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Award Number: W81XWH-11-1-0763

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

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REPORT DATE: October 2013

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE October 2013		2. REPORT TYPE Annual report		3. DATES COVERED 30September2012-29September2013	
4. TITLE AND SUBTITLE Anti-NGF Local Therapy for Autonomic Dysreflexia in Spinal Cord Injury				5a. CONTRACT NUMBER W81XWH-11-1-0763	
				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 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 : none listed					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 22	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			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. The most common source of stimulation that initiates AD is the genitourinary tract including bladder distention. Therefore, the purpose of this project is to investigate the feasibility of local nerve growth factor (NGF) antisense treatment for SCI-induced AD during bladder distention and provides the foundation for future clinical translation of local NGF antisense therapy in people with SCI-induced AD.

II. Body

II.1. Timeline described in the SOW: PITT: University of Pittsburgh

	Year 1			Year 2			Year 3		
	0-2mo	3-8mo	9-12mo	13-16mo	17-20mo	21-24mo	25-28mo	29-32mo	33-36mo
AIM 1 (PITT)	Regulatory approval for animal research	□ Order animals/ □ Prepare protocols/documentation □ Comparison of blood pressure responses during bladder distention between spinal intact and SCI rats	□ Comparison of blood pressure responses during bladder distention in SCI rats with or without capsaicin pretreatment □ Comparison of electrophysiological properties of bladder afferent neurons from spinal intact and SCI rats □ Tissue analysis ELISA histology	Animal study data analysis/ reporting					
		□ Tissue analysis - Optimization and analysis of NGF expression using molecular techniques such as PCR and ELISA in spinal intact and SCI rats				Data analysis/ reporting of NGF expression data			
AIM 2 (PITT)				□ Comparison of blood pressure responses during bladder distention in SCI rats with or without NGF antisense treatment □ Comparison of dosing strategy using one-time or twice application of LP-anti NGF for reducing of blood pressure responses □ Comparison of effects of cationic liposomes vs. amphoteric liposomes (charge-reversible character) on blood pressure responses □ Tissue analysis ELISA histology			□ Comparison of electrophysiological properties of bladder afferent neurons from SCI rats with or without NGF antisense treatment		Data analysis/ reporting
	□ Optimize formulation of NGF antisense □ Manufacture LPs □ Analytical method development			□ Develop LP-NGF antisense using cationic and amphoteric liposomes □ Tissue analysis - Analysis of NGF expression using molecular techniques such as PCR and ELISA in SCI rats with LP-NGF antisense treatment					

II-2. Research Accomplishments

Aim 1 (Year 2)

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

[Accomplishment]

In the second year of the project, we continued to perform experiments to measure blood pressure responses during bladder distention using rats with Th5-level 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.

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. 1), 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 (Fig. 1). These results indicate that SCI (4 weeks) induced AD as evidenced by the earlier onset of arterial blood pressure elevation during bladder distention in SCI rats vs. spinal intact rats and that enhanced AD in SCI rats is dependent of C-fiber afferent activation in the bladder (Yoshizawa et al., 2013 [Appendix]).

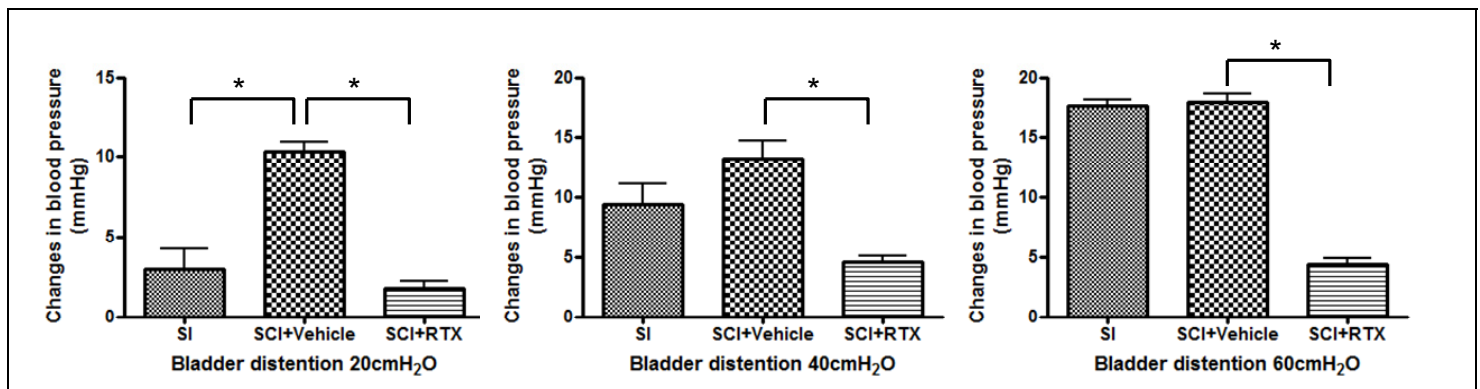


Figure 1. Changes in arterial blood pressure during passive bladder distention to 20 (left), 40 (middle) and 60 cmH₂O (right) in spinal intact (SI), spinal cord injured (SCI+vehicle) and RTX-treated SCI rats (SCI+RTX). Note that blood pressure responses at 20 cmH₂O bladder distention was increased in SCI+vehicle rats vs. SI rats and that RTX-induced C-fiber desensitization significantly reduced blood pressure responses to bladder distention (SCI+vehicle vs. SCI+ RTX). N=4 per each group. *P<0.05

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

[Accomplishment]

In the second year of the project, we continued ELISA experiments to measure NGF protein levels and confirmed that the NGF levels in the mucosa and detrusor of the bladder are significantly increased after SCI (Fig. 2) (Yoshizawa et al., 2013 [Appendix]).

