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Plasticity of lumbosacral propriospinal neurons is associated with the development of autonomic dysreflexia after thoracic spinal cord transection

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Abstract

Complete thoracic (T) spinal cord injury (SCI) above the T6 level typically results in autonomic dysreflexia, an abnormal hypertensive condition commonly triggered by nociceptive stimuli below the level of SCI. Over-expression of nerve growth factor in the lumbosacral spinal cord induces profuse sprouting of nociceptive pelvic visceral afferent fibers that correlates with increased hypertension in response to noxious colorectal distension. After complete T4 SCI, we evaluated the plasticity of propriospinal neurons conveying visceral input rostrally to thoracic sympathetic preganglionic neurons. The anterograde tracer biotinylated dextran amine (BDA) was injected into the lumbosacral dorsal gray commissure (DGC) of injured/non-transected rats immediately after injury (acute) or 2 weeks later (delayed). At 1 or 2 weeks after delayed or acute injections, respectively, a higher density ($p < 0.05$) of BDA⁺ fibers was found in thoracic dorsal gray matter of injured versus non-transected spinal cords. For corroboration, fast blue (FB) or cholera toxin subunit beta (CTb) was injected into the T9 dorsal horns 2 weeks post-injury/non-transection. After 1 week transport, more retrogradely-labeled ($p < 0.05$) DGC propriospinal neurons (T13–S1) were quantified in injured versus non-transected cords. We also monitored immediate early gene, *c-fos*, expression following colorectal distension and found increased ($p < 0.01$) c-Fos⁺ cell numbers throughout the DGC after injury. Collectively, these results imply that, in conjunction with local primary afferent fiber plasticity, injury-induced sprouting of DGC neurons may be a key constituent in relaying visceral sensory input to sympathetic preganglionic neurons that elicit autonomic dysreflexia after high thoracic SCI.

Keywords

dorsal gray commissure; dorsal commissural nucleus; neuronal tracers; c-Fos; hypertension; stereology; colorectal distension

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INTRODUCTION

Autonomic dysreflexia is a potentially life-threatening hypertensive syndrome that develops after spinal cord injury (SCI) above the sixth thoracic (T) spinal segment. It is characterized by severe hypertension due to sudden, massive discharge of the sympathetic preganglionic neurons below the injury site, which when accompanied by baroreflex-mediated bradycardia defines this autonomic syndrome after SCI. Autonomic dysreflexia is commonly triggered by noxious stimuli below the injury site, particularly by the distension of pelvic viscera (bowel and bladder) (Karlsson, 1999; Lindan et al., 1980). Serious cases of autonomic dysreflexia can cause cerebral and spinal subarachnoid hemorrhage, seizures, and pulmonary edema (Snow et al., 1978). It is believed that acute autonomic dysreflexia arises due to loss of bulbospinal sympathetic inhibition (Krassioukov and Weaver, 1995; 1996). This is followed by injury-induced increases in growth factor expression (Brown et al., 2004) that elicit progressive structural and electrophysiological changes in both primary afferents and spinal neurons that coincide with the delayed increased severity of autonomic dysreflexia (Chau et al., 2000; Krenz and Weaver, 1998a; b; Weaver et al., 1997). Importantly, autonomic dysreflexia develops in a time-dependent manner with weak responses to noxious stimuli at 7 days post-injury, which correlates with transient atrophy of the denervated sympathetic preganglionic neurons below the injury site (Krassioukov and Weaver, 1996). However, by 2 weeks post-injury their morphology begins to return to normal, while their hypertensive responses to noxious stimuli become significantly greater (Mayorov et al., 2001).

Experimental autonomic dysreflexia induced by noxious colorectal distension in T4-transected rats correlates with profuse nerve growth factor (NGF)-mediated intraspinal sprouting of calcitonin gene-related peptide (CGRP)⁺ primary afferent fibers (Krenz and Weaver, 1998b; Weaver et al., 2001), particularly into lumbosacral (L/S) spinal segments (termination site of pelvic visceral sensory axons) (Cameron et al., 2006; Rabchevsky, 2006). However, in order to elicit dysreflexic hypertension after SCI, primary afferent input (CGRP⁺ fibers) into the lumbosacral segments is somehow relayed to rostral sympathetic preganglionic neurons located mainly in thoracic segments (Chau et al., 2000; Krassioukov et al., 2002; Petkó and Antal, 2000; Tang et al., 2003).

Primary afferent fibers supplying the pelvic viscera in rats run in the pelvic nerve and distribute mainly to the L6 and S1 dorsal root ganglia (DRG) and spinal cord, while a smaller number of afferents run in either the hypogastric nerve to the T13 and L1 DRG and spinal cord (Ness and Gebhart, 1987) or the vagus nerve to the caudal medulla (Vizzard, 2000). The afferent fibers terminate in the superficial dorsal horns (laminae I-II), the dorsal gray commissure (DGC, see Fig. 1) of lamina X above the central canal (also termed dorsal commissural nucleus) (Hancock and Peveto, 1979; Hosoya et al., 1994), and the lateral laminae V-VIII (region of the sacral parasympathetic nucleus) (Matsushita, 1998; Pascual et al., 1993; Vizzard, 2000). Neurons that reside primarily in the L6/S1 DGC project axons in the medial part of the dorsal columns and relay visceral information rostrally to supraspinal targets (Hirshberg et al., 1996; Matsushita, 1998; Wang et al., 1999). Most of these neurons respond physiologically to visceral stimulation, notably noxious colorectal distension (Al-Chaer et al., 1996). Accordingly, we hypothesize that following SCI the increased local nociceptive afferent input leads to subsequent plasticity of lumbosacral propriospinal DGC neurons which, in turn, relay pelvic visceral sensations to sympathetic preganglionic neurons in the rostral thoracolumbar spinal cord, eliciting dysreflexic hypertension (Rabchevsky, 2006).

To address this hypothesis, in the present study we used both intraspinal anterograde and retrograde neuronal tract tracing, in addition to colorectal distension-induced c-Fos

expression, to establish whether post-traumatic plasticity of DGC propriospinal neurons, as well as increased local cellular activity in response to noxious visceral stimuli, are associated with the manifestation of autonomic dysreflexia. Results showed significantly increased sprouting of ascending propriospinal fibers in T4-transected versus non-transected spinal cords, in conjunction with significantly increased retrograde labeling of L/S DGC neurons projecting to mid-thoracic levels. Moreover, following intermittent colorectal distension, there was significantly enhanced c-Fos immunolabeling throughout the DGC in injured versus nontransected spinal cords. Collectively, our findings imply that post-traumatic sprouting of DGC neurons, accompanied by increased local c-Fos expression, may play a role in relaying noxious visceral input to modulate sympathetic preganglionic neurons to elicit the manifestation of autonomic dysreflexia.

MATERIALS AND METHODS

Animals and surgery

All animal housing conditions, surgical procedures and post-operative care techniques were conducted according to the University of Kentucky Institutional Animal Care and Use Committee and the National Institutes of Health animal care guidelines. Adult female Wistar rats (~200–250g) were anesthetized with a mixture of ketamine (80 mg/kg, i.p.; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg, i.p.; Butler, Columbus, OH). The injured group received complete T4 transection following T3 vertebra laminectomy, in contrast to the non-transected group which received only T3 laminectomy. Respectively, 11 injured and 11 non-transected were used for acute biotinylated dextran amine (BDA) injection, 8 injured and 8 nontransected for delayed BDA injection, 6 injured and 6 non-transected for Fast Blue (FB) injection, 6 injured and 6 non-transected for cholera toxin subunit beta (CTb) injection, and 7 injured and 9 non-transected for c-Fos expression, as detailed in Table 1.

