Original article

Time-dependent progression of neurogenic lower urinary tract dysfunction after spinal cord injury in the mouse model

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Abstract

This study evaluated the time-course changes in bladder and external urinary sphincter (EUS) activity as well as the expression of mechanosensitive channels in lumbosacral dorsal root ganglia (DRG) after spinal cord injury (SCI). Female C57BL/6N mice in the SCI group underwent transection of the Th8/9 spinal cord. Spinal intact mice and SCI mice at 2, 4 and 6 weeks post SCI were evaluated by single-filling cystometry and EUS-electromyography (EMG). In another set of mice, the bladder and L6-S1 DRG were harvested for protein and mRNA analyses. In SCI mice, non-voiding contractions was confirmed at 2 weeks post-SCI, and did not increase over time to 6 weeks. In 2-weeks SCI mice, EUS-EMG measurements revealed detrusor-sphincter dyssynergia (DSD), but periodic EMG reductions during bladder contraction were hardly observed. At 4 weeks, SCI mice showed increases of EMG activity reduction time with increased voiding efficiency (VE). At 6 weeks, SCI mice exhibited a further increase in EMG reduction time. RT-PCR of L6-S1 DRG showed increased mRNA levels of TRPV1 and ASIC1-3 in SCI mice with a decrease of ASIC2-3 at 6 weeks compared to 4 weeks whereas Piezo2 showed a slow increase at 6 weeks. Protein assay

showed the SCI-induced overexpression of bladder BDNF with a time-dependent decrease post SCI. These results indicate that detrusor overactivity is established in the early phase whereas DSD is completed later at 4 weeks with an improvement at 6 weeks post SCI, and that mechanosensitive channels may be involved in the time-dependent changes.

New & Noteworthy

This is the first paper to evaluate the time-course changes of bladder dysfunction associated with mechanosensitive channels in the mice model.

Introduction

Neurogenic lower urinary tract dysfunction (NLUTD) due to spinal cord injury (SCI) is a condition that affects both storage and voiding function (1) (5). In human SCI patients, the normal micturition reflex is initially eliminated in the acute phase, and then the spinal cord reflex activity gradually emerges by time (2). In the chronic phase, functional problems can be divided into storage and voiding dysfunctions. Storage dysfunction is caused by detrusor overactivity (DO), which leads to urinary frequency, often associated with urinary incontinence. Storage dysfunction may also lead to bladder fibrosis and elevation of bladder pressure, causing upper urinary tract dysfunction, which is often managed by intermittent catheterization of the bladder along with medical treatment (10). On the other hand, the major cause of voiding dysfunction in SCI is detrusor sphincter dyssynergia (DSD) that induces disruption of coordinating activity of the bladder and the external urethral sphincter (EUS), resulting in inefficient voiding with increased residual urine (6). Animal models of SCI have been used to study these conditions; however, the adequate timing of evaluation of NLUTD after SCI in the

mouse model has not been well explored. Thus, this study evaluated the time-course changes of bladder and EUS activity as well as expression of mechanosensitive channels in L6-S1 dorsal root ganglia (DRG), which contain bladder and EUS afferent neurons, using 2-6 weeks SCI mice.

Methods

Animal preparation

All experiments were conducted in accordance with National Institutes of Health guidelines and the ARRIVE 10 guidelines, and were approved by the University of Pittsburgh institutional Animal Care and Use Committee. Female C57BL/6N mice at 8 to 9 weeks old (n=40) were used in the study, and housed at 4 or 5 per cage in the air-conditioned room at 22-24°C with free access to food and water. The mice were randomly and equally divided into 4 groups; (1) spinal intact, (2) 2 weeks post SCI, (3) 4 weeks post SCI, and (4) 6 weeks post SCI groups (n=10 each group). Then, each group was further divided equally for functional/molecular evaluation and BDNF protein assay (n=5 per subgroups). All SCI mice underwent complete transection of the Th8/9 spinal cord with microscissors under isoflurane anesthesia (5% induction/2% maintenance). Post-surgical treatments included an analgesic, buprenorphine (0.05mg/kg, s.c.) given every 12 hours for 3 days and an antibiotic, ampicillin (100mg/kg, i.m.) given once daily for 7 days. Also, the bladder was emptied by perineal stimulation

and bladder compression once daily until evaluation to develop the consistent LUTD condition in this SCI model, according to our pervious study (23). SCI mice were monitored daily for postoperative comorbidities along with weekly weight checking.

