been described, as well as reduced detrusor contractility and excessive postvoid residual volumes, which can eventually lead to atonic bladder. These symptoms have been ascribed to altered afferent and efferent neural regulation, resulting from diabetic neuropathy. However, a large proportion of patients demonstrate symptoms of bladder overactivity (rather than underactivity), including urgency, frequency and, ultimately, detrusor overactivity. The pathophysiology involved in this clinical scenario is complex and difficult to understand. A two-step model of diabetic cystopathy progression has been proposed on the basis of experimental animal studies,⁹⁴ which suggest that patients initially develop bladder hypertrophy and overactivity (presumably as an adaptation to polyuria-mediated frequent voiding). As these symptoms resemble those observed in bladders with evidence of BOO or SCI, it seems likely that increased production of neurotrophins might be involved in this phase of diabetic bladder dysfunction. In support of this theory, increased NGF levels have been reported in the bladders of rats with early experimental diabetes mellitus.⁹⁵ In more advanced diabetic bladder disease (for example, the decompensated phase of experimental diabetic cystopathy), bladder and DRG levels of NGF drop⁹⁶ and animals display increased bladder capacity and decreased peak pressures, resulting from diabetic neuropathy and hyperglycemia-induced alterations in detrusor muscles and the urethra.

Loss of NGF production has been implicated in the development of sensory and sympathetic neuronal degeneration associated with diabetic neuropathy.^{97,98} As such, recombinant NGF has been subject to clinical evaluation for the treatment of patients with diabetic polyneuropathy.²⁸ Although data regarding bladder function are lacking for these patients, experimental evidence suggests that NGF improves diabetic bladder dysfunction in the decompensated phase. NGF gene therapy by herpes-virus-mediated NGF gene delivery into the bladder results in long-term elevation of NGF bladder levels, preventing excessive increases in bladder capacity and reducing postvoid residual volume in diabetic rats.⁹⁹ These data provide interventional evidence for a causal role of NGF in the development of diabetic cystopathy. Thus, experimental evidence indicates that NGF levels in the bladder fluctuate during the compensatory and decompensated phases of diabetic bladder dysfunction, possibly actively contributing to functional abnormalities. Diabetic cystopathy represents a progressive disorder in which sensory and contractile bladder dysfunction might be dependent on continually unbalanced neurotrophin production.

Urinary neurotrophins as biomarkers

One of the main problems that urologists face when diagnosing OAB is the lack of an objective diagnostic method. OAB diagnosis relies on the identification of urgency; defined as a sudden compelling desire to void, which is difficult to defer.^{2,3} However, urgency can be easily confused with urge (the normal sensation of bladder fullness, with fully maintained control of bladder function). An objective biomarker—preferably one that is noninvasively detectable in urine with a reasonable sensitivity and specificity—might help clinicians to establish the correct diagnosis in uncertain cases. For instance, patients with stress urinary incontinence (SUI) might mistakenly report urgency to void when urine leaks into the proximal urethra. SUI coexists with OAB in a large fraction of elderly patients with mixed urinary incontinence. In these patients, elevated urinary NGF could be used as a marker to identify patients with OAB-related urgency as SUI does not seem to be associated with changes in urinary NGF excretion.¹⁰⁰ Furthermore, NGF can be employed as an OAB biomarker to assess disease progression. Patients with OAB can develop incontinence (OAB wet), which is associated with higher urinary NGF levels than OAB without incontinence (OAB dry).⁹¹

Importantly, NGF could also be used to monitor therapy efficacy, to establish whether (and when) treatment can be interrupted following symptomatic improvement and to indicate whether asymptomatic patients should resume treatment. Urinary NGF decreases (in parallel with symptomatic improvement) following antimuscarinic treatment and detrusor injections of BoNT/A in patients with OAB or BPS.^{57,58,68,90} Importantly, decreased urinary NGF was only noted in responders to the symptom-relieving therapy. Despite recent efforts, multiple issues still need to be resolved before NGF can become a widely accepted clinical biomarker for OAB or BPS. Of particular note are difficulties regarding the relatively low sensitivity and specificity of the marker and the lack of well-defined normal and cut-off values.^{101,102} Additional studies are needed, involving larger patient cohorts.

Significantly less data are available on the urinary excretion of the other neurotrophins. Interestingly, elevated urinary levels of BDNF are suppressed upon symptomatic improvement following BoNT/A injection in patients with BPS.⁵⁸ Similarly, a small study of patients with OAB demonstrated that increases in urinary BDNF were significantly diminished following therapeutic intervention, including lifestyle modification and antimuscarinic treatment.¹⁰³ Interestingly, BDNF urinary levels strongly correlated with symptom severity in this trial. Another small study reported elevated urinary concentration of NT-3 in patients with BPS,¹⁰⁴ although the relevance of this finding is not yet clear.

Conclusions

The role of NGF as a crucial regulator of bladder sensory function has been now conclusively established. Proof-of-principle clinical evidence involving

the NGF-neutralizing monoclonal antibody tanezumab has confirmed that NGF is a viable therapeutic target for the treatment of BPS.⁶² Moreover, several other anti-NGF monoclonal antibodies—including fulranumab and REGN475—are under investigation in phase II trials for multiple indications associated with chronic pain.^{105–107} However, given the widespread neuromodulatory role of NGF, there is great concern regarding the associated ‘off-target’ adverse effects of anti-NGF antibodies, including paraesthesia and progressive joint degeneration (potentially requiring joint replacement).^{64,108} Although reports of the latter initially led to the suspension of all clinical trials involving anti-NGF treatment, clinical testing has since resumed.¹⁰⁹ It remains unclear whether reported cases of joint degeneration result from direct skeletal effects of NGF or as a consequence of excessive joint overload in the absence of pain (particularly in the case of patients with osteoarthritis).⁶⁴ Eventually, alternative systemic administration routes could be developed for NGF neutralization, such as local bladder injections (which have already been successfully employed for administering BoNT/A to patients with OAB and BPS).¹¹⁰ However, several pharmacokinetic issues will need to be resolved first. Intravesical liposome instillation—which has been tested in 24 patients with BPS to date¹¹¹—might represent another promising approach for the local delivery of NGF-silencing or NGF-sequestering agents to the bladder. Experimental treatments aimed at sequestering NGF or BDNF include recombinant proteins with TrkA or TrkB Ig₂ binding domains, respectively.^{42,61} Low-molecular-weight compounds that prevent specific interactions between NGF or BDNF and their respective receptors have also been investigated.^{112,113} Small

compounds that block the tyrosine kinase activity of TrkA receptors might represent another potential treatment approach.¹¹³ At present, the experimental data that are available for non-NGF neurotrophins are insufficient for these to be considered valid therapeutic targets for bladder dysfunction. Nevertheless, it is likely that these peptides—particularly BDNF—also play an important role in the regulation of bladder sensory function. Unlike neurotrophin blockade, NGF administration and activation of TrkA with low-molecular-weight compounds are unlikely clinical tools for treating diabetic reflexic bladder, as a result of potentially serious nociceptive adverse effects. However, organ-specific delivery of NGF might eliminate or reduce systemic toxicity. Additional studies, involving larger cohorts of patients, are needed to define the sensitivity and diagnostic value of urinary NGF and BDNF markers in patients with OAB-related storage LUTS.

Review criteria

The primary literature was obtained by searching the MEDLINE database for the keyword “urinary bladder” in combination with the following keywords: “neurotrophins”, “NGF” and “BDNF”. In addition, relevant clinical papers on the general aspects of overactive bladder syndrome, bladder pain syndrome (previously known as interstitial cystitis) and diabetic cystopathy were selected, as well as mechanistic publications on the neuromodulatory effects of neurotrophins. Only English-language, full-text papers were selected, with the exception of several abstracts reporting significant mechanistic or clinical advances. Web resources such as clinicaltrials.gov were also reviewed for information on the clinical development of NGF-based interventions.

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Acknowledgements

Work in the laboratories of P. Ochodnický, C. D. Cruz and F. Cruz is supported by the European Community's FP7, HEALTH-F2-2008-223234 InComb grant. Work in the laboratory of N. Yoshimura is supported by the National Institutes of Health (DK057267 and DK088836) and the United States Department of Defense (SC100134 and PR110326). The authors thank Martin C. Michel for his inspiration and help during the preparation of this manuscript.

Author contributions

P. Ochodnický researched data for the article. C. Cruz and P. Ochodnický wrote the article. All authors contributed towards discussions of content, in addition to reviewing and editing the manuscript prior to submission.

Down-Regulation of Nerve Growth Factor Expression in the Bladder by Antisense Oligonucleotides as New Treatment for Overactive Bladder

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Purpose: Nerve growth factor over expression in the bladder has a role in overactive bladder symptoms via the mediation of functional changes in bladder afferent pathways. We studied whether blocking nerve growth factor over expression in bladder urothelium by a sequence specific gene silencing mechanism would suppress bladder overactivity and chemokine expression induced by acetic acid.

Materials and Methods: Female Sprague-Dawley® rats anesthetized with isoflurane were instilled with 0.5 ml saline, scrambled or TYE™ 563 labeled antisense oligonucleotide targeting nerve growth factor (12 μ M) alone or complexed with cationic liposomes for 30 minutes. The efficacy of nerve growth factor antisense treatments for acetic acid induced bladder overactivity was assessed by cystometry. Bladder nerve growth factor expression levels and cellular distribution were quantified by immunofluorescence staining and enzyme-linked immunosorbent assay. Effects on bladder chemokine expression were measured by Luminex® xMAP® analysis.

Results: Liposomes were needed for bladder uptake of oligonucleotide, as seen by the absence of bright red TYE 563 fluorescence in rats instilled with oligonucleotide alone. At 24 hours after liposome-oligonucleotide treatment baseline bladder activity during saline infusion was indistinct in the sham and antisense treated groups with a mean $\pm$ SEM intercontraction interval of 348 ± 55 and 390 ± 120 seconds, respectively. Acetic acid induced bladder overactivity was shown by a decrease in the intercontraction interval to a mean of $33.2\% \pm 4.0\%$ of baseline in sham treated rats. However, the reduction was blunted to a mean of $75.8\% \pm 3.4\%$ of baseline in rats treated with liposomal antisense oligonucleotide ($p < 0.05$). Acetic acid induced increased nerve growth factor in the urothelium of sham treated rats, which was decreased by antisense treatment, as shown by enzyme-linked immunosorbent assay and reduced nerve growth factor immunoreactivity in the urothelium. Increased nerve growth factor in bladder tissue was associated with sICAM-1, sE-selectin, CXCL-10 and 1, leptin, MCP-1 and vascular endothelial growth factor over expression, which was significantly decreased by nerve growth factor antisense treatment ($p < 0.01$).

Conclusions: Acetic acid induced bladder overactivity is associated with nerve growth factor over expression in the urothelium and with chemokine up-regulation. Treatment with liposomal antisense suppresses bladder overactivity, and nerve growth factor and chemokine expression. Local suppression of nerve growth factor in the bladder could be an attractive approach for overactive bladder. It would avoid the systemic side effects that may be associated with nonspecific blockade of nerve growth factor expression.

Key Words: urinary bladder, overactive; nerve growth factor; urothelium; chemokines; liposomes

Abbreviations and Acronyms

AA	= acetic acid
BO	= bladder overactivity
CMG	= cystometrogram
ICI	= intercontraction interval
MCP-1	= monocyte chemoattractant protein-1
NGF	= nerve growth factor
OAB	= overactive bladder
OND	= oligonucleotide
PBS	= phosphate buffered saline
sE-selectin	= soluble endothelial adhesion molecule
sICAM-1	= soluble intracellular adhesion molecule
VEGF	= vascular endothelial growth factor

Accepted for publication February 20, 2013.
Study received animal care and use committee approval.

Supported by National Institutes of Health DK057267 and DK088836, and Department of Defense W81XWH-11-1-0763 and W81XWH-12-1-0565.

* Equal study contribution.

† Financial interest and/or other relationship with Allergan, Astellas, Covic, Lipella, Medtronic and Pfizer.

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PREVIOUS studies by various groups demonstrated that NGF over expression in the bladder and bladder afferent pathways is involved in the emergence of hyperexcitability in bladder C-fiber sensory pathways.^{1,2} C-fiber hyperexcitability is considered responsible for symptoms common to OAB.³ Intrathecal application of NGF antibodies decreased NGF levels in bladder afferent pathways and normalized bladder/urethral function in spinal cord injured rats.⁴

Recently, exogenous over expression of NGF in the urothelium was shown to cause micturition dysfunction and pelvic hypersensitivity in a transgenic mouse model.^{2,5} The major role of NGF in lower urinary tract symptoms encouraged the systemic administration of monoclonal human NGF antibodies in patients with mixed results.⁶ In patients generalized blockade of NGF at sites other than the bladder was associated with the incidence of paresthesia, hypoesthesia and arthralgia.

Therefore, to decrease the intrinsic toxicity of systemic blockade of NGF, we developed what is to our knowledge a novel intravesical therapy for OAB by targeting intracellular synthesis of NGF in the urothelium. We measured the functional efficacy of liposomes complexed with antisense OND targeting NGF, including the effect on the NGF signaling pathway.^{7–10}

METHODS

Reagents

The 18mer phosphorothioated antisense OND was custom made. It had a 5' tag of TYE 563 (bright red fluorescent dye that is a direct substitute for Cy3TM) with the sequence 5'GCCCGAGACGCCTCCCGA3'. A similar length scrambled sequence 5'ACGACCTCGCGACCGGCC3' was designed using GenScript (<https://www.genscript.com/ssl-bin/>

[app/scramble](#)). Cationic liposomes composed of DOTAP were made by the thin film hydration method, in which lipid film was hydrated with nuclease-free water with a final lipid concentration of 7 mM. Lyophilized OND was dispersed in nuclease-free water at a concentration of 12 μ M. It was then complexed with liposomes by incubating the 2 entities together at room temperature for 30 minutes. The molar ratio of OND to lipid in the liposomal complex was 1:10.

Animals

The study was performed in 37 female adult Sprague-Dawley rats weighing 225 to 250 gm divided into 6 study groups of 5 to 8 each. We used 2 group for bladder uptake and the remaining 4 for CMG, which also provided tissue for subsequent immunohistochemistry, NGF and chemokine analysis.

Studies

Bladder uptake. Rats were anesthetized with 2% isoflurane. The bladder was catheterized by a 24 gauge angiocatheter (BDTM) and washed with saline to instill 0.5 ml fluorescent TYE conjugated OND in saline (6 rats) or complexed with liposomes for 30 minutes (6 rats). A purse-string suture was placed around the urethra to occlude for 30 minutes, which was later withdrawn to restart voiding by awake rats in metabolic cages at the end of instillation. Rats were sacrificed 8 and 24 hours (3 per time point per group) after instillation to harvest bladder tissue for cryosectioning into 8 μ m cryosections.

Efficacy. Rats were instilled with 12 μ M NGF antisense (6) or scrambled OND complexed with liposome (5), or with saline as sham treatment (8) after the described bladder uptake studies. CMG was done 24 hours later in the 4 groups, including a control group of 6 rats without prior instillation, under anesthesia using urethane (1.0 gm/kg subcutaneously). A polyethylene-50 catheter was connected by a 3-way stopcock to a pressure transducer and to a syringe pump. The catheter was inserted in the bladder through the dome to record intravesical pressure and infuse solutions into the bladder. Intravesical pres-

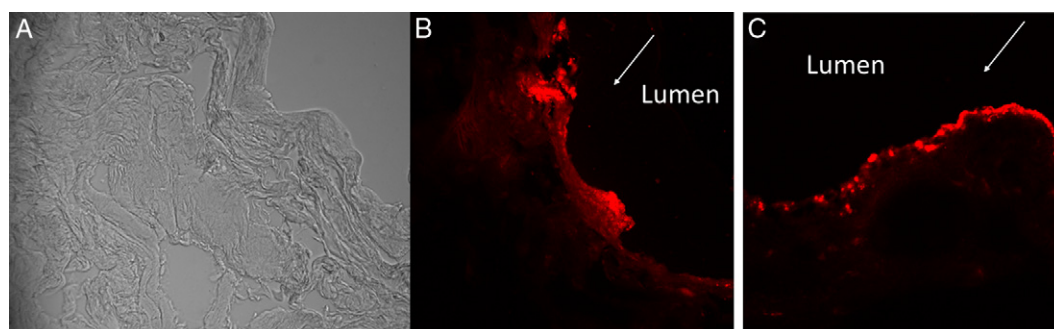


Figure 1. Confocal images show harvested rat bladders instilled with antisense OND with 5' tag of TYE 563 without liposome (A), and with OND complexed with liposomes at 8 (B) and at 24 (C) hours. Bright red fluorescence demonstrates successful uptake and retention in target cells of OND delivered by liposomes (B and C). Fluorescence was more homogenous in urothelium at 24 hours vs discrete localization to lumen surface at 8 hours. Arrow indicates lumen side. Bright field image is also shown since there was no fluorescent signal for image to be taken for group instilled with antisense OND with 5' tag of TYE 563 without liposome (A). Absence of red fluorescence reveals liposome need for successful bladder uptake of OND. Reduced from $\times 40$.

sure was recorded with Chart™ data acquisition software at a sampling rate of 400 Hz on a computer system equipped with an analog-to-digital converter. Body temperature was maintained in the physiological range with a heat lamp.

Control CMG was performed by filling the bladder with saline at 0.04 ml per minute to elicit repetitive voiding for more than 1 hour. Subsequently, bladder irritation was induced by 0.25% AA infusion for more than 3 hours to induce BO, which is considered similar to the phenotype of OAB observed clinically. The ICI of reflex bladder contractions during saline and AA infusion was determined as the time between 2 continuing contraction cycles. We compared the average of at least 3 ICIs measured for more than 30 minutes during saline infusion and 60 minutes after AA infusion, respectively.

Immunohistochemistry

At the end of CMG, a portion of the bladders (5 preparations) from each group were cryopreserved. Cryosections (8 μ m) were washed in PBS and preincubated with PBS containing 20% normal serum (Jackson ImmunoResearch, West Grove, Pennsylvania) and 0.2% Triton™ X-100 for 2 hours at room temperature. Primary polyclonal rabbit H-20 antibody (1:50) (Santa Cruz Biotechnology, Santa Cruz, California) for NGF was applied in PBS containing 5% normal serum and 0.2% Triton X-100 for 16 to 18 hours at 4°C. Sections were washed 4 times in PBS containing 0.1% bovine serum albumin and 0.1% Triton X-100 for 5 minutes each at room temperature. They were then incubated for 2 hours at room temperature with secondary donkey anti-rabbit Alexa Fluor® 488 antibody (1:200). Washing was performed 3 times at room temperature in PBS. Sections were mounted with aqueous mounting medium.

Harvested Bladder NGF

At the end of CMG, mucosa containing the urothelium was surgically separated from the detrusor in a portion of rat bladder from each group (5 preparations), as previously described.¹¹ Tissues were homogenized using the RIPA Lysis Buffer System (Santa Cruz Biotechnology) to isolate protein and measure NGF using antigen capture enzyme-linked immunosorbent assay with the Emax® ImmunoAssay System according to manufacturer instructions, as previously described.¹² Tissue NGF values are expressed as pg/mg protein. Total RNA was isolated from whole bladders in control and antisense treated rats. It was later transcribed into cDNA to measure NGF mRNA levels by quantitative polymerase chain reaction, as previously described.¹³

Effect on Downstream

Effectors of NGF Signaling Pathway

Tissue lysates prepared from whole bladder (5 preparations) were analyzed for the chemokines sICAM-1,¹⁴ sE-selectin, MCP-1,⁹ VEGF,⁸ leptin,⁷ and CXCL-1⁹ and 10 using the Luminex xMAP kit, as previously described.⁹

Statistical Analysis

Results are shown as the mean $\pm$ SEM. Statistical significance between the mean values of different groups was analyzed using 1-way ANOVA, followed by the Tukey post test. The correlation of NGF and chemokine expression was assessed by the Pearson r and Spearman r_s correla-

tion tests. In all statistical tests the minimum criterion chosen to discard the null hypothesis was set at $p < 0.05$.

RESULTS

Bladder Uptake

Rat bladders harvested after OND instillation with or without liposomes were cryosectioned for viewing under a LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany). The fluorescent signal was used as a measure of bladder uptake of OND after instillation. The need for liposomes in successful OND delivery was demonstrated by the lack of bright red fluorescence from the TYE 563 tag of OND in the absence of liposomes (fig. 1, A). Figure 1,

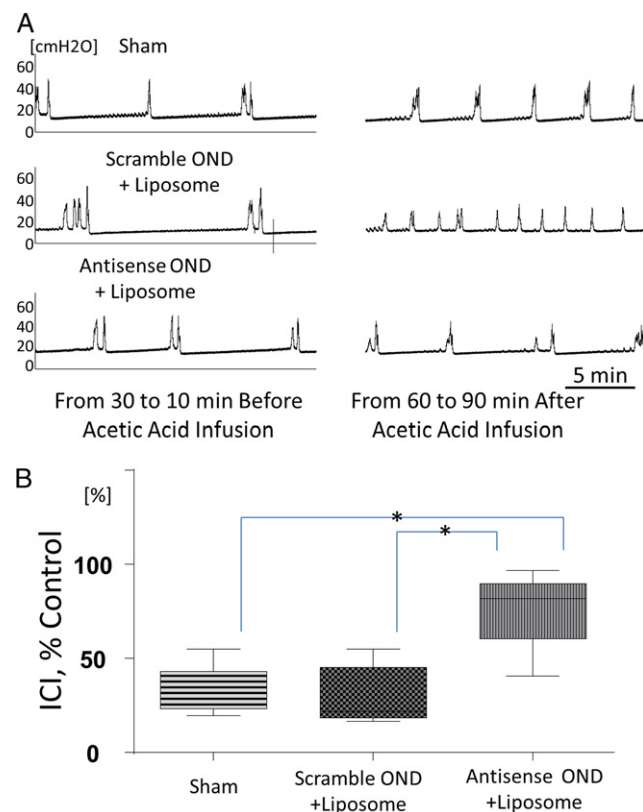


Figure 2. CMG analysis of bladder overactivity in treated groups induced by intravesical application of 0.25% AA. Representative CMG was performed 24 hours after instillation of saline in sham treated group (*Sham*), liposomal complex of scrambled OND or antisense OND (A). Note CMG traces 30 to 10 minutes before and 60 to 90 minutes after AA application. AA-induced reduction in ICI was seen in sham and scrambled OND treated rats but not in group treated with liposomal NGF antisense complex which demonstrates antisense OND protective effect. CMG parameters in absence of AA did not significantly differ in 3 groups. Changes in ICI after intravesical AA application expressed as percent of control ICI before AA (B). AA induced ICI decrease was significantly smaller in liposome-NGF antisense treated group vs sham and liposome-scrambled OND treated groups. Asterisk indicates $p < 0.05$.

B and *C* show the bright field image since there was no fluorescent signal in the images from that group. Liposomes mediated bladder uptake of OND was evident due to intense fluorescence at 8 hours (fig. 1, *B*). Penetration depth was restricted to the urothelium but it increased from 8 to 24 hours (fig. 1, *C*). Fluorescence localization in the urothelium revealed successful bladder uptake and retention of OND in target cells.

Cystometry

Baseline CMG under saline infusion was indistinct between the groups, as evident by the mean ICI of 348 ± 55 and 390 ± 120 seconds in the sham and liposomal antisense treated groups, respectively. AA induced BO was evident in the sham treated group due to the mean percent reduction to $33.2\% \pm 4.0\%$ of baseline ICI (fig. 2, *A*). Pretreatment with antisense OND complexed with liposomes blocked AA induced BO with the mean percent reduction re-

stored to $75.8\% \pm 3.4\%$ of baseline (6 preparations) (fig. 2, *A*). Sequence specificity of NGF antisense was shown by the lack of effect in the group instilled with scrambled OND sequence complexed with liposomes (fig. 2, *A*). ICI was longer in the liposomal antisense treated group than in the sham treated group. Differences were statistically significant (1-way ANOVA followed by the Tukey post test $p < 0.05$, fig. 2, *B*).

Bladder NGF

AA exposure increased NGF production in the sham treated group relative to controls. Pretreatment with NGF antisense OND significantly blocked AA induced NGF over expression in the urothelium as well as in detrusor lysates (fig. 3, *A* and *B*). Changes in NGF protein expression varied in scrambled OND treated rats, resulting in insignificant changes in NGF protein expression. However, it tended to decrease, as shown by the plot of individual values

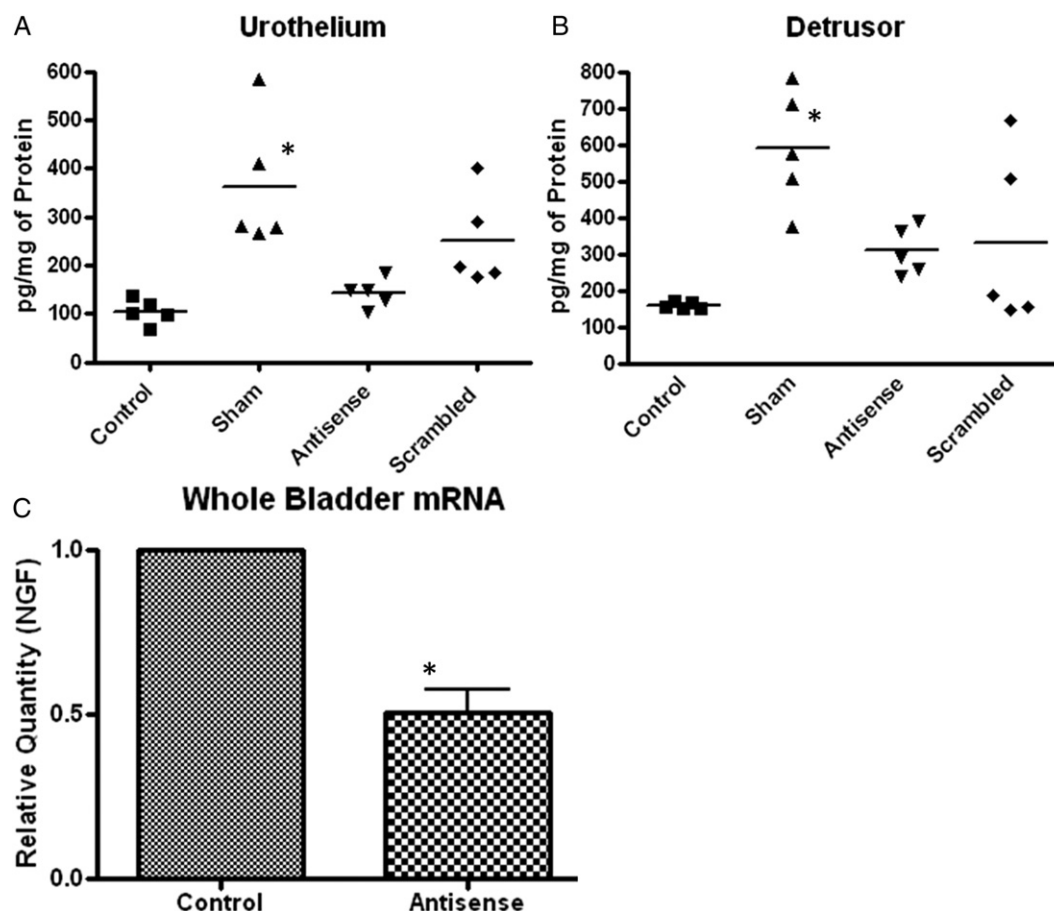


Figure 3. Antisense OND mediated suppression of AA induced NGF protein expression in urothelium (*A*) and detrusor (*B*), and NGF mRNA expression in whole bladder (*C*). AA exposure caused significant increase in NGF in sham treated group (*Sham*) vs untreated controls. NGF antisense OND significantly blunted NGF increase vs sham treated group. Data points represent NGF values of individual rats around mean (horizontal line) of each group. Significant decrease was noted in whole bladder mRNA levels of rats treated with NGF antisense OND complexed with liposomes vs levels in controls (*C*). Normalized NGF levels were compared by ANOVA followed by Tukey test. Asterisk indicates $p < 0.05$.

for each group (fig. 3, A and B). Results could be directly inferred from the urothelial uptake of OND facilitated by liposomes (fig. 3, A), while the effect on detrusor NGF levels may be explained by the downstream effect of NGF expressed in the urothelium.²

NGF mRNA levels were measured in controls. NGF transcript levels in the urothelium were around 25% higher than in the detrusor (data not shown). Tissue damage from 3-hour exposure to AA during CMG hindered successful isolation of mRNA from the bladder tissue of treated groups, except for the control and antisense OND treated groups. A significant reduction in NGF transcript levels was observed in rats treated with NGF antisense OND complexed with liposomes compared to controls ($p < 0.05$, fig. 3, C).

Effect on Downstream

Effectors of NGF Signaling Pathway

Measurement of tissue lysates from different groups revealed that AA exposure increased the expression of chemokines activated by NGF, including sICAM-1, sE-selectin, CXCL-1 and 10, leptin, MCP-1 and VEGF (figs. 4 and 5). sE-selectin and sICAM-1 are adhesion molecules expressed by chemokine activated endothelial cells to mediate leukocyte and lymphocyte adhesion. The involvement of NGF in chemokine expression was shown by an increase in chemokines with scrambled OND and a significant reduction with liposomal NGF antisense (ANOVA followed by the Tukey test $p < 0.05$, $p < 0.01$ or $p < 0.001$). Except for the high leptin levels in sham treated rats, the levels of other chemokines were highest for scrambled OND. AA induced over expression of other chemokines, including sICAM-1, sE-selectin, CXCL-1 and 10, VEGF and MCP-1, was further increased in the scrambled OND treated group vs the sham treated group. This did not rule out any nonspecific induction of chemokine expression by scrambled OND.

When NGF levels were plotted against chemokine levels regardless of treatment group, we noted a positive association with VEGF (Pearson $r = 0.75$, $p < 0.001$) and a negative association with sICAM-1 (Spearman $r_s = -0.5$, $p < 0.01$, fig. 3, A and B). Separate analysis of higher chemokine levels in the scrambled OND group revealed that, apart from VEGF, MCP-1 also positively correlated with NGF ($r = 0.88$, $p < 0.01$).

NGF Immunostaining

Immunoreactivity for NGF (green staining) was noted in the detrusor region in all groups and it was absent in the apical cells of bladder mucosa containing urothelium untreated with AA (fig. 6, A). NGF immunoreactivity appeared to increase several fold in the detrusor. Its presence was distinctly identified in mu-

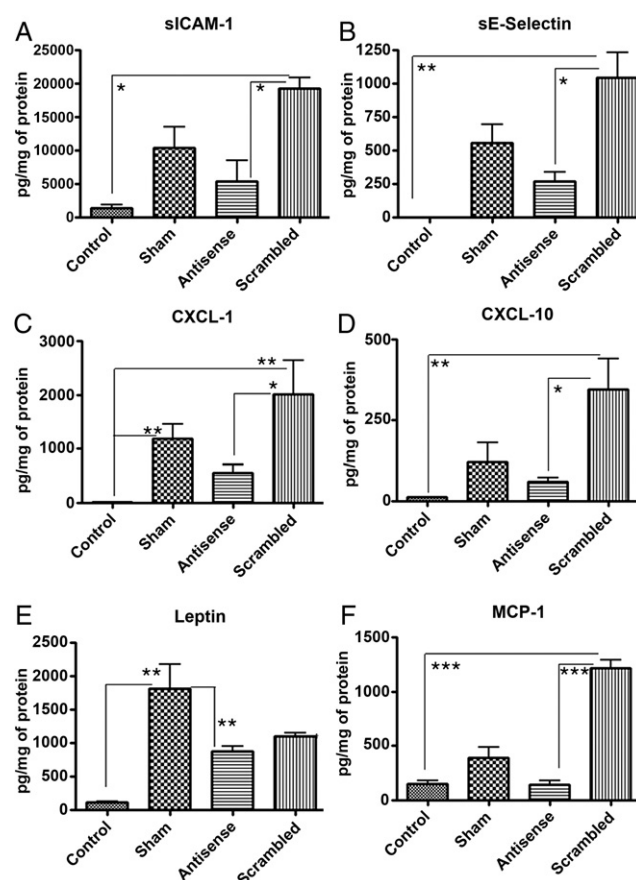


Figure 4. Effect of antisense OND on chemokines activated by NGF signaling pathway. NGF antisense OND significantly reduced chemokine over expression (A to F). Except for leptin, which was highest in sham treated rats (*Sham*), chemokine levels were highest in rat bladders instilled with scrambled sequence OND complexed with liposomes. Bars represent mean $\pm$ SEM. Significance of differences was determined vs sham or scramble treated rats by ANOVA followed by Tukey test. Single asterisk indicates $p < 0.05$. Double asterisks indicate $p < 0.01$. Triple asterisks indicate $p < 0.001$.

cosa containing urothelium after AA infusion in the sham treated group (fig. 6, C). AA induced NGF immunoreactivity in the urothelium was also noted in rats instilled with scrambled OND (fig. 6, B). In contrast, NGF immunoreactivity in the urothelium, including a subpopulation of suburothelial cells, was decreased to levels in controls by pretreatment with liposomal antisense OND (fig. 6, A and D). Control sections incubated in the absence of primary or secondary antibody were evaluated for specificity or background staining. In the absence of primary antibody, no positive immunoreactivity was observed.

DISCUSSION

The bladder is presumed to be the tissue source responsible for increased NGF in the urine of pa-

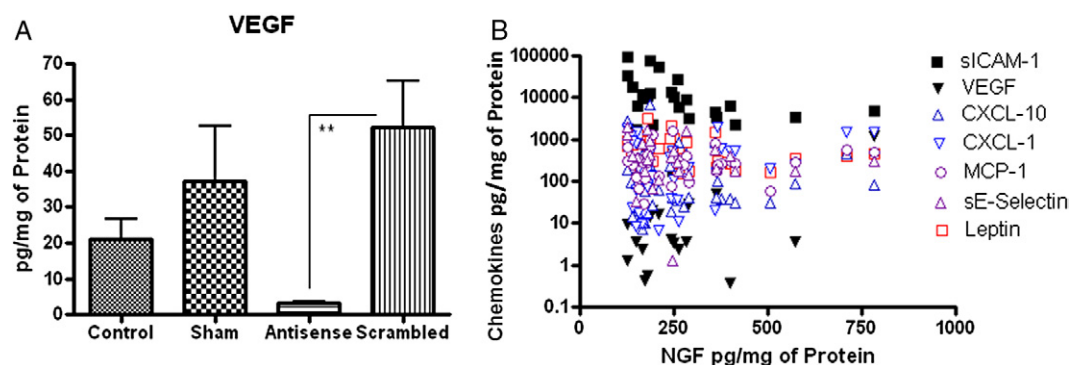


Figure 5. Antisense OND effect on VEGF (A) and NGF plotted with chemokines regardless of treatment group (B). VEGF downstream expression was significantly reduced by antisense OND (A). Decreased VEGF was positively associated with NGF across all treatment groups (Pearson $r = 0.75$, $p < 0.001$) (B). sICAM-1 was negatively associated with NGF levels regardless of treatment group (Spearman $r_s = -0.5$, $p < 0.01$). When only NGF in scrambled group was plotted against respective chemokines, MCP-1 together with VEGF positively correlated with NGF. Asterisks indicate Pearson $r = 0.88$, $p < 0.01$ (A).

tients with OAB or interstitial cystitis/painful bladder syndrome compared to controls.¹⁵ Since NGF over expression in the bladder is implicated as the mediator of symptoms associated with OAB, NGF can be blocked directly by antibodies⁶ or indirectly

by halting the translation of NGF mRNA with sequence specific gene silencing (antisense).

