Increased expression of spinal cord Fos protein induced by bladder stimulation after spinal cord injury

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Received 23 November 1999; accepted in final form 31 January 2000

Vizzard, Margaret A. Increased expression of spinal cord Fos protein induced by bladder stimulation after spinal cord injury. Am J Physiol Regulatory Integrative Comp Physiol 279: R295–R305, 2000.—These studies examined Fos protein expression in spinal cord neurons synthetically activated by stimulation of bladder afferent pathways after spinal cord injury (SCI). In urethan-anesthetized Wistar rats after SCI for 6 wk, intravesical saline distension significantly (P ≤ 0.005) increased the number of Fos-immunoreactive (IR) cells in the rostrolumbar (L1, 38 cells/section; L2, 29 cells/section) and caudal lumbosacral (L6, 140 cells/section; S1, 110 cells/section) spinal cord compared with control animals, but Fos expression in the L5 segment was not altered. The distribution of Fos-IR cells was also altered in the lumbosacral spinal cord. Significantly greater numbers of Fos-IR cells were distributed in the dorsal commissure and medial and lateral dorsal horn after intravesical distension in SCI animals. Large percentages of parasympathetic (75%) and sympathetic (85%) preganglionic neurons also expressed Fos-IR after intravesical distension in SCI animals. These results demonstrate that bladder distension produces increased numbers and an altered distribution pattern of Fos-IR cells after SCI. This pattern resembles that after noxious irritation of the bladder in control animals. Pretreatment with capsaicin significantly reduced the number of Fos-IR cells induced by bladder distension after SCI. These data suggest that SCI can reveal an altered Fos expression pattern in response to a nonnoxious bladder stimulus that is partially mediated by capsaicin-sensitive bladder afferents.

lumbosacral spinal cord; neurotrophic factors; choline acetyltransferase; preganglionic neurons; urinary bladder dysfunction; capsaicin

SPINAL CORD INJURY (SCI) above the lumbosacral spinal cord alters the coordination between the urinary bladder and external urethral sphincter and chronically impairs micturition in humans, cats, and rats (12, 24, 34). SCI produces an initial period of urinary bladder areflexia that persists for several weeks to months depending on the species. This period is followed by the emergence of a micturition reflex at the spinal level and the appearance of spontaneous, involuntary bladder contractions (12). However, the micturition reflex in chronically spinalized animals is characterized by simultaneous bladder and external urethral sphincter contractions (bladder-sphincter dyssynergia) leading to inefficient bladder emptying, large residual urine volumes, and urinary bladder hypertrophy (12, 20, 23).

The changes that occur in spinal voiding reflexes after 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 (LUT) (12, 14). Recent experiments from several laboratories (23, 36–38, 44, 45) have demonstrated that chronic SCI above the lumbosacral level in the rat results in urinary bladder hypertrophy and changes in the neurochemical properties of bladder afferent and spinal cord pathways as well as changes in the electrical properties of bladder afferent cells in the dorsal root ganglia. A major breakthrough has been the recognition that C fiber bladder afferents can trigger bladder hyperactivity after SCI (12, 13, 16, 19). In spinalized cats, the properties of C fiber bladder afferents are altered, so that they become mechanosensitive and now respond to bladder distension (12, 13, 16). In chronic SCI, C fiber afferent-evoked bladder reflexes emerge; however, in cats with an intact spinal cord, myelinated (Aδ) afferents activate the micturition reflex (10, 12, 17). After SCI, dramatic changes in the properties of C fiber afferents have also been detected in the rat (42, 45). These changes suggest considerable reorganization of reflex connections in the spinal cord and marked changes in the properties of micturition reflex pathways after chronic SCI.

Previous studies (3, 4, 39) have used immediate early gene expression as a marker for postsynaptic activation of spinal cord neurons receiving afferent input from the LUT. Studies (3, 4, 39) have revealed that noxious and nonnoxious stimulation of the rat LUT increased the number of Fos-immunoreactive (IR) neurons in discrete regions of the L6-S1 spinal cord, including the superficial lateral and medial dorsal horn (LDH, MDH, respectively), the dorsal commissure (DCM), and the region of the sacral parasympathetic nucleus (SPN). Noxious stimulation activated greater numbers of Fos-IR neurons in the DCM, whereas nonnoxious stimulation elicited a greater response in the...
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SPN (4). Surprisingly, Fos protein expression was not detected in rostralumbar (L1-L2) spinal segments after stimulation (noxious or nonnoxious) of the LUT, although these segments receive afferent input from the urinary bladder (3).

These present studies examined Fos protein expression in spinal cord neurons synaptically activated by stimulation of bladder afferent pathways after chronic SCI. It was hypothesized that after SCI, increased numbers of Fos-IR cells induced by urinary bladder distension (nonnoxious) would be observed in the spinal cord due to 1) central changes in synaptic plasticity and/or membrane excitability, 2) peripheral changes in afferent terminal excitability in the urinary bladder, or 3) absence of tonic descending modulation systems. Our results demonstrate that urinary bladder distension produces increased numbers of Fos-IR cells as well as an altered distribution pattern of Fos-IR cells after SCI. This altered distribution pattern resembles that of noxious irritation (1% acetic acid) of the urinary bladder in control (spinal intact) animals (3, 6, 39).