Single-filling cystometry evaluation

At 2, 4 and 6 weeks after SCI, the mice in each group underwent single-filling cystometry (CMG) and EUS electromyogram (EMG) recordings under an awake condition according to the method previously described(21). Additionally, spinal intact mice without surgical manipulation were used as controls. Briefly, under 2.0% isoflurane anesthesia, the lower abdomen was opened, and the bladder dome was punctured with an 18-gauge needle for insertion of a PE-50 tube (Clay-Adams, Parsippany, JU, USA) with the end flared by heat, which was then tied by a 6-0 silk thread suture. The catheter was externalized from the upper position of the abdominal wound, and the wound was closed with threads. A local anesthetic, EMLA cream containing lidocaine 2.5% and prilocaine 2.5% was applied onto the abdominal wound to reduce surgical pain during CMG

recordings although systemic analgesic administration that may interfere with lower urinary tract function was not used. The mice were then gently placed in a restraining cage (Economy holder 15 to 30g, Kent Scientific, Torrington, CT, USA). First, urodynamic parameters of CMG were evaluated without EUS-EMG recordings in an awake condition. After surgery, isoflurane anesthesia was stopped, and CMG recordings were started 30 min after recovery from anesthesia. The PE-50 tube was connected to a three-way stopcock which was connected to a pressure transducer and to a syringe pump, and CMG was recorded during saline infusion into the bladder at a rate of 0.01ml/min. Saline infusion was continued at least 90 minutes until the bladder activity became stable and, thereafter, the bladder was emptied, and single-filling CMG recordings were performed 3 times after bladder emptying each time. In each single-filling CMG recording, saline infusion was stopped when voiding bladder contraction associated with fluid release from the external urethral meatus was observed. In single-filling CMG recordings, basal bladder pressure, maximum micturition pressure, time until voiding, the number of non-voiding contractions

(NVC) during the storage phase, which was determined as bladder pressure elevation of 8mmHg or higher above the baseline bladder pressure, post-void residual (PVR) volume that was measured by draining through the PE-50 catheter by gravity after voiding was measured. Bladder capacity (BC) was calculated by multiplying the infusion rate by time after starting infusion. The distinction of NVC and micturition contraction was done by observance of any release of fluid from the external meatus of the urethra. Voided volume (VV) was evaluated by subtraction of PVR from BC. Voiding efficiency (VE) was calculated by the following formula ; VE(%)=100×VV/BC.

EUS-EMG measurement

After single-filling CMG measurements, coordinating activities of the bladder and the EUS during the voiding phase were evaluated using simultaneous recordings of CMG and EUS-EMG. The SCI mice were again anesthetized with isoflurane, and epoxy-coated stainless-steel wire electrodes (50μm diameter; M.T.Giken, Tokyo, Japan) were placed into the EUS percutaneously through the perineum using a 30-gauge needle. The EUS-EMG activity was passed through a

discriminator and the output was recorded with an amplifier and data-acquisition software with an analog-to-digital converter (PowerLab, AD Instruments). The electrode was hooked at the tip so that the electrode was anchored into the EUS. After recovery from anesthesia, saline was infused at a rate of 0.01mL/min, and EUS-EMG activity and the intravesical pressure was recorded simultaneously during continuous saline infusion. In the EUS-EMG recordings, voiding contraction time, reduced EMG activity time and the ratio of reduced EMG activity time to voiding contraction time were calculated during the voiding reflex to evaluate DSD. Reduced EMG activity was measured when EMG activity was reduced to the baseline level between tonic firings of EUS-EMG activity during voiding bladder contraction. The voiding contraction time was measured as a duration between the rise of intravesical pressure beyond the threshold pressure and the point at which intravesical pressure returned to the level of threshold pressure.

