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ORIGINAL ARTICLE

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

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

Methods: SCI was produced by the transection of the Th4–5 spinal cord in female Sprague–Dawley rats. At 4 weeks after SCI, changes in blood pressure during graded increases in intravesical pressure (20–60 cm H_2O) were measured in spinal-intact (SI) and SCI rats under urethane anesthesia. In five animals, effects of C-fiber desensitization induced by intravesical application of resiniferatoxin (RTX), a TRPV1 agonist, on the bladder-to-vascular reflex were also examined. Nerve growth factor (NGF) levels of mucosa and detrusor muscle layers of the bladder were measured by enzyme-linked immunosorbent assay. The expression levels of TRPV1 and TRPA1 channels were also examined in laser captured bladder afferent neurons obtained from L6 DRG, which were labeled by DiI injected into the bladder wall.

Results: In SI and SCI rats, systemic arterial blood pressure was increased in a pressure-dependent manner during increases in the intravesical pressure, with significantly higher blood pressure elevation at the intravesical pressure of 20 cm H_2O in SCI rats vs SI rats. The arterial blood pressure responses to bladder distention were significantly reduced by RTX-induced desensitization of C-fiber bladder afferent pathways. SCI rats had higher NGF protein levels in the bladder and higher TRPV1 and TRPA1 mRNA levels in bladder afferent neurons compared with SI rats.

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

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

It has been shown that vascular responses (that is, hypertension) induced by bladder distention are mediated by the activation of TRPV1 (capsaicin receptor)-expressing C-fiber bladder afferent pathways in spinal-intact (SI) rats.[3] In addition, the expression of TRP channels such as TRPV1 and TRPA1 are involved in the sensitization of C-fiber afferent pathways.[4] Previous studies have also indicated that increased levels of nerve growth factor (NGF) in the bladder is one of the key mediators to induce hyperexcitability of C-fiber bladder afferent pathways after SCI, resulting in detrusor overactivity (DO),[5–8] and that intrathecal application of NGF antibodies, which reduces NGF levels in bladder afferent pathways, is effective for DO as well as AD in SCI rats.[9–11] A recent study demonstrated that the intravesical treatment with botulinum toxin also reduces AD induced by bladder contractions, which is associated with a reduction in NGF levels in the whole bladder tissue and dorsal root ganglia (DRG).[12]

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

MATERIALS AND METHODS

Animal preparation

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

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

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

Bladder-to-vascular reflex during bladder distention

Four weeks after spinal cord transection, the bladder-to-vascular reflex during bladder distention was evaluated according to the methods described in our previous study.[3] In brief, PE-50 tubing (Clay-Adams, Parsippany, NJ, USA) was inserted into the bladder through the urethra and tied in place by a ligature around the urethral orifice. Then the PE-50 tubing in the urethra was connected via a three-way stopcock to a pressure transducer and to a syringe pump for recording intravesical pressure and for infusing saline into the bladder, respectively. Mean arterial blood pressure was recorded via a pressure transducer connected to a cannula in the common carotid artery. Intravesical pressure was increased in a stepwise manner to 20, 40 and 60 cm H_2O for the duration of 1 min at each pressure level with 1-min intervals by connecting the urethral catheter through a three-way stopcock to a saline-filled reservoir, the height of which was adjusted to maintain a constant pressure in the bladder. In some SCI rats, a TRPV1 receptor agonist, RTX (10 μM, 0.4 ml) or vehicle was administered intravesically through a urethral catheter, and the solution was kept in the bladder for 30 min to induce desensitization of TRPV1-expressing C-fiber afferent pathways in the bladder 24 h before experiment under isoflurane anesthesia.

