Changes in Galanin Immunoreactivity in Rat Lumbosacral Spinal Cord and Dorsal Root Ganglia after Spinal Cord Injury

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ABSTRACT

Alterations in the expression of the neuropeptide galanin were examined in micturition reflex pathways 6 weeks after complete spinal cord transection (T8). In control animals, galanin expression was present in specific regions of the gray matter in the rostral lumbar and caudal lumbosacral spinal cord, including: (1) the dorsal commissure; (2) the superficial dorsal horn; (3) the regions of the intermediolateral cell column (L1–L2) and the sacral parasympathetic nucleus (L6–S1); and (4) the lateral collateral pathway in lumbosacral spinal segments. Densitometry analysis demonstrated significant increases (P ≤ 0.001) in galanin immunoreactivity (IR) in these regions of the S1 spinal cord after spinal cord injury (SCI). Changes in galanin-IR were not observed at the L4–L6 segments except for an increase in galanin-IR in the dorsal commissure in the L4 segment. In contrast, decreases in galanin-IR were observed in the L1 segment. The number of galanin-IR cells increased (P ≤ 0.001) in the L1 and S1 dorsal root ganglia (DRG) after SCI. In all DRG examined (L1, L2, L6, and S1), the percentage of bladder afferent cells expressing galanin-IR significantly increased (4–19-fold) after chronic SCI. In contrast, galanin expression in nerve fibers in the urinary bladder detrusor and urothelium was decreased or eliminated after SCI. Expression of the neurotrophic factors nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) was altered in the spinal cord after SCI. A significant increase in BDNF expression was present in spinal cord segments after SCI. In contrast, NGF expression was only increased in the spinal segments adjacent and rostral to the transection site (T7–T8), whereas spinal segments (T13–L1; L6–S1), distal to the transection site exhibited decreased NGF expression. Changes in galanin expression in micturition pathways after SCI may be mediated by changing neurotrophic factor expression, particularly BDNF. These changes may contribute to urinary bladder dysfunction after SCI. J. Comp. Neurol. 475:590–603, 2004.

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Indexing terms: micturition; lower urinary tract; neuropeptides; NGF; BDNF

Complete transection of the spinal cord rostral to the lumbosacral level (upper motoneuron injury) eliminates voluntary supraspinal control of voiding (Kuru, 1965; Torrens and Morrison, 1987; de Groat and Kruse, 1993). This period of reflex suppression is followed by the emergence of automatic, involuntary reflex micturition with detrusor hyperreflexia and bladder-sphincter dyssynergia (de Groat et al., 1990, 1993, 1997; de Groat and Kruse, 1993; Kruse et al., 1993). Emergence of automatic micturition after spinal cord injury (SCI) may depend on many factors, including: (1) elimination of bulbospinal inhibitory pathways; (2) strengthening of existing synapses or formation of new synaptic connections due to axonal sprouting in the spinal cord; (3) changes in synthesis, release, or action of neurotransmitters; (4) alteration in afferent input from peripheral organs; or (5) central (spinal cord) and peripheral (urinary bladder) changes in the expression of neurotrophic factors. A number of laboratories have demon-

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Galanin immunostaining in specific regions of the lumbar DRG galanin expression; (2) the topography of intensity of galanin staining in detrusor muscle and urothelium (Drake et al., 1996). A role for nerve growth factor (NGF) in regulating survival or regeneration after neural injury have also been suggested (Liu and Hokfelt, 2002). Galanin expression has been documented in the urinary tract tissues (Bauer et al., 1986; Newton, 1992a,b) and modulatory effects on human bladder (Maggi et al., 1999), neurochemical (Vizzard, 1997, 1999, 2000c; Qiao and Vizzard, 2002b). Briefly, the dorsal T7–T9 vertebrae were removed, and the spinal cord was completely transected. The space between the retracted ends of the spinal cord was packed with Gelfoam (Upjohn, Ontario, Canada), and the incision was sutured. Complete spinal transection was visually confirmed at the time of euthanasia and tissue dissection (see below). Following surgery, the animals were housed in cages lined with Alpha-Dri (Shepherd Specialty Papers, Kalamazoo, MI), and their bladders were manually expressed two to three times a day. An antibiotic (150 mg/kg ampicillin, s.c.) was administered prophylactically 1 day prior to surgery and for 3 days postoperatively. The analgesic buprenorphine (0.01 mg/kg, s.c.) was delivered postoperatively every 12 hours for a total of four doses.

Studies were restricted to female animals, as manual expression of the bladder is more easily accomplished in the female rat because of the shorter and concomitantly smaller region occupied by the external urethral sphincter compared with male rats. Animals were studied <1 week or 6 weeks after spinal transection. All animals studied at 6 weeks after SCI had developed bladder-to-bladder and perineal-to-bladder reflexes. All experimental protocols involving animal use were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC #00-114; 03-148). Animal care was under the supervision of the University of Vermont’s Animal Resource Center in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines. All efforts were made to minimize the potential for animal pain, stress, or distress.

