Available online at www.sciencedirect.com
ScienceDirect
Experimental Neurology xx (2007) xxx–xxx
Experimental Neurology
www.elsevier.com/locate/yexnr

Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury

N.D.T. Zinck [a,*], V.F. Rafuse [b], J.W. Downie [a,c]

[a] Department of Pharmacology, Dalhousie University, Sir Charles Tupper Medical Building, 5850 College Street, Halifax, Nova Scotia, Canada B3H 1X5
[b] Department of Anatomy and Neurobiology, Dalhousie University, Sir Charles Tupper Medical Building, 5850 College Street, Halifax, Nova Scotia, Canada B3H 1X5
[c] Department of Urology, Dalhousie University, Sir Charles Tupper Medical Building, 5850 College Street, Halifax, Nova Scotia, Canada B3H 1X5

Received 2 August 2006; revised 13 November 2006; accepted 5 January 2007

Abstract

Spinal cord injury (SCI) severely disrupts bladder function. What mediates bladder dysfunction after SCI is currently unknown. We investigated the role that primary afferent sprouting in lumbosacral cord may play in emergence of bladder activity after complete spinal cord transection. Rats had a bladder cannula chronically implanted. They were then subjected to complete surgical spinal cord transection at T9/T10. Cystometrographic analysis (0.1 ml/min) after injury revealed that bladder activity emerged in the form of nonvoiding contractions in all rats at approximately 5 days post transection. At 10–14 days after transection nonvoiding contractions remained and voiding contractions emerged that had increased maximal pressures (12–41 vs. 24–57 cmH_2O) but were less efficient (6–15% vs. 79–100%) when compared to control implanted rats. We looked for sprouting 3 days and 8 days post transection, timepoints preceding the emergence of nonvoiding and voiding contractions respectively. Increases in CGRP density and distribution were seen in L6 and S1 spinal cord within lamina groupings of II–IV, V and VI, as well as lamina X at 8 days post transection. This increase remained in most lamina at 21 days post transection. Colocalization with the growth cone marker Gap-43 3 days and 5 days post transection at the level of the lumbosacral preganglionic nucleus verified that CGRP positive afferents were sprouting in L6/S1 spinal cord prior to emergence in bladder activity. These data provide support for the hypothesis that primary afferent sprouting contributes to emergence of bladder activity after spinal cord transection.

Keywords: Micturition; Sprouting; Calcitonin gene-related peptide; Spinal cord injury; Growth associated protein-43; Primary afferents

Introduction

Efficient bladder emptying (micturition) is controlled by complex neuronal circuitry at the level of the brain stem and lumbosacral spinal cord (Kruse et al., 1990; Shefchyk, 2002). Complete suprasacral spinal cord injury initially results in bladder areflexia (Yoshiyama et al., 1999) but reflex bladder activity emerges after some time (de Groat et al., 1990; Kruse et al., 1993; Mallory et al., 1989). This emergent bladder activity is characteristically overactive due to the appearance of nonvoiding contractions. Spinal cord injury also causes inefficient bladder emptying due to dyssynergia between the external urethral sphincter (EUS) and the bladder body (Blaivas et al., 1981). The mechanism mediating the emergence of dysfunctional bladder activity post spinal cord injury has not been established, although it has been suggested that injury-induced neuronal plasticity plays a role (de Groat et al., 1998; Vizzard, 1999).

In rats, the afferent limb of the bladder reflex is initiated by activation of primary afferent neurons located in L6/S1 spinal cord (Shea et al., 2000). CGRP is a neuropeptide found within C and A-delta fibers that terminate in the dorsal horn (Chung et al., 1988). The majority of bladder primary afferents are small diameter unmyelinated C-fibers and contain the neuropeptide calcitonin gene-related peptide (CGRP) (Yoshimura et al., 2003). An increase in density and distribution of CGRP afferent terminals in the spinal cord has been used as a marker for

* Corresponding author. Fax: +1 902 494 1388.
E-mail address: nzinck@dal.ca (N.D.T. Zinck).

0014-4886/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2007.01.011

sprouting after deafferentation (McNeill et al., 1991) or spinal cord injury (Krenz et al., 1999; Mitsui et al., 2005). In addition, this sprouting has been linked to autonomic dysfunctions seen after spinal cord injury. Because most bladder primary afferents contain CGRP any changes in its distribution in lumbosacral cord could have a major impact on bladder function seen after spinal cord injury. It is not known whether lumbosacral afferents sprout after spinal cord transection or if they sprout before the emergence of bladder activity.

Properly guided axonal sprouting is required early in development. Various growth-associated molecules have been implicated in this process (Bruses et al., 2002; de Castro, 2003; McIlvain et al., 2003) and mediate their actions through cell surface interactions with the growth cone, the chemosensitive leading edge of sprouting axons (Gallo and Letourneau, 2004). Growth associated protein (Gap-43) is abundant within growth cone membranes making Gap-43 a good marker for neuronal spouting (Skene et al., 1986). High thoracic spinal cord injury induces increased levels of Gap-43 caudal to the injury site at both early (Ondarza et al., 2003) and late (Mitsui et al., 2005; Ondarza et al., 2003) timepoints after injury. In both studies however, bladder activity had already emerged, leaving the correlation between emergence of bladder activity and sprouting unclear.

The emergence of bladder activity after spinal cord injury develops from initial areflexia to sustained hyperreflexia, characterized by bladder overactivity. Limb spasm also emerges after spinal cord injury, although this occurs prior to the emergence of bladder activity (Ditunno et al., 2004). Although the emergence of limb spasm has been attributed to recovery from "spinal shock" and a deficit in descending inhibition (Ditunno et al., 2004), spinal transection directly interrupts the bladder reflex arc (Morrison et al., 2005). We propose that primary afferents entering the lumbosacral cord sprout after complete spinal cord transection. If primary afferent sprouting contributes to micturition after spinal cord injury then we believe that increases in molecules marking afferent sprouting, Gap-43 and CGRP, should precede the emergence of bladder activity. This study investigates whether spinal cord transection induces sprouting of primary afferents in lumbosacral spinal cord before the emergence of bladder activity.

Methods

Implantation of chronic bladder cannula

Female Wistar rats weighing 200–300 g were anesthetized with isoflurane (2–3%). The experimental protocol met the guidelines of the Canadian Council on Animal Care and was approved by the University Committee on Laboratory Animals, Dalhousie University. A ventral midline abdominal incision was made to expose the urinary bladder. A Silastic cannula, 1.65 mm O.D., was inserted and secured in the bladder dome. Using a trocar, the cannula was tunneled under the skin to exit at the back of the skull. The abdominal incision was closed in two layers with suture and wound clips. The rat was then placed in a stereotaxic apparatus. An area of the skull was freed of attached tissue using blunt dissection and three holes were drilled to accommodate 1.4×3 mm machine screws (Small Parts Inc.). The skull was rinsed with saline to remove debris and blood and allowed to dry. The free end of the cannula was secured to a tube imbedded in a plastic pedestal base (Plastics One) and the whole assembly anchored to the skull with dental acrylic (Caulk Dentsply). Rats were given subcutaneous (s.c.) injections of saline (5 ml), enrofloxacin (Baytril) (2.5 mg/kg) and bupenorphrine (0.03 mg/kg) post operatively. Implanted rats were housed singly and hay was added to the bedding to provide enrichment. All implanted rats received enrofloxacin in their drinking water immediately after cannulation and for the duration of the study.

Assessment of bladder function

All rats in this study underwent cystometrogram (CMG) testing approximately 1 week after bladder cannula implantation, and before spinal cord transection (SCT). This was done to ensure that cannula implantation did not impair normal bladder functioning. Only when rats displayed normal bladder functioning, as defined by the absence of nonvoiding contractions and a voiding efficiency higher than 60%, did they undergo SCT (Fig. 1A). CMGs on awake, unrestrained rats were performed in a metabolic cage. The bladder was initially emptied by urine withdrawal through the implanted line. Room temperature sterile saline was infused through the implanted cannula into the bladder at a rate of 0.1 ml/min by a syringe infusion pump and bladder pressure was measured via a pressure transducer (World Precision Instruments). The infusion rate used in this study is comparable to rates used in similar studies (Pikov and Wrathall, 2001; Yoshiyama et al., 1999). Voided volume was collected in a small, lightweight beaker seated on a strain gauge under the metabolic cage. After the first voiding episode the voided volume was verified for the determination of voiding efficiency. Thereafter, saline infusion and fluid collection were continuous. Both urine weight and bladder pressure were amplified (World Precision Instruments), digitized (MacLab/8, PowerLab Instruments), and displayed using Chart software (PowerLab Instruments). Parameters measured included: voided volume, volume and pressure thresholds for micturition, bladder contraction duration, maximum vesical pressure, volume infused and residual volume (Cruz and Downie, 2005). Voiding efficiency was calculated as (voided volume)*100%/(volume infused).

