Published in final edited form as:
Exp Neurol. 2007 March ; 204(1): 273–282. doi:10.1016/j.expneurol.2006.11.003.

At-level neuropathic pain is induced by lumbosacral ventral root avulsion injury and ameliorated by root reimplantation into the spinal cord

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Abstract

Neuropathic pain is common after traumatic injuries to the cauda equina/conus medullaris and brachial plexus. Clinically, this pain is difficult to treat and its mechanisms are not well understood. Lesions to the ventral roots are common in these injuries, but are rarely considered as potential contributors to pain. We examined whether a unilateral L6-S1 ventral root avulsion (VRA) injury in adult female rats might elicit pain within the dermatome projecting to the adjacent, uninjured L5 spinal segment. Additionally, a subset of subjects had the avulsed L6-S1 ventral roots reimplanted (VRA+Imp) into the lateral funiculus post-avulsion to determine whether this neural repair strategy elicits or ameliorates pain. Behavioral tests for mechanical allodynia and hyperalgesia were performed weekly over 7 weeks post-injury at the hind paw plantar surface. Allodynia developed early and persisted post-VRA, whereas VRA+Imp rats exhibited allodynia only at 1 week post-operatively. Hyperalgesia was not observed at any time in any experimental group. Quantitative immunohistochemistry showed increased levels of inflammatory markers in laminae III-V and in the dorsal funiculus of the L5 spinal cord of VRA, but not VRA+Imp rats, specific to areas that receive projections from mechanoreceptive, but not nociceptive, primary afferents. These data suggest that sustained at-level neuropathic pain can develop following a pure motor lesion, whereas the pain may be ameliorated by acute root reimplantation. We believe that our findings are of translational research interest, as root implantation surgery is emerging as a potentially useful strategy for the repair of cauda equina/conus medullaris injuries.

Keywords

spinal cord injury; neural repair; allodynia; astrocytes; microglia; macrophages; inflammation; primary afferents

Introduction

Intractable neuropathic pain is common in humans with an injury to the conus medullaris portion of the spinal cord and/or cauda equina (Moossy et al., 1987, Sampson et al., 1995, Sindou et al., 2001), as well as following lesions to the brachial plexus (Carlstedt, 1995, Berman

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et al., 1998, Carlstedt et al., 2000, Carlstedt et al., 2004). These spinal injuries are complex, often resulting in the tearing, or avulsion, of ventral and/or dorsal roots from the transitional zone interface between the central and peripheral nervous systems (CNS/PNS) (Moschilla et al., 2001, Hans et al., 2004). Neuropathic pain resulting from these types of injuries can extend one or two segments rostral to the level of injury, and is often referred to as at-level pain (Scheifer et al., 2002, Oatway et al., 2004, Siddall and Middleton, 2006).

As direct injuries to sensory afferents or the spinal cord are thought to be the primary contributors to neuropathic pain, basic research models examining mechanisms of neuropathic pain usually involve injuries to the dorsal roots, mixed nerves, or the spinal cord itself. In contrast, the role of ventral root injury is rarely considered as a contributor to the development of neuropathic pain. There is recent evidence which suggests, however, that a unilateral L5 ventral root transection (VRT) in rats is sufficient to induce hyperalgesia (i.e., a reduced pain threshold for nociceptive stimuli) and allodynia (i.e., a normally innocuous stimulus is perceived as painful) (Li et al., 2002, Sheth et al., 2002, Obata et al., 2004). Thus, a direct injury to the sensory afferents may not be necessary to induce the development of neuropathic pain.

Our laboratory has characterized a lumbosacral ventral root avulsion (VRA) injury model of conus medullaris/cauda equina injury, in which the motor roots are avulsed from the CNS/PNS interface while the sensory afferents remain structurally intact. Although we have extensively examined autonomic and motoneuron cell death (Hoang et al., 2003), as well as inflammation (Ohlsson et al., 2006) in the ventral horn at the level of injury in this model, it is unknown whether VRA alters the processing of sensory information in the dorsal horn at or beyond the injured segment. As neuropathic pain is a consequence of this type of spinal cord injury in the clinical setting, we propose that the VRA injury may contribute to the development of neuropathic pain. Further, we propose that a neural repair strategy in which the avulsed roots are reimplanted into the conus medullaris (Hoang et al., 2006a, Hoang, 2006b) may be efficacious in reducing pain after VRA. As reimplantation of the avulsed ventral roots appear to be neuroprotective (Chai et al., 2000, Hoang et al., 2006a) and promotes functional reinnervation of peripheral targets (Carlstedt et al., 1986, Hallin et al., 1999, Gu et al., 2004, Hoang, 2006b), it is vital to also determine whether this translationally relevant neural repair strategy may influence sensory function.

The objectives of this study, therefore, were to i) determine whether unilateral VRA at the L6-S1 spinal levels induces at-level neuropathic pain (i.e., at the L5 dermatome) associated with cellular plasticity in the L5 dorsal horn, and ii) determine whether reimplantation of the avulsed roots ameliorates or exacerbates pain. As glia and inflammatory cells have been shown to play a role in neuropathic pain (Liu et al., 1998, Liu et al., 2000, Sweitzer et al., 2001, Wieseler-Frank et al., 2004, Nesic et al., 2005), we quantitatively examined immunohistochemical markers for astrocytes, microglia, and macrophages in the L5 dorsal horn region. The present experiments differ from the L5 VRT studies in two important ways. First, VRA is more proximal to the CNS/PNS interface than VRT. Second, our behavioral and morphological analyses occur within the dermatome and spinal segment adjacent and rostral to the lesion site. We show for the first time that VRA results in at-level pain, concomitant with increased activation of glia, microglia, and macrophages in the specific regions of the dorsal horn. Interestingly, VRA-induced neuropathic pain and the activation levels of glia and macrophages are ameliorated by reimplantation of the avulsed roots.

Methods

All animal procedures were carried out according to the standards established by the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals under protocols

approved by the Chancellor's Animal Research Committee at UCLA. Adult female Sprague Dawley rats (BW=208 ± 3 g; Charles River Laboratories, Wilmington, MA) were double housed in standard rat cages (12:12 light/dark cycle; 24±1° C). The three experimental groups included laminectomy controls (Lam; n=7), rats receiving unilateral L6-S1 VRA (n=8; Fig. 1), and rats undergoing unilateral L6-S1 VRA followed by immediate reimplantation of the avulsed roots into the lateral funiculus (VRA+Imp; n=5).