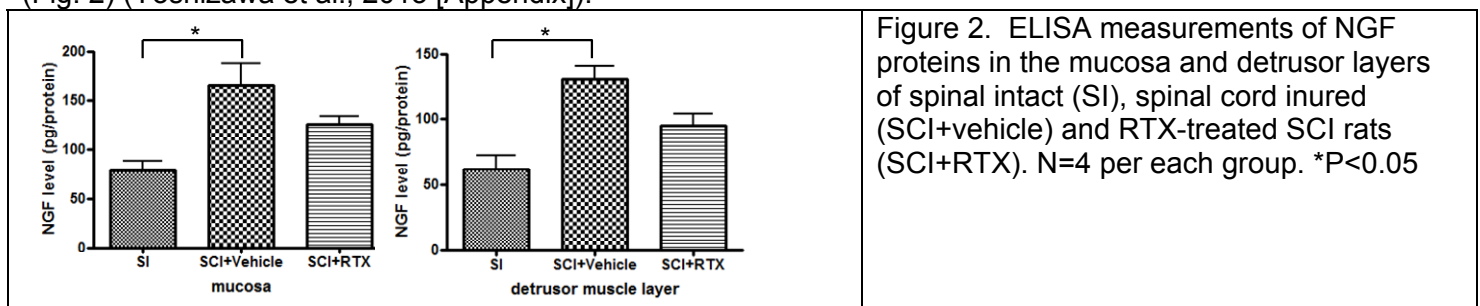


Figure 2. ELISA measurements of NGF proteins in the mucosa and detrusor layers of spinal intact (SI), spinal cord injured (SCI+vehicle) and RTX-treated SCI rats (SCI+RTX). N=4 per each group. *P<0.05

- *Comparison of electrophysiological properties of bladder afferent neurons from spinal intact and SCI rats*
- *Tissue analysis (histology) of dorsal root ganglion (DRG) neurons*

[Accomplishment]

We continued to perform the electrophysiological experiments using patch-clamp recordings and histological analyses of DRG neurons innervating the urinary bladder. We have found that: (1) 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, (2) the peak density of transient A-type K^+ (K_A) currents in capsaicin sensitive bladder afferent neurons from spinal transected rats was significantly less than that from spinal intact rats, (3) the K_A current inactivation curve was displaced to more hyperpolarized levels after spinal transection, and (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. 3) (Takahashi et al., 2013 [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 after SCI.

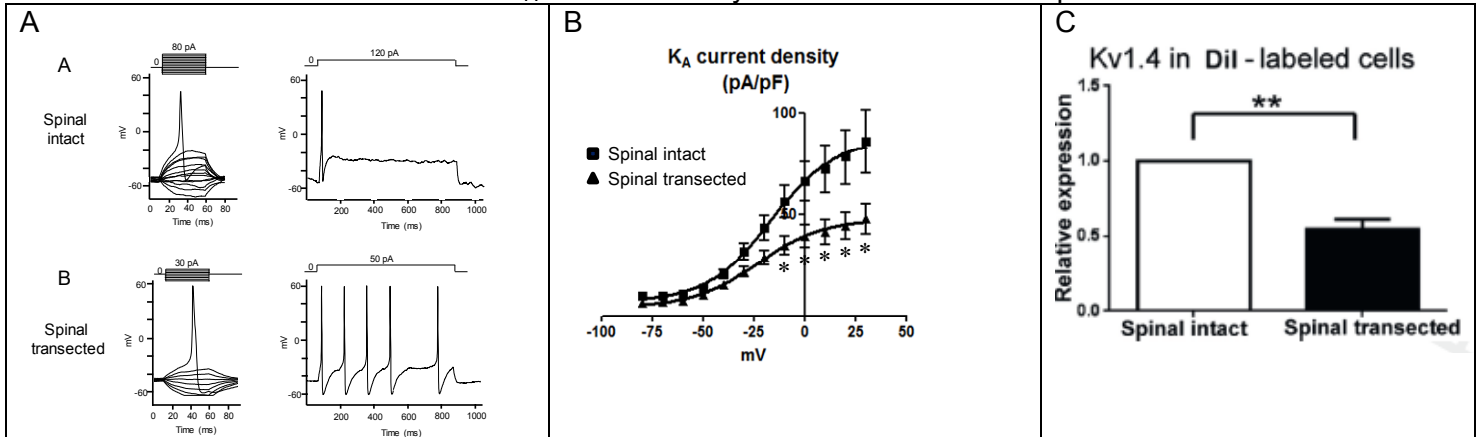


Figure 3. Electrophysiological properties and histological/molecular analysis of bladder afferent neurons from spinal intact and SCI (spinal transected) rats. **A:** Representative recordings of action potentials in capsaicin-sensitive bladder afferent neurons from spinal intact (A) and spinal transected (B) rats. 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 spinal intact and spinal transected 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 spinal intact and transected rats ($n=5$ rats each). ** $p < 0.01$ vs. spinal intact.

Aim 2 (Year 2)

- Develop LP-NGF antisense using cationic and amphoteric liposomes
- Comparison of effects of cationic liposomes vs. amphoteric liposomes (charge-reversible character) on blood pressure responses
- Tissue analysis

[Accomplishment]

In the second year of the project, we continued to perform in-vivo experiments to test the efficacy of LPs conjugated with NGF antisense oligonucleotide (OND). 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, 12 μ M 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 (1.0 g/kg, s.c.).

