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

Katsumi Kadekawa^{1, 3, 4)}, Tsuyoshi Yoshizawa¹⁾, Naoki Wada¹⁾, Takahiro Shimizu¹⁾, Tsuyoshi Majima¹⁾, Pradeep Tyagi¹⁾, William C. de Groat²⁾, Kimio Sugaya³⁾, Naoki Yoshimura^{1, 2)*}.

1) Departments of Urology and 2) Department of Pharmacology & Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA, 3) Southern Knights' Laboratory, Okinawa, Japan, 4) Okinawa Kyodo Hospital, Okinawa, Japan

* Correspondence:

Naoki Yoshimura, MD, PhD
Department of Urology, University of Pittsburgh School of Medicine,
Suite 700, Kaufmann Medical Bldg.
3471 Fifth Ave, Pittsburgh, PA 15213.
Phone: 412-692-4137
Fax: 412-692-4380
E-mail: nyos@pitt.edu

Abstract

Purpose

To examine (1) whether spinal cord injury (SCI) time-dependently increases the severity of autonomic dysreflexia (AD) and expression levels of bladder nerve growth factor (NGF) protein, and (2) whether local suppression of NGF in the bladder improves SCI-induced AD in rats.

Materials and Methods

SCI was produced by the transection of the T2/3 spinal cord in female Sprague-Dawley rats. At 4 or 8 weeks after SCI, differences in the mean arterial blood pressure (Δ MAP) and heart rate (Δ MHR) during graded increases in intravesical pressure to 20, 40 and 60 cm H₂O from those before bladder distention and NGF protein levels in the bladder wall were evaluated in spinal intact and SCI rats under urethane anesthesia. Seven weeks after SCI liposome-NGF antisense conjugates were administered intravesically to the animals. At 1 week after intravesical treatment (8 weeks after SCI), Δ MAP and Δ MHR during bladder distention and bladder NGF protein expression were evaluated.

Results

The Δ MAP and Δ MHR were increased in a graded manner in response to bladder distention at intravesical pressures of 20, 40 and 60 cmH₂O in SCI rats. These AD-like cardiovascular responses and NGF protein expression in the bladder mucosal and muscle layers were increased after SCI in a time-dependent manner. The liposome-NGF antisense treatment significantly reduced the NGF protein overexpression in the mucosal layer of SCI rat bladder and reduced Δ MAP and Δ MHR elicited by bladder distention.

Conclusions

These results indicate that the duration of the post-SCI recovery period affects the severity of AD induced by bladder distention as well as the level of bladder NGF protein, and that local suppression of NGF expression in the bladder reduces SCI-induced AD. Thus, Intravesical application of liposome-NGF antisense conjugates can be a new effective therapy for bladder distention-induced AD after SCI.

Keywords: autonomic dysreflexia, spinal cord injury, liposome NGF antisense, rat

Introduction

Autonomic dysreflexia (AD) is a serious complication in persons with spinal cord injury (SCI) above the mid-thoracic spinal cord level. It is a potentially life-threatening medical emergency that induces uncontrolled sympathetic activity resulting in hypertension and bradycardia, and the source of stimulation initiating AD is bladder distention in up to 85% of cases, followed by colorectal distention in 13% of cases [Furusawa et al., 2011; Khastgir et al., 2007; Krassioukov et al., 2009]. Clinically, episodes of AD usually occur within the first 6 to 12 months after injury [Colachis, 1991], and the blood pressure response during bladder distention induced during urodynamics or cystoscopy, is more severe after post SCI recovery periods longer than 2 years than after recovery periods less than 2 years [Liu et al., 2013; Otani et al., 1985]. Neurogenic lower urinary tract dysfunction, after SCI, presents with detrusor overactivity and detrusor-sphincter dyssynergia (DSD), which result in inefficient voiding and bladder wall tissue remodeling such as hypertrophy and fibrosis [de Groat and Yoshimura, 2010; Kadekawa et al., 2016]. It has been demonstrated that, in SCI rats with spinal cord transection at the T4 level, DSD and AD are correlated, as evidenced by increases of electromyography activity of pelvic floor

1 muscles during voiding contraction, elevations of mean blood pressure and
2 decreases of mean heart rate at cystometric capacity [Rivas et al., 1995].
3 Therefore, inefficient voiding and high intravesical pressure, induced by DSD
4 after SCI, are likely to be involved in the emergence of AD. In addition, the
5 temporal changes in AD during colorectal distention in SCI rats with T3
6 transection has been shown by time-dependent increases in arterial pressure by
7 22 ± 3 and 49 ± 5 mmHg accompanied by bradycardia at 7 and 30 days after
8 SCI, respectively [Krassioukov and Weaver, 1995]. Thus, it is assumed that the
9 severity of AD may be time-dependent after SCI; however, a clinically relevant
10 animal model of bladder distention-induced AD, which shows the
11 time-dependent progression after SCI, has not been established.