Surgeries

The VRA surgical procedures have been previously described at length (Hoang et al., 2003, Hoang et al., 2006a). Briefly, a midline incision was made along the back under general anesthesia (2% isoflurane). In all groups, the lumbar spinal level was identified using bony vertebral landmarks, and an L1-3 hemi-laminectomy was performed, leaving the spinous processes intact. For the Lam group, the dura was opened and the cord and roots were gently manipulated, mimicking exposure of the spinal cord during the VRA procedure. For VRA, the L6 and S1 ventral roots were avulsed from the CNS/PNS interface using a jeweler's forceps to apply constant traction along the normal course of the roots. Finally, in the VRA+Imp group, the L6 and S1 ventral roots were unilaterally avulsed as in VRA rats, then two small incisions were made in the lateral funiculus of the lesioned segments, and the avulsed roots were placed into the incisions. A loose suture was placed around each reimplanted root for later identification purposes. Upon completion of the VRA and implantation procedures, a layer of Gelfoam® (Pharmacia & Upjohn, Kalamazoo, MI) was laid over the laminectomy site, and a thin piece of titanium mesh was secured to vertebral processes at the far ends of the laminectomy site to stabilize the spinal column (Nieto et al., 2005). The muscle and skin were sutured in layers, and the animals were allowed to recover. Buprenex (0.2-0.5 mg/kg) and 0.9% saline (1 cc) were given post-operatively. The bladders were checked daily and manually expressed as needed for the first week after surgery to ensure that bladder function was maintained.

Behavioral testing

All behavioral testing was performed between 8:00 and 12:00, at ambient room temperature (24±1° C). Pre-surgical baseline testing for mechanical allodynia and thermal hyperalgesia at the L5 dermatome region of the plantar surface of the foot (Takahashi et al., 2003) occurred at 4 and 1 days prior to surgery, so that each subject's post-operative pain responses could be expressed relative to its own pre-injury control values. Post-operative tests were performed initially at 5 days after surgery, and weekly thereafter from 2-7 wks. Seven wks was chosen as the endpoint for these studies as we have extensive time course data for motoneuron and parasympathetic preganglionic neuron (PPN) cell death up to 6 wks post-avulsion (Hoang et al., 2003). The experimenter was blinded as to group identification during behavioral testing for allodynia and hyperalgesia. Mechanical allodynia was examined using a variation of a well-established somatosensory test (Chaplan et al., 1994). Rats were acclimated daily for 3 days × 10 min/day to the test environment, a plexiglass box on a metal grid surface, prior to collecting pre-surgical baseline data. On test days, rats were allowed to acclimate to the test environment for 5-10 min. The stimulus, a rigid probe attached to a hand held force transducer (Electronic Von Frey Anesthesiometer, IITC Inc., Woodland Hills, CA) (Cairns et al., 2002) was applied to the medial plantar surface of the hind paw until a hindlimb withdrawal response, accompanied by head turning, biting, and/or licking of the paw were observed. The force transducer automatically recorded the grams of force required to elicit the paw withdrawal (i.e., paw withdrawal threshold; PWT). Both the ipsilateral and the contralateral hindlimbs were tested 3 times/time point, and the average of those three responses was determined. Group averages were then calculated. For pre-surgical data, the 4- and 1-day values were averaged for each subject. Thermal hyperalgesia was examined using the Plantar Analgesia system (IITC Inc.). Rats were placed in a plexiglass box on a glass surface and allowed to acclimate for 5-10

min to their environment. A radiant heat source was focused on the central region of the plantar surface of the foot, eliciting a hindlimb withdrawal response (Hargreaves et al., 1988). Again, accompanying pain behaviors noted above were used to verify that the hind paw movement was not random. The number of seconds from the onset of the stimulus to the time that the hind paw was lifted, or latency to paw withdrawal (PWL), was automatically measured. A cut-off value of 1.5 times the pre-operative baseline data was used to prevent tissue damage. Both hindlimbs of all subjects were tested 3 times at each time point, with at least 5 min in between successive stimuli, and the average of the three responses was taken.

Tissue collection and preservation

One week following the last behavioral data collection (i.e., 8 wks post-surgery), rats were overdosed with sodium pentobarbital and perfused intracardially with 0.1 M phosphate buffer followed by a 4% paraformaldehyde solution. The spinal cords were removed from the spinal column, post-fixed overnight, and rinsed in 0.1 M PBS. The segmental level of the lesion was verified anatomically, and only those spinal cords having a confirmed lesion of the L6 and S1 roots were included in the behavioral and morphological analyses (Lam, n=7; VRA, n=8; VRA +Imp, n=5). The spinal cords were cryoprotected in a 30% sucrose solution for 24 hours, preserved in OCT compound (Sakura Finetek USA, Inc. Torrance, CA), and stored at -80°C. Transverse spinal cord sections were cut at 30 μm on a cryostat and collected as free-floating sections in 0.1 M PBS for immunohistochemistry.

Immunohistochemical analyses

Standard maps of the rat lumbosacral cytoarchitecture was used to determine the segmental location for individual sections (Molander et al., 1984). Adjacent sections (every 5^{th} section, 4-8 sections/rat) in the L5 spinal segment were processed for glial fibrillary acidic protein (GFAP; Chemicon, Temecula, CA), allograft inflammatory factor-1 (AIF-1; Abcam, Inc., Cambridge, MA), or ED-1 (Chemicon), indicating the presence of astrocytes, microglia, and macrophages, respectively. Additionally, as motoneurons are nearly absent at the level of the lesion by 6 wks post-VRA (Hoang et al., 2003), immunohistochemistry for choline acetyltransferase (ChAT) was used to verify the normal and symmetric presence of motoneurons as further verification that the spinal level being examined was rostral to the lesion. Free-floating sections were rinsed in 0.1 M PBS, blocked for 1 hr in 5% normal donkey serum (Jackson Immuno Research Labs, Inc. Westgrove, PA), and incubated in primary antibodies (anti-rabbit GFAP, 1:1000; anti-rabbit AIF-1, 1:000; anti-mouse ED-1 1:500) with 0.3% triton overnight at room temperature. Sections were then rinsed in 0.1 M PBS and incubated in immunofluorescent secondary antibodies for visualization (Alexa Fluor® 488 and Alexa Fluor® 594, 1:500; Molecular Probes, Eugene, OR) for 1 hr at room temperature. Following a final wash, sections were mounted on slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Fluorescent images were captured using a Spot camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon E600 microscope using constant magnification, light intensity, and exposure time. Quantitative densitometric analyses were performed using C-Imaging software (Compix, Inc., Brandywine, PA) to determine the area of immunopositive labeling above a constant threshold. Confocal microscopy was performed using a Leica TCS-SP spectral confocal microscope equipped with argon (488 nm blue excitation) and krypton (568 nm yellow excitation) lasers (Leica, Heidelberg, Germany). For quantitative immunohistochemical analyses, three dorsal horn regions of interest, including the superficial (laminae I-II) and deep (laminae III-V) gray matter, and the dorsal funiculus white matter, were examined. In each region, the immunopositive area within a 10,000 μm^2 area was determined for the ipsilateral and contralateral sides of all groups.