Retrograde labeling of bladder afferent neurons

Five to 7 days prior to perfusion, Fast Blue (FB; 4%, weight/volume; Polyol, Gross Umstadt, Germany) was injected into the bladder to label bladder afferent neurons afferently. As previously described (Vizzard, 2000a,b; Qiao and Vizzard, 2002a,b) a total volume of 40 μl divided into six to eight injections was injected into the dorsal surface of the bladder wall with particular care to avoid injections into the bladder lumen, major blood vessels, or overlying fascial layers. At each injection site, the needle was kept in place for several seconds after injection, and the site was washed with saline to minimize contamination of adjacent organs with FB. Bladder afferent cells in the DRG were only identified in control (spinal intact) and chronic SCI rats.

Materials and Methods

Experimental animals

Adult female Wistar rats [Charles River, Canada; 150–200 g; spinal cord intact (control; n = 9); chronic SCI (6 week; n = 8); acute SCI (<1 week; n = 3)] were used for anatomical studies. For spinal cord neurotrophic factor protein determination, six rats for each group (control, chronic SCI and acute SCI) were used. All animals (control and SCI) were housed together prior to and during the entire study for approximately 4 months. Estrous cycle determination was not performed; however, this was not likely to be a major complicating factor in the present studies. In fact, little variation in galanin staining in the lumbar sacral spinal cord or DRG within control or SCI groups was observed.

Spinal cord transection

Spinal cord transection was performed under isoflurane anesthesia (2–2.5%) 6 weeks or <1 week prior to intracardiac perfusion as previously described (Vizzard, 1997, 1999, 2000c; Qiao and Vizzard, 2002b). Briefly, the dorsal T7–T9 vertebrae were removed, and the spinal cord was completely transected. The space between the retracted ends of the spinal cord was packed with Gelfoam (Upjohn, Ontario, Canada), and the incision was sutured. Complete spinal transection was visually confirmed at the time of euthanasia and tissue dissection (see below). Following surgery, the animals were housed in cages lined with Alpha-Dri (Shepherd Specialty Papers, Kalamazoo, MI), and their bladders were manually expressed two to three times a day. An antibiotic (150 mg/kg ampicillin, s.c.) was administered prophylactically 1 day prior to surgery and for 3 days postoperatively. The analgesic buprenorphine (0.01 mg/kg, s.c.) was delivered postoperatively every 12 hours for a total of four doses.

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Perfusion and tissue harvesting

Tissue processing. After spinalization (<1 week or 6 weeks), animals were deeply anesthetized with isoflurane
(3–4%) and then euthanized via intracardiac perfusion first with oxygenated Krebs buffer (95% O₂, 5% CO₂) followed by 4% paraformaldehyde. After perfusion, the spinal cord and DRG were quickly removed and postfixed for 6 hours. Tissues were then rinsed in phosphate-buffered saline (PBS; 0.1 M NaCl, in phosphate buffer, pH 7.4) and placed in ascending concentrations of sucrose (10–30%) in 0.1 M PBS for cryoprotection. Spinal cord segments were identified based on at least two criteria: (1) the T13 DRG exists after the last rib; and (2) the L6 vertebra is the last moveable vertebra followed by the fused sacral vertebrae.

Another less precise criterion is the observation that the L6 DRG are the smallest ganglia following the largest, L5, DRG. DRG sections from the L1, L2, and L4–S1 spinal segments were sectioned parasagitally at a thickness of 20 μm on a freezing microtome. Some DRG (L1, L2, L6, and S1) were specifically chosen for analysis based on the previously determined segmental representation of urinary bladder circuitry (Donovan et al., 1983; Keast and de Groat, 1992; de Groat et al., 1994; Nadelhaft and Vera, 1995). Bladder afferents are not distributed within the L4–L5 DRG (Donovan et al., 1983; Keast and de Groat, 1992), which contain only somatic afferents, nor are neurons that are involved in urinary bladder function observed in the L4–L5 spinal segments (de Groat et al., 1994; Nadelhaft and Vera, 1995). Thus, the L4–L5 DRG served as internal controls for these studies. Tissues from control animals with an intact spinal cord were handled in an identical manner to that described above.

**Spinal cord tissue harvest and protein extraction.** Acute (<1 week; n = 6) and chronic (6 week; n = 6) SCI rats and spinal intact (control; n = 6) rats were used for protein determinations. Rats were euthanized by isoflurane (4%) plus thoracotomy. The spinal cord and associated DRG were quickly dissected with the animal placed on ice. After dissection, the spinal cord was quickly segmented and pinned on a Sylgard dish placed on ice. Spinal cord segments were grouped and pinned to the site of spinal transection (T8). Thus, spinal cord segments T7–T8, T9–T10, T11–T12, T13–L1, L2–L3, L4–L5, and L6–S1 were grouped and transferred to tubes for weighing and protein extraction. In these studies, the spinal segments T7–T8 were located immediately rostral to the site of injury, and the T9–T10 grouping was located immediately caudal to the site of injury. Animals with a spinal transection at a spinal segment other than the T8 level were excluded from this analysis.