The spinal cord was completely transected with a scalpel blade at the T4 level following the laminectomy, as described previously (Cameron et al., 2006). Subsequent to transection/laminectomy, a second laminectomy was performed at the L1/L2 vertebrae immediately afterwards to expose the L6/S1 segments for BDA injection. Alternatively, a second laminectomy was performed two weeks after transection/non-transection at the T8 vertebrae to expose the T9 segment for FB or CTb injections. After surgical operations were complete, the erector spinae muscles were sutured with 3–0 Vycril (Ethicon, Sommersfield, NJ), the field was disinfected with povidone-iodine solution (Nova Plus, Irving, TX), and the skin was closed with Michel wound clips (Roboz, Gaithersburg, MD). For post-operative care, animals were administered 20 ml lactated Ringer's solution (Baxter Healthcare, Deerfield, IL) and 33 mg/kg cephalosporin (Apothecon, Bristol-Myers Squibb, Princeton, NJ) subcutaneously immediately after surgery, and for injured rats twice daily for up to 10 days to maintain hydration and control infection. Buprenorphine (0.035 mg/kg; Reckitt Benckiser, UK) was also administered subcutaneously once after recovery from anesthesia and twice daily for the next three days to control post-operative pain. Bladders of injured rats were manually expressed twice daily until automatic bladder-emptying reflex developed at about 10 days post injury.

Anterograde tracer injection: BDA

Immediately after laminectomy/transection (acute) or 2 weeks later (delayed), a second laminectomy was done at L1/L2 to expose the dorsal spinal cord at L6/S1 level and the dura matter was incised. For the acute injections, a beveled glass micropipette with a tip diameter of 30–50 μ m was positioned over the right L6/S1 dorsal horn 300 μ m lateral from midline and lowered 500 μ m ventral from the dorsal surface using a micromanipulator (World

Precision Instruments, Sarasota, FL). The micropipette was left undisturbed in the spinal cord to equilibrate for 2 minutes before 200 nl of 5% BDA (10,000 MW dissolved in saline; Molecular Probes, Eugene, OR) was pressure-injected into the spinal cord unilaterally in 50 nl pulses over a period of 1 minute via a nano-injector (World Precision Instruments, Sarasota, FL). The pipette was left in place another 2 minutes to prevent leakage of the tracer upon slow withdrawal of the pipette. In this paradigm, ipsilateral refers to the BDA injection side (Fig. 2A), and animals were perfused to remove their spinal cords for histology after 2 weeks transport. In order to be consistent with the delayed retrograde tracing time course (see below), the delayed BDA injection group was injected bilaterally (200 nl/side) into the dorsal spinal cord at L6/S1 level at 2 weeks post-T4 transection/non-transection using the same coordinates (Fig. 2B). They were then perfused 1 week later to remove their spinal cords for similar densitometry, based on preliminary data from our retrograde tracing experiments with 1 week transport (see below). Notably, in acute versus delayed injection groups, it is possible that the different transportation times after injury/sham can affect the intensity of labeling.

One week prior to perfusion, selected non-transected ($n = 2$) and injured ($n = 2$) rats (acute and delayed BDA injection groups) were given an intraperitoneal injection of FluoroGold (0.2 ml of 1.5% in saline; Biotium, Hayward, CA) to label all sympathetic preganglionic neurons in the intermediolateral cell column (IML) (Anderson and Edwards, 1994).

Retrograde tracer injections: FB & CTb

To corroborate the anterograde tracing results, another cohort of non-transected and T4-transected rats were re-anesthetized two weeks post-laminectomy/transection and a second laminectomy was performed to expose the T9 spinal segment for injections of retrograde tracers into dorsal horns, bilaterally (Fig. 2C). A beveled glass micropipette with a tip diameter of 30–50 μm was positioned 400 μm lateral from midline and lowered 400 μm ventral from dorsal surface. In one group, 100 nl of 2.5% FB (Polysciences, Warrington, PA) diluted in sterile saline was delivered in 50 nl pulses over a period of 30 seconds via a nano-injector. In a separate group, 50 nl of 0.25% CTb (List Biological Laboratories, Campbell, CA) diluted in distilled water was injected into the spinal cord using the same coordinates for FB. After one week of transport, all animals were euthanized for spinal cord dissection.

Activation of c-Fos

The immediate early gene, *c-fos*, and its protein product, c-Fos, are integral components of the complex cellular mechanisms that link stimulation to subsequent increases in cellular activity (Dragunow and Faull, 1989; Minson et al., 1994). c-Fos expression is used as a marker to identify neurons activated by different types of stimuli including injury, neuroactive drugs, and growth factors (Sagar et al., 1988; Xu et al., 2006). To monitor the spatial distribution of neuronal activity throughout the DGC following intermittent colorectal distension, we quantified c-Fos immunolabeling in T4-transected and non-transected rats. To elicit c-Fos expression in the spinal cord (Landrum et al., 2002), colorectal distension was initiated 14 days post-injury, 2 hours before perfusion. Non-transected and injured rats were gently restrained and remained conscious throughout the procedure. A catheter tipped with a latex balloon (Swan-Ganz catheter; Edwards Lifesciences, Irvine, CA) was inserted 2 cm into the rectum and secured to the tail with adhesive tape. The balloon was inflated with 1.5 ml air and held for 30 seconds, after which it was deflated for 60 seconds. This 90 second cycle (30 on, 60 off) was repeated continuously for 1 hour 40 minutes. The animals were then left undisturbed (i.e. with catheter deflated) for 20 minutes and perfused immediately thereafter for spinal cord dissection.

Fixation and dissection

Animals received an anesthetic overdose with sodium pentobarbital (150 mg/kg; Abbott, Chicago, IL) and perfused transcardially with 0.1 M phosphate buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS. The spinal cord from the conus medullaris to the transection site was removed, post-fixed for 4 hours, rinsed in 0.2 M phosphate buffer (PB) overnight, and cryoprotected for at least 48 hours in 20% sucrose in 0.1 M PBS. The caudal and rostral limits of the dissected spinal cords sampled were between 0.5 mm rostral of the conus medullaris (~S4) to 6 cm rostral at the T5 segment. The spinal cords were then divided into two 3 cm portions of caudal (S4-T13) and rostral segments (T12-T5) and embedded in gum tragacanth (Sigma-Aldrich, St. Louis, MO) in 20% sucrose/PBS for cryosectioning, as previously detailed (Cameron et al., 2006). Spinal cord segments were embedded side by side with the rostral ends of each spinal cord evenly aligned within plastic cryomolds (4–6/mold) and the entire mold was snap-frozen in acetone chilled to -40°C and stored at -80°C until sectioning on a cryostat (Microm Laborgerate, Walldorf, Germany).

Tissue processing for quantification

For animals that received BDA injections, both thoracic and lumbosacral segments of spinal cord were serially cryosectioned in the longitudinal, horizontal plane at 50 μm and consecutively mounted onto glass slides (Superfrost plus, Fisher Scientific, Pittsburgh, PA) in 5 series of 5 slides. This rendered 250 μm separations between serial sections within a series on each slide. A role of a die was used to decide which series to evaluate. Another random series was also used for densitometric analysis of enkephalin immunoreactivity (see Immunofluorescent histochemistry).