We have found that; (1) cationic liposomes were needed for bladder uptake of OND, (2) at 24 hours after liposome-oligonucleotide treatment baseline bladder activity during saline infusion was indistinct in the sham and antisense treated groups (Fig. 4B), (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 oligonucleotide ($p < 0.05$), (4) acetic acid induced increased nerve growth factor in the urothelium of sham treated rats, which was decreased

by antisense treatment (Fig. 4A) and (5) 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 ($p < 0.01$) (Kashyap et al., 2013). These results indicate that the cationic LPs-NGF antisense OND conjugate is effective to suppress the urothelial NGF expression and inhibit bladder overactivity induced by bladder afferent sensitization.

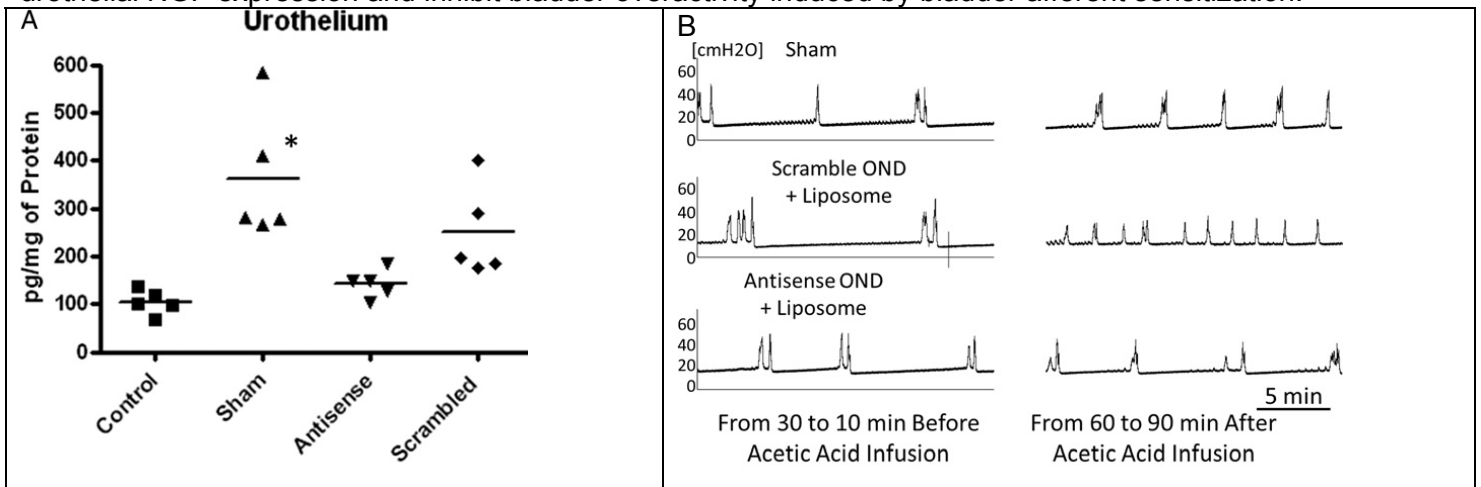


Figure 4. A: Antisense OND mediated suppression of acetic acid (AA) induced NGF protein expression in urothelium. AA exposure caused significant increase in NGF in sham treated group (Sham) vs. untreated controls. Liposome (LP)+NGF antisense OND significantly blunted NGF increase vs. sham treated group. 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 oligo-treated (middle traces) and LP-NGF antisense OND treated rats (lower traces). The cystometrograms before and after are shown in left and right traces, respectively. Note that the AA-induced reduction in intercontraction intervals (ICI) was seen in sham and scramble oligo-treated rats, but not in the rat treated with LP-NGF antisense OND conjugates.

- Comparison of blood pressure responses during bladder distention in SCI rats with or without NGF antisense treatment
- Comparison of dosing strategy using one-time or twice application of LP-anti NGF for reducing of blood pressure responses
- Comparison of effects of cationic liposomes vs. amphoteric liposomes (charge-reversible character) on blood pressure responses
- Tissue analysis

[Accomplishment]

Based on the results described in Figs. 1 & 4, we performed experiments to examine the effects of LP-NGF antisense OND conjugate on AD by measuring blood pressure responses during bladder distention using rats with Th5-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. LP-NGF antisense OND conjugate or saline (control) 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) arterial blood pressure elevation induced by bladder distention at 20, 40 and 60 cmH₂O was remarkably reduced in SCI rats that treated with LP-NGF antisense OND conjugate (Fig. 5A) compared to those without, and (2) NGF levels in the bladder mucosa is elevated in the SCI+ saline group compared to other groups including spinal intact (control) and SCI treated with LP-NGF antisense OND conjugate (SCI-ODN). These results indicate that AD (i.e., enhanced arterial blood pressure elevation during bladder distention) in SCI rats was reduced by the reduction of mucosal NGF expression following one-time intravesical application of LP-NGF antisense OND conjugate. In the third year of the grant, we will increase the animal number to further confirm the results.