12 A major mechanism underlying the emergence of AD is the development
13 of a vesico-cardiovascular reflex triggered by activation of bladder afferents
14 during bladder filling, which facilitates sympathetic nerve outflow via spinal
15 reflexes below the level of injury and causes widespread vasoconstriction and
16 hypertension that then triggers bradycardia due to activation of the vagal
17 nerve-mediated baroreceptor reflex [Blackmer, 2003]. Our recent study in SCI
18 rats also revealed that AD induced by bladder distention is mediated by

1 activation of resiniferatoxin-sensitive C-fiber afferents and is accompanied by
2 increased expression of nerve growth factor (NGF) in the bladder wall and
3 increased transient receptor potential (TRP) V1 channels in bladder afferent
4 neurons [Yoshizawa et al., 2015]. NGF overexpression in the bladder wall as
5 well as in the spinal cord and dorsal root ganglia (DRG) after SCI is thought to be
6 a major contributor to the induction of bladder afferent hyperexcitability and
7 bladder overactivity [Seki et al., 2002; Yoshimura et al., 2006] as well as to AD
8 during colon distention [Krenz et al., 1999]. In addition, intravesical botulinum
9 toxin treatment reportedly reduces arterial pressure responses during bladder
10 contractions in association with a decreased NGF expression in the bladder and
11 DRG of SCI rats [Elkelini et al., 2012]. Taken together, these findings suggest
12 that suppression of NGF expression in the bladder wall after SCI could reduce
13 bladder afferent hyperexcitability, leading to a reduction in AD induced by a
14 vesico-cardiovascular reflex during bladder distention.

15 A recent randomized double-blind placebo controlled phase 2 study,
16 which examined the effects of tanezumab, an NGF monoclonal antibody, on
17 clinical symptoms in patients with bladder pain syndrome showed that systemic
18 application of the antibody significantly improved the symptoms [Evans et al.,

2011]; however, another clinical trial reported systemic adverse effects such as paresthesia, hypoesthesia and arthralgia, which led the FDA to terminate other trials using systemic application of NGF antibodies [Wood, 2010]. Therefore, the site-specific reduction of NGF would be desirable to reduce the intrinsic toxicity from systemic blockade of NGF.

We recently reported that intravesical treatment with liposome-NGF antisense conjugates can deliver the NGF antisense to the bladder urothelium [Kashyap et al., 2013] and suppress the level of urothelial NGF expression as well as bladder overactivity in rats with acetic acid-induced cystitis [Kashyap et al., 2013] or experimental colitis [Kawamorita et al., 2016]. Therefore, we hypothesized that local suppression of NGF expression in the bladder wall after SCI using intravesical liposome-based delivery techniques could also reduce bladder afferent hyperexcitability, leading to the improvement of AD after SCI. Thus, the present study was performed to examine; (1) whether SCI time-dependently increases the severity of vesico-cardiovascular hypertensive reflexes during bladder distention and expression levels of bladder NGF protein and (2) whether local suppression of NGF in the bladder improves bladder distention-induced AD in SCI rats.

Materials and Methods

Animal preparation

Adult female Sprague–Dawley rats weighing 280–368 g (5 to 10 months old) with spinal intact ($n=6$) and spinal cord injury (SCI) ($n=40$) were used according to the experimental protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. In SCI groups, the T2/3 spinal cord was transected under isoflurane anesthesia. Eight SCI rats per each of five experimental groups were prepared because of possible attrition due to premature death of some animals after high thoracic-level SCI. Using sharp microscissors, muscle and fascia between T2 and 3 vertebrae were dissected, and the dura and spinal cord were transected completely with an aid of a surgical stereomicroscope. To ensure complete transection of the spinal cord, the tip of a 23G needle was inserted into the inner space between the exposed vertebrae. The overlying muscles and skin were then sutured. Buprenorphine (Tocris) (0.05 mg/kg, bid) was injected subcutaneously for 3 days postoperatively as an analgesic. Postoperatively the bladder was emptied by abdominal compression twice a day until reflex voiding recovered and once a

day afterwards until the final experiment. The animals were treated with ampicillin (100 mg/kg, subcutaneously) for 5 days after surgery, and this treatment was continued twice a week afterwards to prevent urinary tract infection.

