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Morphological Changes in Different Populations of Bladder Afferent Neurons Detected by Herpes Simplex Virus (HSV) Vectors with Cell Type-Specific Promoters in Mice with Spinal Cord Injury

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Abstract

Functional and morphological changes in C-fiber bladder afferent pathways are reportedly involved in neurogenic detrusor overactivity (NDO) after spinal cord injury (SCI). This study examined the morphological changes in different populations of bladder afferent neurons after SCI using replication-defective herpes simplex virus (HSV) vectors encoding the mCherry reporter driven by neuronal cell type-specific promoters. Spinal intact (SI) and SCI mice were injected into the bladder wall with HSV mCherry vectors driven by the cytomegalovirus (CMV) promoter, CGRP promoter, TRPV1 promoter or neurofilament 200 (NF200) promoter. Two weeks after vector inoculation into the bladder wall, L1 and L6 dorsal root ganglia (DRG) were removed bilaterally for immunofluorescent staining using anti-mCherry antibody. The number of CMV promoter vector-labeled neurons was not altered after SCI. The number of CGRP and TRPV1 promoter vector-labeled neurons was significantly increased whereas the number of NF200 vector-labeled neurons was decreased in L6 DRG after SCI. The median size of CGRP promoter-labeled C-fiber neurons was increased from 247.0 in SI mice to 271.3 μm^2 in SCI mice whereas the

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CONFLICT OF INTEREST

J.C. Glorioso is a founder and consultant of SwitchBio, Inc and NuvoVec srl. None of the other authors has any conflict of interest with any of the data presented in this manuscript.

median cell size of TRPV1 promoter vector-labeled neurons was decreased from 245.2 in SI mice to 216.5 μm^2 in SCI mice. CGRP and TRPV1 mRNA levels of laser-captured bladder afferent neurons labeled with Fast Blue were significantly increased in SCI mice compared to SI mice. Thus, using a novel HSV vector-mediated neuronal labeling technique, we found that SCI induces expansion of the CGRP- and TRPV1-expressing C-fiber cell population, which could contribute to C-fiber afferent hyperexcitability and NDO after SCI.

Keywords

Herpes simplex virus vector; Spinal cord injury; Mouse; Urinary bladder; Dorsal root ganglia; Laser-capture microdissection

INTRODUCTION

The external urethral sphincter (EUS) along with the urinary bladder act in a coordinated effort to control the storage and release of urine. According to de Groat and Yoshimura (2012), certain neural structures in both the brain and the spinal cord are responsible for sensory inputs from both A δ -fiber and C-fiber bladder afferent pathways. In contrast to normal micturition, which is dependent on the incitement of A δ -fiber bladder afferent pathways, chronic spinal cord injury (SCI) occurring above the lumbosacral cord area causes neurogenic detrusor activity (NDO). This condition is partially due to the enhanced excitability of bladder afferent neurons, most especially in the C-fiber population (Yoshimura and de Groat, 1997; de Groat and Yoshimura, 2012; Takahashi et al., 2013).

C-fiber bladder afferent neurons have the ability to synthesize and release different types of neuropeptides, such as tachykinins (substance P and neurokinin A) and calcitonin gene-related peptide (CGRP). Additionally, they also express receptors such as ATP-binding P2 $\times$ 3 or TRPV1 that binds to resiniferatoxin (RTx) or capsaicin (Fowler et al., 2008). On the other hand, A δ bladder afferent neurons express neurofilament (NF) as an indicator of myelination (Yoshimura et al., 1998). Previous studies have shown that following spinal cord injury, C-fiber bladder afferent pathways exhibit various functional, morphological and histological changes that may contribute to NDO. For example, in human bladders, TRPV1- and P2 $\times$ 3-immunoreactivity along with immunoreactivity to PGP9.5 pan-neuronal markers in suburothelial nerves increased in those patients with NDO that resulted from SCI (Brady et al., 2004; Apostolidis et al., 2005). Also, changes in neuropeptide expression, morphology and functioning of C-fiber afferents have been identified in both cats and rats post SCI. Various changes that have been observed include: (1) somal hypertrophy of bladder afferent neurons in L6-S1 dorsal root ganglia (DRG) (Yoshimura and de Groat, 1997; Yoshimura et al., 1998), (2) expansion of CGRP and substance P containing primary afferent fibers in the spinal cord (Zinck et al., 2007; Zhang et al., 2008), (3) increased expression of TRPV1 in bladder afferent neurons from L6-S1 DRG (Yoshizawa et al., 2014), and (4) increased excitability of bladder afferent neurons, which are capsaicin-sensitive, from L6-S1 DRG as revealed by patch clamp recordings (Yoshimura and de Groat, 1997; Takahashi et al., 2013). However, SCI-induced morphological and molecular alterations in bladder afferent neurons have yet to be well characterized in mice, although the mouse model has now become highly

utilized due to its affinity to gene modification. In addition, bladder afferent fibers are carried through hypogastric and pelvic nerves which originate from neurons in rostral lumbar (L1-L2) DRG and lumbosacral (L6-S1), respectively (de Groat et al., 2015). Previous studies mainly focused on SCI-induced changes in the former afferent population using rats.

Therefore, this study was performed using a mouse model of SCI (Kadekawa et al., 2016) to examine the morphological and molecular changes across various populations of hypogastric and pelvic bladder afferent neurons following SCI. For this purpose, we utilized a novel tracing and labeling approach utilizing replication-deficient herpes simplex virus (HSV) vectors, which encoded the mCherry reporter gene product driven by a non-specific promoter (cytomegalovirus [CMV]) and three different neuronal cell type-specific promoters, including CGRP, neurofilament-200 (NF200) and TRPV1, which are able to express the mCherry protein in non-selective CGRP-expressing C-fiber, NF200-expressing A-fiber, and TRPV1-expressing bladder afferent neurons, respectively. Using laser capture microdissection (LCM) and real-time PCR methods, we also investigated any changes in molecular expression in dye-labeled bladder afferent neurons in mice with SCI (Takahashi et al., 2013; Yoshizawa et al., 2015).