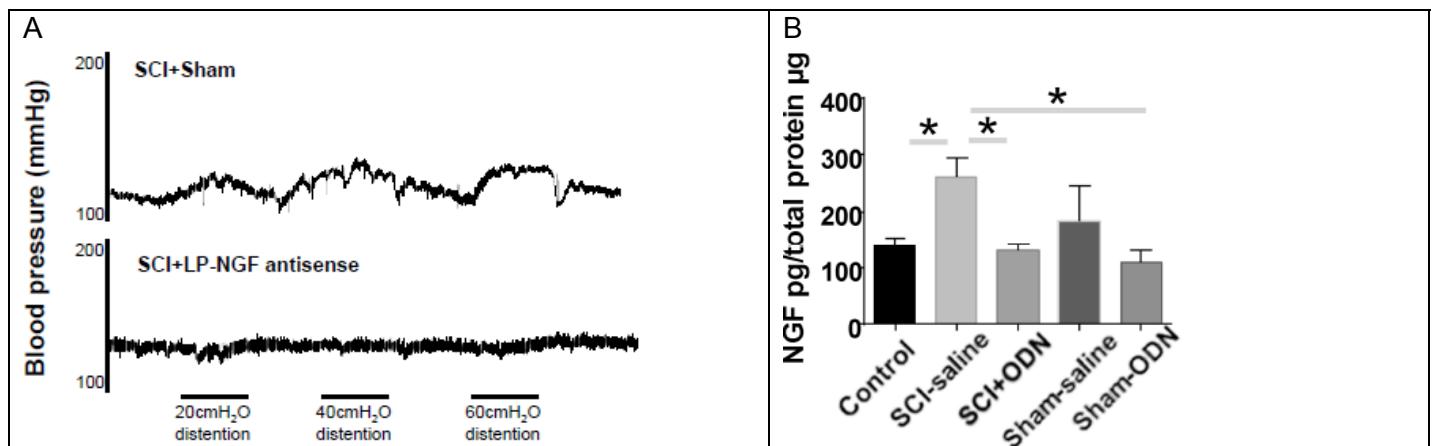


Figure 5. A: Arterial blood pressure responses during passive bladder distention from 20, 40 and 60 cmH₂O for a duration of 2 min each in spinal cord injured (SCI) rats without (SCI+sham; upper panel) or with liposomes-NGF antisense treatment (SCI+LP-NGF antisense; lower panel). Note that blood pressure responses were reduced in liposomes-NGF antisense-treated SCI rats. B: ELISA measurements of NGF proteins in the mucosa of spinal intact (control), SCI with intravesical saline (SCI+saline), SCI with liposomes-NGF antisense conjugate (SCI+ODN), spinal intact with intravesical saline (Sham+ saline) and spinal intact with liposomes-NGF antisense (Sham+ODN) groups. N=5 per each group. *P<0.05. Note that the NGF upregulation in the mucosa of SCI rats (SCI+ saline) was reduced by liposomes-NGF antisense treatment (SCI+ODN).

III. Key research accomplishments

- Confirmation of autonomic dysreflexia (AD) during bladder distention associated with NGF upregulation in the bladder urothelium of SCI rats
- Detection of hyperexcitability of bladder afferent neurons due to the reduction of A-type K⁺ channel activity and Kv1.4 subunit expression in SCI rats
- Formulation and optimization of cationic liposomes-NGF antisense conjugates
- Detection of the reduction of AD associated with decreased NGF expression in the bladder in SCI rats by intravesical treatment of liposomes-NGF antisense conjugates

IV. Reportable outcomes

Published abstract:

1. 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.
2. 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 AUA meeting.
3. 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.

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*, in press, 2013.

V. Conclusions

In the second year of the project, we successfully completed the works listed in the SOW (Year 2). Based on the results obtained in the first & second years of 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 C-fiber bladder afferent pathways, in SCI rats. Secondly, our formulation of cationic liposomes conjugated with NGF antisense successfully suppresses bladder overactivity induced by AA-induced C-fiber sensitization as well as SCI-induced AD in association with the reduction in NGF expression in the bladder urothelium. In the third year of the project, we will continue to investigate the pathogenesis of SCI-induced AD and the therapeutic effects of liposome-NGF antisense conjugates on SCI-induced AD, as proposed in the SOW (Year 3 timeline).

VI. References

- 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.
- 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*, in press, 2013.
- 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.

VII. Appendices

PDF files of the following publications are appended.

1. 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.
2. 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.
3. 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.
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, in press, 2013.

2013 AUA abstract

13-5525

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

Tsuyoshi Yoshizawa, Satoru Yoshikawa, Ryosuke Takahashi, Pittsburgh, PA, Satoru Takahashi, Tokyo, Japan, Naoki Yoshimura, Pittsburgh, PA*

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

Increased excitability of bladder afferent neurons associated with reduced expression of Kv1.4 α -subunit in rats with spinal cord injury*Ryosuke Takahashi*, Takakazu Yunoki, Seiji Naito, Fukuoka, Japan, Naoki Yoshimura, Pittsburgh, PA*

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

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

Naoki Kawamori^a*, Mahendra Kashyap, Vikas Tyagi, Yoshio Sugino, Pittsburgh, PA, Michael Chancellor, Royal Oak, MI, Naoki Yoshimura, Pradeep Tyagi, Pittsburgh, PA