**Preparation of ELISA samples.** Adult rats were euthanized and the bladder (n = 6 for each time point; n = 8 for control) was rapidly dissected and weighed. Individual bladders were solubilized in T-PER Tissue Protein Extraction Reagent (1 g tissue/20 ml; Pierce Biotechnology, Woburn, MA) with Complete (protease inhibitor cocktail tablets; Roche, Germany). Bladder tissue was disrupted with a Polytron homogenizer until it was homogeneous and then centrifuged (10,000 rpm for 5 min). The supernatants were used for NGF and BDNF quantification as previously described (Vizzard, 2000b; Vizzard et al., 2000). Total protein was determined by the Coomassie Plus Protein Assay Reagent Kit (Pierce). According to the manufacturer, the NGF E-max immunoassay system (Promega, Madison, WI) demonstrates very low cross-reactivity with structurally related growth factors [recombinant human BDNF (rhBDNF), neurotrophin 3 (rhNT3), and neurotrophin 4 (rhNT4)] at concentrations as high as 10 ng/ml as indicated by the manufacturer. According to the manufacturer (Promega), the BDNF E-max immunoassay system shows less than 3% cross-reactivity to NGF, NT-3, and NT-4 at concentrations as high as 100 ng/ml.

The NGF standard provided with this system generated a linear standard curve from 7.8 to 500 pg/ml (r² = 0.997, P ≤ 0.001). Similarly, the BDNF standard provided with this system generated a linear standard curve from 7.8 to 500 pg/ml (r² = 0.998, P ≤ 0.001). Absorbance values of standards and samples were corrected by subtraction of the background value (absorbance due to nonspecific binding). Samples were diluted to bring the absorbance values onto the linear portion of the standard curve. No samples fell below the minimum detection limits of the assay. Curve fitting of standards and evaluation of NGF or BDNF content of samples was performed using a least squares fit. All values are means ± SEM. Comparisons of NGF or BDNF protein in urinary bladder samples in control (spinal intact) and SCI rats were made using analysis of variance. When F ratios exceeded the critical value (P ≤ 0.05), Dunnett’s test was used to compare the control mean with the experimental mean.

**Whole mount bladder preparation.** After SCI had been performed 3 weeks or <1 week before, animals were euthanized as described above. Prior to intracardiac perfusion with fixative, the urinary bladder was dissected and placed into Krebs solution (119.0 mmol NaCl, 4.7 mmol KCl, 24.0 mmol NaHCO₃, 1.2 mmol KH₂PO₄, 1.2 mmol MgSO₄ · 7H₂O, 11.0 mmol glucose, 2.5 mmol CaCl₂) (Zvarova et al., 2003). The bladder was cut open through the urethra in the midline and pinned flat on a Sylgard-coated dish. After maximal stretch of the tissue, the bladder was incubated for 1.5 hours at room temperature in cold fixative (2% paraformaldehyde + 0.2% picric acid), and the urothelium was removed. Urothelium and bladder musculature were examined separately for galanin immunoreactivity (IR) by a free-floating technique.

**Galanin immunohistochemistry** Spinal cord sections, detrusor, and urothelium for both control and SCI animals were processed for galanin-IR by using a free-floating method. DRG were immunostained using an on-slide processing technique. Groups of control animals and experimental animals were processed simultaneously to decrease the incidence of variation in staining and background that can occur between sections and between animals. Spinal cord sections, detrusor, or urothelium were incubated overnight at room temperature with rabbit anti-galanin antibody (1:3,000; Phoenix Pharmaceuticals, Belmont, CA) in 1% goat serum and 0.1 M KPBS and then washed (3 × 10 minutes) with 0.1 M KPBS, pH 7.4. Tissue was then incubated with Cy3-conjugated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature. After several rinses with 0.1 M KPBS, slides were coverslipped using Citifluor (Citifluor, London, UK). Control tissues incubated in the absence of primary or secondary antibody were also processed and evaluated for specificity or background staining levels. In the absence of primary antibody, no positive immunostaining for galanin was observed.

**Data analysis**

Tissues were examined under an Olympus fluorescence photomicroscope for visualization of Cy3 and FB. Cy3 was
visualized with a filter with an excitation range of 560–596 nm and an emission range from 610 to 655 nm. In DRG from spinal intact and SCI rats, galanin-IR cell profiles were counted in 10–15 sections of each selected DRG (L1, L2, and L4–S1). Only cell profiles with a nucleus were quantified. DRG sections with FB-labeled cells were viewed using a filter with an excitation wavelength of 340–380 nm and an emission wavelength of 420 nm. Cells colabeled with FB + galanin-IR were similarly counted. Numbers of galanin-IR cell profiles per DRG section are presented (mean ± SEM). The percentage of presumptive bladder afferent cells (FB-labeled) expressing galanin-IR in each DRG examined is also presented (mean ± SEM). The results are not corrected for double counting. Comparisons between spinal intact and SCI groups were made using analysis of variance. Percentage data were arcsinus-transformed to meet the requirements of this statistical test. Animals, processed and analyzed on the same day, were tested as a block in the analysis of variance. Thus, day was treated as a blocking effect in the model. Two variables were being tested in the analysis: (1) experimental manipulation versus control situation; and (2) the effect of day [i.e., tissue from groups (experimental and control) of animals were processed on different days]. When F ratios exceeded the critical value (P < 0.05), the Newman-Keuls test was used for multiple comparisons among means.

