Neuroscience 152 (2008) 338–345

REIMPLANTATION OF AVULSED LUMBOSACRAL VENTRAL ROOTS IN THE RAT AMELIORATES INJURY-INDUCED DEGENERATION OF PRIMARY AFFERENT AXON COLLATERALS IN THE SPINAL DORSAL COLUMNS

A. J. BIGBEE, T. X. HOANG AND L. A. HAVTON*

Department of Neurology, David Geffen School of Medicine at UCLA, Neuroscience Research Building, 635 Charles E. Young Drive South, Los Angeles, CA 90095-7334, USA

Abstract—Injuries to the cauda equina/conus medullaris portion of the spinal cord can result in motor, sensory, and autonomic dysfunction, and neuropathic pain. In rats, unilateral avulsion of the motor efferents from the lumbosacral spinal cord results in at-level allodynia, along with a corresponding glial and inflammatory response in the dorsal horn of the spinal cord segments immediately rostral to the lesion. Here, we investigated the fate of intramedullary primary sensory projections following a motor efferent lesion. The lumbosacral (L6 and S1) ventral roots were unilaterally avulsed from the rat spinal cord (VRA; *n*=9). A second experimental group had the avulsed roots acutely reimplanted into the lateral funiculus (Imp; *n*=5), as this neural repair strategy is neuroprotective, and promotes the functional reinnervation of peripheral targets. A laminectomy-only group served as controls (Lam; *n*=7). At 8 weeks post-lesion, immunohistochemical examination showed a 42% reduction (*P*<0.001) in the number of RT97-positive axons in the ascending tracts of the dorsal funiculus of the L4-5 spinal segment in VRA rats. Evidence for degenerating myelin was also present. Reimplantation of the avulsed roots ameliorated axon and myelin degeneration. Axons in the descending dorsal corticospinal tract were unaffected in all groups, suggesting a specificity of this lesion for spinal primary sensory afferents. These results show for the first time that a lesion restricted to motor roots can induce the degeneration of intramedullary sensory afferents. Importantly, reimplantation of the lesioned motor roots ameliorated sensory axon degeneration. These data further support the therapeutic potential for reimplantation of avulsed ventral roots following trauma to the cauda equina/conus medullaris.

Key words: spinal cord injury, cauda equina/conus medullaris, neuroprotection, motor lesion, neurofilament, myelin.

Injury to the cauda equina/conus medullaris (CE/CM) portion of the spinal cord may have devastating consequences for afflicted persons, including motor, autonomic, and sensory dysfunction, and neuropathic pain (Moossy et al., 1987; Sampson et al., 1995; Sindou et al., 2001). We have developed a rat model of CE/CM spinal cord injury (SCI) that approximates many of the behavioral and physiological deficits that are associated with this type of injury in humans. In this model, the lumbosacral L6 and S1 ventral motor roots are torn, or avulsed (ventral root avulsion; VRA) from the spinal cord at the CNS and peripheral nervous system (PNS) interface. The result is a progressive and significant death of autonomic and motor neurons at the injured spinal segments, denervation of peripheral targets, and subsequent Wallerian degeneration of the avulsed motor nerve roots (Hoang et al., 2003). Importantly, acute reimplantation of the avulsed roots into the injured spinal segments promotes cell survival, stimulates reinnervation of the implant, and facilitates some physiological restoration of bladder function (Hoang and Havton, 2006; Hoang et al., 2006a,b).

In light of studies that have shown that transecting a spinal motor root can result in neuropathic pain and/or sensory plasticity in the CNS (Holmberg and Kellerth, 2000; Li et al., 2002; Sheth et al., 2002; Havton and Kellerth, 2004; Obata et al., 2004), we recently decided to examine whether VRA injury might also influence sensory plasticity. As such, we showed that a unilateral L6–S1 VRA injury induces at-level allodynia, concomitant with glial and macrophage activation in the adjacent L5 deep dorsal gray matter and dorsal funiculus (DF) ipsilateral to the lesion at 8 weeks post-injury (Bigbee et al., 2007). Importantly, acute reimplantation of the avulsed lumbosacral roots into the lateral funiculus ameliorated VRA-induced neuropathic pain and inflammation in the dorsal horn (Bigbee et al., 2007). The regions of specificity of the inflammatory response in the dorsal horn of the VRA group warrants further investigation. The objective of the present experiment, therefore, was to examine the late effects of VRA or VRA combined with the reimplantation neural repair strategy (Imp) on the intramedullary sensory afferent projections in the lumbosacral spinal cord. To that end, we examined morphological features of sensory afferent collaterals in the DF of spinal segments rostral to the L6–S1 lesion and repair at 8 weeks post-lesion. The DF region is of interest, as it primarily contains large myelinated sensory afferents that carry proprioceptive information to more