Statistical analyses

For the behavioral outcome measures, the mean PWT and mean PWL percent changes from pre-surgical baseline levels were determined. An absolute difference score for each time point was computed as follows to include relative differences between the ipsilateral and contralateral sides for comparison between groups:

$$\text{Absolute Difference Score} = \left(\text{Pre}_{\text{ipsi}} - \text{Post}_{\text{ipsi}}\right) - \left(\text{Pre}_{\text{contra}} - \text{Post}_{\text{contra}}\right)$$

where “pre” refers to the pre-surgical values, “post” refers to each time point post-surgery, and “ipsi” and “contra” refer to the injured and non-injured sides of the spinal cord, respectively. Data were compared using two way (group × time) repeated measure analysis of variance (ANOVA) methods with group as the between animal factor and time as the within animal factor. The repeated measure analysis of variance was implemented using SAS Procedure MIXED (SAS Inc, Cary, NC). Statistical significance, determined at $p \leq 0.05$, was assessed using the Tukey-Fisher post hoc mean comparison criteria. For immunohistochemical data, the percentage immunopositive area of the 10,000 μm^2 region of interest was presented as the ratio of ipsilateral to contralateral values within groups. Between group analyses were performed using the non-parametric Kruskal-Wallis ANOVA with Dunn’s post-hoc test, with $p \leq 0.05$ considered significant.

Results

Pain behavior

The present experiments were designed to determine whether a lumbosacral motor efferent lesion, in the absence of injury to sensory afferents, might cause atlevel neuropathic pain at the L5 dermatome, i.e., beyond the territory of innervation by the L6-S1 injury. To that end, the uninjured L5 dermatome region was tested for allodynia and hyperalgesia (Fig. 1). The behavioral tests used to measure allodynia and hyperalgesia at the L5 dermatome rely on intact motor behaviors. Comprehensive anatomical studies showed that while the L6 segment contributes to semitendinosus (Nicolopoulos-Stournaras and Iles, 1983) and medial gastrocnemius (<9%; (Peyronnard et al., 1986) innervation, it is a major contributor to pelvic musculature innervation, including the external sphincter uerthrae, and the ischiocavernosus, bulbocavernosus, and sphincter ani muscles (Schroder, 1980). From our own behavioral observations from this study, locomotor function is normal to the point where it is difficult for a blind observer to distinguish lesioned from non-lesioned subjects having the unilateral L6-S1 VRA injury. Further, published locomotor evidence (obtained using the BBB Scale) that showed that the much more severe L5-S2 bilateral VRA injury resulted only in minor hindlimb movement deficits, exhibited as limited active range of motion at the ankle (Hoang, 2006b).

For allodynia, the PWT response to a blunt probe was used as a measure of sensitivity to a non-noxious stimulus after Lam, VRA, or VRA+Imp. The VRA absolute difference score for allodynia was significantly reduced relative to Lam at 2,3,5,6, and 7 wks ($p<0.05$), indicating increased sensitivity to the non-noxious stimulus, while VRA+Imp was significantly reduced relative to Lam only at the 1 wk time point (Fig. 2A). As a measure of hyperalgesia, the PWL was measured to determine whether unilateral VRA or VRA+Imp altered sensitivity to a noxious heat stimulus. The absolute difference score for hyperalgesia, determined in same manner as for allodynia, showed no significant difference between groups at any time point (Fig. 2B).

Immunohistochemistry for astrocytes, microglia, and macrophages

In order to examine a potential role for non-neuronal inflammatory cells in the development of pain after VRA or VRA+Imp, immunohistochemistry for GFAP, AIF-1, and ED-1 was performed to identify astrocytes, microglia, and macrophages, respectively. Some important qualitative observations were made regarding the specificity of the injury-related changes to the spinal cord. First, the physical integrity of the ipsilateral dorsal horn was preserved even after the extensive laminectomy, suggesting that pain was likely due primarily to the VRA injury, and not compression of the cord resulting from the laminectomy (Fig. 3). Second, the effect of the L6-S1 ventral root lesion clearly had an effect on astrocytes and microglia that was restricted to the ipsilateral dorsal horn (Fig. 3).

Using quantitative immunohistochemical densitometry, ipsilateral and contralateral immunoreactivity (IR) was compared across groups in three dorsal horn regions: the superficial gray matter (laminae I-II), deep gray matter (laminae III-V), and the dorsal funiculus white matter (Fig. 4A). The regions of interest examined were 10,000 μm^2 in all cases. As contralateral immunohistochemical levels were not different between groups for any marker, quantitative data are presented as the ratio of ipsilateral to contralateral values. Qualitatively, GFAP was prominent in the VRA deep gray matter and dorsal funiculus relative to Lam and VRA+Imp (Figs. 3A; 4B-D) as exhibited by swollen cell bodies and processes relative to Lam, indicative of reactive astrocytes (Figs. 4E-F). Quantitatively, GFAP IR was not significantly different between groups in either laminae I-II or laminae III-V (Fig. 4G). In the dorsal funiculus Lam GFAP IR increased by 10-fold relative to its contralateral values, suggesting that the laminectomy alone may have some effect on local gliosis. VRA GFAP IR was, however, significantly increased relative to Lam ($p<0.05$), while VRA+Imp was not.