Spinal cord transection

At least 1 week post cannulation, depending on bladder function, rats were anesthetized using isoflurane (2–3%). A dorsal midline incision was made to expose T8–T13 vertebral processes. Fascia and muscle were removed from T9 and T10 vertebrae. A laminectomy was performed to visualize the whole width of the dural sac. Complete SCT was performed at T10 using fine dissecting scissors. Gelfoam (Upjohn) was placed between the cut ends of the cord and the incision was closed using sutures and skin clips. Spinal rats were given s.c. injections

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

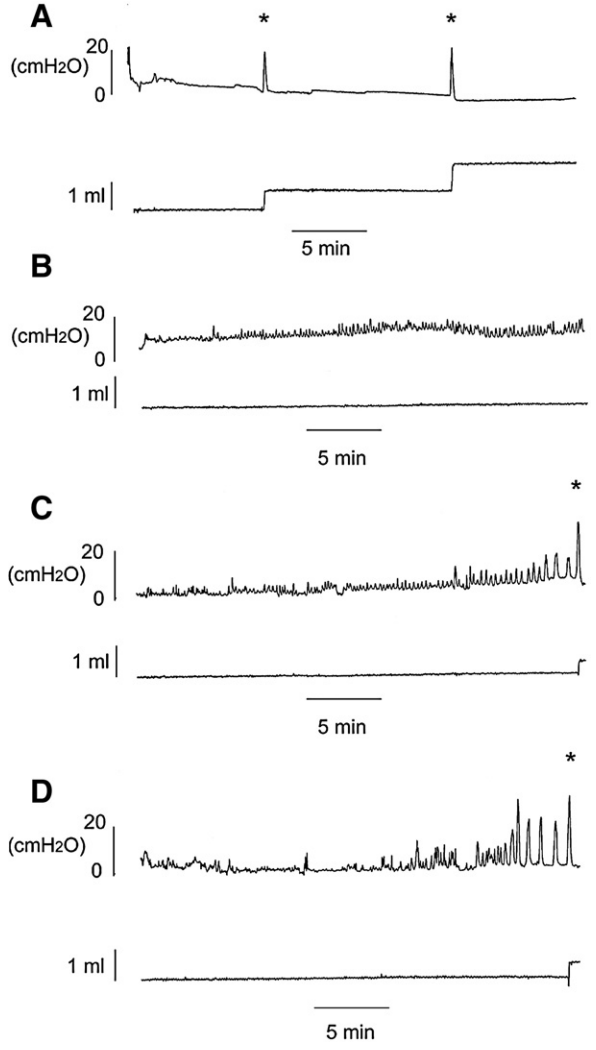


Fig. 1. Bladder function is significantly altered following spinal cord injury. (A) Bladder function in a control rat demonstrates voiding occurring signified by large amplitude bladder contractions that are coupled with voiding responses seen as increases in the volume trace. (B) 5 days post SCT there is an absence of large amplitude contractions and the appearance of frequent small amplitude nonvoiding bladder contractions. (C–D) 14 (C) and 21 (D) days post transection, nonvoiding contractions give rise to a large amplitude contraction that facilitates a voiding response. All CMG's (A–D) were conducted at an infusion rate of 0.1 ml/min. * Indicates contractions that are coupled to a voiding response.

of 0.9% saline (5 ml), Baytril (2.5 mg/kg) and bupenorphrine (0.03 g/kg) immediately after surgery. Rats received s.c. injections of saline (2.5 ml) and bupenorphrine (0.03 mg/kg) every 8 to 12 h for 24–48 h after SCT. Rats were housed individually and were monitored daily for any signs of distress, autotomy, and failure to thrive. Out of 59 spinally transected rats in this study, 2 had to be euthanized due to autotomy.

Bladder management after SCT

Bladders were drained through the chronic indwelling cannula. Polyethylene tubing was attached to the post of the headcap and the bladder was evacuated using a 3-cc syringe. Bladders were drained at approximately 8 h intervals and the urine volumes retrieved were recorded. Only in circumstances when cannula lines were blocked and thus unable to facilitate draining were bladders manually expressed. Six rats required manual expression of the bladder at some point during the course of the study. Of these 6 rats, three required manual expression up to 2 times, two rats required manual expression between 5 and 6 times, and one rat required manual expression 12 times.

Perfusions and tissue processing

Tissue for immunohistochemistry was taken from cannula-implanted (non-injured) rats, and from rats 3, 8 and 21 days ($n=5$ per group) after SCT. The timepoints selected for tissue harvesting were determined from the functional results we obtained. Bladder activity emerges in the form of nonvoiding and voiding contractions 5 and 10–14 days post transection. We therefore looked for sprouting in lumbosacral cord at 3 and 8 days; timepoints just preceding observed functional changes. Rats were deeply anesthetized with sodium pentobarbital (Euthanyl) and perfused through the left ventricle with 300 ml of 0.9% saline followed by phosphate buffered 4% paraformaldehyde. L6/S1 vertebral columns and bladders were removed and the completeness of transection confirmed by inspection. After perfusion, the spinal cord, still within the vertebral column, was postfixed overnight in 4% paraformaldehyde. The next day the spinal cord was removed and returned to 4% paraformaldehyde for an additional 1 h post fixation. Dura was removed from the spinal cord and the spinal cord was cryoprotected in ascending concentrations to 30% sucrose. Spinal cords were embedded in O.C.T. Compound (Tissue-Tek) and 20 μm sections were cut on a cryostat in four sequential series in coronal or longitudinal planes. Longitudinal sections ($n=2$ for each group) were reacted with primary monoclonal antibody rabbit anti-CGRP (Peninsula Labs, 1:1000) on slide at room temperature for 3 days. All primary antisera solutions were made in phosphate buffered saline that included 10% normal goat serum as a blocking agent and 0.3% Triton-X to prevent tissue degradation. Sections were rinsed three times with 0.05 M phosphate buffered saline pH 7.4 and placed in biotinylated goat anti-rabbit (Jacksons Immuno Research Laboratories Inc., 1:500) secondary antibody overnight at room temperature. In this same reaction solution primary monoclonal anti-Gap-43 (Chemicon, 1:1000) was added. After rinsing sections again in PBS sections were incubated in streptavidin Alexa 568 conjugate (1:300) (Molecular Probes) and FITC goat anti-mouse (1:200) (Chemicon). Sections were rinsed and cover-slipped with Citifluor to preserve fluorescence. Immunohistochemistry on coronal sections ($n=3$ for each group) was against CGRP only and used the same antibodies and concentrations mentioned above.

Bladder histology

Bladders from control implanted and spinal cord transected rats were removed after perfusion and post fixed for at least 48 h before cutting transversely to separate the dome and the trigone regions. Bladders were imbedded in paraffin and sectioned at 5 μm. Hematoxylin and eosin staining was conducted to allow

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

for assessment of inflammation within the three layers of the bladder (epithelium, lamina propria, and lamina muscularis). Each bladder was given an overall inflammation score based on congestion, leukocyte infiltration and on the integrity of the epithelium. Each of these three criteria was given a score out of three, where 0 showed no visible signs and 3 implied a severely inflamed state. Statistical analysis set at a confidence level of $p < 0.05$ using nonparametric one-way ANOVA was used to assess significance.

In addition, the thicknesses of the epithelium, lamina propria, and lamina muscularis were measured separately to investigate any changes in the integrity of bladder wall components after spinal cord injury. The bladder was placed in a microscopic field containing four quadrants and at each quadrant a measurement was made of the thickness of each three layers. An average was taken of all four measurements to represent the overall thickness of each layer. Significance in layer thickness was assessed by performing parametric one-way ANOVA statistical analysis set at a confidence level of $p < 0.05$.

Spinal cord densitometry

The density and distribution pattern of CGRP positive primary afferent axons in L6/S1 spinal cord in laminas I–VI and X was analyzed for control rats, and for SCT rats 3 days, 8 days and 21 days post transection ($n = 3$ per group). SCION imaging software (http://www.scioncorp.com) was used to conduct the analysis. Digital images of coronally cut lumbosacral spinal cord were captured using a Leica fluorescent microscope and Nikon camera through the 5× objective. Image brightness, contrast as well as exposure time was held constant for all images analyzed. A scanned image of L6 and S1 spinal cord profiles with Rexed's lamina marked (Coimbra et al., 1974; Molander et al., 1984) was overlayed on 8 randomly selected sections of both L6 and S1 spinal cord segments for each rat of each group to allow for specific density measurements of each lamina studied. Density analysis was conducted on gray scale images with intensities ranging from white (0) to black (256). Upper and lower thresholds were set and used to assess positive staining for all sections analyzed. Individual lamina was grouped to represent 4 different regions in the dorsal horn. We analyzed lamina II on its own which represented superficial dorsal horn. Laminas III and IV were grouped and represented mid dorsal horn. Lamina V and VI represented deep dorsal and lamina X represented the region surrounding the central canal. Unpaired student *t*-tests set at a confidence level of $p < 0.05$ were used to assess statistical significances between the different lamina and animal groupings for both L6 and S1. Because CGRP immunoreactivity reached saturation in lamina I it was not used in this analysis.

DRG counts

L6 DRG were removed from control and 8 day post transection rats ($n = 3$). The left DRG from each rat was cut on a cryostat at a thickness of 40 μm. DRG were reacted with antibody to CGRP using the same protocol as described previously. Every section of DRG was analyzed for positive CGRP neurons allowing for a total cell count to be made of CGRP positive neurons in L6 spinal cord for control and 8 days post transection rats.

Confocal microscopy

Spinal cords from rats 3 and 5 days post transection were cut on a cryostat in a longitudinal orientation at a thickness of 15 μm. Analysis was conducted 5 days post transection because it was imperative to know if the CGRP positive fibers traversing the empty space between SPN expressed Gap-43. Confocal analysis was not conducted 8 days post transection because it was already known that Gap-43 was not expressed at this time. Spinal cords were reacted with antibody to CGRP and Gap-43 using the same protocol as in the above section. Spinal cords were imaged using a laser-scanning confocal microscope (LSM 510 META; Zeiss, Thornwood, NY) and viewed using LSM Image Browser (Zeiss).