Pretreatment with capsaicin (Cap; C fiber neurotoxin) significantly reduced the number of Fos-IR spinal cord cells induced by urinary bladder distension after SCI. These data suggest that SCI above the lumbar sacral spinal cord can reveal an altered Fos expression pattern in the spinal cord in response to a nonnoxious bladder stimulus that is partially mediated by Cap-sensitive (presumptive C fibers) bladder afferents.

MATERIALS AND METHODS

SCI. Spinal cord transections were performed under isoflurane anesthesia (2%) 6 wk (n = 21) before intravesical saline distension (see below) or tissue harvest as previously described (36, 38). The dorsal T8-T10 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, Don Mills, Ontario, Canada), and the incision was sutured. An antibiotic (150 mg/kg ampicillin sc) with Gelfoam (Upjohn, Don Mills, Ontario, Canada), and the irritant was allowed to leak out the urethral orifice to minimize irritation of the perineum. Mineral oil was applied to the area around the urethral orifice to minimize irritation of the perineum.

Cap treatment. Birder and de Groat (4) demonstrated that pretreatment of rats with Cap markedly suppressed Fos protein expression in the spinal cord induced by noxious stimulation (1% acetic acid) of the LUT; however, Cap pretreatment did not affect the distribution or numbers of Fos-IR cells in the spinal cord after distension of the urinary bladder by saline infusion (nonnoxious). Thus it was concluded (4) that a specific population of nociceptors (C fiber afferents) exists and responds to chemical irritation of the LUT. To determine if Fos protein expression in the spinal cord induced by distension of the urinary bladder by saline in SCI animals was mediated by a Cap-sensitive population of nerve fibers, some animals (n = 6) were pretreated 4–5 days before the experiment with the neurotoxin Cap to achieve desensitization of small-diameter primary afferents, as previously described (38, 40).

As described previously (39, 41), Cap solution containing 20 mg/ml Cap (Sigma) in 10% ethanol, 10% Tween 80, and 80% physiological saline was injected (125 mg/kg sc) in divided doses on 2 consecutive days: 25 and 50 mg/kg at a 12-h interval on the first day and 50 mg/kg on the second day. Thirty-six to forty-eight hours after the last injection, the animals exhibited a negative eye-wipe test, which involved applying a drop of dilute Cap solution (20 μg/ml) to the surface of the eye and counting the number of eye wiping movements. This test indicates the extent of desensitization to Cap (33).

Euthanasia and tissue handling. Two hours after infusion of saline or acetic acid into the urinary bladder, while still under urethan anesthesia, animals were killed by intracardiac perfusion first with oxygenated Krebs buffer (95% O2, 5% CO2) followed by 4% paraformaldehyde. Previous studies determined that Fos protein expression in the spinal cord is maximal 2 h after intravesical infusion (3–6). After perfusion, the spinal cords were quickly removed and postfixed for 2–6 h. Tissue was then rinsed in 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 (L1, L2, L5-S1) were sectioned in the transverse plane at a thickness of 40 μm on a freezing microtome.

Control experiments. Multiple control groups were used for these studies (Table 1).

Pan-Fos immunohistochemistry. As previously described (38, 39), alternate spinal cord sections (L1, L2, L5-S1) were incubated for 72 h at 4°C with pan-Fos antiserum (1:10,000; Genosys Biotechnologies, The Woodlands, TX) diluted in potassium PBS (KPBS) plus 0.4% Triton X-100. This antibody recognizes Fos oncoproteins and, as such, detects Fos and Fos-related proteins. After the incubation, the antibody was visualized with an avidin-biotin horseradish-peroxidase complex using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Tissue sections were then mounted on gelatin-coated slides, dehydrated in graded ethanol rinses, cleared in xylene, and placed under a coverslip with Permount. All sections were examined with bright-field microscopy. Tissue from control (no treatment, sham, or vehicle treatment) and SCI animals was processed at the same time. Control tests run without the primary or secondary antisera or with anti-
sera preabsorbed with Fos protein (whole peptide, Santa Cruz Biotechnology, Santa Cruz, CA), eliminated Fos staining. Fos-IR was also detected by using an alternative c-Fos antisera (H-125; 1:3,000; Santa Cruz Biotechnology). Although the Fos-IR was comparable in number and distribution using either Fos antisera, visualization of Fos antigen by indirect immunofluorescence was only successful when using the pan-Fos antisera (see below). Thus the pan-Fos antisera was the preferred antisera, and quantification of Fos-IR in this study is based on this antisera. However, the dilution of the pan-Fos antisera was increased (see below) for indirect immunofluorescence, presumably because the Cy3-streptavidin was less sensitive than the peroxidase reaction.