Microglial IR was examined using AIF-1 (Fig. 5). AIF-1 is indicative of microglial/ macrophage activation, and has been proposed to be present in pre-phagocytic microglia in the spinal cord after injury (Schwab et al., 2001). A basal level of AIF-1 positive microglia was observed in Lam gray and white matter (Fig. 5A). In VRA, AIF-1 IR was visibly increased both in laminae III-V and the dorsal funiculus (Figs. 5B, D). Also visible in the dorsal funiculus were AIF-1 positive cells that were morphologically more rounded and without processes (Fig. 5E), similar to macrophages labeled by ED-1 (Fig. 6). AIF-1 staining in VRA+Imp was qualitatively similar to Lam, and no macrophage-like morphology was observed in the dorsal funiculus (Fig 5C). Quantitative analyses showed that VRA AIF-1 IR was significantly increased relative to Lam in both the deep gray and dorsal funiculus ($p<0.05$), while VRA+Imp was not (Fig. 5F). There were no changes in AIF-1 IR in laminae I-II between groups. ED-1 was used to identify activated macrophages in the dorsal horn after VRA and VRA+ Imp. ED-1 positive total cell counts were performed in the same 10,000 μm^2 regions of the dorsal horn as densitometry for GFAP and AIF-1. As ED-1 positive macrophages were virtually absent from the contralateral side, only the ipsilateral data are shown (Fig. 6). ED-1 positive macrophages were rarely observed in any of the three regions in Lam (Figs. 6A, D). In VRA rats, few macrophages were present in the L5 laminae I-II. There were, however, significantly more ED-1 positive macrophages in laminae III-V and in the dorsal funiculus relative to Lam (3.2 ±0.8 vs. 0.3±0.1 cells, and 11.8±2.6 vs. 0.3±0.1 cells, respectively) (Figs. 6B, D). In VRA+Imp, some ED-1 positive macrophages were present in laminae III-V and the dorsal funiculus (Fig. 6C, D), but the numbers of cells were not significantly different from Lam (1.2±0.2 vs 0.3±0.1 cells, and 4.2±1.4 vs. 0.3±0.1 cells, respectively).

Discussion

In the present study, we used a unilateral VRA injury model to determine whether lesioning of the motor and autonomic efferents at the L6-S1 spinal levels, while sparing sensory afferents, may contribute to the development of at-level neuropathic pain. We showed here for the first

time that a unilateral lumbosacral VRA injury results in at-level allodynia ipsilateral to the injury, in the absence of hyperalgesia. Consistent with the development of allodynia, activation of astrocytes, microglia, and macrophages were observed in the VRA deep gray matter and dorsal column white matter regions receiving mechanoreceptive, large diameter myelinated sensory afferent projections. The behavioral and immunohistochemical data combined suggest that (i) avulsion of the L6-S1 ventral roots at the CNS/PNS interface results in sensory plasticity and the development of neuropathic pain affecting the uninjured segment immediately rostral to the level of injury, and (ii) this effect is particularly prominent within regions that receive projections from a specific subset of afferents, namely those that carry mechanoreceptive, non-noxious sensory information. Importantly, from a therapeutic standpoint, reimplantation of the avulsed roots into the conus medullaris ameliorated pain, concomitant with a reduction in astrocytes, microglia, and macrophages in the L5 dorsal horn at 8 wks post-surgery.

A motor lesion can induce sensory plasticity beyond the level of injury

The present results, suggesting that a motor lesion can influence sensory plasticity, are not unprecedented. Our observation that a motor lesion can elicit sensory plasticity is in agreement, at least in part, with studies showing that an L5 VRT can result in neuropathic pain (Li et al., 2002, Sheth et al., 2002, Obata et al., 2004). However, there are some important differences between the VRA and VRT models that should be considered. First, a VRA injury occurs at the CNS/PNS interface, resulting in marked motoneuron and autonomic cell death within the spinal cord at the level of injury over several weeks (Hoang et al., 2003), while VRT is a non-lethal, peripheral injury (Vizzard et al., 1995, Anneser et al., 2000) in which axotomized motoneurons and autonomic neurons survive. Second, in the present study, only allodynia was observed, suggesting that VRA-induced plasticity may be specific to mechanoreceptive afferents, whereas a unilateral L5 VRT resulted in both allodynia and hyperalgesia at the L5 dermatome (Li et al., 2002), raising the possibility that small diameter non- or thinly myelinated afferents carrying noxious sensory information may have been affected by VRT, but not by VRA in the present study. One physiological consideration with respect to these data is that of ventral root afferents, which we propose played a minimal role, if any, in the development of allodynia in the present study. Ventral root afferents are largely unmyelinated or thinly myelinated (Coggeshall et al., 1973, Fang, 1987). While we cannot rule out the possibility that VRA induced some plasticity of small, unmyelinated afferents, our behavioral and immunohistochemical data strongly support the involvement of primarily large diameter myelinated afferents. Further, evidence suggests that the presence of unmyelinated afferents in the ventral root is minimal as the CNS/PNS interface is approached (Risling et al., 1984). As our lesion was performed at the CNS/PNS interface, the likelihood of injured ventral root afferents contributing to pain is minimal. In the VRT, however, the lesion is more distal to the CNS/PNS interface, and therefore more likely to injure ventral root afferents, possibly contributing to the development of hyperalgesia in those studies. Finally, in our VRA injury model, the lesion is caudal to the dermatome being tested, so that even if 10% of the unmyelinated fibers in the L6 ventral root were sensory (Coggeshall et al., 1980), direct injury to sensory projections from the L5 dermatome would be absent.

Our data also suggest that sensory plasticity at the cellular level extends beyond the injured segment. In agreement with these findings, there is a plethora of data supporting the notion that sensory plasticity can occur in segments adjacent to the level of injury that are, themselves, uninjured. It was recently shown that monosynaptic reflex efficacy is enhanced at the L6 spinal cord level following either L7 VRT (Havton and Kellerth, 2004) or VRA (Holmberg and Kellerth, 2000) in cats, consistent with the idea that sensory plasticity can occur within non-noxious sensory afferents adjacent to the level of a motor injury. Following a peripheral nerve injury, such as an L5-L6 spinal nerve ligation, electrophysiological (Gold et al., 2003) and cellular (Hudson et al., 2001, Schafers et al., 2003, Obata et al., 2004, Shortland et al., 2006)

plasticity has been shown to occur in the uninjured L4 dorsal root ganglia. There is also evidence that these adjacent, uninjured afferents may contribute to the development and maintenance of neuropathic pain (Wu et al., 2001, Gold et al., 2003, Lee et al., 2003). One proposed mechanism is that Wallerian degeneration in mixed roots may result in the release of degenerative by-products and subsequent changes in the cellular and electrophysiological properties of the adjacent uninjured afferents, thereby resulting in pain (Yoon et al., 1996, Li et al., 2000). In the present model, for example, as the L6 fibers travel with L5 afferents to some degree in the sciatic nerve, it is possible that by-products of L6 efferent axonal degeneration may affect the functional properties of the L5 afferents (Wu et al., 2001). A second possibility regarding the development of pain in the L5 dermatome is that the loss of motoneuron targets in the L6-S1 segments resulted in afferent sprouting or plasticity from the injured segments into adjacent uninjured segments in search of new targets. This possibility is seemingly consistent with those studies showing enhanced synaptic efficacy in the L6 feline spinal cord following an L7 VRA (Holmberg and Kellerth, 2000) or VRT (Havton and Kellerth, 2004), but is less convincing in light of the development of pain in the VRT injury models (Li et al., 2002, Sheth et al., 2002, Obata et al., 2004), as motoneurons do not die. Clearly, additional studies are needed to characterize the effects of degenerating efferent axons on intact sensory afferents.