*Corresponding author. Tel: +1-310-206-5757; fax: +1-310-206-5533.
E-mail address: lhavton@mednet.ucla.edu (L. A. Havton).

Abbreviations: AIF-1, allograft inhibitory factor-1; ANOVA, analysis of variance; CE/CM, cauda equina/conus medullaris; CST, corticospinal tract; DF, dorsal funiculus; DRG, dorsal root ganglia; GFAP, glial fibrillary acidic protein; IHC, immunohistochemistry; Imp, implanted; Lam, laminectomy; MBP, myelin basic protein; NF, neurofilament; NF^+, neurofilament-immunopositive; PBS, phosphate-buffered saline; PNS, peripheral nervous system; SCI, spinal cord injury; VRA, ventral root avulsion.

0306-4522/08$32.00+0.00 © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2007.11.043

rostral spinal and supraspinal centers (Chung et al., 1987; Smith and Bennett, 1987; Yamamoto and Ohnishi, 1996). The present results show a significant loss of intramedullary sensory axons in the DF rostral to the lesion at 8 weeks post-VRA, concomitant with degenerating myelin. Axonal loss was restricted to the ascending sensory tracts of the DF, as axon counts were unchanged in the descending dorsal corticospinal tract (CST) region. Acute reimplantation of the lesioned roots ameliorated VRA-induced degeneration of sensory afferent projections. These data provide new insight into the surprising sensory consequences of a spinal motor root lesion, and provide further support for the promising translational therapeutic potential of nerve reimplantation following spinal nerve root avulsion injuries.

EXPERIMENTAL PROCEDURES

Animals and surgery

Animal procedures were performed according to the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals standards under protocols approved by the UCLA Chancellor's Animal Research Committee. All efforts were made to minimize the number of animals used and their suffering. Adult female Sprague–Dawley rats (BW=208±3 g; Charles River Laboratories, Wilmington, MA, USA) were housed in standard rat cages (12-h light/dark cycle; 24±1 °C). Experimental groups included: 1) sham-operated controls receiving a hemilaminectomy (Lam; $n=7$); 2) rats receiving unilateral avulsion of the L6–S1 ventral roots (VRA; $n=9$); and 3) rats undergoing unilateral L6–S1 VRA followed by immediate implantation of the avulsed roots into the lateral funiculus (Imp; $n=5$). The surgical procedures for the unilateral VRA procedure have been previously described (Bigbee et al., 2007). Briefly, a midline incision was made over the lumbar spine under anesthesia (2% isoflurane), and an L1-3 Lam was performed. In all rats, the dura was opened and the spinal cord and nerve roots were gently manipulated. In VRA rats, the L6 and S1 ventral roots were unilaterally avulsed at the CNS/PNS interface using forceps to apply traction along the course of each root. Imp rats underwent VRA, and then the avulsed roots were reinserted into a longitudinal slit made into the lateral funiculus of the spinal cord. In all groups, Gelfoam® (Pharmacia & Upjohn, Kalamazoo, MI, USA) was placed over the spinal cord, and a titanium mesh cage was secured to the vertebral processes to stabilize the spinal column (Nieto et al., 2005). Finally, the muscle and skin were sutured. Buprenorphine (0.2–0.5 mg/kg) and 0.9% saline (1 ml) were given s.c. after surgery, and bladders were checked twice daily and manually expressed until bladder function recovered.