Western blotting

Fresh L6/S1 spinal cord was removed from deeply anesthetized control rats, and rats 3 days, 8 days and 21 days post transection ($n = 3$ per group). Cords were quickly frozen with liquid nitrogen and stored at −70 °C. The spinal cords were placed in extraction buffer (1000 mM HEPES, 5000 mM NaCl, 500 mM EDTA and 100% NP40, 2 mM PMSF) and homogenized with a Douce glass homogenizer. Tissue was then sonicated using a pin point sonicator and centrifuged (100 000×*g*) for 1 h at 4 °C. The amount of protein in the supernatant was quantified using the Bradford method. Protein samples were then separated on polyacrylamide gel and transferred via electrophoresis to nitrocellulose membrane. After blocking in 4% milk for 1 h at 4 °C, blots were incubated in primary monoclonal anti-Gap-43 antiserum (Chemicon, 1:1000) overnight at 4 °C. Primary antiserum was removed and the blot was washed 3 times in Tris buffered saline–Tween 20. Horseradish peroxidase conjugated goat anti-mouse IgG (1:1000) secondary antibody (Chemicon) in 4% milk was applied to the blot for 1 h at room temperature. After washing, blots were incubated in ECL (PerkinElmer) and exposed to X-ray film (Kodak). Film was then developed (Kodak X-Omat 1000A) and relative protein quantities determined via densitometry (NIH imaging software). Immunoblotting against actin (Chemicon) was used as a loading control.

Results

Bladder contractions emerge over time after spinal cord transection

To test bladder activity after spinal injury awake rats had their bladders infused with saline and bladder pressure as well as voided volume monitored. During filling in control rats bladder pressure increased very little until a sharp increase in

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

bladder pressure occurred marking a bladder contraction that was always coupled to a voiding response (Fig 1A). Testing spinally injured rats in the same fashion for the first 4 days post transection revealed that the bladder was areflexic, presumably because the normal reflex pathway controlling micturition was directly interrupted by SCT (Morrison et al., 2005). At 5 days post transection, small, high frequency, bladder contractions appeared during CMG analysis. The contractions reached amplitudes >4 cmH_2O in 67% of rats (Fig. 1B, Table 1). These contractions were not associated with urine expulsion and will be referred to as nonvoiding contractions. Others have adopted a criterion of 8 cmH_2O or greater for defining nonvoiding contractions (Yoshiyama et al., 1999). By this criterion, we found that 17% of spinal rats had nonvoiding contractions 5 days after transection (Table 1). At 8 days post SCT, the pressures reached during nonvoiding contractions had increased such that all 11 rats tested reached pressures >4 cmH_2O and 73% of rats had nonvoiding contraction pressures that reached >8 cmH_2O. The volume threshold for inducing nonvoiding contractions >4 cmH_2O was also significantly lower at 8 days than at 5 days after SCT (Table 1). Rats at 5 ($n=12$) and 8 ($n=11$) days post transection never showed voiding contractions during CMG analysis.

Within 10–14 days post SCT, bladder filling evoked bladder contractions that resulted in fluid expulsion in all rats tested (Fig. 1C) ($n=7$). This time frame agrees with other studies (Kruse et al., 1993; Mallory et al., 1989; Yoshiyama et al., 1999). Throughout this study we emptied the rats bladders via the implanted cannula and recorded the amount of urine evacuated each day. Emergence of voiding on CMG was accompanied by decreases in urine volumes retrieved per day, confirming that the rats were voiding, partially emptying their bladders between drainings (Fig. 2). All rats in the present study exhibited nonvoiding contractions (type I voiding; Kruse et al., 1993) that increased in amplitude until the volume threshold for voiding was reached (Figs. 1C, D). Other studies (Kruse et al., 1993; Yoshiyama et al., 1999) have shown that about 50% of rats 14–21 days after complete SCT do not demonstrate nonvoiding contractions (type II voiding). We found no rats exhibiting this pattern.

Table 1
Incidence and volume threshold of nonvoiding contractions in rats after spinal cord injury

Days post transection	Presence of nonvoiding contractions >4 cmH_2O	Presence of nonvoiding contractions >8 cmH_2O	VT for nonvoiding contraction >4 cmH_2O (ml)	VT for nonvoiding contractions >8 cmH_2O (ml)
5	8/12	2/12	1.6±0.9	1.7±0.2
8	11/11	8/11	0.31±0.19**	0.76±0.75
10–14	7/7	7/7	0.59±0.75*	0.77±0.98
20–30	5/5	5/5	0.27±0.74*	0.85±0.74

Incidence expressed as rats with nonvoiding contractions/total number of rats assessed. Volume threshold (VT) for induction of nonvoiding contractions reaching either 4 or 8 cmH_2O at 5, 8, 10–14 and 20–30 days post spinal cord transection. *$p<0.05$, **$p<0.01$ unpaired t-test compared to value at 5 days post transection.

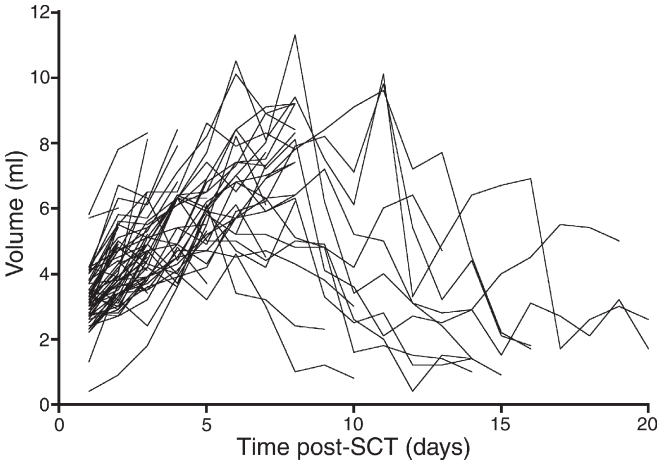


Fig. 2. Mean values of volumes of urine collected from bladders by draining through implanted bladder cannula from rats with spinal cord transection. Amount of urine evacuated from bladder fluctuates with time after transection. Points are averages of the three daily bladder drainings.

CMG analysis on control and spinal rats allowed for a comparison of bladder activity by assessing parameters such as maximum pressure reached during void, void volume, and voiding efficiency. It is known that various bladder pathologies such as bladder outlet obstruction and inflammation will cause changes in these parameters (Hu et al., 2005; Igawa et al., 1994). It is also known that spinal cord injury severely compromises voiding efficiency both in humans and in rats. At both 10–14 and 20–30 days post transection, voiding contractions had significantly greater maximum pressure and contraction duration compared to the preceding nonvoiding contraction (Table 2). The presence, threshold, frequency and amplitude of nonvoiding contractions and the overall voiding pattern remained the same between 10–14 days and 20–30 days post transection (Fig. 1D, Table 1). Mean voiding times in our rats 10–14 days after SCT were similar to those reported for rats that demonstrate type I voiding (Kruse et al., 1993). By contrast, others have reported that spinal rats that do not generate nonvoiding contractions (rats with type II voiding) had a significantly longer voiding contraction, sometimes lasting for over 2 min (Kruse et al., 1993).

The micturition that emerged after SCT also was quantitatively different compared to voiding prior to spinal cord injury in unanaesthetized rats (Table 3). SCT caused a significant increase in maximum pressure reached during voiding, volume

Table 2
Comparison of nonvoiding and voiding bladder contractions in rats after spinal cord injury

Days post transection	MP (cmH_2O)		CD (s)	
	Voiding	Nonvoiding	Voiding	Nonvoiding
10–14 ($n=7$)	32.9±6.4*	17.9±11.2	29.9±6.9	21.6±6.7
20–30 ($n=5$)	31.8±5.1**	17.8±5.3	31.8±5.1	18.8±5.3

Mean maximum pressure (MP) (cmH_2O) and contraction duration (CD) (s)±S.E. of the voiding contraction and the preceding nonvoiding contraction 10–14 and 20–30 days post transection. n=number of animals per group. *$p<0.05$, **$p<0.01$ paired t-test.

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

Table 3
Characteristics of voiding contractions in rats at different times after spinal cord injury

Days post transection	MP (cmH_2O)	CD (s)	PT (cmH_2O)	VT (ml)	VE (%)	RV (ml)	BC (ml/cmH_2O)
Control ($n=5$)	24±11.3 (12–41)	22.2±7.5 (15–35)	12.6±2.41 (10–16)	1.5±0.6 (0.8–2.1)	95.2±9.1 (79–100)	0.9±0.2 (0–0.41)	0.13±0.06 (0.06–0.19)
10–14 ($n=5$)	35±6.3* (24–57)	28.5±7.5 (19–28)	13.6±6.7 (6–21)	3.6±1.6* (1.6–6)	10.7±3.9** (5.5–14.8)	3.2±1.4** (1.3–5.2)	0.36±0.36 (0.13–0.99)
Control ($n=5$)	19.6±8.6 (9–31)	24.4±7.6 (15–34)	12.8±3.4 (10–17)	1.5±0.8 (0.7–3.4)	93.3±8.8 (78.6–100)	0.06±0.08 (0–0.18)	0.13±0.07 (0.41–0.14)
20–30 ($n=5$)	31.8±5.1* (27–40)	30.8±5.5* (24–37)	9.6±3.6 (4–12)	3.5±0.52** (2.8–4.1)	16.8±2.7**∫ (14–20)	2.96±0.45** (2.5–3.5)	0.37±0.3* (0.23–0.98)

Mean values for maximum pressure (MP) (cmH_2O), contraction duration (CD) (s), pressure threshold (PT) (cmH_2O), volume threshold (VT) (ml), voiding efficiency (VE) (%), residual volume (RV) (ml) and bladder compliance (BC) (ml/cmH_2O)±S.E. compared between control (uninjured) and SCT rats at 10–14 and 20–30 days post transection. *N* indicates the number of animals used. Numbers in parentheses=(minimum–maximum) values measured for that parameter. *$p<0.05$, **$p<0.01$ paired *t*-test compared to control. ∫$p<0.05$ unpaired *t*-test compared to 10–14 days post transection.

threshold for voiding, and post voiding residual volume in 10–14 and 20–30 day groups. In contrast, voiding efficiency was much lower in both groups of SCT rats compared to uninjured rats. 20–30 day spinal rats had increased efficiency compared to rats 10–14 days post transection. Furthermore, contraction duration and bladder compliance were significantly increased over control at 20–30 days post SCT. If sprouting is contributing to the emergence of bladder activity, this process might still be having an influence on activity as long as 3 weeks after injury. Despite increases in efficiency seen between the two spinal groups in this study all rats had large bladders as confirmed visually at the time of perfusion.