Real time RT-PCR

After CMG and EMG evaluation, bilateral L6-S1 DRG were harvested to measure

mRNA expression of TRP channels and mechanosensitive ion channels such as ASICs and Piezo2 by real-time RT-PCR. The specimens were frozen in liquid nitrogen and stored at -80℃ immediately until the experiment. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad CA) and two micrograms of RNA were revers-transcribed back to c-DNA measured by MX3000P real-time PCR system (Stratagene, La Jolla, CA) in a 12μL volume by SYBR Green PCR Master Mix (QIAGEN, Valencia, CA). The primers were obtained from QIAGEN (β-actin: Rn_Actb_1_SG, TRPV1: Mm_Trpv1_1_SG, ASIC1: Mm_Asic1_1_SG, ASIC2: Mm_Asic2_1_SG, ASIC3: Mm_Asic3_1_SG, Piezo2: Mm_Piezo2_1_SG) and diluted as described in the manufacturer's instruction. The ratio of each gene to β-actin mRNA was used for statistical analysis. Data was analyzed by the difference in crossing points method as R=2^(Cp sample-Cp control) to evaluate the expression ratio of the specific gene compared to β-actin.

Protein assay

Using another set of SCI mice and SI mice (n=5 each group), the bladder was

harvested for protein assay evaluation. The bladder was opened, and the mucosa was separated from the muscle layer under microscopic vision and was preserved at -80°C until the experiment. BDNF Emax ImmunoAssay Systems (Promega Co, Ltd., Madison, WI) was used for measurement of the BDNF concentration in the bladder mucosa. The assayed BDNF value was standardized by the total protein concentration of the bladder mucosa measured by the BCA protein assay kit (Thermo Fisher Scientific, Waltham MA).

Statistical analysis

Multiple researchers were involved in the analysis of CMG and EMG charts for data blinding. Following the evaluation of equal distribution of the data among groups using F-test, one-way ANOVA followed by Tukey`s test was used for statistical comparison of the results using the PRISM software. $P < 0.05$ was considered statistically significant.

Results

Single filling CMG parameters

None of the animals were excluded from the study. Representative CMG charts and parameters are shown in Fig 1 and Table 1, respectively. Compared to SI mice (Fig. 1-A), NVC during bladder filling were already observed at 2 weeks post SCI, and the number of NVC per min did not increase at 4 weeks and 6 weeks post SCI (Fig. 1-B, C, D, F). Bladder capacity was significantly increased at 6 weeks post SCI, compared to 2 and 4 weeks post SCI (Fig. 1-D,E). Basal bladder pressure and maximal micturition pressure were not significantly different among groups (Table 1).

EUS-EMG measurement

Representative EMG charts and parameters of a SI mouse and 4-week post SCI mice is shown in Fig. 2-A and 2-C. In SI mice, the EUS activity was mostly suppressed during voiding contraction, indicative of synergistic activity of bladder and EUS (Fig. 2-A), whereas in SCI mice, the EUS was tonically active during voiding contraction with intermittent EUS reductions (Fig. 2-C). By observing the

196 EUS-EMG chart and fluid elimination from the external urethral meatus,
197 intermittent EUS reductions during voiding contraction can be divided into 2
198 types; EUS reductions with notch-like decreases in intravesical pressure, which
199 were observed as pressure drops during bladder contractions, leading to
200 urination and those without notch-like intravesical pressure decreases or
201 urination. Thus, in our study, we measured the EUS activity reduction associated
202 with notch-like pressure deceases during voiding bladder contractions as a
203 parameter to evaluate the DSD condition post SCI.
204 The comparison of SI and 2-, 4- and 6-weeks post SCI mice are shown in Fig. 2-
205 A, B, C and D. DSD evident as tonic EUS activity during bladder contraction was
206 observed at 2 weeks, but periodic EMG reductions during bladder contraction,
207 resulting in urination, were not observed in most 2-weeks SCI mice, thereby
208 leading to urinary retention (Fig. 2-B). At 4 weeks, SCI mice showed increases of
209 the EMG activity reduction time during bladder contraction (Fig. 2-C, H) in
210 association with increased VV and VE (Fig. 2-E,G; Table 1). At 6 weeks, SCI
211 mice exhibited a further increase in the EMG reduction time and VV compared to