The primary impediment to developing intravesical antisense therapy is inefficient bladder uptake of OND across the urothelium. Bladder uptake of fluorescent OND without liposomes is deficient due to poor intracellular passage of OND across the urothelium (fig. 1, A). Uptake of anionic OND is probably limited by the size of the OND and the charge interaction with the urothelial anionic glycosaminoglycan layer. Our prior studies of liposome interaction with urothelial cells showed liposome adsorption and endocytosis.¹⁶ Cationic liposomes were successfully used to deliver siRNA after intravesical administration in the murine bladder.¹⁷ The current study supports the use of cationic liposomes as an OND carrier.

The rapid increase in NGF protein levels noted in sham treated rats after AA exposure could be blunted by pretreatment with NGF antisense OND (figs. 3 and 6, C). Down-regulation of NGF mRNA expression is in agreement with decreased protein levels and suppressed NGF-like immunoreactivity in the urothelium (figs. 3, C and 6, D). CMG data, NGF levels and chemokine suppression together support our hypothesis that NGF released from the urothelium is an important chemical mediator responsible for changes in bladder function (figs. 2 to 6).¹⁸

Rats pretreated with scrambled OND were devoid of any functional treatment response on CMG or any chemokine suppression (figs. 2, 4 and 5). However, scrambled OND was associated with variable but insignificantly reduced NGF protein compared to sham treated levels (fig. 3, A and B). The disparity in chemokine and NGF levels in the treated groups indicates interaction between chemokine and NGF

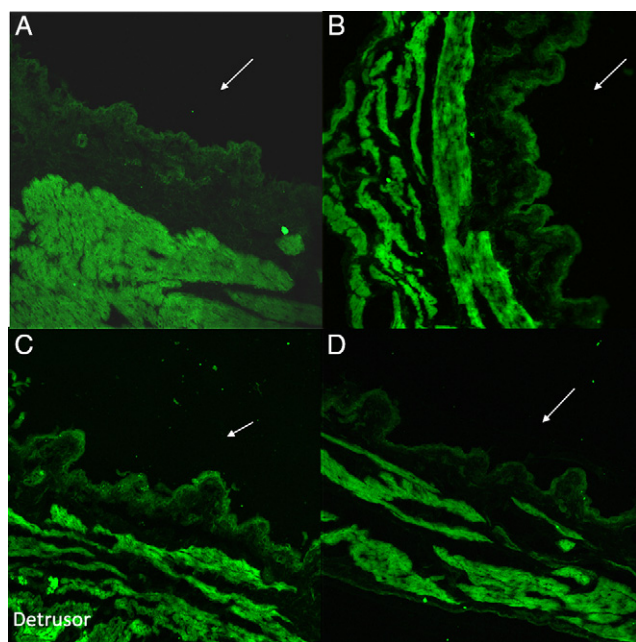


Figure 6. Representative immunofluorescence labeling in rat bladder cross sections reveals that density of NGF immunoreactivity (green areas) was highest in detrusor in all groups. It increased after AA treatment in accordance with NGF expression in muscle. NGF immunoreactivity in bladder mucosa containing urothelium cell layer was absent in rats untreated with AA (A). It was noted in sham (C) and scrambled OND (B) treated rats. NGF immunoreactivity in urothelial cell layer was reduced in rats treated with antisense OND complexed with liposomes (D) to levels comparable to controls (A). Reduced from $\times 20$.

expression in the bladder after intravesical AA infusion. Increased chemokine expression with scrambled OND indicates aggravated tissue inflammation, which may cause mRNA and protein decay. mRNA degradation may partially explain the inability to perform mRNA analysis in some groups, although it is unclear whether protein degradation had a role in the reduced NGF protein levels in different groups (fig. 3). Future studies in animals that over express the NGF gene may clarify the disparity in NGF and chemokine levels caused by antisense OND.

Increased NGF expression in the urothelium and detrusor combined with dense NGF immunoreactivity in the detrusor confirmed them as 2 major sources of bladder NGF (figs. 3, A and B, and 6).¹⁹ Considering our bladder uptake data (fig. 1), we cannot rule out the modulation of detrusor NGF levels by freshly produced NGF in the urothelium.¹⁰ NGF immunoreactivity in the absence of AA exposure may be due to receptor bound NGF that is not available to act on the detrusor. The decrease in NGF protein levels and immunoreactivity after antisense treatment is similar to the decreased urinary excretion of NGF protein in patients with OAB after antimuscarinic or Botox® therapy.^{11,20}

It was previously reported that BO caused by exposure to irritants (turpentine), akin to the AA induced BO that we studied, involves a rapid increase in the bladder content of NGF, which occurs within 2 hours of irritant exposure.²¹ Later studies showed that within 30 minutes of exposure to insults such as lipopolysaccharide, the bladder responds by up-regulating the genes of NGF and sE-selectin, and the receptor of MCP-1.¹⁰ These earlier results agree with our findings of the reported increase in the protein levels of NGF, MCP-1, sE-selectin, sICAM-1, leptin, CXCL-1 and 10, and VEGF in bladder tissue after 3-hour AA exposure (figs. 3 to 5).

NGF is a paracrine messenger involved in physiological and pathological signaling² that activates several downstream effectors to manifest signaling changes.^{7,8,10,14} Chemokines are one of several downstream effectors activated by NGF.^{7,8,10,14} Interestingly, chemokine receptors are widely expressed in neural and nonneural elements of the nociceptive pathways responsible for visceral and somatic pain sensation.²² MCP-1²³ and CXCL-10²⁴ are constitutively expressed in neurons (fig. 4, D and F), where they participate in the excitability of primary afferent neurons via transactivation of transient receptor channels and nociceptor sensitization.²⁴ Chemokine localization in neuron synaptic vesicles is consistent with their ability to act as excitatory neurotransmitters after AA exposure.²⁵

It was recently reported that NGF binding to its high affinity TrkA receptor controls sICAM-1 expression on target cells¹⁴ and inhibition of NGF expression significantly down-regulates ICAM-1 expression.¹⁴ The negative association of NGF with sICAM-1 in all treatment groups corroborates the reported regulating effect of NGF on sICAM-1 expression (figs. 4, A and 5, B).¹⁴ The reported activation of VEGF expression by NGF⁸ is consistent with the positive association of VEGF and NGF levels in all treatment groups (fig. 5, B).

AA exposure is not known to selectively induce NGF expression and the involvement of other mediators, such as prostaglandins,²⁶ in the BO model tested cannot be ruled out. The role of prostaglandins in AA induced BO may explain the variable expression of chemokines in the different groups. Prostaglandins induce mRNA coding for CXCL-1²⁷ and MCP-1,²⁸ while at the same time decreasing leptin mRNA levels (fig. 4, C, E and F).²⁸ NGF dependent leptin expression⁷ is presumed to emerge from bladder adipocytes associated with afferent neurons.²⁹ Compared to the short biological half-life of prostaglandins, chemokines are long acting downstream effectors and may be better suited to track treatment response in tissue or urine.^{9,30}

Taken together, our data support the hypothesis that increased bladder NGF content after AA irritation can be blocked by local instillation of antisense OND complexed with liposomes. These observations are consistent with the presumed role of NGF in OAB symptoms.²⁰ However, to our knowledge it remains to be determined how NGF expression blockade in the urothelium affects the excitability of bladder afferents leading to BO. Future steps in the drug development of this strategy will be the duration of effect and the effect in other models of bladder irritation and overactivity.

CONCLUSIONS

NGF down-regulation as novel treatment for BO also suppresses the downstream signaling cascades activated by NGF leading to reduced chemokine expression. The intravesical route is the most appropriate choice for anti-NGF therapy since the bladder is the putative source of NGF responsible for increased C-fiber afferent nerve excitability and BO. Liposomes represent a delivery platform for the local delivery of antisense based therapy that may avoid systemic toxicity.

ACKNOWLEDGMENTS

OND was made at Integrated DNA Technologies, San Diego, California.

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Hyperexcitability of Bladder Afferent Neurons Associated with Reduction of Kv1.4 α -Subunit in Rats with Spinal Cord Injury

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Abbreviations and Acronyms

Dil = 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DRG = dorsal root ganglion
DTX = α -dendrotoxin
FITC = fluorescein isothiocyanate
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
 K_A = A-type K^+
 K_{DR} = delayed rectifier-type K^+
Kv = voltage-gated K^+
NDO = neurogenic detrusor overactivity
PBS = phosphate buffered saline
PCR = polymerase chain reaction
SCI = spinal cord injury
TTX = tetrodotoxin
 V_h = half-maximal conductance

Purpose: To clarify the functional and molecular mechanisms inducing hyperexcitability of C-fiber bladder afferent pathways after spinal cord injury we examined changes in the electrophysiological properties of bladder afferent neurons, focusing especially on voltage-gated K channels.

Materials and Methods: Freshly dissociated L6-S1 dorsal root ganglion neurons were prepared from female spinal intact and spinal transected (T9-T10 transection) Sprague Dawley® rats. Whole cell patch clamp recordings were performed on individual bladder afferent neurons. Kv1.2 and Kv1.4 α -subunit expression levels were also evaluated by immunohistochemical and real-time polymerase chain reaction methods.

Results: Capsaicin sensitive bladder afferent neurons from spinal transected rats showed increased cell excitability, as evidenced by lower spike activation thresholds and a tonic firing pattern. The peak density of transient A-type K^+ currents in capsaicin sensitive bladder afferent neurons from spinal transected rats was significantly less than that from spinal intact rats. Also, the K_A current inactivation curve was displaced to more hyperpolarized levels after spinal transection. The protein and mRNA expression of Kv1.4 α -subunits, which can form transient A-type K^+ channels, was decreased in bladder afferent neurons after spinal transection.

Conclusions: Results indicate that the excitability of capsaicin sensitive C-fiber bladder afferent neurons is increased in association with reductions in transient A-type K^+ current density and Kv1.4 α -subunit expression in injured rats. Thus, the Kv1.4 α -subunit could be a molecular target for treating overactive bladder due to neurogenic detrusor overactivity.

Key Words: urinary bladder, overactive; spinal cord injuries; potassium channels, voltage-gated; afferent pathways; nerve fibers, unmyelinated

SPINAL cord injury above the lumbosacral level eliminates voluntary and supraspinal control of voiding, leading initially to areflexic bladder and urinary retention, followed by the slow development of automatic micturition

and NDO mediated by spinal micturition reflex pathways.¹ Electrophysiological studies in animal models revealed that excitability of the afferent limb of the micturition reflex is increased after SCI, which was

Accepted for publication July 19, 2013.
Study received University of Pittsburgh institutional animal care and use committee approval.

Supported by National Institutes of Health Grants P01DK093424, DK088836, DOD W81XWH-11-1-0763 and PVA 2793.

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proposed as an important pathophysiological basis of NDO with SCI.¹

Bladder afferent pathways consist of 2 types of axons, ie A δ and C fibers. In the normal condition A δ fibers, which are mechanosensitive and respond to bladder distention, initiate the micturition reflex. In chronic SCI rats capsaicin sensitive C-fiber afferents show increased excitability² and must be responsible for initiating NDO because desensitizing C-fiber afferents by systemic capsaicin administration suppresses nonvoiding contractions in SCI rats.¹

Mechanisms underlying the hyperexcitability of C-fiber bladder afferent pathways after SCI were previously investigated using whole cell patch clamp recordings in DRG neurons innervating the rat bladder.² The density of TTX sensitive Na⁺ currents in bladder afferent neurons significantly increased, while TTX resistant Na⁺ current density decreased after SCI. This indicated that SCI induces a switch in the expression of Na⁺ channels from the TTX resistant to the TTX sensitive type. Since TTX sensitive Na⁺ currents have a lower threshold for action potential activation than TTX resistant currents, it is assumed that these changes in the expression of Na⁺ channels in bladder afferent neurons after SCI contribute to a low threshold for spike activation in these neurons.

Although Na⁺ channels are a major determinant of neuronal excitability, Kv channel activation is also an important factor for spike threshold and firing frequency control. Kv currents in sensory neurons are divided into 2 major categories, ie sustained K_{DR} and transient K_A currents.³ A reduction in K_{DR} and/or K_A currents is involved in the hyperexcitability of afferent pathways under various pathological conditions,⁴ including chronic bladder inflammation, which increases the excitability of capsaicin sensitive bladder DRG neurons by decreasing K_A currents without affecting K_{DR} currents.^{5,6}

However, little is known about functional changes in Kv currents after SCI. In addition, to our knowledge the molecular mechanism responsible for changes in Kv currents after SCI remains to be elucidated. Therefore, we sought to clarify the mechanisms inducing bladder afferent neuron hyperexcitability in SCI rats, especially focusing on Kv channels.

MATERIALS AND METHODS

Animal Preparation

Experiments were performed in spinal intact and spinal transected adult female Sprague Dawley rats weighing 170 to 220 gm. All animal experiments were done in accordance with institutional guidelines and approved by

the University of Pittsburgh institutional animal care and use committee.

Spinal cord transected rats were prepared by complete transection of the Th8-Th9 spinal cord, as previously described.² The population of DRG neurons that innervates the bladder was labeled by retrograde axonal transport of the fluorescent dye Fast Blue (1% weight per volume, PolyScience®) or DiI (1% weight per volume, Invitrogen™). Dye was injected in the bladder wall in isoflurane anesthetized animals 7 to 10 days before dissociation, as described in our previous study.⁷

Cell Dissociation and Whole Cell Patch Clamp Recordings

Four weeks after spinal cord transection dissociated L6-S1 DRG cells were prepared as previously described.⁷ Since 80% of C-fiber bladder afferent neurons are sensitive to capsaicin but only 5% of A δ -fiber bladder neurons are capsaicin sensitive,⁸ capsaicin sensitive neurons were selected for C-fiber population evaluation. Whole cell patch clamp recordings were performed at room temperature (20°C to 22°C) on each Fast Blue positive neuron within 10 hours after dissociation. The internal solution contained 140 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 11 mM EGTA, 10 mM HEPES and 2 mM Mg adenosine triphosphate, adjusted to pH 7.4 with KOH. Patch electrodes had 2 to 4 M Ω resistance when filled with the internal solution. Neurons were superfused at a flow rate of 2.0 ml per minute with an external solution containing 150 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES and 10 mM D-glucose, adjusted to pH 7.4 with NaOH.

To isolate K⁺ currents after evaluating action potential characteristics we changed the external solution to one containing 150 mM choline Cl, 5 mM KOH, 0.03 mM CaCl₂, 10 mM HEPES, 3 mM Mg(OH)₂ and 10 mM D-glucose, adjusted to pH 7.4 with tris base. Slow K_A current inactivation and activation characteristics were examined using the same membrane voltage paradigm described in our previous study.⁷

Immunohistochemistry

Spinal intact and transected rats were anesthetized with pentobarbital (80 mg/kg) intraperitoneally. They were perfused through the left ventricle with 300 ml cold oxygenated PBS, followed by fixative solution consisting of 4% paraformaldehyde in 0.1 M PBS. L6 DRGs were then removed and postfixed for 12 hours in the same fixative solution. Tissues were placed in PBS containing increasing concentrations of sucrose (10%, 20% and 30%) at 4°C for cryoprotection, frozen in mounting medium and sectioned at 10 μ m. After mounting on slides the sections were washed and incubated with antibodies for Kv1.2 or Kv1.4 α -subunits (Alomone Labs, Jerusalem, Israel) for 18 hours at 4°C, followed by incubation with anti-rabbit IgG antibody conjugated to FITC for 1.5 hours at room temperature. Images were obtained with a fluorescence microscope. We confirmed that there was no positive staining above background when primary antibody was omitted (data not shown).

In 6 randomly selected DRG sections from each of 3 rats we measured FITC fluorescence intensity in

individual neurons using ImageJ (<http://rsbweb.nih.gov/ij/>). The mean labeling intensity of Kv α -subunits was calculated in dye labeled bladder afferent neurons and in unlabeled DRG neurons. We determined the ratio of mean labeling intensity of bladder afferent neurons to that of unlabeled neurons in each DRG section. The staining density ratio (dye labeled vs unlabeled cells) per section was then averaged in randomly selected DRG sections from each rat. The mean ratio in each rat was again averaged in the spinal intact or spinal transected groups. These analytical methods of Kv α -subunit staining were used to minimize variations in staining intensity between different DRG sections, which might occur due to different staining conditions and nonlinear fluorescent signal decay among sections.

Laser Capture Microdissection and Real-Time PCR

Using isoflurane anesthesia, L6 DRGs were removed in a separate group of 5 spinal intact and 5 transected rats that received DiI injection in the bladder wall 1 week earlier. L6 DRGs were embedded in Tissue-Tek® O.C.T.™ Compound and stored at −80C until use. Samples were sectioned at 8 μ m and sections were mounted on PEN membrane slides (Leica Microsystems, Wetzlar, Germany). The tissue was air-dried. Laser capture microdissection was performed using an LMD6000 (Leica Microsystems) to separately dissect DiI labeled and unlabeled bladder afferent neurons. Excised cells were individually captured in the caps of 0.5 ml Eppendorf tubes and lysed. RNA isolation, reverse transcription and real-time PCR were performed using a Cells Direct™ One-Step qRT-PCR Kit.

Gene specific primers and TaqMan® probes crossing exon-exon junctions were designed for the Kv1.2 and Kv1.4 α -subunits using Primer3 (<http://primer3.sourceforge.net/>) (table 1). Probes contained FAM fluorophore and TAMRA quencher. Primer-probe combinations were optimized within suitable ranges for efficiency and correlation coefficients using standard curve dilutions. Data output was done on a StepOnePlus™ thermocycler. cDNA was amplified under the conditions of 1 cycle at 50C for 45 minutes and 95C for 2 minutes, followed by 50 cycles at 95C for 15 seconds and 60C for 1 minute. Reactions were analyzed in triplicate and normalized relative to GAPDH.

Real-time PCR data were analyzed by the difference in CPs (crossing points) method using the equation, $R = 2^{(CP_{sample} - CP_{control})}$, where R represents the expression ratio of each target gene relative to that of GAPDH. We also determined specificity to cDNA using real-time PCR to verify that our primer-probe sets did not amplify genomic DNA. All primer-probe combinations showed 100% to 104% efficiency (table 1).

Statistical Analysis

Data are shown as the mean $\pm$ SEM. The unpaired Student t-test was used to determine statistical differences between 2 groups with $p < 0.05$ considered significant.

RESULTS

Spinal Transected Rats

Increased excitability of bladder afferent neurons.

Figure 1 shows representative recordings of action potentials in capsaicin sensitive bladder afferent neurons from the 2 groups of rats. The resting membrane potential of capsaicin sensitive bladder afferent neurons did not differ between spinal intact and transected rats (table 2). However, the mean threshold for eliciting action potentials from spinal transected rats was significantly lower than that from spinal intact rats. Also, the number of action potentials during 800-millisecond membrane depolarization in capsaicin sensitive bladder afferent neurons from spinal transected rats was significantly greater than that in neurons from spinal intact rats when current intensity was set to the value just above the threshold for inducing spike activation with a 50-millisecond pulse (table 2). These results indicate that capsaicin sensitive bladder afferent neurons became hyperexcitable in SCI rats. In addition, the diameter and cell input capacitance of capsaicin sensitive bladder afferent neurons from spinal transected rats were significantly greater than those from spinal intact rats, indicating that spinal transection induced somal hypertrophy of bladder afferent neurons, as previously noted.^{2,9}

Slow K_A current reduction. We estimated the density of slow K_A currents by measuring the difference in currents activated by a depolarizing voltage pulse from −120 and −40 mV holding potentials (fig. 2, A and B). This method is useful because our previous studies showed that slow K_A currents in C-fiber bladder afferent neurons were activated by depolarizing voltage steps from hyperpolarized membrane potentials but they were almost completely inactivated when membrane potential was maintained at a depolarized level of greater than −40 mV.^{5,10}

We calculated the peak density of slow K_A currents evoked by depolarization to 0 mV from the

Table 1. Primer and TaqMan probe sequences

	Kv1.2	Kv1.4	GAPDH
Primer:			
5'	CCTGTCTATCACCCAGGAACA	TGACCTACTGCCACAGGATG	AGACAGCCGCATCTCTTGT
3'	TTGAGGAGAGTGGAGCTTGG	GGTTTCGAAGCGTAGACCA	GATACGGCCAAATCCGTT
Probe	CATCTGCAAGGGCAACGTCACA	CAGTGACTGTTGTGAACGCTGGT	GCAGTGCCAGCCTCGTCTCA
% Efficacy	99.8	104.4	104.0

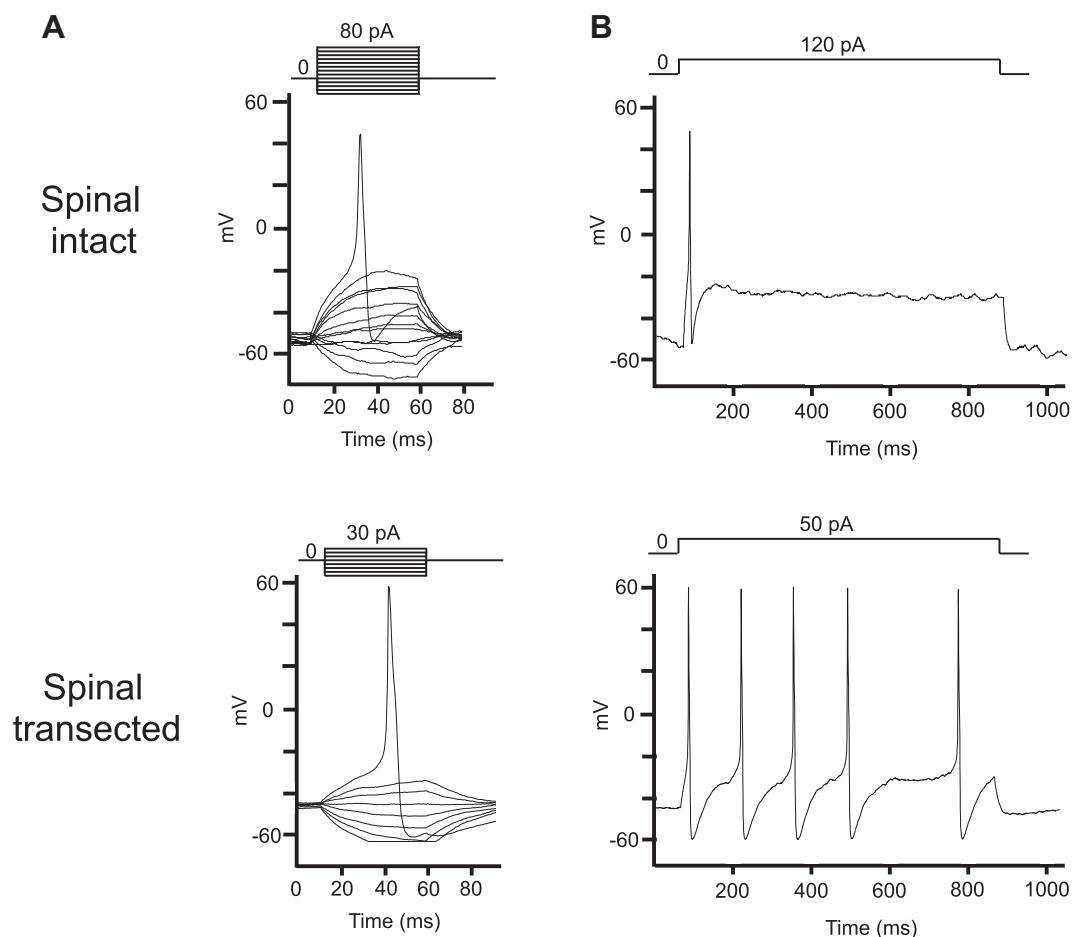


Figure 1. Representative action potential recordings in capsaicin sensitive bladder afferent neurons from spinal intact and transected rats. *A*, action potentials evoked by 50-millisecond (*ms*) depolarizing current pulses injected through patch pipettes during current clamp recording. *B*, firing patterns during sustained 800-millisecond membrane depolarization.

difference in K^+ currents activated from holding potentials of -40 and -120 mV, and sustained K_{DR} currents evoked by depolarization to 0 mV from a holding potential of -40 mV. Density was increased during membrane depolarization in capsaicin sensitive bladder afferent neurons from spinal intact and transected rats (fig. 2, *C*). However, slow K_A peak current density was lower in neurons from spinal transected rats than those from spinal intact rats. We detected significant differences in current density at depolarizing pulses greater than -10 mV. However, the peak current density of sustained K_{DR} currents in capsaicin sensitive bladder afferent neurons did not differ between spinal intact and transected rats.

Bladder Afferent Neurons

Steady-state activation and inactivation characteristics of K_A currents in spinal intact and spinal transected rats. K_A current in spinal intact rats started to inactivate at membrane potentials positive to -120 mV and were almost totally inactivated by

depolarizing prepulses to -40 mV. Data were well fitted by the modified Boltzmann equation with a V_h of -87.1 mV (8 cells from 6 rats). This inactivation curve indicated that 10% to 15% of maximum

Table 2. Electrophysiological properties of capsaicin sensitive bladder afferent neurons

	Control	SCI
No. cells/rats	17/13	19/13
Mean $\pm$ SEM diameter (μ m)	25.8 ± 0.6	$32.5 \pm 0.9^*$
Mean $\pm$ SEM input capacitance (pF)	23.2 ± 1.4	$45.0 \pm 3.4^*$
Mean $\pm$ SEM membrane potentials (mV):		
Resting	-47.8 ± 1.5	-46.8 ± 1.0
Spike threshold	-21.8 ± 0.9	$-26.4 \pm 1.3^*$
Peak	38.0 ± 2.1	40.8 ± 2.1
Mean $\pm$ SEM spike duration (msecs)	4.3 ± 0.2	4.2 ± 0.3
Mean $\pm$ SEM No. 800-msec depolarization action potentials	1.3 ± 0.1	$4.7 \pm 0.7^*$
Density:		
No. cells/rats	20/12	17/10
Mean $\pm$ SEM intensity K_A (pA/pF)	68.6 ± 6.3	$38.1 \pm 4.6^*$
Mean $\pm$ SEM intensity K_{DR} (pA/pF)	54.0 ± 7.0	48.6 ± 6.6

* $p < 0.01$ vs control.

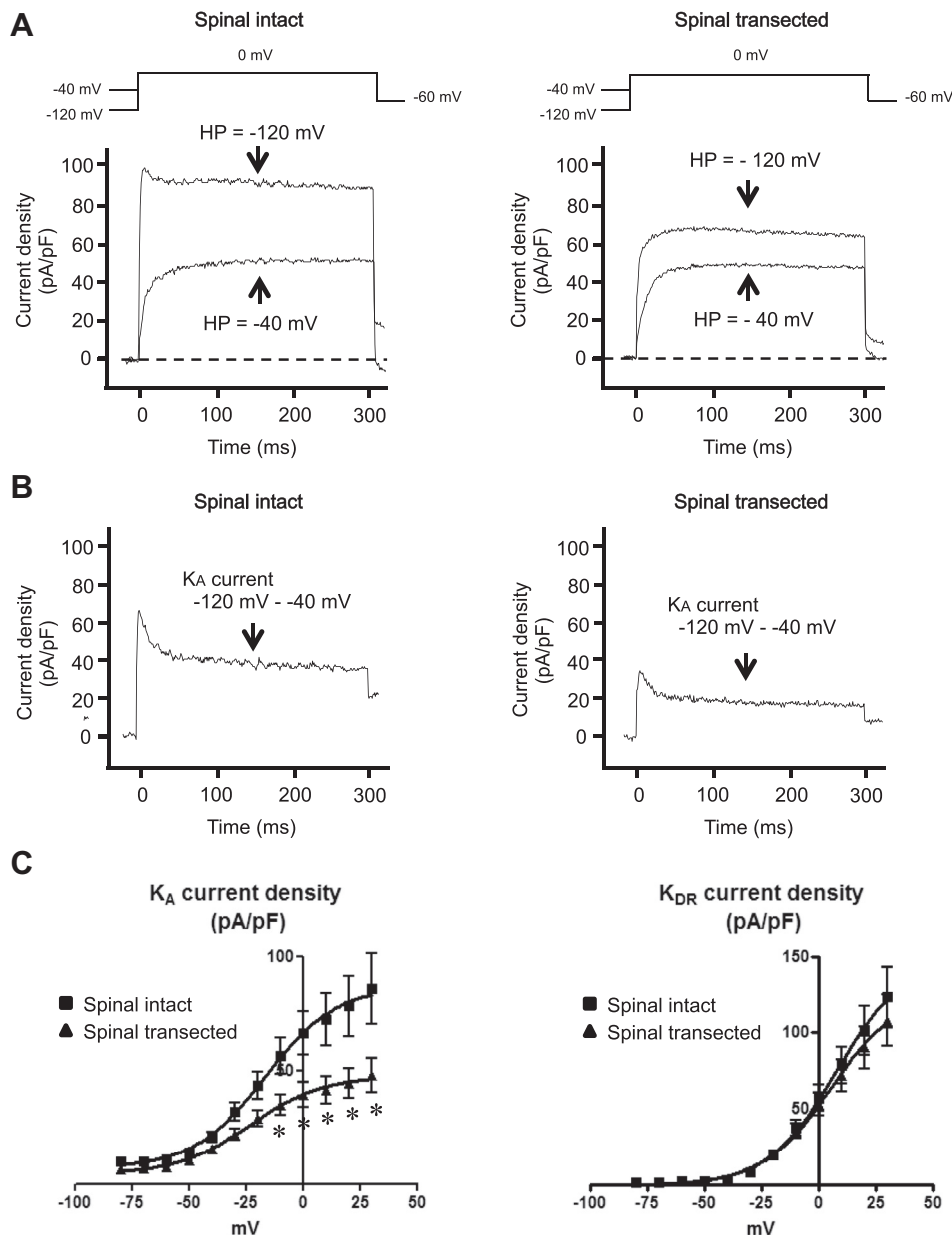


Figure 2. Changes in capsaicin sensitive bladder afferent neuron K^+ currents in rats without vs with spinal cord transection. **A**, representative recordings show superimposed outward K^+ currents evoked by voltage steps to 0 mV from -120 and -40 mV holding potentials (HP). **B**, K_A currents were obtained by subtracting K^+ currents evoked by depolarization to 0 mV from -40 and -120 mV holding potentials. **C**, mean $\pm$ SEM K_A and K_{DR} current-voltage relationships in 20 cells from 12 spinal intact rats and 17 from 10 spinal transected rats. Asterisk indicates $p < 0.05$ vs spinal intact.

current could be elicited at membrane potentials in the range of -60 to -50 mV, equivalent to resting membrane potential (fig. 3, A). In contrast, K_A currents in spinal transected rats were almost negligible when holding membrane potential was in the range of -60 to -50 mV. In contrast to the inactivation curve in neurons from spinal intact rats, the inactivation curve for spinal transected rats was displaced to more hyperpolarized levels by approximately 10 mV. The V_h for K_A current

inactivation in spinal transected rats was -98.3 mV (7 cells from 7 rats) (fig. 3, A).

In contrast to the difference in inactivation characteristics, the voltage dependence of K_A current activation did not differ between spinal intact and transected rats. K_A current in bladder afferent neurons from spinal intact rats was elicited by membrane depolarizations higher than -60 mV with V_h occurring at the membrane potential of -32.2 mV according to the modified Boltzmann

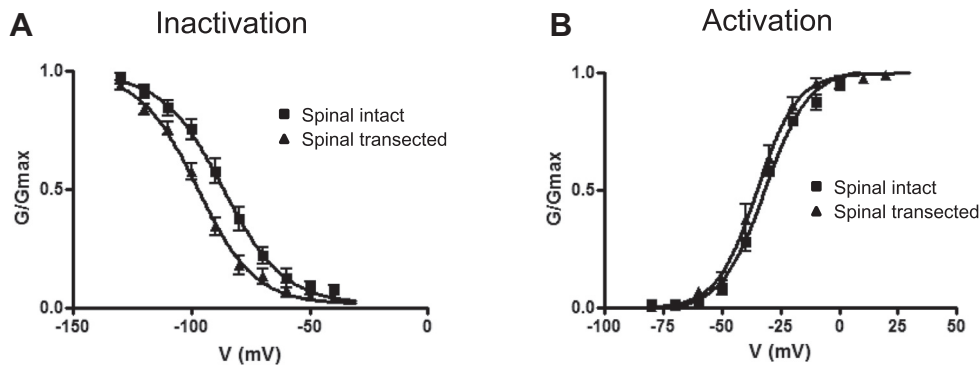


Figure 3. Mean $\pm$ SEM steady-state activation and inactivation characteristics of K_A currents in capsaicin sensitive bladder afferent neurons, shown as relative peak conductance of K_A currents normalized to maximal K_A current conductance (G/G_{max}) plotted against membrane potentials. *A*, inactivation characteristics in 8 cells from 6 spinal intact rats and 7 from 7 spinal transected rats. *B*, activation characteristics in 7 cells from 7 spinal intact rats and 12 from 10 spinal transected rats.

equation (7 cells from 7 rats). Similarly, the V_h of K_A current activation of bladder afferent neurons from spinal transected rats was -35.4 mV (12 cells from 10 rats) (fig. 3, *B*).

Reduction of Kv1.4 α -subunit expression in spinal transected rats. The ratio of Kv1.4 α -subunit staining density in bladder afferent and unlabeled DRG neurons was significantly lower in spinal transected than in spinal intact rats (112 and 132 cells from 3 rats each, 0.79 vs 1.01) (fig. 4, *A*) in which there was similar Kv1.4 α -subunit staining in Fast Blue labeled and unlabeled neurons. In contrast, in each group we noted similar Kv1.2 α -subunit staining density in Fast Blue labeled and unlabeled neurons (121 and 114 cells from 3 spinal intact and 3 spinal transected rats, respectively) (fig. 4, *B*).

Changes in mRNA levels of Kv1.2 and Kv1.4 α -subunits were also examined in laser captured DRG neurons. The relative expression level of Kv1.2 in DiI labeled and unlabeled neurons (30 cells per rat) did not differ between 5 spinal intact and 5 spinal transected rats (fig. 5, *D* and *E*). However, relative Kv1.4 expression was significantly lower (0.53) in DiI labeled bladder afferent neurons (30 cells per rat) from 5 spinal transected rats compared to those from 5 spinal intact rats (fig. 5, *F*). There was no significant difference between the 2 groups in Kv1.4 mRNA levels in unlabeled neurons (fig. 5, *G*).

DISCUSSION

Our results indicate that 1) capsaicin sensitive bladder afferent neurons in spinal transected rats show hyperexcitability, as evidenced by lower spike activation thresholds and tonic firing pattern, 2) slow K_A current density was decreased and the K_A current inactivation curve of spinal transected rats was displaced to more hyperpolarized levels

compared to those of spinal intact rats and 3) protein and mRNA expression of the Kv1.4 but not the Kv1.2 α -subunit was decreased in bladder afferent neurons after SCI. To our knowledge this is the first report of a direct association of functional and molecular changes in the Kv channels responsible for the hyperexcitability of bladder afferent neurons in SCI rats.

Kv currents in sensory neurons are divided into 2 major categories, ie sustained K_{DR} and transient K_A currents.³ Transient K_A currents in sensory neurons, including DRG cells, can be further subdivided into at least 2 subtypes based on inactivation kinetics, ie fast and slow decaying K_A currents.⁴ The slow decaying K_A current is preferentially expressed in small, capsaicin sensitive bladder afferent neurons. A reduction in slow K_A currents by the application of the K_A channel blocker 4-aminopyridine increased the excitability of these neurons, as evidenced by lower spike activation thresholds and tonic firing.^{5,10}

In this study we confirmed that the current density of slow K_A currents was decreased in bladder afferent neurons from spinal transected rats compared to that in spinal intact rats (fig. 2). In addition, the inactivation curve of spinal transected rats was displaced by about 10 mV to more hyperpolarized levels compared to the inactivation curve in spinal intact rats. These results suggest that a reduction in slow decaying K_A currents is a key event resulting in the hyperexcitability of capsaicin sensitive bladder afferent neurons after SCI in rats.