Enzyme-linked immunosorbent assay measurements of NGF in the bladder

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

LCM and real-time PCR analysis

The population of DRG neurons, which innervate the urinary bladder, was labeled by retrograde axonal transport of a fluorescent dye, DiI (1% w/v; Invitrogen, Carlsbad, CA, USA), injected into the bladder wall of SI and SCI rats ($n=5$ each) under isoflurane anesthesia, as previously reported.[14] Seven days after DiI injection, L6 DRG were removed and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) and stored at −80 °C until use. Samples were sectioned at 8-μm thickness and the sections were mounted on PEM membrane slides (Leica Microsystems, Wetzlar, Germany). The tissue was air-dried, and LCM was performed using a Leica LMD6000 (Leica Microsystems) to dissect DiI-labeled bladder afferent neurons and unlabeled neurons separately. Excised cells were individually captured into the caps of 0.5 ml Eppendorf tubes and lysed, and RNA isolation, reverse transcription and real-time PCR were performed using a Cells DirectTM One-Step qRT-PCR kit (Invitrogen). Gene-specific primers and TaqMan probes crossing exon/exon junctions (Invitrogen) were designed for TRPV1 and TRPA1 using the Primer 3 Software (Primer 3, Totowa, NJ, USA; Table 1). Probes contained FAM fluorophore (Invitrogen) and TAMRA quencher (Invitrogen). The primer and probe combination were optimized within suitable ranges for efficiency and correlation coefficient using standard curve dilutions and data output on an ABI Step-One Plus thermocycler (Applied Biosystems, Foster City, CA, USA). Amplification of complementary DNA was performed under the following conditions: one cycle of 50 °C for 45 min and 95 °C for 2 min, followed by 50 cycles of 15 s at 95 °C, 1 min at 60 °C. The reactions were analyzed in triplicate and normalized relative to GAPDH. Real-time PCR data were analyzed by the DCp (difference in crossing points) method as $R=2^{(\text{Cp sample}-\text{Cp control})}$ to generate the relative expression ratio (R) of each target gene relative to that of GAPDH. We also determined specificity to complementary DNA using real-time PCR to verify that our primer/probe sets did not amplify the genomic DNA. All of our primer/probe combinations showed efficiencies of 98–104% (Table 1).

Statistical analyses

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

RESULTS

Bladder-to-vascular reflex during bladder distention

Arterial pressor responses shown by the changes in mean arterial blood pressure were elicited by bladder distention with increasing distention pressures in a graded manner to 20, 40 and 60 cm H_2O in

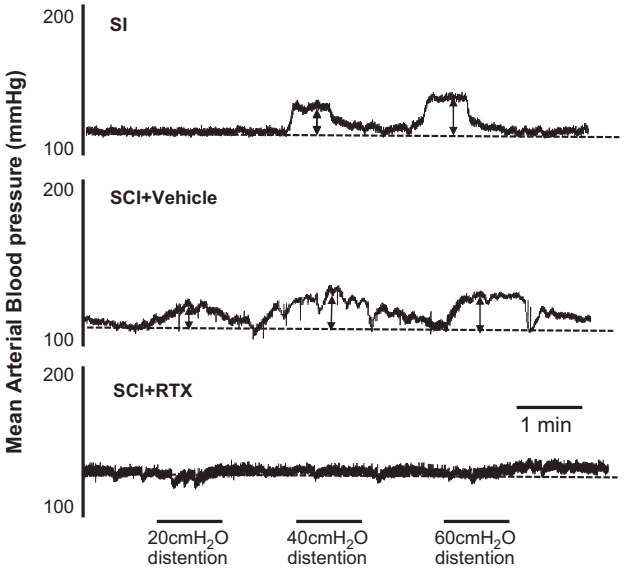

Figure 1 Recordings of mean arterial blood pressure during bladder distention in a stepwise manner to intravesical pressure at 20, 40 and 60 cm H_2O for a duration of 1 min at each pressure level with 1-min intervals in SI (upper panel) and SCI rats without (SCI+ vehicle; middle panel) or with TRPV1-espressing C-fiber desensitization induced by the intravesical RTX pretreatment (SCI+ RTX; lower panel). Break lines and bidirectional arrows indicate the baseline mean arterial blood pressure and the increases of mean arterial blood pressure during bladder distention, respectively.

