

Research report

Nerve root injury severity differentially modulates spinal glial activation in a rat lumbar radiculopathy model: considerations for persistent pain

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Abstract

Nerve root deformation magnitude affects behavioral sensitivity and spinal cytokine expression in a lumbar radiculopathy model. Despite evidence suggesting spinal glia play a role in persistent pain, no study has examined the relationship between injury severity in painful radiculopathy and spinal glial activation. This study quantified local *in vivo* biomechanics for nerve root injury, describing effects on temporal glial activation. Sham rats had only nerve root exposure; ligation rats received a tight L5 nerve root ligation with silk suture. Using image analysis, the magnitude of nerve root compressive strain was calculated at the time of injury. Mechanical allodynia was assessed from days 1 to 14 following injury and spinal microglial and astrocytic expression were evaluated using immunohistochemistry on days 1, 3, 7, and 14. More severe ligations produced greater microglial activation, indicating injury severity modulates spinal microglial activation. However, astrocytic activation levels did not demonstrate any relationship with the degree of initial injury severity. While allodynia decreased slightly over time following injury, the temporal changes in mechanical allodynia were not significant. Microglial activation levels were maintained temporally, and in some cases increased over time; whereas, changes in astrocytic activation levels were not temporally or injury-related. While initial nerve root injury severity likely modulates spinal OX-42 (CR3/CD11b) expression, OX-42 staining does not directly correlate with nerve root injury-induced mechanical allodynia.

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1. Introduction

Clinically, mechanical compression of lumbar spinal nerve roots, either by disc prolapse or spinal stenosis, is a common mechanism of painful lumbar radiculopathy [7,18]. As such, a variety of animal models mimicking compression of these structures in the lumbar spine have demonstrated a relationship between neural compression and altered neurologic function [2,6,8,10,14,22–24]. Typically, compressive insults are delivered to the lumbar

nerve roots and nerves in these studies by applying a ligation [2,6,8,10,22–24] that produces behaviors suggestive of persistent pain. Behavioral sensitivity has been produced in radiculopathy models with nerve root ligations using both silk [6,23,24] and chromic gut [6,22] suture material. A typical behavioral response includes mechanical allodynia (increased sensitivity to a non-noxious stimulus) and is observed in the innervated hind paw of the injured nerve or nerve root [6,22]. Such behavioral sensitivity is also a characteristic clinical sign observed in lumbar radiculopathy and provides a gauge of nociceptive responses and persistent pain.

In addition to the behavioral sensitivity responses well described in these animal models, a host of central immune changes has been reported as contributing to the onset and

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maintenance of persistent pain [2,3]. Neuroimmune activation involves the activation of endothelial cells, microglia and astrocytes, which leads to production of cytokines [3]. Both immune cell activation and upregulation of pro- and anti-inflammatory cytokines in the central nervous system (CNS) have been previously demonstrated in rat models of lumbar radiculopathy using nerve root ligation [2,6,8,22,23]. While microglial activation using the OX-42 antibody to CR3/CD11b has not been directly correlated with pain behaviors in peripheral nerve injury models, astrocytic activation has been reported to follow mechanical allodynia for up to 7 days following injury [1]. However, spinal glial activation is accepted as having a role in the CNS response leading to hypersensitivity and persistent pain [6,20,21]. For example, glia have been shown to directly influence neuronal activity in the spinal cord through the release of neurotransmitters and pain mediators [9,11,19,21], including pro-inflammatory cytokines, nitric oxide, prostaglandins and glutamate. In addition, activation of these cells can enhance the release of synaptic Substance P and regulate neurotransmitter uptake in the spinal cord [21]. In these ways it is clear that glial activation and their subsequent cytokine release and synaptic modulation have a key pain-modulating effect. However, while glial activation has been qualitatively characterized for the lumbar radiculopathy model in this study [6], it has not been established whether the degree of nerve root deformation at the time of injury modulates the resulting spinal glial activation or whether this glial modulation is responsible for behavioral hypersensitivity patterns in this painful radiculopathy model.

For this radiculopathy model using ligation of the L5 dorsal and ventral nerve roots, it has been shown quantitatively that the severity of the initial mechanical tissue injury is directly correlated with both the amount of resulting mechanical allodynia and the degree of spinal cytokine upregulation following injury [23]. Using the same *in vivo* imaging techniques presented herein, injury severity was estimated by calculations of applied tissue deformation (strain) due to nerve root ligation [23]. The calculated applied strain measurement provides a quantifiable measure of the amount of compression delivered to the nerve roots at the time of injury. While previous data suggest a graded relationship between local injury severity, behavioral sensitivity, and increases in specific cytokine mRNA [23,24], no investigation has characterized the relationship between the degree of root injury and the ensuing spinal glial activation which may contribute to central hypersensitization, a pathologic correlate of persistent pain.