A reduction in slow decaying K_A channel activity was associated with decreased expression of Kv1.4 α -subunit protein and mRNA in bladder afferent neurons. Kv channels are composed of homotetramers or heterotetramers of α -subunits that form K^+ ion conducting pores.¹¹ Previous reports indicated that Kv1 α -subunits, including Kv1.1, Kv1.2

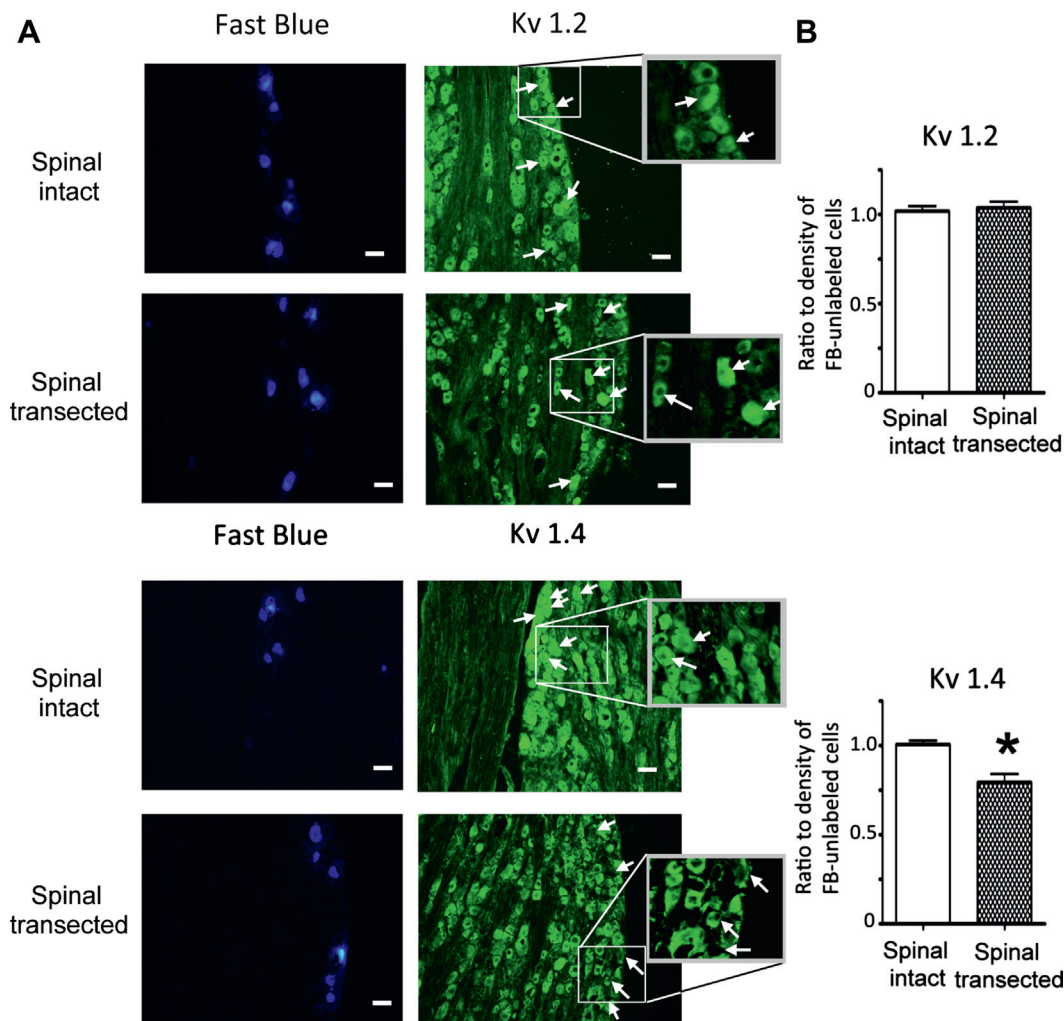


Figure 4. Kv α -subunit immunoreactivity in L6 DRGs after spinal cord transection. **A**, photomicrographs show same Fast Blue, Kv1.2 and Kv1.4 stained sections from spinal intact and transected rats under fluorescence illumination with ultraviolet (blue areas) and FITC (green areas) filters. Arrows indicate neuronal profiles identified by Fast Blue labeling. Rectangles show insets at higher magnification. Scale bars indicate 50 μ m. **B**, mean $\pm$ SEM expression shown as ratio of FITC immunoreactivity intensity in Fast Blue labeled vs unlabeled cells, including Kv1.2 in 121 cells from 3 spinal intact rats and 114 from 3 spinal transected rats, and Kv1.4 in 132 cells from 3 spinal intact rats and 112 from 3 spinal transected rats. Asterisk indicates $p < 0.05$ vs spinal intact.

and Kv1.4, could be major components of Kv channels in DRG neurons.¹² The homotetramer of Kv1.4 α -subunits shows rapid, prominent inactivation processes but it is insensitive to DTX, a specific inhibitor of Kv1.1 and Kv1.2.¹³ The heteromeric channels containing Kv1.4 and DTX sensitive Kv1.1 and Kv1.2 α -subunits show inactivation that is much slower than the Kv1.4 homomeric channels and similar to slow decaying K_A in DRG neurons.¹⁴

We previously reported that DTX partially suppressed slow decaying K_A currents in capsaicin sensitive, small DRG neurons.¹⁵ Therefore, it seems reasonable that the assembly of Kv1.4 with other DTX sensitive Kv α -subunits, such as Kv1.1 and/or Kv1.2, contributes to the formation of slow decaying K_A channels. Also, decreased Kv1.4 α -subunit expression after SCI might be a molecular

mechanism responsible for the reduction in slow decaying K_A currents, leading to hyperexcitability of capsaicin sensitive C-fiber bladder afferent neurons.

We previously reported that SCI induces a switch in the expression of Na^+ channels from the TTX resistant to the TTX sensitive type.² In contrast to neurons from spinal intact rats, in which approximately 70% of bladder afferent neurons show high threshold TTX resistant action potentials, 60% of bladder afferent neurons from SCI rats show low threshold TTX sensitive action potentials.² We also previously reported that applying the K_A channel blocker 4-aminopyridine significantly decreased the spike threshold and increased the firing number during sustained membrane depolarization.⁵ These results suggest that changes in Na^+ channel

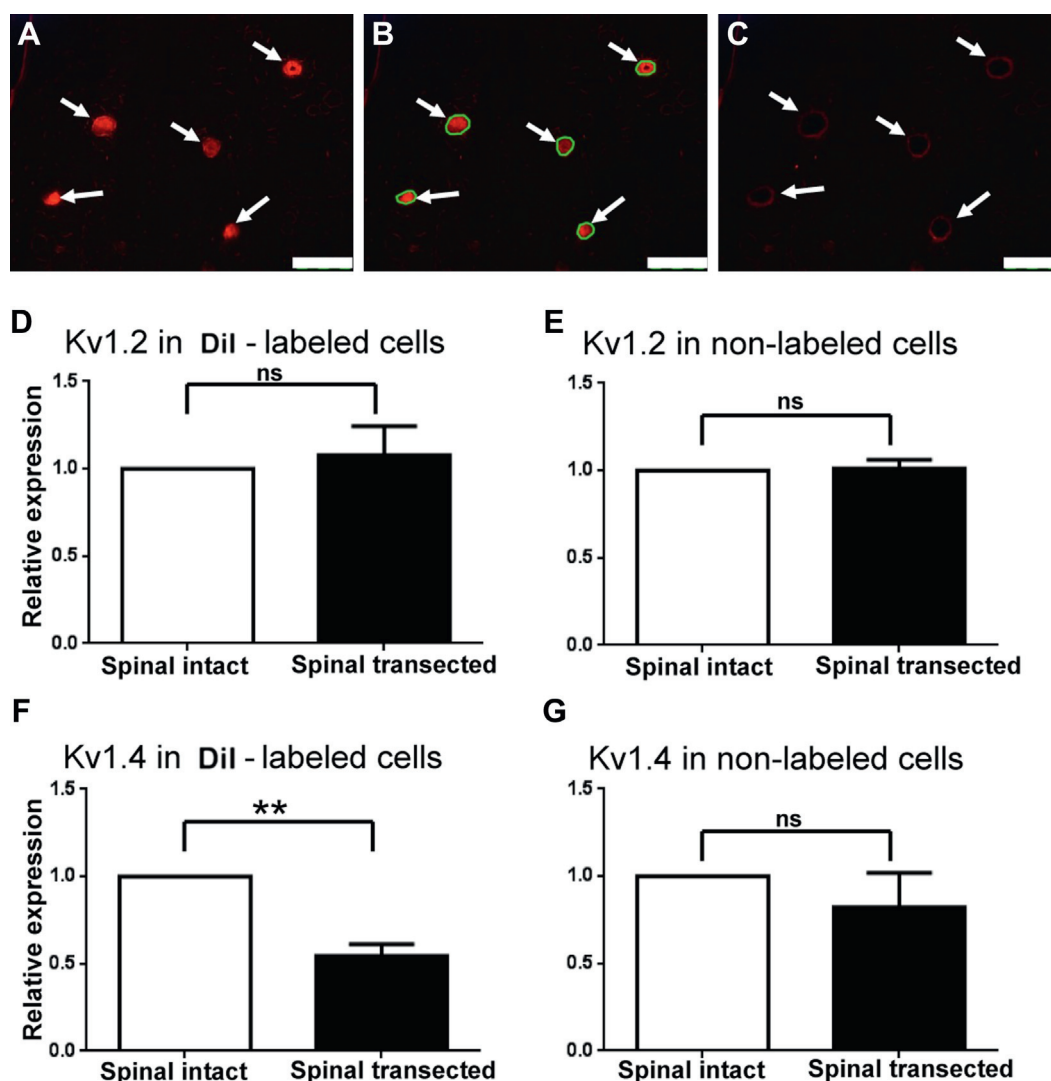


Figure 5. Kv α -subunit mRNA expression after spinal cord transection. A to C, photomicrographs show single L6 DRG section during laser capture microdissection of Dil labeled bladder afferent neurons. Arrows indicate neurons positively stained with Dil. Scale bars represent 100 μ m. A, before microdissection. B, before microdissection. Green circles indicate laser captured areas. C, after microdissection. D to G, mean $\pm$ SEM levels in 30 neurons from each of 5 spinal intact and 5 spinal transected rats. ns, not significant. Asterisks indicate $p < 0.01$ vs spinal intact.

property contribute to a lower spike activation threshold and changes in K^+ channel property contribute to a lower threshold for spike activation and tonic firing pattern in these neurons. Therefore, it is reasonable that changes in the expression of Na^+ channels and K^+ channels in bladder afferent neurons after SCI contribute to the hyperexcitability of C-fiber afferent pathways.

CONCLUSIONS

The current study provides direct evidence that the excitability of capsaicin sensitive C-fiber bladder afferent neurons is increased in association with decreases in K_A current density and Kv1.4 α -subunit expression in SCI rats. Thus, the Kv1.4 α -subunit could be a molecular target for treating overactive bladder due to neurogenic detrusor overactivity.

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Review Article

Intravesical Liposome and Antisense Treatment for Detrusor Overactivity and Interstitial Cystitis/Painful Bladder Syndrome

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Received 18 July 2013; Accepted 24 October 2013; Published 16 January 2014

Academic Editors: S. Mingmalairak, T. W. Stone, and S.-N. Wu

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Purpose. The following review focuses on the recent advancements in intravesical drug delivery, which brings added benefit to the therapy of detrusor overactivity and interstitial cystitis/painful bladder syndrome (IC/PBS). **Results.** Intravesical route is a preferred route of administration for restricting the action of extremely potent drugs like DMSO for patients of interstitial cystitis/painful bladder syndrome (IC/PBS) and botulinum toxin for detrusor overactivity. Patients who are either refractory to oral treatment or need to mitigate the adverse effects encountered with conventional routes of administration also chose this route. Its usefulness in some cases can be limited by vehicle (carrier) toxicity or short duration of action. Efforts have been underway to overcome these limitations by developing liposome platform for intravesical delivery of biotechnological products including antisense oligonucleotides. **Conclusions.** Adoption of forward-thinking approaches can achieve advancements in drug delivery systems targeted to future improvement in pharmacotherapy of bladder diseases. Latest developments in the field of nanotechnology can bring this mode of therapy from second line of treatment for refractory cases to the forefront of disease management.

1. Introduction

Intravesical therapies have demonstrated varying degrees of efficacy and safety in treatment of interstitial cystitis/painful bladder syndrome (IC/PBS) [1] and overactive bladder OAB [2]. Pharmacotherapy by this route provides high local drug concentrations in the bladder with low risk of systemic side effects [3]. Conventional therapies for OAB and detrusor overactivity (DO), either neurogenic or idiopathic, have limited efficacy and acceptability. Anticholinergic medications, which are currently the mainstay of the treatment of OAB, are not always effective and often have undesirable side effects such as dry mouth and constipation [4]. Therefore, search for alternative therapies directed against local targets with fewer side effects is encouraged.

The IC/PBS syndrome is characterized by pelvic pain and urinary storage symptoms (e.g., urinary urgency and frequency). The O'Leary-Sant symptom and problem score

(interstitial cystitis symptom index (ICSI) and problem index (ICPI)) is recognized as one of the most reliable and valid instruments to identify the extent of bothersome symptoms and the most prominent voiding and painful symptoms in IC/PBS patients [5, 6]. Pentosan polysulfate, PPS, is a synthetic sulphated polysaccharide and is the only approved oral drug for IC/PBS, but it requires daily administration for 6 months before any benefit accrues for the patients [7, 8]. Less than one-tenth of oral dose is excreted into the urine of IC/PBS patients, which is considered to replenish the damaged glycosaminoglycan GAG layer and reduce the influx of potassium back into bladder from urine.

Orally administered agents are often unable to create effective luminal drug concentration due to low urinary excretion of drugs, which justifies bladder instillation. Replacement of GAG layer by intravesical administration of hyaluronic acid has been successfully tried in IC/PBS patients [9]. Intravesical therapy also holds the potential to facilitate

the separation of therapeutic actions from side effects by involving a diverse array of novel chemical, pharmacological, and formulation strategies. However, drug delivery by intravesical route is constrained by the impermeability of the urothelium, short duration of action, and the need for frequent administration.

The urinary bladder lining is the most impermeable barrier in the human body [10, 11]. Therefore, therapies delivered directly to the bladder lumen have limited opportunity for systemic distribution, which typically leads to fewer side effects. In addition, the mechanisms for locally delivered therapies can be independent of existing oral therapies, and in some cases, an additive effect can provide compelling comarketing opportunities [12]. We will review the current understanding of urothelium structure and role of intravesical drug delivery in unmasking the pharmacological function of different receptors expressed on its luminal surface.

2. Urothelium

Recent investigations have revealed that the urothelium is not just a physical barrier between blood and urine but can express a host of receptors having a functional significance in micturition reflex. The recent identification of a cannabinoid, nicotinic, neurokinin receptors and potassium ion channels in urothelium [13–16] have revealed the role of urothelium as an excitable cell layer in bladder that responds to stretch and convey messages to underlying afferents in bladder. There is also mounting evidence to demonstrate expression of adrenergic, bradykinin, and transient receptor potential (TRP) receptors in urothelium and in proximity of afferent nerves [17, 18]. Urothelium is the primary nonneuronal source for the release of molecules such as adenosine triphosphate (ATP), acetylcholine, and nitric oxide, which are known to affect micturition [19, 20]. Intravesical therapy can be used to unravel the pharmacology of these receptors and paracrine messengers released from urothelium [21, 22].

3. Muscarinic Receptors

It is widely accepted that oral antimuscarinics act on muscarinic receptors in the detrusor for managing the symptoms of DO and OAB. Conventional wisdom largely ignores the role of muscarinic receptors expressed on urothelium [23]. Muscarinic receptors expressed on urothelium are believed to be involved in afferent signaling for micturition [23]. The afferent signals are believed to be generated from the basal nonneuronal acetylcholine released during the storage phase from urothelium to enhance the myogenic contractile activity of the detrusor [19, 20].

Theoretically speaking, not only can receptors expressed on the urothelium be influenced by antimuscarinics via the bloodstream, but also few selected antimuscarinics and their active metabolites can affect the muscarinic receptors from the luminal side following their excretion into urine [12]. Alternative mode of action for two antimuscarinic drugs, trospium and solifenacin, was demonstrated by our group [21, 22]. DO in the rat was mimicked by intravesical carbachol

[21, 22]. Urine collected from human volunteers who took trospium and solifenacin was then instilled into rat bladder to determine the effect of the drug fraction excreted into urine. Therefore, intravesical therapy can assist in elucidating the yet unexplored mechanisms for improvement of OAB symptoms by antimuscarinics.

4. Liposomes

Liposomes were earliest prototype of nanoparticles (particles with one of the dimensions in nanometers) that are described as lipid vesicles composed of concentric phospholipid bilayers enclosing an aqueous interior [24, 25]. The lipid vesicles comprise either one or several aqueous compartments delineated by either one (unilamellar) or several (multilamellar) phospholipid bilayers [26]. Liposomes have been widely studied as drug carriers for a variety of chemotherapeutic agents (approximately 40,000 scientific articles have been published on the liposomes use so far) [25, 27]. Liposomes improved the delivery of chemotherapeutic agents by altering pharmacokinetics and reducing toxicity [26, 28, 29]. The success of liposomes in the clinic has been attributed to the nontoxic nature of the lipids used in their formulation.

5. Empty Liposomes

Empty liposomes itself can act as a topical healing agent and same has been demonstrated in treatment of dry eye [30, 31]. Either empty or with entrapped drugs, liposomes have also been used in ophthalmology to ameliorate keratitis, corneal transplant rejection, uveitis, endophthalmitis, and proliferative vitreoretinopathy [32]. These reports encouraged investigation of empty liposomes as a therapeutic agent for bladder injury. Interaction of liposomes with cultured urothelium cells suggested that liposomes can be adsorbed and be endocytosed [33]. Previous studies showed that binding of large multilamellar liposomes to the bladder cells was stronger than of sonicated small size liposomes [34, 35].

5.1. Preclinical Studies. A rat model of bladder injury induced by protamine sulfate [36] was used to assess efficacy of empty liposomes. Instillation of liposomes protected bladder irritation induced by protamine sulfate instillation into rat bladder [37] (Figure 1). In earlier study, empty liposomes were used as controls against capsaicin delivery study, which reported tolerance of empty liposomes in uninjured bladder [38]. Bladder tolerance was investigated by cystometry and histology [38].

Physiological effect of liposomes on bladder irritation model induced by protamine sulfate was studied in separate studies [39, 40]. Cystometric studies involved bladder injury induced by infusion of protamine sulfate for an hour followed by irritation caused by infusion of high concentration of potassium chloride solution [39, 40]. Post-treatment of liposomes demonstrated the protective effect in this model [39, 40], which involved coadministration of liposomes with potassium chloride to mimic the clinical disease condition. The comparative efficacy of liposomes was evaluated against

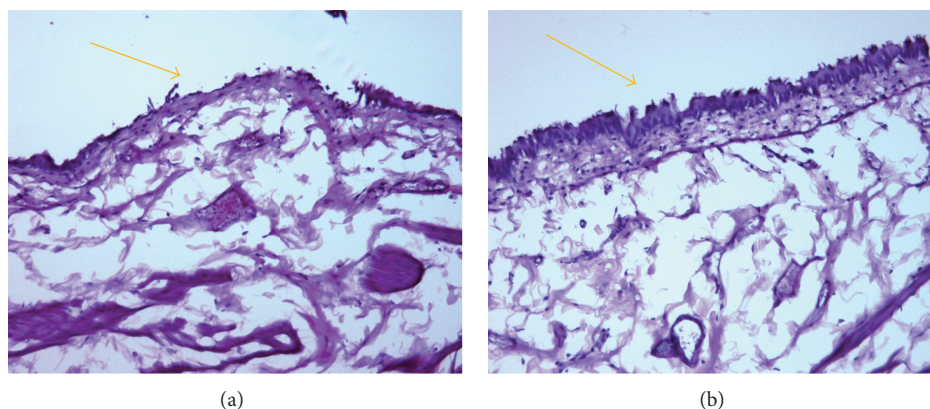


FIGURE 1: Protective effect of liposomes evaluated by histology in rat bladder injured by protamine sulfate. Focal loss of apical layer and ulcerated areas were noted in saline treated rats (a). Liposome post-treatment protected rats from ulceration induced by PS as evident from photograph in (b) taken on bladder harvest at 8 h after liposome instillation.

FDA approved therapies of dimethyl sulfoxide (DMSO) and intravesical instillation of PPS [1].

Clinically, DMSO (RIMSO-50) is the only FDA approved intravesical treatment for PBS/IC, [41] but off-label instillation of PPS has also been pursued [42]. The efficacy of various treatments was evaluated in chemically induced bladder hyperactivity in rats by sequential infusion of protamine sulfate and potassium chloride. Bladder reflex activity of female Sprague-Dawley rats before and after treatment was evaluated by continuous cystometry under urethane anaesthesia (1.0 g/kg). Intravesical liposomes were effective in doubling the intercontractile interval (ICI) compared with PPS, while acute instillation of DMSO failed to produce any protective effect in this animal model [43].

Recently, Lee et al. [44] further improved this model of DO induced by intravesical infusion by combining it with systemic metabolic alteration through fructose feeding. Metabolic syndrome created by feeding of fructose to rats can lead to DO and urination frequency [44]. Cystometric bladder capacity of fructose fed rats can be further decreased by instillation of acidic ATP solution, which provokes reflex micturition via afferent noise. Evidence suggests that increased expression or release of neurotransmitters in the mucosal layer of the bladder can generate afferent noise via C-fiber pathway and result in DO [45]. Compared to infusion with normal saline, ATP solution decreased bladder capacity and increased phasic contractions. Addition of liposomes to the ATP solution partially reversed the ATP solution-induced response [46]. Capsaicin induced desensitization also blunted the provocation of ATP in this model to demonstrate the role of afferent noise via C-fibers.

5.2. Clinical Studies. Encouraged by the exciting preclinical efficacy of empty liposomes as a therapeutic agent for intravesical therapy of IC/PBS, Chuang et al. recently published the clinical safety and efficacy of liposomes in IC/PBS patients [47]. In an open label prospective study on 24 IC/PBS patients, the effect of intravesical liposomes was compared against oral PPS. Patients were equally divided into the two treatment arms, administered either intravesical

liposomes (80 mg/40 cc distilled water) once weekly or oral PPS (100 mg) 3 times daily for 4 weeks each. Ten possible responses to treatment were monitored at 3 time points, including baseline, and at weeks 4 and 8.

Comparable efficacy of liposomes to oral PPS was demonstrated by statistically significant decreases in urinary frequency and nocturia in both treatment arms. Liposome treated patients showed statistically significant decreases in pain, urgency, and the O'Leary-Sant symptom score, with the effect being most profound on urgency. None of the treated patients in the study reported urinary incontinence, retention, or infection due to liposome instillation and there were no unanticipated adverse events and no significant worsening of symptoms during followup. Intravesical instillation of liposomes in IC/PBS patients was found to be safe with potential improvement after 1 course of therapy for up to 8 weeks. The study design was suboptimal, with lack of blinding and two treatment arms assigned to different routes of drug administration and regimen. Still once weekly liposome therapy was able to demonstrate efficacy comparable to thrice daily oral administration of PPS.

Subsequently, Lee et al. assessed the safety and efficacy of twice weekly administration of liposomes on IC/PBS symptoms [48]. Five patients were given twice a week treatment of liposomes for 4 weeks. The primary outcome was the change in the O'Leary-Sant symptom/problem score and O'Leary-Sant total score from baseline to week 4 and week 8. The O'Leary-Sant symptom/problem score, O'Leary-Sant total score, and pain score at the 4-week followup showed significantly greater improvement from baseline with biweekly instillation than once a week instillation. Tolerability of liposomes remains unchanged from once a week regimen to twice a week regimen. The followup at 8 weeks was also similar for both treatment regimens. The incidence of urinary incontinence, retention, or unanticipated adverse changes was not noted with any regimen. Intravesical liposomes appear to be a promising new treatment for IC/PBS and future large-scale placebo controlled studies are needed to verify these results from a pilot study.

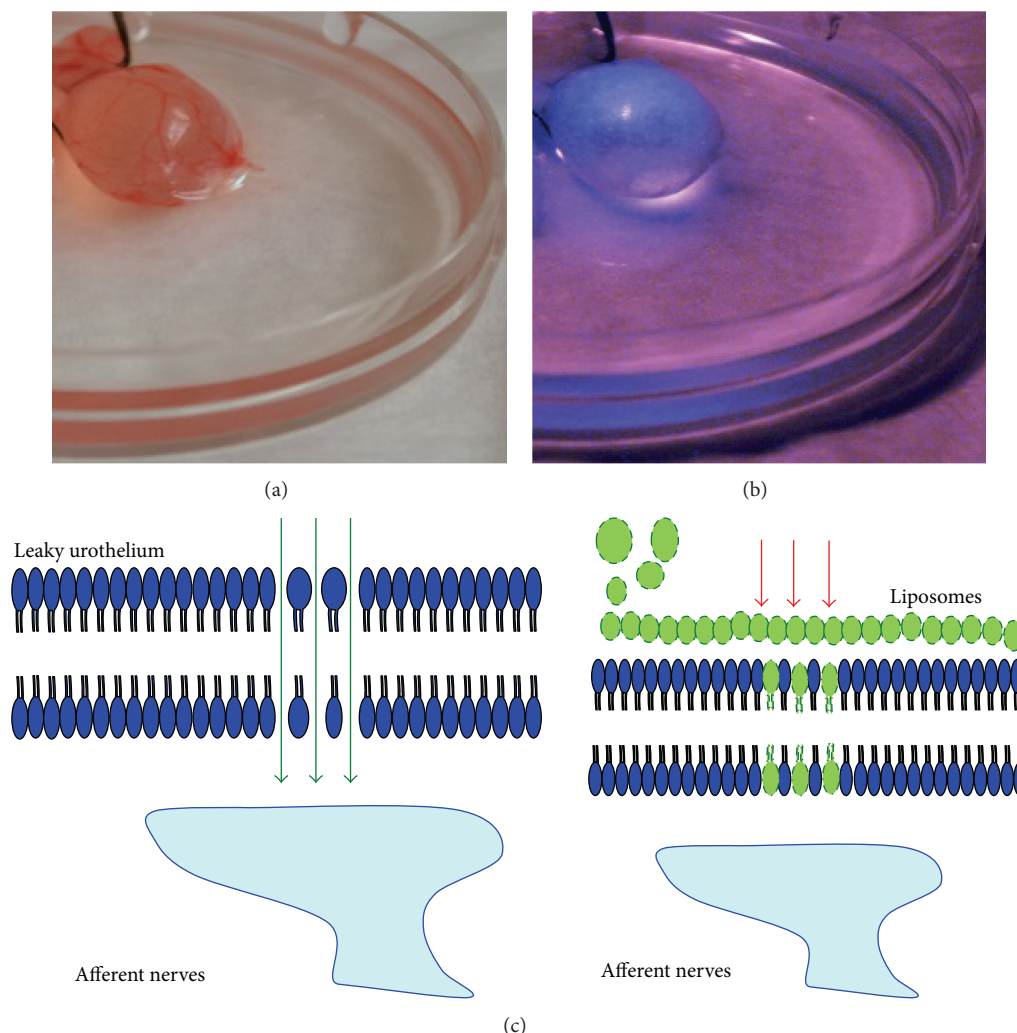


FIGURE 2: *Ex vivo* images of rat bladder taken in visible and near-infrared light indicate the coating formed by instilled liposomes on bladder surface. Liposomes carry a trace amount of near-infrared (NIR) lipophilic carbocyanine dye 1,1'-di-octadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) that fluoresces on exposure to NIR light. Rat bladder filled with urine was tied at the base with thread prior to harvest. Liposomes coating the bladder surface are invisible in visible light photograph (a) but is indicated by blue colored coating on the bladder luminal surface in NIR light (b). NIR imaging *in vivo* can allow noninvasive repeat objective measurement for bladder residence time of instilled treatments. (c) Schematic illustration of liposome coating the bladder surface. Given the chemical affinity of phospholipids of instilled liposomes with lipids in cells lining the bladder surface, the liposomes form a protective film coating on the injured bladder lumen surface and assist in the repair of leaky and inflamed uroepithelium.

The exact mechanism of action for liposomes in IC/PBS remains to be established, but protective coating effect based on preclinical studies cannot be ruled out as illustrated in Figure 2.

6. Liposomes as a Delivery Platform

Not only is the lining of the urinary bladder the most impermeable tissue in the human body, but it is also the most compliant. As a bladder lining expands, additional membrane material is added to its cells to help retain impermeability [49]. Therefore, vesicular trafficking may provide a favorable environment for drug delivery and therefore it is worth investigating whether vesicle nature of liposomes can aid in

improving the delivery of cargo across the bladder permeability barrier.

In the field of neurourology, instillation of neurotoxins into bladder is an accepted approach to achieve chemical neuromodulation of afferent neurotransmission underlying neurogenic bladder and IC/PBS [50]. Existing approaches of chemical neuromodulation by intravesical neurotoxins are suboptimal due to vehicle toxicity for capsaicin [51] or degradation of botulinum toxin (BoNT) in urine. Possible reasons underlying the lack of efficacy from BoNT instillation in bladder can be protein degradation by proteases and proteinases in urine, dilution in urine, or poor uptake of the BoNT solution into the urothelium.

Liposomes have been previously studied as a carrier of toxins to enhance efficacy at lower doses [52]. In the context of

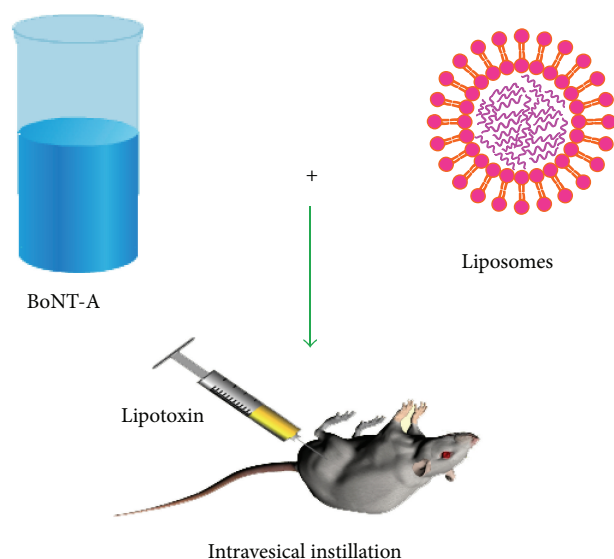


FIGURE 3: Scheme for formulation of BoNT into liposomes for bladder instillation. Freeze dried BoNT is resuspended in distilled water and added to vials containing a desiccated liposomal preparation made of phospholipids. This reconstituted suspension is allowed to hydrate at room temperature for 30–60 min before instillation.

toxins instilled in the bladder, fat-soluble neurotoxin such as capsaicin can be integrated into the phospholipid bilayer [38] and water-soluble neurotoxin such as BoNT can be protected inside the aqueous compartment(s) of liposomes delimited by the phospholipid bilayer(s) [53] (Figure 3). Cystoscope guided injections are the current standard practice in the clinic for administering BoNT to the bladder. But in recent years, studies have also assessed the potential for intravesical instillation of BoNT alone in animal models of bladder irritation [54].

Development of instillation as a mode for administering BoNT in patients will drastically bring down the cost of treatment for patients with refractory overactive bladder. Other groups have recently reported the use of DMSO for instillation of BoNT instead of injection [55]. DMSO does not afford the natural state of the BoNT as a protein and need to be formulated immediately before instillation. Dissolving BoNT in DMSO may not be advisable owing to concerns of BoNT uptake into the systemic circulation of patient. Moreover, biochemical studies have demonstrated that metalloproteolytic activity of the BoNT is strongly enhanced by the presence of lipid membranes [56].

Recent studies reaffirmed the potential of liposomes as a promising vehicle for delivery of neurotoxins to the bladder [38, 53]. The transport of BoNT into urothelium from liposomes was confirmed by detection of its unique effect on neurotransmitters and proteolysis of synaptosomal-associated protein SNAP-25 through immunohistochemistry [53]. The protection of BoNT entrapped inside liposomes from degradation in urine without compromising efficacy was demonstrated by attenuation of acetic acid induced bladder irritation in rats [53]. Similar results were obtained in pre-clinical studies with liposomes encapsulating capsaicin [38].

Liposomes have proven themselves as biocompatible delivery agents in the bladder.

7. Intravesical Antisense Therapeutics

The term “antisense” therapeutics emerged from seminal studies done 4 decades using a short synthetic oligonucleotide for sequence-specific gene silencing [57]. Gene silencing involves introduction of short strands of DNA (termed as antisense) with sequences complementary to the mRNA encoding a particular gene inside the cell with the intent to block gene expression through either translational inhibition or enzymatic cleavage of the mRNA target [3].

Oligonucleotide ODN binds specifically and strongly to the mRNA target through Watson-Crick base pairing. ODN can be basically categorized into those that direct cleavage of the target mRNA as caused by small interfering RNAs (siRNAs) and those that alter mRNA translation without causing mRNA cleavage. Recent discovery of small interfering RNAs (siRNAs) and the elucidation of the RNA interference (RNAi) pathway has also brought a sea change in the control of posttranscriptional gene expression. siRNA takes advantage of endogenous cellular pathways to potentially silence the expression of specific genes (Figure 4).

Antisense mechanism is a promising approach for developing therapeutics based on rational gene-based drug design. Antisense therapeutics have been under clinical investigation for more than 30 years [58]. However, drug development of this approach has been hampered by inefficient intracellular delivery and cellular uptake of the ODN. The translation of basic antisense research into therapeutics is also impeded by intracellular stability of ODN and potential for “off-target” gene silencing, immunostimulation, and other side effects.

A vast array of chemical modifications to ODN has been developed to overcome the therapeutically limiting features by altering internucleotide phosphate linkages, backbone sugars, or nucleobases. One such modified ODN is peptide nucleic acid, which replaces phosphodiester bond between nucleobases with a peptide bond. Replacement of phosphodiester bond by a phosphorothioate linkage is another method to improve the stability of ODN against nucleases.

Great progress has been made in translating antisense research into clinical therapies based on local injection into eye [58]. The field recently progressed further with a systemic injection therapy for treating familial hypercholesterolemia [59]. Antisense therapeutics have been used for exon skipping to optimize the functionality of a truncated dystrophin protein in dog model of Duchenne muscular dystrophy [60].

Nevertheless, applied research for bladder diseases has lagged behind, considering that the anatomical architecture of bladder provides ease of local administration with restricted systemic side effects due to lower serum uptake of antisense ODN. Bladder instillation of antisense ODN or their chemically modified mimics can therefore be an efficient means to control the expression of therapeutically relevant genes. Antisense agents can also be used to elucidate the role of newly discovered genes in bladder function.