For animals that received FB or CTb injections or colorectal distension-induced c-Fos expression, the thoracic and lumbosacral segments were similarly aligned and embedded, but for serial transverse cryosectioning. Approximately 15 consecutive rows of 20 μm transverse cryosections from each block (up to 6 spinal cords per block) were placed onto each of 10 adjacent slides in two series, as previously detailed (Cameron et al., 2006). In this manner, sections on adjacent slides were separated by 100 μm , resulting in 1 mm separation of adjacent sections within a series on each slide. Every other section in each slide series was analyzed beginning with the first or second section, determined by a coin flip. If a section was missing or damaged in such a way that it could not be measured, the same numbered section from an adjacent slide in the same series was measured (100 μm separation). All mounted slides were stored at -20°C until staining procedures.

Immunofluorescent histochemistry

The complete information of all primary antibodies used is detailed in Table 2. To label BDA⁺ fibers, slides with mounted longitudinal sections were removed from the freezer to thaw prior to rehydration in 0.1 M PBS. They were then pre-incubated in PBS containing 0.5% Triton-X for 1 hour before exposure to streptavidin-fluorescein (FITC) (Vector, Burlingame, CA; 5 $\mu\text{g}/\text{ml}$ in 0.1 M PBS and 0.5% Triton-X) for 3 hours at room temperature, followed by final rinses (3×10 minutes) in PBS. To label enkephalinergic (Enk⁺) fibers in longitudinal sections, mouse anti-rat enkephalin (Enk) (1:200) was added on sections overnight at 4°C after blocking in PBS containing 0.5% Triton-X and 5% normal donkey serum for 1 hour. Slides were then rinsed and incubated with donkey anti-goat conjugated to Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:200, 7.5 $\mu\text{g}/\text{ml}$) for 3 hours at room temperature, followed by final rinses (3×10 minutes) in PBS.

For CTb-injected spinal cords, slides with mounted coronal sections were thawed and pre-incubated in 0.1 M PBS containing 0.5% Triton-X and 5% normal donkey serum (Vector

Laboratories, Burlingame, CA) for 1 hour, followed by incubation with goat anti-CTb (List Biological Laboratories, Campbell, CA, 1:1500) in same buffer overnight at 4°C. The slides were then rinsed before applying donkey anti-goat conjugated to FITC (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:200, 7.5 µg/ml) overnight at 4 °C. Alternatively, for FB-injected spinal cords, rinsed slides were directly coverslipped using Vectashield mounting medium for visualizing FB-labeled cells under UV fluorescence.

For c-Fos immunolabeling, thawed slides with mounted cross sections were rehydrated and pre-incubated in 0.1 M PBS containing 3% normal donkey serum and 0.3% triton-X for 1 hour at room temperature before applying goat anti-c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA; 2 µg/ml) overnight at 4°C. Slides were then thoroughly rinsed and incubated with donkey anti-goat conjugated to Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:200, 7.5 µg/ml) for 3 hours at room temperature.

After final rinses, slides were coverslipped using Vectashield mounting medium (Vector, Burlingame, CA) containing 5 µM Hoechst 33342 nuclear dye (Sigma-Aldrich, Saint Louis, MI) and sealed with Cutex nail hardener (Jackson, WY) for visualizing cellular distribution under an Olympus BX51 microscope (Olympus Corp. Melville, NY) equipped with epifluorescent illumination connected to an Olympus 'MagnaFire' digital camera to capture images.

For phenotypic characterization of retrogradely-labeled cells, immunohistochemical staining was conducted for GABAergic [rabbit anti-rat γ -aminobutyric acid (GABA)], glutamatergic [rabbit anti-rat glutamate], cholinergic [mouse anti-rat choline acetyltransferase (ChAT)] or enkephalinergic [mouse anti-rat Enk] immunolabeling in coronal sections (see Table 2). Sections were pre-incubated in 0.1 M PBS containing 0.5% Triton-X and 5% normal donkey serum for 1 hour at room temperature. The concentrations of secondary antibodies were, individually, donkey anti-rabbit conjugated to Texas-Red (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:200, 7.5µg/ml) for glutamate or donkey anti-mouse conjugated to Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:200; 7.5 µg/ml) for GABA, ChAT and Enk. All control slides had the primary antibody omitted before applying the secondary. The slides were rinsed and coverslipped with Vectashield containing 5 µM Hoechst 33342 nuclear dye for visualizing cellular distribution under an Olympus IX81 spinning confocal microscope (Olympus Corp. Melville, NY). Photomicrographs in all figures were optimized for final production by adjusting only the brightness and contrast using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA). Notably, since BDA⁺ immunofluorescence intensity was weak at low magnifications, we constructed a collage of higher magnification photographs, adjusting equivalent levels of brightness and contrast in sequential overlaid images. All the figure graphs were created with DeltaGraph 5.4 (Red Rock Software, Inc., Salt Lake City, UT).

Antibody specificity

All primary antibodies used in this study were previously characterized and are summarized in Table 2. The specificity of the ChAT antiserum (Chemicon; #AB305) was verified by western blot analysis of rat brain tissue, in which the antibody recognized a single band at 67 kD (Dietz and Salvaterra, 1980). For the anti-GABA antibody (Sigma-Aldrich; #A2052), the antiserum was raised in rabbit against GABA-bovine serum albumin (BSA) conjugate. According to the manufacturer, the antibody was isolated from the antiserum by immunopurification to remove essentially all rabbit serum proteins, including immunoglobulins that do not specifically bind to GABA. In dot blot assays, the specificity of this antiserum showed positive binding to GABA and GABA-keyhole limpet hemocyanin (KLH) and negative binding to BSA. Importantly, *in vivo* testing correctly identified GABAergic interneurons in the olfactory bulb of the silkworm moth (Seki and Kanzaki, 2008). The

glutamate antibody from Abcam (Cat. #ab9440) was raised in rabbit against L-glutamate conjugated to glutaraldehyde as the immunogen peptide. This antibody was characterized against a spectrum of antigens to assure hapten selectivity. The manufacture's information shows no measurable cross-reactivity detected against glutamate in peptides or proteins, but modest cross-reactivity against D-glutamate. The specificity of this antibody was confirmed *in vivo* by immunostaining rat retinal tissue (Sun et al., 2007). For enkephalin (Chemicon; #MAB350), the antiserum was raised in mouse against L⁵-enkephalin conjugated to BSA. Dot blot immunoassay showed specific binding for Met⁵- and Leu⁵-enkephalin, but displayed minimal cross-reactivity to other endogenous peptides. *In vivo* testing in rat brain using both radioimmunoassay and immunocytochemistry showed specific immunoreactivity for enkephalin (Cuello et al., 1984). The c-Fos antiserum (Santa Cruz Biotechnology, #sc-52-G) was developed against a synthetic peptide sequence corresponding to the N-terminus of human c-Fos (Table 2). The specificity of this antibody was confirmed by immunoblotting in Syrian Hamster Embryonic cell line (Preston et al., 1996) and subsequently verified with western blot analysis of human T cells (Whisler et al., 1997).