Table 1 Primer and TaqMan probe sequences

	5′′primer	*3′primer*	*TaqMan probe*	*Efficacy (%)*
TRPV1	5′-CCGGAGAGATCTTGTCTGTG-3′	5′-ACCAGCATGAACAGCGACT-3′	5′-TATTTCCTGCAGAGGCGACCATCC-3′	95.7
TRPA1	5′-ATCAGGAGACCCTGCTTCAC-3′	5′-GTTGATGTCTGCTCCCACTG-3′	5′-TGATCATCATGACCTGGCAGACTACC-3′	101.3
GAPDH	5′-AGACAGCCGCATCTTCTTGT-3′	5′-GATACGGCCAAATCCGTTC-3′	5′-GCAGTGCCAGCCTCGTCTCA-3′	104.0

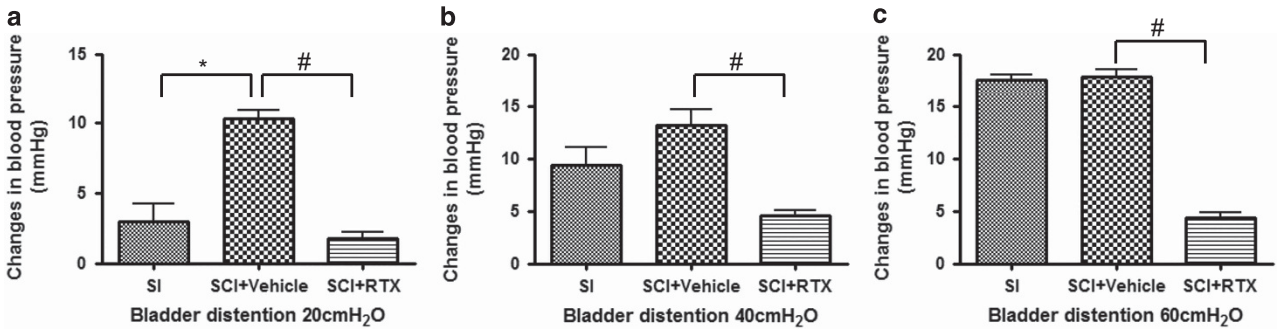

Figure 2 Changes in mean arterial blood pressure responses to bladder distention at 20, 40 and 60 cm H_2O of intravesical pressure (**a**–**c** respectively) in SI rats ($n=5$) and SCI rats without (SCI+vehicle, $n=5$) or with RTX-induced C-fiber desensitization (SCI+RTX, $n=5$). $^{*}P<0.05$: SI vs SCI+vehicle, $^{\#}P<0.05$: SCI+vehicle vs SCI+RTX.

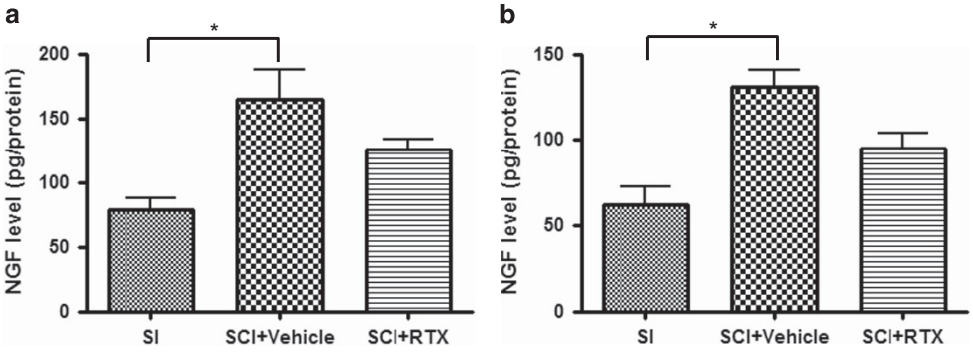

Figure 3 Expression of NGF protein in mucosa (**a**) and detrusor layers (**b**) of the bladder in SI ($n=5$) rats and SCI rats without (SCI+vehicle, $n=5$) or with RTX-induced C-fiber desensitization (SCI+RTX, $n=5$). $^{*}P<0.05$: SI vs SCI+vehicle.

