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

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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

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<td>AIM 1 (PITT)</td>
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- Regulatory approval for animal research
- Order animals/
  Prepare protocols/
  documentation
- Comparison of blood pressure responses during bladder distention between spinal intact and SCI rats
- Tissue analysis
  Optimization and analysis of NGF expression using molecular techniques such as PCR and ELISA in spinal intact and SCI rats

| AIM 2 (PITT) |
- 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

|  |
|---|---|
| 0-2mo | Comparison of blood pressure responses during bladder distention in SCI rats with or without capsaicin pretreatment |
| 3-8mo | Comparison of electrophysiological properties of bladder afferent neurons from spinal intact and SCI rats |
| 9-12mo | Tissue analysis |
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| 21-24mo | Data analysis/reporting of NGF expression data |
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| 33-36mo | Data analysis/reporting of NGF expression data |
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 distension was significantly reduced in SCI rats when C-fiber bladder afferent pathways were desensitized by intravesical application of resiniferatoxin (RTX; 10μM) 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 inured (SCI+vehicle) and RTX-treated SCI rats (SCI+RTX). Note that blood pressure responses at 20 cmH₂O bladder distension 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](image)

- **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 inured (SCI+vehicle) and RTX-treated SCI rats (SCI+RTX). N=4 per each group. *P<0.05](image)

- **Comparison of electrophysiological properties of bladder afferent neurons from spinal intact and SCI rats**
- **Tissue analysis (histology) of dorsal root ganglion (DRG) neurons**
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⁺ (Kₐ) currents in capsaicin sensitive bladder afferent neurons from spinal transected rats was significantly less than that from spinal intact rats, (3) the Kₐ 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 Kₐ 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 Kₐ 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 Kₐ 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 ± s.e.m. *; p < 0.05 compared with the spinal intact rats at corresponding membrane potentials. C: Kv1.4 mRNA levels in DiI-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

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, 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 (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.
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:

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.


Refereed articles:


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
VII. Appendices
PDF files of the following publications are appended.
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 viscero-vascular 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-sensitization 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±1.3mV) and the increased number of action potentials (4.7±0.7 spikes) during a 800 msec depolarizing pulse compare to control rats (-21.8±0.9mV and 1.3±0.1 spikes). The peak density of KA currents during membrane depolarizations to 0mV 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 ~10mV 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, DK88936, DOD SC100134 and PVA 2793
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 µM) 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 TYE563 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±4.0% of baseline values, compared to 75.8±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

Ryosuke Takahashi, Tsuyoshi Yoshizawa, Takakazu Yunoki, Pradeep Tyagi, Seiji Naito, William C. de Groat and Naoki Yoshimura*

From the Departments of Urology (RT, TT, PT) and Pharmacology and Chemical Biology (WCdG), University of Pittsburgh School of Medicine (NY), Pittsburgh, Pennsylvania, and Department of Urology, Graduate School of Medical Sciences, Kyushu University (RT, TY, SN), Fukuoka, Japan

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⁺ 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 lumbar-sacral 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'-tetramethylindocarbocyanine perchlorate
Dil = Dil
DRG = dorsal root ganglion
DTX = α-dendrotoxin
FITC = fluorescein isothiocyanate
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
Kₐ = A-type K⁺
K₀ = 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
Vₜₘ = half-maximal conductance

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

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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 Aδ and C fibers. In the normal condition Aδ 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 KvDR and transient KvA currents.³ A reduction in KvDR and/or KvA 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 KvA currents without affecting KvDR 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 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 disociation, 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 Aδ-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 dissocation. 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 MΩ 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 KV 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 µm. 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 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 μm 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, \( R = 2^{CP_{sample} - CP_{control}} \), 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 ± 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 from spinal transected rats did not differ from that of 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 KA current reduction.** We estimated the density of slow KA 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 KA 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 KA currents evoked by depolarization to 0 mV from the

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<table>
<thead>
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<th>Table 1. Primer and TaqMan probe sequences</th>
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<td><strong>Kv 1.2</strong></td>
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<td>Primer:</td>
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<td>5'</td>
</tr>
<tr>
<td>3'</td>
</tr>
<tr>
<td>Probe</td>
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<td>% Efficacy</td>
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We calculated the peak density of slow KA currents evoked by depolarization to 0 mV from the
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

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**Table 2. Electrophysiological properties of capsaicin sensitive bladder afferent neurons**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SCI</th>
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<tbody>
<tr>
<td>No. cells/rats</td>
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<td>19/13</td>
</tr>
<tr>
<td>Mean ± SEM diameter (μm)</td>
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<td>32.5 ± 0.9*</td>
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<tr>
<td>Mean ± SEM input capacitance (pF)</td>
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<td>45.0 ± 3.4*</td>
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<td>Mean ± SEM membrane potentials (mV):</td>
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<tr>
<td>Resting</td>
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<td>-48.8 ± 1.0</td>
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<tr>
<td>Spike threshold</td>
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<td>Peak</td>
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<tr>
<td>Mean ± SEM spike duration (msec)</td>
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<td>4.2 ± 0.3</td>
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<tr>
<td>Mean ± SEM No. 800-msec depolarization action potentials</td>
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<tr>
<td>Density:</td>
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<tr>
<td>No. cells/rats</td>
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<td>17/10</td>
</tr>
<tr>
<td>Mean ± SEM intensity $K_A$ (pA/pF)</td>
<td>68.8 ± 6.3</td>
<td>38.1 ± 4.6*</td>
</tr>
<tr>
<td>Mean ± SEM intensity $K_{DR}$ (pA/pF)</td>
<td>54.0 ± 7.0</td>
<td>48.6 ± 6.6</td>
</tr>
</tbody>
</table>

*p < 0.01 vs control.

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**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.
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 equation (fig. 3, B).

**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 ± 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.
HYPEREXCITABILITY OF BLADDER AFFERENT NEURONS ASSOCIATED WITH SPINAL CORD INJURY

Figure 3. Mean ± 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, i.e. sustained $K_{DR}$ and transient $K_A$ currents. Transient $K_A$ currents in sensory neurons, including DRG cells, can be further subdivided into at least 2 subtypes based on inactivation kinetics, i.e. fast and slow decaying $K_A$ currents. The slow decaying $K_A$ current is preferentially expressed in small, capsaicin sensitive bladder afferent neurons. A reduction in slow $K_A$ currents by 4-aminopyridine increased the excitability of these neurons, as evidenced by lower spike activation and tonic firing thresholds.

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.

Previous reports indicated that Kv1.1 $α$-subunits, including Kv1.1, Kv1.2
and Kv1.4, could be major components of Kv channels in DRG neurons. 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. 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 KA in DRG neurons. We previously reported that DTX partially suppressed slow decaying KA currents in capsaicin sensitive, small DRG neurons. 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 Kₐ channels. Also, decreased Kv1.4 α-subunit expression after SCI might be a molecular mechanism responsible for the reduction in slow decaying Kₐ 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. 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. We also previously reported that applying the KA channel blocker 4-aminopyridine significantly decreased the spike threshold and increased the firing number during sustained membrane depolarization. These results suggest that changes in Na⁺ channel
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 KA current density and Kv1.4 a-subunit expression in SCI rats. Thus, the Kv1.4 a-subunit could be a molecular target for treating overactive bladder due to neurogenic detrusor overactivity.

REFERENCES