To determine the type of spinal neuron (interneuron vs. preganglionic neuron) expressing Fos-IR in the region of the IML (L1), DCM (L1), or SPN (L6), cholinergic preganglionic neurons in the L1 (n = 6) or L6 (n = 6) segment were identified by an antibody against choline acetyltransferase (ChAT). For this analysis, Fos-IR was detected using indirect immunofluorescence, and this analysis was restricted to the L1 and L6 spinal segments because these segments exhibited the greatest number of Fos-IR cells. Tissue sections were first incubated with pan-Fos antisera (1:4,000; Genosys Biotechnologies) for 72 h at 4°C. After several rinses with KPBS for 30 min, sections were incubated with biotinylated rabbit antiserum to sheep antibody (1:500, Vector Laboratories) for 2 h at room temperature. Subsequently, tissue was incubated with Cy3-streptavidin (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA) for an additional 2 h at room temperature. After additional rinses with KPBS, tissue was incubated with an antibody to ChAT (1:500, Chemicon International, Temecula, CA) diluted in KPBS plus 0.4% Triton X-100 for 48 h at 4°C. After several rinses with KPBS for 30 min, sections were incubated with Cy2-conjugated donkey anti-goat IgG (1:500; Jackson ImmunoResearch Laboratories) for 2 h at room temperature. Sections were again washed before being placed under covergals with Citifluor (Citifluor Limited, London, UK). Control tests run without the primary or secondary antisera (Fos or ChAT) or with antisera preabsorbed with Fos protein (whole peptide, Santa Cruz Biotechnology, Santa Cruz, CA) eliminated staining. Sections were examined under a fluorescence photomicroscope with a multiband filter set for simultaneous visualization of Cy3 and Cy2 fluorophores. Cy2 was viewed by using a filter with an excitation range of 447–501 nm and an emission range from 510–540 nm; Cy3 was visualized with a filter with an excitation range of 560–596 nm and an emission range from 610–655 nm. In all cases, Fos-IR was visualized as red fluorescence and ChAT-IR was visualized as yellow-green fluorescence.

**Determination of urinary bladder weight after SCI.** Urinary bladders were harvested from SCI and control animals euthanized by intracardiac perfusion but not subjected to any urinary bladder distension protocol. These bladders were blotted dry, and the weights were recorded.

**Quantification and statistical analysis.** The goals of the present study were 1) to determine the relative distribution of Fos-IR cells in specific spinal cord regions (see below) and 2) to determine the percentage of Fos-IR cells exhibiting ChAT-IR in the lateral horn of the L1 and L6 spinal segments. In this study, the absolute number of Fos-IR cells was not determined. Thus the number of cells exhibiting Fos-IR was estimated from 15 to 20 spinal cord sections from L1, L2, and L5 to S1 segmental levels in four spinal cord regions for each animal: 1) MDH, 2) LDH, 3) DCM in L6-S1 and dorsal commissural nucleus (DCN) in L1-L2, and 4) lateral laminae V-VII, including the SPN in L6-S1 or the IMl in L1-L2. Fos-IR cells in the lateral horn region also exhibiting ChAT-IR were similarly counted. These spinal cord regions were the same as those examined in previous studies examining Fos distribution after LUT stimulation (3, 4, 39). These regions were divided as follows: 1) the vertical boundary between the MDH and LDH was set by determining the midpoint between the medial and lateral borders of the dorsal horn of the spinal section examined, 2) the horizontal boundary denoting the ventral limit of the MDH and LDH was determined by drawing a line from the neck of the dorsal horn to the middle of the spinal cord section examined, 3) the vertical boundary between the SPN (or IMl) and DCM (or DCN) was set by determining the midpoint between the midline of the spinal cord and the neck of the dorsal horn, 4) the horizontal boundary denoting the ventral limit of the SPN (or IMl) and DCM (or DCN) was set by determining the midpoint between the central canal and the midline gray matter of the section examined and extending this point to the lateral edge of the spinal cord. Spinal cord sections used for counts were separated by 120 μm to reduce the possibility of double counting. Counts of Fos-IR cells are presented as

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The various control experiments performed are detailed. Capsaicin (Cap) vehicle is corresponding volume of distilled water in 10% ethanol, 10% Tween 80, and 80% physiological saline injected subcutaneously on 2 consecutive days. Cap treatment and spinal cord transection procedures are more fully described in methods. SCI, spinal cord injured; Sham bladder infusion and Bladder infusion, bladder exposed via abdominal incision and needle placed in bladder dome without and with, respectively, infusion of fluids.
average numbers of cells per section or percentage change in
numbers of cells per section. Comparisons between the num-
ber of Fos-IR spinal neurons in control or experimental sit-
uations were made using analysis of variance. Animals, pro-
cessed and analyzed on the same day, were tested as a block
in the analysis of variance. Thus processing day was treated
as a blocking effect in the model. Two variables were being
tested in the analysis: 1) experimental manipulation vs.
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
($P \leq 0.05$), the Newman-Keuls test was used for multiple
comparisons among means. All values are expressed as
means ± SE.

Figure preparation. Tissue sections were examined and
photographed with Ektachrome Elite II film using an Olym-
pus photomicroscope. Photographic slides were subsequently
scanned into Photoshop 3.0 with the aid of a Polaroid Sprint-
Scan 35. Images were minimally adjusted for brightness and
contrast and appropriately cropped. Images were imported
into Canvas 6.0, where groups of images were assembled and
labeled. Composite figures were printed on a Codonics NP-
1600 Photographic Network printer (Middleburg Heights,
OH) or an Epson Stylus Color 900 Printer (Torrance, CA).