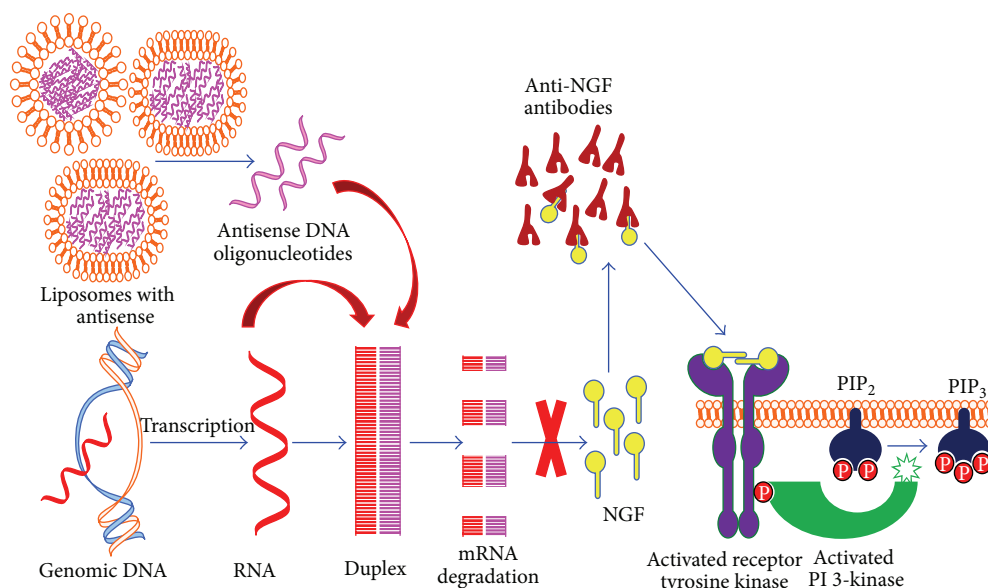


FIGURE 4: Illustration of mechanisms for blocking NGF expression by either NGF antibodies or downregulation of NGF mRNA expression. NGF antibody sequesters the freely available NGF and prevents it from binding with the cognate TrkA receptors. NGF antisense selectively binds with NGF mRNA inside the cells to block the translation of corresponding mRNA. siRNA takes advantage of endogenous cellular pathways to degrade NGF mRNA and control the posttranscriptional NGF gene expression.

8. NGF Expression in Bladder

Several studies have reported that patients with OAB or IC/PBS excrete more nerve growth factor (NGF) in their urine relative to asymptomatic controls [61]. Higher expression of NGF in bladder of patients [62], with corresponding lower serum levels of NGF, makes the bladder tissue the likely source for the elevated NGF in urine [63]. Previous studies have indicated that increased levels of NGF in the bladder and bladder afferent pathways are directly involved in the emergence of hyperexcitability of C-fiber bladder sensory pathways leading to the pathology of DO and OAB (Figure 4) [64].

In addition, intrathecal application of NGF antibodies reduced NGF levels in bladder afferent pathways and normalized bladder/urethral function in spinal cord injured (SCI) rats [65]. Because it is likely that the major pathology of OAB is driven by NGF, targeting the intracellular synthesis of NGF molecule in bladder is a promising therapeutic alternative.

9. NGF Expression a Drug Target

Overexpression of NGF can be blocked either directly by antibodies [66] or by blocking the synthesis of NGF protein from mRNA [67]. Systemic administration of monoclonal human NGF antibodies (tanezumab) has been explored for therapeutic outcomes in IC/PBS patients but not without encountering safety concerns such as paresthesia, hypoesthesia, and arthralgia [66]. Generalized blockade of NGF activity at sites other than bladder by anti-NGF antibodies may not be the preferred outcome, because NGF is an essential housekeeping growth factor necessary for the survival and growth of neurons [68]. The physiological necessity of NGF

action at those sites may explain the incidence of paresthesia, hypoesthesia, and arthralgia in patients treated with systemic anti-NGF antibodies [66].

9.1. Peptide Nucleic Acid (PNA). Therefore, to reduce the toxicity of systemic blockade of NGF, we sought to develop a novel intravesical therapy of OAB by targeting the intracellular synthesis of NGF in the urothelium. Antisense ODN needed to be able to cross cell membrane to act as a drug and negatively charged ODN will not pass through a lipid layer such as cell membranes. Bladder uptake of ODN is limited by the anionic charge and size of the ODN as well as anionic glycosaminoglycan layer of the inner bladder surface. Therefore, primary impediment to be overcome in the development of intravesical antisense therapeutics is inefficient bladder uptake of the ODN across urothelium. Previous studies showed that heparan sulfate proteoglycans expressed on cell surface act as receptors for extracellular TAT uptake [69]. Therefore, it was reasoned that GAG layer on bladder surface can facilitate bladder uptake of peptide nucleic acid PNA, if it could be conjugated with synthetic TAT peptide.

Water insoluble peptide nucleic acid targeting NGF was conjugated with cell penetrating cationic peptide TAT for intracellular delivery of antisense moiety to demonstrate efficacy in animal models. Studies showed that PNA conjugated with TAT suppressed cyclophosphamide cystitis following local instillation of antisense against NGF [67]. There was negligible uptake of peptide nucleic acid in absence of TAT conjugation. Prior to determination of *in vivo* efficacy of conjugate, suitable target sequence on NGF mRNA was determined by predicted folding structure and cell transfection experiments. Successful intravesical delivery of peptide

nucleic acid in bladder at the same time of cyclophosphamide injection protected against the cystitis by blocking the rise in bladder contraction frequency and inflammation.

9.2. Phosphorothioate-Linked Analogues. Considering the difficulty and possible nonspecific toxicity of peptide nucleic acid and TAT, recent developments focused on simplifying the approach using water-soluble phosphorothioated ODN, which have an increased resistance to exo- and endonucleases for improved stability [70]. As alluded to in the above text, success of antisense therapeutics is largely dependent on the development of a delivery vehicle that can efficiently deliver antisense ODN in bladder. Preliminary studies showed that liposomes can be far better biocompatible effective carriers for local gene silencing of NGF gene in bladder cells.

Cationic liposomes and mimics have emerged as the most popular nonviral method to deliver nucleic acids in therapeutic applications. Easy and reversible complex formation of cationic liposomes with ODN at room temperature allows their use as carriers. Electrostatic attraction between the cationic lipid, DOTAP, and the polyanionic antisense ODN is responsible for the complex formation. The efficacy of liposome delivered siRNA by intravesical route has been previously demonstrated in preclinical models of bladder cancer [71]. Residence of ODN in the rat bladder after intravesical instillation was demonstrated to be longer than 24 h using fluorescent tagged ODN complexed with liposomes [72]. Fluorescent probe was localized in bladder urothelium cells 24 h following instillation. The 24 h residence time was also demonstrated for siRNA in mouse bladder [73]. Bladder uptake of fluorescent ODN without liposomes in normal rat bladder is poor. Previous studies have shown that very high concentration of phosphorothioated ODN can deliver ODN without liposomes to bladder cells of mice having bladder cancer [74]. The urothelium barrier may be slightly compromised in cancerous condition and the strategy of loading bladder with high dose of ODN may not work in noncancerous diseased condition of bladder with intact barrier.

In order to evaluate the efficacy of NGF antisense ODN, acetic acid infusion was used to cause a rapid rise of NGF protein levels. A single dose instillation of ODN complexed with liposomes protected against bladder overactivity (BO) induced by acetic acid. Together with data of bladder uptake studies using fluorescent ODN, it is demonstrated that ODN is readily available to the bladder after intravesical instillation. It has been previously reported that, within 2 h of exposure to irritants such as turpentine and acetic acid, there occurs a rapid rise in bladder content of NGF [75]. Later studies found that bladder responds to insults with upregulation in the genes for NGF, sE-Selectin and receptor for monocyte chemoattractant protein-1 (MCP-1) within 30 min of exposure to lipopolysaccharide (Figure 5) [76]. The acetic acid induced overexpression of NGF was blunted by pretreatment with NGF antisense ODN with phosphorothioate linkage prior to exposure of acetic acid. The downregulation of NGF mRNA expression was in agreement with reduced protein levels and suppressed NGF-like immunoreactivity in the urothelium.

9.3. Antisense and Downstream Signalling of NGF. Drug development of intravesical antisense for NGF was also able to unmask the downstream signaling [77] involving NGF following exposure to acetic acid. Experiments supported the role of NGF as a paracrine messenger [77], which is known to activate several downstream effectors to manifest physiological and pathological signaling changes linked to it [76, 78–80]. The bladder injury set off by acetic acid initiates the signalling cascades that upregulate the expression of NGF and other chemokines, MCP-1, CXCL-1, and CXCL-10, and prostaglandins [76, 78–80]. As reported elsewhere, localization of chemokines within synaptic vesicles in neurons [81] is consistent with their ability to act as excitatory neurotransmitters following AA exposure.

Chemokines are one of several downstream effectors activated by NGF [76, 78–80] and interestingly chemokine receptors are widely expressed in neural and nonneural elements of the nociceptive pathways that are responsible for visceral and somatic pain sensation [82]. MCP-1 [83] and CXCL-10 [84] are constitutively expressed in neurons, where they participate in excitability of primary afferent neurons through transactivation of transient receptor channels and nociceptor sensitization [84]. Overexpression of NGF is likely to drive the expression of VEGF from neurons and leptin [78] from adipocytes covering neurons [85].

Cooperative expression of NGF and MCP-1 is able to induce hyperexcitability in neurons by activating TRPV1 receptor. In addition, MCP-1, CXCL-1, and CXCL-10 cause chemoattraction of monocytes, neutrophils, and lymphocytes, respectively, to mediate the bladder injury set off by acetic acid. Extravasation is an essential prerequisite for infiltration of monocytes, neutrophils, and lymphocytes, which requires the expression of adhesion molecules like E-selectin and intracellular adhesion molecule ICAM-1 [76, 86]. E-selectin is a cytokine-inducible adhesion molecule that supports the rolling and stable arrest of leukocytes on activated vascular endothelium. Expression of E-selectin has been linked to adherence of neutrophils to bladder microvascular endothelial cells and to cyclophosphamide cystitis [87, 88]. E-selectin gene expression is activated by the NF- κ B and MAP kinase signal transduction pathways [76, 86].

Activation and recruitment of leukocytes to acetic acid induced bladder injury also require expression of intracellular adhesion molecule ICAM-1 [89]. ICAM-1 interacts specifically with its receptors of the integrin family to induce reversible cell-cell interactions involving adhesion. Expression of ICAM-1 was found to be increased in patients with IC/PBS and reduced in patients responding to instillation of hyaluronic acid [90]. Expression of ICAM-1 and VCAM-1 was also noted in a recent study on biopsy tissue of IC/PBS patients [91]. Increased expression of ICAM-1 subsequent to exposure with acetic acid in animal model is therefore clinically relevant.

Antisense experiments supported the earlier report that inhibition of NGF expression significantly downregulates the expression of ICAM-1 [80]. It is reported that binding of NGF to its high-affinity TrkA receptor controls the sICAM-1 expression on target cells [80]. Expression of ICAM-1 was

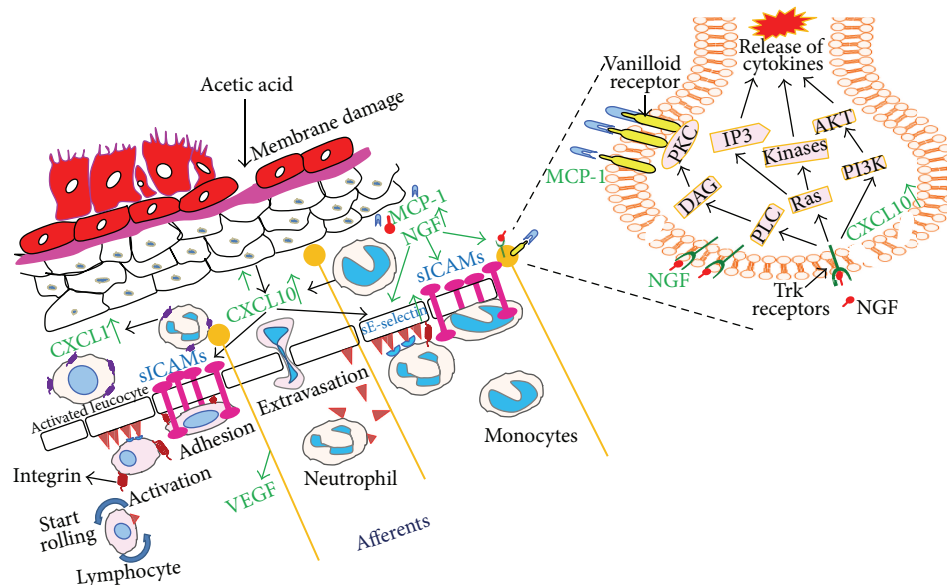


FIGURE 5: Signalling cascades induced by NGF overexpression following exposure to acetic acid. Injury from exposure to acetic sets off the signalling cascades that upregulate the expression of NGF, MCP-1, prostaglandins, CXCL-1, and CXCL-10. Overexpression of NGF drives the expression of VEGF from neurons and leptin from adipocytes covering neurons. NGF and MCP-1 also induce hyperexcitability in neurons by activating TRPV1 receptor. MCP-1, CXCL-1, and CXCL-10 cause chemoattraction of monocytes, neutrophils, and lymphocytes, which requires extravasation across endothelium from blood through the cooperation of adhesion molecules E-selectin and ICAM-1.

negatively associated with NGF expression in antisense experiments. Ultimately, expression of ICAM-1 is regulated at the level of transcription by one of several signaling cascades NF- κ B, JAK/STAT, IFN- γ (CXCL-10), AP, MAP kinase, and PKC [92].

Intravesical route can allow selective exposure of antisense ODN to the NGF producing cells in urothelium and avoid systemic side effects from genetic manipulation of NGF expression. Intravesical route can also prove to be cost effective given the cost of ODN or siRNA. Contrary to the goal of loss of function in bladder with antisense ODN, in recent studies liposomes have also been used to deliver ODN for gain of function in bladder [93].

10. Imaging of Intravesical Therapy

Drug development for DO and IC/PBS relies heavily on subjective outcomes for predicting efficacy and safety in early clinical development. Although there are many validated measurement tools used in DO and IC/PBS research, they are usually burdensome and do not capture all symptoms related to the lower urinary tract. Moreover, improvement in symptoms scores following an intervention does not always correlate with patient expectations, satisfaction, and goal achievement, which are critically important for successful management of DO and IC/PBS.

There is underdevelopment and underutilization of urine biomarkers and imaging methods for investigational drugs given by intravesical route. Overreliance on subjective impressions of patients for therapeutic response limits clinical relevance due to weak correlation with patient satisfaction and can also impede continued scientific progress in assessing

study outcomes relative to symptom bother. Symptom scores may also be related to the cause of high heterogeneity in clinical response in DO and IC/PBS patients.

Imaging can offer the possibility to provide region-specific information in situations where serum and urine assays may not reflect the true state of bladder [94–96]. Urine chemokines have been reported to be associated with symptom severity of IC/PBS patients [97]. Application of fluorescent microscopy for imaging of pelvic floor has been limited due to the issues pertaining to light absorption by tissues, scattering, and autofluorescence [98].

Recent studies have shown that dyes fluorescing in the near-infrared (NIR) band can overcome this handicap in deep-tissue imaging of experimental animals [99]. Selected NIR fluorochromes emit light with tissue penetration approaching 10–15 cm [100]. The light emission from probes in this spectrum encounters low background autofluorescence and minimal attenuation of signal due to light absorption by tissue components [98]. Therefore, NIR imaging yields high signal-to-noise ratios and is well suited for studying the distribution of instilled treatments in bladder (Figure 2(b)).

Preliminary studies suggest that imaging of mouse pelvic floor in NIR spectrum can be a viable option [101]. NIR imaging easily allows visualization and quantification of bladder distribution by tracing the migration of fluorescence-labeled liposomes in anaesthetized mouse. Compared with other biological assays, imaging could provide objective endpoint for patients with ulcerative cystitis to indicate its high translation potential. Other benefits of optical imaging include its relatively low cost compared with more traditional imaging systems such as MRI and PET. The instrument

consists of two near-infrared lasers emitting at 685 nm and 785 nm, camera, and novel optics.

Furthermore, preferential binding of liposomes and nanoparticles to bladder lesions of IC/PBS after instillation can be assessed by NIR imaging of bladder, where it can theoretically provide anatomically specific information about the real time status of ulcer/lesion in bladder surface or tissue abnormality [102]. Therefore, future studies can substitute a response variable that is continuous in nature instead of a subjective clinical outcome.

11. Conclusions

Adoption of forward-thinking approaches can achieve advancements in drug delivery systems targeted to improve pharmacotherapy of bladder diseases in the future. Latest developments in the field of nanotechnology can bring this mode of therapy from second line of treatment for refractory cases to the forefront of disease management. Liposomes are an attractive drug delivery platform by virtue of their biodegradability, biocompatibility, low toxicity, and simple and mild preparation methods.

Conflict of Interests

All authors except Dr. Michael Chancellor declare that there is no conflict of interests regarding the publication of this paper. Dr. Michael Chancellor owns stocks in the start-up liposome company, Lipella.

Acknowledgments

This work was supported by the National Institutes of Health (DK057267 and DK088836) and the Department of Defense (W81XWH-11-1-0763 and W81XWH-12-1-0565).

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Neural Mechanisms Underlying Lower Urinary Tract Dysfunction

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This article summarizes anatomical, neurophysiological, and pharmacological studies in humans and animals to provide insights into the neural circuitry and neurotransmitter mechanisms controlling the lower urinary tract and alterations in these mechanisms in lower urinary tract dysfunction. The functions of the lower urinary tract, to store and periodically release urine, are dependent on the activity of smooth and striated muscles in the bladder, urethra, and external urethral sphincter. During urine storage, the outlet is closed and the bladder smooth muscle is quiescent. When bladder volume reaches the micturition threshold, activation of a micturition center in the dorsolateral pons (the pontine micturition center) induces a bladder contraction and a reciprocal relaxation of the urethra, leading to bladder emptying. During voiding, sacral parasympathetic (pelvic) nerves provide an excitatory input (cholinergic and purinergic) to the bladder and inhibitory input (nitric) to the urethra. These peripheral systems are integrated by excitatory and inhibitory regulation at the levels of the spinal cord and the brain. Therefore, injury or diseases of the nervous system, as well as disorders of the peripheral organs, can produce lower urinary tract dysfunction, leading to lower urinary tract symptoms, including both storage and voiding symptoms, and pelvic pain. Neuroplasticity underlying pathological changes in lower urinary tract function is discussed.

Keywords: *Detrusor overactivity; Lower urinary tract; Nerve growth factor; Overactive urinary bladder*

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Article History:

received 14 January, 2014
accepted 27 January, 2014

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INTRODUCTION

The storage and periodic elimination of urine depend on the coordinated activity of two functional units in the lower urinary tract (LUT): (1) a reservoir (the urinary bladder) and (2) an outlet consisting of the bladder neck, the urethra, and the urethral sphincter [1]. Coordination between these organs is mediated by a complex neural control system located in the brain, spinal cord, and peripheral ganglia [2]. Thus, urine storage and release are highly dependent on central nervous system pathways. This distinguishes the LUT from many other visceral structures (e.g., the gastrointestinal tract and cardiovascular system) that maintain a certain level of function even after extrinsic neural input has been eliminated.

The LUT is also unusual in its pattern of activity and organization of neural control mechanisms. For example, the urinary bladder has only two modes of operation: storage and elimination. Thus, many of the neural circuits have switch-like or phasic patterns of activity [3,4], unlike the tonic patterns characteristic of the autonomic pathways to cardiovascular organs. Micturition also requires the integration of autonomic and somatic efferent mechanisms to coordinate the activity of visceral organs (the bladder and urethra) with that of urethral striated muscles [2,4].

Owing to the complexity of the neural mechanisms regulating the LUT, micturition is sensitive to a wide variety of injuries, diseases, and chemicals that affect the nervous system. Thus, neurologic mechanisms are an important

consideration in the diagnosis and treatment of voiding disorders. This article reviews (1) the innervation of the LUT, (2) the organization of the reflex pathways controlling urine storage and elimination, and (3) neurogenic dysfunctions of the LUT.

NEUROPHYSIOLOGY OF THE LOWER URINARY TRACT

1. Peripheral nerves controlling micturition

The reciprocal function of the bladder and urethra, i.e., storage and voiding, is peripherally coordinated by three sets of nerves, the parasympathetic, sympathetic, and somatic peripheral nerves, that are components of intricate efferent and afferent circuitry derived from the brain and the spinal cord [5,6] (Figs. 1, 2). These nerves also carry sensory information in afferent fibers from the LUT to the lumbosacral spinal cord, which is composed of myelinated A δ -fibers and unmyelinated C-fibers [6-10] (Fig. 3). A δ -Fibers, which are located primarily within the detrusor smooth muscle layer, respond primarily to detrusor stretching during bladder filling and convey sensations of fullness. Unmyelinated sensory C-fibers are more widespread and reside in the muscle, close to the urothelium in the mucosa and directly adjacent to the urothelial cells themselves [6,11,12].

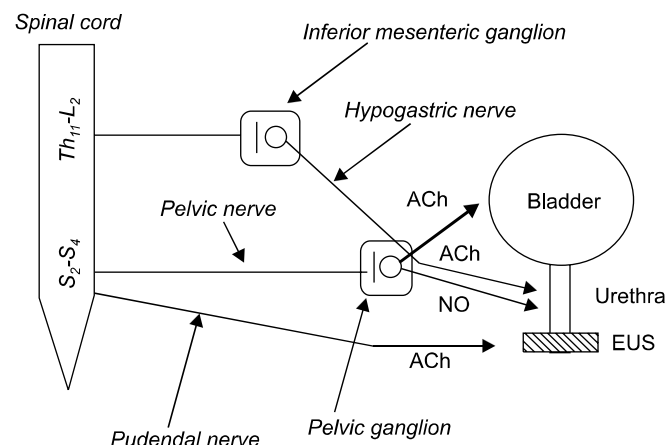


FIG. 1. Major preganglionic and postganglionic neural pathways from the spinal cord to the lower urinary tract. The sympathetic hypogastric nerve, emerging from the inferior mesenteric ganglion, stimulates urethral smooth muscle. The parasympathetic pelvic nerve, making synapses onto postganglionic neurons in the pelvic ganglion, stimulates bladder detrusor muscle and inhibits urethral smooth muscle. The somatic pudendal nerve stimulates striated muscle of the external urethral sphincter (EUS). ACh, acetylcholine; NE, norepinephrine; NO, nitric oxide; S2-S4, sacral segments of the spinal cord; T10-L2, thoracolumbar segments of the spinal cord. Adapted from Yoshimura et al. Naunyn Schmiedeberg Arch Pharmacol 2008;377:437-48 [4].

2. Interacting reflexes controlling micturition

1) Storage phase

Until the volume of urine in the bladder reaches a critical threshold for voiding, the detrusor is quiet, the bladder having low and relatively constant levels of internal pressure during filling [6]. During this phase, the intrinsic viscoelasticity of detrusor muscles permits the bladder wall to adjust to increasing volume by stretching and the stimulatory parasympathetic pathway is quiescent. However, there are also neurogenic contributions toward maintaining an inactive bladder during the storage phase [6,13-15]

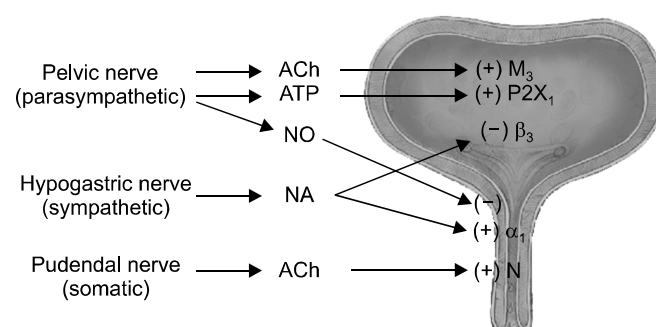


FIG. 2. Innervation of the lower urinary tract. The parasympathetic pelvic nerve stimulates the bladder detrusor muscle, mediated by muscarinic receptors (M₃) being activated by acetylcholine (ACh) and purinergic receptors (P2X₁) being activated by adenosine triphosphate (ATP), and relaxes the urethral smooth muscle, mediated by nitric oxide (NO). The sympathetic hypogastric nerve stimulates urethral smooth muscle and inhibits bladder detrusor, mediated by α_1 -adrenergic and β_3 -adrenergic receptors, respectively. The somatic pudendal nerve stimulates striated muscle of the external urethral sphincter, mediated by ACh activating nicotinic (N) receptors. NA, noradrenaline. Plus and minus signs in parentheses indicate neural stimulation and inhibition, respectively.

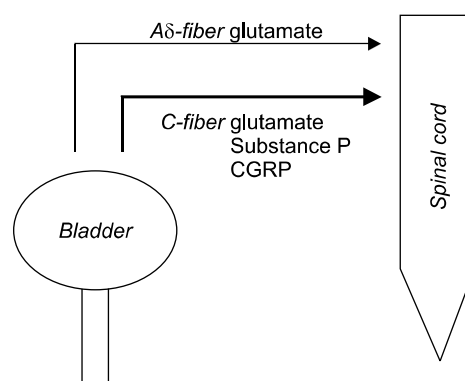


FIG. 3. Afferent fibers transmitting sensory information from the lower urinary tract to the spinal cord. Glutamate is a neurotransmitter present in both the A δ - and C-fibers; additional neurotransmitters in the C fibers include substance P and calcitonin gene-related peptide (CGRP). Adapted from Yoshimura et al. Naunyn Schmiedeberg Arch Pharmacol 2008;377:437-48 [4].

(Fig. 4).

The bladder-to-external urethral sphincter (EUS) reflex, the guarding reflex, is initiated by distension of the bladder during filling, which activates stretch-sensitive mechanoreceptors in the bladder wall, in turn generating afferent signals to the sacral spinal cord where pudendal motoneurons are activated. The pudendal nerve efferents stimulate EUS contractions, thereby maintaining outlet resistance and urinary continence. The guarding reflex increases in intensity as the bladder volume increases (Fig. 4).

The bladder-to-sympathetic pathway reflex is also triggered by bladder distension. Stimulated bladder afferents activate an intersegmental pathway from sacral cord to thoracolumbar sympathetic nerves. The activated sympathetic nerves stimulate contraction of the internal urethral sphincter and inhibit bladder activity [14] (Fig. 4).

The reflexes involved in urine storage are predominantly integrated in the spinal cord and seem to function normally in animals with supraspinal transections. However, voluntary control of micturition is impaired in many patients with lesions that interrupt brain stem pathways. Thus, although the storage reflex seems to be established within the spinal cord, maintaining a stable urethral resistance apparently requires supraspinal input [6,16]. It is known that the pontine urine storage center located in the dorsolateral pons provides descending inputs that activate pudendal motoneurons and thus increase urethral resistance (Fig. 4).

2) Elimination phase

The essential first step in micturition is relaxation of the EUS muscles. In adult humans with normal LUT function,

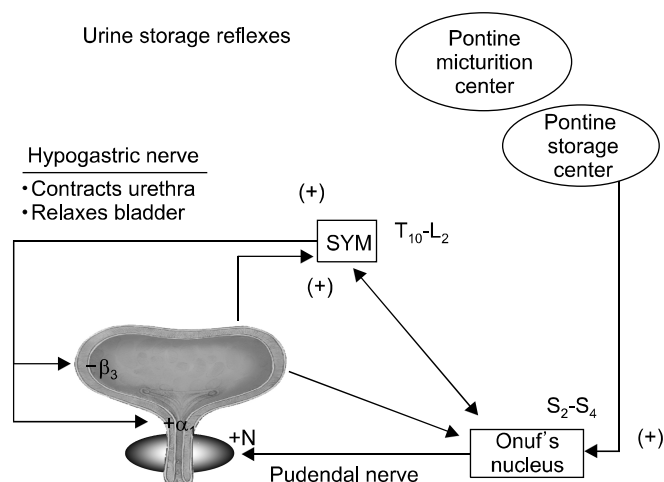


FIG. 4. Major reflex pathways to the lower urinary tract initiating and maintaining urine storage. α₁, α-adrenergic receptors; β₃, β-adrenergic receptors; N, nicotinic receptors; S₂-S₄, sacral segments of the spinal cord; T₁₀-L₂, thoracolumbar segments of the spinal cord; SYM, sympathetic nerve. Plus and minus signs indicate neural stimulation and inhibition, respectively. Adapted from Yoshimura et al. *Naunyn Schmiedebergs Arch Pharmacol* 2008;377:437-48 [4].

the individual has sensory awareness of a full bladder, and the guarding reflex is intensified until voluntary elimination is possible. Initiation as well as normal completion of the elimination process depends on input from the brain [6,16] (Fig. 5).

In order for the guarding reflex to be reversed and the EUS relaxed, a final inhibitory signal is generated from the pontine micturition center (PMC). Bladder afferent fibers in the pelvic nerve form synapses in the spinal cord, and axons from the second-order neurons travel rostrally to the micturition center. The center integrates this sensory information with signals from more rostral brain regions and ultimately generates inhibitory input to the sympathetic and somatic centers in the spinal cord and stimulatory input to the parasympathetic center (Fig. 5). This spino-bulbo-spinal reflex results in relaxation of the EUS and internal urethral sphincter, followed by contraction of detrusor muscles, increase in bladder pressure, and flow of urine [4].

DISEASE-INDUCED NEUROGENIC CHANGES IN MICTURITION

1. Spinal cord injury and neuropathic bladder

Spinal cord injury (SCI) rostral to the lumbosacral level eliminates voluntary and supraspinal control of voiding, leading initially to an areflexic bladder and complete urinary retention followed by a slow development of automatic

Micturition reflexes

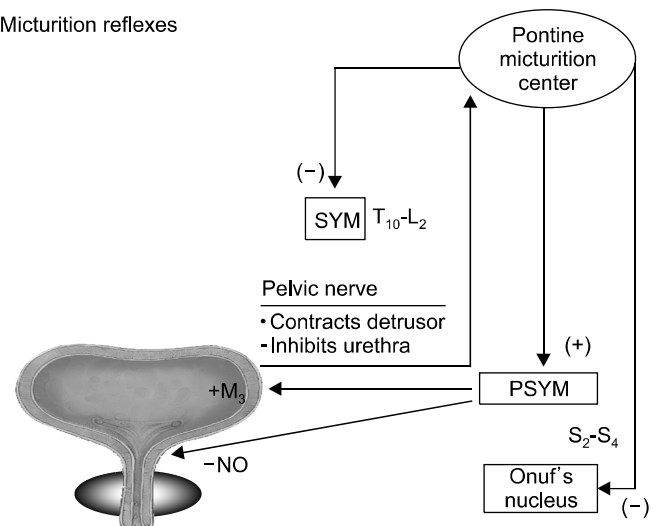


FIG. 5. Major reflex pathways from the pontine micturition center in the brainstem to the lower urinary tract via the spinal cord regulating micturition. M₃, muscarinic receptors; S₂-S₄, sacral segments of the spinal cord; T₁₀-L₂, thoracolumbar segments of the spinal cord; SYM, sympathetic nerve; PSYM, parasympathetic nerve. Plus and minus signs indicate neural stimulation and inhibition, respectively. Descending inputs from the pontine micturition center inhibit neuronal activity in the sympathetic nervous system and Onuf's nuclei in the spinal cord. Adapted from Yoshimura et al. *Naunyn Schmiedebergs Arch Pharmacol* 2008;377:437-48 [4].

micturition and bladder overactivity mediated by spinal reflex pathways [17-24]. However, voiding is commonly inefficient owing to simultaneous contractions of the bladder and urethral sphincter (detrusor-sphincter-dyssynergia, or DSD). Electrophysiologic studies in animals have shown that the micturition reflex pathways in spinal-intact animals and in chronic spinal-injured animals are markedly different [25,26]. In cats with an intact spinal cord, myelinated A δ afferents activate the micturition reflex, whereas in cats with chronic thoracic spinal cord transection, micturition is induced by unmyelinated C-fiber axons. In normal cats, capsaicin does not block reflex contractions of the bladder or the A δ -fiber-evoked bladder reflex. However, in cats with chronic spinal injury, capsaicin, a neurotoxin known to disrupt the function of C-fiber afferents, completely blocks C-fiber-evoked bladder reflexes [19,26]. Fig. 6 depicts the hypothetical mechanisms inducing lower urinary tract symptoms (LUTS) and bladder overactivity following bladder dysfunction induced by pathological conditions including SCI.

Chronic SCI in humans also causes the emergence of an unusual bladder reflex that is elicited in response to infusion of cold water into the bladder [27]. Studies in animals indicate that cold temperature activates receptors in bladder C-fiber afferents and urothelial cells [28,29]. Contribution of afferent hyperexcitability to the emergence of bladder overactivity in SCI has also been identified by clinical studies using neurotoxins such as botulinum toxin and resiniferatoxin (RTX). For example, suppression of bladder afferent activity with botulinum toxin effec-

tively treats detrusor overactivity (DO), mitigates urgency in both neurogenic DO in SCI patients, and, with sustained therapy, reduces the expression of the capsaicin receptor (TRPV1) and the purinergic receptor in suburothelial nerve fibers [30,31]. In patients with SCI-induced DO, the clinical response to intravesical therapy with the C-fiber afferent toxin RTX is accompanied by a marked decrease in the density of nerve fibers staining positively for PGP9.5 and TRPV1 [32,33].

The emergence of C-fiber bladder reflexes seems to be mediated by several mechanisms, including changes in central synaptic connections and alterations in the properties of the peripheral afferent receptors that lead to sensitization of the 'silent' C fibers and the unmasking of responses to mechanical stimuli [22-24,34] (Fig. 6). In rats, it has been shown that bladder afferent neurons undergo both morphologic (neuronal hypertrophy) [35] and physiologic changes, including a shift from the high-threshold, tetrodotoxin (TTX)-resistant Na⁺ channel type to the low-threshold, TTX-sensitive Na⁺ channel type [21,36]. Other physiologic changes include the down-regulation of low-threshold A-type K⁺ channels, which is associated with decreased expression of Kv1.4 α -subunit following SCI [37].

1) Role of neurokinins

Destruction of lumbosacral spinal neurons expressing neurokinin-1 (NK-1) receptors using substance P conjugated with saporin does not affect reflex voiding in normal rats, but reduces the bladder irritant effects of intravesical capsaicin [38] and reduces nonvoiding contractions in SCI rats [39]. Similarly, intrathecal administration of a selective NK-1 receptor antagonist (L-733060) does not affect the micturition reflex in spinal-intact rats but blocks the micturition reflex in SCI rats [40]. These data coupled with the increased expression of substance P in the region of the sacral parasympathetic nucleus in SCI rats [40] suggest that activation of NK-1 receptors in the spinal cord plays a role in SCI-induced DO.

2) Role of GABA

Reduced γ -aminobutyric acid (GABA)-ergic inhibition could contribute to the development of neurogenic DO because mRNA levels of GAD67, an enzyme involved in GABA synthesis, are decreased in the spinal cord after SCI in rats [41]. A possible therapy for neurogenic DO has emerged from experimental studies, in which a herpes simplex virus vector encoding the *GAD* gene was injected into the bladder of SCI rats to increase GAD expression in bladder afferent nerves. This treatment reduces DO and DSD in SCI rats [42,43].