Assessment of positively stained DRG cells. Staining observed in experimental tissue was compared with that observed from experiment-matched negative controls. DRG cells exhibiting immunoreactivity that was greater than the background level observed in experiment-matched negative controls were considered positively stained. Positively counted cells were not further divided into categories of different staining intensities.

Spinal cord densitometry. The density of galanin-IR in specific regions of spinal cord was determined with densitometry analysis (Image-Pro express, version 4.0, Media Cybernetics, L.P., Silver Spring, MD) as previously described (Vizzard, 1999; Vizzard, 2000e). Spinal cord segments were sectioned entirely from rostral to caudal. Every third and sixth tissue section was then processed for galanin-IR. Of these tissue sections, every first and fifth tissue section was then processed for semiquantitative analysis of galanin-IR. We did not select sections based on staining intensity, and no sections were discarded from analysis because of low staining. Because stratification does not take the periodicity of the staining into account, it is ranked with respect to staining intensity. The following regions of spinal cord from both experimental and control animals were analyzed: the superficial lateral dorsal horn (LDH) and medial dorsal horn (MDH), the dorsal commissure (DCM) region, the region of the sacral parasympathetic nucleus (SPN; L6, S1), the region of the intermediolateral cell column (IML; L2), and the region of lateral collaterals pathway (LCP; L6, S1) (Fig. 1Aa). Five randomly chosen sections from each spinal segment examined were viewed with a 4× objective and captured through a video camera attachment to the microscope with exposure time, brightness, and contrast being held constant. The image was converted into pixels on the computer monitor according to a gray scale that ranged in intensity from 0 (white) to 255 (black). The spinal cord section was centered in the field, and a standard size square was overlaid on the areas of interest (LDH, MDH, DCM, SPN, IML, and LCP regions). The labeled area within the square was measured (Fig. 1Ac). Transmission (t) was calculated as t = (gray level + 1/256). Optical density (OD) was derived from OD = −log t. Comparisons among control and experimental groups were made using analysis of variance. When F ratios exceeded the critical value (P < 0.05), Dunnett’s test was used to compare the control mean with the experimental mean.

Figure preparation

Digital images were obtained using a CCD camera (MagnaFire SP; Optronics; Optical Analysis, Nashua, NH) and LG-3 frame grabber attached to an Olympus microscope (Optical Analysis). Exposure times, brightness, and contrast were held constant when images were acquired from spinal intact and SCI animals; images were processed and analyzed on the same day. Images were imported into Adobe (San Jose, CA) Photoshop 7.0, and groups of images were assembled and labeled.

RESULTS

Galanin-IR in spinal cord: distribution and general characteristics.

In spinal intact animals in all segmental levels (L1, L2, and L4–S1) examined, galanin-IR was expressed in distinct regions of the rat spinal cord. Some galanin-IR was unique to specific segmental levels (i.e., rostral lumbar, L1–L2 or lumbosacral spinal cord, L6–S1), and other staining was similar in all segmental levels (L1, L2, and L4–S1) examined. In all segmental levels examined in control animals, galanin-IR was expressed in the superficial dorsal horn (medial and lateral laminate I–II; Fig. 1A–D). Galanin-IR was observed in the region of the dorsal commissure located dorsal to the central canal (Fig. 1Aa,Ba,Ca,Da). In the rostral lumbar (L1–L2; Fig. 1Aa,Ba) or lumbosacral (L6–S1; Fig. 1Ca,Da) spinal cord, galanin-IR was also expressed in the region of the IML (Fig. 1Aa) or in the region of the SPN, respectively (Fig. 1Ca,Da). Little if any galanin-IR was observed in the ventral horn of any segmental level examined. Some galanin-IR was observed in control animals in the lateral collaterals pathway in the L6–S1 segments (Fig. 1Ca,Da). The LCP of Lissauer refers to the central projections of visceral afferents in the pelvic nerve that have been labeled in the rat and cat by axonal transport of horseradish peroxidase (Morgan et al., 1981; Steers and de Groat, 1988; Steers et al., 1991a). In the spinal cord, galanin-IR was not expressed in neuronal cell bodies.

Changes in galanin-IR in the spinal cord following SCI

Following SCI, galanin-IR was expressed in identical spinal cord regions observed for spinal intact animals. However, the intensity and the overall distribution of the staining were increased in specific spinal cord segments and regions (see below). In the L1 segment, decreases in galanin expression were observed. As seen in spinal intact animals, no galanin-IR was present in neuronal cell bodies in the spinal cord following SCI (Fig. 1Ab,Bb,Cb,Db). Galanin-IR was restricted to nerve fibers in the spinal cord following SCI (Fig.1Ab,Bb,Cb,Db).
Fig. 1. Galanin immunoreactivity (IR) is altered in specific regions of the lumbosacral [L1 (A), L2 (B), L6 (C), S1 (D)] spinal cord. Fluorescence images of galanin-IR in L1, L2, L6, and S1 spinal segments in control (spinal intact; Aa,b; Ba,b; Ca,b; Da,b) or after spinal cord injury (SCI; Ab,d; Bb,d; Cb,d; Db,d). The image was converted to a gray scale that ranges in intensity from 0 (white) to 255 (black) (Ac,d; Bc,d; Cc,d; Dc,d). The spinal cord section was centered in the field, and a standard size square was overlaid on the areas of interest: LDH, lateral dorsal horn; MDH, medial dorsal horn; DCM, dorsal commissure; SPN, sacral parasympathetic nucleus; IML, intermediolateral cell column; LCP, lateral collateral pathway. The labeled area within the square was measured (Ac; see Fig. 2). Scale bar = 150 μm in Bb (applies to all).
Galanin-IR is altered in rostral lumbar spinal cord (L1–L2) following SCI