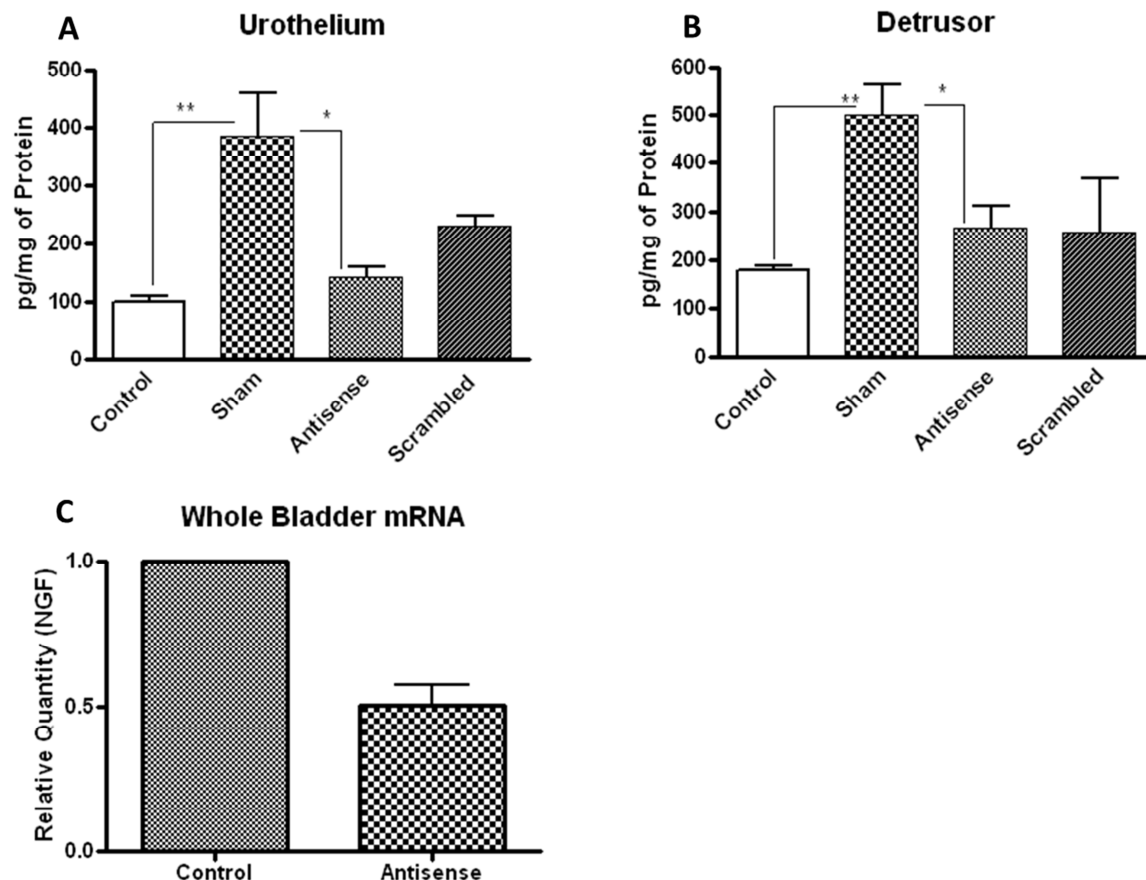
INTRODUCTION AND OBJECTIVES: Overexpression of NGF in the urothelium has been shown to play a role in symptoms of OAB by mediating functional changes of bladder afferents. We studied whether the blockade of NGF overexpression in the urothelium by a sequence-specific gene-silencing mechanism suppresses 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 $\pm$ 55.0 sec and 390.8 $\pm$ 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



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

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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 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

Abbreviations and Acronyms

Dil	= 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
Dil	= Dil
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^+
K_V	= 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

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 DK057267, 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, K_V channel activation is also an important factor for spike threshold and firing frequency control. K_V 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 K_V currents after SCI. In addition, to our knowledge the molecular mechanism responsible for changes in K_V 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 K_V 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 were labeled by retrograde axonal transport of the fluorescent dye Fast Blue (1% weight per volume, PolyScience®) or Dil (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 $K_V \alpha$ -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 $K_V \alpha$ -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 Dil injection in the bladder wall 1 week earlier. L6 DRGs were embedded in Tissue-Tek® O.C.T.™ Compound and stored at -80°C until use. Samples were sectioned at $8\ \mu\text{m}$ 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 Dil labeled and unlabeled bladder afferent neurons (fig. 5, A to C). 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 certain conditions, including 1 cycle at 50°C for 45 minutes and at 95°C for 2 minutes, followed by 50 cycles at 95°C for 15 seconds and at 60°C 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, $R = 2^{(CP_{\text{sample}} - CP_{\text{control}})}$, 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).

Table 1. Primer and TaqMan probe sequences

	Kv 1.2	Kv 1.4	GAPDH
Primer:			
5'	CCTGTCTATCACCCAGGAACA	TGACCTACTGCCACAGGATG	AGACAGCCGCATCTTCTTGT
3'	TTGAGGAGAGTGGAGCTTGG	GGTTTCGAAGCGTAGACCAG	GATACGGCCAAATCCGTTTC
Probe	CATCTGCAAGGGCAACGTCACA	CAGTGACTGTTGTGAACGCGTGGT	GCAGTGCCAGCCTCGTCTCA
% Efficacy	99.8	104.4	104.0