Evaluation of vesico-cardiovascular reflexes during bladder distention after SCI

The rats were divided into 3 groups; (1) spinal intact (n=6), (2) 4 weeks after SCI (SCI 4 weeks, n=6) and (3) 8 weeks after SCI (SCI 8 weeks, n=7) groups. Two rats died prematurely in the 4 weeks group and one in the 8 weeks groups. These rats were anesthetized with urethane (0.9 g/kg subcutaneously) aided with isoflurane anesthesia. The dose of urethane was determined by our previous study [Yoshizawa et al., 2015] and preliminary experiments to suppress the body movement in spinal intact rats and spastic movements of the hind limbs in SCI rats during bladder distention. A 3Fr catheter (Hakko Co., Ltd., Nagano, Japan) filled with saline containing heparin was inserted into the common carotid artery to record the mean arterial pressure (MAP) and the mean heart rate (MHR). The bladder was exposed via a lower abdominal incision, and bilateral

1 ureters were cut and the distal ends were ligated to prevent vesicoureteral reflux
2 during bladder distention [Sugata, 1980]. A polyethylene catheter (PE-160,
3 Clay-Adams, Parsippany, NJ) was inserted through the bladder dome and a
4 purse suture was placed tightly around the catheter. The urethral orifice was
5 closed with glue to prevent fluid leakage (Fig. 1-A). Thereafter, isoflurane
6 anesthesia was turned off and the rats were placed in restraining cages (W
7 80 mm × L 300 mm × H 150 mm, Yamanaka Chemical Ind., Ltd. Osaka, Japan).
8 Intravesical pressure was kept constant by connecting a bladder catheter to a
9 saline reservoir and a pressure transducer via three-way stopcocks. The
10 reservoir was mounted on a metered vertical pole for controlled height
11 adjustment. Intravesical pressure was abruptly increased to 20, 40 and 60
12 cmH₂O by elevating the reservoir and maintained at each pressure level for 2
13 min [Yoshizawa et al., 2015]. Between pressure elevations, the reservoir was
14 returned to 0 cmH₂O for 2 min. The MAP was electronically averaged from
15 pressure values measured for 2 minutes before and during bladder distention
16 using Chart software (AD Instruments, Colorado Springs, CO) (Fig. 1-B). Using
17 a faster scale of arterial pressure tracing, heart beats were also counted for 10
18 seconds and the MHR was averaged for 1 minute (Fig. 1-C). Arterial pressure

and heart rate responses are shown as Δ MAP and Δ MHR by the difference of mean values measured before and during bladder distention.

Local suppression of NGF in the bladder after SCI

Cationic liposomes composed of DOTAP were made by the thin film hydration method [Fraser et al., 2003], during which a lipid film is hydrated with nuclease-free water to a final lipid concentration of 7mM. The NGF antisense oligonucleotide (5'GCCCGAGACGCCTCCCGA3') was dispersed in nuclease-free water at a concentration of 12 μ M, and then conjugated with liposomes by incubating the two entities together at room temperature for 30 minutes. The molar ratio of NGF antisense oligonucleotide to lipid in the liposomal complex was 1:10. Seven weeks after SCI the rats were divided into 3 groups; (1) vehicle (saline) (n=6), (2) liposome-only (n=8) and (3) liposome-NGF antisense (n=8) treated groups because 2 out of 8 vehicle-treated SCI rats died prematurely. In each group, a polyethylene catheter (PE-50, Clay-Adams, Parsippany, NJ) was inserted through the urethral orifice under isoflurane anesthesia, and then 1 ml of saline, liposomes only or liposomes conjugated with NGF antisense was administered intravesically, and the solution

was kept in the bladder for 30 minutes [Kashyap et al., 2013]. At 1 week after intravesical treatment (8 weeks after SCI), Δ MAP and Δ MHR during bladder distention were evaluated as mentioned above.