MATERIALS AND METHODS

Vectors

The replication-defective mCherry reporter expression vectors were designed with complete deletion of essential immediate early genes ICP4 and ICP27 (Miyagawa et al., 2015) from the KOS-37 BAC vector genome (Gierasch et al., 2006). The internal repeat region was also deleted resulting in the vector genome being stabilized by losing the ability to isomerize with the deletion of over 25 Kb of total sequence (Miyagawa et al., 2015). For ease of insertion of a wide variety of promoter-reporter gene constructs into the vector genome, a Gateway-Destination cassette was inserted into the remaining latency associated transcript (LAT) locus, replacing the LAT promoter elements (Fig. 1A) between the CTCF sequences (Amelio et al., 2006; Bloom et al., 2010). To generate targeted expression vectors, mCherry was cloned into a modified pENTR1A plasmid, containing attL sites for recombination with the attR-containing vector backbone (Fig. 1B) using the Gateway LR Clonase II enzyme mix (Life Technologies, Carlsbad, CA). pENTR1A was modified to delete the CCDB gene and insert the highly-stable α Globin 3'UTR and SV40 polyadenylation signal. A *PmeI* linker was inserted into the *DraI* site, upstream of the mCherry insertion site, so that this 8-bp restriction site would remain unique after the insertion of the mCherry ORF. The mCherry ORF was amplified by PCR using a forward primer incorporating a Kozak consensus translation initiation sequence and then inserted into the unique *HincII* site of this vector downstream from the unique *PmeI* site. Each of the above transcriptional/translational elements was confirmed by sequencing. Finally, the promoters were inserted into the unique *PmeI* site as blunt-end digested fragments from other plasmids of known sequence.

The 4 different (CMV, CGRP, TRPV1, NF200) promoter-mCherry reporter gene expression vectors were engineered by Gateway recombineering of the pENTR1A plasmids (Fig. 1B) into the LAT locus (Wolfe et al., 2010) with the 932 bp CGRP and 970 bp NF200 promoter sequences (SwitchGear Genomics, Carlsbad CA), the 553 bp CMV (Thomsen et al., 1984;

Stinski and Roehr, 1985), and 1284 bp TRPV1 (Xue et al., 2007; personal communication with Mark Schumacher, UCSF) promoter sequences. The identity of each promoter-mCherry reporter pENTR plasmid was verified by sequence analysis. Isolates of each of the 4 promoter-reporter vectors that were purified by 3 rounds of limiting analysis (Goins et al., 2002) were subjected to FIGE analysis (Wolfe et al., 2010) and sequencing of the inserted cassette into the LAT region to confirm the structure of the 4 vectors (Fig. 1B). The 11 Kb BAC sequences were subsequently excised from all the isolates since the BAC sequences contribute to reduced vector growth (Miyagawa et al., 2015), on U2Os-4/27/Cre cells (Miyagawa et al., 2015) via Cre expression. Analysis of the BAC sequence removal was provided using X-gal staining (Goins et al., 2002) for detection of *lacZ* expression that is lost upon BAC excision.

All vector stocks were propagated on a human U2OS-ICP4/27 complementing cell line (Miyagawa et al., 2015). Twenty-four hours before infection, 1×10^8 U2OS-ICP4/27 cells were seeded as a 50% confluent monolayer to obtain a confluence of about 90–100% the day after in 10-layer cell factories (Nunc/Thermo-Fisher, Pittsburgh, PA). The cell monolayers were infected at a multiplicity of infection (MOI) of 0.001 PFU/cell for 1 hour at 37°C in serum-free DMEM (Gibco-Invitrogen). After the 1-hour infection period, DMEM containing 5% FBS (SIGMA, St. Louis, MO) was added and the cells re-incubated at 37°C. The next day, infected cells were then shifted to 33°C in 5% CO₂ until harvest. After 5–7 days post infection, once the infection reaches about 100% cytopathic effect (CPE), 5M NaCl (SIGMA) is added to the cell factories to a final concentration of 0.45M and placed onto a rocker platform for 2 hours. The supernatant was collected, cells and cell debris pelleted by low-speed centrifugation at $2,060 \times g$ at 4°C for 10 min and the supernatant the low-speed spin was filtered through a 0.8- μ m CN vacuum filter (Fisher), and the virus concentrated by high-speed centrifugation. Finally, the viral pellet is re-suspended in phosphate-buffered saline (dPBS), glycerol (SIGMA) is added to 5% and aliquots stored at –80°C (Goins et al., 2014).

DNA sequence of the CGRP, CMV, NF200 and TRPV1 promoters

The CGRP, CMV, NF200 and TRPV1 promoters were sequenced prior to cloning into the pENTR1A plasmids, after cloning into the pENTR1A plasmids and after insertion into the replication-defective HSV BAC genome (Appendices).

Animal preparation for HSV tracing study

All experiments were conducted in accordance with institutional guidelines and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). A total of 24 8–9 week-old female C57BL/6N mice (Envigo, Frederick, MD) weighing 18–20 g were used, and SCI mice underwent complete transection of Th8/9 spinal cord after induction of anesthesia by intraperitoneal (i.p.) injection of pentobarbital (50 mg/kg), according to the methods described previously (McCarthy et al., 2009; Ikeda et al., 2012; Kadekawa et al., 2016). SCI animals were treated with ampicillin (100 mg/kg, subcutaneously) for 5 days post-SCI and this treatment was continued twice a week afterwards. The bladder of SCI animals was emptied by manual bladder compression and perineal stimulation daily out to 4 weeks post-SCI. Spinal intact (SI) mice underwent sham

operation without spinal cord transection. SI and SCI mice were then divided into 8 groups; (1) SI mice with CMV promoter-mCherry HSV vector ($n = 3$), (2) SI mice with CGRP promoter-mCherry HSV vector ($n = 3$), (3) SI mice with TRPV1 promoter-mCherry HSV vector ($n = 3$), (4) SI mice with NF200 promoter-mCherry HSV vector ($n = 3$), (5) SCI mice with CMV promoter-mCherry HSV vector ($n = 3$), (6) SCI mice with CGRP promoter-mCherry HSV vector ($n = 3$), (7) SCI mice with TRPV1 promoter-mCherry HSV vector ($n = 3$), and (8) SCI mice with NF200 promoter-mCherry HSV vector ($n = 3$).