Following SCI, galanin-IR decreased in several regions in the rostral lumbar L1 spinal cord compared with control (Fig. 1Ab,Ad). The density of galanin-IR was significantly decreased, by twofold ($p \leq 0.001$) in the superficial laminae (I–II) of the dorsal horn (Figs. 1Ab,Ad, 2A). No significant changes in galanin-IR were observed in the region of the dorsal commissure, but a significant decrease in galanin-IR was exhibited in the LCP following SCI. In contrast, galanin-IR was only increased in the IML of the L2 segment (Figs. 1Ba–d, 2B).

Galanin-IR is increased in sacral spinal cord (S1) following SCI

For the most part, galanin-IR was unchanged in the L4–L6 segments (Figs. 1Ca–d, 2C–E) following SCI; however, galanin-IR was increased in the DCM region of the L4 segment (Fig. 2C). In contrast, significant ($p \leq 0.001$) changes in galanin-IR were detected in the S1 spinal cord following SCI (Figs. 1Db,Dd, 2F). In the S1 spinal segment, galanin-IR was increased in the dorsal commissure (1.2-fold), LCP (1.4-fold), and SPN (1.4-fold; Fig. 2F).

Galanin-IR in lumbosacral dorsal root ganglia (DRG)

In contrast to galanin-IR in the spinal cord, galanin-IR in the DRG (L1–S1) was expressed by neuronal cell bodies and fibers throughout each DRG examined (Fig. 3A1,B1). In control animals, galanin-IR was present in modest numbers of cells in the L1–S1 DRG (Figs. 3A1,B1, 4A). The number of galanin-IR cells among the DRG examined was comparable (range 12–29 galanin-IR cell profiles/section). Following SCI (6 weeks), galanin-IR was significantly ($p \leq 0.001$) increased in the rostral lumbar (L1) and sacral (S1) DRG (Fig. 4A). Both small (17.5 ± 4.2 μm) and medium (23.5 ± 3.5 μm) sized DRG cells expressed galanin-IR in control animals and following SCI. No change in numbers of cells expressing galanin-IR was observed in the L2 and L4–L6 DRG following SCI (Fig. 4A). We examined galanin-IR after acute SCI (<1 week) to determine whether we had missed an earlier increase in galanin expression that might have returned to control levels by 6 weeks after SCI. Galanin-IR in the DRG after acute SCI (<1 week) was not different from control (Fig. 4A).

Galanin-IR in bladder afferent cells is increased following SCI

To determine whether galanin-IR was expressed in bladder afferent cells, FB was injected into the urinary bladder to label bladder afferent cells retrogradely in the L1, L2, L6, and S1 DRG (Fig. 3A2,B2). In control animals, approximately 1.5% of bladder afferent cells in the L1, L2, L6, or S1 DRG exhibited galanin-IR (Figs. 3B2, 4B). Following SCI (6 weeks), the percentage of bladder afferent cells exhibiting galanin-IR significantly ($p \leq 0.001$) increased in the L6 (4.5 ± 0.7%) and S1 DRG (18.4 ± 7.5%) and in the L1–L2 DRG (L1, 4.6 ± 0.5%; L2, 7.2 ± 2.2%; Fig. 4B). The number of galanin-IR cell profiles/section in DRG (L1–S1) examined from control animals or SCI animals was similar with or without the presence of FB (data not shown).

Galanin-IR in the detrusor muscle and urothelium in control and SCI rats

In spinal intact animals, galanin-IR nerve fibers were present in the detrusor and urothelium. In the detrusor, prominent galanin-IR fibers were observed just dorsal to the point of ureter insertion (Fig. 5A). Galanin-IR fibers were also present in the bladder dome (Fig. 5B) and urothelium (Fig. 5E) facing the detrusor. After either acute (Fig. 5C) or chronic SCI (Fig. 5D), galanin-IR was decreased throughout the detrusor including the region dorsal to ureter insertion (Fig. 5C) and absent in the urothelium (Fig. 5F). No galanin-IR fibers were present on the adventitia portion of the detrusor.

NGF and BDNF expression in spinal cord in control rats and after SCI

NGF was increased in the spinal segments immediately rostral to the spinal injury site 6 weeks after SCI (Fig. 6). In contrast, NGF was decreased in the spinal segments immediately caudal to the spinal injury site within 1 week after SCI (Fig. 6). Other changes were limited to the T13–L1 and L6–S1 segments, where significant decreases in NGF content were detected with both acute (<1 week) and chronic (6 weeks) SCI (Fig. 6). Increases in BDNF content were detected in all spinal cord segments both rostral and caudal to the site of injury except for the L2–L3 spinal segments (Fig. 7). Increases in BDNF content were demonstrated following acute or chronic SCI, and the increases were of similar magnitude (two- to threefold; Fig. 7). BDNF content increased only acutely in the T11–T12 and L4–L5 spinal segments (Fig. 7).