Enzyme-linked immunosorbent assay (ELISA) measurements of NGF in the bladder

NGF immunoassay of the bladder was performed in spinal intact and SCI rats with or without intravesical treatment (n=6 each group). After measurement of the vesico-cardiovascular reflex during bladder distention, the bladder was harvested from 6 spinal intact rats and 6 SCI rats per group, which were randomly selected when the number of animals per group was more than six, and the mucosa and detrusor layers were separated by microscissors. Tissues were then homogenized using the RIPA Lysis Buffer System (Santa Cruz Biotechnology, Dallas, TX, USA) to isolate protein and measure NGF using an ELISA kit (Abnova, CA, USA) according to the manufacturer's instructions. Tissue NGF values are expressed as pg per mg protein.

Statistical analyses

Results are reported as the mean $\pm$ standard error. Comparisons between the groups with the same intravesical pressure during bladder distention were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Comparisons of the values at 40 or 60 cmH₂O with those at 20 cmH₂O bladder distention in the same group were performed by two-way ANOVA followed by Dunnett's multiple comparisons test. $P < 0.05$ was considered to indicate statistical significance.

Results

Severity of vesico-cardiovascular reflexes and bladder NGF expression after SCI

In SCI 4 and 8 weeks groups, Δ MAP was increased in a graded manner in response to bladder distention at 20, 40 and 60 cmH₂O levels, and Δ MAP during 60 cmH₂O of intravesical pressure elevation was significantly higher than Δ MAP at 20 cmH₂O. When compared among groups, Δ MAP at 40 and 60 cmH₂O in the SCI 8 weeks group (25.9 ± 12.9 and 36.2 ± 16.2 mmHg, respectively) was significantly higher than in other 2 groups (Fig. 2-A and B).

1 The MHR during bladder distention in the spinal intact group increased, resulting
2 in positive Δ MHR values, but it decreased in the SCI 4 and 8 weeks groups to
3 show the negative Δ MHR values. In the spinal intact and the SCI 8 weeks
4 group, Δ MHR at 60 cmH₂O bladder distention was significantly greater than
5 Δ MHR at 20 cmH₂O. When compared among groups, Δ MHR values at 40 and
6 60 cmH₂O in the SCI 4 and 8 weeks groups were significantly lower than those
7 in the spinal intact group (Fig. 2-C).

8 SCI 4 and 8 weeks groups showed significantly higher levels of NGF protein
9 expression in the mucosa layer of the bladder, and the SCI 8 weeks group also
10 showed significantly higher levels in the muscle layer compared to the spinal
11 intact group (Fig. 2-D).

12 13 **The effect of local suppression of NGF in the bladder**

14 In all groups, Δ MAP values at 40 and 60 cmH₂O of intravesical pressure
15 elevation were significantly higher than those at 20 cmH₂O. When compared
16 among groups, the increases of MAP at 40 and 60 cmH₂O were significantly
17 reduced in the liposome-NGF antisense group compared to the vehicle and
18 liposome-only groups (Fig. 3-A and B).

1 Δ MHR values at 60 cmH₂O were significantly greater than those at 20 cmH₂O in
2 the vehicle and the liposome-only groups; however, there was no significant
3 difference in Δ MHR values at three different intravesical pressures in the
4 liposome-NGF antisense group. When compared among groups, the decrease
5 of MHR at 40 and 60 cmH₂O in the liposome-only group was significantly
6 reduced in the liposome-NGF antisense group (Fig. 3-C).

7 There were no significant differences in NGF expression in either the mucosa or
8 muscle layers between vehicle and liposome-only groups; however, the
9 liposome-NGF antisense treatment significantly reduced the increase of NGF
10 expression in the mucosa layer (Fig. 3-D).

11

12 **Discussion**

13 The results of this study using Th2/3 SCI rats demonstrated that; (1) MAP and
14 MHR were elevated and decreased, respectively, in a pressure-dependent
15 manner during increases in the intravesical pressure as well as in a
16 time-dependent manner after SCI in association with an increase of NGF protein
17 expression in the bladder wall, and (2) the intravesical treatment with
18 liposome-NGF antisense, which decreased NGF expression in the bladder

mucosa, reduced the MAP elevation and the MHR decrease in response to bladder distention in SCI rats.