Quantification

All quantifications were conducted in a blinded fashion using the Bioquant® image analysis program (Nova Prime, V6.70.10; Bioquant Image Analysis Corp., Nashville, TN) on each series of immunolabeled longitudinal and coronal sections viewed under an Olympus BX51 microscope (Olympus Corp. Melville, NY) using established methods (Cameron et al., 2006; McCullers et al., 2002; Pandya et al., 2007; Rabchevsky et al., 2007). Live images were captured using an Optronics digital video camera (Optronics Corp., Goleta, CA) in conjunction with Microcode II stage encoders (Boeckler Instruments, Tucson, AZ) for densitometric or stereological analyses.

Densitometry of BDA⁺ fibers—To assess BDA⁺ fiber density in sections from acute or delayed injected spinal cords, densitometric quantification using Bioquant® image analysis program was chosen to obtain efficient and unbiased quantification of labeled fibers compared to manual measurements (Grider et al., 2006). From each animal, 3 longitudinal, horizontal thoracic and lumbosacral sections, each separated by 250 µm, between the central canal and superficial dorsal horns, were randomly selected for BDA⁺ fiber density quantification. For standardization, the injection sites were first delineated (see below), and the entire gray matter area starting 1.5 mm rostral to the injection site in each lumbosacral and thoracic section extending up to the T5 gray matter was analyzed, bilaterally, at 10× objective magnification (10× eye piece). Thus, the data represent whole gray matter areas measured, not random sampling.

Threshold measurements of BDA⁺ fibers were performed using a “live” image capture procedure, in which the perimeter of the section (for reference), as well as regions of gray matter, bilaterally, were outlined and converted into area measurements by the program. Approximately 10–15 contiguous “captured” images were generated per side, corresponding to adjoining gray matter areas within each of the two contiguous segments. The variability in the number of images “captured” is based on differential gray matter area depending upon lumbosacral versus thoracic gray matter segments and/or the plane of sectioning (i.e. dorsal versus medial gray matter). To minimize BDA⁺ fiber threshold variability through the focal plane, all density measurements were taken 20 µm ventral from the surface of each section. For each manually “captured” image, the brightness, contrast and exposure times were set to be constant for all analyses.

The total BDA⁺ gray matter area per section was first determined, unilaterally, by summing all quantified “captured” threshold images in both thoracic and lumbosacral segments. For

acute unilateral injections, the mean BDA⁺ fiber density in gray matter was calculated by dividing total BDA⁺ fiber area by total gray matter area for each side of each spinal cord segment (i.e. thoracic and lumbosacral, 3 sections each). For delayed bilateral injections, density was calculated by dividing total BDA⁺ fiber area by total gray matter for both sides of each spinal cord segment (i.e. thoracic and lumbosacral, 3 sections each). Thus, in the acute injection group the mean BDA⁺ fiber density in gray matter was expressed as ipsi- and contralateral with respect to the injection site, whereas in the delayed group it was expressed bilaterally.

We estimated the BDA injection volumes and the rostra-caudal diffusions to assess possible variability between these groups. The area with the brightest intensity in each section was carefully outlined and quantified. The mean BDA injection area corresponding to each of 3 sections was determined and multiplied by the distance between the central canal and superficial dorsal horns (750 μ m) to estimate volume, and the mean diffusion of BDA was derived from the rostral to caudal margins of the injection sites.

Densitometry of Enk⁺ fibers—Using similar analysis methods as described above, Enk⁺ fiber density in regions of gray matter within 2 mm rostral and caudal of the L6/S1 segmental boundary, bilaterally, was quantified for 3 sections per animal from the acute BDA injection group. The Enk⁺ fiber density was calculated by dividing total area of Enk⁺ fiber immunolabeling by total L6/S1 gray matter area, bilaterally.

Quantification of FB or CTb retrogradely-labeled neurons—Animals were excluded from the study if the injections of CTb or FB were localized too ventral, lateral or midline to the dorsal horns. All retrograde tracer injections were spread throughout the dorsal horns no deeper than lamina VII, and the rostro-caudal spread of FB or CTb injections was approximately 3–4 mm. To assess the extent of FB or CTb-labeling in the DGC of non-transected and injured spinal cords, the total number of labeled neurons was determined in the DGC at 3 weeks post-injury (after 1 week tracer transport). For each animal, all labeled DGC neurons in 18 equally spaced systematically sampled coronal sections were counted from T13/L1 to S1/S2 spinal level. This procedure was processed on the computer screen and each labeled cells was confirmed by observing its nucleus. The anatomical boundaries of the DGC in lamina X on each section were defined based on previous reports (Hancock and Peveto, 1979; Yaici et al., 2002a; Yaici et al., 2002b), as shown in Figs. 1 and 3A. Because counts of profiles in sections inevitably produce an overcount, correction factors were used to rectify potential errors. Once the thickness of sections and the diameter of labeled cells were measured, the “real” number of labeled cells in the sampled sections was obtained according to Abercrombie’s Formula: the “real” cell number equals the “observed” cell number multiplied by $T/T+h$, where T = section thickness and h = mean diameter of the labeled cells along the axis perpendicular to the plane of the section (Abercrombie and Johnson, 1946; Guillery, 2002).

Stereology of c-Fos⁺ cells—To estimate total c-Fos⁺ cells in the DGC and dorsal horn from T13-S1 in cross sections from each spinal cord (Fig. 3B), we employed the optical fractionator technique (West et al., 1991) to 11 evenly spaced cryosections at 2 mm intervals. The optical fractionator technique involves the combining of optical disector counting with fractionator sampling that is unaffected by tissue shrinkage due to tissue loss (McCullers et al., 2002). A uniform counting grid was placed over the bordered region with a counting frame setting of $150 \times 150 \mu\text{m}$ ($22,500 \mu\text{m}^2$). Since the injuries were complete transections, the cell counts were calculated for each spinal cord, unilaterally (left side). The disector area was set at $50 \times 50 \mu\text{m}$ ($2,500 \mu\text{m}^2$) and the height of the disector was set at 10 μm , with a 2 μm thick guard zone on the top and bottom. This generated 15–25 optical disectors depending on the spinal level. To count labeled cells, each disector was viewed

under 40× objective magnification (10× eye piece). In brief, all cells within the counting frame or touching the upper or right inclusive sides of the frame were counted through the depth of the disector. Cells touching the left or lower forbidden sides of the frame were not counted. Labeled cells with the nuclei in the plane of focus within the top 2 µm guard zone were not counted, but all cells with nuclei in focus within the 10 µm disector were counted. The total number of c-Fos⁺ cells in each cord were calculated using our specific parameters, detailed above, using the published equation (McCullers et al., 2002). Coefficient of error (CE) and coefficient of variation (CV) were calculated to determine intra-animal variation and inter-animal variation, respectively.

Statistical analysis

Statistical analyses between spinal-transected and non-transected rats were performed using Statview (SAS Institute, Cary, NC). Unpaired Student's t-test employing Bonferroni correction factors were used between non-transected and injured groups. Significance throughout all experiments was set at $p < 0.05$. Data is represented as mean $\pm$ SD.