Possible cellular mechanisms underlying the development of at-level pain

Activation of astrocytes and microglia may occur in the dorsal horn following direct trauma to the spinal cord (Popovich et al., 1997, Sroga et al., 2003, Nesic et al., 2005), and in response to spinal nerve injury (Gilmore et al., 1990, Murray et al., 1990, Eriksson et al., 1997, Coyle, 1998, Hashizume et al., 2000, Winkelstein et al., 2001, Ma and Quirion, 2002) or dorsal root injury (Murray et al., 1990, Winkelstein et al., 2001, Kozlova, 2003). However, some controversy remains as to the diverse roles that these cells may play in the spinal cord following PNS and CNS injuries. On one hand, astrocytes and microglia are known to release cytokines, nitric oxide synthase, and other molecules involved in cell excitability, cell death, and axonal degeneration (Popovich et al., 2002, Coutaux et al., 2005, Inoue, 2006). They may therefore be important mediators in the development and maintenance of neuropathic pain (Stuesse et al., 2001, Sweitzer et al., 2001, Jin et al., 2003, Tsuda et al., 2005). On the other hand, astrocytes may provide a tissue-protective function support to a damaged region (Aldskogius, 2001, Faulkner et al., 2004). In all of the studies just mentioned, however, sensory afferent damage was inherent to the models being examined. We previously showed that reactive astrocytes, microglia, and macrophages are prominent in the ventral horn at the level of injury after VRA and VRA+Imp, and speculated that their presence was a direct result of neuronal injury (Ohlsson et al., 2006). We now show for the first time that an L6-S1 VRA injury results in significant activation of astrocytes, microglia, and macrophages in the ipsilateral deep dorsal horn gray matter and in the dorsal funiculus of the spinal cord segment immediately rostral to the level of injury, relative to Lam control levels, in spite of the absence of a direct injury to the primary afferents. The observation that a laminectomy alone can have an effect on experimental measures, exhibited here as increased gliosis in the Lam dorsal funiculus, emphasizes the importance of including a sham-surgery control, However, as glial changes were not different between groups in the superficial gray matter, and allodynia was not present in the Lam group, we speculate that behavioral changes were related primarily to cellular plasticity in the deep dorsal horn gray matter and dorsal funiculus, above and beyond plasticity seen in Lam alone. It is important to note in this context that the VRA-induced inflammatory responses in the deep dorsal horn gray matter are associated with projection areas of mechanoreceptive primary afferents. The VRA-induced glial and macrophage activation in the dorsal funiculus was surprising, as no direct injury to the sensory afferents occurred. However, our findings are consistent with evidence that suggests that ascending sensory fibers in the dorsal funiculus that project to the gracile nucleus may play a role in tactile allodynia (Miki et

al., 2000, Ha et al., 2001, Sun et al., 2001, Ossipov et al., 2002, Back et al., 2003) as well as visceral pain (Palecek and Willis, 2003) after a peripheral nerve injury.

Ventral root reimplantation ameliorates at-level neuropathic pain and inflammation in the dorsal horn

Avulsed ventral roots have been successfully reimplanted into the cervical spinal cord following brachial plexus injury in humans, and anecdotal reports suggest the potential for the recovery of some motor and sensory function (Carlstedt et al., 2000). Although previous studies demonstrated that peripheral nerve transfers may reduce pain following brachial plexus injuries in humans (Berman et al., 1998), to our knowledge, no systematic studies have evaluated the effects of directly reimplanting avulsed ventral roots into the spinal cord on pain behavior in conjunction with the underlying associated mechanisms of sensory reorganization. The present study supports the concept that, while the reimplantion process acutely resulted in allodynia, it may have a beneficial therapeutic effect with respect to the recovery of more normal-like sensation by just a few weeks post-surgery, as allodynia observed at later timepoints after VRA was ameliorated by reimplantation. We speculate that the early allodynia may have resulted from the white matter incisions that were made to reinsert the ventral roots. Nonetheless, allodynia was ameliorated by 2 wks relative to Lam, and remained so throughout the remainder of the timecourse examined. Microglia staining intensity and the numbers of macrophages in the deep gray and dorsal funiculus white matter were also reduced towards Lam levels following implantation, providing further support for the notion that VRA injury-induced neuropathic pain and inflammation responses in the dorsal horn may be associated. Thus, VRA +Imp not only is neuroprotective, and promotes functional reinnervation of peripheral targets, it may also alleviate allodynia caused by VRA. One possible mechanism could be that the implanted root provides neurotrophic support to the lumbosacral spinal cord, as an injury to a peripheral axon results in Schwann cell proliferation and an increased expression of nerve growth factor (NGF), glial-derived neural growth factor (GDNF), brain-derived neurotrophic factor (BDNF), and neurotrophin 4/5 (Salzer and Bunge, 1980, Meyer et al., 1992, Funakoshi et al., 1993). In this context it is interesting to note that neurotrophic factors may also act as peripheral pain mediators, particularly in inflammatory pain states (Pezet and McMahon, 2006). Further studies are needed to clarify which specific interactions between neurotrophic factors and sensory projections may occur in the VRA and repair model.

In conclusion, the present data showed that a lesion solely to the L6-S1 motor roots causes sensory plasticity beyond the injured segment and leads, consequently, to the development of at-level mechanical allodynia, but not hyperalgesia, out to 7 wks post-VRA. Inflammatory changes were localized in the dorsal horn regions receiving inputs from the large-diameter fibers carrying mechanoreceptive information. Importantly, the reimplantation procedure reduced behavior associated with pain and injury-induced activation of astrocytes, microglia, and macrophages in the dorsal horn, suggesting an overall beneficial therapeutic effect. While further investigation into the physiological mechanisms of neuropathic pain induced by VRA is warranted, reimplantation of avulsed ventral roots emerges as a potentially efficacious therapeutic intervention and should be considered in the ongoing development of treatment strategies for cauda equina and brachial plexus spinal cord injury.