2 and 4 weeks post SCI (Fig. 2-D, E, H; Table 1). However, because of increased bladder capacity and post-void residual volume, VE at 6 weeks was not significantly different from that at 4 weeks post SCI (Fig. 1-D, Fig. 2-F, G; Table 1). In addition, there was no significant difference in maximal micturition pressure in 4- and 6-weeks post SCI mice (Table 1), which may have contributed to the similar VE level in these 2 SCI groups, while VV was increased in 6-weeks vs. 4-weeks post SCI due to the improved EMG reduction time.

Real time PCR

RT-PCR of L6-S1 DRG showed increased mRNA levels of TRPV1 and ASIC1-3 in SCI mice vs SI mice, along with a decrease of ASIC2-3 at 6 weeks compared to 4 weeks post SCI, whereas Piezo2 mRNA levels showed a significant increase later at 6 weeks compared to SI mice (Fig. 3).

BDNF protein assay

Protein assay of BDNF in the bladder mucosa showed an increase of BDNF in all 3 groups of SCI mice compared to SI mice (Fig. 4). Also, BDNF levels showed a time-dependent decrease at 6 weeks compared to those at 2 weeks

228 post SCI (Fig. 4).

229

Discussion

The results of this study indicate that; (1) DO evident as NVC is established in the early phase (2 weeks) whereas DSD is established later at 4 weeks with an improvement evident as increased EMG reduction time at 6 weeks post-SCI, (2) ASIC2/3 and Piezo2 mechanosensitive channels expressed in L6-S1 DRG, which contain bladder and EUS afferent neurons, could be involved in the progression of DSD in early (2-4 weeks) and late phases (4-6 weeks) of SCI, respectively and (3) the protein expression of BDNF in the bladder mucosa was increased significantly after SCI along with a gradual decrease towards 6 weeks post SCI. Our recent study in mice with SCI up to 30 days has shown that the number of NVC per entire voiding cycle was time-dependently increased along with the enlarged bladder capacity and that bladder BDNF levels were increased early at 5 days after injury and gradually decreased during 30 days post SCI (24). The present study confirmed these BDNF data, and additionally showed that bladder BDNF is still significantly upregulated at 6 weeks post SCI. Furthermore, we found that SCI-induced DO is stably observed up to 6 weeks post SCI as

246 shown by the similar number of NVC per min at 2-6 weeks post SCI although
247 enlarged bladder capacity during the post injury period up to 6 weeks increased
248 the number of NVC per voiding cycle (Fig. 1), as similarly observed in the
249 previous study using 30-days SCI mice (24).
250 In previous reports, the difference of EUS activity in different species post SCI
251 have been demonstrated (8). In SCI rats, the EUS shows intermittent bursting
252 activities during voiding, which contribute to urethral pumping function to enhance
253 bladder emptying. On the other hand, SCI mice rather show periodic relaxations
254 of EUS activity during voiding, which enables urine to flow out through the urethra
255 although voiding efficiency is low in SCI mice compared to SCI rats (8). When
256 considering the EUS activity in humans including SCI patients, who do not exhibit
257 the bursting EUS activity during voiding, the mouse SCI model would be more
258 suitable for investigating the pathophysiological mechanisms of DSD seen in SCI
259 patients.
260 It has been reported that bladder afferent pathways controlling the micturition
261 reflex consist of C-fiber and Aδ-fiber afferents. In the spinal intact condition, the