RESULTS

Anterograde tract tracing: BDA

For acute BDA injections, unilateral injection sites were identified in coronal (Fig. 4A) and longitudinal (Fig. 4B) sections by examining the fluorescence intensity in both T4-transected and non-transected spinal cords. Injections were located predominantly in the ipsilateral L6/S1 DGC in both non-transected and T4-transected spinal cords, and the distribution of labeled axons appeared similar proximal to the injections. BDA⁺ fibers were seen throughout the extent of the dorsal gray matter, bilaterally, up to thoracolumbar regions, but the density of labeled ascending projections gradually decreased with distance from the injection site (Fig. 5A,B), as previously reported in normal rats (Matsushita, 1998). However, in injured rats the thoracic spinal segments appeared to have increased BDA⁺ fibers throughout the dorsal gray matter, bilaterally (Fig. 5C,D). Statistical analysis showed that BDA⁺ fiber density in the lumbosacral segments was not significantly ($p > 0.1$) different in either ipsi- or contralateral gray matter between the non-transected and injured groups (Fig. 6A). This is likely due to proximity of the L6/S1 injection site and/or the acute timing of injection (2 weeks pooling and transport of tracer). Conversely, in thoracic segments of the transected spinal cords there was significantly greater BDA⁺ fiber density in both the ipsi- ($p < 0.01$) and contralateral ($p < 0.05$) gray matter compared to non-transected cords (Fig. 6B).

To rule out potential artifactual labeling after acute injections post-injury (Steward et al., 2007), in another cohort of animals BDA was injected 2 weeks post-injury (delayed) to label putative established sprouts. One week after delayed injections, there was significantly greater BDA⁺ fiber density in both the lumbosacral ($p < 0.05$) (Fig. 7A) and thoracic ($p < 0.05$) (Fig. 7B) gray matter of injured spinal cords compared to non-transected cords. Notably, however, the overall BDA⁺ fiber density in the acute injection paradigm was orders of magnitude greater compared to delayed BDA injections in both lumbosacral and thoracic spinal cord segments, despite bilateral injections in the delayed group (compare Figs. 5 and 6). Potential explanations for such discrepancies are discussed in Methodological considerations.

To begin addressing this disparity, we compared BDA injection volumes and rostro-caudal diffusion from the injection site, as well as total gray matter area measurements to obviate tissue-related variation between injured/non-transected groups. In the acute injection group, BDA volume and diffusion, as well as total gray matter area (an index for tissue shrinkage),

showed no significant differences between non-transected and injured groups in either lumbosacral or thoracic segments ($p>0.1$) (Table 3). Similarly, in the delayed injection group, BDA volume and diffusion, as well as total gray matter area measured, showed no significant differences ($p>0.1$) between non-transected and injured groups in either lumbosacral or thoracic segments ($p>0.1$) (Table 3). Therefore, we standardized our BDA⁺ fiber threshold measurements to start 1.5 mm rostral to each injection site at specified depth into the plane

Notably, peripheral injection of FluoroGold one week before histological evaluation in the acute and delayed BDA injection groups, which labeled sympathetic preganglionic neurons in the IML, revealed juxtaposition of BDA⁺ fiber projections among labeled neurons in thoracolumbar segments of both non-transected and injured groups ($n = 2$ per group). However, such appositions appeared more prominent after injury in the acute injection group due to the significant increase in BDA⁺ fiber density in thoracic segments compared to non-transected cords (Fig. 8). On the contrary, few such appositions were seen in the injured delayed injection groups, in which the BDA labeling intensity was markedly reduced throughout the spinal cord. Potential explanations for such discrepancies are discussed in Methodological considerations.

Retrograde tract tracing: FB & CTb

In retrograde tracing studies, FB or CTb injections were localized bilaterally in dorsal horns of the T9 spinal segment, including laminae VII, X and IML. All tracer injections were spread throughout the dorsal horns no deeper than lamina VII (Fig. 9). FB- or CTb-labeled neurons were distributed throughout the gray matter caudal to the injection site. At the T13/L1 spinal level, they were primarily located in laminae I-VII and X. The number of labeled neurons in laminae I-V gradually decreased caudally, and virtually none were observed at L4. In contrast, the density of labeled cells in the DGC appeared to progressively increase caudal to the injections, reaching maximum levels between L5-S2 segments. Labeled neurons were small to medium in size in the L6/S1 DGC, with some large cells scattered in the ventral horn. A similar distribution of retrogradely-labeled cells was observed in the gray matter of T4-transected and non-transected rats, but the density of labeled cells appeared increased in injured rats (Fig. 10). Following stereological sampling in the DGC (see Fig. 3A), statistical analysis showed that the total number of retrogradely-labeled neurons from T13-S1 levels of T4-transected spinal cords was significantly greater versus non-transected cords for both FB ($p<0.05$) and CTb ($p<0.05$) injection groups (Fig. 11).

Phenotypes of retrogradely-labeled DGC neurons

Phenotypic analysis of FB or CTb retrogradely-labeled neurons under confocal microscopy in both T4-transected and non-transected spinal cords revealed co-localization with glutamate, GABA and ChAT throughout the DGC, but not Enk (Fig. 12).

Enk⁺ fiber sprouting

We have reported that post-traumatic sprouting of CGRP⁺ afferent fibers into the lumbosacral spinal cord correlates temporally with colorectal distension-induced autonomic dysreflexia (Cameron et al., 2006). In this study, we also found that NGF-over-expression markedly altered the spatial distribution pattern for intraspinal Enk⁺ fibers similar to sprouting CGRP⁺ fibers, although there was no co-localization (unpublished observation). We reasoned, therefore, that perhaps the increased afferent fiber spouting after injury alters the distribution of Enk⁺ fibers. We found that the density of Enk⁺ fibers appeared prominently in the L6/S1 gray matter of both non-transected and T4-transected spinal cords (Fig. 13A,B). However, the extent of Enk⁺ fiber immunolabeling was noticeably increased

in injured spinal cords (Fig. 13C,D). Densitometric analysis confirmed significantly ($p < 0.05$) greater Enk⁺ fiber density in the L6/S1 gray matter after T4 transection (Fig. 13E).

c-Fos immunolabeling

Stereological quantification of c-Fos⁺ cells induced by intermittent noxious colorectal distension prior to euthanasia was assessed in the DGC and the superficial dorsal horns (see Fig. 3B). c-Fos⁺ cells were far more prominent in injured spinal cords versus non-transected cords, the latter of which showed scant labeling and distribution throughout the gray matter (Fig. 14). Statistical analysis revealed significantly ($p < 0.01$) more c-Fos⁺ cells in lumbosacral segments of T4-transected versus non-transected cords (Fig. 15A). Furthermore, the increased number of c-Fos⁺ cells was significantly ($p < 0.05$) greater at each level from T13-S1 segments (Fig. 15B). Intra-animal variation (CE) in cell counts was 0.09 for injured and 0.24 for non-transected cords, and inter-animal variation (CV) in cell counts was 0.42 for injured and 0.84 for non-transected cords. Notably, the variation in values was higher in non-transected cords due to the low numbers of c-Fos⁺ cells after colorectal distension.

DISCUSSION

Utilizing anterograde and retrograde neuronal tract tracing methods, the major findings in the present study include significant increases in axon density of lumbosacral DGC propriospinal fibers in the weeks following complete high thoracic spinal cord transection. In addition, there was a significant increase in the number of c-Fos⁺ cells in the lumbosacral gray matter of injured animals following noxious intermittent colorectal distension. Therefore, the current results corroborate neuroanatomically the electrophysiological evidence for a significant role of propriospinal plasticity (Krassioukov et al., 2002), as well as afferent fiber sprouting (Cameron et al., 2006; Krenz and Weaver, 1998b), in the development of autonomic dysreflexia after complete high thoracic SCI. The increases in the density of BDA-labeled axons and numbers of retrogradely-labeled DGC neurons are consistent with sprouting of ascending projections, but there are caveats and methodological issues that do not completely exclude other possibilities.