Acknowledgements

Sincere thanks to Dr. Peter Ohara, UCSF, for helpful discussions on the behavioral aspects of this study, Dr. Jun Wu, UCLA, for technical support, Dr. Jeff Gornbein, UCLA, for statistical consulting, and Lynne Olson, UCLA Photographic Services. This work was supported by the NIH (5T32 NS07449:06 to AJB; NS042719 to LAH), the Roman Reed Spinal Cord Injury Research Fund of California (LAH), and the Adelson Program in Neural Repair and Rehabilitation of the Dr. Miriam and Sheldon Adelson Medical Foundation (LAH).

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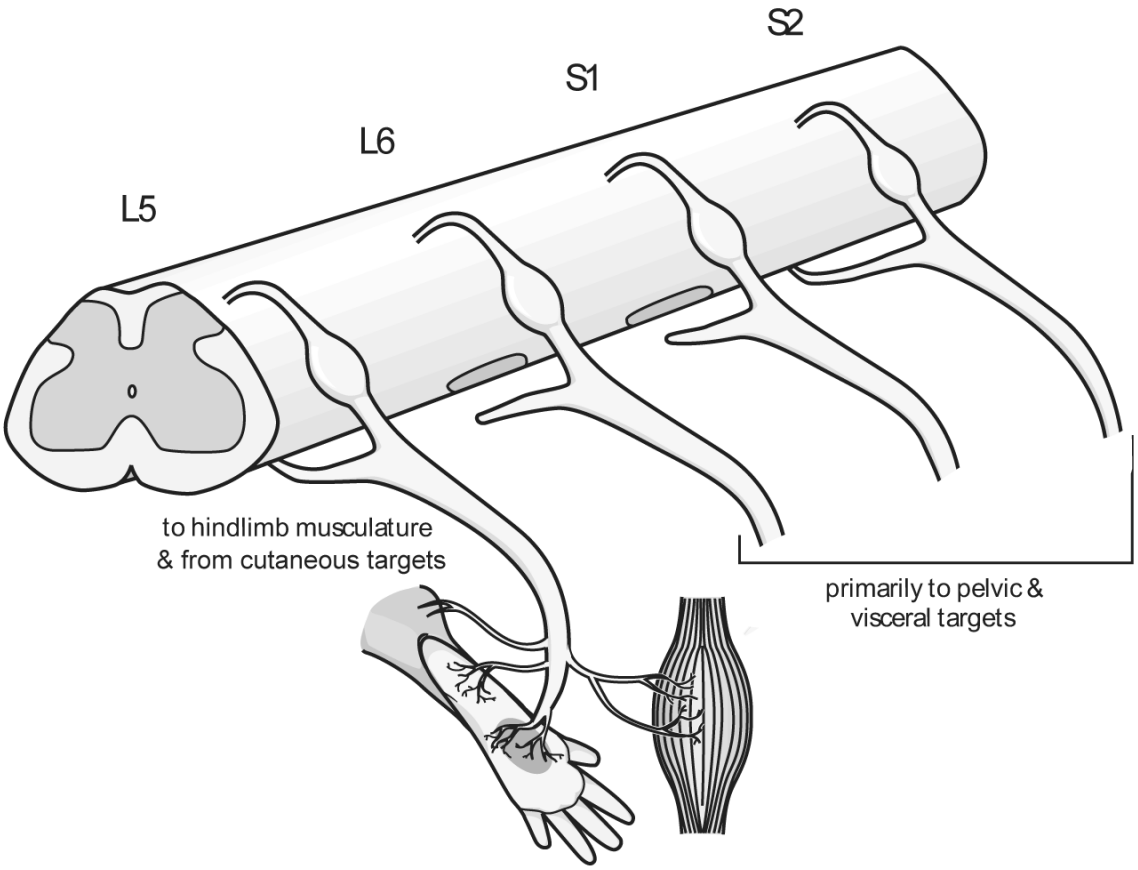

Figure 1. The unilateral lumbosacral ventral root avulsion (VRA) model

The L6 and S1 ventral roots were avulsed unilaterally from the CNS/PNS interface of the spinal cord. For VRA+Imp rats, both ventral roots were reimplanted into the lateral funicuclus (not shown). Note that the injured L6 and S1 efferents project primarily to pelvic floor and visceral targets, while the intact L5 efferents project more so to hindlimb musculature and receive afferents from cutaneous targets. Behavioral tests for allodynia and hyperalgesia were performed at the L5 dermatome on the central footpads of the hindlimbs (gray shaded region).