normal micturition reflex is triggered by Aδ-fiber bladder afferent pathways whereas in the post SCI condition, increased excitability of C-fiber bladder afferents significantly contributes to DO (3) (4) (6). Previous studies also showed that treatments targeting C-fiber afferent pathways such as capsaicin pre-treatment that desensitizes capsaicin-sensitive bladder afferents improve DO and TRPV1 up-regulation in L6-S1 DRG although DSD was not improved (7). In this study, TRPV1 up-regulation, which continued up to 6 weeks post SCI, further suggests that hyperexcitability of TRPV1-expressing C-fiber afferent pathways contribute to the long-term DO in SCI. Furthermore, the results of this study may indicate that DSD can be caused by afferent pathways other than capsaicin-sensitive C-fiber afferents. Previous studies have demonstrated that neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) play an important role in LUTD and changes afferent excitability after SCI. For example, in recent studies, anti-NGF antibody treatment reduced DO and hyperexcitability of capsaicin-sensitive C-fiber bladder afferent neurons in 4-weeks SCI mice (18) (21) whereas anti-BDNF antibody treatment improved

DSD and inefficient voiding in association with a reduction of upregulated ASIC channels in 4-weeks SCI mice (22) (24). It has been shown that trkB, a BDNF-binding receptor, is expressed in relatively large-sized bladder afferent neurons, which typically represent the Aδ-fiber afferent neuronal cells (12) (13). Thus, it is reasonable to assume that BDNF-sensitive Aδ-fiber bladder afferent pathways are involved in the emergence of DSD after SCI although NGF-sensitive C-fiber afferent pathways play an important role in SCI-induced DO. Furthermore, our previous study showed that anti-BDNF antibody treatment ameliorated DSD in the same mouse model of SCI, as evidenced by an increase in the duration of notch-like reductions of bladder contraction pressure during voiding after BDNF inhibition (22) (24), Thus, it is assumed that a gradual reduction in mucosal BDNF expression during 4-6 weeks post SCI may play a role in the partial improvement of DSD shown by increases of EMG reduction time during bladder contraction in the present study.

In our study, mechanosensitive channels such as ASIC and Piezo2 showed time-sensitive changes in L6-S1 DRG after SCI. ASICs were initially characterized

as ion channels that respond to the extracellular pH environment, but later found to have mechanosensitive functions (11) (20). Further studies also showed the relation of ASICs and BDNF as BDNF levels are correlated with changes of ASIC expression in the mouse DRG (11). In addition, Piezo2 is an ion channel receptor that is mechanically activated and expressed in a subpopulation of sensory neurons, which is classified as low-threshold mechanosensory neurons (LTMR) that detect the direction of stimulus movements (14) (19). Recent studies also showed that a decrease of BDNF expression is associated with morphological polarization of Aδ-LTMR, leading to failure of exhibiting direction-selective responses (15) (16). Moreover, a recent study showed that Piezo 2 channels expressed in the bladder epithelium and afferent pathways can control low-threshold bladder-stretch sensing and micturition reflexes in spinal intact mice (9). Thus, it seems likely that changes of BDNF expression in the bladder and mechanosensitive ASIC/Piezo2 channel expressions in L6-S1 DRG, which contain bladder and EUS afferent neurons, are involved in the emergence of DSD in SCI, possibly due to the enhancement of bladder-to-EUS reflexes via Aδ-fiber

afferent pathways. In addition, these two different mechanosensitive channels, ASICs and Piezo2 could be involved in different phases of DSD after SCI because ASICs showed the early increase during 2-4 weeks post SCI, but Piezo2 increased the later phase at 6 weeks post SCI in this study.

There are some limitations in this study. First, we did not measure the expression changes in mechanosensitive channels in bladder or EUS-specific afferent neurons although we previously reported that the TRPV1 mRNA level of laser-captured bladder afferent neurons labeled with Fast Blue was significantly increased in SCI mice compared to SI mice (17). Therefore, further studies are needed to determine the specific afferent cell population responsible for SCI-induced DSD. Second, we measured the mRNA levels of TRPV1, ASICs and Piezo2 of the DRG, but not the protein levels; therefore, further studies are needed to clarify whether the changes of mRNA levels are relevant to those of protein expression of these channels. Finally, we did not examine whether therapies targeting mechanosensitive channels such as ASICs or Piezo2 can improve DSD or inefficient voiding after SCI; thus, the direct correlation of LUTD

and mechanosensitive channel expression in afferent pathways is not known. Further research would enable us to understand the underlying progression mechanisms of SCI-induced NLUTD including DSD.