RESULTS

Increase in urinary bladder weight after SCI. The
urinary bladders of chronic SCI (6 wk) rats were mark-
edly distended, and the walls were thickened 6 wk
after spinalization. There was a 6.9-fold increase ($P \leq
0.005$) in urinary bladder weight after chronic SCI
[5.85±57 vs. 85.2±16 mg (control)]. All animals
exhibited reflex urinary bladder contractions with in-
travesical infusion of saline. Animals were studied 6
wk after spinal transection when they had developed
bladder-to-bladder and perineal-to-bladder reflexes. At
least by 6 wk after SCI, all animals had largely empty
urinary bladders as assessed by manual compression
and volume voided. Thus it was inferred that animals
were spontaneously voiding via a spinal mediated,
bladder-to-bladder reflex. Before manual compression
was started, animals were tested for the presence of
perineal-to-bladder reflexes by rapidly stroking the
skin around the urethral outlet with a cotton swab for
~20–30 s to induce micturition. All animals at 6 wk
after SCI exhibited spontaneous bladder-to-bladder
and spinal micturition reflexes elicited by perigenital
stimulation.

Control experiments. Urethane-anesthetized control
animals not subjected to any experimental procedures
(control group 1) or urethane-anesthetized control ani-
mals receiving Cap vehicle or Cap treatment without
any further experimental procedure exhibited low
numbers of Fos-IR cells (<2 cells/section) in the spinal
segments examined (L1, L2, L5–S1) (control groups 3
and 6) (Table 1). Urethane-anesthetized SCI animals
with or without Cap vehicle treatment or with or with-
out Cap treatment and without any further experiment-
mental procedure (control groups 9, 10, 13) (Table 1)
also exhibited low numbers of Fos-IR cells (<2 cells/section)
in the spinal segments examined (L1, L2, L5–S1).
Sham-operated control animals in which the urinary
bladder was exposed through a midline abdominal
incision and a needle placed through the dome of the
urinary bladder (control group 2) or sham-operated
animals receiving Cap vehicle or Cap (control groups 4
and 7) exhibited low numbers of Fos-IR cells (<3 cells/
section) in the L5–S1 spinal segments examined (Table
1). Sham-operated SCI animals in which the urinary
bladder was exposed through a midline abdominal
incision and a needle placed through the dome of the
urinary bladder receiving Cap vehicle or Cap (control
groups 11 and 14) exhibited low numbers of Fos-IR
cells (<3 cells/section) in the L5–S1 spinal segments
examined (Table 1). However, as previously noted (4, 8)
greater numbers of Fos-IR cells (5–10 cells/section)
were observed after sham surgery in the rostral-lumbar
(L1–L2) spinal segments that receive afferent input
from the abdominal wall. There was no effect of pro-
cessing day on the numbers of Fos-IR cells observed.

Fos protein expression induced by nonnoxious stim-
ulation of the LUT. Urethane-anesthetized animals
with a constant infusion (0.12 ml/min) of saline
through a needle inserted into the bladder dome (control
group 15) or urethane-anesthetized animals receiving
Cap vehicle or Cap (control groups 5 and 8) with a
constant intravesical infusion of saline exhibited sig-
ificantly ($P \leq 0.005$) greater numbers of Fos-IR cells
in the lumbar-sacral (L6–S1) spinal cord (Fig. 1). No
differences in the numbers of Fos-IR cells in the L6–S1
spinal cord were observed among these groups (groups
5, 8, and 15). There was no effect of day on the numbers
of Fos-IR cells observed. Thus data from these animals
are presented together as control distension data. In-
fusion of saline induced repeated micturition reflexes
at 3- to 5-min intervals during the duration (2 h) of
the experiment. In agreement with previous studies (4),

Fig. 1. Histogram showing the segmental distribution of Fos-immu-
noreactive (IR) cells/section (s) in the rat spinal cord (L1, L2, L5–S1)
after intravesical saline distension in control animals, in animals
with spinal cord injury (SCI) above the lumbosacral spinal cord, and
in animals pretreated with the C fiber neurotoxin capsaicin (Cap)
after SCI. *$P \leq 0.005$ vs. Control Distension and SCI + Distension
groups; $n = 15$ for Control Distension and SCI + Distension
groups; $n = 12$ for the SCI + Cap + Distension groups.
intravesical saline infusion induced the expression of Fos-IR cells in the L6 (65.5 ± 1.7 Fos-IR cells/section) and S1 (47.0 ± 3.6 Fos-IR cells/section) spinal cord, whereas few Fos-IR cells were observed in the L1, L2, or L5 spinal segments (range 5–7 cells/section) (Fig. 1). Fos-IR cells were observed in specific regions of the L6-S1 spinal cord. The largest number (39.6 ± 1.2 Fos-IR cells/section) of Fos-IR cells were observed in the L6 spinal segment. In the L6 spinal segment, the majority of Fos-IR cells was distributed in the DCM (64.0 ± 2.2 Fos-IR cells/section), with smaller numbers distributed in the MDH and LDH (Figs. 4 and 5). The topographic distribution of Fos-IR cells in the L2 spinal cord was similar to that observed in the L1 spinal segment.

Fos-IR cells were present in the DCM (18.0 ± 1.7 Fos-IR cells/section) in L6, 12.2 ± 1.3 Fos-IR cells/section in S1), MDH (9.5 ± 2.0 Fos-IR cells/section in L6, 4.8 ± 1.0 Fos-IR cells/section in S1), and LDH (5.1 ± 1.6 Fos-IR cells/section in L6, 2.4 ± 0.8 Fos-IR cells/section in S1) (Figs. 2 and 3). The largest number of Fos-IR cells in the L1 spinal cord were distributed in the MDH, with significantly smaller numbers being fairly equally distributed in the region of the IML, DCN, and LDH (Figs. 4 and 5). The distribution of Fos-IR cells in the L2 spinal cord after intravesical saline infusion was similar to that observed in the L1 segment.