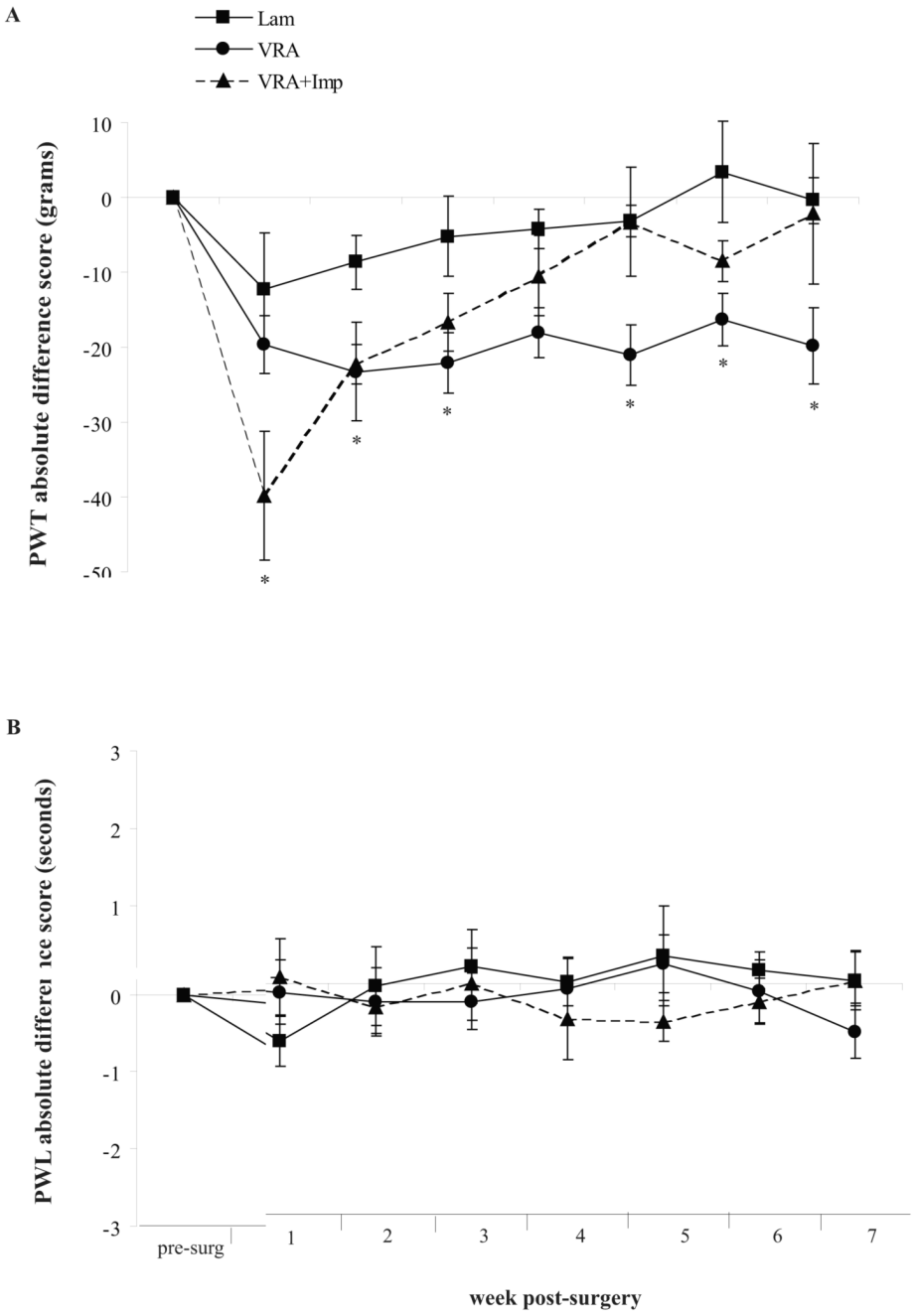

Figure 2. Neuropathic pain following unilateral L6-S1 VRA and VRA + Imp

The paw withdrawal thresholds (PWT; in grams of force) and paw withdrawal latencies (PWL; in seconds) were determined as indicators of mechanical allodynia **(A)** and hyperalgesia **(B)**, respectively, prior to surgery and weekly thereafter for 7 wks. Data are presented as an absolute difference score (i.e., the ipsilateral minus the contralateral values normalized to presurgical baseline levels). **A)** For mechanical allodynia, VRA is significantly reduced relative to Lam at 2,3,5,6 and 7 weeks, while VRA+Imp differs from Lam only at 1 wk. **B)** For hyperalgesia, no differences between groups were observed. Data shown are the mean values ± SEM. *, significantly different from Lam within each time point at $p<0.05$.

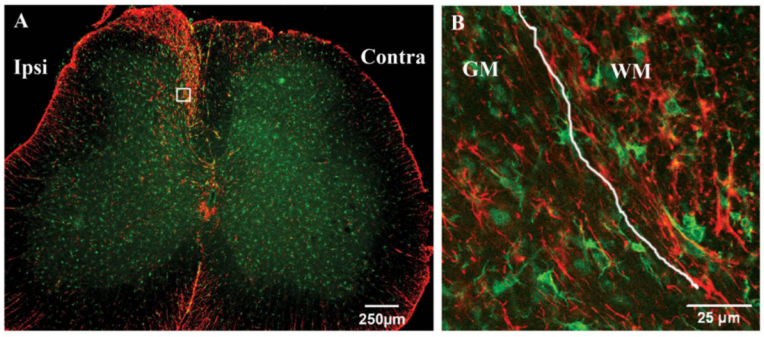

Figure 3. Unilateral L6-S1 VRA elicits an inflammatory response in the L5 ipsilateral dorsal horn

A) Astrocytes (GFAP; red) and microglia (AIF-1; green) in the L5 spinal cord at 8 wks post L6-S1 VRA. Note that the inflammatory response is restricted to the ipsilateral dorsal horn quadrant. A magnified region from the inset is shown in **B)**, where astrocytes (red) and microglia (green) are found in both the gray matter (GM) and white matter (WM), and exhibit characteristic morphological properties. Calibration bars are 250- and 25 μm, respectively. The white line in **(B)** indicates the WM/GM border.

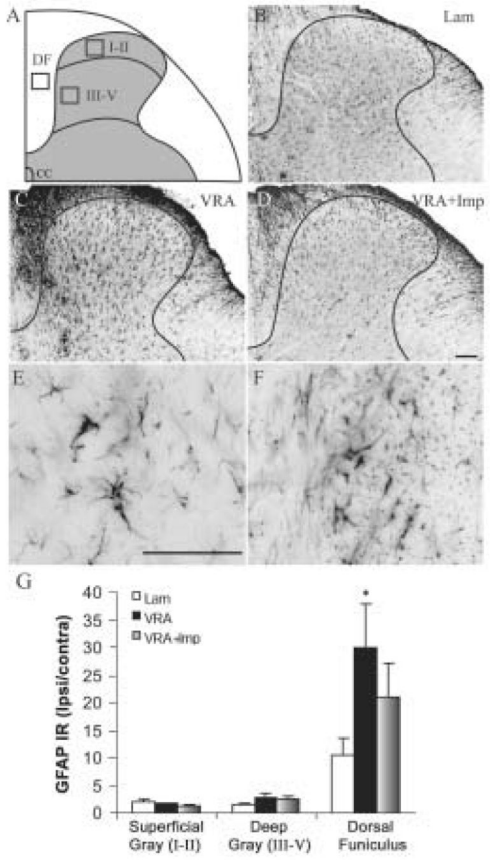

Figure 4. Increased activation of astrocytes in the ipsilateral L5 dorsal horn at 8 wks following unilateral L6-S1 VRA

A) Three regions of interest for quantitative immunohistochemistry were examined in the superficial gray matter (laminae I-II), deep gray matter (laminae III-V), and the white matter dorsal funiculus (DF). The central canal (CC) is also shown for orientation purposes. The boxes represent an area of 10,000 μm^2. (**B-D)** GFAP immunoreactivity (IR) is shown in the ipsilateral L5 dorsal horn quadrants for Lam **(B)**, VRA **(C)**, and VRA+Imp subjects **(D)**. Note the prominent upregulation of GFAP in the VRA DF **(B)** relative to Lam **(A)**, while VRA+Imp is qualitatively similar to Lam. The black border identifies the gray matter/white matter border. Representative high magnification (40x) images for GFAP from VRA gray matter **(E)** and white matter **(F)** are also shown. Calibration bars are 100 μm. **(G)** VRA GFAP IR is significantly increased in the DF relative to Lam ($p<0.05$), while VRA+Imp is not different from Lam. GFAP IR in laminae I-II and laminae III-V did not significantly differ between groups. Values represent the area of GFAP-positive IR / 10,000 μm^2 for the ipsilateral region relative to the same region on the contralateral side. Data shown are the mean values ± SEM. *, significantly different from Lam within each time point at $p<0.05$.