Spinal cord transection changes bladder morphology

Within the first 3 days after spinal cord transection, the bladder distended to accommodate over twice the amount of urine compared to a control bladder (Fig. 2). At this time the thicknesses of the urothelium, lamina propria or the muscularis propria did not significantly differ from those of control rats (Fig. 3A). At later time points (8, 10–14 and 20–30 days post SCT) however, the thickness of both the bladder muscle and urothelial cell layers were increased (Figs. 3A,B). The thickness of the lamina propria did not significantly differ from control values at any time point post transection.

Each bladder layer was also assessed for signs of inflammation by using a points scale based on the level of congestion, leukocyte infiltration and on the integrity of the epithelium. Another study from this laboratory showed that bladders 3 days after irritation with lipopolysaccharide had noticeable signs of inflammation and inflammation scores in the range of 5–7 (N. Power, R. Gupta and J. Downie, unpublished). The total scores of 1–1.25 in bladders 3, 8, 10–14 and 20–30 days post transection did not significantly differ from control implanted or control non-implanted rats (Fig. 3C). In addition, the two control groups did not significantly differ in inflammation scores. These results demonstrate that bladder hyperactivity seen after spinal cord injury was caused by a mechanism other than bladder inflammation. It is more likely that neuronal activity at the level of the spinal cord rather than at the level of the bladder is mediating bladder hyperreflexia seen in our spinal rats.

CGRP distribution increases in L6/S1 dorsal horn over time after spinal cord transection

Our functional results indicated that 3 days is a time point just preceding the appearance of nonvoiding contractions and that the emergence of voiding contractions occurs after the 8 day timepoint. To assess if sprouting might be a contributing factor to emergence of dysfunctional bladder activity after spinal injury, sprouting in L6/S1 spinal cord was assessed in coronal sections 3 and 8 days after SCT. We assessed the level of CGRP positive fibers within specific laminar groupings; lamina II, III and IV, V and VI, and X. We set lamina II to represent superficial dorsal horn where the majority of primary afferents terminate, laminas III and IV to represent mid dorsal

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

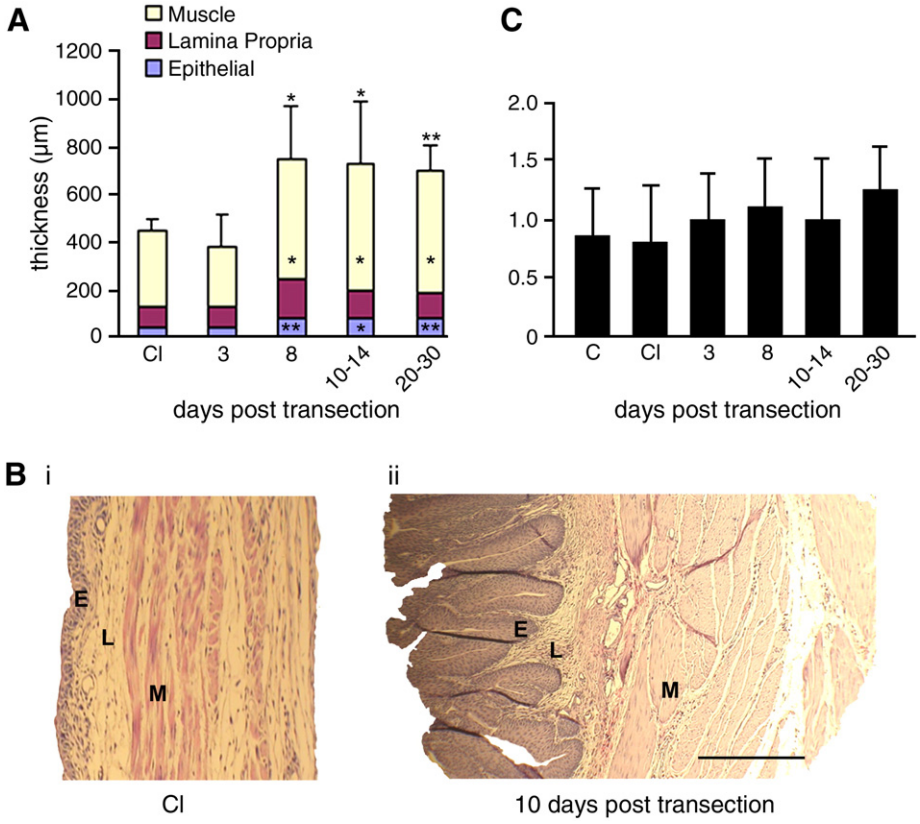


Fig. 3. Morphological changes occur to the bladder wall following SCT. (A) Stacked histogram of thicknesses of muscle (M), lamina propria (L) and epithelial layers (E) from bladders of control implanted (CI) and rats 3, 8, 10–14 and 20–30 days post transection. Error bars represent S.E. from the mean total thickness of the bladder wall. Significant increases were seen in thickness of the epithelial and lamina propria as well as overall thickness in rats 8, 10–14, and 20–30 days post transection when compared to CI. No significant difference was measured between the thickness of the lamina propria and control implanted rats at any timepoint after transection. No significant difference was measured in thickness of any bladder layer between control implanted and 3 days post SCT. (B) Hematoxylin and Eosin staining of a bladder from a control implanted rat (i) and from a rat 10 days post transection (ii) showed thickening of the E and L. C, Histogram of inflammation scores from rats that had no bladder cannula implanted (C), CI rats, and rats 3, 8, 10–14, and 20–30 days post transection showed no significant difference in scores of inflammation between any group measured. Scale bar represents 200 μm. $*p < 0.05$, $**p < 0.01$ vs. CI thickness of M, L and E in untransected rats; paired *t*-test.

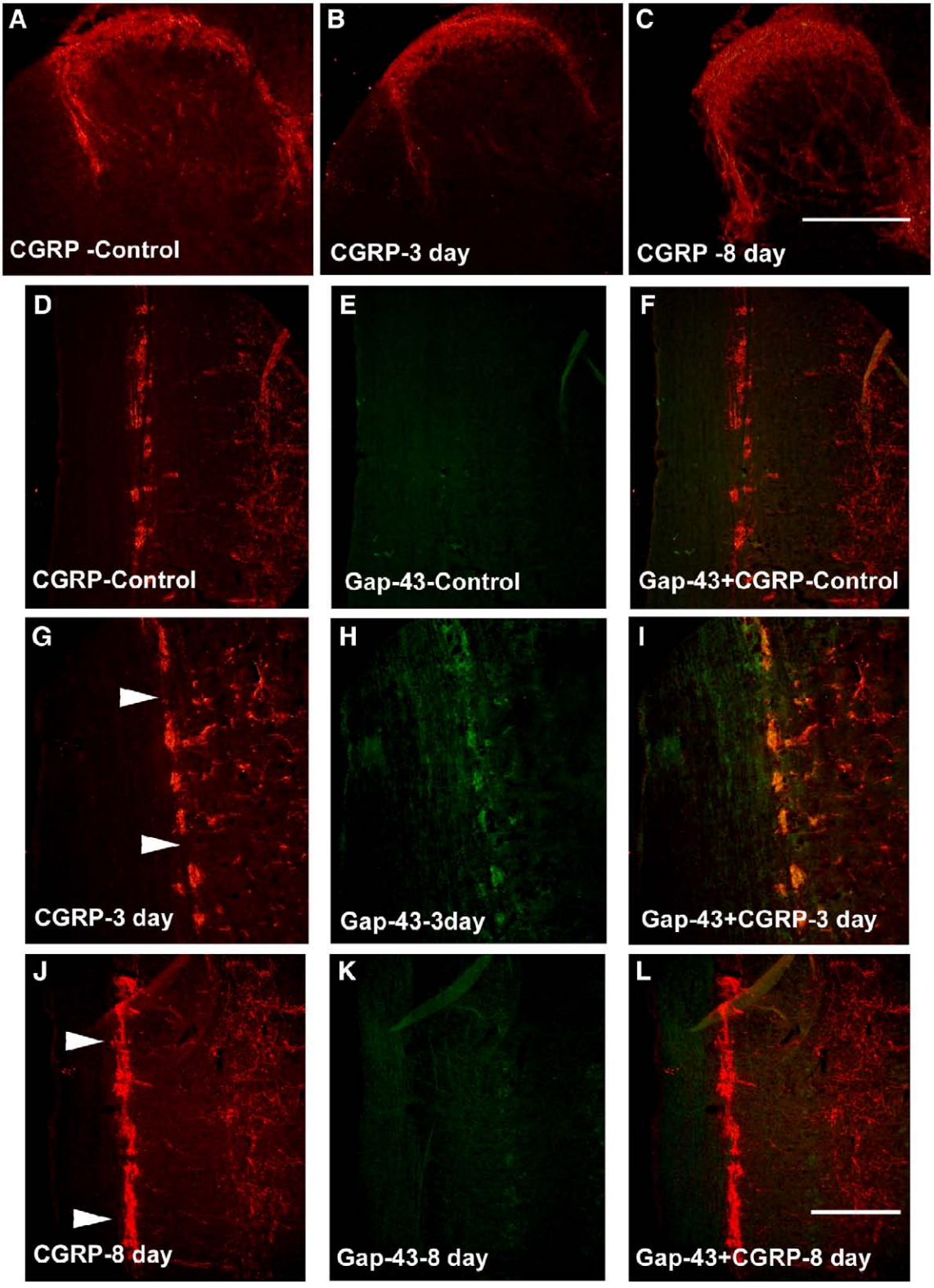
horn. Laminas V and VI represented intermediate regions of the dorsal horn containing some portion of the lateral collateral pathway followed by pelvic nerve afferents as well as the intermediolateral cell column containing bladder preganglionic neurons. Lamina X surrounds the central canal and also contains some portion of the medial collateral pathway also followed by pelvic nerve afferents. Control rat L6/S1 spinal cord was found to express CGRP extensively within lamina II but also at lower levels within lamina III–VI and X (Fig. 4A). Thoracic SCT did not increase CGRP distribution within any lamina of L6 or S1 analyzed at 3 days post transection (Figs. 4B and 5A–D). In fact, there appeared to be a trend toward decreases in CGRP at 3 days when compared to control. In contrast, 8 days post SCT there was a significant increase in CGRP density in all laminar groupings analyzed (Figs. 4C and 5A–D). At 21 days after SCT, the increase in CGRP density seen at 8 days in L6/S1 lamina V and VI remained elevated (Fig. 5). The expression of CGRP within lamina X of L6 and S1 at 20–30 days post transection was not different from untransected control rats. Pelvic primary afferents enter the spinal cord in a rostrocaudal periodic termination pattern (Nadelhaft and Booth, 1984). CGRP positive primary afferents also terminate in lumbosacral cord in a periodic pattern when viewed in longitudinal sections (Zinck and Downie, 2005). CGRP expression within lumbosacral cord remained periodic in nature 3 days post SCT (Figs. 4D, G). At 8 days post transection however, periodicity was lost, evidenced by a filling in of the gaps between CGRP dense staining in the region of the pelvic preganglionic nucleus (Fig. 4J). This is consistent with the CGRP increase in lamina V/VI that was conducted on coronal cut L6/S1 spinal cord. Total CGRP positive cell counts of L6 DRG neurons in control and in rats 8 days post transection showed no significant difference in the number of CGRP positive neurons. Total CGRP positive cell count values for control rats were 1209, 1306 and 1326 whereas positive cell count values for rats 8 days post transection were 1214, 1361 and 1422.