Fos protein expression induced by nonnoxious stimulation of the LUT after SCI. In urethan-anesthetized animals after SCI, a constant infusion of saline through a needle inserted into the bladder dome significantly (P = 0.0005) increased the number of Fos-IR cells observed in both the rostral lumbosacral (L1, 38.3 ± 2.5 cells/section; L2, 29.5 ± 2.5 cells/section) and caudal lumbosacral (L6, 140.6 ± 8.5 cells/section; S1, 110.3 ± 6.2 cells/section) spinal cord, but Fos protein expression in the L5 segment (7 ± 2.5 cells/section) was not altered (Fig. 1). In addition to an increase in the magnitude of Fos protein expression after intravesical saline infusion, the topographical distribution of Fos-IR cells was also altered in animals with chronic SCI (Figs. 1–5). In the rostral lumbosacral (L1) spinal cord, the majority of Fos-IR cells was fairly equally distributed in the IML (11.4 ± 1.5 Fos-IR cells/section) and DCN (15.5 ± 1.2 Fos-IR cells/section), with smaller numbers distributed in the MDH and LDH (Figs. 4 and 5). The topographic distribution of Fos-IR cells in the L2 spinal cord was similar to that observed in the L1 spinal segment.

Nonnoxious stimulation of the LUT in SCI animals compared with noxious stimulation of the LUT in control animals. The magnitude of Fos-IR cells in the lumbosacral spinal cord and the change in the topographic distribution of Fos-IR cells in the lumbosacral spinal cord after intravesical saline infusion in SCI animals was similar to that previously reported for control animals with a constant intravesical irritant infusion (3, 4). To confirm these previous studies and to provide a direct comparison for the present studies, control animals (spinal cord intact) were continuously infused with 1% acetic acid for 2 h through a needle placed through the dome of the urinary bladder (Fig. 2). In confirmation of previous results (3, 4, 39), the majority of Fos-IR cells in the L6 spinal cord was distributed in the DCM (55 ± 3.7%), with smaller percentages in the SPN (22 ± 6.5%), MDH (15 ± 5.6%), and LDH (8 ± 3.6%) (Fig. 2). Smaller numbers of Fos-IR cells (10–15 Fos-IR cells/section), comparable to

Fig. 2. Bright-field photographs from sections (40 μm) of the L6 spinal cord showing the distribution of Fos-IR cells after intravesical saline distension in control (spinal cord intact) animals (A), animals with chronic SCI (B), and animals pretreated with the neurotoxin Cap after SCI (D). C: distribution of Fos-IR cells in the L6 spinal segment after lower urinary tract irritation with 1% acetic acid in control animals. MDH, medial dorsal horn; LDH, lateral dorsal horn; DCM, dorsal commissure; CC, central canal; SPN, sacral parasympathetic nucleus. Calibration bar represents 110 μm.
that observed with intravesical saline infusion, were observed in the rostral (L1-L2) or L5 spinal segments. Thus the topographic distribution of Fos-IR cells in the lumbosacral spinal cord after either noxious stimulation of the LUT in control animals or nonnoxious stimulation of the LUT in SCI animals was similar. In contrast, the large number of Fos-IR cells in the rostral (L1-L2) spinal segments induced by nonnoxious stimulation of the LUT in SCI animals (Figs. 1, 4, 5) does not parallel that observed for noxious stimulation of the LUT in control animals where few Fos-IR cells are observed (4). There was no effect of processing day on the numbers of Fos-IR cells observed.

**Preganglionic neurons** (ChAT-IR) exhibiting Fos protein after intravesical saline distension after SCI. We wanted to determine if Fos-IR cells in autonomic centers in the L1 (IML and DCN) and L6 (SPN) spinal cord also exhibit ChAT-IR and represent either sympathetic (L1) or parasympathetic (L6) preganglionic neurons. In control animals with constant saline infusion through a needle placed in the urinary bladder, no Fos-IR cells in the region of either the IML or DCN in the L1 segment exhibited ChAT-IR (Table 2). Similarly, no Fos-IR cells in the region of the SPN in the L6 spinal segment exhibited ChAT-IR (Table 2). In chronic SCI animals with a constant intravesical saline infusion, a significant percentage of Fos-IR cells in three regions examined (IML, 52.5 ± 4.5%; DCN, 38.3 ± 3.9%; SPN, 35.7 ± 6.2%) exhibited ChAT-IR (Table 2). In SCI animals, 75.6 ± 8.2% of preganglionic neurons (ChAT-IR) in the L6 SPN were Fos-IR. Fos-IR cells expressing ChAT-IR (presumptive preganglionic neurons) were located ventral to cells exhibiting only Fos-IR (presumptive interneurons or projection neurons) (Table 2, Fig. 6). Thus in the region of the SPN, interneurons or projection neurons (nonpreganglionic neurons) are located dorsal to the more ventrally located preganglionic neurons (Fig. 6). There was no effect of processing day on the numbers of Fos-IR, ChAT-IR, or Fos ChAT-IR cells observed.