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

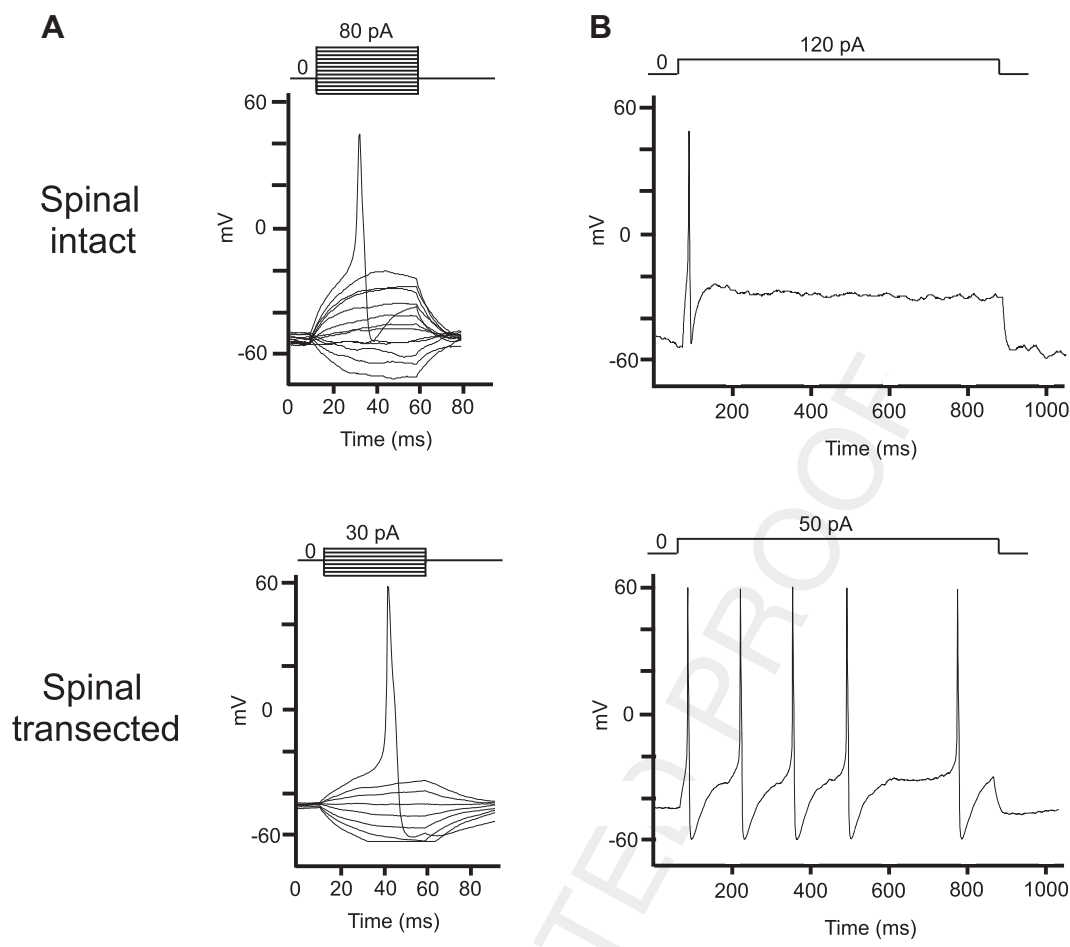


Figure 1. Representative action potential recordings in capsaicin sensitive bladder afferent neurons from spinal intact and transected rats. **A**, action potentials evoked by 50-millisecond (ms) depolarizing current pulses injected through patch pipettes during current clamp recording. **B**, firing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