Gap-43 distribution increases in L6/S1 dorsal horn and is colocalized within CGRP terminals after spinal cord transection

To determine if the increase in CGRP density and distribution was a result of sprouting or whether this increase

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

was due to an increase in CGRP expression in existing primary afferent terminals, we examined the expression of Gap-43 in L6/S1 cord 3 and 8 days post SCT. Gap-43 was constitutively expressed in control spinal cord in superficial dorsal horn regions of lumbosacral spinal cord (not shown). Gap-43 expression was assessed in horizontal sections of control and transected spinal cords at the level of the L6/S1 preganglionic nucleus (Figs. 4E, H and K). In both control (Fig. 4F) and 8 day (Fig. 4L) spinal rats, Gap-43 was not detectable at the level of the preganglionic nucleus in L6/S1 spinal cord. However, 3 days post SCT, Gap-43 was detected in this region (Fig. 4H). Overlayed images of L6/S1 spinal



Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

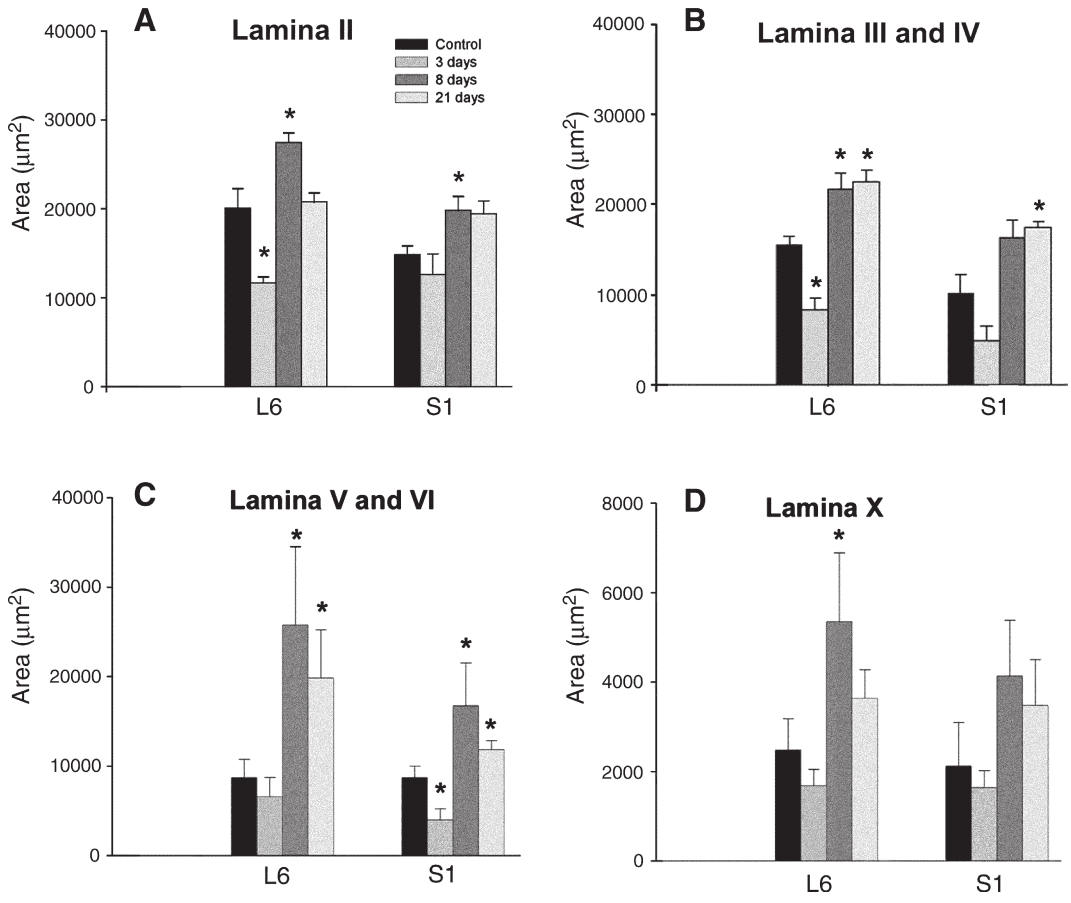


Fig. 5. CGRP positive afferent terminals show increased distribution in deeper dorsal horn regions after SCT. (A–D) Histograms representing mean ± S.E. area (um^2) immunoreactive for CGRP within laminar groupings of II, (A), III and IV (B), V and VI (C) and X (D) of L6 and S1 spinal cord.*$p < 0.05$ vs. control level of CGRP in untransected spinal cord; unpaired *t*-test.

cord double-labeled for CGRP and Gap-43 imply that increases in Gap-43 3 days post SCT occurred in CGRP containing primary afferent terminals (Fig. 4I). Confocal microscopy verified that afferent sprouting was occurring in lumbosacral cord. At the level of the preganglionic nucleus it is clear that Gap-43 is colocalized with CGRP at both 3 (Fig. 6A) and 5 days post transection (Fig. 6C). At 5 days post transection fibers expressing Gap-43 and CGRP (sprouting fibers) can be seen running between preganglionic nuclei (Fig. 6C). This was not seen 3 days post transection (Fig. 6A). Higher magnification (100×) of individual nuclei 3 (Fig. 6B) and 5 (Fig. 6D) days post transection show more clearly in the x/y plane the colocalization seen between Gap-43 and CGRP.

To quantify increases seen in Gap-43 by immunohistochemistry Western blotting was conducted. Protein homogenates of whole L6/S1 spinal cord from control, 3 and 8 days post transections were reacted with Gap-43 antibody. This method of analysis showed increased Gap-43 levels 3 days after SCT (Fig. 7A) and a return to control levels by 8 days post transection (Fig. 7B). It should be noted that these Western blots reveal the presence of constitutive levels of Gap-43 within control spinal cord, likely derived from superficial dorsal horn.

Discussion

Our results show that primary afferent sprouting occurred in L6/S1 spinal cord, some 8 spinal cord segments below the site

Fig. 4. CGRP-positive afferents in lumbosacral spinal cord dorsal horn sprout after SCT. (A–C) Coronal sections of lumbosacral spinal cord. (A) Control, uninjured spinal cord expresses CGRP at high levels within superficial dorsal horn regions including lamina I and II as well as deeper dorsal horn regions of lamina V and X. (B) 3 days post injury there is no difference in dorsal horn CGRP distribution. (C) 8 days post injury CGRP positive terminals increase in deeper dorsal horn regions including lamina II, regions surrounding the SPN (lamina V) and the DGC (lamina X). (D–L) Horizontal sections of lumbosacral spinal cord taken at the level of the preganglionic nucleus and DGC from control uninjured (D–F), 3 days (G–I), and 8 days post SCT (J–L). (D–L) CGRP is expressed in a rostrocaudal periodic nature in regions of the preganglionic nucleus and is also expressed within the DGC. (E) Gap-43 is not expressed in control spinal cord at this level. (F) Overlaying (D and E) shows no expression of Gap-43 by CGRP afferents in control tissue. (G) 3 days following SCT (G–I) CGRP still shows a periodic nature of distribution in the cord as well as immunopositive fibers in the DGC. (H) Gap-43 is now expressed by neurons in regions around the preganglionic nucleus. (I) Overlaying images of panels G and H show that the same afferents expressing CGRP also express Gap-43. 8 days post transection (J–L) CGRP terminals increase their distribution within the preganglionic nucleus giving a more filled in appearance as opposed to periodicity (J). Arrow heads on panel G show the absence of CGRP terminals between nuclei whereas in panel J CGRP terminals have filled in the empty spaces creating a continuous column of fibers. (K) Gap-43 is no longer expressed 8 days post transection. (L) Overlaying panels J and K show that CGRP afferents do not express Gap-43 in lumbosacral spinal cord 8 days following SCT. Scale bar represents 100 µm.