DISCUSSION

The present studies demonstrate significant changes in galanin-IR in specific regions of the lumbosacral spinal cord after chronic (6-week) SCI. Although decreases in galanin expression were observed in the L1 spinal segment, other (L2, L4, and S1) spinal cord segments examined exhibited increased galanin expression. The number of galanin-IR cells also increased in the L1 and S1 DRG after acute (<1 week) and/or chronic (6-week) SCI. To determine which DRG cells represented bladder afferent DRG cells, the conventional tracer Fast Blue was injected into the urinary bladder smooth muscle. In all DRG examined (L1, L2, L6, and S1), the percentage of bladder afferent cells expressing galanin-IR significantly increased (4–19-fold) after chronic SCI. In contrast, galanin expression in nerve fibers in the urinary bladder detrusor and urothelium was decreased or eliminated after acute or chronic SCI. To determine whether the expression of neurotrophic factors, NGF and BDNF, was altered in the spinal cord after acute or chronic SCI, NGF and BDNF protein content was determined in spinal cord segments adjacent and distal to the spinal transection site. A significant increase in BDNF expression was present in the majority of spinal cord segments examined after SCI. In contrast, NGF expression was only increased in the spinal segments adjacent and rostral to the transection site (T8–T9), whereas spinal segments (T13–L1 and L6–S1) distal to the transection site exhibited decrease NGF expression.

Transaction of the spinal cord that interrupts the spinobulbospinal micturition reflex pathway, blocks voluntary voiding and initially produces an areflexic bladder...
Fig. 2. Histograms representing optical density (O.D.) measurements of galanin immunoreactivity (IR) in specific regions of the L1 (A), L2 (B), L4 (C), L5 (D), L6 (E), and S1 (F) spinal segments in control (spinal intact) and SCI rats 6 weeks after complete transection of the spinal cord. Intensity of galanin-IR was determined in the medial dorsal horn (MDH), lateral dorsal horn (LDH), dorsal commissure (DCM), and lateral collateral pathway (LCP), and in the region of the intermediolateral cell column (IML) and sacral parasympathetic nucleus (SPN). A: Significant decreases ($P < 0.001$) in galanin-IR were demonstrated in the MDH, LDH, and LCP regions of the L1 spinal segment. B,C: Significant increases ($P < 0.001$) in galanin-IR were detected in the IML of the L2 spinal segment (B) and in the DCM of the L4 spinal segment (C). D,E: No changes in galanin-IR were detected in the L5 (D) or L6 (E) spinal segments. F: Significant ($P < 0.001$) increases were demonstrated in the DCM, LCP, and SPN regions of the S1 spinal segment.
with complete urinary retention (Kuru, 1965; Torrens and Morrison, 1987; de Groat et al., 1993). However, depending on the species, reflex bladder activity slowly recovers over the course of weeks or months. In chronic spinally injured animals, reflex mechanisms in the lumbosacral spinal cord are capable of duplicating many of the functions performed by reflex pathways in the spinal cord intact animal and can induce bladder hyperreflexia (de Groat and Ryall, 1969; de Groat, 1975). However, the bladder does not empty efficiently due to a loss of bladder-sphincter coordination (McGuire and Brady, 1979; Morrison, 1987; Wyndaele, 1987; de Groat et al., 1993; de Groat and Kruse, 1993; Kruse et al., 1995).

In contrast to normal animals in which the sphincter relaxes during voiding, SCI animals exhibit sphincter contractions (bladder-sphincter dysynergia) during voiding, an increase in urethral outlet resistance, urinary retention, bladder hyperreflexia, bladder overdistension, and
an increase in bladder afferent cell size (McGuire and Brady, 1979; Morrison, 1987; Wyndaele, 1987; de Groat et al., 1993; de Groat and Kruse, 1993; Kruse et al., 1995). Changes in electrophysiological (Yoshimura and de Groat, 1993a; Yoshimura and de Groat, 1997; Yoshimura, 1999) or neurochemical properties of bladder afferent cells in the DRG could contribute to the emergence of the spinal micturition reflex (de Groat et al., 1993; de Groat and Kruse, 1993), bladder hyperreflexia, and/or changes in the pharmacologic responses of reflex pathways (Yoshimura and de Groat, 1993a; Yoshimura and de Groat, 1997; Yoshimura, 1999) in the lumbosacral spinal cord after SCI.