Viral vector administration

At 2 weeks after SCI or sham operation, after a laparotomy under pentobarbital (50 mg/kg, i.p.) anesthesia, a total of 20- μ L HSV viral suspension containing 3×10^7 plaque-forming units [PFU] of CMV promoter-mCherry, CGRP promoter-mCherry, NF200 promoter-mCherry or TRPV1 promoter-mCherry vector was injected into the bladder wall at four sites (5- μ L per site) using a 31-gauge Hamilton syringe. After the vector inoculation, the abdominal wound was closed with absorbable sutures. We confirmed in preliminary experiments that 20- μ L saline solution with cresyl violet covered the bladder wall almost entirely after injection (data not shown).

Immunohistochemistry

At 2 weeks after vector inoculation, SI and SCI mice were anesthetized with pentobarbital (80 mg/kg, i.p.) and perfused through the left ventricle with 100 mL cold oxygenated phosphate-buffered saline (PBS). L1 and L6 DRG were then removed and post-fixed overnight in the same fixative solution. The tissues were placed in PBS containing increasing concentrations of sucrose (10, 20, and 30%) at 4°C for cryoprotection, frozen in mounting medium, and sectioned at 10- μ m thickness. After mounting on slides, the sections were washed three times with PBS and incubated with monoclonal antibodies for mCherry (#16D7, 1:500 dilution, Thermo Fisher, USA) for 48 hour at 4°C, followed by incubation with secondary antibodies conjugated to Alexa Fluor 594 (1:1000 dilution, Thermo Fisher) for 2 hours at room temperature. The sections were then washed three times with PBS, and cover-slipped. We confirmed that there was no positive staining above background when the primary antibody was omitted (data not shown).

Histological analysis

Images were taken with a fluorescence microscope (BX51, Olympus America Inc, Center Valley, PA) and digitized by a Magnafire camera (Olympus). DRG sections (13–17 sections per DRG, $n = 3$ mice) were randomly selected, and histological analyses of mCherry-positive bladder afferent neurons were performed on every third section to avoid duplicate analysis of cells. We counted as a positive cell when more than 80% of the cell cytoplasm was positively stained above the background intensity with its nucleus being clearly seen (Fig. 2). The number of positive cells was counted on each section and averaged in one DRG. Then, the mean cell number per section in DRGs of either SI or SCI mice was used for the statistical comparison between SI and SCI groups. For the neuronal cell size analysis, cell size area (μm^2) of each mCherry-positive neuron was also measured using *Image J software* (<http://imagej.nih.gov/ij/>). Thereafter, the median cell size area of positive neurons was calculated in each of SI and SCI groups and compared between two groups.

Animal preparation for laser capture microdissection (LCM)

A total 28 female C57BL/6N mice were used. At 4 weeks after SCI or sham operation, L6 DRGs were removed bilaterally in a separate group of SI ($n = 8$) and SCI ($n = 5$). To label bladder afferent neurons, under isoflurane anesthesia, animals received an injection of a total of 20- μ L Fast Blue (FB) (1.5% w/v in water, Polysciences Inc., Warrington, PA, USA) at four sites in the bladder wall (5- μ L per site) using a 31-gauge Hamilton syringe into the bladder wall, 1 week prior to tissue removal.

LCM and Real-Time PCR

L6 DRG were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Torrance, CA) and stored at -80°C until use. Samples were sectioned at 10- μ m thickness, and sections were mounted on PEN membrane slides (Leica Microsystems, Wetzlar, Germany). Tissue sections were dipped sequentially in 75%, 95% and 100% ethanol each for 30 seconds, followed by xylene for 2 minutes. The sections were then air-dried. LCM was performed using an LMD6000 (Leica Microsystems) to separately dissect FB-labeled bladder afferent neurons (Fig. 6A–D). Excised cells were individually captured in the caps of 0.5 mL Eppendorf tubes and lysed. RNA isolation was performed using RNeasy® Plus Micro Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Real-time PCR was performed using the MX3000P (Stratagene, La Jolla, CA, USA). Primers sequences used were as follow: Actb (NM_007393.5 forward primer GCCCTGAGGCTCTTTTCCAG, reverse primer TGCCACAGGATTCCATACCC), CGRP (NM_001305616.1 forward primer CCACTGGGTGAGGAGAAAGTC, reverse primer AAGCAAGACTAGAAGCTCTACTAGG) and TRPV1 (NM_001001445.2 forward primer TACTTTTCTTTGTACAGTCACT, reverse primer TCAATCATGACAGCATAGAT). The cycle conditions comprised 10 minutes polymerase activation and 45 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds followed by dissociation from 55 to 95°C . The reactions were analyzed in triplicate and, relative quantities of CGRP and TRPV1 mRNA were normalized to the neuronal cell control gene β -actin (Actb) mRNA. 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 Actb. We also determined specificity of the cDNA using real-time PCR to verify that our primer/probe sets did not amplify genomic DNA.

Statistical analysis

The number of labeled neurons per DRG section was expressed as mean $\pm$ standard error (SEM) whereas median $\pm$ interquartile range (IQR) was used for the cell size area due to its skewed data distribution over the different cell sizes. Mann–Whitney U-test was used to calculate the statistical significance between groups because the equal variances of the data were not found. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

The number of bladder afferent neurons labeled by HSV vectors with neuronal subtype-specific promoters driving mCherry reporter

The specificities of the various neuronal subtype specific promoters (CGRP, TRPV1, NF200) were compared to the non-specific CMV promoter at 2 weeks post-injection of the CMV promoter-mCherry (Fig. 2A), CGRP promoter-mCherry (Fig. 2B), TRPV1 promoter-mCherry (Fig. 2C) and NF200 promoter-mCherry (Fig. 2D) vectors in normal SI mice prior to a comparison in spinal cord injured (SCI) mice to provide background promoter-reporter activity levels. At 2 weeks after HSV vector inoculation (i.e., 4 weeks after SCI or sham operation), the number of bladder afferent neurons labeled by HSV vectors with non-specific CMV promoter driving mCherry (Fig. 3A) expression per L1 DRG section was 6.6 ± 0.4 ($n = 83$ sections from 5 DRG, $n = 3$ SI mice) and 5.7 ± 0.4 cells ($n = 107$ from 6 DRG, $n = 3$ SCI mice), and the number per L6 DRG section was 16.4 ± 1.0 ($n = 79$ sections from 6 DRG, $n = 3$ SI mice) and 14.0 ± 0.8 cells ($n = 84$ sections from 6 DRG, $n = 3$ SCI mice). There were no statistically significant differences in these numbers of non-specifically labeled neurons in L1 or L6 DRG sections between SI and SCI mice (Fig. 3A).