both SI and spinalized rats (Figures 1 and 2). The arterial pressor responses during the intravesical pressure elevation to 40 and 60 cm H_2O were not different between the two groups (Figure 2). However, an increase in mean arterial blood pressure of spinalized rats during bladder distention of 20 cm H_2O was significantly greater ($P<0.05$) than in SI (Figure 2), demonstrating that SCI enhances the bladder-to vascular reflex, which is indicative of SCI-associated AD.

We then examined the effect of C-fiber desensitization in the bladder on arterial pressor responses during bladder distention of spinalized rats. We observed that the pretreatment with intravesical RTX in spinalized rats, 24 h before experiment caused a significant reduction in the increase of mean arterial blood pressure induced by the bladder distention of 20, 40 and 60 cm H_2O (Figures 1 and 2), indicating that the bladder-to-vascular reflex is dependent on the activation of C-fiber bladder afferent pathways in SCI.

Enzyme-linked immunosorbent assay measurements of bladder NGF

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

LCM and real-time PCR analysis

Changes in mRNA levels of TRPV1 and TRPA1 were examined in laser captured DRG neurons. The TRPV1 expression relative to GAPDH was significantly higher (1.98) in DiI-labeled bladder afferent neurons (30 cells per rat) isolated from five spinal-transected rats compared with those from five SI rats. Similarly, relative TRPA1 expression was significantly higher (1.96) in DiI-labeled bladder afferent neurons (30 cells per rat) isolated from five spinal-transected rats compared with five SI rats. In contrast, there was no significant difference in the TRPV1 or TRPA1 mRNA levels in unlabeled neurons isolated from the groups of SI and transected rats (Figure 4).

DISCUSSION

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

We have previously reported that bladder distention induces intravesical pressure-dependent arterial pressor responses in SI rats, which are reduced by intravesical RTX treatment that desensitizes the TRPV1-expressing C-fiber afferent pathways.[3] These earlier results indicated that the bladder-to-vascular reflex is induced by the activation of C-fiber bladder afferent pathways during bladder distention of normal rats. The current study extends the earlier findings to rats with SCI induced by complete Th4–5 spinal cord transection. We observed that arterial pressor responses to bladder distention at 20 cm H_2O in spinalized rats were greater than in SI rats, which indicate that the bladder-to-vascular reflex during bladder distention at a low pressure range is enhanced in spinalized rats. These findings demonstrate that the clinical relevance of this model as the condition of AD, which is a hypertensive response often induced