Methodological considerations

Discerning possible artifactual from genuine labeling has been the stumbling block of previous studies, as highlighted recently (Steward et al., 2007). To ensure the consistency of BDA injections at the L6/S1 level for anterograde tracing, the dura matter was always cut and this leads to local cerebrospinal fluid (CSF) leakage. If BDA managed to diffuse and reach the transection site along the central canal in the CSF and labeled injured or cut fibers, we would expect to see numerous labeled fibers near the transection site in the thoracic spinal level. In fact, the number of labeled projections gradually decreased from the lumbosacral (injection site) to the thoracic segments (Matsushita, 1998). We did not observe any labeled fibers near the transection site, illustrating that it is improbable that BDA diffusion produced artifactual labeling via the transection site. At high magnification, the BDA labeling seen along the central canal was ascending or descending fibers, as previously reported (Matsushita, 1998). Assessments of BDA injection volume and diffusion, as well as total gray matter area analyzed in both transected and non-transected spinal cords revealed no significant differences between these two groups (Table 3).

Unexpectedly, BDA⁺ fiber density in the delayed experiments was orders of magnitude lower than after acute BDA injection. After careful consideration, this discrepancy is likely due to differential BDA transportation time. In the acute group, BDA was injected immediately after T4-transection/non-transection and animals were perfused 2 weeks later.

In contrast, in the delayed group the animals were injected 2 weeks post-T4-transection/non-transection and perfused 1 week later. According to the transportation rate of BDA (2.2 mm/day) (Ye et al., 2000), the 2 weeks survival time in the acute injection group would be expected to have greater density of labeled fibers due to increased pooling and transport of BDA compared to delayed injections with 1 week transport (Kratskin et al., 1997). If we had allowed 2 weeks of BDA transport in the delayed injection group, sufficient tracer would have reached sprouting collaterals to increase the labeling density of BDA⁺ fibers, and further demonstrated juxtaposition with FluoroGold-labeled cells. This is consistent with the report that, with increasing survival time post injection, BDA⁺ fiber labeling becomes more intense distally (Novikov, 2001).

Importantly, identical procedures for densitometry were employed, including standardization of gray matter/BDA⁺ fiber sampling beginning at 1.5 mm rostral to the injection site on each section. The fact that more retrograde labeling occurred after injury following delayed injections, despite that BDA labeling was markedly reduced with the same transportation time, supports the phenomenon of BDA pooling and differential transport rates of the tracers. Moreover, the reduction of BDA labeling intensity in the thoracic gray matter segments after delayed injections (1 week transport) versus acute injections (2 weeks transport) indicates insufficient time for transport from L6/S1 to T9 (~25 mm), which would require a minimum of 10 days.

Intraspinal plasticity of propriospinal neurons

Elevation in spinal NGF levels following SCI (Brown et al., 2004) and the subsequent intraspinal sprouting of CGRP⁺ afferent fibers are reported to be contributing factors underlying the development of experimental autonomic dysreflexia (Krenz and Weaver, 1998b; Marsh et al., 2002). The consequent afferent barrage into the dorsal horns causes massive, sympathetic discharge below the level of the lesion, resulting in vasoconstriction of the muscular, splanchnic, and cutaneous vascular beds that leads to dysreflexic hypertension (Chau et al., 2000; Weaver et al., 2001). We showed previously that after T4 transection, intraspinal over-expression of NGF in the lumbosacral dorsal horns, which receive sensory input from the pelvic viscera, significantly enhanced local CGRP⁺ afferent fiber sprouting (Cameron et al., 2006). This was directly correlated with exacerbation of hypertensive autonomic dysreflexia, while the opposite conditions occurred with over-expression of chemorepulsive semaphorin 3A. This confirms a crucial role for CGRP⁺ afferent fiber sprouting in modulating the severity of autonomic dysreflexia, but what remains unknown is how primary afferent input from the pelvic viscera is conveyed rostrally to sympathetic preganglionic neurons in the IML of the thoracolumbar spinal cord. (Al-Chaer et al., 1996). It is our hypothesis that increased local nociceptive afferent sprouting following SCI elicits plasticity of lumbosacral propriospinal DGC neurons.

Previous elegant electrophysiology studies showed that spinal neurons are activated by pelvic visceral distension and relay primary afferent information rostrally to supraspinal targets (Chau et al., 2000; Tang et al., 2003). The DGC, as a part of lamina X, is a continuous gray matter region within the spinal cord midline above the central canal, and in the lumbosacral segments it relays information from pelvic viscera (Lu et al., 2001; Molander et al., 1984; Molander et al., 1989). Propriospinal neurons are known to be the predominant cell population of the spinal cord (Chung and Coggeshall, 1983; Masson et al., 1991). Germane to the current study is the report of propriospinal neurons connecting lamina X throughout the rat spinal cord up to cervical levels after injecting BDA into the central gray at L6-S2 (Matsushita, 1998), indicating that long-ranging propriospinal neurons exist in the lumbar gray matter. The dense network made up of ascending axons throughout the length of lamina X is thought to sub serve modulation of noxious as well as visceral

input based upon the neurochemical substances, receptors, and supraspinal projections of lamina X neurons (Matsushita, 1998).

Therefore, we injected BDA into the L6/S1 DGC, unilaterally, immediately following complete T4 transection/non-transection (acute) and found significant increases in propriospinal labeling in thoracic spinal segments 2 weeks after injury. Accordingly, we also observed more prominent appositions between BDA⁺ fibers and FluoroGold-labeled sympathetic preganglionic neurons in the thoracic spinal cord after injury compared to non-transected cords (Fig. 8). Following delayed, bilateral BDA injections the fiber density was significantly greater after injury versus non-transected cords in both thoracic and lumbosacral segments. However, the magnitude of BDA⁺ fiber density was markedly reduced compared to acute injections, as were the appositions with FluoroGold-labeled sympathetic preganglionic neurons (see Methodological considerations). While BDA-labeled corticospinal tract axons are reported to be more closely apposed to FluoroGold-labeled sympathetic preganglionic neurons in spinal lesioned rats versus non-transected cords (Pan et al., 2005), these additional synapses did not increase sympathetic activity after SCI at the level of electrophysiology following cortical stimulation (Pan et al., 2007). Therefore, without differential tissue processing and subsequent EM analysis we can only speculate whether our observed BDA⁺ fiber appositions have physiological influences on sympathetic preganglionic neurons and/or interneurons.

The current results imply that plasticity of lumbosacral DGC propriospinal neurons is correlated with the development of autonomic dysreflexia. Nevertheless, it remains uncertain whether these projections reflect sprouting of existing fiber tracts or plasticity of more DGC propriospinal neurons activated by injury-induced afferent fiber sprouting. Taking into consideration the significant increase in BDA-labeled fibers after transection, and the insufficient time for extensive regeneration (~25 mm from L6/S1 to T9), it is likely that the increase in retrogradely labeled neurons after injury is due to the sprouting of existing fibers. Such sprouting distal to the injection site is expected to greatly increase the likelihood of tracer uptake by collaterals with subsequent transport to the soma, thus increasing the probability of the cell filling with the tracers and being observed by fluorescent illumination (see Methodological considerations).

c-fos is a proto-oncogene that functions to couple short-term extracellular signals, which is rapidly expressed in neurons following various noxious stimulations (Morgan and Curran, 1991). Therefore, the protein product, c-Fos, is used as an indirect indicator of cellular activity or neuronal responses to SCI (Landrum et al., 2002; Xu et al., 2006). Importantly, our hypothesis is supported by significant increases in c-Fos immunolabeling in the lumbosacral DGC and dorsal gray matter of injured animals following intermittent colorectal distension. While noxious colorectal distension did activate sparse c-Fos expression in non-transected spinal cords, there was significantly less immunolabeling compared to the injured rats, probably due to inhibition from supraspinal levels in intact animals (Vizzard, 2000).