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

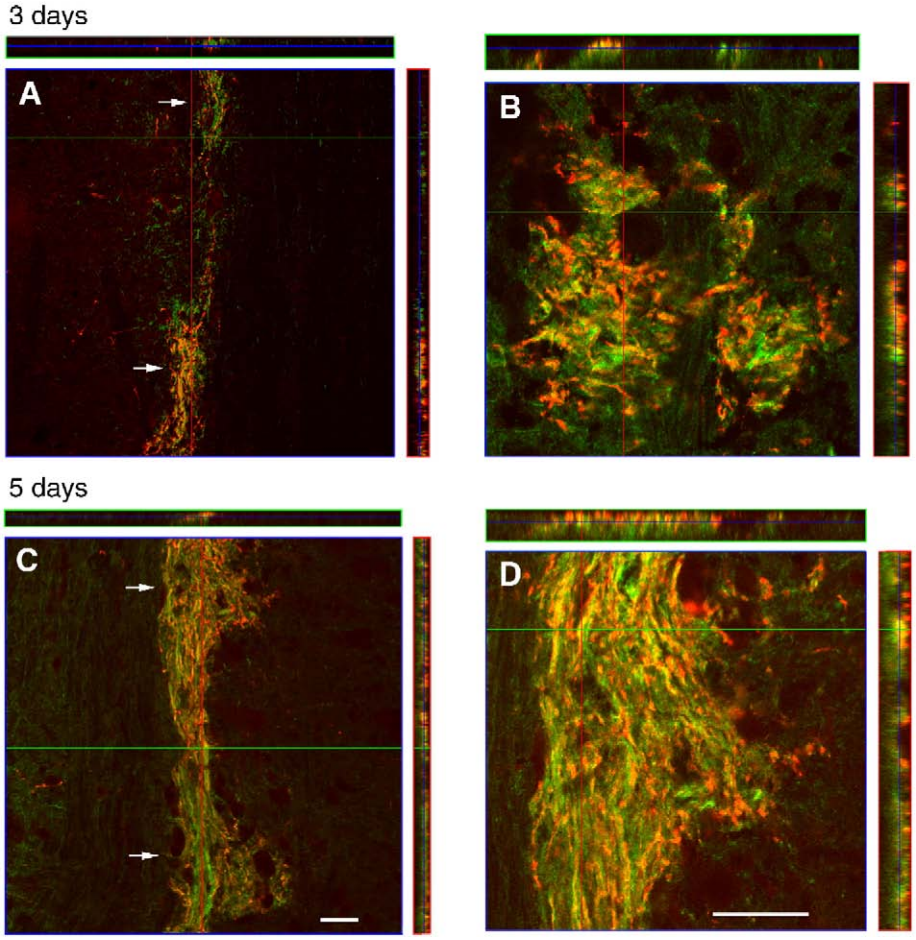


Fig. 6. Orthogonal views of horizontal sections of L6/S1 spinal cord immunopositive for CGRP and Gap-43 3 days post transection (A and B) and 5 days post transection (C and D). Arrows in panels A and C indicate the preganglionic nucleus. Scale bar in panel C represents 20 μm for panels A and C. Panels B and D were taken at the level of an individual preganglionic nucleus. Scale bar in panel D represents 20 μm for both panels B and D.

of complete spinal cord transection. The conclusion that sprouting of CGRP positive afferents occurred is based on several findings. First, the distribution of CGRP afferents in lumbosacral cord increased after spinal cord transection. Secondly, there was no change in the number of CGRP positive cells in L6 DRG 8 days post transection when compared to control DRG. Thus, a contribution from phenotypic switching to the increase in CGRP seen in the dorsal horn seems unlikely.

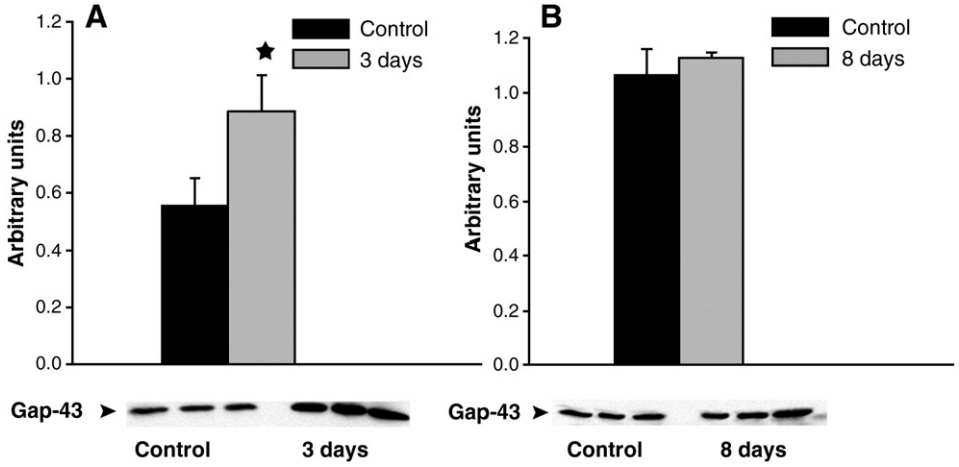


Fig. 7. Gap-43 increases in lumbosacral spinal cord 3 days after SCT. (A) Histogram of western blots (seen below histogram) of Gap-43 expression±S.E. of control (noninjured) whole lumbosacral spinal cord (black bar) compared to lumbosacral cord taken from rats 3 days post SCT (gray bar) and (B) 8 days post transection. Actin was the loading control.

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

In addition, we have shown colocalization of a growth cone marker Gap-43 with CGRP. Because this colocalization precedes the increased distribution of CGRP in the dorsal horn we believe CGRP afferents sprouted. We showed for the first time that this sprouting response occurs just prior to the emergence of bladder activity. Afferent sprouting has been shown to have functional consequences in the development of autonomic dysreflexia (Krenz et al., 1999) after spinal cord injury. Our findings indicate that the time frame for primary afferents sprouting in the lumbosacral spinal cord is consistent with this process contributing to bladder function changes post SCT.

Changes in bladder activity post SCT

Spinal cord injury-induced morphological changes in the bladder may contribute to altered function seen after spinal cord injury (Apodaca et al., 2003). Our study has shown significant increases in thicknesses of both the epithelial and muscle layers 8–30 days post spinal cord transection. Alterations in the epithelium due to spinal cord injury are thought to disrupt urothelium-to-primary afferent communication possibly modulating emergent micturition (Birder, 2005). Thickening of the bladder musculature is thought to contribute to decreased compliance seen in many spinal cord injured patients (Hackler et al., 1989). However, there is evidence showing that changes in bladder musculature elastin and collagen contribute to increased compliance after spinal cord injury in rats (Nagatomi et al., 2004). We believe that this may be the mechanism by which we see increased bladder muscle thickness with increased compliance in our study.

The first evidence of emergent bladder activity after spinal injury in our rats was the development of nonvoiding contractions. Nonvoiding contractions appeared consistently by 5 days post transection and are considered to be abnormal because they are also found in rats with outlet obstruction (Igawa et al., 1994) and bladder inflammation (Hu et al., 2005). However, the presence of nonvoiding contractions after spinal cord injury in our study is unlikely to be due to inflammation because inflammation scores were very low, likely due to prophylactic antibiotic treatment. Thus the bladder hyperreflexia seen in this study was induced by spinal cord injury.

In agreement with other studies (Kruse et al., 1993; Mallory et al., 1989; Yoshiyama et al., 1999) all rats tested by CMG showed contractions that were coupled to urine expulsion (voiding contractions) at 10–14 days post transection. The major difference in CMG pattern for rats in the present study is that all rats exhibited nonvoiding contractions prior to a void (type I voiding). We attribute this difference to our practice of draining via the implanted bladder cannula, rather than by the more commonly used method of manual expression. This method of bladder draining was chosen because it most closely reflects the way in which spinal cord injured patients manage their bladders, through intermittent catheterization rather than abdominal compression. We believe that repeatedly provoking high bladder pressures by manual expression causes changes in bladder muscle properties similar to those seen with urethral outlet obstruction (Nagatomi et al., 2004; Peters et al., 1997). In addition, increasing the frequency of complete bladder emptying (3/day in this study vs. 2/day usually) may also reduce the likelihood or magnitude of significant distension-induced changes in bladder wall morphology. The presence of type II voiding (absence of nonvoiding contractions) in other studies (Yoshiyama et al., 1999) implies that manual expression may damage the bladder possibly compromising its ability to contract.

Bladder contractility was not compromised in our rats as increases in contraction duration and higher maximum pressures were observed in rats between 2 and 3 weeks post injury. However, voiding efficiency was low in all voiding spinal rats. Low efficiency is usually attributed to lack of coordination between the external urethral sphincter and the bladder (Kruse et al., 1993; Mallory et al., 1989; Pikov and Wrathall, 2001; Yoshiyama et al., 2000). Because voiding efficiency was significantly greater 20–30 days compared to 10–14 days post transection, we conclude that there may be an ongoing process that slightly improves bladder activity beyond 2 weeks after spinal injury.