The numbers of bladder afferent neurons labeled by HSV vectors with CGRP promoter driving mCherry (Fig. 3B) per L1 DRG section were 5.0 ± 0.3 ($n = 101$ sections from 6 DRG, $n = 3$ SI mice) and 8.8 ± 0.6 cells ($n = 94$ sections from 6 DRG, $n = 3$ SCI mice), and the numbers per L6 DRG section were 6.8 ± 0.6 ($n = 83$ sections from 6 DRG, $n = 3$ SI mice) and 9.5 ± 0.5 cells ($n = 94$ sections from 6 DRG, $n = 3$ SCI mice). The number of CGRP promoter vector-labeled cells in both L1 and L6 DRG sections from SCI mice was significantly greater ($p < 0.001$) than those from SI mice (Fig. 3B).

The numbers of bladder afferent neurons labeled by HSV vectors with TRPV1 promoter driving mCherry (Fig. 3C) per L1 DRG section were 4.7 ± 0.4 ($n = 58$ sections from 4 DRG, $n = 3$ SI mice) and 10.6 ± 0.6 cells ($n = 110$ sections from 6 DRG, $n = 3$ SCI mice), and the numbers per L6 DRG section were 5.7 ± 0.3 ($n = 83$ sections from 6 DRG, $n = 3$ SI mice) and 10.3 ± 0.6 cells ($n = 70$ sections from 6 DRG, $n = 3$ SCI mice). The number of TRPV1 promoter vector-labeled cells in both L1 and L6 DRG sections from SCI mice was significantly greater ($p < 0.001$) than those from the SI mice (Fig. 3C).

The numbers of bladder afferent neurons labeled by HSV vectors with NF200 promoter driving mCherry (Fig. 3D) per L1 DRG section were 5.1 ± 0.4 ($n = 82$ sections from 6 DRG, $n = 3$ SI mice) and 4.5 ± 0.3 cells ($n = 75$ sections from 6 DRG, $n = 3$ SCI mice), and the numbers per L6 DRG section were 6.3 ± 0.3 ($n = 89$ sections from 6 DRG, $n = 3$ SI mice) and 4.6 ± 0.3 cells ($n = 94$ sections from 6 DRG, $n = 3$ SCI mice). The number of NF200 promoter vector-labeled cells in L6 DRG sections from SCI mice was significantly smaller than those from SI mice ($p < 0.001$) whereas the number of labeled cells in L1 DRG section were not statistically different between SI and SCI groups (Fig. 3D).

In addition, we confirmed that there were very few HSV vector-labeled afferent neurons (0–2 cells/section) in L3–L5 DRG (data not shown), as reported in previous studies, which used

other retrograde axonal tracing techniques (Yoshikawa et al., 2015). This indicates that systemic spread of HSV vectors after bladder wall injection was negligible.

Cell size distribution of bladder afferent neurons labeled by HSV vectors with CMV or neuronal subtype-specific promoters

The histogram of cell size distribution of the CGRP promoter vector-labeled C-fiber and NF200 promoter vector-labeled A-fiber bladder afferent neurons shows an increase in the number of C-fiber bladder afferent neurons labeled by the CGRP promoter-mCherry vector in both L1 (Fig. 4B) and L6 DRG after SCI (Fig. 4A). The median cell size of bladder afferent neurons labeled by HSV vectors with CGRP, TRPV1 or NF200 promoter driven mCherry vectors is shown in Fig. 5. The median cell size of CGRP promoter vector-labeled bladder afferent neurons shifted significantly to a larger size from $247.0 \mu\text{m}^2$ in the SI group to $271.3 \mu\text{m}^2$ in the SCI group within the L6 DRG, and from $244.2 \mu\text{m}^2$ in the SI group to $252.4 \mu\text{m}^2$ in the SCI group within the L1 DRG (Fig. 5B). In contrast, the median cell size of the TRPV1 promoter vector-labeled bladder afferent neurons shifted significantly to a smaller size from $245.2 \mu\text{m}^2$ in the SI group to $216.5 \mu\text{m}^2$ in the SCI group within the L6 DRG, and from $258.8 \mu\text{m}^2$ in the SI group to $233.4 \mu\text{m}^2$ in the SCI group within the L1 DRG (Fig. 5C). The median cell size of the NF200 promoter vector-labeled bladder afferent neurons shifted to a larger size from $281.2 \mu\text{m}^2$ in the SI group to $326.6 \mu\text{m}^2$ in the SCI group within the L1 DRG without significant changes in cell size within the L6 DRG after SCI (Fig. 5D). In addition, the cell size of the total population of bladder afferent neurons labeled by the CMV promoter-mCherry vector was also significantly increased from $295.12 \mu\text{m}^2$ in the SI group to $314.1 \mu\text{m}^2$ in the SCI group within the L1 DRG, and $289.88 \mu\text{m}^2$ in the SI group to $308.29 \mu\text{m}^2$ in the SCI group within the L6 DRG (Fig. 5A).