We cannot eliminate the possibility that DGC neurons die following transection. However, this is unlikely based on our FB and CTb retrograde tracing results. The local increased expression of c-Fos, accompanied by plasticity of DGC propriospinal neurons following SCI, potentially conveys CGRP⁺ nociceptive pelvic visceral input rostrally from the lumbosacral spinal cord. We hypothesize that this signal may, in turn, excite thoracolumbar sympathetic preganglionic neurons to elicit dysreflexic hypertension. Whether this stems from direct stimulation of sympathetic neurons or indirectly via sympathetically-correlated interneurons (Krassioukov et al., 2002; Tang et al., 2003) is a matter of ongoing investigations.

Phenotypes of propriospinal neurons

Spinal cholinergic neurons are mainly located in the IML (sympathetic preganglionic neurons), parasympathetic preganglionic nucleus, the DGC, as well as the ventral horn (Hancock and Peveto, 1979). Cholinergic sympathetic preganglionic neurons are subject to both excitatory and inhibitory influences from neurons located in the spinal cord, brainstem and higher centers. A loose network of transversely or longitudinally oriented cholinergic dendrites forms a longitudinal plexus throughout the DGC (Hosoya et al., 1994). Together with the dendrites of the sympathetic preganglionic neurons, lateral oriented dendrites of the DGC converge and form the transverse dendritic bundles in the intermediate zone that connect the DGC and the IML (Hosoya et al., 1994). In the central nervous system, L-glutamate is the major excitatory neurotransmitter (Llewellyn-Smith et al., 1998) and γ -aminobutyric acid (GABA) is the main inhibitory neurotransmitter which is synthesized from glutamate by the glutamate decarboxylase (GAD) enzyme (Schwartz-Bloom and Sah, 2001). In the spinal cord and higher centers, glutamate and GABA have been implicated in the control of blood pressure (Dampney, 1994). Our phenotypic analysis of DGC propriospinal neurons revealed a heterogeneous population containing glutamatergic, GABAergic and cholinergic neurons. Under normal conditions, glutamate-immunoreactivity occurs in about two-thirds of the terminals that synapse on sympathoadrenal neurons (Llewellyn-Smith et al., 1992), and GABA-immunoreactivity is present in about one-third of these terminals (Bacon and Smith, 1988). In the spinal cord, glutamate acts as the main excitatory synaptic neurotransmitter of primary sensory fibers (De Biasi and Rustioni, 1988), while GABAergic neurons are proposed to presynaptically inhibit primary sensory boutons (Todd and McKenzie, 1989).

It is plausible that one of the main triggers of sympathetic preganglionic neurons discharge following SCI could be an imbalance between glutamatergic and GABAergic inputs from the lumbosacral DGC neurons or interneurons proximal to the IML. Reduced glutamate and increased GABA neurotransmission has been reported in both acute and delayed stages after SCI (Diaz-Ruiz et al., 2007; Rodriguez et al., 2005). Since increased inhibitory GABAergic input is unlikely to trigger sympathetic preganglionic neurons discharge directly, a secondary inhibitory interneuron may, instead, inhibit GABAergic signaling to the IML, thus leading to dysreflexic hypertension. Such putative interactions are currently being investigated with transynaptic retrograde tracing experiments.

Enkephalinergic fibers

Enk⁺ fibers in the thoracolumbar spinal cord are of both supraspinal and intraspinal origin, as spinal cord transection reduces the descending contribution to specific sympathetic nuclei, but does not eliminate Enk⁺ fibers in the thoracolumbar spinal cord (Romagnano et al., 1987). Some studies report a general decrease in Enk immunoreactivity in all autonomic areas caudal to spinal transection (Holets and Elde, 1982), while others show increased Enk⁺ fiber density in sacral segments of transected spinal cords (Thor et al., 1994). In the present study, spinalized rats showed significantly more Enk immunoreactivity in the lumbosacral spinal cord 2 weeks after injury compared to non-transected cords. Since our immunohistochemical analysis showed that FB or CTb retrogradely-labeled DGC propriospinal neurons are not Enk⁺, this implies that plasticity of both DGC propriospinal neurons and Enk⁺ fibers following SCI are independent processes. In relation to the manifestation of autonomic dysreflexia, however, the putative interactions among CGRP⁺ afferent sprouts, altered intraspinal Enk⁺ fiber distribution and plasticity of propriospinal projections remain to be established.

In conclusion, the present study demonstrates that, in addition to injury-induced local primary afferent fiber sprouting, plasticity of lumbosacral DGC propriospinal neurons may

be a key neuronal substrate which relays noxious visceral input to stimulate thoracolumbar sympathetic preganglionic neurons that elicit autonomic dysreflexia. Future studies will focus on employing site-specific over-expression of various neurotrophic factors (other than NGF), along with transynaptic retrograde tract tracing methods, in an attempt to elucidate whether differentially altering primary afferent and/or DGC propriospinal plasticity acts to augment or mitigate the development of autonomic dysreflexia after T4 transection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Nissl stained cross sections showing the dorsal gray commissure (DGC, boxed regions) at different spinal levels, spanning the regions that were examined (T13/L1-L6/S1). Scale bar = 500 μ m.

Figure 2.

Schematic diagrams illustrating the pathways under investigation and the tract tracer injection sites. (A) Acute (immediately after T4-transection/non-transection) unilateral biotinylated dextran amine (BDA) injection at the L6/S1 dorsal gray commissure (DGC) for anterograde labeling in ipsi- and contralateral gray matter, (B) delayed (2 weeks after T4-transection/non-transection) bilateral BDA injections for anterograde labeling in gray matter, bilaterally and (C) delayed (2 weeks after T4-transection/non-transection) fast blue (FB) or cholera toxin subunit beta (CTb) injections at T9 dorsal horns to retrogradely label lumbosacral DGC neurons. Arrows indicate direction of tracer transport.

Figure 3. Photomicrographs demonstrating the regions of interest (*shaded insets*) at L6/S1 level for stereological quantification of (A) retrogradely-labeled cells in the dorsal gray commissure (DGC) or (B) c-Fos-immunoreactive cells in the left dorsal gray matter. Scale bar = 500 μ m. (CC central canal)

Figure 4.

Representative photomicrographs demonstrate the distribution of unilateral BDA injection sites (right) in the L6/S1 dorsal gray commissure (DGC) in (A) coronal and (B) longitudinal, horizontal sections. The circumscribed area in B represents an example of the spatial extent of BDA diffusion used to calculate BDA injection volumes. Scale bars = 500 μ m. (CC central canal)

Figure 5. BDA-labeled propriospinal pathways in (A, B) lumbosacral and (C, D) thoracic spinal cord segments 2 weeks after acute injection at L6/S1 in non-transected (Non-Tx) and T4-transected (T4-Tx) rats. Photomicrographs show apparent increased BDA⁺ fiber density in lumbosacral and thoracic gray matter in T4-transected compared to non-transected spinal cords. Scale bars = 1 mm (B), 100 μ m (D).