Conclusion

Differences in time-dependent progression of NLUTD such as DO and DSD are identified in the mouse model of SCI. In addition, the late development of DSD might be related to changes in expression of mechanosensitive channels such as ASICs and Piezo2, which showed the different timing of upregulation after SCI.

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425 **Figure Legends**

426 Figure 1

427 Representative cystometry (CMG) traces of a (A) spinal intact (SI) mice and
428 spinal cord injury (SCI) mice at (B) 2 weeks post injury, (C) 4 weeks post injury
429 and (D) 6 weeks post injury. At 6 weeks post injury, SCI mice had increased
430 bladder capacity compared to 2 and 4 weeks post SCI mice (E). Non-voiding
431 contractions (NVC) were observed at 2 weeks, and the number of NVC per
432 minute (NVCs/min) did not show further changes at 4 and 6 weeks post SCI (F).
433 ＊: P<0.05 vs SI. †: P<0.05 vs 2week. ∩: P<0.05 vs 4week.

434

435 Figure 2

436 Representative cystometry and EUS-EMG traces of (A) spinal intact, (B) 2 weeks
437 post spinal cord injury (SCI), (C) 4 weeks post SCI, and (D) 6 weeks post SCI
438 during voiding contraction.
439 In SI mice, EUS-electromyography (EMG) activity was mostly silent during
440 voiding contraction whereas, in SCI mice, the EUS showed increased tonic

activity during bladder contraction along with periodic, intermittent reductions of EUS activity shown by arrows in B, C and D. When observing the external meatus and EUS activity, intermittent EUS reductions during voiding contraction can be divided into 2 types; (1) EUS reductions with notch-like deceases in intravesical pressure, leading to urination, shown by the black arrow and (2) those without notch-like intravesical pressure deceases or urination, shown by the white arrow. In 2-weeks post SCI mice, EUS activity did not show apparent reductions, leading to urinary retention. At 4 weeks post SCI, periodic EUS reductions during bladder contraction were increased to induce urination, which corresponded to notch-like intravesical pressure reductions shown by the black arrow. At 6 weeks post SCI, periodic EUS reductions were further increased, leading to an increase of notch-like reductions of intravesical pressure. Comparison of 2 weeks, 4 weeks and 6 weeks post SCI, voided volume and EMG reduction time were increased in a time-dependent manner (E, H). However, due to larger bladder capacity at 6 weeks post SCI, residual volume did not show differences among the groups (F). Voiding efficiency showed an improvement at 4 weeks compared to 2 weeks post

SCI, but did not show further changes at 6 weeks post SCI (G). ∗: *P*<0.05 vs SI. †: *P*<0.05 vs 2week. ∩: *P*<0.05 vs 4week.

Figure 3

Results of RT-PCR of L6-S1 DRG. mRNA levels of TRPV1 and ASIC1 showed an increase in 2, 4 and 6 weeks post spinal cord injury (SCI) compared to spinal intact (SI). ASIC2 showed an increase at 2 and 4 weeks post SCI, and a decrease at 6 weeks. ASIC3 showed an increase at 4 weeks post SCI and a decrease at 6 weeks. Piezo2 showed an increase at 6 weeks post SCI. ∗: *P*<0.05 vs SI. †: *P*<0.05 vs 2week. ∩: *P*<0.05 vs 4week.

Figure 4

Results of the BDNF protein level in the bladder mucosa by ELISA. BDNF showed a significant increase at 2, 4 and 6 weeks post spinal cord injury (SCI) compared to spinal intact mice. Upregulated BDNF in SCI mice showed a time-dependent decrease as 6-weeks post SCI mice showed a significant decrease of

473 BDNF compared to 2-weeks post SCI mice. ＊: $P<0.05$

474