Changes in CGRP and TRPV1 mRNA levels in FB-labeled bladder afferent neurons

Because the HSV vector-mediated tracing studies revealed increases in the numbers of bladder afferent neurons labeled by the CGRP or TRPV1 promoter-mCherry reporter vectors after SCI, we determined if CGRP and TRPV1 expression was also increased using RT-PCR analysis on laser-captured bladder afferent neurons labeled by FB injected into the bladder wall (Fig. 6A–D). FB-labeled bladder afferent neurons were randomly selected from L6 DRG sections, and 90–100 bladder afferent neurons per sample were laser-captured for the measurement of TRPV1 mRNA levels. For the CGRP mRNA measurement, 400–450 bladder afferent neurons per sample were laser-captured because of low mRNA levels of CGRP in DRG neurons. One or two samples were obtained from L6 DRG sections of each SI or SCI mouse, and the relative expression to the housekeeping Actb gene was calculated in each sample and averaged in 8 SI mice and 7 SCI mice for CGRP expression and in separate groups of 8 SI mice and 5 SCI mice for TRPV1 expression. The mRNA expression of both CGRP and TRPV1 in FB-labeled bladder afferent neurons from SCI rats was significantly higher compared to the levels in DRG from SI mice (Fig. 6 E–F).

DISCUSSION

The present study demonstrated that: (1) mCherry reporter gene expression driven by neuronal subtype-specific promoters in the context of replication-defective HSV vectors can

be used to detect CGRP-expressing C-fiber and NF200-expressing A-fiber populations of bladder afferent neurons, (2) SCI increases the number of CGRP and TRPV1-expressing C-fiber bladder afferent neuron populations in L1 and L6 DRG, (3) SCI respectively increases and decreases the median cell size of CGRP and TRPV1-expressing C-fiber bladder afferent neuron populations in L1 and L6 DRG, (4) the number of NF200-expressing A-fiber bladder afferent neurons decreased in L6 DRG, and the cell size increased in L1 DRG and (5) mRNA levels of CGRP and TRPV1 increased in bladder afferent neurons from L6 DRG of SCI mice. Because the CMV promoter vector infects DRG neurons in a non-specific manner, the number of CMV promoter-mCherry positive DRG neurons corresponds roughly to the total population of bladder afferent neurons whereas CGRP promoter TRPV1 promoter vectors can label subpopulations of bladder afferent neurons that express CGRP or TRPV1, respectively. Thus, our data indicate that the total number of bladder afferent neurons identified by CMV promoter vectors was not altered after SCI; however, CGRP or TRPV1 expressing bladder afferent neurons change their expression profiles after SCI. In addition, one should note that the data regarding cell number changes in DRG sections may only apply to our study and not to the general population of mice because we used a fixed-effect analysis with multiple data points from each DRG instead of using the animal as the random variable.

SCI rostral to the lumbosacral level induces NDO following an initial period of bladder areflexia, as well as uncoordinated contractions of EUS during voiding (i.e., DSD), resulting in high intravesical pressure (IVP), inefficient voiding and bladder wall tissue remodeling such as hypertrophy and fibrosis (de Groat and Yoshimura, 2012). One of the major mechanisms inducing NDO after SCI is the morphological and functional plasticity of C-fiber bladder afferent pathways, which is evident as; (1) cell hypertrophy of lumbosacral bladder afferent neurons (Kruse et al., 1995; Yoshimura et al., 1998), (2) sprouting of CGRP- and substance P-positive C-fiber afferent central terminals in the spinal cord (Zinck et al., 2007; Zhang et al., 2008) and (3) increased expression of TRP channels such as TRPV1 and TRPA1 in lumbosacral bladder afferent neurons (Yoshizawa et al., 2014), as shown in previous studies using rats. Increased TRPV1 immunoreactivity in bladder suburothelial nerve fibers has also been detected in SCI patients with NDO (Brady et al., 2004; Apostolidis et al., 2005). These morphological and histological changes in bladder afferent pathways are also accompanied by functional plasticity, which is evident as increased excitability of capsaicin-sensitive bladder afferent neurons from L6-S1 DRG in rats (Yoshimura and de Groat, 1997; Takahashi et al., 2013). However, changes in the characteristics of bladder afferent neurons in the mouse SCI model have not previously been well clarified. Because this study showed an expansion of CGRP and TRPV1-expressing bladder afferent neurons at both rostral lumbar (L1) and lumbosacral (L6) levels, along with upregulation of CGRP and TRPV1 gene transcripts in bladder afferent neurons from SCI mice, C-fiber bladder afferents are likely to contribute to the emergence of the spinal micturition reflex pathways and NDO in mice as they do in humans, cats and rats after SCI (de Groat and Yoshimura, 2012).

Furthermore, the current study revealed that CGRP-expressing bladder afferent neurons increased in size whereas TRPV1-expressing neuron size decreased after SCI although the number of these two neuronal populations increased after SCI compared to SI control mice.

Because the number of NF200-expressing, large-sized A-fiber bladder afferent decreased, especially in L6 DRG, without changing the number of overall neurons labeled by the non-specific CMV promoter-mCherry vector, it is possible that some A-fiber neurons that express neurofilament under normal conditions switch their phenotype to express CGRP after SCI whereas the expansion of TRPV1-expressing neurons after SCI might occur within small-sized C-fiber bladder afferent neurons, which do not express TRPV1 under normal conditions, but change after SCI.

In our previous study using SCI rats, we discovered that the number of neurofilament-positive bladder afferent neurons from L6-S1 DRG increased after SCI (Yoshimura et al., 1998) in contrast to the data in this mouse study showing a decrease in NF200-expressing bladder afferent neurons in L6 DRG. Thus, it is possible that the contribution of A-fiber bladder afferents to SCI-induced lower urinary tract (LUT) dysfunction might be different between mice and rats. In this regard, we recently reported that the behavior of EUS during the voiding reflex, which is still dependent on capsaicin-insensitive A-fiber afferents after SCI (Cheng and de Groat, 2004), is quite different in rats and mice (Kadekawa et al., 2016) although C-fiber dependent NDO during urine storage is similarly observed between these two species after SCI (McCarthy et al., 2009; Wada et al., 2017). In SCI rats, EUS bursting occurs during voiding bladder contractions, which coincides with small-amplitude IVP oscillations during cystometry. Furthermore, α -bungarotoxin, a neuromuscular blocking agent, reduces voiding by suppressing EUS bursting activity, which is also necessary for efficient urine elimination in normal rats (Yoshiyama et al., 2000), indicating that EUS bursting activity recovers after SCI in rats and is able to induce relatively efficient voiding (Kadekawa et al., 2016). However, SCI mice do not exhibit clear EUS bursting or IVP oscillations during voiding, but rather exhibit periods of reduced EUS activity during voiding accompanied by a slowly developing large-amplitude reduction in IVP (Kadekawa et al., 2016). These differences in voiding between SCI mice and rats might be related to a difference in the post-SCI changes of neurofilament-expressing bladder afferent neurons in the L6 DRG between the two species and in turn a difference in A-fiber-dependent reflex activity during voiding although further studies are needed to clarify this point