Figure 6.

Quantitative comparisons of BDA-labeled fiber density between non-transected and T4-transected spinal cords throughout the dorsal gray matter after unilateral *acute* injections. **(A)** In lumbosacral segments, BDA⁺ fiber density was not significantly different in either ipsi- or contralateral gray matter between injured and non-transected spinal cords. **(B)** On the contrary, in distal thoracic segments BDA⁺ fiber density in both ipsi- and contralateral gray matter was significantly greater in injured spinal cords compared to non-transected cords. Bars represent mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$

Figure 7.

Quantitative comparisons of BDA-labeled fiber density between non-transected and T4-transected spinal cords throughout the dorsal gray matter after bilateral *delayed* injections. In both (A) lumbosacral and (B) distal thoracic segments, BDA⁺ fiber density was significantly greater in gray matter of injured spinal cords, bilaterally, compared to non-transected cords. Bars represent mean ± SD. * $p < 0.05$.

Figure 8. Peripheral i.p. injection of FluoroGold (FG) at 1 week post-BDA injection (acute) shows juxtaposition of (A) FG-labeled sympathetic preganglionic neurons (*arrows*) in the intermediolateral cell column of thoracolumbar segments with (B) BDA⁺ fiber projections. Scale bar = 100 μ m.

Figure 9.

Representative photomicrographs demonstrating retrograde tracer injection sites made 2 weeks after T4 transection/non-transection (delayed). (A) FB or (B) CTb were injected into dorsal horn gray matter at the T9 level, bilaterally, and allowed to transport for 1 week to retrogradely label lumbar dorsal gray commissure (DGC) neurons. Scale bar = 500 μ m. (CC central canal)

Figure 10.

Photomicrographs of L6/S1 cross sections showing FB retrogradely-labeled propriospinal neurons in the dorsal gray commissure (DGC). (**A**) Compared to non-transected cords (Non-Tx), more DGC neurons appeared labeled in (**B**) T4-transected (T4-Tx) spinal cords 1 week after FB injections at T9, bilaterally. **C** and **D** are higher magnifications of boxed regions in **A** and **B**, respectively. Scale bars = 500 μm (**B**); 200 μm (**D**). (**DGC** dorsal gray commissure, **CC** central canal)

Figure 11.

Quantification of FB or CTb retrogradely-labeled dorsal gray commissure (DGC) neurons (T13-S1 segments) between non-transected and T4-transected spinal cords (see Figs. 1 and 3A for regions of interest). For each tracer, retrogradely-labeled DGC neurons were significantly greater in injured spinal cords compared to non-transected cords. Bars represent mean $\pm$ SD. * $p < 0.05$

Figure 12.

Phenotypic characterization of CTb retrogradely-labeled dorsal gray commissure (DGC) neurons at L6/S1 level. Confocal photomicrographs show dual immunohistochemical staining (*arrows*) for CTb in conjunction with (A-C) γ -aminobutyric acid (GABA), (D-F) glutamate (Glut), or (G-I) choline acetyltransferase (ChAT) in coronal sections. Scale bar = 10 μ m. Note: Magenta-green copy of images available as supplementary figure.

Figure 13.

Photomicrographs demonstrate enkephalin (Enk)⁺ fiber plasticity 2 weeks after T4 transection in longitudinal, horizontal sections. Compared to non-transected (Non-Tx) spinal cords (A), immunohistochemical staining for Enk⁺ fibers showed greater density in T4-transected (T4-Tx) spinal cords (B) in the L6/S1 segments. C and D represent higher magnifications of boxed regions in A and B, respectively. (E) Densitometric analysis of Enk⁺ fiber density in the L6/S1 gray matter, bilaterally, showed significantly more immunoreactivity in T4-transected spinal cords compared to non-transected cords. Scale bars = 1 mm (B); 500 μ m (D). Bars represent mean $\pm$ SD. * $p < 0.05$

Figure 14.

Representative photomicrographs demonstrating c-Fos⁺ neurons in the T13/L1 (**A, B**) and L6/S1 (**C, D**) dorsal gray matter from both non-transected (Non-Tx) and T4-transected (T4-Tx) spinal cords following 2 hours of intermittent colorectal distension prior to perfusion and fixation. Compared to Non-Tx spinal cords, c-Fos⁺ cells were conspicuously increased throughout the dorsal horns and dorsal gray commissure 2 weeks after injury. Scale bar = 200 μ m. (**DGC** dorsal gray commissure, **CC** central canal)

Figure 15.

(A) Stereological quantification, in unilateral dorsal horns and dorsal gray commissure (DGC) from T13-S1 (see Fig. 3B for region of interest), showed that the total number of c-Fos⁺ cells was significantly increased in T4-transected spinal cords compared to non-transected cords. (B) Stereological comparisons across lumbosacral segments revealed that the number of c-Fos⁺ cells in T4-transected cords was significantly greater than non-transected cords at each level. Bars and symbols represent means $\pm$ SD. ** $p < 0.01$

Table 1

Number of rats used in the experimental paradigms

Groups	T4-Transection	Non-Transection (Sham)
Anterograde tract tracing		
<i>Acute BDA injection</i> (n = 22)	11	11
<i>Delayed BDA injection</i> (n = 16)	8	8
FB retrograde tract tracing (n = 12)	6	6
CTb retrograde tract tracing (n = 12)	6	6
c-Fos expression (n = 16)	7	9

Table 2

Complete information and immunogen of primary antibodies applied

Antiserum	Species	Clonality	Working Dilution	Source Catalog/Lot Number	Immunogen
ChAT	Mouse	Monoclonal	1:100	Chemicon, Temecula, CA AB305/0704058097	Choline acetyltransferase purified from rat brain
GABA	Rabbit	Polyclonal	1:1000	Sigma-Aldrich, Saint Louis, MO A2052/106K4753	GABA-BSA
Glutamate	Rabbit	Polyclonal	1:50	Abcam, Cambridge, MA ab9440/180238	L-glutamate conjugated to glutaraldehyde
Enkephalin	Mouse	Monoclonal	1:200	Chemicon, Temecula, CA MAB350/0509010613	Leu ⁵ -enkephalin conjugated to BSA
c-Fos	Goat	Polyclonal	1:100	Santa Cruz Biotechnology, Santa Cruz, CA sc-52-G/G0706	A synthetic peptide corresponding to the N-terminus residues 3–16 of human c-Fos molecule: FSGFNADYEASSR

Table 3

Assessment of BDA volume, rostro-caudal diffusion and analyzed gray matter area of the spinal cord

	BDA Injection		Gray Matter Area (mm²)	
	Volume (mm³)	Diffusion (mm)	Lumbosacral	Thoracic
<i>Acute BDA injection</i>				
T4-transection	0.11 ± 0.04	0.60 ± 0.12	18.89 ± 4.52	18.81 ± 3.04
Sham	0.11 ± 0.02	0.64 ± 0.10	19.21 ± 4.16	17.29 ± 3.97
<i>Delayed BDA injection</i>				
T4-transection	0.29 ± 0.06	1.32 ± 0.24	22.83 ± 3.58	15.99 ± 1.51
Sham	0.29 ± 0.11	1.27 ± 0.30	23.87 ± 1.83	16.62 ± 2.66

Data expressed as mean ± SD