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INTRASPINAL SPROUTING OF UNMYELINATED PELVIC AFFERENTS AFTER COMPLETE SPINAL CORD INJURY IS CORRELATED WITH AUTONOMIC DYSREFLEXIA INDUCED BY VISCERAL PAIN

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Abstract—Autonomic dysreflexia is a potentially life-threatening hypertensive syndrome following high thoracic (T) spinal cord injury (SCI). It is commonly triggered by noxious pelvic stimuli below the injury site that correlates with increased sprouting of primary afferent C-fibers into the lumbosacral (L/S) spinal cord. We have recently demonstrated that injury-induced plasticity of (L/S) propriospinal neurons, which relay pelvic visceral sensations to thoracolumbar sympathetic preganglionic neurons, is also correlated with the development of this syndrome. To determine the phenotype of pelvic afferent fiber sprouts after SCI, cholera toxin subunit beta (CTb) was injected into the distal colon 2 weeks post–T4 transection/sham to label colonic visceral afferents. After 1 week of transport, the (L/S) spinal cords were cryosectioned and immunohistochemically stained for CTb, the nociceptive-specific marker calcitonin gene-related peptide (CGRP), and the myelinated fiber marker RT97. Quantitative analysis showed that the density of CGRP$^+$ afferent fibers was significantly increased in the L/S dorsal horns of T4-transected versus sham rats, whereas RT97$^+$ afferent fiber density showed no change. Importantly, CTb-labeled pelvic afferent fibers were co-localized with CGRP$^+$ fibers, but not with RT97$^+$ fibers. These results suggest that the sprouting of unmyelinated nociceptive pelvic afferents following high thoracic SCI, but not myelinated fibers, contributes to hypertensive autonomic dysreflexia induced by pelvic visceral pain.

Key words: distal colon, cholera toxin subunit beta, pelvic primary afferent, neuronal plasticity.

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 syndrome (Finestone and Teasell, 1993; Zagon and Smith, 1993). Autonomic dysreflexia is commonly triggered by noxious stimuli below the injury site, particularly by the distension of pelvic viscera (bowel and bladder) (Lindan et al., 1980; Karlsson, 1999). 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 increased severity of autonomic dysreflexia (Maiorov et al., 1997; Weaver et al., 1997; Krenz and Weaver, 1998a,b; Chau et al., 2000). Moreover, studies from our laboratory have shown that following complete T4 spinal transection, both primary afferent fiber sprouting into lumbosacral (L/S) dorsal horns (Cameron et al., 2006) and plasticity of L/S propriospinal neurons in the dorsal gray commissure (DGC) (Hou et al., 2008) correlate temporally with the development of autonomic dysreflexia.

Nevertheless, there is still uncertainty regarding which branch of the pelvic primary afferents, the myelinated or unmyelinated fibers, contributes to the development of autonomic dysreflexia. The unmyelinated pelvic afferent fibers, which convey thermal and nociceptive information, have been shown to contain calcitonin gene-related peptide (CGRP) (Keast and De Groat, 1992). Alternatively, the monoclonal antibody for 200 kDa neurofilament subunit in phosphorylated form, RT97, which is exclusively expressed in A-fiber afferent neurons, is a marker for myelinated primary afferent fibers in both somatic and visceral nerves (Perry et al., 1991; Sann et al., 1995; Wang et al., 1998). Yoshimura et al. (1998) reported that capsaicin-sensitive neurons (unmyelinated fibers) in L6/S1 dorsal root ganglia (DRG) were dramatically reduced in spinal-transected rats compared with shams. Conversely, neurofilament-rich DRG neurons (myelinated fibers) were detected at a significantly greater percentage after spinal cord transection (Yoshimura et al., 1998). On the contrary, we and others have shown that experimental autonomic dysreflexia induced by noxious colorectal distension (CRD) in spinal-transected rats correlates with profuse nerve growth factor (NGF)–mediated intraspinal sprouting of CGRP$^+$ primary afferent fibers into L/S spinal segments (termination sites of pelvic visceral sensory axons) (Krenz

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Abbreviations: CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; CRD, colorectal distension; CTb, cholera toxin subunit beta; DGC, dorsal gray commissure; DRG, dorsal root ganglia; HR, heart rate; HRP, horseradish peroxidase; LCP, lateral collateral projection; L/S, lumbosacral; LT, Lissauer's tract; MAP, mean arterial pressure; MCP, medial collateral projection; NGF, nerve growth factor; PBS, phosphate buffered saline; SCI, spinal cord injury; SPN, sacral parasympathetic nucleus; T, thoracic.

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and Weaver, 1998a; Weaver et al., 2001; Cameron et al., 2006; Rabchevsky, 2006).

Primary sensory afferent fibers innervating the descending colon in rats run in the pelvic and hypogastric/lumbar colonic nerves, distributing mainly to the L6/S1 spinal level (Ness and Gebhart, 1987; Al-Chaer and Traub, 2002). To characterize the relative contribution of myelinated versus unmyelinated sensory fiber sprouting to the development of autonomic dysreflexia, cholera toxin subunit beta (CTb) was injected into the distal colon of selected T4-transected versus sham rats to label distal colonic afferents and their terminal arbors within the L/S spinal cord. Our results demonstrate, for the first time, that following high T SCI, the sprouting of unmyelinated nociceptive pelvic afferents into the L/S spinal cord, but not myelinated fibers, is correlated with dysreflexic hypertension induced by visceral pain.