This study also showed that there are some differences in SCI-induced changes in NF200-expressing bladder afferent neurons in L1 and L6 DRG, including: (1) an increase in cell size of these neurons in L1 DRG, but not in L6 DRG and (2) a decreased number of these neurons in L6 DRG, but not in L1 DRG. Previous studies in rats demonstrated that rostral lumbar bladder afferents which pass through the hypogastric nerve have a larger population of unmyelinated axons compared to those arising in L6-S1 DRG and passing through the pelvic nerve. (Nadelhaft and Vera, 1991) and a higher percentage of cells with CGRP immunoreactivity (70–90%) compared to those in L6-S1 DRG (50–60%) (Su et al., 1986; Bennett et al., 2003). However, in this study, neither SI or SCI mice showed a significant difference between L1 and L6 DRG in the number of bladder afferent neurons labeled by CGRP or NF200 promoter vectors (Fig. 3), indicating that the two bladder afferent pathways have a similar composition of C-fibers and A-fibers in mice. In addition, it has been previously shown that mechanosensitive properties of single bladder afferent fibers are different in the hypogastric and pelvic nerve pathways in SI mice (Xu and Gebhart, 2008). Thus, it is possible that bladder afferents at two different spinal cord levels might respond

differently to neurological disease such as SCI; therefore, future studies using HSV vectors employing neuronal subtype-specific promoters to drive expression of the mCherry reporter gene could further compare the contribution of phenotype-specific afferent populations to lower urinary tract dysfunction between these two afferent pathways.

Replication-defective recombinant HSV vectors, which lack multiple essential gene functions and are non-toxic in vivo (Samaniego et al., 1995; Marconi et al., 1996; Krisky et al., 1997; Samaniego et al., 1997; Miyagawa et al., 2015), have been generated to increase the overall safety and efficacy for clinical applications. These vectors can be prepared to high titer and purity without contamination by the wild-type virus (Jiang et al., 2004; Ozuer et al., 2002a; Ozuer et al., 2002b). Furthermore, because of the natural ability of the HSV vectors to efficiently transduce primary afferent neurons and rapidly establish a “latent-like” state in the infected cells without altering the normal cell properties, they are suitable for gene delivery to the target organ-specific afferent pathways after local inoculation. We previously shown that bladder wall inoculation of HSV vectors encoding glutamic acid decarboxylase (GAD), the GABA synthesizing enzyme, driven from the ubiquitous CMV promoter effectively increased the GABA level in bladder afferent neurons and reduced NDO and DSD in SCI rats (Miyazato et al., 2009; 2010). The current study further demonstrated that gene delivery to phenotype-specific subpopulations of bladder afferent pathways can be achieved using newly engineered replication-defective HSV vectors with neuronal subtype-specific promoters such as CGRP, TRPV1 or NF200. These novel vectors and this new technique will allow us to perform future studies using cell type-specific promoter HSV vectors encoding therapeutic genes such as GAD in order to develop new modalities for targeting phenotype-specific afferent subpopulations for the treatment of neurogenic lower urinary tract dysfunction.

CONCLUSION

To our knowledge, this is the first study investigating morphological changes in different populations of bladder afferent neurons in mice using mCherry-encoding replication-defective HSV vectors with neuronal cell subtype-specific promoters. We found that SCI can induce morphological changes in bladder afferent pathways, especially in the C-fiber cell population, along with upregulation of CGRP and TRPV1 expression in bladder afferent neurons after SCI in mice. These findings provide further insights into the mechanism of neurogenic lower urinary tract dysfunction associated with SCI.

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APPENDICES

DNA sequences of the CGRP, CMV, NF200 and TRPV1 promoters

A. CGRP Promoter Sequence (932 bp)

5'TAATGGATCTGAAGAGTACCCCGGGACAGTCCGGGGAGATGGAGATTCCGAAA
 GTATCCATGGAGATCTTACAGAATCCCCTGTGCGGACCAGGAACTCTTGTAATC
 CCTGCCTATCTGAGGCCAGGCGCTGGGCTGTTTCTCACAATATTCCTTCAAGATG
 AGATTGTGGTCCCCATTTCAAAGATGAGTACACTGAGCCTCTGTGAAGTTACTTGC
 CCATGATCACACAACCAGGAATTGGGCCAACTGTAATTGAACTCCTGTCTAACAA
 A
 GTTCTTGCTCCCAGCTCCGTCTCTTGTTCCTCCACGAGCCCTGGCCCTCTGTGGGTA
 ATACCAGCTACTGGAGTCAGATTTCTTGGGCCAGAACCCACCCTTAGGGGCATTA
 ACCTTTAAATCTCACTTGGGCAGGGGTCTGGGATCAGAGTTGGAAGAGTCCCTA
 CAATCCTGGACCCTTTCCGCCAAATCGTGAAACCAGGGGTGGAGTGGGGCGAGG
 GTTCAAACCAGGCCGACTGAGAGGTGAAATTCACCATGACGTCAAACCTGCCC
 T
 CAAATCCCGCTCACTTTAAGGGCGTTACTTGTGGTGCCCCCACCATCCCCCACC
 ATTTCCATCAATGACCTCAATGCAAATACAAGTGGGACGGTCTGCTGGATCCTCC
 AGGTTCTGGAAGCATGAGGGTGACGCAACCCAGGGGCAAAGGACCCCTCCGCCC
 ATTGGTTGCTGTGCACTGGCGGAACCTTTCCCGACCCACAGCGGCGGGAATAAGAG
 CAGTCGCTGGCGCTGGGAGGCATCAGAGACACTGCCCAGCCCAAGTGTGCGCCG
 CGCTTCCACAGGGCTCTGGCTGGACGCCGCCGCCGCTGCCACCGCCTCTGA
 TCCAAGCCACCTCCCGCCAGGTGAGCCCCGAGATCCTGGCTCAGGTATAT3'