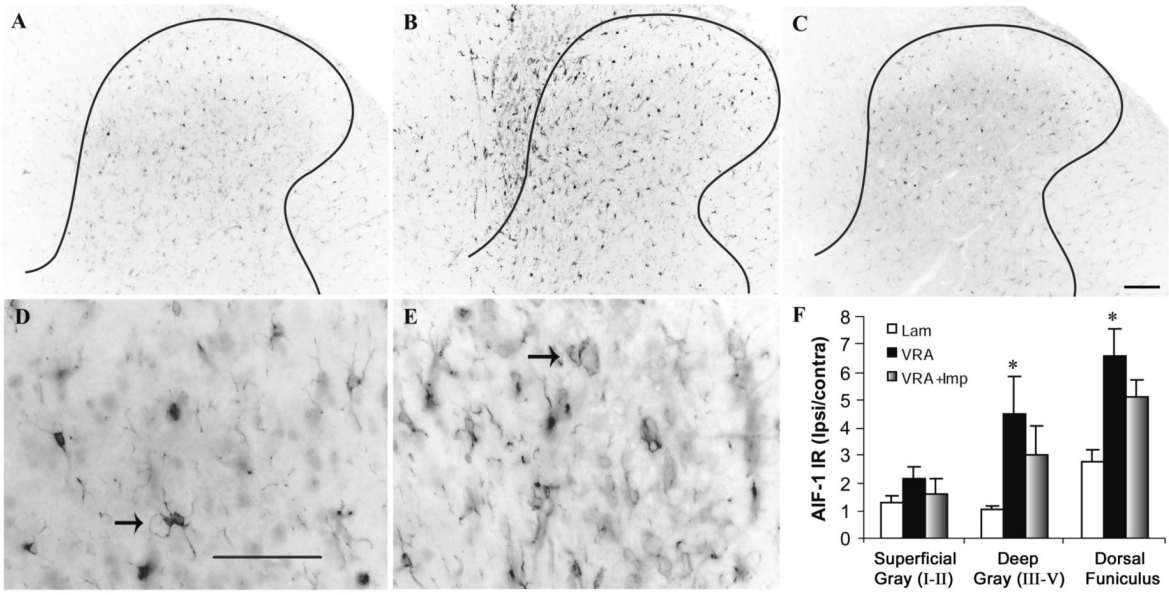

Figure 5. Increased microglial activation in the ipsilateral L5 dorsal horn at 8 wks following unilateral L6-S1 VRA

AIF-1 IR identifies microglia in the ipsilateral dorsal horn in Lam, VRA, and VRA+Imp rats. Note the low-intensity levels of staining in Lam **(A)** and VRA+Imp rats **(C)**, while the intensity is substantially increased in VRA rats **(B)** in both laminae III-V and the DF. **(D)** AIF-1 positive microglia in the VRA laminae III-V show characteristic morphology with extended processes (arrow), while in the DF white matter **(E)**, many AIF-1 positive cells show a more macrophage-type morphology (arrow) indicative of an active inflammatory response. Calibration bars are 100 μm. **(F)** In the VRA group, AIF-I IR was significantly increased in laminae III-V and in the DF relative to Lam, while VRA+Imp was not. There were no differences between groups in laminae I-II. Data shown are the mean values ± SEM. *, significantly different from Lam within each time point at $p<0.05$.

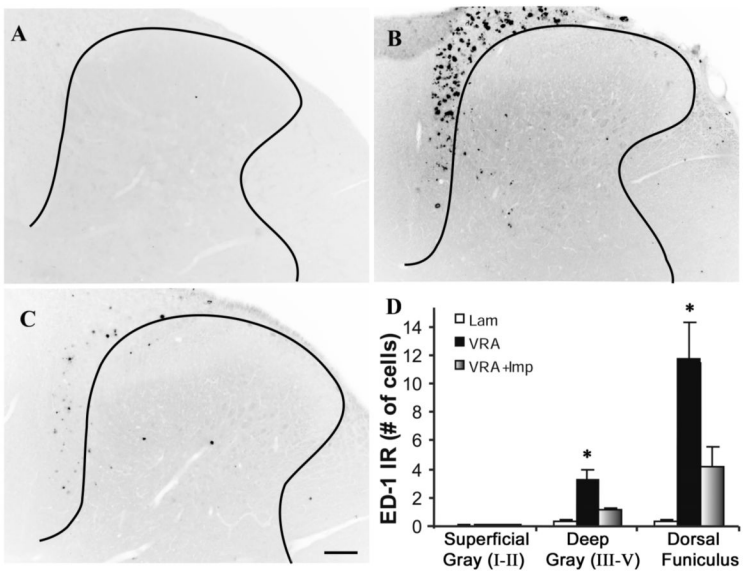

Figure 6. Macrophage response in the L5 ipsilateral dorsal horn of VRA rat at 8 wks post-avulsion of the L6-S1 ventral roots

(A) Few, if any, ED-1 positive macrophages were observed in Lam. **(B)** Macrophage staining was prominent in the L5 DF of VRA rats, and also, but to a lesser degree, in laminae III-V. **(C)** The macrophage response was ameliorated in the VRA+Imp rats. The calibration bar is 100 μm. **(D)** The number of ED-1 positive macrophages in VRA rats was significantly increased in both laminae III-V and in the DF relative to Lam ($p<0.05$), while VRA+Imp was not different from Lam in those regions. There were no group differences in laminae I-II. Data shown are the mean number of ED-1 positive cells / 10,000 μm^2 region of interest ± SEM. *, significantly different from Lam within each time point at $p<0.05$.