MAP and MHR were increased and decreased, respectively, in a graded manner in response to bladder distention. Δ MAP in SCI 4 and 8 weeks groups and Δ MHR in the SCI 8 weeks group during 60 cmH₂O of intravesical pressure elevation were significantly higher and lower, respectively, than Δ MAP and Δ MHR values at 20 cmH₂O. Thus, our methodology using the graded elevation of intravesical pressure seems to be suitable for evaluating the vesico-cardiovascular reflex induced by bladder distention to assess AD in SCI.

In addition, AD in human SCI was defined by an increase in systolic blood pressure greater than 20 mmHg above baseline according to the International Standards [Krassioukov et al., 2012], which is in line with our results showing that Δ MAP was over 20 mmHg at 40 or 60 cmH₂O of intravesical pressure elevation in the SCI 8 weeks group (Fig. 2). We also observed that Δ MAP in the SCI 8 weeks group were greater than in the spinal intact and SCI 4 weeks groups, and that Δ MHR, which was a positive value in spinal intact rats, became negative values after SCI, indicating that SCI induces hypertension and bradycardia during bladder distention. Therefore, the SCI rat with Th2/3 spinal

cord transection is a clinically relevant model of AD because AD is induced by bladder distention and worsens at longer periods after SCI in patients with SCI above mid-thoracic spinal cord levels [Colachis, 1991; Liu et al., 2013; Otani et al., 1985]. This is also in line with the results of a previous study showing that AD becomes more prominent after the establishment of neuroplastic changes within the spinal cord and aberrant inappropriate connections within the central and peripheral nervous system [Ramer et al., 2012]. However, in our current study, we did not directly evaluate whether progressive changes in the degree of bladder distension-induced AD correspond to anatomical or physiological changes in bladder afferent pathways and/or in the spinal cord over time post-injury. Thus further studies, such as anatomical evaluation of the SCI-induced sprouting of C-fiber bladder afferents in the spinal cord, are needed to clarify these points.

Our model reliably assesses bladder distention-induced AD by controlling the magnitude of intravesical pressure, using a saline reservoir connected with a relatively large intravesical catheter (PE-160), which allows the rapid and stable distention of the bladder at a desired pressure. Therefore, this model seems appropriate to investigate the pathophysiology of bladder

1 distension-related AD. However, there are some limitations compared to other
2 models of AD induced by colorectal distention. First, because our model is
3 terminal, it is not possible to repeatedly analyze AD conditions in the same
4 animal, as has been done in the colorectal distention model, which uses
5 telemetry of hemodynamic parameters and colorectal balloon catheter via anus
6 under a conscious condition [Mayorov et al., 1998; Cormier et al., 2010].
7 Secondly, the possibility that anesthesia may influence the bladder
8 distention-induced AD cannot be excluded, although, in our model, urethane
9 anesthesia is needed to suppress body movements in spinal intact rats and
10 spastic movements of the hind limbs in SCI rats during bladder distention in
11 order to obtain stable blood pressure and heart rate recordings.

12 The expression levels of NGF protein in the bladder mucosal and muscle
13 layers were also increased time-dependently after SCI in the present study. NGF
14 has been implicated as a chemical mediator of pathology-induced changes in
15 C-fiber afferent nerve excitability and reflex bladder activity [de Groat and
16 Yoshimura, 2010; Lamb et al., 2004; Yoshimura et al., 2006; Zvara and Vizzard,
17 2007]. Also, AD induced by bladder or colorectal distention is mediated by
18 C-fiber afferent pathways in SCI rats [Lamb et al., 2004; Schnegelsberg et al.,

2010; Yoshizawa et al., 2015]. Since NGF levels in the bladder of rats are similarly increased after partial obstruction of the urethral outlet and SCI [Steers and Tuttle, 2006; Yoshizawa et al., 2015], NGF overexpression after SCI is thought to be induced at least in part by functional urethral obstruction, reduced voiding efficiency and high intravesical pressure due to DSD [de Groat and Yoshimura., 2012].

In the present study, the intravesical treatment of liposome-NGF antisense reduced the vesico-cardiovascular reflex during bladder distention and the NGF overexpression in the bladder mucosa in SCI rats. A transgenic mouse model of chronic NGF overexpression in the bladder shows bladder overactivity association with neuronal proliferation and focal increases in urinary bladder mast cells [Schnegelsberg et al., 2010]. Intrathecal delivery of NGF in spinal intact rats induces bladder overactivity as well as somal hypertrophy and hyperexcitability of bladder afferent neurons [Yoshimura et al., 2006]. Intrathecal application of NGF antibody in SCI rats reduces bladder overactivity accompanied by a decrease in NGF levels in spinal cord and DRG [Seki et al., 2002]. These findings suggest that NGF overexpression in the bladder after SCI is a major factor in inducing hyperexcitability of bladder afferent pathways.