Tissue preservation and immunohistochemistry (IHC)

At 8 weeks post-surgery, rats were overdosed with sodium pentobarbital and intracardially perfused with 0.1 M phosphate buffer, followed by 4% paraformaldehyde. The spinal cords were removed, post-fixed overnight, and rinsed in 0.1 M phosphate-buffered saline (PBS). The injury level was anatomically verified, and only those spinal cords having the L6 and S1 ventral roots avulsed were included in the analyses ($n=7$, 9, and 5 for the Lam, VRA, and Imp groups, respectively). The spinal cord tissue was cryoprotected in a 30% sucrose solution for 24 h, preserved in OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA), and stored at −80 °C. Transverse sections were cut at 30 μm on a cryostat and collected as free-floating sections in 0.1 M PBS. Standard maps of the rat lumbosacral cytoarchitecture were used to determine the segmental location (Molander et al., 1984). It should be noted that the tissues used in these analyses are from subjects used in our recent study showing VRA-induced neuropathic pain, glial activation, and inflammation (Bigbee et al., 2007). Sections from the L4–L5 spinal segments were used for IHC analyses of axons and myelin using antibodies against the phosphorylated 200 kD neurofilament (NF) variant (RT97; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), and myelin basic protein (MBP; Chemicon, Temecula, CA, USA), respectively. Additionally, sections from the more rostral T13–L1 spinal segments were analyzed for astrocytes and microglia/macrophages using antibodies for glial fibrillary acidic protein (GFAP; Chemicon), and allograft inhibitory factor-1 (AIF-1; Abcam, Cambridge, MA, USA), respectively, to determine whether VRA-induced cellular changes also occurred at segments multiple levels rostral to the injury. Free-floating, adjacent spinal cord sections (3 to 7 sections/rat) were rinsed in 0.1 M PBS, blocked for 1 h in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and incubated in primary antibody (anti-mouse RT97, 1:2000; anti-rat MBP, 1:400; anti-rabbit GFAP, 1:1000; anti-goat AIF-1, 1:1000) with 0.3% triton overnight at room temperature. Additionally, double labeling for NF and myelin was performed using anti-mouse RT97 (1:2000) and anti-rat MBP (1:400) with 0.3% triton overnight at room temperature. Sections were then rinsed in 0.1 M PBS, incubated for 1 h at room temperature in immunofluorescent secondary antibody for visualization (Alexa Fluor® 594 or 488, 1:500; Molecular Probes, Eugene, OR, USA), and mounted on slides for also DAPI (1.5 μg/ml) visualization with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Data analysis and statistics

A Spot camera (Diagnostic Instruments, Sterling Heights, MI, USA) attached to a Nikon E600 microscope (Nikon Instruments Inc., Melville, NY, USA) was used to capture fluorescent images of the ipsilateral (i.e. lesioned) and contralateral (i.e. uninjured internal control) DF for all groups under constant conditions. Myelin immunoreactivity and presence of DAPI-positive nuclei were examined qualitatively. Quantitative IHC analyses were performed using C-Imaging software (Compix, Inc., Brandywine, PA, USA) to determine the total area of neurofilament (NF) immunoreactivity within a 2500 μm^2 region of interest, as well as the number of NF-positive profiles within the same region (Fig. 1A). Statistical tests for axon quantification were performed using Student's *t*-test to identify differences between the ipsilateral and contralateral DF within each group. Additionally, in order to examine inter-group comparisons, the ratio of the ipsilateral to contralateral values was compared across groups using analysis of variance (ANOVA) followed by Tukey's post hoc test. Significance for all statistical tests was determined at $P<0.05$.

RESULTS

VRA-induced sensory afferent degeneration in the DF is ameliorated by root reimplantation

Qualitative IHC analyses in Lam rats showed intramedullary neurofilament-immunopositive (NF^+) axons in cross-section surrounded by myelin sheaths in the DF (Fig. 1B; Fig. 2A–D). At 8 weeks post-VRA, NF^+ labeling in the ipsilateral DF was sparse and irregular (Fig. 1C; Fig. 2E–H). Additionally, many of the remaining NF^+ profiles appeared to be unmyelinated, and myelin debris in the absence of NF was also commonly observed. We should note, however, that the most definitive way to confirm demyelination is by electron microscopy. Furthermore, rats of the VRA series also exhibited a qualitative increase in DAPI-positive nuclei in the DF (Fig. 2). This increased cellularity may