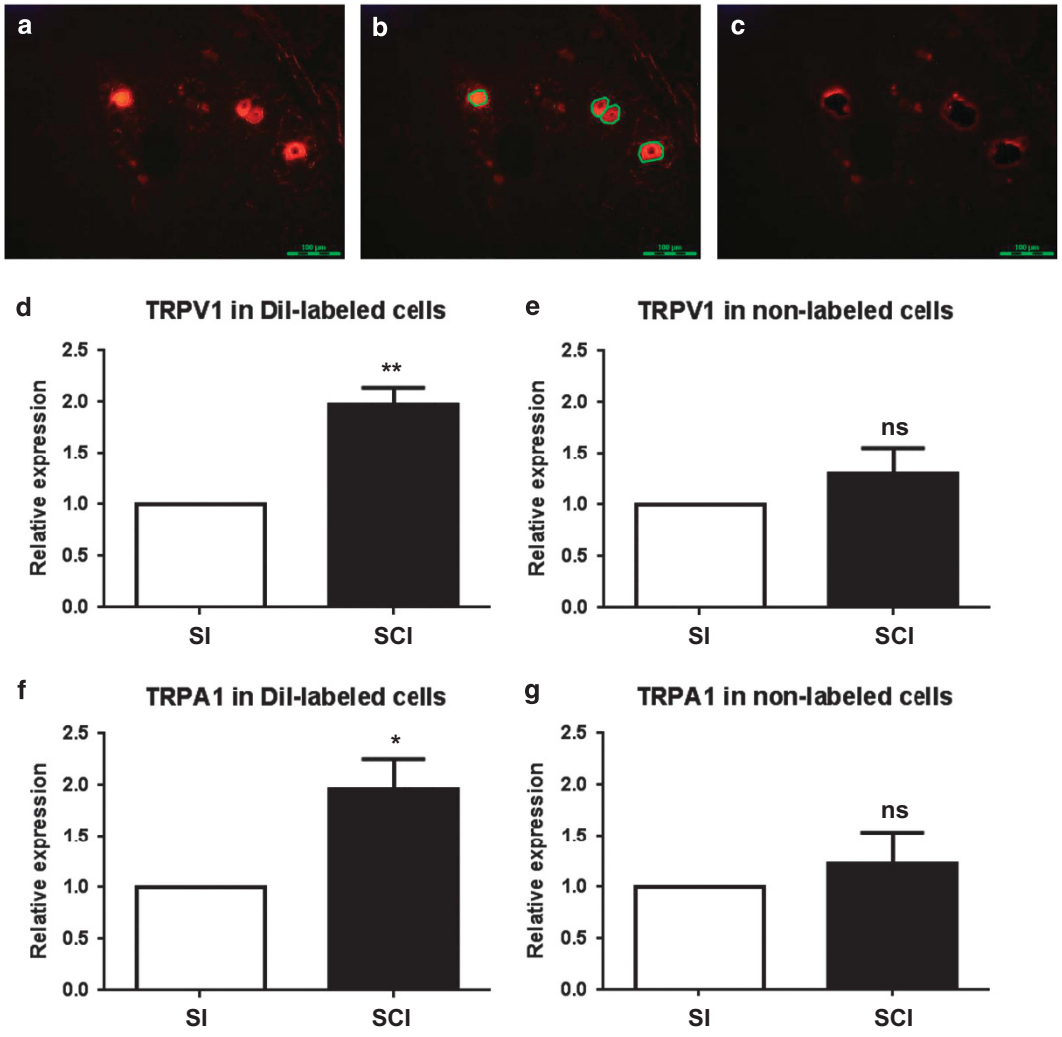

Figure 4 (**a**–**c**) Photomicrographs of the same L6 DRG section taken during LCM of DiI-labeled bladder afferent neurons before (**a** and **b**) and after LCM (**c**). Arrows point to neurons positively stained with DiI. Green-line circles in **b** indicate the areas that were laser captured **b**.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

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

However, the arterial pressor responses to higher bladder distentions (40 and 60 responses to higher bladder distention H_2O) were not different between SI and spinalized rats in this study. The findings at higher bladder distention are in line with the results of a previous study showing that changes in arterial blood pressure during bladder distention that raises the intravesical pressure to 35 H_2O are not different in control and spinalized rats.[15] In addition, a recent study showed that arterial pressor responses induced by reflex bladder contractions during filling cystometry in spinalized rats were greater compared with SI rats and that intravesical administration of botulinum toxin reduced the enhanced arterial pressor responses induced by bladder contractions.[12] However, after careful review of the study, it was noted that maximal voiding pressure was also higher in spinalized rats than in SI rats due to bladder overactivity after SCI,[12] suggesting the possibility that high bladder contraction pressure as a consequence of bladder overactivity leads to an increased blood pressure elevation during bladder contractions in spinalized rats. In this regard, we believe that our methodology using graded elevation of the intravesical pressure is better suited for evaluating the relationship between arterial pressor responses and bladder distention crucial for assessing AD in SCI.

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

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

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

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

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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