1 Therefore, in the present study, it seems reasonable to assume that local
2 suppression of NGF expression in the bladder reduces the bladder afferent
3 activity, resulting in the improvement of sympathetic nerve outflow and a
4 reduction in the vesico-cardiovascular reflex after SCI.

5 In this study, Δ MAP values were significantly reduced in liposome-NGF
6 antisense-treated SCI rats compared to both groups of vehicle-treated and
7 liposome only-treated SCI rats, whereas Δ MHR values during bladder distention
8 in the liposome-NGF antisense treated group were significantly reduced
9 compared to the liposome-only treated group, but not to the vehicle treated
10 group. Because bradycardia in AD events after SCI is induced secondarily by a
11 vagal nerve-mediated reflex that is induced via activation of baroreceptors
12 located at the aortic arch in response to hypertension [Blackmer, 2003], the
13 Δ MHR might be less sensitive to the liposome-NGF antisense treatment than the
14 Δ MAP, which is more directly induced by a vesico-vascular reflex during bladder
15 distention. In addition, the present study showed that MHR responses in the
16 liposome-only treated group were slightly greater than in the vehicle-treated
17 group although they were not significantly different (Fig. 3), which might reflect
18 the statistically insignificant results of Δ MHR between vehicle-treated and

1 liposome-NGF antisense treated groups. Because a previous study reported
2 that the liposome-only treatment can modulate bladder activity in rats with
3 intravesical administration of acetic acid or potassium chloride/potassium sulfate
4 [Fraser et al., 2003], it is possible that liposome itself might affect
5 vesico-cardiovascular reflex activity during bladder distention although further
6 studies are needed to clarify this point. Nevertheless, the current study clearly
7 showed the significant improvement of AD-like cardiovascular responses such
8 as Δ MAP after the NGF antisense treatment in SCI animals.

9 There are several clinical studies showing the improvement of
10 bladder-related AD. Intravesical instillation with capsaicin, but not vehicle
11 instillation, acutely worsened AD induced by bladder distention, but, one month
12 later, it reduced bladder-related AD and urinary incontinence, suggesting that
13 capsaicin-sensitive bladder afferents are involved in bladder-related AD [Igawa
14 et al., 2003]. A recent open-label clinical trial revealed that intradetrusor
15 injections of onabotulinumtoxin-A decreased the severity and frequency of AD
16 during urodynamics and during bladder-related events, and improved quality of
17 life in patients with SCI [Fougere et al., 2016]. In SCI rats, onabotulinumtoxin-A
18 is also shown to reduce bladder-related AD in association with a decrease in

1 bladder NGF expression [Elkelini et al., 2012]. Therefore, capsaicin or
2 onabotulinumtoxin-A treatments are likely to reduce AD, at least in part, through
3 chemodenervation of C-fiber bladder afferents, which are sensitized by NGF
4 overexpression in the bladder. Our present study further supports this
5 assumption by providing a proof of concept for the future development of a direct
6 anti-NGF therapy, for bladder-related AD in SCI patients, or in combination with
7 other treatments such as onabotulinumtoxin-A. However, the current study
8 only looked at the effect of liposome-based NGF antisense instillation after one
9 week in SCI animals at 8-weeks; therefore, it is not known how long the
10 treatment effects last. Thus, future studies will be planned to examine the effects
11 of intravesical NGF antisense treatment at different time points following SCI.

12 Furthermore, it is not known whether this bladder-targeting NGF
13 antisense treatment can mitigate AD, induced by colorectal distention, which is
14 another important source of stimulation initiating AD after SCI [Furusawa et al.,
15 2011]. Pelvic organ cross sensitization between the colon and the bladder is
16 thought to contribute to the overlapping symptoms in chronic pelvic pain
17 syndrome [Pezzone et al., 2005]; also, our recent study revealed that
18 experimental colitis induced NGF overexpression in the bladder and bladder

1 overactivity, which were reduced by intravesical application of liposome-NGF
2 antisense conjugates [Kawamorita et al 2016]. These data suggest that NGF
3 overexpression in the bladder, induced by colon-to-bladder cross sensitization
4 following colitis, likely stimulates bladder afferent pathways to induce bladder
5 overactivity. Thus, it is possible that anti-NGF treatment in one organ (i.e.,
6 bladder) affects afferent activity in another organ (i.e., colon), and it may improve
7 AD induced by not only bladder distention, but also colorectal distention in SCI.
8 Therefore, further studies are necessary to clarify this point.