3) Role of TRP receptors

The number of suburothelial nerve fibers expressing TRPV1 receptors, which are predominantly expressed in C-fiber afferent pathways, is increased in patients with neurogenic DO compared to controls [32]. In rats with SCI,

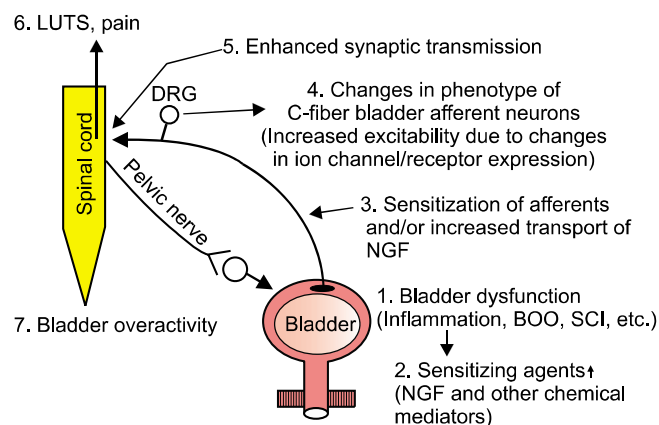


FIG. 6. Diagram showing hypothetical mechanisms inducing bladder overactivity, LUTS, and pelvic pain. Bladder dysfunction (1) causes increased production of neurotrophic factors such as NGF or other chemical mediators in the bladder wall (2). NGF is taken up by afferent nerves and transported to DRG cells (3) where it changes gene expression, which leads to changes in functional properties of ion channels and receptors and increased neuronal excitability (4). Increased afferent nerve activity results in enhanced synaptic transmission in the spinal cord (5), leading to LUTS or pelvic pain (6) and bladder overactivity (7). BOO, bladder outlet obstruction; DRG, dorsal root ganglion; LUTS, lower urinary tract symptoms; NGF, nerve growth factor.

duodenal administration of a TRPV1 antagonist (GRC 6211) reduces bladder contraction frequency in SCI rats [44]. In addition, intravenous administration of a TRPA1 antagonist (HC-030031) or intrathecal treatment with antisense oligodeoxynucleotide of TRPA1 receptors is effective in suppressing DO in SCI rats [45]. These results along with the increased TRPA1 expression in the bladder and L6-S1 dorsal root ganglion (DRG) in SCI rats [45] suggest that TRPV1 and TRPA1 channels are involved in the emergence of C-fiber bladder afferent hyperexcitability that contributes to neurogenic DO in SCI.

4) Role of neurotrophic factors

It has been speculated that SCI-induced neuroplasticity is mediated by the actions of neurotrophic factors such as nerve growth factor (NGF) released within the urinary bladder or the spinal cord (Fig. 6). In clinical studies, NGF production is elevated in the bladder and in urine samples of SCI patients with DO and can be reduced along with symptom improvements after intradetrusor botulinum toxin treatment [46,47]. Animal studies have also demonstrated that the production of neurotrophic factors including NGF increases in the bladder after SCI [48]. Furthermore, chronic administration of NGF into the spinal cord or into the bladder wall in rats induces bladder overactivity and increased excitability of bladder afferent neurons [49-51]. Increased transport of NGF to DRG cell bodies or central NGF production in the injured spinal cord could modulate the micturition pathway at the spinal level. Intrathecal delivery of an NGF monoclonal antibody diminishes neurogenic DO and DSD in rats with SCI [39,52]. Thus, a combination of peripheral and central NGF actions is likely to be involved in the emergence of neurogenic DO.

2. Bladder outlet obstruction

Bladder outlet obstruction (BOO), which can occur in patients with benign prostatic hyperplasia, often produces detrusor hypertrophy and DO [5,53].

1) Role of altered neural activity

BOO alters neural networks in the central nervous system to induce bladder dysfunction (Fig. 6). BOO in rats enhances a spinal micturition reflex [54] and clinically enhances the bladder ice water test, which is mediated by a C-fiber afferent-dependent spinal micturition reflex. This finding in rats is consistent with a considerable upregulation of C-fiber afferent mechanisms in BOO patients with benign prostatic hyperplasia [55-57]. Within the spinal cord, obstruction stimulates an increased expression of GAP-43, an effect that is often associated with axonal sprouting after injury [58]. These observations suggest an enhancement or de novo development of new spinal circuits after obstruction.

2) Role of altered myogenic activity

Increased myogenic activity of detrusor smooth muscles is another important mechanism inducing overactive blad-

der and DO, which seems to be more applicable to patients with BOO. Partial BOO increases intravesical pressure and induces bladder hypertrophy and partial denervation of the bladder smooth muscle, leading to various functional changes in smooth muscles. These changes include denervation supersensitivity of cholinergic (muscarinic) receptors [59], increases in purinergic receptor-mediated contractile responses as well as expression of purinergic receptors such as P2X₁ [60,61], and changes in the cell-to-cell communication in detrusor muscles owing to upregulation of gap-junction proteins such as connexin 43 [62,63]. In addition, another population of cells in the bladder known as interstitial cells has been proposed for a pacemaking role in spontaneous activity of the bladder [6,10]. It has been reported that the number of interstitial cells is increased in guinea pig and rat models of BOO [64,65] and that c-kit tyrosine kinase inhibitors, which inhibit interstitial cell activity, decrease the amplitude of spontaneous contractions in the guinea pig and human bladder [66,67]. These findings suggest that interstitial cells may also be involved in the emergence of DO as the result of enhanced autonomous detrusor muscle activity.

3) Role of neurotrophic factors

Men with overactive bladder symptoms and BOO caused by benign prostatic hyperplasia display increased levels of NGF in bladder tissues [68] and increased levels of urinary NGF [69]. NGF content is also increased in obstructed bladders in BOO animals [68,70]. Moreover, blockade of NGF action using autoantibodies prevents the neural plasticity and urinary frequency after obstruction and, in rats with persistent urinary frequency after relief of obstruction, NGF remains elevated in the bladder [68]. These findings suggest a cause-and-effect relationship between NGF-mediated changes in bladder afferents and an enhanced spinal micturition reflex and urinary frequency associated with BOO.

3. Inflammation and bladder pain syndrome

Bladder pain syndrome/interstitial cystitis (BPS/IC) is a disease with LUTS such as urinary frequency with bladder pain related to bladder filling. Although the etiology of BPS/IC is still not known, increasing evidence suggests that the disorder is associated with urothelial dysfunction and afferent hyperexcitability due to neurogenic bladder inflammation [71-73] (Fig. 6). In a rat model of chronic cystitis induced by cyclophosphamide or hydrochloric acid, it was shown that capsaicin-sensitive bladder afferent neurons increase their excitability owing to decreased density of A-type K⁺ (K_A) currents, associated with the decreased expression of the Kv1.4 α -subunit [74,75]. Increased afferent hyperexcitability in BPS/IC is also supported by previous clinical findings that C-fiber desensitization induced by intravesical application of high-dose capsaicin or RTX is effective for treating painful symptoms in patients with IC [76,77]. However, a recent prospective, randomized clinical trial using intravesical RTX application failed to show

a significant improvement of symptoms in patients with IC [78].

1) Role of TRP receptors

In patients with BPS/IC, there is a significant increase in suburothelial nerve fibers expressing TRPV1 and the increase is correlated with pain scores [79]. There is also evidence that chronic bladder inflammation in animal models can induce changes in functional properties of chemosensitive receptors such as TRPV1 in sensory neurons. In rat bladders, increased expression of anandamide, which can activate TRPV1 receptors, has been proposed as one mechanism that could contribute to the bladder overactivity elicited by cyclophosphamide-induced cystitis [80]. In addition, bladder overactivity elicited in rats by lipopolysaccharide-induced cystitis is inhibited by intraduodenal administration of a TRPV1 antagonist (GRC-6211) [81] and is prevented in TRPV1-knockout mice [82]. Therefore, it is assumed that enhanced activity of TRPV1 receptors in bladder afferent pathways contributes to bladder pain in BPS/IC.

2) Role of neurotrophic factors

In patients with BPS/IC, increased levels of neurotrophic factors, including NGF, neurotrophin-3, and glial-derived neurotrophic factor, have been detected in the urine [83]. Increased expression of NGF is also present in bladder biopsies from women with BPS/IC [84]. It has also been demonstrated that exogenous NGF can induce bladder nociceptive responses and bladder overactivity in rats when applied acutely into the bladder lumen [85,86] or chronically to the bladder wall or intrathecal space [49,50]. Thus, target organ-neural interactions mediated by an increase of neurotrophic factors in the bladder and increased transport of neurotrophic factors to the neuronal cell bodies in afferent pathways may contribute to the emergence of bladder pain and other symptoms, such as urinary frequency, in BPS/IC.

In clinical studies, the monoclonal NGF neutralizing antibody, tanezumab, has been tested, and encouraging results of the Phase II efficacy study were obtained [87]. Although proof-of-concept evidence has been provided for the effectiveness of systemic targeting of the NGF system in the treatment of BPS/IC, clinical studies were put on hold following reports of bone necrosis requiring total joint replacements in clinical trials for osteoarthritis (www.clinicaltrials.gov). Therefore, site-specific reduction of NGF would be desirable to reduce the intrinsic toxicity from systemic blockade of NGF. In this regard, a recent study using rats showed that treatment with intravesical liposomal antisense suppresses NGF expression in the urothelium as well as bladder overactivity and chemokine up-regulation in a model of acetic acid-induced bladder overactivity [88]. Thus, local suppression of NGF in the bladder using intravesical liposome-based delivery techniques could be an attractive approach for BPS/IC treatment. Such an approach could avoid the systemic side effects that may be associated with nonspecific blockade of NGF

expression.

4. Diabetes mellitus and detrusor underactivity

A large percentage (50%–70%) of patients with diabetes mellitus (DM) exhibit LUTS [89,90]. The most common urodynamic findings, classically referred to as diabetic cystopathy, include impaired sensation of bladder fullness, increased bladder capacity, reduced bladder contractility, and increased residual urine [90–92]. However, DO is also common in patients with DM, especially when they present with LUTS [89,93].

The pathophysiology of DM-associated LUT complications is multifactorial and can be a result of an alteration in the physiology of the detrusor smooth muscle cell, the peripheral innervation reflex mechanisms, or urothelial function [92]. A two-step model of diabetic cystopathy progression has been proposed on the basis of experimental animal studies. This model suggests that patients initially develop bladder hypertrophy and overactivity, which is presumably a process of adaptation to polyuria-mediated frequent voiding, followed by the decompensated phase inducing classical diabetic cystopathy associated with detrusor underactivity [94]. DM neuropathy also reportedly affects urethral function in streptozotocin-induced DM rats. DM rats exhibit a reduction in nitric oxide-mediated relaxation and an enhancement of $\alpha 1$ -adrenoceptor-mediated contraction of the urethral smooth muscle during reflex bladder contractions, both of which may contribute to increased bladder outlet resistance resulting in impaired bladder emptying in DM [95,96].

1) Role of neurotropic factors

A two-step model of diabetic cystopathy progression (i.e., initial overactivity followed by underactivity) is also supported by changes in NGF levels in the bladder and axonal transport of NGF. Increased NGF levels have been reported in the bladders of rats with early experimental DM [97], whereas in more advanced diabetic bladder disease (for example, the decompensated phase of experimental diabetic cystopathy), bladder and DRG levels of NGF drop and animals display increased bladder capacity and decreased peak pressures [98]. Loss of NGF production has been implicated in the development of sensory and sympathetic neuronal degeneration associated with diabetic neuropathy [99,100].

CONCLUSIONS

Storage and periodic release of urine is dependent on a complex neural network located at various levels of the peripheral and central nervous system that coordinates the activity of smooth and striated muscles of the bladder and urethra. Afferent pathways that trigger storage and voiding reflexes as well as the sensations of bladder filling transmit activity from mechanoreceptors in the bladder through second order neurons in the spinal cord to various central processing areas in the brain.

Owing to the complexity of the neural mechanisms that regulate urine storage and voiding, these processes are sensitive to various neural injuries and diseases. As the underlying mechanism inducing LUT dysfunction, several types of peripheral and central neuroplasticity have been identified. These include the following: (1) emergence of primitive neonatal micturition reflexes and (2) remodeling of spinal circuitry and sensitization of bladder silent C-fiber afferents leading to the emergence of a spinal micturition reflex. NGF has been implicated in this plasticity because NGF levels increase in the bladder and spinal cord. Furthermore, intrathecal administration of NGF antibodies suppresses neurogenic LUT dysfunction and afferent sensitization in animal models. Increased levels of neurotrophic factors have also been detected in other types of bladder disorders. Therefore, the role of these agents in bladder pathophysiological mechanisms is a very active research field in neurourology.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

ACKNOWLEDGMENTS

The authors' research is supported by NIH grants (P01DK093424, DK088836), DOD grants (W81XWH-11-1-0763, W81XWH-12-1-0565), and PVA 2793.

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Differential contribution of Kv4-containing channels to A-type, voltage-gated potassium currents in somatic and visceral dorsal root ganglion neurons

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Submitted 16 January 2014; accepted in final form 13 August 2014

Yunoki T, Takimoto K, Kita K, Funahashi Y, Takahashi R, Matsuyoshi H, Naito S, Yoshimura N. Differential contribution of Kv4-containing channels to A-type, voltage-gated potassium currents in somatic and visceral dorsal root ganglion neurons. *J Neurophysiol* 112: 2492–2504, 2014. First published August 20, 2014; doi:10.1152/jn.00054.2014.—Little is known about electrophysiological differences of A-type transient K⁺ (K_A) currents in nociceptive afferent neurons that innervate somatic and visceral tissues. Staining with isolectin B4 (IB4)-FITC classifies L6-S1 dorsal root ganglion (DRG) neurons into three populations with distinct staining intensities: negative to weak, moderate, and intense fluorescence signals. All IB4 intensely stained cells are negative for a fluorescent dye, Fast Blue (FB), injected into the bladder wall, whereas a fraction of somatic neurons labeled by FB, injected to the external urethral dermis, is intensely stained with IB4. In whole-cell, patch-clamp recordings, phrixotoxin 2 (PaTx2), a voltage-gated K⁺ (Kv)4 channel blocker, exhibits voltage-independent inhibition of the K_A current in IB4 intensely stained cells but not the one in bladder-innervating cells. The toxin also shows voltage-independent inhibition of heterologously expressed Kv4.1 current, whereas its inhibition of Kv4.2 and Kv4.3 currents is voltage dependent. The swapping of four amino acids at the carboxyl portion of the S3 region between Kv4.1 and Kv4.2 transfers this characteristic. RT-PCRs detected Kv4.1 and the long isoform of Kv4.3 mRNAs without significant Kv4.2 mRNA in L6-S1 DRGs. Kv4.1 and Kv4.3 mRNA levels were higher in laser-captured, IB4-stained neurons than in bladder afferent neurons. These results indicate that PaTx2 acts differently on channels in the Kv4 family and that Kv4.1 and possibly Kv4.3 subunits functionally participate in the formation of K_A channels in a subpopulation of somatic C-fiber neurons but not in visceral C-fiber neurons innervating the bladder.

A-type K channel; dorsal root ganglion; nociceptive C-fiber; phrixotoxin 2; Kv4 channel

PRIMARY AFFERENT NEURONS RESPONSIBLE for pain sensation contain lightly myelinated or unmyelinated fibers with medium- to small-sized cell bodies. Neurons with unmyelinated fibers (C-fiber neurons) are responsible for chronic and intractable pain with clinical significance. Neurochemically, C-fiber neurons are divided further into two major subtypes. One group contains neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P, whereas the other group lacks peptides but contains fluoride-resistant acid phosphatase activity

and binds to the *Griffonia simplicifolia* isolectin B4 (IB4) (Nagy and Hunt 1982; Silverman and Kruger 1990). Although the presence of two C-fiber neuron types is well established, their differences in innervating tissues remain uncertain. For example, retrograde labeling indicated that dorsal root ganglion (DRG) neurons, innervating the skin and bladder, consist of both IB4-positive and -negative cells (Bennett et al. 1996). More IB4-positive cells are found in skin afferents than bladder afferents, suggesting that the two types of C-fiber neurons may preferentially innervate somatic or visceral tissues (Bennett et al. 1996). However, the proportion of IB4-positive cells in somatic or visceral afferent pathways varies among studies (Ambalavanar et al. 2003; Bennett et al. 1996; Dang et al. 2005; Hwang et al. 2005; Ivanavicius et al. 2004; Lu et al. 2001; Yoshimura et al. 2003).

Voltage-gated K⁺ (Kv) currents are major determinants of neuronal excitability. Kv currents in sensory neurons are divided into two major categories: sustained, delayed rectifier-type K⁺ (K_{DR}) and transient A-type K⁺ (K_A) currents (Gold et al. 1996; Hall et al. 1994; Kostyuk et al. 1981; Yoshimura et al. 1996). K_A currents in DRG cells consist of at least two different components, based on their inactivation kinetics (i.e., fast- and slow-inactivating K_A currents) (Akins and McCleskey 1993; Everill et al. 1998; McFarlane and Cooper 1991). The slow-inactivating K_A current in DRG neurons is partially inhibited by α -dendrotoxin, a Kv1 channel blocker (Yang et al. 2004). Furthermore, chronic bladder inflammation increases excitability of capsaicin-sensitive C-fiber bladder afferent neurons due to reductions in the slow-inactivating K_A current and Kv1.4 channel expression (Hayashi et al. 2009; Yoshimura and de Groat 1999). Thus Kv1.4-containing Kv1 family channels are responsible, at least in part, for the K_A current in visceral afferent neurons. Other potential molecular correlates of the K_A current include Kv4 family channels. Kv4.x subunits constitute a K_A-like, rapidly inactivating channel (Birnbbaum et al. 2004). Kv4.3-immunoreactive proteins were found in the somata of a subset of nociceptive DRG neurons (Chien et al. 2007). We also reported recently that Kv4.1 mRNA is expressed in all sizes of rat DRG neurons, using in situ hybridization techniques (Matsuyoshi et al. 2012). Thus multiple Kv subunits may significantly participate in forming K_A channels, yet molecular correlates of K_A currents in distinct target tissues, cell morphologies, and other properties still remain unclear.

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We therefore set out to identify cellular and electrophysiological characteristics of K_A channels in DRG neurons innervating somatic and visceral tissues. Here, we show that IB4 intensely positive neurons innervate the somatic tissue but not the bladder. Moreover, phrixotoxin 2 (PaTx2), a Kv4 channel blocker, exhibits distinct voltage-dependent inhibitions of heterologously expressed Kv4.x currents. With the use of this toxin, we identify further the functional contribution of Kv4.1/4.3 subunits to the K_A channel in IB4 intensely positive C-fiber neurons that contain somatic afferent cells.

Glossary

τ act	Time constants of current activation
τ decay	Time constants of current decay
D	Concentration of toxins (M)
G/G max (control)	Peak K^+ conductance relative to the maximum conductance in control
h	Hill coefficient
I/I max	Normalized peak K^+ current amplitude for inactivation curve analysis
I_c	Mean amplitude of depolarization-induced current in control (in the absence of toxins)
k	Slope factor
K_i	Apparent dissociation constant
L	Fractional current amplitude remaining when the affinity sites are fully occupied
V_h	Voltage at half-maximal conductance

MATERIALS AND METHODS

Animal Preparation and DRG Cell Dispersion

Adult female Sprague-Dawley rats (200–250 g; Hilltop, Scottdale, PA) were used. All animal experiments were carried out in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and with the guidelines by the U.S. National Institutes of Health regarding the care and use of animals for experimental procedures. Fast Blue (FB; 2% w/v; EMS-Chemie, Zürich, Switzerland) was injected into the bladder wall to identify afferent neurons innervating the bladder as visceral neurons by retrograde axonal transport of the fluorescent dye, as described previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Briefly, the bladder was exposed by a midline lower-abdominal incision, and the dye was injected with a 30-gauge needle at four to six sites (20 μ l total vol) on the surface of the bladder under isoflurane anesthesia (1–2%). At each injected site, the needle was kept in place for 20–30 s, and any leakage of dye was removed by application of cotton swab. The injection site was then rinsed with saline, and the incision was closed. In a separate group of animals, we injected FB into the dermis surrounding the external urethral orifice at four sites (20 μ l total vol) to identify afferent neurons innervating the skin as somatic neurons. After 6–9 days, under isoflurane anesthesia, the L6 and S1 DRG were removed, minced, and incubated for 25 min at 37°C in DMEM (Sigma-Aldrich, St. Louis, MO), containing 0.3 mg/ml trypsin (type III; Sigma-Aldrich), 1 mg/ml collagenase (type I-A; Sigma-Aldrich), and 0.1 mg/ml deoxyribonuclease (type IV; Sigma-Aldrich). Trypsin inhibitor (type II-S; Sigma-Aldrich) was then added to neutralize the enzyme activity. Individual DRG cell bodies were isolated by trituration and plated on poly-D-lysine-coated, 8 mm round coverglasses and incubated at 37°C overnight before patch-clamp experiments. Just before the experiments, single cells were stained by

G. simplicifolia IB4-FITC (3 μ g/ml; Axxora, San Diego, CA) for 5 min. The staining condition of IB4-FITC does not influence Kv currents in DRG neurons, including K_A currents (Vydyanathan et al. 2005).

Morphological Characterization in Native DRG Neurons

The coverglass with cultured cells was mounted on a cell chamber for patch-clamp recordings. Microscopic characterization of cells was performed by measuring cell diameter and fluorescence intensities of FB and IB4-FITC by visual inspection (done by one examiner, T.Y.). The degree of IB4 staining in the cell membrane was divided into three grades: *grade 0*, weakly or nonstained (including cytosol-stained cells); *grade 1*, moderately stained on the whole circle of the cell membrane; *grade 2*, intensely stained on the whole circle of the cell membrane.

We also verified this visual grading using the fluorescence intensity of individual cells. Briefly, we quantitatively measured the staining intensity of 92 DRG neurons, randomly photographed during experiments using ImageJ software. The lowest and highest intensities of IB4 staining were rated as 0% and 100%, respectively. With the use of this scale, all visually classified *grades 0*, *1*, and *2* cells fell into the measured fluorescence intensity: <20% of the highest intensity (32 of 92 cells; 34.8%), between 20 and 70% (41 of 92 cells; 44.6%), and >70% (19 of 92 cells; 20.6%), respectively.

Constructions and Transfection

We used Kv4.x channels containing a Myc tag at the N-terminus for this study. Addition of the tag allowed the easy detection of channel protein expression. Myc-tagged Kv4.x, Kv1.4, and Kv2.1 cDNAs were constructed previously in our laboratory (Ren et al. 2003). We targeted the four-amino acid portions in the carboxyl terminal of the S3 region (S3b) to test its roles in voltage dependence of the toxin effects (see Fig. 6). Replacement of the four-amino acid sequences between Kv4.1 and Kv4.2 was done using a two-step, overlapped PCR with primers containing a part of the other channel subunit sequence. Obtained constructs were verified by DNA sequencing.

Chinese hamster ovary (CHO)-K1 cells on an 8-mm coverslip were transfected with expression constructs for Myc-tagged mouse Kv4.1, rat Kv4.2, and rat Kv4.3 at the cDNA ratio 1:4–1:8 using Lipofectamine PLUS (Invitrogen, Life Technologies, Grand Island, NY). We also transfected rat Kv1.4 and rat Kv2.1 on CHO-K1 cells. A small amount of Emerald-C1 (10 ng/dish) was included in transfection for visualization of transfected cells (Takimoto et al. 2002). One or 2 days after transfection, at ~10% confluence, the membrane currents of the cells were recorded using patch-clamp methods.

Whole-Cell, Patch-Clamp Recording Procedure

The setup of the patch-clamp experimental system used was essentially the same as described previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Briefly, a whole-cell, voltage-clamp recording was performed on dispersed, native DRG neurons or cultured CHO-K1 cells with an Axopatch-700B patch-clamp amplifier, and data were acquired and analyzed by pCLAMP 8 software at a sampling rate of 1,000/s (Axon Instruments, Union City, CA). The filter was set to –3 dB at 2,000 Hz, and the P/N protocol was used to subtract leak currents. Whole-cell input capacitance was neutralized directly from the amplifier. Current traces were low-pass filtered by the digital filter of the data acquisition program (pCLAMP 8). Patch electrodes were fabricated from borosilicate capillary tubing and had resistances of 3–7 M Ω when filled with the internal solution. During recordings, isolated DRG neurons and CHO-K1 cells were superfused with bath solution at a flow rate of 2.0 ml/min in a chamber with a 0.30-ml vol at room temperature (20–22°C). Patch pipettes were filled

with a solution containing the following (in mM): 140 KCl, 1.0 CaCl₂, 2.0 MgCl₂, 11 EGTA, 2.0 ATP, 0.40 GTP, and 10 HEPES, titrated to pH 7.4 with Tris base.

For patch-clamp experiments in native DRG neurons, we selected two populations of single cells: 1) FB-positive bladder afferent neurons smaller than 30 μ m in diameter and 2) intensely stained cells with IB4-FITC. Note that there was no overlap in these two populations (see Table 3). We obtained outward K_{DR} currents by depolarizing step pulses from a holding potential of -40 mV for 500 ms, and the outward K_A current was obtained by subtracting K_{DR} from the outward currents activated from the holding potential of -120 mV (I_{-120}), as established previously (Hayashi et al. 2009; Yoshimura and de Groat 1999).

The extracellular solution, which contained the following (in mM): 150 choline-Cl, 5.0 KCl, 0.03 CaCl₂, 3.0 MgCl₂, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4), was used to suppress inward Na⁺ and Ca²⁺ currents. At the end of experiments, all neurons investigated their capsaicin (1 μ M) sensitivity by switching the bath solution to the following solution (in mM): 150 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4). Only the capsaicin-sensitive cells were enrolled into analysis.

In Kv channel-expressed CHO cells, Kv currents were evoked by depolarizing voltage pulses from holding potentials of -100 mV. The extracellular solution included the following (in mM): 150 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4).

Drugs

All drugs were dissolved directly into the extracellular solution. PaTx2 was applied to the cell by injection into the bath solution. Recombinant PaTx2 was purchased from Alomone Labs (Jerusalem, Israel). The rest of the chemicals were purchased from Sigma-Aldrich.

Equation for Curve Fitting of the Steady-State Activation and Inactivation of K_A and Kv4 Currents

The peak outward current amplitude, evoked by depolarization from the holding potential of -100 mV to various potentials, from -60 to $+50$ mV every 10 mV, was recorded to estimate the peak K⁺ conductance. The duration from a pulse to the next pulse was 5 s. The steady-state activation curve was plotted as the G/G max (control) vs. the test potential (V) concerning the theoretical equilibrium potential of K⁺ in 20°C. The data were fitted by the modified Boltzmann equation

$$G/G \text{ max (control)} = 1/(1 + \exp [(Vh - V)/k])$$

Steady-state inactivation was determined by depolarizing voltage steps to $+20$ mV, following 1 s conditioning prepulses from -120 to $+20$ mV. The inactivation curve was plotted as the I/I max vs. the potential of the conditioning prepulse (V). The data were fitted by the modified Boltzmann equation

$$I/I \text{ max} = 1/(1 + \exp [(V - Vh)/k])$$

Table 1. PCR primers for DRG RNAs

Gene	GenBank #	Sequence	Position	Size, bp
Kv4.1	XM_217601	ACTAGCGGGTCTTCGGAGGA	1,683–1,702	271
Kv4.2	NM_031730	TCACAGGGAAGAGATCTTGA	1,953–1,934	579
		TCCGCCACATCCTCAACTTC	828–847	
		CATCCTCATTTGCTGTCTCATC	1,406–1,385	
Kv4.3	NM_031739	GCAAGACCACGTCACTCATC	1,455–1,474	217 (S)
		AGGTGCGTGGTCTTCTTGCT	1,671–1,652	274 (L)
GAPDH	NM_017008	TATGATGACATCAAGAAGTGG	787–808	219
		CACCACCCTGTTGCTGTA	1,005–988	

DRG, dorsal root ganglion; Kv, voltage-gated K⁺; S, short splicing isoform of Kv4.3; L, long splicing isoform of Kv4.3.

Equation for Curve Fitting of the Effects of PaTx2 on Expressed Kv4 Currents

The peak inward current amplitude, evoked by depolarization from the holding potential of -100 mV to $+30$ mV for a duration of 300 ms every 15 s (0.067 Hz), was measured to obtain a concentration-response relationship of toxins. The mean amplitude (I) of two outward currents, just before application of toxins, was taken as the control level, and the amplitude of current (mean of two stable depolarization-induced currents) during toxin application (4–7 min after application) was estimated. The curve was drawn by fitting the equation using the least squares method

$$I/I_c = L + (1 - L)/[1 + (D/K_i)^b]$$

RT-PCR Analysis

Total RNAs were isolated from L6 and S1 DRGs and forebrains, and RT reaction and PCR analyses are performed as described previously (Takimoto et al. 2006). We also performed PCR analyses for Kv4.1 and Kv4.3 mRNA expression in laser-captured DRG neurons. L6 DRGs were removed from rats that had been injected with FB into the bladder wall, 1 wk earlier, and serially sectioned at 8 μ m thickness. Every third section was mounted onto polyethylene naphthalate membrane slides (Leica Microsystems, Wetzlar, Germany) to avoid repeated excision of cells. Slides were then stained with IB4-FITC conjugate (50 μ g/ml in PBS) for 5 min, rinsed with PBS, and air dried. The slides were observed using a fluorescence microscope with the appropriate filter, and laser capture microdissection (LCM) was performed using the Leica LMD6000. DRG cells, labeled with FB or stained with IB4 ($n = 20$ cells each/animal), were laser captured into 0.5 ml Eppendorf tube caps. RNA was isolated from captured individual tissue specimens, followed by DNase treatment. RT and real-time PCR were performed using the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Carlsbad, CA; Tables 1 and 2). Gene-specific primers and TaqMan probes were designed with Primer3 Software (<http://biocompute.bmi.ac.cn/CZlab/Primer3Plus/primer3plusAbout.cgi>). Probes contained 6-carboxyfluorescein (FAM) fluorophore and tetramethylrhodamine (TAMRA) quencher. The primer and probe combination was optimized within suitable ranges for efficiency and correlation coefficient using standard curve dilutions and data output on the ABI StepOne Plus thermocycler (Applied Biosystems). Amplification of cDNA was performed under the following conditions: one cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The reactions were analyzed in triplicate and normalized to GAPDH. Real-time PCR data were analyzed by the difference in crossing point (Cp) method, as $R = 2^{(Cp \text{ sample} - Cp \text{ control})}$ to generate the relative expression ratio (R) of each target gene relative to GAPDH.

Statistical Analysis

Statistical analyses were performed with nonparametric Mann-Whitney test (see Table 5) and nonparametric Wilcoxon signed-rank

Table 2. Real-time PCR primers and probes for laser-captured cells

Gene	GenBank #		Sequence	Position
Kv4.1	NM_001105748	5' Primer	GCACCAGCAAGACCAACTTC	1,049–1,068
		3' Primer	GTACAGGCAAGGCAATGACC	1,213–1,194
		Probe	TGGTGCCTAGCACCATCGCA	1,133–1,152
Kv4.3	NM_031739	5' Primer	CAAGACCACGTCACCTCATCG	1,490–1,509
		3' Primer	AGAGAAGGGCTTCTGGTGGGA	1,642–1,623
		Probe	TGAGCAGAACTGCATGGAGAGCTCA	1,583–1,607
GAPDH	NM_017008	5' Primer	AGACAGCCGCATCTTCTTGT	29–48
		3' Primer	GATACGGCCAAATCCGTTT	112–94
		Probe	GCAGTGCCAGCCTCGTCTCA	49–68

test (see Figs. 3 and 7, and see Table 6). Changes were considered significant at $*P < 0.05$, and $**P < 0.01$. Data are expressed as mean with the SE, except those (see Table 5) that were expressed as mean with SD.

RESULTS

Characterization of Bladder and Cutaneous DRG Neurons

We first examined IB4-FITC binding to acutely dissociated, live L6-S1 DRG neurons from animals that had been injected with FB at the bladder walls. Dissociated, single DRG neurons (530 cells) were observed as round- to oval-shaped cells, in which 11% (59 cells) were FB-positive bladder afferent neurons (Fig. 1A). Staining with IB4-FITC provided various fluorescence intensities. We classified cells based on the IB4-staining level into three categories: cells showed no or negligible fluorescent staining (IB4:0), whereas others exhibited moderate to intense signals (moderate, IB4:1; intense, IB4:2; Fig. 1 and Table 3). The analysis of 530 cells from three rats in this fashion indicated that $>1/3$ of cells were moderately stained with IB4, whereas $\sim 20\%$ of cells were intensely stained (Table 3). IB4-stained cells were distributed in various

sizes of DRG neurons (Fig. 1B). We also verified visual evaluation of IB4-FITC signal intensity by measuring the fluorescence intensity of 92 DRG neurons, randomly photographed during experiments. The fluorescence intensity of grades 0, 1, and 2 cells, judged by visual evaluation, was $<20\%$ (32 of 92 cells; 34.8%), between 20 and 70% (41 of 92 cells; 44.6%), and $>70\%$ (19 of 92 cells; 20.6%) of the highest value among DRG neurons, respectively, demonstrating that our visual evaluation method is appropriate to classify DRG neurons based on IB4-staining intensity. FB-positive bladder afferent neurons showed only negative to weak (IB4:0, 35/59 cells = 59%) or moderate (IB4:1, 24/59 cells = 41%) IB4-FITC staining and were never intensely stained with IB4-FITC (Fig. 1A).

L6-S1 DRG also contains somatic afferent neurons innervating the external skin area surrounding the urethra through the pudendal nerve. Therefore, we analyzed a total of 260 cells from three rats that had been injected with FB at the external urethral dermis (Fig. 2 and Table 4). Among those, 34 cells (13%) were FB-positive cutaneous afferent neurons. As expected, three IB4-staining categories represented similar proportions to the above experiments. Likewise, no apparent

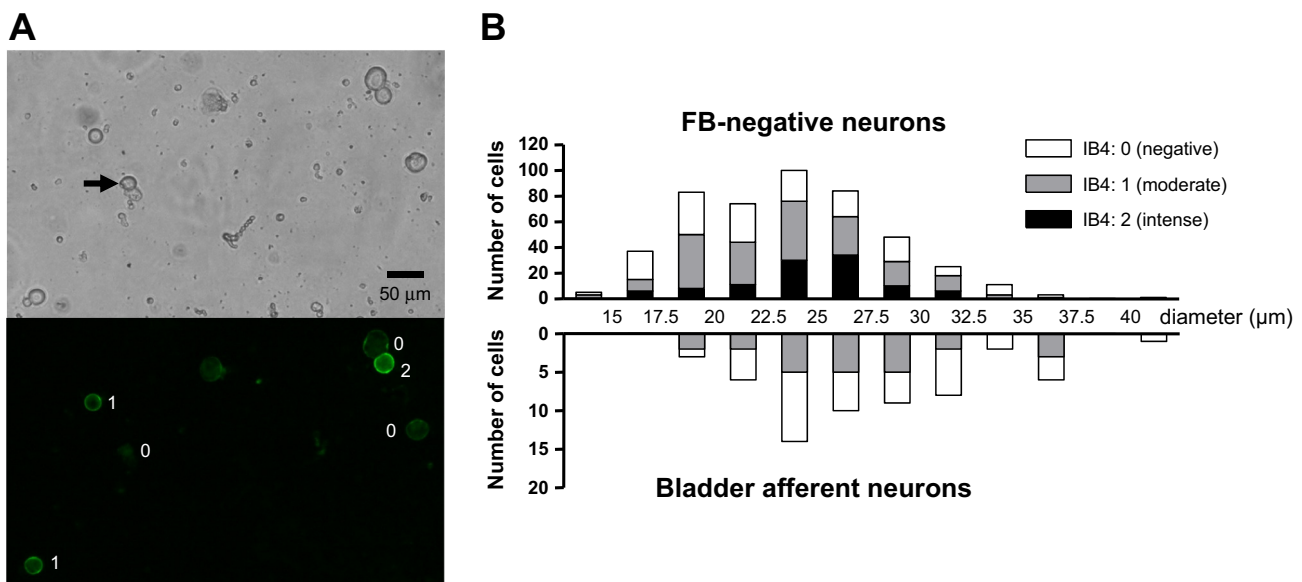


Fig. 1. Morphological characterization of dissociated L6-S1 dorsal root ganglion (DRG) neurons from rats injected with Fast Blue (FB) into the bladder wall. A: single DRG neurons in the same field, observed by light-field and fluorescent microscope, are shown in the top and bottom, respectively. Top: an arrow indicates a FB-positive bladder afferent neuron identified under the fluorescent light. Bottom: single DRG neurons with various green-staining intensities of isolectin B4 (IB4)-FITC. The numbers (0–2) beside the cells indicate the intensity of staining: 0, no or weak (negative) staining; 1, moderate staining; 2, intense staining. B: cell-size distributions of FB-positive and -negative DRG cells with different IB4 staining intensities (grades 0–2). Note that there is no grade 2-stained cells in FB-positive bladder afferent neurons.