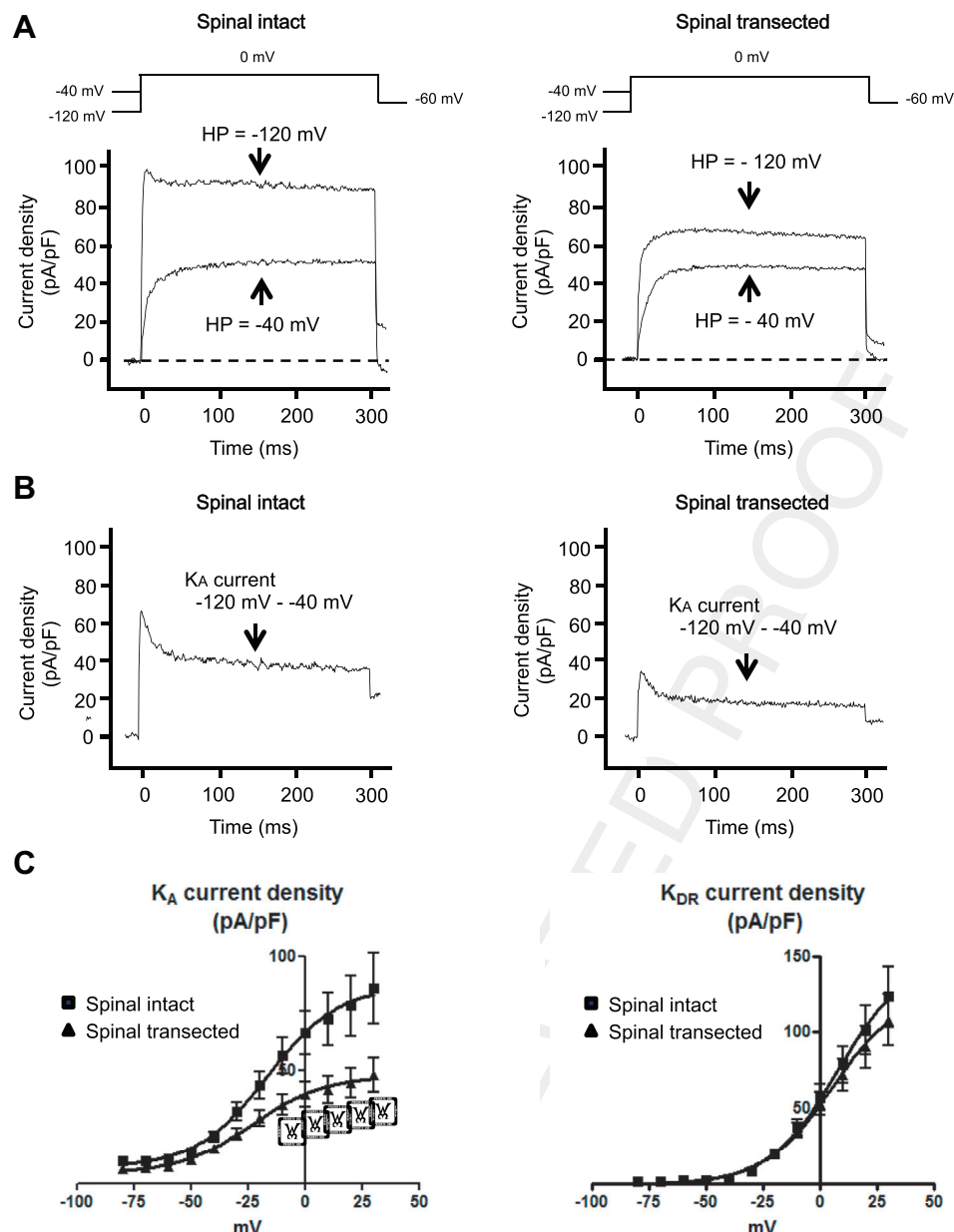


Figure 2. Changes in capsaicin sensitive bladder afferent neuron K^+ currents in rats without vs with spinal cord transection. **A**, representative recordings show superimposed outward K^+ currents evoked by voltage steps to 0 mV from -120 and -40 mV holding potential (HP). ms, milliseconds. **B**, 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

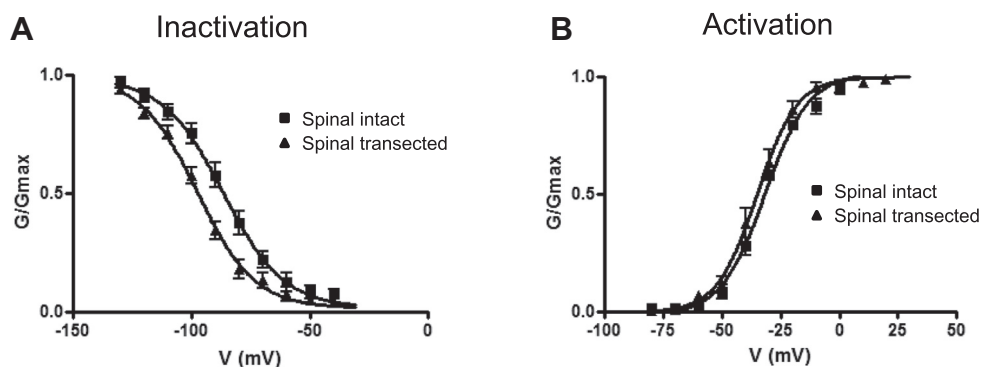


Figure 3. Mean $\pm$ SEM steady-state activation and inactivation characteristics of K_A currents in capsaicin sensitive bladder afferent neurons, shown as relative peak conductance of K_A currents normalized to maximal K_A current conductance (G/G_{max}) plotted against membrane potentials. *A*, inactivation 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*). 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 and tonic firing pattern thresholds, 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 that 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 immunohistochemical changes in the K_V channels responsible for the hyperexcitability of bladder afferent neurons in SCI rats.

K_V 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 and tonic firing thresholds.^{5,10}

In this study we confirmed that the current density of slow K_A currents were 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. K_V 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