Recovery from spinal shock is not a likely explanation for emergence of bladder dysfunction. Although the mechanisms responsible for recovery from spinal shock are not fully understood, it is implied that there is an initial interruption of neuronal signaling, “neuronal shock”, that is overcome in time. Complete spinal cord transection will not allow for recovery from neuronal shock of bladder function because all ascending and descending fibers controlling bladder function are lost. The direct interruption of the micturition reflex arc by spinal cord transection (Morrison et al., 2005) demands an alternative explanation for the emergence of bladder activity after the lesion.

To summarize, bladder activity emerges after spinal cord injury, after an initial 4 day period of areflexia. Activity emerges first in the form of nonvoiding contractions 5 days post injury. At 10–14 days post transection, voiding emerges but is always preceded by multiple nonvoiding contractions. Although voiding contractions return after spinal cord injury, they generate greater maximal pressures in the bladder but have significantly diminished efficiency when compared to uninjured rats.

Primary afferent sprouting in L6/S1 spinal cord

CGRP expression is often used to assess the distribution of primary afferent terminals within the dorsal horn. Injury to the thoracic spinal cord increases dorsal horn CGRP distribution both rostral and caudal to the site of injury (Christensen and Hulsebosch, 1997; Krenz and Weaver, 1998; Ondarza et al., 2003; Weaver et al., 1997). In our experiments, CGRP distribution in lamina V was first increased at 8 days after spinal transection. This is after the nonvoiding contractions appeared but just precedes the emergence of voiding contractions. It is also apparent that the periodic distribution of CGRP positive terminals in lamina V in control animals is lost at 8 days after spinal transection. A previous study has shown similar results after pudendal nerve transection using neuronal tracing as a technique to label primary afferents (Thor et al., 1986). The

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

more continuous column of CGRP positive terminals implies that the terminals have extended into new areas, and are making new synaptic contacts. Due to the timing of CGRP afferent reorganization in the cord it is probable that the new synaptic contacts being made may be responsible for bladder activity after SCT.

CGRP containing afferents rely on signaling of nerve growth factor through its tyrosine kinase receptor A (trkA) for survival and outgrowth. Nearly 90% of bladder primary afferents possess trkA and therefore respond to NGF (Yoshimura et al., 2003). Moreover, NGF increases in the bladder (Vizzard, 2000), dorsal root ganglia (Qiao and Vizzard, 2002) and in L6/S1 spinal cord (Krenz and Weaver, 2000) after SCT. Exogenous application of NGF to lumbosacral cord increases CGRP density and distribution of dorsal horn afferents (Tuszynski et al., 1994). In addition, immunoneutralization of lumbosacral NGF suppresses both nonvoiding contractions (Seki et al., 2002) and detrusor sphincter dyssynergia associated with spinal cord injury (Seki et al., 2004). Based on these studies, the increases in lumbosacral afferent CGRP demonstrated here are likely to have a significant impact on bladder activity after spinal cord transection.

Gap-43 is expressed in areas of the central and peripheral nervous systems undergoing synaptic remodeling (Jacobson et al., 1986; Skene et al., 1986; Van der Zee et al., 1989). Studies showing co-localization between Gap-43 and CGRP have been used to strengthen the hypothesis that changes in the distribution of CGRP positive axons is in fact due to plasticity of peptidergic primary afferents (Ondarza et al., 2003). Most recently, Mitsui et al. (2005) have shown increases in Gap-43 and CGRP in lumbosacral spinal cord 8 weeks after a thoracic contusion injury. However, we have shown that the important timepoints corresponding to the emergence of bladder activity occur between 5 and 14 days after spinal cord injury. We examined whether sprouting occurred at 3 days, preceding the appearance of nonvoiding contractions, and at 8 days, preceding the emergence of voiding contractions.

Gap-43 expression was significantly increased at 3 days post transection in deep regions of the dorsal horn (lamina V), before significant changes in CGRP distribution were detected. This increased Gap-43 was verified by confocal microscopy to be colocalized within CGRP positive terminals. At 5 days post transection, Gap-43 remained increased in lamina V and was now found colocalized with CGRP between preganglionic neuron clusters. Thus, CGRP positive terminals redistributed after injury, bridging the area between SPN nuclei as seen 8 days post transection. Gap-43 returned to low levels in primary afferents before emergence in bladder activity implying that the neurons had stopped sprouting and had made functional connections. This finding is compatible with the interpretation that sprouting of L6/S1 afferents has an impact on emergent bladder activity.

There was a significant increase in voiding efficiency from 2 weeks to 3 weeks post transection. Likewise, in some lamina there was a difference in the level of primary afferent sprouting seen between these two time points. During development extensive neuronal outgrowth is followed by dieback of about half of the total neuronal population in adulthood (Lo et al., 1995). This process is thought to be beneficial in establishing a properly functioning nervous system. Our observation of decreased primary afferent sprouts 21 days after injury may also be the result of axonal dieback and could be responsible for improving voiding efficiency over the period from 14 to 21 days after SCT.

Relevance of sprouting to bladder function

Anterograde tracing from Barrington's nucleus revealed fibers that travel directly to the pelvic preganglionic neurons (lamina V) and the dorsal gray commissure (lamina X) (Blok et al., 1997; Holstege et al., 1979; Loewy et al., 1979). Electrical stimulation of these regions stimulates voiding in cats (Blok et al., 1998), but a comparable study has not been conducted in rats. Our study demonstrated significant increases in CGRP density in both lamina V and X after spinal cord transection, suggesting an increase in afferent termination in regions containing efferent nuclei controlling micturition.

This study provides evidence that sprouting occurs prior to emergence of micturition after spinal cord injury and ceases after the establishment of bladder functional changes. More specifically, sprouting occurs in regions known to coordinate bladder function, thereby contributing to the theory that a local circuit controls bladder activity after spinal cord injury.

Acknowledgments

This work was supported by grants from the Canadian Institutes for Health Research and the Nova Scotia Health Research Foundation. We thank Leslie Ingraham for her technical assistance throughout the study as well as Stephen Whitefield and Angela Gamouras for their assistance with confocal microscopy. The assistance of Carleton Animal Care Facility staff is greatly appreciated.

References

Apodaca, G., Kiss, S., Ruiz, W., Meyers, S., Zeidel, M., Birder, L., 2003. Disruption of bladder epithelium barrier function after spinal cord injury. Am. J. Physiol.: Renal, Physiol. 284, F966–F976.

Birder, L.A., 2005. More than just a barrier: urothelium as a drug target for urinary bladder pain. Am. J. Physiol.: Renal, Physiol. 289, F489–F495.

Blaivas, J.G., Sinha, H.P., Zayed, A.A.H., Labib, K.B., 1981. Detrusor-external sphincter dyssynergia: a detailed electromyographic study. J. Urol. 125, 545–548.

Blok, B.F., de Weerd, H., Holstege, G., 1997. The pontine micturition center projects to sacral cord GABA immunoreactive neurons in the cat. Neurosci. Lett. 233, 109–112.

Blok, B.F., van Maarseveen, J.T., Holstege, G., 1998. Electrical stimulation of the sacral dorsal gray commissure evokes relaxation of the external urethral sphincter in the cat. Neurosci. Lett. 249, 68–70.

Bruses, J.L., Chauvet, N., Rubio, M.E., Rutishauser, U., 2002. Polysialic acid and the formation of oculomotor synapses on chick ciliary neurons. J. Comp. Neurol. 446, 244–256.

Christensen, M.D., Hulsebosch, C.E., 1997. Spinal cord injury and anti-NGF treatment results in changes in CGRP density and distribution in the dorsal horn in the rat. Exp. Neurol. 147, 463–475.

Chung, K., Lee, W.T., Carlton, S.M., 1988. The effects of dorsal rhizotomy and spinal cord isolation on calcitonin gene-related peptide-labeled terminals in the rat lumbar dorsal horn. Neurosci. Lett. 90, 27–32.

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

Coimbra, A., Sodre-Borges, B.P., Magalhaes, M.M., 1974. The substantia gelatinosa Rolandi of the rat. Fine structure, cytochemistry (acid phosphatase) and changes after dorsal root section. J. Neurocytol. 3, 199–217.

Cruz, Y., Downie, J.W., 2005. Sexually dimorphic micturition in rats: relationship of perineal muscle activity to voiding pattern. Am. J. Physiol.: Regul., Integr. Comp. Physiol. 289, R1307–R1318.

de Castro, F., 2003. Chemotropic molecules: guides for axonal pathfinding and cell migration during CNS development. News Physiol. Sci. 18, 130–136.

de Groat, W.C., Kawatani, M., Hisamitsu, T., Cheng, C.L., Ma, C.P., Thor, K., Steers, W., Roppolo, J.R., 1990. Mechanisms underlying the recovery of urinary bladder function following spinal cord injury. J. Auton. Nerv. Syst. 30, S71–S78.

de Groat, W.C., Araki, I., Vizzard, M.A., Yoshiyama, M., Yoshimura, N., Sugaya, K., Tai, C., Roppolo, J.R., 1998. Developmental and injury induced plasticity in the micturition reflex pathway. Behav. Brain Res. 92, 127–140.

Ditunno, J.F., Little, J.W., Tessler, A., Burns, A.S., 2004. Spinal shock revisited: a four-phase model. Spinal Cord 42, 383–395.

Gallo, G., Letourneau, P.C., 2004. Regulation of growth cone actin filaments by guidance cues. J. Neurobiol. 58, 92–102.