9 In conclusion, the present study shows that: (1) SCI time-dependently
10 increased the severity of AD during bladder distention in association with the
11 increase of NGF protein expression in the bladder wall, and (2) local
12 suppression of NGF in the bladder improves bladder distention-induced AD in
13 SCI rats. Thus, local suppression of NGF expression in the bladder using
14 liposome-NGF antisense conjugates might be an effective therapy for AD
15 induced by bladder distention after SCI.

16 17 18 **Abbreviations**

- 1 AD autonomic dysreflexia
- 2 ANOVA analysis of variance
- 3 DRG dorsal root ganglia
- 4 MAP mean arterial blood pressure
- 5 MHR mean heart rate
- 6 NGF nerve growth factor
- 7 SCI spinal cord injury
- 8 TRP transient receptor potential

9

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14

15 **Disclosures**

16 No conflicts of interest, financial or otherwise, are declared by the
17 authors.

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Figure legends

Fig. 1 Experimental set-up and recordings of arterial pressure and heart rate during bladder distention.

(A) A 3Fr catheter was inserted into the common carotid artery to record the mean arterial pressure (MAP) and mean heart rate (MHR). A polyethylene catheter (PE-160) was inserted through the bladder dome and the urethral orifice was closed with glue to prevent fluid leakage. Intravesical pressure was kept constant by connecting a bladder catheter to a saline reservoir. (B) Intravesical pressure was abruptly increased to 20, 40 and 60 cmH₂O by elevating the reservoir and maintained at each pressure level for 2 min. Mean arterial pressure was averaged from pressure values measured for 2 min before and during bladder distention. (C) Using a faster scale of arterial pressure tracing, heart beats were also counted for 10 seconds and then averaged for 1 min.

Fig. 2 Comparisons of the magnitude of vesico-cardiovascular reflexes elicited by bladder distention and the NGF protein expression between spinal intact and 4 or 8 weeks of SCI rats.

(A) Representative traces of arterial pressure responses during bladder distention. (B and C) The changes of mean arterial pressure (B) and heart rate (C) during bladder distention to intravesical pressures of 20, 40 and 60 cmH₂O. (D) NGF expression levels in the mucosa and detrusor layers of the bladder. SCI rats exhibit time dependent progression in changes of mean arterial pressure (A and B) and heart rate (C), which were elevated and decreased, respectively, in a graded manner in response to bladder distention at 20, 40 and 60 cmH₂O levels. NGF protein expression in the bladder mucosa and detrusor layers (D) was also increased after SCI in a time-dependent manner.

* p<0.05, ** p<0.01, *** p<0.001: vs. 20 cmH₂O in the same group (two-way ANOVA followed by Dunnett's multiple comparisons test). # p<0.05, ## p<0.01, ### p<0.001: vs. other groups (one-way ANOVA followed by Tukey's multiple comparisons test).

Fig. 3 Comparison of the effects of intravesical vehicle, liposome-only or liposome-NGF antisense treatments on vesico-cardiovascular reflexes induced by bladder distention and on NGF protein expression in SCI 8 weeks rats.

(A) Representative traces of arterial pressure responses during bladder distention. (B and C) The changes of mean arterial pressure (B) and heart rate (C) during bladder distention to intravesical pressures of 20, 40 and 60 cmH₂O. (D) NGF expression levels in the mucosa and detrusor layers of the bladder.

The liposome-NGF antisense treatment in comparison to the vehicle and/or liposome-only treatment significantly reduced the elevation of mean arterial pressure (A and B) and the decrease of mean heart rate (C) elicited by bladder distention at 40 and 60 cmH₂O, and significantly reduced NGF protein overexpression in the mucosal layer of SCI rat bladders. (D)

* p<0.05, ** p<0.01, *** p<0.001: vs. 20 cmH₂O in the same group (two-way ANOVA followed by Dunnett's multiple comparisons test). # p<0.05, ## p<0.01, ### p<0.001: vs. other groups (one-way ANOVA followed by Tukey's multiple comparisons test).

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