**Pharmacological evaluation of the Fos protein response to nonnoxious stimuli of the LUT in SCI animals with Cap.** Previous studies (4) demonstrated that pretreatment with Cap (100 mg/kg sc) significantly suppressed the expression of Fos protein induced by noxious (acetic acid) stimulation of the LUT. In contrast, Cap treatment did not affect the Fos protein expression induced by nonnoxious stimulation of the LUT.
induced by nonnoxious (saline) stimulation of the LUT. To determine if Cap-sensitive (presumptive C fibers) fibers are contributing to the expression of Fos protein after nonnoxious (saline) stimulation of the LUT after SCI, animals pretreated with Cap (125 mg/kg sc) were used in the present studies. In the present studies, pretreatment with Cap in a dose that produced complete desensitization to the eye-wipe test significantly (P ≤ 0.005) decreased, but did not eliminate, the number of Fos-IR cells induced by intravesical saline distension observed in the rostralumbar (L1-L2) and lumbo-sacral (L6-S1) spinal cord of SCI animals (Figs. 1, 2, 4, 5). Pretreatment with Cap in SCI animals also altered the topographic distribution of Fos-IR cells in the L1-L2 and L6-S1 spinal cord (Figs. 3 and 5). The greatest number of Fos-IR cells were distributed in the SPN region for both the L6 (34.5 ± 2.6 Fos-IR cells/section) and S1 (31.2 ± 1.8 Fos-IR cells/section) spinal segments, with smaller numbers being distributed in the DCM (L6, 21.2 ± 2.2 Fos-IR cells/section; S1, 15.6 ± 1.3 Fos-IR cells/section), MDH (L6, 15.5 ± 1.8 Fos-IR cells/section; S1, 9.5 ± 1.2 Fos-IR cells/section), and LDH (L6, 6.1 ± 1.8 Fos-IR cells/section; S1, 3 ± 0.9 Fos-IR cells/section) (Figs. 2 and 3). In the L1 segment, the greatest number of Fos-IR cells were distributed in the MDH (11.7 ± 1.8 Fos-IR cells/section), with smaller numbers being distributed in the IML, DCN, and LDH (Figs. 4 and 5).

DISCUSSION

The present study demonstrates that urinary bladder distension produces increased numbers of Fos-IR cells as well as an altered distribution pattern of Fos-IR cells after chronic SCI for 6 wk. This chronic SCI is associated with a significant increase in urinary bladder weight. The altered distribution pattern of Fos protein resembles that after noxious irritation (1% acetic acid) of the urinary bladder in control (spinal cord intact) animals (4, 6). A significant percentage (75%) of parasympathetic preganglionic neurons in the SPN exhibited Fos-IR after saline distension of the urinary bladder after SCI, whereas no parasympathetic PGNs exhibited Fos-IR in control (spinal cord intact) animals after intravesical distension. Pretreatment with Cap (C fiber neurotoxin) significantly reduced the number of Fos-IR spinal cord cells induced by urinary bladder distension after SCI. These data suggest that chronic SCI can reveal an altered Fos expression pattern in the spinal cord in response to a nonnoxious bladder stimulus that is partially mediated by Cap-sensitive (presumptive C fibers) bladder afferents.

The present study has demonstrated that after chronic SCI, urinary bladder distension results in an increased expression of Fos-IR in spinal neurons as well as an altered expression pattern of Fos-IR in specific regions of the rostralumbar and caudal lumbo-sacral spinal cord that play a role in micturition reflexes. The spinal cord regions (LDH, SPN, DCM) that exhibit Fos protein induced by intravesical saline distension after SCI correspond to those regions previously shown to exhibit increased growth-associated protein (GAP-43) immunoreactivity after SCI (36). Results from the GAP-43 experiments (36) together with the present Fos-IR results suggest a significant alteration in processing and organization of micturition reflexes after SCI above the lumbosacral spinal cord. Animal studies (11–15, 20, 22) have indicated that SCI in the rat alters micturition reflexes that are characterized by urinary bladder hyperreflexia, incomplete voiding, and bladder-sphincter dyssynergia. These changes in urinary bladder function may involve alterations in urinary bladder afferent fibers and central projections and their sites of termination in the spinal cord (LDH, lateral collateral pathway, SPN, and DCM).
Table 2. Fos-IR and Fos + ChAT-IR cells in autonomic nuclei in control and SCI animals after intravesical saline distension