Hackler, R.H., Hall, M.K., Zampieri, T.A., 1989. Bladder hypocompliance in the spinal cord injury population. J. Urol. 141, 1390–1393.

Holstege, G., Kuypers, H.G., Boer, R.C., 1979. Anatomical evidence for direct brain stem projections to the somatic motoneuronal cell groups and autonomic preganglionic cell groups in cat spinal cord. Brain Res. 171, 329–333.

Hu, V.Y., Zvara, P., Dattilio, A., Redman, T.L., Allen, S.J., Dawbarn, D., Stroemer, R.P., Vizzard, M.A., 2005. Decrease in bladder overactivity with REN1820 in rats with cyclophosphamide induced cystitis. J. Urol. 173, 1016–1021.

Igawa, Y., Mattiasson, A., Andersson, K.E., 1994. Micturition and premicturition contractions in unanesthetized rats with bladder outlet obstruction. J. Urol. 151, 244–249.

Jacobson, R.D., Virag, I., Skene, J.H., 1986. A protein associated with axon growth, GAP-43, is widely distributed and developmentally regulated in rat CNS. J. Neurosci. 6, 1843–1855.

Krenz, N.R., Weaver, L.C., 1998. Sprouting of primary afferent fibers after spinal cord transection in the rat. Neuroscience 85, 443–458.

Krenz, N.R., Weaver, L.C., 2000. Nerve growth factor in glia and inflammatory cells of the injured rat spinal cord. J. Neurochem. 74, 730–739.

Krenz, N.R., Meakin, S.O., Krassioukov, A.V., Weaver, L.C., 1999. Neutralizing intraspinal nerve growth factor blocks autonomic dysreflexia caused by spinal cord injury. J. Neurosci. 19, 7405–7414.

Kruse, M.N., Noto, H., Roppolo, J.R., de Groat, W.C., 1990. Pontine control of the urinary bladder and external urethral sphincter in the rat. Brain Res. 532, 182–190.

Kruse, M.N., Belton, A.L., de Groat, W.C., 1993. Changes in bladder and external urethral sphincter function after spinal cord injury in the rat. Am. J. Physiol. 264, R1157–R1163.

Lo, A.C., Houenou, L.J., Oppenheim, R.W., 1995. Apoptosis in the nervous system: morphological features, methods, pathology, and prevention. Arch. Histol. Cytol. 58, 139–149.

Loewy, A.D., Saper, C.B., Baker, R.P., 1979. Descending projections from the pontine micturition center. Brain Res. 172, 533–538.

Mallory, B., Steers, W.D., De Groat, W.C., 1989. Electrophysiological study of micturition reflexes in rats. Am. J. Physiol. 257, R410–R421.

McIlvain, V.A., Robertson, D.R., Maimone, M.M., McCasland, J.S., 2003. Abnormal thalamocortical pathfinding and terminal arbors lead to enlarged barrels in neonatal GAP-43 heterozygous mice. J. Comp. Neurol. 462, 252–264.

McNeill, D.L., Carlton, S.M., Hulsebosch, C.E., 1991. Intraspinal sprouting of calcitonin gene-related peptide containing primary afferents after deafferentation in the rat. Exp. Neurol. 114, 321–329.

Mitsui, T., Shumsky, J.S., Lepore, A.C., Murray, M., Fischer, I., 2005. Transplantation of neuronal and glial restricted precursors into contused spinal cord improves bladder and motor functions, decreases thermal hypersensitivity, and modifies intraspinal circuitry. J. Neurosci. 25, 9624–9636.

Molander, C., Xu, Q., Grant, G., 1984. The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. J. Comp. Neurol. 230, 133–141.

Morrison, J.F., Birder, L.A., Craggs, M., de Groat, W.C., Downie, J.W., Drake, M.J., Fowler, C.J., Thor, K.B., 2005. Neural control. In: Abrams, P., Cardozo, L., Khoury, S., Wein, A.J. (Eds.), Incontinence. Health Publications Ltd, Paris, pp. 363–422.

Nadelhaft, I., Booth, A.M., 1984. The location and morphology of preganglionic neurons and the distribution of visceral afferents from the rat pelvic nerve: a horseradish peroxidase study. J. Comp. Neurol. 226, 238–245.

Nagatomi, J., Gloeckner, D.C., Chancellor, M.B., DeGroat, W.C., Sacks, M.S., 2004. Changes in the biaxial viscoelastic response of the urinary bladder following spinal cord injury. Ann. Biomed. Eng. 32, 1409–1419.

Ondarza, A.B., Ye, Z., Hulsebosch, C.E., 2003. Direct evidence of primary afferent sprouting in distant segments following spinal cord injury in the rat: colocalization of GAP-43 and CGRP. Exp. Neurol. 184, 373–380.

Peters, C.A., Freeman, M.R., Fernandez, C.A., Shepard, J., Wiederschain, D.G., Moses, M.A., 1997. Dysregulated proteolytic balance as the basis of excess extracellular matrix in fibrotic disease. Am. J. Physiol. 272, R1960–R1965.

Pikov, V., Wrathall, J.R., 2001. Coordination of the bladder detrusor and the external urethral sphincter in a rat model of spinal cord injury: effect of injury severity. J. Neurosci. 21, 559–569.

Qiao, L., Vizzard, M.A., 2002. Up-regulation of tyrosine kinase (Trka, Trkb) receptor expression and phosphorylation in lumbosacral dorsal root ganglia after chronic spinal cord (T8–T10) injury. J. Comp. Neurol. 449, 217–230.

Seki, S., Sasaki, K., Fraser, M.O., Igawa, Y., Nishizawa, O., Chancellor, M.B., de Groat, W.C., Yoshimura, N., 2002. Immunoneutralization of nerve growth factor in lumbosacral spinal cord reduces bladder hyperreflexia in spinal cord injured rats. J. Urol. 168, 2269–2274.

Seki, S., Sasaki, K., Igawa, Y., Nishizawa, O., Chancellor, M.B., De Groat, W.C., Yoshimura, N., 2004. Suppression of detrusor-sphincter dyssynergia by immunoneutralization of nerve growth factor in lumbosacral spinal cord in spinal cord injured rats. J. Urol. 171, 478–482.

Shea, V.K., Cai, R., Crepps, B., Mason, J.L., Perl, E.R., 2000. Sensory fibers of the pelvic nerve innervating the rat's urinary bladder. J. Neurophysiol. 84, 1924–1933.

Shefchyk, S.J., 2002. Spinal cord neural organization controlling the urinary bladder and striated sphincter. Prog. Brain Res. 137, 71–82.

Skene, J.H., Jacobson, R.D., Snipes, G.J., McGuire, C.B., Norden, J.J., Freeman, J.A., 1986. A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. Science 233, 783–786.

Thor, K., Kawatani, M., de Groat, W.C., 1986. Plasticity in the reflex pathways to the lower urinary tract of the cat during postnatal development and following spinal cord injury. In: Goldberger, M.E., Gorio, A., Murray, M. (Eds.), Development and Plasticity of the Mammalian Spinal Cord, Vol. III section II, pp. 65–80.

Tuszynski, M.H., Peterson, D.A., Ray, J., Baird, A., Nakahara, Y., Gage, F.H., 1994. Fibroblasts genetically modified to produce nerve growth factor induce robust neuritic ingrowth after grafting to the spinal cord. Exp. Neurol. 126, 1–14.

Van der Zee, C.E., Nielander, H.B., Vos, J.P., Lopes da Silva, S., Verhaagen, J., Oestreicher, A.B., Schrama, L.H., Schotman, P., Gispen, W.H., 1989. Expression of growth-associated protein B-50 (GAP43) in dorsal root ganglia and sciatic nerve during regenerative sprouting. J. Neurosci. 9, 3505–3512.

Vizzard, M.A., 1999. Alterations in growth-associated protein (GAP-43) expression in lower urinary tract pathways following chronic spinal cord injury. Somatosens Motor Res. 16, 369–381.

Vizzard, M.A., 2000. Changes in urinary bladder neurotrophic factor mRNA and NGF protein following urinary bladder dysfunction. Exp. Neurol. 161, 273–284.

Weaver, L.C., Cassam, A.K., Krassioukov, A.V., Llewellyn-Smith, I.J., 1997. Changes in immunoreactivity for growth associated protein-43 suggest reorganization of synapses on spinal sympathetic neurons after cord transection. Neuroscience 81, 535–551.

Yoshimura, N., Seki, S., Erickson, K.A., Erickson, V.L., Chancellor, M.B., Groat, W.C., 2003. Histological and electrical properties of rat dorsal root ganglion neurons innervating the lower urinary tract. J. Neurosci. 23, 4355–4361.

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011

Yoshiyama, M., Nezu, F.M., Yokoyama, O., de Groat, W.C., Chancellor, M.B., 1999. Changes in micturition after spinal cord injury in conscious rats. Urology 54, 929–933.

Yoshiyama, M., deGroat, W.C., Fraser, M.O., 2000. Influences of external urethral sphincter relaxation induced by alpha-bungarotoxin, a neuromuscular junction blocking agent, on voiding dysfunction in the rat with spinal cord injury. Urology 55, 956–960.

Zinck, N.D.T., Downie, J.W., 2005. Plasticity in the injured spinal cord: can we use it to advantage to reestablish effective bladder voiding and continence? Prog. Brain Res. 152, 147–162.

Please cite this article as: Zinck, N.D.T., et al., Sprouting of CGRP primary afferents in lumbosacral spinal cord precedes emergence of bladder activity after spinal injury, Exp. Neurol. (2007), doi:10.1016/j.expneurol.2007.01.011