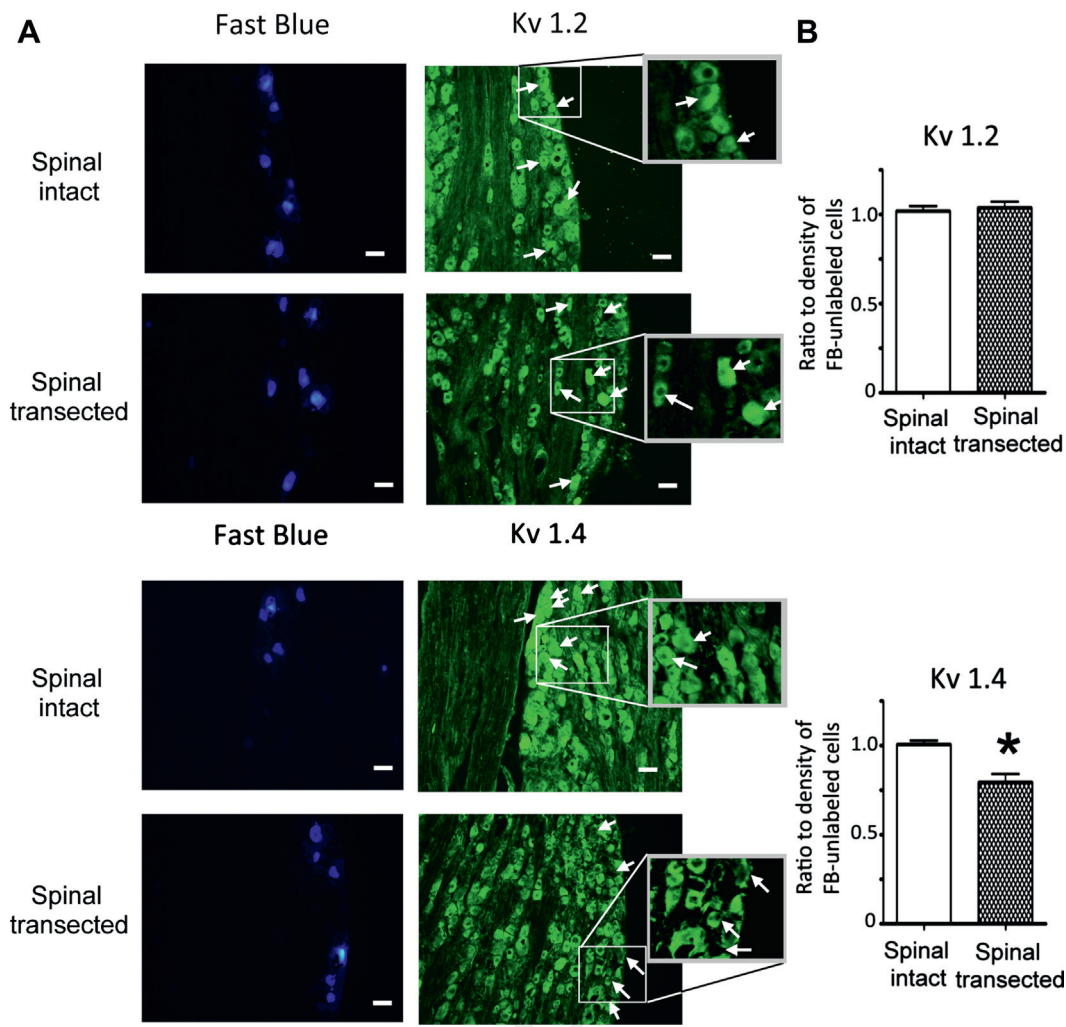


Figure 4. Kv α -subunit immunoreactivity in L6 DRGs after spinal cord transection. **A**, photomicrographs show same Fast Blue, Kv1.2 and Kv1.4 stained sections from spinal intact and transected rats under fluorescence illumination with ultraviolet (blue areas) and FITC (green areas) filters. Arrows 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

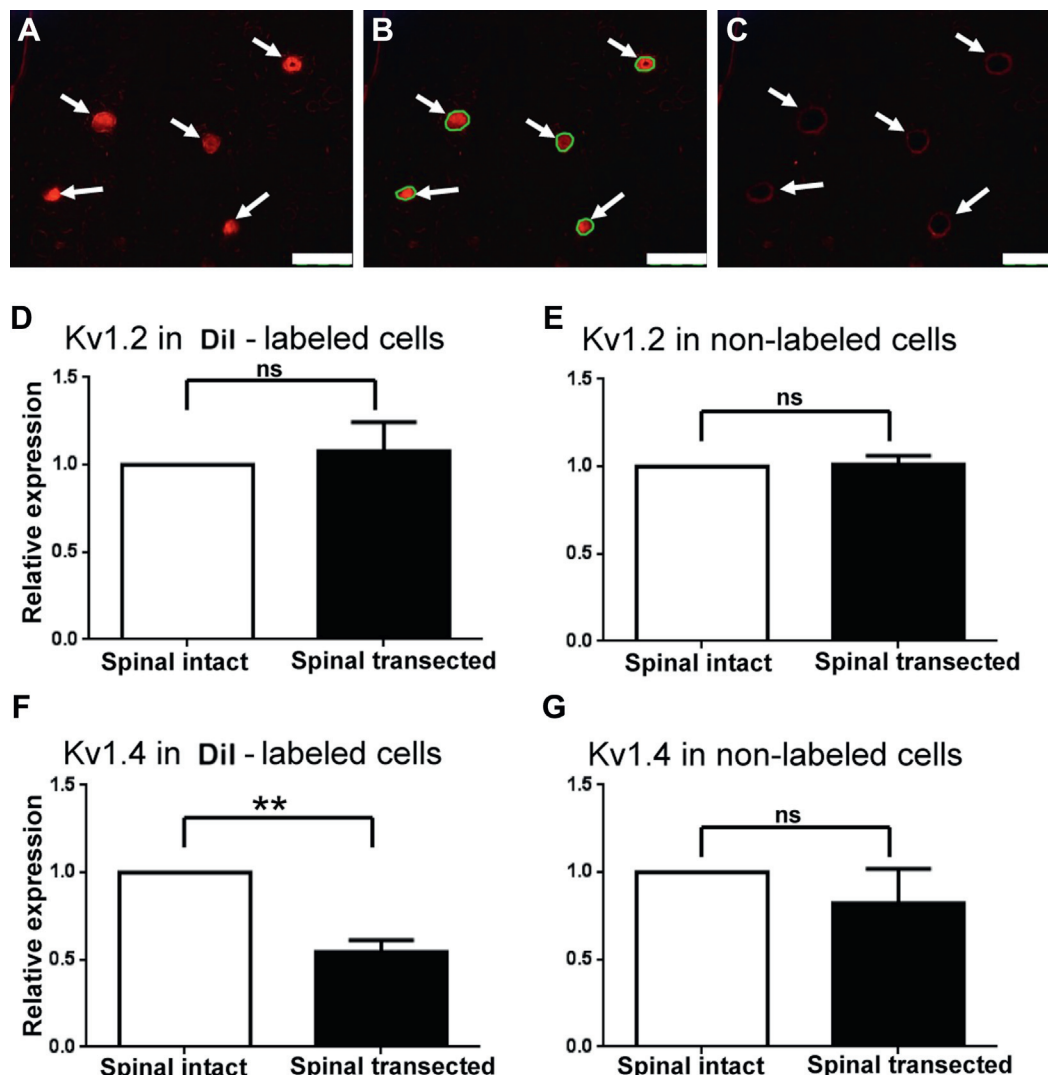


Figure 5. Kv α -subunit mRNA expression after spinal cord transection. A to C, photomicrographs show single L6 DRG section during laser capture microdissection of Dil labeled bladder afferent neurons. Arrows indicate neurons positively stained with Dil. Scale bars represent 100 μ m. A, before microdissection. 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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