Table 3. IB4 staining intensity of bladder afferent neurons

	IB4:0	IB4:1	IB4:2
FB-negative neurons	168 (32%)	198 (37%)	105 (20%)
Bladder afferent neurons	35 (6.6%)	24 (4.5%)	0 (0%)
Total neurons	203 (38%)	222 (42%)	105 (20%)

Total 530 cells. Fast Blue (FB) was injected into the bladder wall at 4–6 sites (20 μ l total vol) in each animal. IB4, isolectin B4.

correlation between IB4-staining intensity and cell size was observed. However, FB-positive cutaneous afferent neurons included many IB4-stained cells, and $>1/4$ of FB-positive cells were intensely stained with IB4-FITC (IB4:2, 9/34 cells). Thus intense IB4-positive cells constitute a subpopulation of somatic afferent neurons innervating the urethral external skin, whereas visceral afferent neurons innervating the bladder consist of IB4-negative to moderately stained cells. Hence, afferent neurons with distinct IB4-staining intensities may preferentially innervate either cutaneous or visceral tissues.

Distinct Effects of PaTx2 on K_A Currents in Bladder Afferent and IB4-Positive Neurons

K_A currents play important roles in controlling activity of excitable cells, including sensory neurons. We wished to identify functional differences in K_A currents between cutaneous and visceral afferent neurons. We chose two populations of L6-S1 DRG neurons, <30 μ m in diameter, from rats injected with FB in the bladder: 1) IB4 intensely stained cells and 2) FB-positive cells. The former represents a subpopulation of somatic afferent neurons, whereas the latter corresponds to a fraction of bladder afferent neurons. There was no overlap in these two neuron types. All of the tested cells in the two groups showed the response to capsaicin (1 μ M) to induce inward current, indicating that they were capsaicin-sensitive C-fiber afferent cells. The peak current density of the capsaicin-induced inward current was 80 ± 4.7 pA/pF ($n = 24$) in IB4 intensely stained neurons and 89 ± 12 pA/pF in bladder afferent neurons ($n = 11$).

Kv currents were recorded using two holding potentials to obtain K_{DR} and K_A current components. K_{DR} currents were

Table 4. IB4 staining intensity of skin afferent neurons

	IB4:0	IB4:1	IB4:2
FB-negative neurons	90 (35%)	96 (37%)	40 (15%)
Skin afferent neurons	11 (4.2%)	14 (5.4%)	9 (3.5%)
Total neurons	101 (39%)	110 (42%)	49 (19%)

Total 260 cells. FB was injected into the skin surrounding the external urethral orifice at 4 sites (20 μ l total vol) in each animal.

evoked from a holding potential of -40 mV, whereas K_A currents were obtained by subtracting K_{DR} currents from I_{-120} , as we reported previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Peak amplitudes of K_A and K_{DR} were larger in IB4 intensely stained cells than those in bladder afferent cells ($P < 0.01$, Mann-Whitney test; Table 5). The V_h in steady-state activation and inactivation was lower in IB4 intensely stained cells than those in bladder afferent neurons ($P < 0.01$ for steady-state activation, and $P < 0.01$ for steady-state inactivation, Mann-Whitney test; Table 5). These differences indicate that the bladder afferent cells possess a typical slowly inactivating K_A current, as we have reported previously (Yoshimura and de Groat 1999; Yoshimura et al. 1996), whereas IB4 intensely positive cells display K_A currents with faster kinetics.

Recent studies indicate that Kv4.x subunits are significantly expressed in DRG neurons (Matsuyoshi et al. 2012; Phuket and Covarrubias 2009). To identify the functional contribution of Kv4 channels to K_A currents in DRG neurons, we tested the effect of a Kv4 blocker, PaTx2, on the currents. Application of PaTx2 at 1 μ M significantly inhibited the K_A current in IB4 intensely positive cells ($P < 0.01$ for PaTx2, 500 nM; $P < 0.01$ for PaTx2, 1,000 nM; Wilcoxon signed-rank test) without significant influence on the K_{DR} current ($96 \pm 0.85\%$ of the predrug value for PaTx2, 1,000 nM, $n = 13$; Fig. 3, A, B, and D). In contrast, PaTx2 produced no effect on the K_A currents in FB-positive bladder afferent neurons (Fig. 3, C and D). These results indicate that Kv4 family channels contribute to the K_A current in IB4 intensely stained afferent neurons that represent a subpopulation of somatic afferent cells. In contrast, these

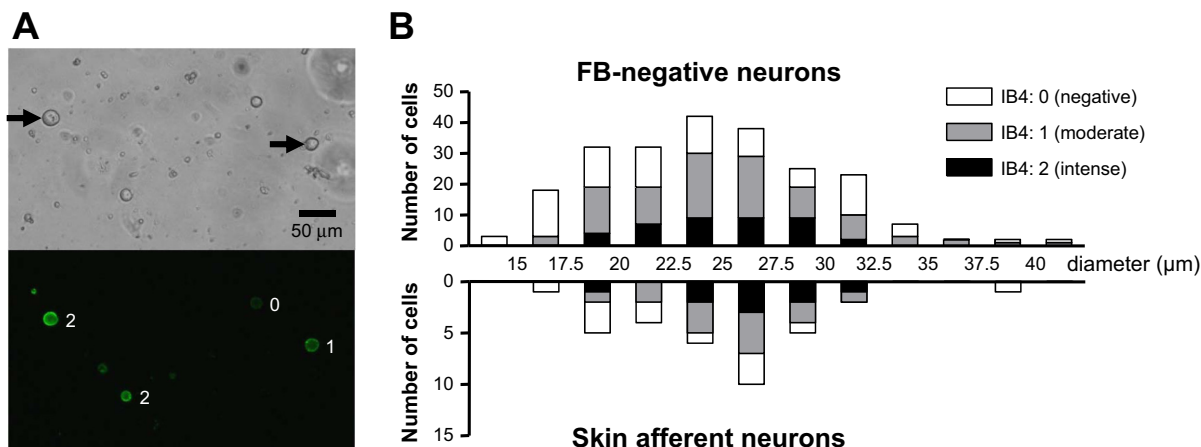


Fig. 2. Morphological characterization of dissociated L6-S1 DRG neurons from rats injected with FB into the skin around the urethral orifice. A: single DRG neurons in the same field observed by light-field and fluorescent microscope are shown in the top and bottom, respectively. Top: arrows indicate FB-positive cutaneous afferent neurons identified under the fluorescent light. Bottom: single DRG neurons with various green-staining intensities of IB4-FITC. The numbers (0–2) beside the cells indicate the intensity of staining, as described in Fig. 1. B: cell-size distributions of FB-positive and -negative DRG cells with different IB4-staining intensities (grades 0–2). Note that grade 2-stained cells are observed in FB-positive cutaneous afferent neurons.

Table 5. Electrophysiological properties of IB4 intensely positive and bladder afferent neurons

	IB4 Intensely Positive Cells	Bladder Afferent Cells
Number of cells	21	9
Diameter, μm	27 ± 2.1	$24 \pm 2.2^*$
Input capacitance, pF	35 ± 3.7	$28 \pm 3.3^*$
I_A peak at 0 mV, nA	2.5 ± 0.63	$0.73 \pm 0.14^*$
I_{DR} peak at 0 mV, nA	1.3 ± 0.39	$0.75 \pm 0.22^*$
Steady-state activation V_h , mV	-58 ± 4.4	$-39 \pm 5.0^*$
Steady-state inactivation V_h , mV	-89 ± 4.3	$-75 \pm 4.3^*$
τ act at 0 mV, ms	1.9 ± 0.20	$3.5 \pm 0.72^*$
τ decay at 0 mV, ms	160 ± 19	$210 \pm 17^*$

Values are means $\pm$ SD. I_A , A-type transient K^+ current; I_{DR} , delayed rectifier-type K^+ current. $^*P < 0.01$ vs. IB4 intensely positive cells, Mann-Whitney test.

channels do not contribute significantly to the K_A current in bladder afferent neurons.

PaTx2 is closely related to the gating-modifying toxins that act on Kv2 and/or Kv4 channels, such as hanatoxins and heteropoda toxins (Diochot et al. 1999; Escoubas et al. 2002; Sanguinetti et al. 1997; Swartz and MacKinnon 1995). These toxins usually exhibit voltage-dependent inhibition of target channel currents. We found that PaTx2 similarly inhibited the K_A current in IB4 intensely positive DRG neurons at all

depolarizing pulses (Fig. 4A). No apparent voltage shift was seen in the normalized steady-state activation curves before and after application of PaTx2 (Fig. 4B). The toxin produced no significant changes in time constants for activation and inactivation, although it tended to slow both processes at all voltages (Fig. 4, C and D). Thus PaTx2 voltage independently blocks the K_A current in IB4 intensely stained cells without apparent effects on activation or inactivation.

Distinct Effects of PaTx2 on Heterologously Expressed Kv4 Channels

The lack of voltage dependence of the toxin action on the native K_A current stimulated us to test whether the toxin might similarly inhibit heterologously expressed Kv4 currents in a voltage-independent fashion. We expressed N-terminally Myc-tagged rat Kv4.1, Kv4.2, or Kv4.3 channels in CHO cells. Transient outward K^+ currents were evoked by depolarizing voltage pulses in channel cDNA-transfected cells (Fig. 5, A–C). The inactivation time courses were slightly slower than those obtained with the corresponding channels without a tag (time constants of inactivation at 30 mV were 56.3 ± 6.8 ms for wild-type Kv4.3 and 104.7 ± 22.3 ms for Myc-tagged Kv4.3; unpublished observation). The toxin at 500 nM significantly reduced the peak amplitude of all Kv4.x currents ($P < 0.01$ for all Kv4.x currents evoked with depolarization to 0 mV

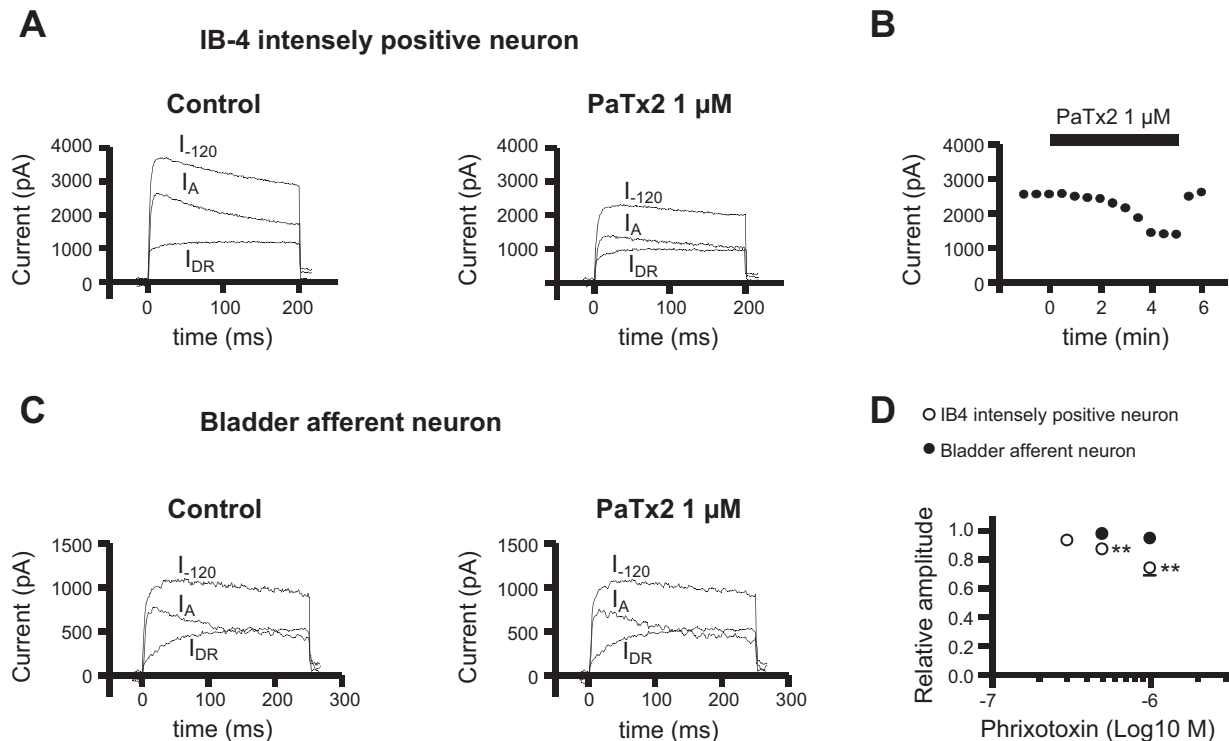


Fig. 3. Effects of phrixotoxin 2 (PaTx2) on A-type transient K^+ (K_A) currents in IB4 intensely stained and bladder afferent neurons. K_A current (I_A) trace was obtained by subtracting the delayed rectifier-type K^+ (K_{DR}) current (I_{DR}) from the outward currents activated from the holding potential of -120 mV (I_{-120}). I_{DR} and I_{-120} were evoked by a depolarizing voltage step to 0 mV from the holding potential of -40 mV and -120 mV, respectively. A and C: representative I_{-120} , I_{DR} , and I_A before (Control) and after application of 1 μM PaTx2 in an IB4 intensely positive neuron (A) and a bladder afferent neuron (C). B: the time course of PaTx2 effects on peak I_A amplitudes in an IB4 intensely positive neuron. D: the concentration-response relationship of PaTx2. Relative peak I_A amplitudes were determined using the level before application of PaTx2 (control) as 1 from the same cell. In IB4 intensely positive cells, PaTx2 decreased the K_A current in a concentration-dependent manner: 300 nM, 0.93 ± 0.024 ; 500 nM, 0.87 ± 0.019 ; 1,000 nM, 0.74 ± 0.040 ($n = 4-18$). Peak I_A amplitudes at 500 and 1,000 nM PaTx2 were significantly different from the control level in IB4 intensely positive cells ($^{**}P < 0.01$ for PaTx2, 500 nM; $^{**}P < 0.01$ for PaTx2, 1,000 nM, Wilcoxon signed-rank test). In contrast, PaTx2 exhibited no significant changes in peak I_A amplitudes in bladder afferent neurons: 500 nM, 0.98 ± 0.0050 ; 1,000 nM, 0.94 ± 0.023 ($n = 6-11$). The data are expressed as means $\pm$ SE.

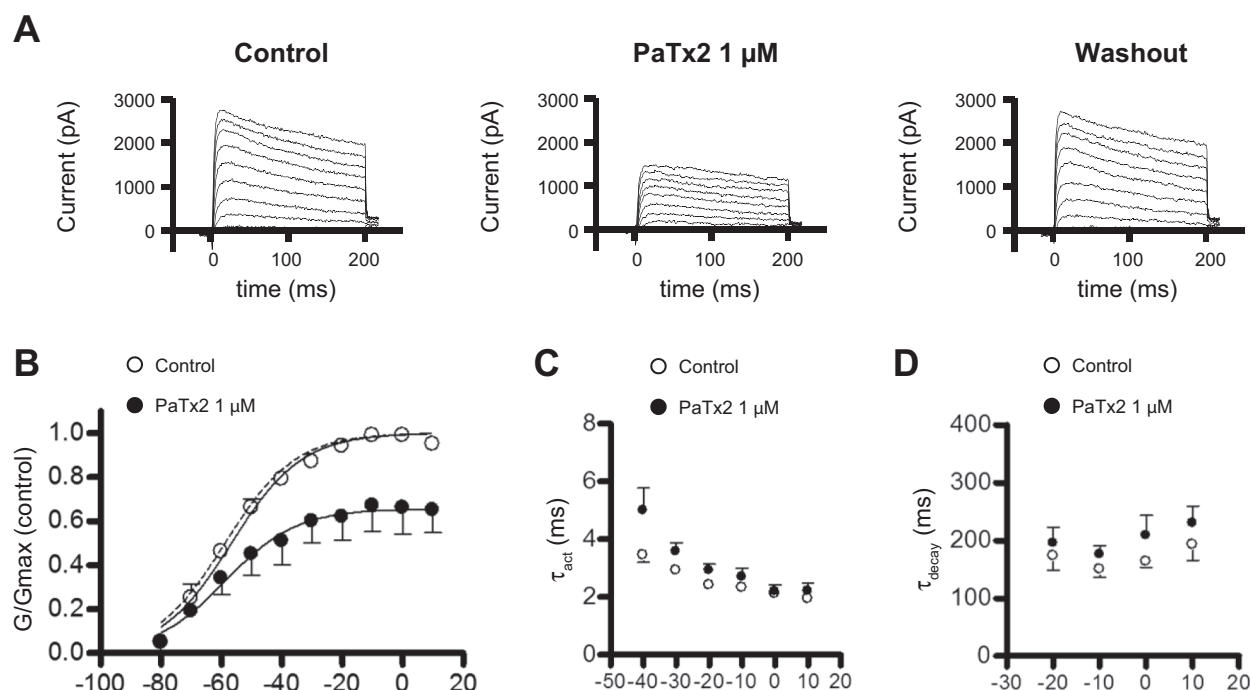


Fig. 4. Effects of PaTx2 on steady-state kinetic parameters of I_A in IB4 intensely stained neurons. I_A current trace was obtained by subtracting K_{DR} from I_{-120} . Membrane depolarization was evoked by test pulses from -80 to $+10$ mV with a 10 -mV interval from the holding potential of -40 mV (K_{DR}) and -120 mV (I_{-120}). A: representative I_A current traces show inhibition by PaTx2 and its recovery. I_A in IB4 intensely positive DRG cells was suppressed by application of $1 \mu\text{M}$ PaTx2 and was almost totally recovered after washing PaTx2 by perfusion. B: relative conductance levels (G/G_{max} control) are plotted against the depolarizing test potential (V). Open and filled circles indicate the values before and after application of PaTx2, respectively. Solid lines were obtained with the relative conductance levels in the absence and presence of $1 \mu\text{M}$ PaTx2. A broken line was generated with the normalized PaTx2 data using the maximum conductance value. C: τ_{act} are plotted against V. D: τ_{decay} are plotted against V. Note that time-constant (τ) values for activation and inactivation were obtained using a single exponential equation. The data are expressed as means $\pm$ SE ($n = 5$).

and $+30$ mV, Wilcoxon signed-rank test; Fig. 5, A–C). The estimated K_i values were 230 nM, 120 nM, and 110 nM for Kv4.1, Kv4.2, and Kv4.3 currents evoked with depolarization to $+30$ mV, respectively (Table 6). No detectable effects were seen with Kv1.4 or Kv2.1 currents at 500 nM (data not shown). Hence, PaTx2 is specific for Kv4 family channels in this concentration range.

Importantly, we found that PaTx2 produced distinct inhibitory actions on Kv4 currents. The toxin similarly reduced Kv4.1 current elicited by pulse voltages at 0 and $+30$ mV, whereas it caused more pronounced inhibition of Kv4.2 and Kv4.3 currents at the lower pulse voltage (Fig. 5D). The toxin did not apparently change the voltage dependence of steady-state activation (Fig. 5A) or inactivation (Fig. 5E) for the Kv4.1 current. In addition, no significant changes in the time constants for activation or inactivation were observed at any pulse voltages for the current (Fig. 5A). In contrast, the toxin caused clear changes in these parameters for Kv4.2 and Kv4.3 currents. The steady-state activation (Fig. 5, B and C) and inactivation (Figs. 5E) curves were shifted to more positive voltages with significant changes in the time constants for activation and inactivation (Fig. 5, B and C). These results indicate that the toxin voltage independently and dependently inhibited Kv4.1

and Kv4.2/Kv4.3 currents, respectively. It should be noted, however, that Kv4.3 current exhibited a complex behavior upon the toxin treatment and might involve, in part, voltage-independent inhibition by the toxin. For instance, the current showed a less obvious, positive shift in the steady-state inactivation (Fig. 5E).

The observed different inhibitory modes of Kv4 channels might arise from sequence differences in their interaction site with PaTx2. Specifically, the latter part of the third transmembrane S3 is a major interaction site with gating modifier toxins. Sequence alignment of Kv4 polypeptides revealed divergence of four amino acids in this portion (Fig. 6A). Therefore, we set out to test if swapping this portion between Kv4 proteins might transfer voltage dependence of the toxin action. Since Kv4.1 and Kv4.2 channels show apparent differences in the toxin inhibition, we generated the two-channel proteins with the four-amino acid sequence of the other at the corresponding site (Kv4.1-VMTD and Kv4.2-FVPK). Kv4.1-VMTD exhibited most of the characteristics of the wild-type Kv4.2 channel in the toxin inhibition. The toxin produced larger inhibition at lower pulse voltage (Fig. 6D) and shifted steady-state activation and inactivation curves to the right (Fig. 6, B and E). Time constants for inactivation were also reduced at lower voltages.

Fig. 5. Effects of PaTx2 on expressed voltage-gated K^+ (Kv)4 currents. Kv4.1, Kv4.2, and Kv4.3 currents were recorded in Chinese hamster ovary-K1 cells transfected with Myc-tagged rat Kv4.x expression vectors in a whole-cell configuration. K^+ currents were elicited by 300 ms test pulses from a holding potential of -100 mV, before and after application of 200 nM PaTx2, except that the data with 500 nM toxin are shown in A, B, C, and E. Open and closed circles represent before and after application of 200 nM PaTx2 ($n = 5$ – 8). A–C, *i*: representative Kv4 current traces; *ii*: steady-state activation curves; *iii*: τ_{act} ; and *iv*: τ_{decay} . Broken lines (in *ii*) were generated with the PaTx2 data normalized to the maximum conduction value. τ Values for activation and inactivation were obtained using a single exponential equation. D: PaTx2 dose-dependent inhibition of Kv4 currents at 0 mV and 30 mV. E: steady-state inactivation.

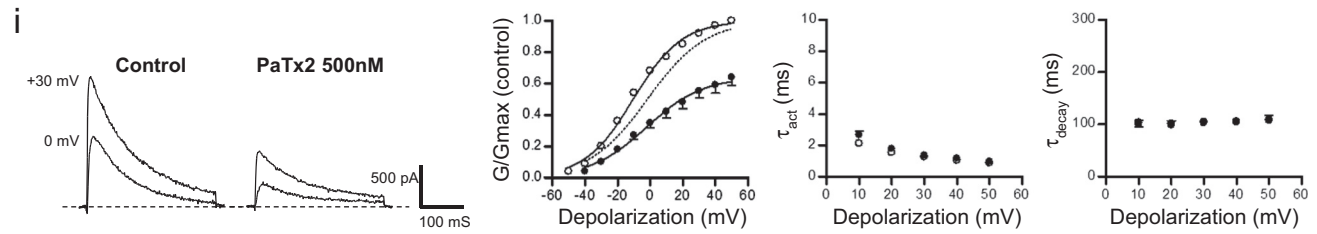
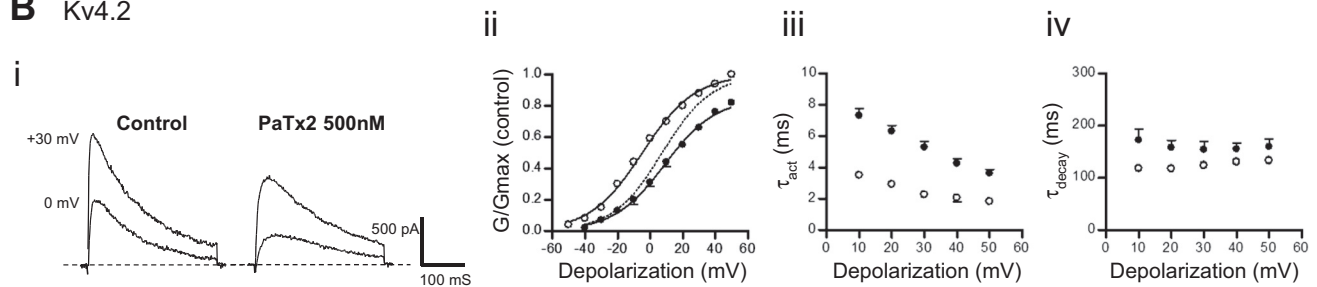
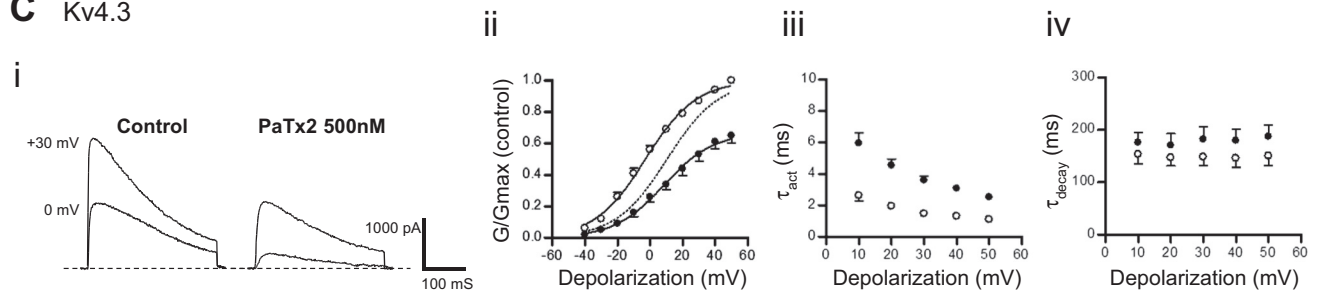
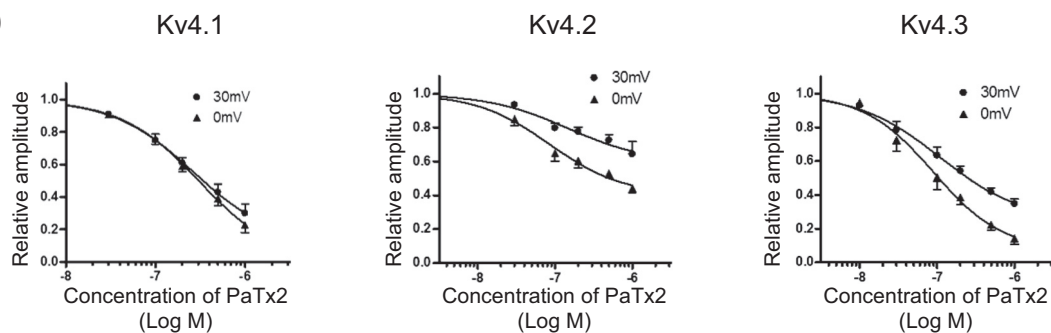
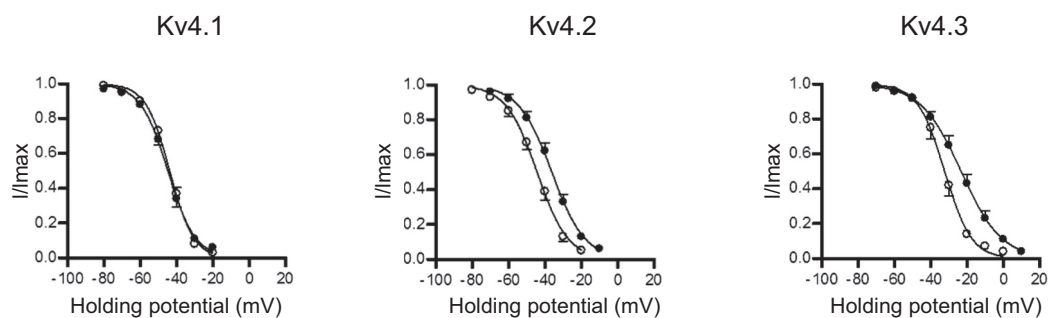
A Kv4.1**B** Kv4.2**C** Kv4.3**D****E**

Table 6. Effects of PaTx2 on Kv4 channels expressed in CHO cells

		Kv4.1	Kv4.2	Kv4.3	Kv4.1 VMTD	Kv4.2 FVPK
K_i value, nM	0 mV	250	80	89	55	650
	30 mV	230	120	110	36	810
Steady-state activation V_h , mV	Control	-11 ± 1.7	-4.8 ± 1.4	-3.1 ± 1.6	-2.4 ± 2.2	-2.9 ± 1.9
	PaTx2, 200 nM	$-3.8 \pm 2.0^*$	$7.6 \pm 1.7^*$	$12 \pm 3.3^*$	$12 \pm 2.9^\dagger$	$3.4 \pm 2.8^{*§}$
Steady-state activation slope factor	Control	15 ± 0.55	16 ± 0.53	15 ± 0.34	15 ± 0.58	16 ± 0.40
	PaTx2, 200 nM	$18 \pm 0.37^*$	15 ± 0.55	17 ± 0.81	14 ± 0.73	$17 \pm 0.50^*$
Steady-state inactivation V_h , mV	Control	-44 ± 0.90	-44 ± 1.8	-32 ± 1.7	-36 ± 1.7	-42 ± 1.2
	PaTx2, 500 nM	-45 ± 1.3	$-36 \pm 1.8^*$	$-23 \pm 2.3^\dagger$	$-18 \pm 1.9^*$	-44 ± 0.88
Steady-state inactivation slope factor	Control	6.4 ± 0.26	8.3 ± 0.46	6.7 ± 0.37	5.5 ± 0.14	8.4 ± 0.27
	PaTx2, 500 nM	7.5 ± 0.47	8.8 ± 0.71	$10 \pm 0.56^\dagger$	$8.3 \pm 0.41^*$	9.2 ± 0.55

CHO, Chinese hamster ovary. Values are means $\pm$ SE ($n = 5-11$). $^*P < 0.05$, and $^\dagger P < 0.01$ vs. respective control values, Wilcoxon signed-rank test; § values obtained after application of phrixotoxin 2 (PaTx2), 500 nM.

Similarly, Kv4.2-FVPK showed voltage-independent inhibition by the toxin. The toxin similarly decreased Kv4.2-FVPK current at different pulse voltages (Fig. 6D) without an apparent shift in the steady-state inactivation curve and time constants (Fig. 6, E and B). These results indicate that the four-amino acid portion at the end of S3 determines voltage dependence of the inhibition by PaTx2.

Expression of Kv4 mRNAs in DRG Neurons

We performed RT-PCRs to detect the expression of Kv4 mRNAs. Abundant expression of Kv4.1 and the long isoform of Kv4.3 mRNAs were seen in L6-S1 DRGs (Fig. 7A). In contrast, a very low level of the Kv4.2 message was detected. These findings indicate that DRG neurons are largely devoid of Kv4.2.

We then compared the expression level of Kv4.1 and Kv4.3 mRNAs between bladder afferent neurons and IB4-stained DRG neurons using LCM methods (Fig. 7B). Although the difference in IB4-staining intensity (e.g., intense vs. moderate staining) is less obvious in DRG sections (Fig. 7B) compared with the cell culture system (Figs. 1 and 2), we laser captured DRG cells, which were not labeled by FB injected into the bladder wall, with relatively strong IB4 staining. Real-time PCR analysis detected higher expression of Kv4.1 and Kv4.3 mRNAs in laser-captured, IB4-stained neurons than in FB-labeled bladder afferent neurons ($n = 6$ rats; $P < 0.05$ for both Kv4.1 and Kv4.3, Wilcoxon signed-rank test; Fig. 7, D and E).

Taken together, the findings suggest that selective expression of the pore-forming Kv4.1 and possibly Kv4.3 in IB4 intensely stained afferent neurons contributes to the observed voltage-independent inhibition of the K_A current.

DISCUSSION

IB4 Staining of Bladder and Cutaneous Afferent Neurons in Rats

Several groups have reported various percentages of IB4-positive cells in adult rat bladder afferents (L6-S1): 14% (adult

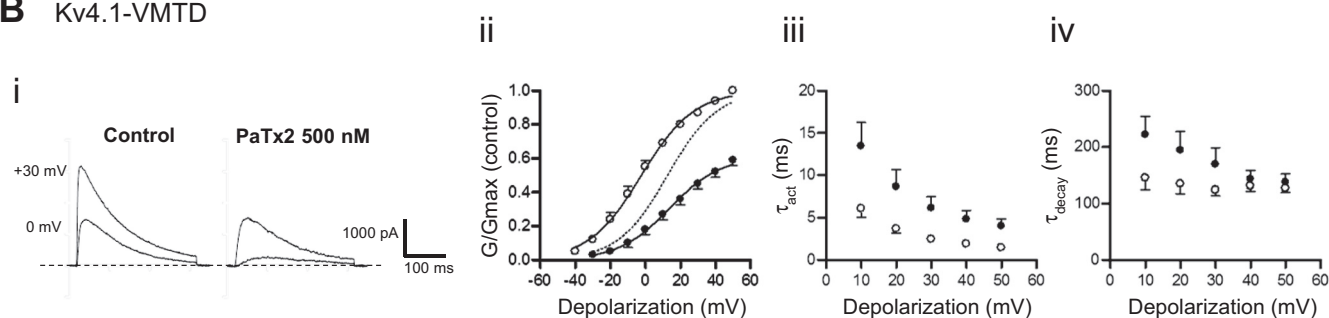
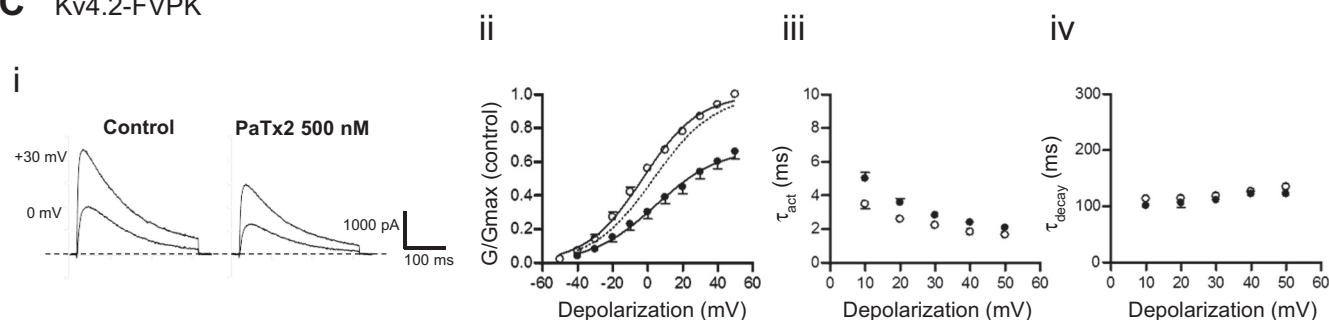
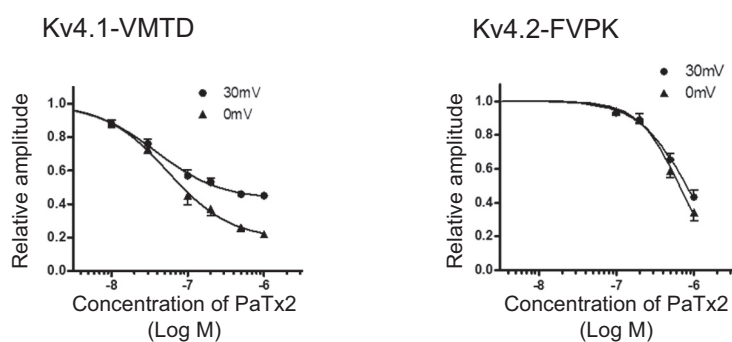
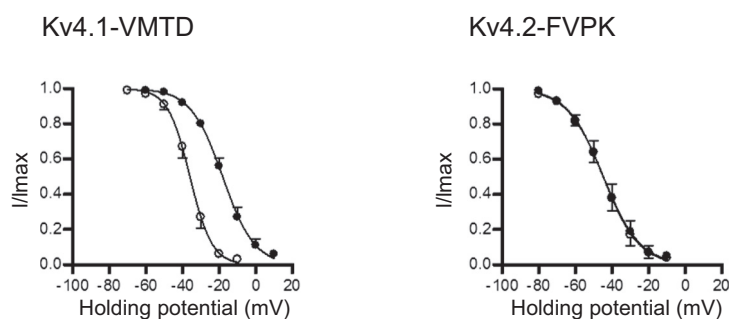
female) (Yoshimura et al. 2003), 29% (adult male) (Bennett et al. 1996), 48% (adult male) (Hwang et al. 2005), and 61% (adult male) (Dang et al. 2005). Dang et al. (2005) reported the highest rate of IB4 staining in bladder afferents, possibly due to the high-intensity staining by IB4-Alexa Fluor 488 used in their study, whereas IB4-FITC was used in other studies, including our current study. The former two groups also reported that the percentage of IB4-positive cells is higher in somatic afferent neurons than in bladder afferents: 27% (distal urethra, L6-S1 DRG) (Yoshimura et al. 2003) and 43% (medial ankle, L3 DRG) (Bennett et al. 1996). Other studies have also found high percentages of IB4-positive cells in cutaneous somatic afferent neurons from lumbar DRG of adult rats: 48% (knee) (Ivanavicius et al. 2004), 44% (vibrissal pad area) (Ambalavanar et al. 2003) using IB4-FITC, and 70% (footpads) (Lu et al. 2001) using IB4-Cy3.