<table>
<thead>
<tr>
<th>SCI Group</th>
<th>Fos-IR, Cells/Sec</th>
<th>ChAT-IR, Cell Profiles/Sec</th>
<th>Fos + ChAT, %Colabel</th>
<th>ChAT + Fos, %Colabel</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control distension</td>
<td>0.5 ± 0.3</td>
<td>11.5 ± 2.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DCN</td>
<td>0.6 ± 0.5</td>
<td>8.3 ± 4.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SCI+distension</td>
<td>10.2 ± 1.4</td>
<td>12.2 ± 2.5</td>
<td>52.5 ± 4.5*</td>
<td>85.4 ± 6.8*</td>
</tr>
<tr>
<td>IML</td>
<td>15.2 ± 3.8</td>
<td>9.4 ± 3.5</td>
<td>38.3 ± 3.9*</td>
<td>45.4 ± 4.7*</td>
</tr>
<tr>
<td>L6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control distension</td>
<td>17.5 ± 4.0</td>
<td>12.5 ± 2.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SPN</td>
<td>30.4 ± 7.2</td>
<td>10.2 ± 3.7</td>
<td>35.7 ± 6.2*</td>
<td>75.6 ± 8.2*</td>
</tr>
<tr>
<td>SCI+distension</td>
<td>4.0 ± 1.0</td>
<td>7.2 ± 1.2</td>
<td>4.5*</td>
<td>85.4</td>
</tr>
<tr>
<td>SPN</td>
<td>6.8*</td>
<td>45.4</td>
<td>45.4*</td>
<td>45.4*</td>
</tr>
<tr>
<td>LDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IML</td>
<td>4.1*</td>
<td>38.3</td>
<td>4.1*</td>
<td>38.3</td>
</tr>
<tr>
<td>DCN</td>
<td>3.9*</td>
<td>45.4</td>
<td>3.9*</td>
<td>45.4</td>
</tr>
</tbody>
</table>
| LDH values are means ± SE of no. of Fos immunoreactive (IR) cells and/or choline acetyltransferase (ChAT)-IR cell profiles/section (sec) induced by intravesical saline distension in control or SCI animals in the intermediolateral cell column (IML), dorsal commissural nucleus (DCN), or sacral parasympathetic nucleus (SPN) regions of the L1 and L6 spinal cord, respectively. After chronic SCI and intravesical saline distension, a significant increase in the percentage of Fos-IR cells exhibiting ChAT-IR was observed (Fos + ChAT). In addition, there was a significant increase in the percentage of sympathetic or parasympathetic preganglionic neurons exhibiting Fos-IR (ChAT + Fos). *P ≤ 0.005.

In confirmation of previous studies (4, 39), the present studies demonstrated that Fos protein is induced by intravesical saline distension in spinal neurons located predominantly in the DCM and SPN regions of the L6-S1 spinal cord of the control animals. The present studies demonstrated that the Fos expression pattern and magnitude of Fos expression induced by intravesical saline distension is altered after SCI. After SCI, the number of Fos-IR cells is significantly (P ≤ 0.05) increased in the region of the DCM, MDH, and LDH in the lumbosacral (L6-S1) spinal cord as well as in the DCN and IML regions of the L1-L2 spinal cord. Previous studies (39) demonstrated a similar change in the number and distribution of Fos-IR cells induced by intravesical saline distension after cyclophosphamide (CYP)-induced cystitis.

The altered Fos distribution pattern in SCI animals after intravesical saline distension resembles that after noxious irritation (1% acetic acid or acute CYP treatment) of the urinary bladder in control (spinal intact) animals (4, 25, 39). Recent studies by Al-Chaer et al. (1) demonstrated the presence of cells adjacent to the central canal that respond selectively to visceral (colonic) stimulation, including mustard oil-induced inflammation, but not to cutaneous stimulation. Al-Chaer et al. (1) suggested that these postsynaptic dorsal column neurons may provide the physiological basis for primary visceral hyperalgesia. Interestingly, in the present study, larger numbers of cells in the DCN and IML regions of the L1-L2 spinal cord, regions complementary to that described by Al-Chaer (1), express Fos protein after saline distension of the urinary bladder in SCI animals or after irritation of the LUT (4, 39). Our previous studies involving a rat model of urinary bladder inflammation induced by CYP also demonstrated that greater numbers of Fos-IR cells are distributed in the DCN after intravesical saline distension (39). Thus some of these cells may provide a neuroanatomical substrate for visceral (i.e., urinary bladder) pain.

Pretreatment with Cap (C fiber neurotoxin), significantly reduced the number of Fos-IR spinal cord cells in the lumbosacral spinal cord induced by urinary bladder distension after SCI. These data suggest that chronic SCI can reveal an altered Fos expression pattern in the spinal cord in response to a nonnoxious bladder stimulus that is partially mediated by Capsensitive (presumptive C fibers) bladder afferents. The present results further demonstrate a prominent sensitization of rostrolumbar spinal cord pathways after SCI. In control animals, neither noxious nor nonnoxi-
ious stimulation of the LUT induces Fos protein expression in rostralumbar spinal neurons. However, after SCI, saline bladder distension does induce Fos protein expression in spinal neurons in the L1-L2 spinal segments. Clinical application of intravesical Cap for voiding dysfunction was first reported by Maggi et al. (26), and the use of Cap and resiniferatoxin therapy for overactive bladder treatment has recently been reviewed (9). To our knowledge, the present results are among the first to demonstrate the involvement of Cap-sensitive bladder afferents in the expression of Fos protein induced by intravesical distension after chronic SCI in the rat.