EXPERIMENTAL PROCEDURES

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 to ensure minimizing the number of animals used and any potential suffering. Adult female Wistar rats (~200–250 g) were anesthetized with a mixture of ketamine (80 mg/kg, i.p.; Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (10 mg/kg, i.p.; Butler, Columbus, OH, USA). The injured group received complete T4 transection following T3 vertebral laminectomy (n=18), in contrast to the sham group which received only T3 laminectomy (n=15).

The spinal cord was completely transected with a #11 scalpel blade at the T4 level following the laminectomy, as described previously (Cameron et al., 2006). After surgical operations were complete, the erector spinae muscles were sutured with 3-0 Vycril (Ethicon, Sommerfield, NJ, USA), the field was disinfected with povidone–iodine solution (Nova Plus, Irving, TX, USA), and the skin was closed with Michel wound clips (Roboz, Gaithersburg, MD, USA). For post-operative care, animals were administered 20 ml lactated Ringer's solution (Baxter Healthcare, Deerfield, IL, USA) and 33 mg/kg cephazolin (Apothecon, Bristol-Myers Squibb, Princeton, NJ, USA) s.c. 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; Hospira, Inc., Lakeforest, IL, USA) was also administered s.c. once after recovery from anesthesia and twice daily for the next 3 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 postinjury.

CTb injections and tracing

Two weeks after injury/sham, selected T4-transected (n=7) and non-transected (n=7) rats were reanesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) to inject the distal colon with CTb, as previously described with modifications (Valentino et al., 2000). Briefly, a laparotomy was made to expose the pelvic viscera. With a Hamilton microsyringe (33-gauge needle; Hamilton, Reno, NV, USA), 6 μl of CTb solution (1% in dH_2O; List Biological Laboratories, Campbell, CA,USA) was injected circumferentially beneath the serosal layer in the distal colon, 1–2 cm proximal to the anus. The injections were made in six adjacent sites surrounding the colon (1 μl/site). Injection sites were sealed with a drop of tissue adhesive (3 M Vetbond™, St. Paul, MN, USA) to minimize leakage of tracer before the wound was sutured. To control for CTb labeling specificity, CTb solution was topically applied on the ventral surface of the distal colon of an additional sham and an injured rat (n=1 per group). After 1 week post-CTb injection to allow for transport, animals were perfused and spinal cords were harvested for histology.

Assessing autonomic dysreflexia with CRD

Two weeks after T4 spinal transection, femoral cannulas were implanted in three randomly selected rats to verify the incidence of autonomic dysreflexia during noxious CRD the following day, as detailed previously (Cameron et al., 2006). An injured rat was regarded as dysreflexic if CRD, produced by inflation of a cardiac catheter balloon at a pressure that is known to stimulate the nociceptive fibers, created a rise in mean arterial pressure (MAP) and a concomitant decrease in heart rate (HR) for as long as the period of CRD.

Dissection and tissue processing

Three weeks after T4-transection/sham (n=33), including 1 week post-CTb injections in the tracing cohort (n=7 per group), animals were overdosed with sodium pentobarbital (150 mg/kg; Abbott, Chicago, IL, USA) and perfused transcardially with 0.1 M phosphate buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS. A 6 cm long spinal cord extending from the conus medullaris to the transection site was removed, post-fixed for 4 h, rinsed in 0.2 M phosphate buffer (PB) overnight, and cryoprotected in 20% sucrose in 0.1 M PBS. The 3 cm caudal segments (~T12–S3) were embedded in gum tragacanth (Sigma-Aldrich, St. Louis, MO, USA) in 20% sucrose/PBS for cryosectioning, as previously detailed (Cameron et al., 2006). Embedded spinal cord segments were, snap-frozen in acetone chilled to −40 °C and stored at −80 °C until sectioning on a cryostat (Microm Laborgerate, Walldorf, Germany). Approximately 30 consecutive rows of 20 μm transverse cryosections separated by 100 μm from each spinal cord were placed onto each of 10 adjacent glass slides (Superfrost plus, Fisher Scientific, Pittsburgh, PA, USA) in two series, as previously detailed (Cameron et al., 2006). 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 1. For CTb staining, 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, USA) for 1 h, followed by incubation with goat

Table 1. Complete information and immunogen of primary antibodies applied

Antiserum	Species	Clonality	Working dilution	Source catalog/lot number	Immunogen
CTb	Goat	Polyclonal	1:1500	List Biological Laboratories, 703/7032A5	CTb (choleragenoid)
CGRP	Rabbit	Polyclonal	1:2000	Sigma-Aldrich, C8198/101K4846	CGRP-KLH
RT97	Mouse	Monoclonal	1:400	Chemicon, CBL212/TR1418670	200 kDa Neurofilament polypeptide
ChAT	Goat	Polyclonal	1:100	Chemicon, AB144P/0608037072	ChAT purified from human placental enzyme