Fig. 5. Galanin immunoreactivity (IR) in urinary bladder detrusor (D) and urothelium (U) from control and spinal cord-injured (SCI) rats. Galanin-IR was present throughout the detrusor. In the detrusor, prominent galanin-IR fibers were present in the trigone in control rats (A). These thick fibers extend from the trigone to the bladder body. After SCI (5 days), a reduction in galanin-IR was observed in this same area (C). Galanin-IR fibers were also present in the bladder detrusor dome (B) and urothelium (E). After either acute (5-day SCI; C) or chronic SCI (3-week SCI; D), galanin-IR was decreased throughout the detrusor including the bladder body (D) and absent in the urothelium (U; F). Scale bar = 100 μm.
The changes that occur in spinal voiding reflexes following SCI appear to be similar in humans and experimental animals and are beginning to provide important insights into a variety of neurogenic disorders of the lower urinary tract (de Groat et al., 1993; de Groat and Kruse, 1993). A major breakthrough has been the recognition that C-fiber bladder afferents can trigger bladder hyperactivity (de Groat et al., 1981, 1990, 1993; Fall et al., 1990). In spinalized cats, the properties of C-fiber bladder afferents are altered, so they become mechanosensitive and now respond to bladder distension (de Groat et al., 1981, 1990, 1993; Fall et al., 1990). In chronic spinal cord injury, C-fiber afferent-evoked bladder reflexes emerge; however, in cats with an intact spinal cord, myelinated (A-/H9254) afferents activate the micturition reflex (de Groat and Ryall, 1969; de Groat, 1975; de Groat et al., 1993). de Groat and colleagues (de Groat et al., 1990, 1993; de Groat and Kruse, 1993) have demonstrated that systemically administered capsaicin, a C-fiber neurotoxin, blocked bladder hyperreflexia in the chronic paraplegic cat but was without effect in spinal intact cats. In the rat, both the spinal and supraspinal micturition reflexes are activated by capsaicin-resistant Aδ afferents (Mallory et al., 1989); however, capsaicin-sensitive afferents do appear to modulate micturition under certain conditions (Maggi, 1991, 1993).

The mechanisms underlying the emergence of the C-fiber-evoked reflex are unknown; however, recent experiments have begun to examine changes in the electrical properties of afferent neurons innervating the urinary bladder of the adult rat before and after SCI (Yoshimura and de Groat, 1993a, 1997; Yoshimura, 1999). These studies suggest an ionic mechanism underlying the relative inexcitability of C-fiber bladder afferents in normal animals and the increased excitability of these afferents after SCI (Yoshimura and de Groat, 1993a, 1997; Yoshimura, 1999). Changes in the electrophysiological properties of bladder afferent neurons after SCI may occur concomitantly with changes in neurochemical properties described in this and previous studies (Yoshimura and de Groat, 1993b; Vizzard, 1997, 1999, 2000c; Vizzard et al., 2001). Urinary bladder hyperreflexia after SCI may reflect a change in the balance of neuroactive compounds in bladder reflex pathways. Our previous studies have examined changes in neuronal nitric oxide synthase (nNOS; Vizzard, 1997) and pituitary adenylate cyclase-activating polypeptide (PACAP; Vizzard et al., 2001, 2003) immunoreactivity in micturition reflex pathways after SCI. After chronic SCI, bladder afferent neurons in the DRG significantly increased expression of both nNOS and PACAP-IR (Vizzard, 1997; Vizzard et al., 2001, 2003). We have previously suggested that increases in the expression of these two neuroactive compounds contribute to urinary bladder hyperreflexia after SCI (Vizzard, 1997; Vizzard et al., 2001, 2003). In support of this hypothesis, intrathecal administration of a PAC1 receptor PACAP antagonist significantly reduced bladder hyperreflexia after SCI (Vizzard et al., 2003). Previous studies have demonstrated that galanin has a potent neuromodulatory action on isolated human detrusor where galanin suppresses the cholinergic component of the response to electric field stimulation (Maggi et al., 1987). Thus, an inhibitory action for galanin on neurotransmitter release has been suggested in smooth muscle tissues and may also pertain to the urinary bladder (Maggi et al., 1987). Increases in galanin expression in bladder afferent cells in the DRG may there-

Fig. 6. Changes in total spinal cord nerve growth factor (NGF) as detected with an NGF ELISA after spinal cord injury (SCI; <1 week or 6 weeks). The line drawing at the top represents the spinal cord, and the vertical line indicates the position of the spinal cord transection at T8. A significant increase in total NGF from the T7–T8 spinal segments was present 6 weeks after SCI. Significant decreases in total NGF in the T9–T10, T13–L1, and L6–S1 spinal segments were present after acute or chronic SCI compared with control values. *, P ≤ 0.001.
fore act to oppose the actions of PACAP and nNOS, as well as others still to be identified, in micturition reflex pathways after SCI (Vizzard, 1997; Vizzard et al., 2001, 2003).

Galanin may also play a modulatory role in central micturition reflexes after SCI because significant changes in galanin expression were observed in specific regions of the L1, L2, L4, and S1 spinal segments. Previous studies have suggested a potential role for galanin in lumbosacral spinal cord pathways because of its localized expression (Newton, 1992a,b). In addition, a sexual dimorphism has been demonstrated, with male rats expressing greater levels of galanin in lumbosacral spinal cord compared with females (Newton, 1992a,b). In female rats, galanin expression has been shown to vary with the estrous cycle (Newton, 1992a). In the present study, very little variation in galanin immunostaining was observed within female control or female SCI groups (Figs. 2, 4).