B. CMV Promoter Sequence (553 bp)

5' ATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTT
 ACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCGCCCAT
 TGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGA
 CGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTA
 TCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGG
 CATTATGCCAGTACATGACCTTATGGGACTTTCTTACTTGGCAGTACATCTACGTA
 TTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGG
 ATAGCGGTTTGACTCACGGGGATTTCGAAGTCTCCACCCATTGACGTCAATGGG
 A
 GTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACCTCCGC
 C CCAATTGACGCAAATGGGCGGTAGGCGGTACGGTGGGAGGTCTATATAAGC3'

C. NF200 Promoter Sequence (970 bp)

5'GTAGGTTCTCTGCCCCTCAAACCTCAGCCCAGCTTTCTCCTGCCTGTTTACAGGGGA
CCTTCTGCCCCGTTTCGCTGAGGGTCCGTCCCCTTTACTGGGGCTGGCAGCAGGGT
CTCCCATCTCCTCTCTCGGGGGCCACTGCAGACTTTTTAGAGAACGCCTTGCCTCC
CCCCAACCCACCCATCCGGGGTTCCTCTCTCCATCCTCTGCAGTGTCTCCATA
CCCCATTACAGGGTAGCCTTGCTATTCTCCCCAACTCCAGGTCCCCCTTCATCTAT
TCCGGGGCTGGCCGCGGAGTTTCTGAGCGCTCTCCAAGTGGGTCTCTAGATGT
TAGGAGAACACTGTACCTCCCCCGTTCAGGGGTCTCCTGTCTCCGTTCTATGGAG
CGTCCATGCTCCCATTCAGGACTGCCTTGCTCCCTCCTCTGTTCCGGGGCTGGCTG
CACAGTCTCTGCACCCCCTATCCTGAAAGCCTCTCTTAACTATTTGGAAAGCCTC
GTGTCTGTCTCATAAGGGATCCCCCTATCCTAATGACTGCAATCTTCCATTGCT
CCATCCCGAGGGCATCCTGCCCCATTCCCATCAGGTTTCTCCTTGCTCTCCCT
GTTTCAAGTCCCCTTTCTTATTCGAACACACTCGCAGGCTCTTCCGACGCGCACC
CGGGGGTCTCTACTGGCCCACTCCGGGAGTCTCTGCCCCGTTCCCCGACCTCG
AGGGTCTCCTCTGACGCAGCGTCGATTCCCCCTCCCTCCTCGGTCCCCTGCCCG
CCCCCTCTACTGCGGCGGAGCCGGTCGGCCGGGGGGCCGCAGGGGAGGAGGC
GGAGAGGGCGGGGCCCTCCTCCCCACCCTCTCACTGCCAAGGGGTGGACCCGG
CCGCGGCGGCTATAAAAGGGCCGGCGCCCTGGTGCTGCCGCAGTGCCTCCCGCC
CCGTCCCGGCTCGCGCACCTGCTCAGGCCATGAT3'

D. TRPV1 Promoter Sequence (1284 bp)

5'CAGCTTTTGTACTTCTCCAGGCGGAGCTGCCGTGGCTGCTCCACTGGAGCAGTG
TCTGAAAAAATAAAAAAGGAAAGAAAAGGACATGACTGTTTTTCGGTGCGGT
GG
AAGAGAAAGTTTATTGTAGATAAAGGGGGAGCATAGACAGAGGCAGACATGTCTG
GGAGAGCCAGAGTGGTTGTGACCCTGAGCCATATGGAGAGGTGGGGTGAGGGGT
GGCAGAGAGGGATCGAGAGAGGAGAGAGGGGAACCAGATGTAGCAGCCAGGAG
GCCAAAGGTACAAAAGGGGTGGGTAACCAAAATGTCTGGATTATATAAAAAAGAG
C
CAGAGGTCAGGCCCCACTTTGATATGTTAAATAGGCACCTCAGCCATTTATCCAGGT
TTGAAATGTAATATAATTTACATCCCCCTGGCTTCCTAGAGACCGTTGTTTAGACGG
ATGACCTCTGCAGAATGTTTGAGGGTGCAGTCTTGCATGTACTCCCTGGTGGGCTT
TCTTGGGCAGGATCTGGGCAGGAATGGGCTTGTTCAGTACCCACTGCGTATGA
TGGATGAACCCGCTTCCTAGTAGTTAGGATGGCACTGGGGGAGGCGAGAAATTAG
CACACGTAACGTTTCTTGTGTTCTATTGTTCACTAAGGGACCCCACTCAAGCAAG
ACTGGGCCTTGGAAGACCTAGAGACCACCAACCTAATCTCTACCCCGGTCTGA
GTACACAGGGACTCAGAGTCCCAAAGGGGGCAGGGCCTCCAGACAGGTGGCTCA
GAGGTCCCAGTCCTTTGGAAACATGGCATCTTCAGGACACTGGGCTTTGCATCTC
T
GGCTGTGACAGTCCTTTAAAGGGAGCTACTCCTCAGACATACAGGAGAGATGGTTT
GGAAAGTCCGAGATCCAAAGCCTGGTTCAGGCTGGACTGGGCTGCAGGCTGCTA
A
GTGCTCCTCTGCCCTGGCATGGCTGGGGGTGGGGCATTGGCTGTGGTTTCTGAAA
AAGGGCAAAAATGATGGGAAAAGCTTTGGGATCCTCTGGGAATCGGAGCCGTGG

T
AACAGCAGCTGCTGCCATTGCTGCAAATGTTTCCTTGAGTGCCAGAGTATGCCCCA
G AGCCCATCCCTGCCGTACGCCAGGGGAGGGGCGAGGACCCTCACAGAGGCAGG
GAGGCCGGCCACTCTTACCACACAGCAGCCTGGCTCTCCCACAAAGGACAGCTCC
AAGGCACTTGCTCATTGTTGGGGTGTAAGGGCGAATTCTGCAGATATCCATCAGCTG
GCGGCCGCTCGAGATC3'

Glossary

SCI	spinal cord injury
LUTD	lower urinary tract dysfunction

Highlights

- A new HSV vector method is useful to identify bladder afferent subpopulations
- CGRP-expressing bladder afferent neurons increase their size and number after SCI
- TRPV1-expressing neurons increase the number, but decrease the size after SCI
- A-fiber bladder neurons may switch their phenotype to express CGRP after SCI
- SCI-induced changes in A-fiber afferents may be different between mice and rats

Figure 1.