Although Cap pretreatment significantly reduced the number of Fos-IR cells induced by saline distension of the urinary bladder in SCI animals, these effects were incomplete. Recent experiments in the rat demonstrated a complex organization of pelvic nerve afferent fibers innervating the urinary bladder (28). In the rat, Aδ- and C fiber bladder afferent fibers show considerable overlap in the response threshold as well as in the pressure/volume characteristics to which each fiber subfraction responds (29). The following types of pelvic afferent fibers have been identified in the rat (29): 1) low-threshold mechanoreceptors with small myelinated axons (Aδ-) and unmyelinated axons (C fibers), 2) high-threshold mechanoreceptors with small myelinated axons (Aδ-) and unmyelinated axons (C fibers), and 3) “silent” receptors with small myelinated axons (Aδ-) and unmyelinated axons (C fibers) that are mechanically unresponsive but may be chemosensitive. In the present studies, the Cap data suggest the possibility that both Aδ- and C fiber bladder afferents are activated by urinary bladder distension in the SCI animals. Incomplete effects of Cap treatment are attributed to the Cap insensitivity of the Aδ bladder afferents. In light of the above characteristics of pelvic visceral afferents (29), it is also possible that, in the rat, some Aδ- and C fiber bladder afferents are normally silent or inactive. After SCI, these fiber types may become sensitized or awakened and contribute to the altered Fos protein expression. Previous studies (4) suggested that a specific population of nociceptors (C fiber afferents) exists and responds to chemical irritation of the LUT in control rats. The present results suggest that two populations of nociceptors (Aδ- and C fiber afferents) may contribute to the altered Fos pattern induced by saline distension of the urinary bladder after SCI. Recent studies involving a CYP-induced model of urinary bladder inflammation also suggested the involvement of both Aδ- and C fiber bladder afferents in altered spinal cord Fos protein expression induced by intravesical saline distension (39), although the involvement of Aδ-fibers appears somewhat greater in bladder dysfunction originating from bladder inflammation compared with SCI. Therefore, in the rat, Aδ- and C fiber bladder afferents may contribute to altered micturition reflexes after various forms of urinary bladder dysfunction.

In both control animals and SCI animals, saline bladder distension induced Fos protein expression in the region of the SPN. Because the SPN contains interneurons and projection neurons as well as preganglionic neurons (PGNs) (5, 6, 27), the question arises as to which cell populations in the region of the SPN are expressing Fos-IR. Whereas the IML region has traditionally been viewed as a “closed” nucleus containing only sympathetic PGNs, recent studies using transneuronal tracing with pseudorabies virus have revealed groups of interneurons intermingled with PGNs (2). Thus the possibility exists that the IML also contains interneurons and projection neurons in addition to PGNs. In the present experiments, no Fos-IR cells in the region of the IML or SPN in control animals after saline bladder distension were identified as PGNs on the basis of neurochemical phenotype (ChAT-IR). In contrast, after SCI, a significant percentage of sympathetic PGNs (85% in L1 IML, 45% in L1 DCN) expressed Fos-IR after saline bladder distension. Similarly, a large percentage (75%) of parasympathetic PGNs in the L6 SPN of SCI animals exhibited Fos-IR after saline bladder distension. This percentage (75–85%) is greater than that observed after noxious distension of the proximal colon in a rat model (27). Colon distension induced the expression of Fos protein in 40% of parasympathetic PGNs (identified on the basis of NADPH diaphorase histochemistry) in the S1 spinal segment (27). In contrast, acetic acid irritation of the urinary bladder in control animals induced the expression of Fos protein in 20% of parasympathetic PGN, identified on the basis of retrograde labeling from the major pelvic ganglion (6). Thus, despite a similar distribution of Fos-IR cells and a similar magnitude of Fos protein expression after acetic acid infusion into the urinary bladder and saline distension of the urinary bladder in SCI animals, these two stimuli may, in fact, be quite different in terms of the chemical phenotype of the Fos-IR cells activated in the spinal cord.

A possible mechanism underlying the increased expression of Fos-IR in spinal neurons in LUT pathways after SCI may involve neurotrophic factors (NTFs) (18, 28) or neural activity arising in the bladder. According to this hypothesis (18, 28), SCI can change organ function that in turn can change afferent pathways from the organs. It is now widely accepted that target organs can influence the neurons that innervate them (30, 32, 35). Previous experiments have demonstrated the influence of target organ-neuron interactions in the adult animal (30, 32, 35). Previous studies (21, 30–32) have suggested that neurotrophic factors released in the hypertrophied bladder are partly responsible for the change in neuronal morphology and neurochemistry of bladder afferent projections after partial urethral obstruction. Similarly, alterations in Fos protein expression and the efficacy of the micturition reflexes may also be modulated by neurotrophic factors in the urinary bladder after SCI (37).

Recent studies demonstrated sudden and dramatic alterations in urinary bladder neurotrophic factor mRNA after both acute (4 days) and chronic SCI (4–6 wk) (37). These changes in neurotrophic factor mRNA include significant increases in nerve growth factor
(NGF) mRNA, but also indicate that other neurotrophic factors (brain-derived neurotrophic factor, glial-derived neurotrophic factor, ciliary neurotrophic factor, neurotrophin-3, neurotrophin-4) may also contribute to changes in LUT function after SCI. These studies (37) suggest that neurotrophic factors other than NGF may contribute to changes in the neurochemical, electrophysiological, and organizational properties of the LUT after SCI. The contribution of NGF or other neurotrophic factors to the alterations in Fos-IR observed in the present study is not known but is a focus of future studies.

We originally hypothesized that after SCI, increased numbers of Fos-IR cells induced by urinary bladder distension (nonnoxious) would be observed in the spinal cord due to 1) central changes in synaptic plasticity and/or membrane excitability, 2) peripheral changes in afferent terminal excitability in the urinary bladder, or 3) absence of tonic descending modulation systems. The present results support the involvement of peripheral changes in bladder afferent terminal excitability (C and A-δ-fibers) in the urinary bladder after SCI as a contributor to altered spinal cord Fos protein expression induced by intravesical saline distension. However, these studies do not rule out contributory roles for changes in central plasticity or the absence of descending modulation systems for the observed changes in Fos expression after SCI.

This study was supported by grants from the Paralyzed Veterans Association/Spinal Cord Research Foundation (1993–01/02) and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-51369.

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