In our current experiments with single, live DRG neurons, membrane staining of IB4-FITC was compared very clearly with an image of fixed and sliced sections. This condition with live cells allowed us to classify the level of IB4 staining. Instead of defining individual neurons as positive or negative, we therefore divided cells into three categories: *grades 0* (no or weak staining), *1* (moderate staining), and *2* (intense staining). According to this classification, we found that bladder afferent neurons lack the IB4 intensely stained (*grade 2*) cells. On the contrary, skin afferent neurons contained significant IB4 intensely stained (*grade 2*) cells (24%). These results strongly suggest that IB4 intensely stained (*grade 2*) cells are not visceral afferents but represent a subpopulation of somatic afferent neurons. On the other hand, IB4 moderately stained (*grade 1*) cells were included in both bladder and skin afferent populations. Taken together, various percentages of IB4-positive cells in visceral and somatic DRG neurons in the previous studies might be attributable to different criteria used to identify IB4-positive cells. In particular, the previous studies might include or exclude in the positive category IB4 moderately stained cells, which were categorized as *grade 1* in the current study.

Fig. 6. Effects of PaTx2 on Kv4 chimeric channels. A: amino acid sequences of the S3 region of rat Kv4.x pore-forming subunits are aligned. A box represents the 4-amino acid portion that is diverged among the Kv4 family channels. Bold letters indicate the amino acids that were swapped between Kv4.1 and Kv4.2. B and C: data were obtained using 500 nM PaTx2, except for B, *ii-iv*, in which 200 nM PaTx2 was used. Open and closed circles represent before and after application of PaTx2 ($n = 7-11$). *i*: representative current traces before and after application of 500 nM PaTx2; *ii*: steady-state activation curves with broken lines generated with normalization using PaTx2 data; *iii*: τ act; and *iv*: τ decay. D: PaTx2 dose-dependent inhibition of Kv4 currents at 0 mV and 30 mV. E: steady-state inactivation.

A

	S2	S3	S4
rKv4.1	LFAAPSRRCFLRSVMSLIDVVAILPYYIGL	FVPKNDDVSGAFVTLRV	296
rKv4.2	LAAAPSRYRFVRSVMSIIDVVAILPYYIGL	VMTDNEDVSGAFVTLRV	294
rKv4.3	LFAAPSRYRFIRSVMSIIDVVAIMPYYIGL	VMTNNEDVSGAFVTLRV	291

B Kv4.1-VMTD**C** Kv4.2-FVPK**D****E**

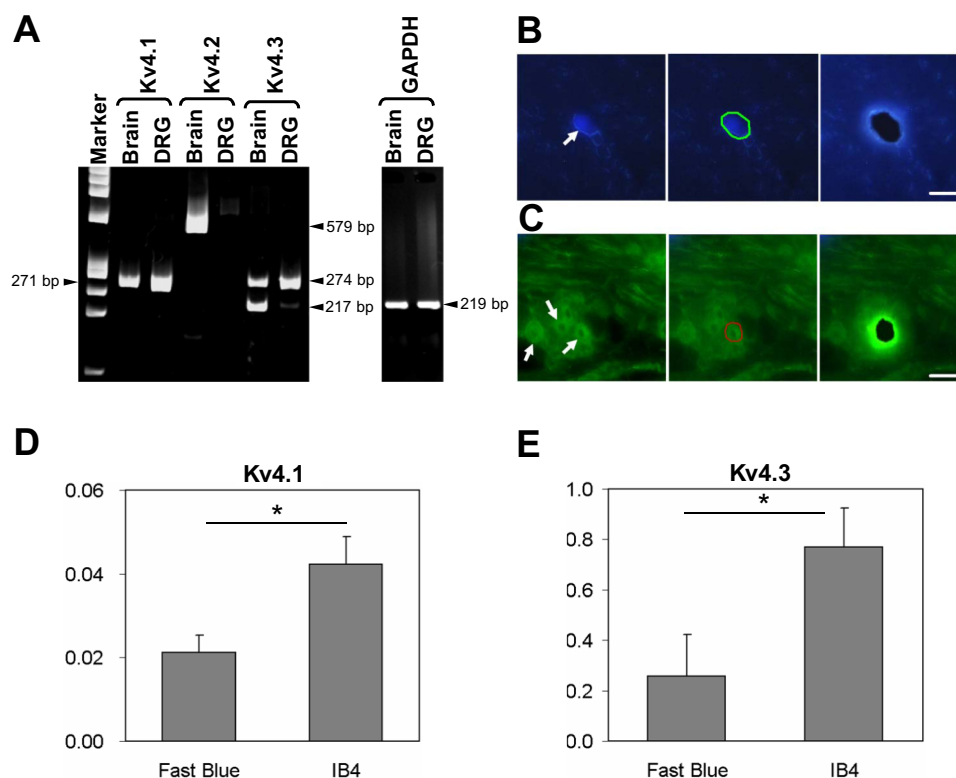


Fig. 7. Expression of Kv4 mRNAs in DRG. **A**: RT-PCR data with tissue samples. PCR was performed with primers for Kv4.x designed to detect splicing variants and the housekeeping gene, GAPDH. The 2 bands with distinct sizes in Kv4.3 correspond to the short and long splicing isoforms (Kv4.3S and Kv4.3L) (Takimoto et al. 1997). The left lane indicates molecular size markers (ϕ X174 DNA digested with *Hae*III). Note that DRGs contain abundant Kv4.1 and Kv4.3L but not Kv4.2. **B**: photomicrographs of the same L6 DRG section taken during laser-captured microdissection (LCM) of FB-labeled bladder afferent neuron before (left and middle) and after (right) LCM. An arrow indicates a laser-captured bladder afferent neuron labeled with FB injected into the bladder wall (left). A green-line circle represents the area captured by this procedure. **C**: photomicrographs of the same L6 DRG section taken during LCM of IB4-stained DRG neurons before (left and middle) and after (right) LCM. Arrows point to DRG neurons positively stained with IB4 (left). A red-line circle indicates the area that was laser captured (middle). **D**: Kv4.1 mRNA levels in laser-captured, FB-labeled bladder afferent neurons and IB4-stained DRG neurons. **E**: Kv4.3 mRNA levels in laser-captured, FB-labeled bladder afferent neurons and IB4-stained DRG neurons. **D** and **E**: data are expressed as relative values normalized with GAPDH mRNA as a control. Note that Kv4.1 and Kv4.3 mRNA levels are significantly higher in IB4-stained cells than those in FB-labeled bladder afferent neurons. * $P < 0.05$ for both Kv4.1 and Kv4.3, Wilcoxon signed-rank test.

Electrical and Pharmacological Properties of K_A Currents in Bladder Afferent and IB4 Intensely Positive Neurons

The IB4 staining results indicate that DRG cells, intensely stained with IB4-FITC, are a subpopulation of somatic afferent neurons but not visceral afferent neurons innervating the bladder. Thus the combination of FB injection into the bladder wall with IB4 staining of live, dissociated L6-S1 DRG cells allowed us to examine the differences in potassium currents in the same preparation. In this preparation, FB-positive cells were bladder-innervating visceral afferent neurons, whereas IB4 intensely stained neurons should represent a fraction of IB4-positive somatic afferent neurons. We found the following using these techniques: that 1) IB4 intensely stained DRG neurons exhibit a larger amplitude of K_A currents than FB-labeled bladder afferent neurons, 2) activation and inactivation of K_A currents in IB4 intensely stained DRG neurons are faster with more hyperpolarized, half-maximum potentials than those in bladder afferent neurons, and 3) the Kv4 channel blocker PaTx2 suppresses K_A currents in IB4 intensely stained DRG neurons but has no effect on the current in bladder afferent neurons. Thus bladder afferent and IB4-positive neurons contain the K_A current with distinct kinetic and pharmacological properties. Bladder afferent neurons

appear to contain an almost exclusive, slow-inactivating current, whereas IB4-positive neurons contain fast-inactivating currents. The results with PaTx2 further suggest that Kv4 channels are responsible for the fast-inactivating current in IB4-positive afferents that corresponds to a subpopulation of somatic afferents.

Previous studies have shown that K_A currents in sensory neurons, including DRG cells, consist of slow- and fast-inactivating components (Akins and McCleskey 1993; Everill and Kocsis 1999; Everill et al. 1998; Gold et al. 1996; McFarlane and Cooper 1991; Yoshimura et al. 1996). Slow-inactivating K_A currents are seen in capsaicin-sensitive C-fiber afferent neurons and are sensitive to dendrotoxin, a blocker of Kv1.1- and Kv1.2-containing channels. Furthermore, Kv1.4 subunits are significant in small-sized DRG neurons, including bladder afferent neurons (Hayashi et al. 2009; Rasband et al. 2001; Takahashi et al. 2013; Yang et al. 2004). Therefore, Kv1 family channels, including the inactivating, ball-containing Kv1.4 subunit, are thought to carry the slow-inactivating K_A current in C-fiber DRG neurons. The slow kinetics and insensitivity to PaTx2 of the K_A current in bladder afferents are consistent with this idea. In contrast, the fast-inactivating K_A current is generally seen in capsaicin-insensitive, myelinated A-fiber bladder afferent neurons (Yoshimura et al. 1996). We

show here that IB4 intensely stained cells that respond to capsaicin exhibit prominent K_A currents with faster kinetics and PaTx2 sensitivity. Thus an IB4 intensely positive subpopulation of somatic C-fiber DRG neurons appears to possess fast-inactivating K_A currents carried by Kv4 family channels.

Voltage-Dependent and -Independent Inhibition of Kv4 Channels by PaTx2

PaTx2 is a member of gating-modifying toxins that include hanatoxins and heteropoda toxins (Diocot et al. 1999; Escoubas et al. 2002; Sanguinetti et al. 1997; Swartz and MacKinnon 1995). These toxins act on a nonpore region to exhibit voltage-dependent inhibition of target channel currents (Corzo and Escoubas 2003; Norton and Pallaghy 1998; Swartz 2007; Zarayskiy et al. 2005). With the use of heterologously expressed Kv4.x currents, we found that PaTx2 shows distinct voltage dependencies for their inhibition. The toxin altered the steady-state activation and inactivation curves of the Kv4.2 current to a positive potential. The toxin also increased time-constant values in the raising and decaying phases of Kv4.2 current. These results indicate that the inhibition of Kv4.2 current by the toxin is mainly voltage dependent. On the other hand, the toxin caused little shift in the normalized, steady-state activation and inactivation curves of the Kv4.1 current without apparent changes in the time-constant values. Therefore, the toxin inhibits the Kv4.1 current in a voltage-independent fashion. The inhibition of the Kv4.3 current by the toxin seemed to include both inhibitory mechanisms. A similar voltage-dependent and -independent blockade of Kv4.x currents has been reported recently with heteropoda toxin 2 (Desimone et al. 2011). Similar to this report, we found that four amino acids at the end of S3 primarily determine voltage dependence of the toxin action. Thus gating-modifying toxins, including PaTx2, detect subtle differences in pore-forming subunit sequences at the specific region to produce distinct inhibitory mechanisms. Hence, these toxins appear to be useful tools to identify molecular correlates of the native currents.

We took advantage of the two inhibitory fashions by PaTx2 to determine molecular correlates of the K_A current in IB4 intensely positive afferent DRG neurons that correspond to a subpopulation of somatic afferent cells. PaTx2 did not shift the normalized steady-state activation curve with minor changes in the time constants. Thus the toxin inhibition of the native K_A current is mostly voltage independent. These findings support the idea that the K_A channel in IB4 intensely stained DRG cells contains Kv4.1 and possibly Kv4.3 subunits. Our RT-PCR data are consistent with a less-obvious contribution of Kv4.2 in DRG neurons and revealed higher Kv4.1 and Kv4.3 mRNA levels in laser-captured, IB4-positive neurons than in bladder afferent neurons. There are certainly limitations in this interpretation. For instance, different Kv4.x subunits can form heteromeric channel complexes. The inhibitory fashion of heteromeric Kv4.x channel currents by the toxin might not linearly reflect the subunit composition. However, PaTx2 is considered to bind to each subunit in the tetrameric channel. Therefore, the obtained voltage-independent inhibition of the K_A current likely represents the overall subunit ratio in the native, IB4 intensely positive afferent cells.

Previous immunochemical studies indicate the presence of Kv4.3 subunits in DRG cells. For example, Kv4.3-immunore-

active proteins were found selectively in the somata of a subset of nonpeptidergic (i.e., CGRP-negative), nociceptive DRG neurons (Chien et al. 2007). Although less is known about the protein expression of the Kv4.1 subunit, due to the lack of a high-quality commercial antibody against this protein, the expression of Kv4.1 mRNA has been reported in small-sized DRG neurons using a single-cell RT-PCR method (Phuket and Covarrubias 2009). We also recently reported that Kv4.1 mRNA is expressed, not only in small-sized cells but also in all sizes of rat DRG neurons using in situ hybridization techniques (Matsuyoshi et al. 2012). Our LCM study demonstrated further that Kv4.1 and Kv4.3 mRNA expression is higher in IB4-positive afferent neurons than that in bladder afferent neurons. However, the obtained Kv4 subunit mRNA levels do not necessarily indicate the functional contribution of Kv4.1 or Kv4.3 subunits to the K_A current. In this regard, our electrophysiological data demonstrate the presence of the PaTx2-sensitive K_A current in IB4 intensely stained neurons constituting a subpopulation of somatic afferent cells. The lack of voltage-dependent inhibition of this K_A current supports a large contribution of Kv4.1 subunits to this current. It is important to note that IB4 intensely positive cells constitute a smaller fraction of somatic afferent neurons than those moderately stained. Thus further studies with IB4-negative and -moderately stained cells are needed to obtain a more complete view on molecular correlates of K_A currents between somatic and visceral afferent neurons.

In conclusion, the reduction of K^+ channel activity, including slow- and fast-inactivating K_A currents, is one of the important mechanisms for hyperexcitability and chronic pain. The present study identified that Kv4.1 and possibly Kv4.3 subunits functionally contribute to K_A channels in IB4 intensely stained neurons that correspond to a subpopulation of somatic afferent cells. Molecular correlates of K_A channels in different afferent pathways could help to develop suitable molecular targets for the treatment of pain conditions of somatic and visceral organs.

GRANTS

Support for this work was provided by grants from the National Institutes of Health (Grants DK088836 and P01DK093424) and the Department of Defense (Grants W81XWH-11-1-0763 and W81XWH-12-1-0565).

DISCLOSURES

There is no conflict of interest and no commercial affiliation in the present study.

AUTHOR CONTRIBUTIONS

Author contributions: K.T., H.M., S.N., and N.Y. conception and design of research; T.Y., K.K., Y.F., and R.T. performed experiments; T.Y. and Y.F. analyzed data; T.Y., K.T., and N.Y. interpreted results of experiments; T.Y. prepared figures; T.Y. and N.Y. drafted manuscript; K.T. and N.Y. edited and revised manuscript; K.T. and N.Y. approved final version of manuscript.

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ORIGINAL ARTICLE

Mechanisms inducing autonomic dysreflexia during urinary bladder distention in rats with spinal cord injury

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Objectives: This study investigated the mechanisms inducing autonomic dysreflexia due to enhanced bladder-to-vascular reflexes in rats with spinal cord injury (SCI).

Methods: SCI was produced by the transection of the Th4–5 spinal cord in female Sprague–Dawley rats. At 4 weeks after SCI, changes in blood pressure during graded increases in intravesical pressure (20–60 cm H₂O) were measured in spinal-intact (SI) and SCI rats under urethane anesthesia. In five animals, effects of C-fiber desensitization induced by intravesical application of resiniferatoxin (RTX), a TRPV1 agonist, on the bladder-to-vascular reflex were also examined. Nerve growth factor (NGF) levels of mucosa and detrusor muscle layers of the bladder were measured by enzyme-linked immunosorbent assay. The expression levels of TRPV1 and TRPA1 channels were also examined in laser captured bladder afferent neurons obtained from L6 DRG, which were labeled by Dil injected into the bladder wall.

Results: In SI and SCI rats, systemic arterial blood pressure was increased in a pressure-dependent manner during increases in the intravesical pressure, with significantly higher blood pressure elevation at the intravesical pressure of 20 cm H₂O in SCI rats vs SI rats. The arterial blood pressure responses to bladder distention were significantly reduced by RTX-induced desensitization of C-fiber bladder afferent pathways. SCI rats had higher NGF protein levels in the bladder and higher TRPV1 and TRPA1 mRNA levels in bladder afferent neurons compared with SI rats.

Conclusions: The bladder-to-vascular reflex induced by TRPV1-expressing C-fiber afferents during bladder distention is enhanced after SCI in association with increased expression of NGF in the bladder and TRP channels in bladder afferent neurons.

Spinal Cord advance online publication, 23 December 2014; doi:10.1038/sc.2014.233

INTRODUCTION

Among various complications of spinal cord injury (SCI), autonomic dysreflexia (AD) is a potentially life-threatening medical emergency that occurs in persons with SCI at or above the mid-thoracic spinal cord segment as a consequence of uncontrolled sympathetic activity resulting in hypertension, due to enhanced viscerovascular reflexes.^{1,2} The most common source of stimulation that initiates AD is the genitourinary tract including bladder distention, followed by colorectal distention.¹

It has been shown that vascular responses (that is, hypertension) induced by bladder distention are mediated by the activation of TRPV1 (capsaicin receptor)-expressing C-fiber bladder afferent pathways in spinal-intact (SI) rats.³ In addition, the expression of TRP channels such as TRPV1 and TRPA1 are involved in the sensitization of C-fiber afferent pathways.⁴ Previous studies have also indicated that increased levels of nerve growth factor (NGF) in the bladder is one of the key mediators to induce hyperexcitability of C-fiber bladder afferent pathways after SCI, resulting in detrusor overactivity (DO),^{5–8} and that intrathecal application of NGF antibodies, which reduces NGF levels in bladder afferent pathways, is effective for DO as well as AD in SCI rats.^{9–11} A recent study demonstrated that the intravesical treatment with botulinum toxin also reduces AD induced

by bladder contractions, which is associated with a reduction in NGF levels in the whole bladder tissue and dorsal root ganglia (DRG).¹²

Thus, the present study was performed to examine the following: (1) whether arterial pressor responses induced by bladder distention are enhanced after SCI, (2) whether C-fiber desensitization by the intravesical treatment with resiniferatoxin (RTX), a TRPV1 channel agonist, can reduce bladder distention-induced vascular responses in a rat model of SCI, (3) whether the expression of TRPV1 and TRPA1 channels is altered after SCI in individually identified afferent neurons innervating the bladder using laser capture microdissection (LCM) methods and (4) whether the expression levels of NGF are increased in different bladder layers (mucosa and detrusor) of SCI rats.

MATERIALS AND METHODS

Animal preparation

Experiments were performed on SI ($n=5$) and spinal-transected ($n=10$) adult female Sprague–Dawley rats (Hilltop, Pittsburgh, PA, USA). All animal experiments were in accordance with the institutional guidelines approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Spinal cord transection was performed at the level of Th4–5 with the rat under isoflurane anesthesia. After Th4–5 laminectomy, the dura and spinal cord were cut with scissors and a sterile Gelform sponge was placed between

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Received 1 September 2014; revised 29 October 2014; accepted 19 November 2014

the cut ends of the spinal cord. The overlying muscle and skin were then sutured. The bladder of spinalized rats was manually emptied twice daily after spinalization until the experiment. Sham-operated rats without spinalization were used as controls.

Bladder-to-vascular reflex during bladder distention

Four weeks after spinal cord transection, the bladder-to-vascular reflex during bladder distention was evaluated according to the methods described in our previous study.³ In brief, PE-50 tubing (Clay-Adams, Parsippany, NJ, USA) was inserted into the bladder through the urethra and tied in place by a ligature around the urethral orifice. Then the PE-50 tubing in the urethra was connected via a three-way stopcock to a pressure transducer and to a syringe pump for recording intravesical pressure and for infusing saline into the bladder, respectively. Mean arterial blood pressure was recorded via a pressure transducer connected to a cannula in the common carotid artery. Intravesical pressure was increased in a stepwise manner to 20, 40 and 60 cm H₂O for the duration of 1 min at each pressure level with 1-min intervals by connecting the urethral catheter through a three-way stopcock to a saline-filled reservoir, the height of which was adjusted to maintain a constant pressure in the bladder. In some SCI rats, a TRPV1 receptor agonist, RTX (10 μ M, 0.4 ml) or vehicle was administered intravesically through a urethral catheter, and the solution was kept in the bladder for 30 min to induce desensitization of TRPV1-expressing C-fiber afferent pathways in the bladder 24 h before experiment under isoflurane anesthesia.

Enzyme-linked immunosorbent assay measurements of NGF in the bladder

NGF immunoassay of the bladder was performed in separate groups of SI or SCI rats with or without RTX treatment 4 weeks ($n=5$ each) after sham operation or spinal cord transection, respectively. The bladder was harvested under isoflurane anesthesia, and the mucosa and detrusor layers were separated by microscissors. Tissues were then homogenized using the RIPA Lysis Buffer System (Santa Cruz Biotechnology, Dallas, TX, USA) to isolate protein and measure NGF using an enzyme-linked immunosorbent assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, as previously described.^{11,13} Tissue NGF values are expressed as pg per mg protein.

LCM and real-time PCR analysis

The population of DRG neurons, which innervate the urinary bladder, was labeled by retrograde axonal transport of a fluorescent dye, Dil (1% w/v; Invitrogen, Carlsbad, CA, USA), injected into the bladder wall of SI and SCI rats ($n=5$ each) under isoflurane anesthesia, as previously reported.¹⁴ Seven days after Dil injection, L6 DRG were removed and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) and stored at -80°C until use. Samples were sectioned at 8- μ m thickness and the sections were mounted on PEM membrane slides (Leica Microsystems, Wetzlar, Germany). The tissue was air-dried, and LCM was performed using a Leica LMD6000 (Leica Microsystems) to dissect Dil-labeled bladder afferent neurons and unlabeled neurons separately. Excised cells were individually captured into the caps of 0.5 ml Eppendorf tubes and lysed, and RNA isolation, reverse transcription and real-time PCR were performed using a Cells Direct™ One-Step qRT-PCR kit (Invitrogen). Gene-specific primers and TaqMan probes crossing exon/exon junctions (Invitrogen) were designed for TRPV1 and TRPA1 using the Primer 3 Software (Primer 3, Totowa, NJ, USA; Table 1). Probes contained FAM fluorophore (Invitrogen) and TAMRA quencher (Invitrogen). The primer and probe combination were optimized within

suitable ranges for efficiency and correlation coefficient using standard curve dilutions and data output on an ABI Step-One Plus thermocycler (Applied Biosystems, Foster City, CA, USA). Amplification of complementary DNA was performed under the following conditions: one cycle of 50°C for 45 min and 95°C for 2 min, followed by 50 cycles of 15 s at 95°C , 1 min at 60°C . The reactions were analyzed in triplicate and normalized relative to GAPDH. Real-time PCR data were analyzed by the DCp (difference in crossing points) method as $R = 2^{(Cp \text{ sample} - Cp \text{ control})}$ to generate the relative expression ratio (R) of each target gene relative to that of GAPDH. We also determined specificity to complementary DNA using real-time PCR to verify that our primer/probe sets did not amplify the genomic DNA. All of our primer/probe combinations showed efficiencies of 98–104% (Table 1).

Statistical analyses

All analyses of the experiments were performed using GraphPad Prism 4.01 (GraphPad Software, Inc., San Diego, CA, USA). All experimental values are presented as the mean $\pm$ s.e.m. Statistical differences were analyzed using unpaired Student's t -test, and a P -value $<5\%$ was regarded as significant.

RESULTS

Bladder-to-vascular reflex during bladder distention

Arterial pressor responses shown by the changes in mean arterial blood pressure were elicited by bladder distention with increasing distention pressures in a graded manner to 20, 40 and 60 cm H₂O in

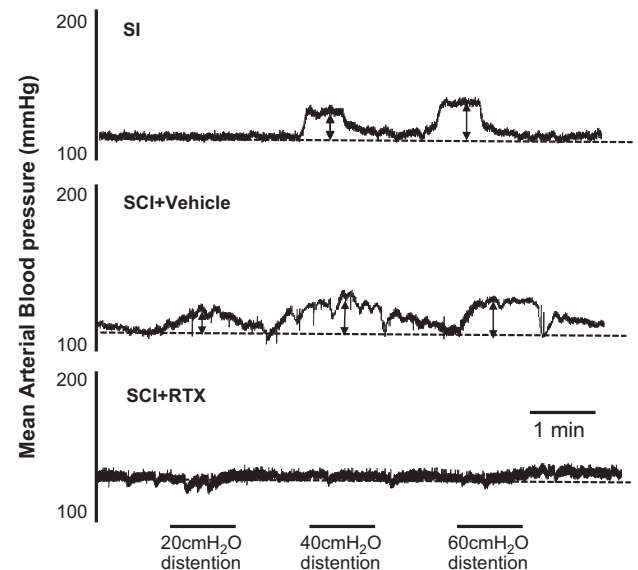


Figure 1 Recordings of mean arterial blood pressure during bladder distention in a stepwise manner to intravesical pressure at 20, 40 and 60 cm H₂O for a duration of 1 min at each pressure level with 1-min intervals in SI (upper panel) and SCI rats without (SCI+ vehicle; middle panel) or with TRPV1-expressing C-fiber desensitization induced by the intravesical RTX pretreatment (SCI+ RTX; lower panel). Break lines and bidirectional arrows indicate the baseline mean arterial blood pressure and the increases of mean arterial blood pressure during bladder distention, respectively.

Table 1 Primer and TaqMan probe sequences

	5' primer	3' primer	TaqMan probe	Efficacy (%)
TRPV1	5'-CCGGAGAGATCTTGCTGTG-3'	5'-ACCAGCATGAACAGCGACT-3'	5'-TATTTCTGCGAGAGCGACCATCC-3'	95.7
TRPA1	5'-ATCAGGAGACCCTGCTTAC-3'	5'-GTTGATGCTGCTCCACTG-3'	5'-TGATCATCATGACCTGGCAGACTACC-3'	101.3
GAPDH	5'-AGACAGCCGCATCTTCTGT-3'	5'-GATACGGCCAAATCCGTTCT-3'	5'-GCAGTGCCAGCCTCGTCTCA-3'	104.0

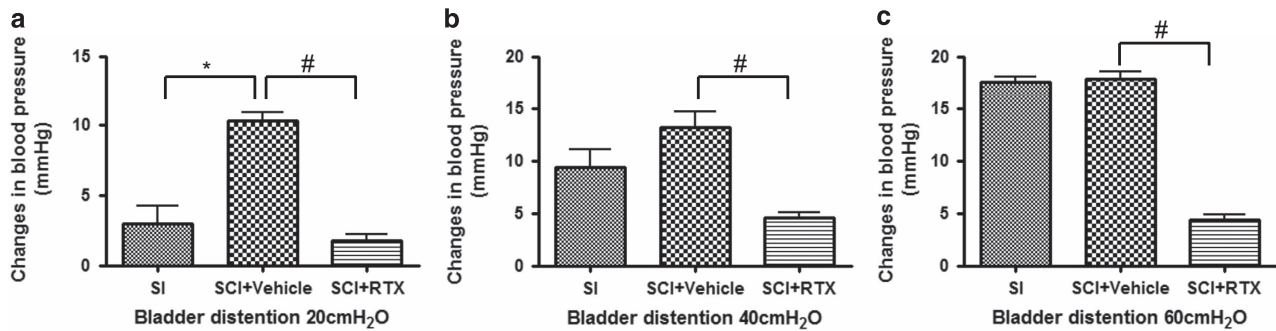


Figure 2 Changes in mean arterial blood pressure responses to bladder distention at 20, 40 and 60 cm H₂O of intravesical pressure (a–c respectively) in SI rats ($n=5$) and SCI rats without (SCI+vehicle, $n=5$) or with RTX-induced C-fiber desensitization (SCI+RTX, $n=5$). * $P<0.05$: SI vs SCI+vehicle, # $P<0.05$: SCI+vehicle vs SCI+RTX.

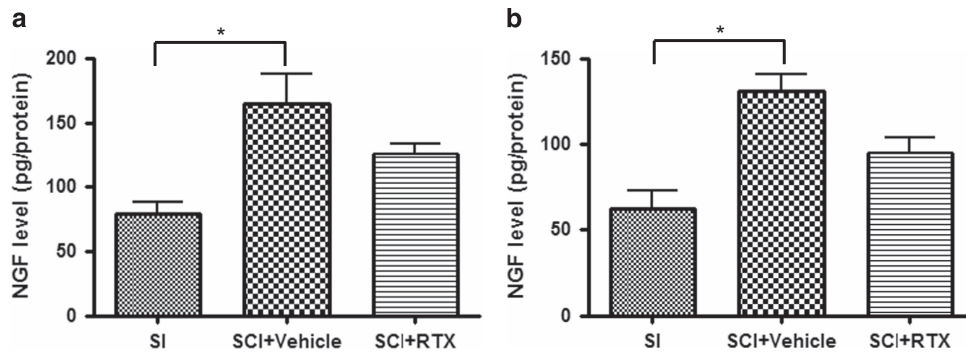


Figure 3 Expression of NGF protein in mucosa (a) and detrusor layers (b) of the bladder in SI ($n=5$) rats and SCI rats without (SCI+vehicle, $n=5$) or with RTX-induced C-fiber desensitization (SCI+RTX, $n=5$). * $P<0.05$: SI vs SCI+vehicle.

both SI and spinalized rats (Figures 1 and 2). The arterial pressor responses during the intravesical pressure elevation to 40 and 60 cm H₂O were not different between the two groups (Figure 2). However, an increase in mean arterial blood pressure of spinalized rats during bladder distention of 20 cm H₂O was significantly greater ($P<0.05$) than in SI (Figure 2), demonstrating that SCI enhances the bladder-to-vascular reflex, which is indicative of SCI-associated AD.

We then examined the effect of C-fiber desensitization in the bladder on arterial pressor responses during bladder distention of spinalized rats. We observed that the pretreatment with intravesical RTX in spinalized rats, 24 h before experiment caused a significant reduction in the increase of mean arterial blood pressure induced by the bladder distention of 20, 40 and 60 cm H₂O (Figures 1 and 2), indicating that the bladder-to-vascular reflex is dependent on the activation of C-fiber bladder afferent pathways in SCI.

Enzyme-linked immunosorbent assay measurements of bladder NGF

Spinalized rats showed higher levels of NGF protein expression in both mucosa and detrusor muscle layers of the bladder compared with SI rats (Figure 3). Increased NGF expression in mucosa and detrusor layers of spinalized rat bladders was not affected by C-fiber desensitization induced by the intravesical application of RTX.

LCM and real-time PCR analysis

Changes in mRNA levels of TRPV1 and TRPA1 were examined in laser captured DRG neurons. The TRPV1 expression relative to GAPDH was significantly higher (1.98) in DiI-labeled bladder afferent neurons (30 cells per rat) isolated from five spinal-transected rats

compared with those from five SI rats. Similarly, relative TRPA1 expression was significantly higher (1.96) in DiI-labeled bladder afferent neurons (30 cells per rat) isolated from five spinal-transected rats compared with five SI rats. In contrast, there was no significant difference in the TRPV1 or TRPA1 mRNA levels in unlabeled neurons isolated from the groups of SI and transected rats (Figure 4).

DISCUSSION

The results of this study demonstrated that (1) arterial pressor responses during bladder distention, which are mediated by the activation of TRPV1-expressing C-fiber bladder afferent pathways, are enhanced in SCI rats, (2) protein expression of NGF is increased in bladder mucosa and detrusor layers after SCI and (3) mRNA expression levels of TRPV1 and TRPA1 are increased in bladder afferent neurons after SCI.