HSV expression vector schematic. **A:** The vector backbone vLG was generated from the HSV KOS strain in which BAC sequences were inserted into the TK locus (UL23). Deletions were introduced in the internal repeat region and the genes encoding immediate early proteins ICP4 and ICP27, rendering the vector replication-defective. The Gateway destination cassette was inserted into the remaining latency locus, replacing the latency promoter elements while maintaining a wild-type copy of ICP0 and the surrounding CTCF chromatin boundary elements. **B:** To generate PNS neuronal subtype targeted expression vectors, transgenes were recombined into the vector backbone via the Gateway cassette. Individual promoter sequences (1284 bp TRPV1 promoter [TRPV1p], 932 bp CGRP promoter [CGRPp], 553 bp CMV promoter [CMVp], and 970 bp NF200 promoter [NF200p]) incorporating a Kozak consensus translation initiation sequence were inserted into the pENTR1A transfer vector which contains *attL* sites for site-directed recombination

with the *attR*-containing vector backbone. These Gateway plasmids were then recombined into the vLG vector backbone to generate the experimental vectors. The structure of the recombinant vector genomes were verified by FIGE analysis and the promoters and mCherry reporter gene were all sequenced verify the authenticity of the reagents.

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Figure 2. mCherry immunostaining. Positively stained neurons, in an L6 DRG section are indicated by arrows, 2 weeks after bladder wall injection of HSV virus encoding mCherry from **A**: CMV promoter, **B**: CGRP promoter, **C**: TRPV1 promoter and **D**: NF200 promoter vectors in spinal intact (SI) mice. Scale bar = 50 μ m.

Figure 3.

The average number of mCherry-positive neurons per DRG section at 2 weeks after bladder wall injection of HSV virus encoding mCherry from different subtype promoters in SI and SCI mice. **A:** The number of CMV promoter (CMVp)-mCherry HSV vector-labeled bladder afferent neurons (L1: U=3714, SI=83, SCI=107, $p=0.053$, L6: U=2887, SI=79, SCI=84, $p=0.152$). **B:** The numbers of CGRP promoter (CGRPp)-mCherry HSV vector-labeled bladder afferent neurons (L1: U=3311.5, SI=101, SCI=94, $p<0.001$, L6: U=5222, SI=83, SCI=94, $p<0.001$). **C:** The number of TRPV1 promoter (TRPV1p)-mCherry HSV vector-labeled bladder afferent neurons (L1: U=3978, SI=58, SCI=110, $p=0.022$, L6: U=1255, SI=88, SCI=70, $p<0.001$). **D:** The number of NF200 promoter (NF200p)-mCherry HSV vector-labeled bladder afferent neurons (L1: U=3159.5, SI=83, SCI=94, $p=0.767$, L6: U=2819, SI=89, SCI=96, $p<0.001$). Significance of $p<0.001$ determined by Mann-Whitney test indicated by a double asterisk. Data shown represent mean $\pm$ SEM. The number of DRG sections is described below each bar of the graph.

Figure 4.

Cell size distribution of CGRP promoter (CGRPp) and NF200 promoter (NF200p) -mCherry HSV vector-labeled neurons. **A:** L6 DRG. **B:** L1 DRG. Filled and open columns represent cell-size distribution of CGRP promoter-mCherry and NF200 promoter-mCherry HSV vector-labeled bladder afferent neurons, respectively. Bin width = 50 μm^2

Figure 5.

Cell size distribution of neuronal subtype vector-labeled bladder afferent neurons from L6 and L1 DRG. **A:** CMV promoter (CMVp)-mCherry HSV vector-labeled neurons (L6: U=700768, SI=1286, SCI=1154, $p=0.018$, L1: U=244049, SI=549, SCI=824, $p=0.013$). **B:** CGRP promoter (CGRPp)-mCherry HSV vector-labeled neurons (L6: U=260904, SI=558, SCI=872, $p=0.021$, L1: U=223004, SI=506, SCI=824, $p=0.033$). **C:** TRPV1 promoter (TRPV1p)-mCherry HSV vector-labeled neurons (L6: U=145547, SI=478, SCI=718, $p<0.001$, L1: U=130034, SI=266, SCI=1104, $p=0.004$). **D:** NF200 promoter (NF200p)-mCherry HSV vector-labeled neurons (L6: U=117770, SI=556, SCI=441, $p=0.285$, L1:

U=57829, SI=404, SCI=345, $p<0.001$). Shaded and filled columns represent cell-size distribution of bladder afferent neurons from SI and SCI mice, respectively. Bin width = 50 μm^2 . Insets in A–D show the comparison of median cell size of bladder afferent neurons labeled by different promoter-mCherry HSV vectors. Data shown represent median $\pm$ IQR. Significance of $p<0.05$ indicated by a single asterisk, and significance of $p<0.01$ indicated by a double asterisk determined using the Mann-Whitney test.

Figure 6.

Laser capture microdissection (LCM) and real time RT-PCR of Fast Blue-labeled bladder afferent neurons. **A-D:** Photomicrographs show the same L6 DRG section during LCM of Fast Blue-labeled bladder afferent neurons. **A:** Neurons before microdissection. **B:** Bladder afferent neurons outlined for LCM. **C:** Neurons after microdissection. **D:** Neurons after microdissection under a bright field. **E:** CGRP mRNA expression of captured bladder neurons from SI and SCI mice. **F:** TRPV1 mRNA expression of captured bladder neurons from SI and SCI mice. The number of mice is indicated under each bar of the graph in E and F. Data are shown as relative values of mRNA expression in SCI mice vs. SI mice whereas

the statistical analysis was performed using the CGRP or TRPV1 mRNA expression ratio against the house keeping gene (β -actin) calculated in each mouse. Significance of $p < 0.05$ indicated by a single asterisk using the Mann-Whitney test.

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