Previous studies have demonstrated a reduction in a number of neuroactive compounds, including galanin, in the human detrusor and suburothelial plexus after SCI (Drake et al., 2000). The present studies also confirm a dramatic reduction or disappearance of galanin expression in nerve fibers in rat detrusor and urothelium after acute (<1-week) or chronic (3-week) SCI. Although bladder afferent cells in the lumbosacral DRG increase expression of galanin after SCI, there is not a corresponding increase in galanin expression in peripheral nerve fibers in the urinary bladder. It has been suggested (Drake et al., 2000) that the suburothelial denervation observed after SCI decreases the likelihood that nonvoiding bladder contractions originate from exaggerated bladder afferent activity after SCI. However, if the role of galanin is inhibitory, as previously suggested (Maggi et al., 1987), then the absence of galanin in the urinary bladder could result in an excitatory shift (i.e., disinhibition) in the balance of afferent activity and could contribute substantially to bladder hyperreflexia and nonvoiding bladder contractions after SCI.

A large number of studies have demonstrated that pathological changes in a target organ after SCI can alter the neurochemical (Vizzard, 1997, 1999; Yoshimura et al., 1998; Yoshimura, 1999; Vizzard et al., 2001, 2003), electrical (Yoshimura and de Groat, 1993a,c, 1997; Yoshimura, 1999), and organizational (Vizzard, 2000c) properties of micturition reflex pathways. A possible mechanism underlying these changes may involve neurotrophic factors and/or neural activity arising in the bladder. Previous experiments have demonstrated target organ to neuron interactions in the adult animal (Steers and de Groat, 1988; Steers et al., 1991a,b, 1996; Tuttle et al., 1994; Zvara et al., 2002). Furthermore, a recent study from this laboratory has demonstrated changes in mRNA and/or protein expression of neurotrophic factors in the urinary bladder after complete SCI including, bNGF, BDNF, glial-derived neurotrophic factor (GDNF), NT-3, and NT-4 (Vizzard, 2000b). Both acute and chronic SCI result in significant increases in NGF, BDNF, GDNF, NT-3, and NT-4 transcript expression as well as increased NGF protein expression in urinary bladder 4–6 weeks after SCI (Vizzard, 2000b). It has also been reported that NGF levels modestly increase in the transected spinal cord (Frisen et al., 1992; Krenz and Weaver, 2000) after SCI.
The present studies have also demonstrated a significant increase in NGF content in spinal segments adjacent to the transection site. However, other spinal segments (T9–T10, T13–L1, and L6–S1) exhibit decreased NGF protein content with acute or chronic SCI. In contrast, BDNF protein content significantly increased in the majority of spinal segments examined. No spinal segments exhibited a decrease in BDNF protein content after SCI, in contrast to that seen for NGF. Thus, bladder afferent neurons may have at least two potential sources of increased BDNF following SCI: (1) central terminals in the spinal cord; and (2) peripheral terminals in the urinary bladder (Vizzard, 2000b). The results of these studies may focus new attention on the potential role of BDNF in micturition reflex plasticity after SCI.

Changes in neurotrophic factor expression in lower urinary tract (LUT) tissues after SCI may be associated with changes in the neurochemical properties of LUT tissues. At sites of tissue injury, inflammation, or target organ hypertonphy, upregulation of cytokines and growth factors is seen, which can result in the upregulation of NGF (Lindholm et al., 1987; Lewin and Mendell, 1993; Meller et al., 1994; Dray, 1995; Woolf et al., 1997). NGF is then transported from its source (e.g., urinary bladder or spinal cord) to afferent cells in the DRG, where it then binds to its receptor, tyrosine kinase A (TrkA). Recent studies (Qiao and Vizzard, 2002b) from this laboratory have demonstrated significant increases in the percentage of bladder afferent neurons in the DRG that express the tyrosine kinase membrane receptors TrkA or TrkB. Excess NGF within the DRG may induce increased production of neurotrophins [i.e., substance P, calcitonin gene-related peptide (CGRP), and PACAP] in sensory neurons (Donnerer et al., 1992; Donnerer and Stein, 1992; Gary and Hargreaves, 1992; Woolf et al., 1997).

An increase in the levels of neuroactive compounds (e.g., enkephalin (Lewin and Mendell, 1993), dynorphin (Ruda et al., 1988), CGRP (Donnerer and Stein, 1992; Gary and Hargreaves, 1992; Woolf et al., 1997; Vizzard, 2001), substance P (Ruda et al., 1988; Gary and Hargreaves, 1992; Lewin and Mendell, 1993; Vizzard, 2001), neuropeptide Y (Lewin and Mendell, 1993), nNOS (Vizzard et al., 1995; Vizzard and de Groat, 1996; Vizzard, 1997), and PACAP (Jongsma et al., 2000; Vizzard, 2000c) following noxious peripheral stimulation, cyclophosphamide-induced cystitis (Vizzard and de Groat, 1996; Vizzard, 2000d,e, 2001), or SCI (Vizzard and de Groat, 1996; Vizzard, 1999, 2000c; Vizzard et al., 2001) has also been demonstrated in DRG cells as well as in spinal cord neurons. Furthermore, intravesical administration of exogenous NGF in animals may facilitate afferent firing and induce bladder hyperreflexia that is blocked by anti-NGF (Dmitrieva et al., 1997). Thus, changes in the expression of neurotrophic factors after SCI may result in changes in the neurochemical phenotype of bladder afferent cells in DRG as well as contribute to urinary bladder dysfunction.

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