We have previously reported that bladder distention induces intravesical pressure-dependent arterial pressor responses in SI rats, which are reduced by intravesical RTX treatment that desensitizes the TRPV1-expressing C-fiber afferent pathways.³ These earlier results indicated that the bladder-to-vascular reflex is induced by the activation of C-fiber bladder afferent pathways during bladder distention of normal rats. The current study extends the earlier findings to rats with SCI induced by complete Th4–5 spinal cord transection. We observed that arterial pressor responses to bladder distention at 20 cm H₂O in spinalized rats were greater than in SI rats, which indicate that the bladder-to-vascular reflex during bladder distention at a low pressure range is enhanced in spinalized rats. These findings demonstrate that the clinical relevance of this model as the condition of AD, which is a hypertensive response often induced

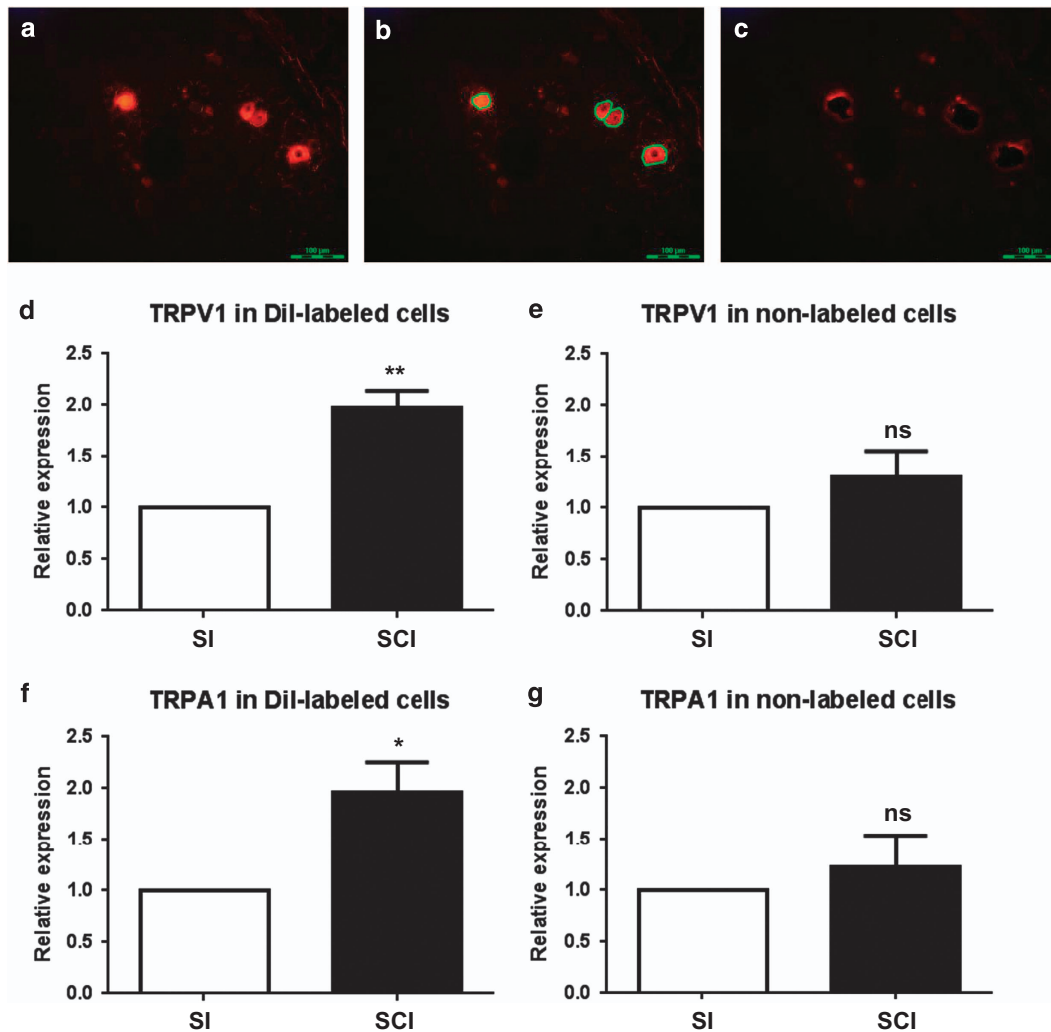


Figure 4 (a–c) Photomicrographs of the same L6 DRG section taken during LCM of DiI-labeled bladder afferent neurons before (a and b) and after LCM (c). Arrows point to neurons positively stained with DiI. Green-line circles in b indicate the areas that were laser captured b. Scale bar: 100 μ m. (d) TRPV1 mRNA levels in DiI-labeled neurons (30 cells per rat) from SI and transected rats (SCI; $n=5$ rats each). (e) TRPV1 mRNA levels in unlabeled neurons (30 cells per rat) from SI and SCI rats ($n=5$ rats each). (f) TRPA1 mRNA levels in DiI-labeled neurons (30 cells per rat) from SI and SCI rats ($n=5$ rats each). (g) TRPA1 mRNA levels in unlabeled neurons (30 cells per rat) from SI and SCI rats ($n=5$ rats each). Data are expressed as mean $\pm$ s.e.m. * $P<0.05$ and ** $P<0.01$, compared with the SI group. ns: not significant.

during bladder distention in patients of SCI at or above mid-thoracic spinal cord levels,^{1,2} can be reproduced in spinalized rats.

However, the arterial pressor responses to higher bladder distentions (40 and 60 responses to higher bladder distention H₂O) were not different between SI and spinalized rats in this study. The findings at higher bladder distention are in line with the results of a previous study showing that changes in arterial blood pressure during bladder distention that raises the intravesical pressure to 35 H₂O are not different in control and spinalized rats.¹⁵ In addition, a recent study showed that arterial pressor responses induced by reflex bladder contractions during filling cystometry in spinalized rats were greater compared with SI rats and that intravesical administration of botulinum toxin reduced the enhanced arterial pressor responses induced by bladder contractions.¹² However, after careful review of the study, it was noted that maximal voiding pressure was also higher in spinalized rats than in SI rats due to bladder overactivity after SCI,¹² suggesting the possibility that high bladder contraction pressure as a consequence of bladder overactivity leads to an increased blood pressure elevation

during bladder contractions in spinalized rats. In this regard, we believe that our methodology using graded elevation of the intravesical pressure is better suited for evaluating the relationship between arterial pressor responses and bladder distention crucial for assessing AD in SCI.

Increased excitability of C-fiber bladder afferent pathways is shown to be involved in neurogenic DO in animals and humans with SCI.^{6,14,16} Also, it has been documented that AD induced by colorectal distention is mediated by C-fiber afferent pathways in SCI rats.¹⁷ The present study further revealed that TRPV1 channel-expressing C-fiber bladder afferents are responsible for SCI-associated AD during bladder distention because the enhanced bladder-to-vascular reflex induced by bladder distention in SCI rats was significantly reduced by RTX-induced C-fiber desensitization. Although various mediators could contribute to afferent hyperexcitability after SCI, neurotrophic factors such as NGF released within the urinary bladder or the spinal cord reportedly have an important role in SCI-induced neuroplasticity.^{6,11} Animal studies demonstrated that immunoneutralization of NGF in

the spinal cord and afferent pathways can suppress DO,¹¹ as well as AD induced by colon distention in spinalized rats.⁹ A recent study also demonstrated that a reduction in enhanced arterial pressor responses during bladder contractions after intravesical botulinum toxin treatment was associated with a decreased NGF expression in the bladder and DRG of spinalized rats.¹² Taken together, these results suggest that NGF overexpression in the bladder is the major source that induces hyperexcitability of bladder afferent pathways, leading to AD induced by bladder distention following SCI.

The current study showed that the upregulation of NGF in the bladder occurs in both the mucosa that contains the urothelium and the detrusor layer. Our recent study using rats also showed that treatment with intravesical liposomal antisense suppresses NGF expression in the urothelium, as well as bladder overactivity and chemokine upregulation in a model of acetic acid-induced bladder overactivity.¹³ Thus, local suppression of NGF in the bladder urothelium using intravesical liposome-based delivery techniques could be an effective treatment of SCI-induced AD during bladder distention, although further studies are needed to clarify this point.

In this study, TRPV1 and TRPA1 channels are upregulated in dye-labeled bladder afferent neurons after spinal cord transection using LCM methods (Figure 4), which allow us to obtain individual neurons innervating a specific target organ (that is, the bladder in this study). The number of suburothelial nerve fibers expressing TRPV1 receptors is increased in patients with neurogenic DO compared with controls.¹⁸ In SCI rats, duodenal administration of a TRPV1 antagonist (GRC 6211) reduces the bladder contraction frequency.¹⁹ In addition, the increased TRPA1 expression has been reported in the bladder and L6-S1 DRG and that intravenous administration of a TRPA1 antagonist (HC-030031) is effective in suppressing DO in SCI rats.²⁰ Taken together, these results suggest that TRP channels such as TRPV1 and TRPA1 are involved in the emergence of C-fiber bladder afferent hyperexcitability and that TRPV1 and TRPA1 could be effective targets for the treatment of not only neurogenic DO but also AD in SCI.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Department of Defense (W81XWH-11-1-0763), Paralyzed Veterans of America (2793) and National Institutes of Health (DK088836 and P01 DK093424).

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1202631

Increased excitability of bladder afferent neurons in rats with spinal cord injury: a role of A-type voltage-gated potassium channels

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INTRODUCTION AND OBJECTIVE: Although the etiology of overactive bladder (OAB) seems to be multifactorial, afferent sensitization is considered to contribute to OAB symptoms such as urgency. Also, increased excitability of C-fiber afferent pathways has been proposed as an important pathophysiological basis of neurogenic detrusor overactivity (DO) in humans and animals with spinal cord injury (SCI). However, the functional mechanisms inducing hyperexcitability of C-fiber bladder afferent neurons (B-AN) after SCI are not fully elucidated. We therefore examined changes in electrophysiological properties of B-AN obtained from SCI rats, especially focusing on voltage-gated potassium channels, using patch-clamp recording techniques.

METHODS: SCI was produced by transection of the spinal cord at the level of T9-T10 in female SD rats. After 4 weeks, L6-S1 dorsal root ganglia (DRG) were removed from spinal intact and SCI rats, and freshly dissociated DRG neurons were prepared with enzymatic methods. Whole cell patch-clamp recordings were performed on individual B-AN, which were labeled by retrograde axonal transport of a fluorescent dye, Fast Blue (FB), injected into the bladder wall 7 days earlier and identified with a fluorescent microscope. Since the majority of C-fiber B-AN are sensitive to capsaicin, FB-labeled cells that exhibited inward currents in response to capsaicin (500nM) application were selected for evaluation.

RESULTS: Capsaicin-sensitive B-AN from SCI rats exhibited lower thresholds for spike activation (-26.4 ± 1.3 mV) than those from control rats (-21.8 ± 0.9 mV) and did not exhibit membrane potential relaxation during membrane depolarization. The number of firing during a 800 msec depolarizing pulse was significantly increased after SCI (4.7 ± 0.7 spikes) compared to control rats (1.3 ± 0.1 spikes). The peak density of A-type potassium (K_A) currents during membrane depolarizations to 0 mV in capsaicin-sensitive B-AN of SCI rats was significantly smaller (38.1 ± 4.6 pA/pF) than that from control rats (68.6 ± 6.3 pA/pF), and the inactivation curve of the K_A current was displaced to more hyperpolarized levels by ~ 10 mV after SCI. On the other hand, the sustained delayed-rectifier potassium current density was not altered after SCI.

CONCLUSIONS: These results suggest that reduced K_A channel activity is involved in hyperexcitability of capsaicin-sensitive C-fiber B-AN after SCI. Thus, the K_A channel could be a potential target for treating OAB due to neurogenic DO.

Source of funding: NIH DK57267, DK68557 and DOD SC100134

2013 AUA abstract

13-5525

Mechanisms inducing autonomic dysreflexia during bladder distention in rats with spinal cord injury

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INTRODUCTION AND OBJECTIVES: Autonomic dysreflexia (AD) is a potentially life-threatening medical emergency, and can be induced by viscerovascular reflexes during bladder distention, resulting in uncontrolled sympathetic activity and hypertension, in patients with spinal cord injury (SCI). This study investigated the mechanisms inducing AD in SCI due to enhanced bladder-to-vascular reflexes in rats, especially focusing on capsaicin-sensitive C-fiber afferents and nerve growth factor (NGF) expression.

METHODS: SCI was produced by transection of the Th4-5 spinal cord in female rats. After 4 weeks, changes in blood pressure during graded increases in intravesical pressure (20 to 80 cmH₂O) were measured in spinal intact (SI) and SCI rats under urethane anesthesia. In some animals, effects of C-fiber desensitization induced by capsaicin-pretreatment (125mg/kg, s.c.) on the bladder-to-vascular reflex were also examined. NGF levels of mucosa and detrusor muscle layers of the bladder, and L6-S1 dorsal root ganglia (DRG) were measured by RT-PCR and ELISA. Patch-clamp recordings were also performed in capsaicin-sensitive bladder afferent neurons dissociated from L6-S1 DRG, which were labeled by Fast Blue injected into the bladder wall.

RESULTS: In SI and SCI rats, systemic arterial blood pressure was increased in pressure-dependent fashion during increases in intravesical pressure, with blood pressure elevation started at lower intravesical pressure (20 cmH₂O) in SCI rats compared to SI rats. In addition, these arterial blood pressure responses to bladder distention were significantly reduced by desensitization of capsaicin-sensitized C-fiber afferent pathways. Patch-clamp recordings showed hyperexcitability of bladder afferent neurons from SCI rats as evidenced by lower thresholds for action potential activation in SCI rats compared to SI rats (-26.4±1.3 vs. -21.8±0.9mV). SCI rats also had higher NGF mRNA and protein levels of the bladder and DRG compared to SI rats.

CONCLUSIONS: These results indicate that the bladder-to-vascular reflex induced by capsaicin-sensitive C-fiber afferents is enhanced in association with increased NGF expression after SCI, suggesting that suppression of C-fiber bladder afferent activity and/or NGF expression in the bladder could be effective for reducing AD episodes during bladder distention in patients with SCI.

Source of Funding: DOD SC100134 and PVA 2793

2013 AUA abstract

13-1297

Increased excitability of bladder afferent neurons associated with reduced expression of Kv1.4 α -subunit in rats with spinal cord injury

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INTRODUCTION AND OBJECTIVES: Hyperexcitability of C-fiber bladder afferent pathways has been proposed as an important pathophysiological basis of neurogenic detrusor overactivity with spinal cord injury (SCI). However, the molecular mechanisms inducing hyperexcitability of C-fiber bladder afferent neurons (B-AN) after SCI are not fully elucidated. We therefore examined changes in electrophysiological properties of B-AN obtained from SCI rats, especially focusing on voltage-gated potassium channels and the expression levels of α -subunits, which can form A-type K⁺ (KA) channels.

METHODS: Fresh dissociated L6-S1 dorsal root ganglia (DRG) neurons were prepared from female spinal intact and SCI (T9-T10 transection) SD rats. Whole cell patch-clamp recordings were performed on individual B-AN, which were labeled by retrograde axonal transport of a fluorescent dye, Fast Blue (FB) injected into the bladder wall 7 days prior the dissociation. Since the majority (80%) of C-fibre bladder afferent neurons are known to be sensitive to capsaicin, capsaicin-sensitive neurons were selected for evaluation. The expression levels of Kv1.2 and 1.4 α -subunits were evaluated using immunohistochemical methods.

RESULTS: Capsaicin-sensitive B-AN from SCI rats exhibited increased cell excitability evidenced by lower thresholds for spike activation (-26.4 ± 1.3 mV) and the increased number of action potentials (4.7 ± 0.7 spikes) during a 800 msec depolarizing pulse compare to control rats (-21.8 ± 0.9 mV and 1.3 ± 0.1 spikes). The peak density of KA currents during membrane depolarizations to 0 mV in capsaicin-sensitive B-AN of SCI rats was significantly smaller (38.1 ± 4.6 pA/pF) than that from control rats (68.6 ± 6.3 pA/pF), and the inactivation curve of the KA current was displaced to more hyperpolarized levels by ~ 10 mV after SCI. On the other hand, the sustained delayed-rectifier K⁺ current density was not altered after SCI. The expression of Kv1.4 α -subunits, which can form KA channels, was reduced in B-AN from SCI rats compared to control rats.

CONCLUSIONS: These results indicate that the excitability of capsaicin-sensitive C-fiber B-AN is increased in association with reductions in KA current size and Kv1.4 α -subunit expression in SCI rats. Thus, the Kv1.4 α -subunit could be a potential molecular target for treating OAB due to neurogenic detrusor overactivity.

Source of Funding: NIH DK57267, DK88836, DOD SC100134 and PVA 2793

2013 AUA abstract

13-2598

Downregulation of NGF Expression in the Bladder by Antisense Oligonucleotides as New Treatment of Overactive Bladder

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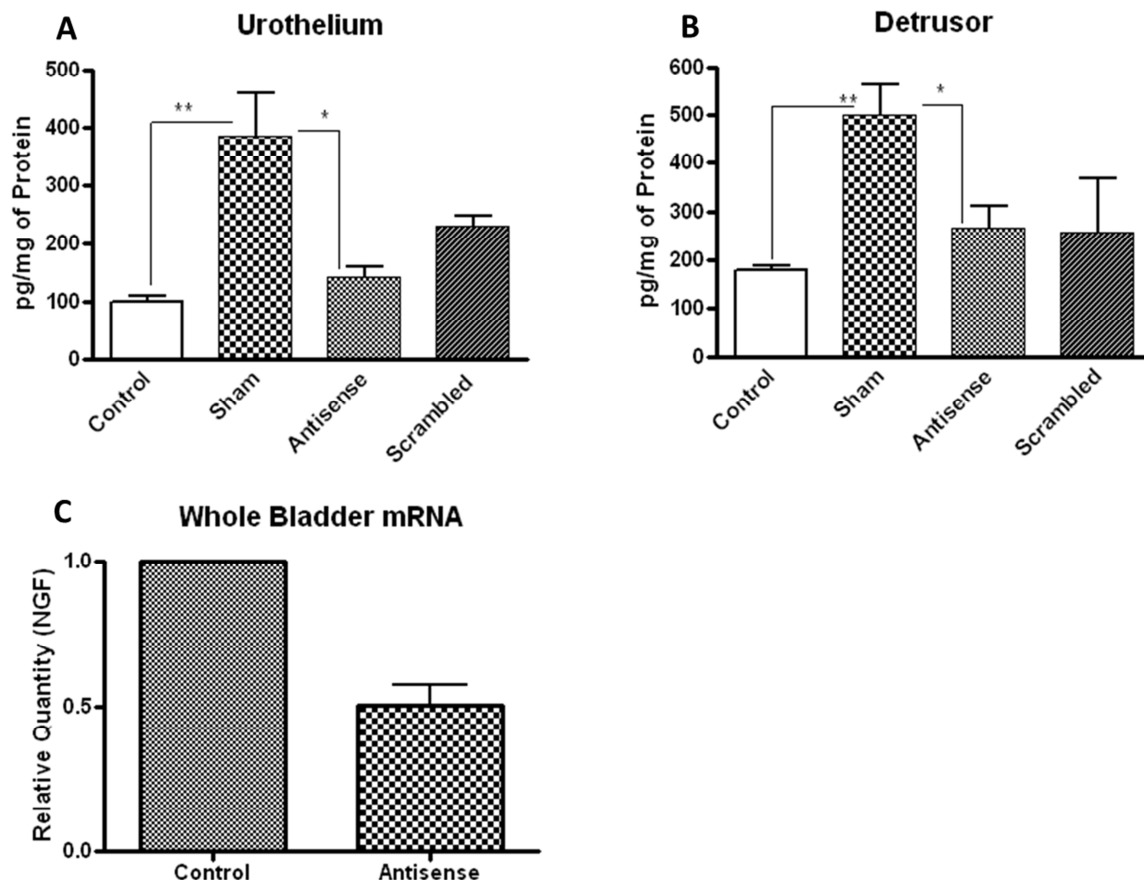
INTRODUCTION AND OBJECTIVES: Overexpression of NGF in the urothelium has been shown to play a role in symptoms of OAB by mediating functional changes of bladder afferents. We studied whether the blockade of NGF overexpression in the urothelium by a sequence-specific gene-silencing mechanism suppresses acetic acid (AA) induced bladder overactivity.

METHODS: Female Sprague-Dawley rats anaesthetized with isoflurane were instilled with 0.5mL of either saline, scrambled or TYE563-labelled antisense oligonucleotide (ODN) targeting NGF (12 μ M) alone or complexed with cationic liposomes for 30min. 24h later, the efficacy of antisense treatment was assessed by saline and AA infused cystometry under urethane anesthesia (1.2g/kg, s.c.). The expression levels and the bladder distribution of NGF after treatment were quantified by immunofluorescence staining and ELISA.

RESULTS: Liposomes were necessary for bladder uptake of ODN as revealed by absence of bright red fluorescence of TYE 563 in rat bladder instilled with ODN alone. 24h after instillation, the baseline CMG under saline infusion was indistinct with mean intercontraction interval (ICI) of 348.4 ± 55.0 sec and 390.8 ± 120.7 sec in the sham and antisense treated groups, respectively. In sham rats instilled with vehicle, AA infusion reduced the ICI to $33.2 \pm 4.0\%$ of baseline values, compared to $75.8 \pm 3.4\%$ of baseline values (n=6) in rats instilled with liposomal antisense ODN. AA induced elevation of NGF in the urothelium and detrusor of sham rat was also reduced by antisense treatment as revealed by ELISA (panel A&B) and reduced density of NGF immunoreactivity in the urothelium. Expression of NGF mRNA in rats treated with antisense ODN was also downregulated to 50% of control rats (panel C).

CONCLUSIONS: Our findings demonstrate that bladder overactivity induced by bladder irritation is associated with overexpression of NGF in the urothelium and that pretreatment with intravesical antisense suppresses expression of urothelial NGF and AA-induced bladder overactivity. Thus, local suppression of NGF in the bladder could be an attractive approach for the treatment OAB that can avoid systemic side effects due to non-specific peripheral blockade of NGF expression.

Source of Funding: NIH DK057267, DK088836; DOD SC100134 and PR110326



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HYPEREXCITABILITY OF BLADDER AFFERENT NEURONS ASSOCIATED WITH REDUCED EXPRESSION OF POTASSIUM CHANNEL KV1.4 α -SUBUNIT IN RATS WITH SPINAL CORD INJURY

Hypothesis / aims of study

Hyperexcitability of C-fibre bladder afferent pathways has been proposed as an important pathophysiological basis of neurogenic detrusor overactivity with spinal cord injury (SCI). However, the functional and molecular mechanisms inducing hyperexcitability of C-fibre bladder afferent neurons after SCI are not fully elucidated. We therefore examined changes in electrophysiological properties of bladder afferent neurons obtained from spinal transected rats, especially focusing on voltage-gated potassium (K^+) channels and the expression levels of α -subunits, which can form A-type K^+ (K_A) channels. Activation of K_A channels is known to reduce excitability of capsaicin-sensitive C-fibre bladder afferent neurons from spinal intact rats [1].

Study design, materials and methods

Freshly dissociated L6-S1 dorsal root ganglia (DRG) neurons were prepared from female spinal intact and spinal transected (4 weeks after T9-T10 transection) SD rats. Whole cell patch-clamp recordings were performed on individual bladder afferent neurons, which were labelled by retrograde axonal transport of a fluorescent dye, Fast Blue (FB) injected into the bladder wall 7 days prior the dissociation. Since the majority (80%) of C-fibre bladder afferent neurons are known to be sensitive to capsaicin, capsaicin-sensitive neurons were selected for evaluation. The expression levels of Kv1.2 and 1.4 α -subunits were evaluated using immunohistochemical methods.

Results

Capsaicin-sensitive bladder afferent neurons from spinal transected rats exhibited increased cell excitability evidenced by lower thresholds for spike activation (-26.4 ± 1.3 mV) and the increased number of action potentials (4.7 ± 0.7 spikes) during a 800 msec depolarizing pulse compare to spinal intact rats (-21.8 ± 0.9 mV and 1.3 ± 0.1 spikes, respectively) (Fig. 1). The peak density of slowly-inactivating K_A currents during membrane depolarizations to 0 mV in capsaicin-sensitive bladder afferent neurons of spinal transected rats was significantly smaller (38.1 ± 4.6 pA/pF) than that from spinal intact rats (68.6 ± 6.3 pA/pF) (Fig. 2), and the inactivation curve of the K_A current was displaced to more hyperpolarized levels by ~ 10 mV after spinal transection (Fig. 3). On the other hand, the sustained delayed-rectifier K^+ current density was not altered after spinal transection (Fig. 2). The expression of Kv1.4 α -subunits, which can form K_A channels, was reduced in bladder afferent neurons from spinal transected rats compared to spinal intact rats (Fig. 4).

Interpretation of results

These results indicate that: (1) capsaicin-sensitive bladder afferent neurons in spinal transected rats show hyperexcitability as evidenced by lower thresholds for spike activation and tonic firing pattern, (2) the density of slowly-inactivating K_A currents was reduced and the inactivation curve of K_A currents for spinal transected rats was displaced to more hyperpolarized levels in comparison with spinal intact rats, (3) the expression of Kv1.4 α -subunits in bladder afferent neurons was decreased after spinal transection. Thus, it is assumed that the excitability of capsaicin-sensitive C-fiber bladder afferent neurons is increased in association with reductions in K_A current size and Kv1.4 α -subunit expression in spinal transected rats.

Concluding message

The reduction in K_A channel activity due to downregulation of the Kv1.4 α -subunit could contribute to hyperexcitability of C-fibre bladder afferent pathways, leading to detrusor overactivity in SCI. Thus, the Kv1.4 α -subunit could be a potential molecular target for treating OAB due to neurogenic detrusor overactivity.

Fig.1

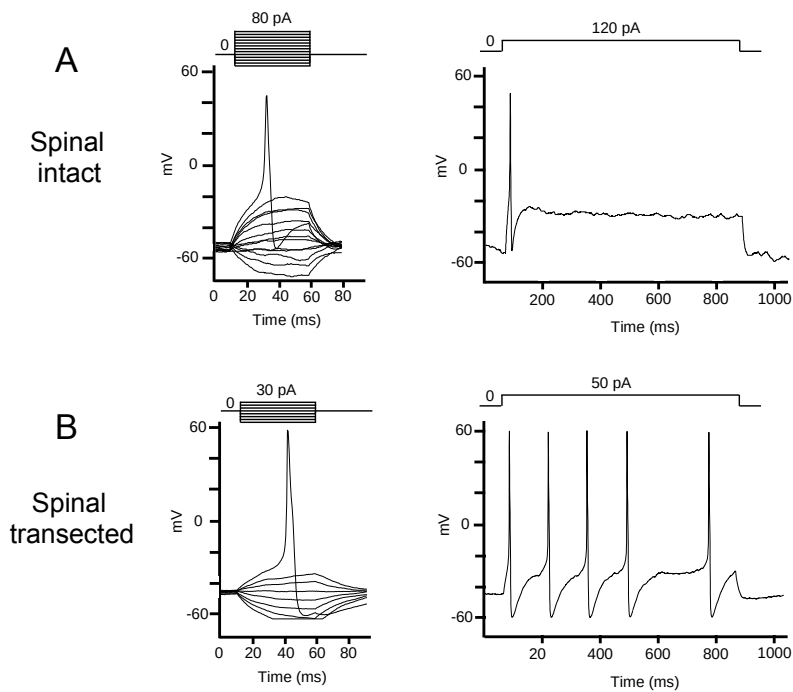


Fig.2

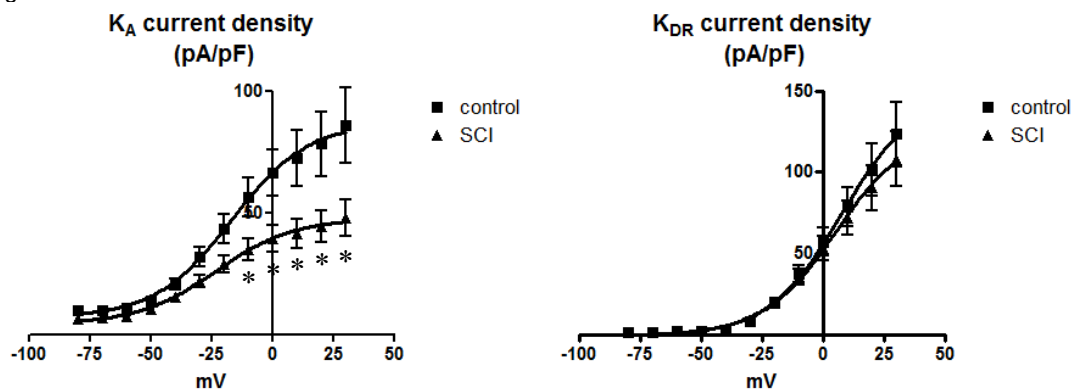
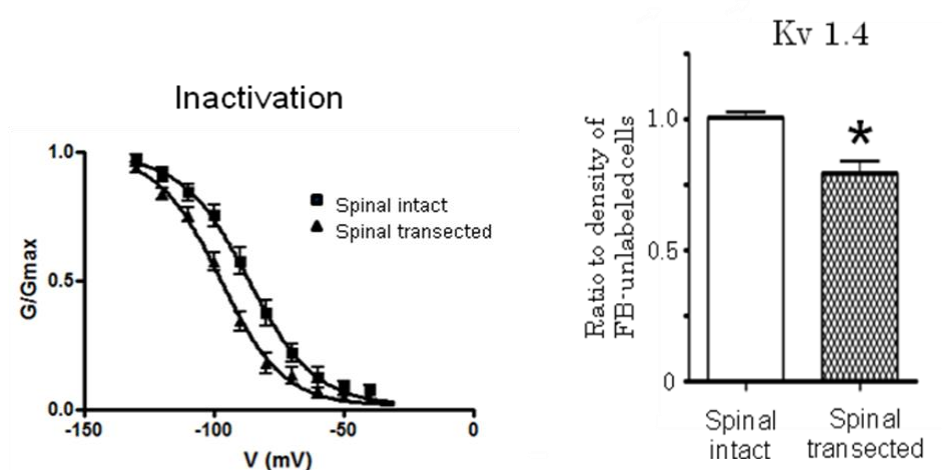


Fig.3

Fig.4



References

1. Yoshimura and de Groat, Journal of Neuroscience, 19: 4644-4653, 1999

Disclosures

Funding: NIH DK57267, DK88836, DOD W81XWH-11-1-0763 and PVA 2793 **Clinical Trial:** No **Subjects:** ANIMAL **Species:** Rat **Ethics Committee:** Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh

2014 AUA abstract

14-6490

Therapeutic effects of intravesical application of liposome conjugated with antisense oligonucleotide targeting nerve growth factor on neurogenic detrusor overactivity in rats with spinal cord injury

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INTRODUCTION AND OBJECTIVES: We recently reported that instillation of liposome conjugated with antisense oligonucleotide (OND) targeting nerve growth factor (NGF) into the bladder suppressed bladder overactivity in a rat model of acute cystitis (Kashyap et al., J Urol, 2013). Therefore, this study investigated whether instillation of liposome with NGF-targeting antisense OND into the bladder can suppress neurogenic detrusor overactivity shown by non-voiding contractions (NVCs) during the storage phase in rats with spinal cord injury (SCI).

METHODS: Adult female Sprague-Dawley rats were used. SCI was produced by complete transection of the Th8-9 spinal cord. After 3 weeks, SCI rats were divided into three groups; (a) Control group (no treatment), (b) Liposome without antisense OND group, (c) Liposome with NGF-targeting antisense OND group, in which. NGF antisense-liposome solution (1 ml) containing 6 μ l of anti-NGF oligonucleotide (2mM) in 994 μ l of liposome (7mM) was injected into the bladder and retained for 30 min. At 4 weeks after SCI, awake cystometry was performed during continuous infusion of saline (0.08 ml/min) into the bladder. Cystometric parameters evaluated included the amplitude (cmH₂O) and frequency (number/min) of NVCs, maximal voiding pressure (MVP) and residual urine volume (RV).

RESULTS: There was no any significance in the amplitude and frequency of NVCs between control (no treatment) and liposome without antisense OND groups of SCI rats. However, in the liposome with NGF-targeting antisense OND group, the amplitude and frequency of NVCs were significantly reduced compared to control (no treatment) (38 and 55% reductions, respectively) and liposome without antisense OND groups (35 and 58% reductions, respectively) of SCI rats. MVP and RV were also significantly reduced in the NGF-targeting antisense OND group compared to other groups.

CONCLUSIONS: These results indicate that intravesical treatment with liposome conjugated with antisense OND targeting NGF effectively reduces neurogenic detrusor overactivity as evidenced by the reduction of NVCs in SCI rats. Because NGF upregulation in the bladder is shown to be one of the key mechanisms inducing bladder overactivity, the intravesical liposome-NGF antisense delivery could be a novel option for the treatment of neurogenic detrusor overactivity in SCI.

Source of Funding: DOD W81XWH-11-1-0763, W81XWH-12-1-0565 and NIH DK088836, P01 DK093424

2015 AUA abstract

15-3882

Effects of liposome-based local suppression of nerve growth factor in the bladder on autonomic dysreflexia during urinary bladder distention in rats with spinal cord injury

Katsumi Kadekawa*, Tsuyoshi Yoshizawa, Pradeep Yyagi, Pittsburgh, PA, Kimio Sugaya, Okinawa, Japan, Naoki Yoshimura, Pittsburgh, PA

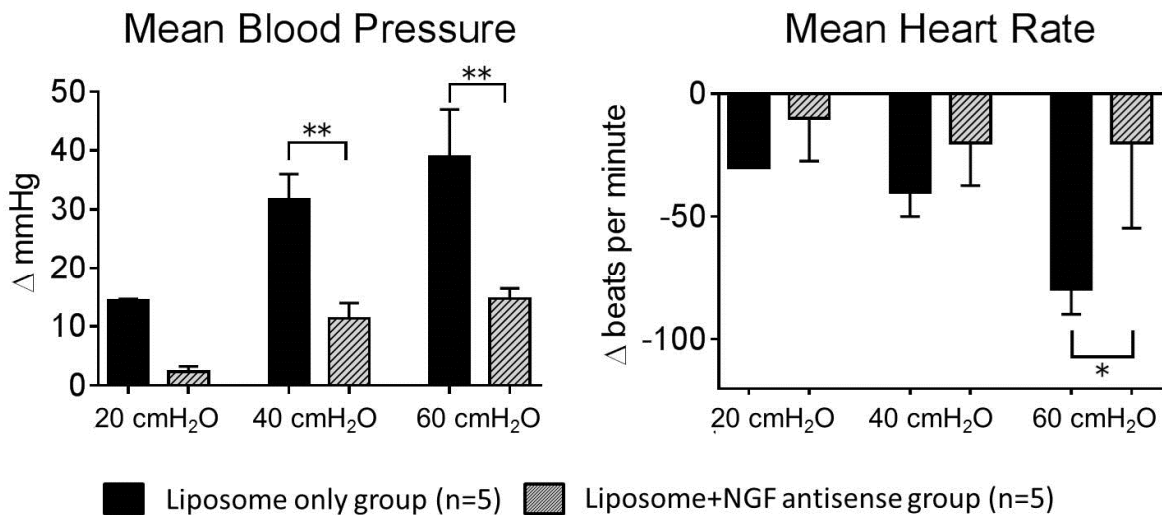
INTRODUCTION AND OBJECTIVES: Among various complications of spinal cord injury (SCI), autonomic dysreflexia (AD) is a potentially life-threatening medical emergency that induce hypertension and bradycardia due to enhanced viscerovascular reflexes. However, no effective pharmacotherapy for preventing AD is available. Nerve growth factor (NGF) in the bladder is one of the key mediators to induce hyperexcitability of bladder afferent pathways after SCI. In order clarify whether local suppression of NGF in the bladder improve viscerovascular reflexes, we examined the effect of intravesical administration of liposome-NGF antisense conjugates on AD in rats with SCI.

METHODS: Female Sprague-Dawley rats (6 months age, n=10) with SCI produced by complete transection of the Th2-3 spinal cord were used. Seven weeks after spinalization, liposomes only or liposomes conjugated with NGF antisense were administered intravesically. At 1 week after intravesical treatment, changes in blood pressure and heart rates during graded increases in intravesical pressure (20 to 60 cmH₂O) were measured under urethane anesthesia.

RESULTS: The mean arterial blood pressures was elevated and the mean heart rate was decreased during bladder distension with increasing distension pressures in a graded manner to 20, 40 and 60 cmH₂O in the liposome only-treated group. The liposome-NGF antisense treatment suppressed the increases of mean blood pressures (2.3 $\pm$ 0.8 mmHg; 83% decrease at 20 cmH₂O, 11.3 $\pm$ 2.5 mmHg; 64% decrease at 40 cmH₂O, 14.7 $\pm$ 1.7 mmHg; 62% decrease at 60 cmH₂O) and the decreases of mean heart rates (-10 $\pm$ 10 bpm; 66% increase at 20 cmH₂O, -20 $\pm$ 10 bpm; 50% increase at 40 cmH₂O, -20 $\pm$ 20 bpm; 75% increase at 60 cmH₂O) compared to the liposome only-treated group.

CONCLUSIONS: These results indicate that liposome-based local suppression of NGF in the bladder improved viscerovascular reflexes after SCI. Since the expression of NGF in the bladder is increased after SCI based on previous studies, intravesical application of liposome NGF antisense conjugates may be an effective therapy for AD induced by bladder distension.

Source of Funding: DOD W81XWH-11-1-0763, W81XWH-12-1-0565 and NIH DK088836, P01 DK093424



One-way ANOVA followed by Tukey's multiple comparisons test, * p<0.05, ** p<0.01