Therefore, the purpose of this study was to utilize an *in vivo* method for quantifying local nerve root mechanical injury in an existing painful lumbar radiculopathy rat model to simultaneously quantify injury severity and the resulting spinal glial activation following injury. Injury severity was quantified as nerve root strain applied through

tight ligation and allodynia was monitored postoperatively. Spinal microglial and astrocytic activation were examined at four postoperative time points following injury using immunohistochemistry, in the context of injury severity, and examined as may be related to behavioral hypersensitivity (mechanical allodynia).

2. Materials and methods

2.1. Animal subjects and surgery

Experiments were performed using male Holtzman rats, each weighing 225–275 g at the start of surgery. Animals were housed individually under USDA and AAALAC-approved conditions with a 12–12 h light–dark cycle and free access to food and water. All experimental procedures were approved by the Dartmouth College Institutional Animal Care and Use Committee. All surgical procedures were performed under inhalation anesthesia (4% halothane for induction, 2% halothane for maintenance). Lumbar radiculopathy was induced according to an established nerve root injury model [6,23,24]. At the time of surgery, animals were divided into two groups: a sham group in which the left L5 dorsal and ventral roots were exposed only and a ligation group in which the left L5 dorsal and ventral nerve roots were ligated together using a single 6–0 silk suture with varying degrees of tightness. Following surgery, the wound was closed with 3–0 polyester suture and surgical staples and animals were recovered in room air.

2.2. Imaging and strain approximation techniques

In vivo imaging techniques were employed to quantify applied tissue deformation at the time of injury, as previously described [23,24]. Briefly, the surgical operating scope (LFS 200, Carl Zeiss Inc.) was equipped with a digital camera (Model DP10, Olympus Optical Co.), having 1024×1280 pixel resolution, to image the surgical exposure and neural tissue. A minimum of two marks was made on the L5/L6 bony facet surfaces using acrylic black paint. These bony reference markers provide positional data defining a local origin and orientation for each image of a given animal's surgical exposure. Images were acquired for the initial undeformed nerve root configuration and for the deformed ligation configuration of the nerve roots. Additional images were acquired to provide geometric calibration and to accommodate any differences in magnification between images. Locations of the bony marks were digitized using ImageTool Software (UTHSCSA, San Antonio, TX); point locations and the orientation of the line defined by them were used to transform image orientations to the reference coordinate system. Nerve root boundaries were also digitized along the outer most edges of the structure, providing a set of

contours describing the nerve root in its in vivo unligated and immediately ligated geometries [23,24]. Cubic polynomials were used to fit each series of digitized nerve root boundary points.

To estimate tissue deformation at the time of injury, radial strain was quantified. Using the digitized boundaries, the nerve root was approximated as a cylinder with a circular cross-section and variable radius along its length. Radial strain (ϵ_R) was calculated along the length for the exposed nerve root using the change in measured nerve root radii (Δr) divided by the radius (r_{ref}) of the undeformed configuration (before ligation) [4], where: $\epsilon_R = \Delta r / r_{ref}$. This calculation was performed in the region of maximal nerve root compression. A single digitizer who was blinded to all other measurements of this study performed all digitization.

2.3. Mechanical allodynia

Tactile sensitivity in the ipsilateral hind paw was measured as the frequency of foot withdrawals elicited by mechanical stimulation to the hind paw. Each animal was monitored at days 1, 3, 5, 7, 10, and 14 postoperatively until the time of euthanization. Mechanical allodynia was measured as the number of paw withdrawals elicited by a defined non-noxious mechanical stimulus. In each testing session, rats were subjected to three rounds of ten tactile stimulations to the plantar surface of the hind paw using 2 and 12 g von Frey filaments (Stoelting, Wood Dale, IL). Animals were previously acclimated to the testing environment and the tester and baseline measurements were determined for each animal prior to the initial surgical procedure.

2.4. Immunohistochemical staining

Immunohistochemistry was performed on L5 spinal cord tissue harvested from animals at different postoperative time points ($n=4$ ligation; $n=1$ sham at each time point). Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneally) and subsets of rats were euthanized at days 1, 3, 7, and 14 following injury by transcardiac perfusion. Harvested lumbar spinal cord sections were post-fixed for 30 min in 4% paraformaldehyde. The L5 portion of the lumbar spinal cord was determined by dissecting the L5 nerve roots to their region of entry into the spinal cord. Immunohistochemistry was performed on 20 μ m, free-floating L5 spinal sections. Optimal dilutions and incubation time periods for each antibody and lot were determined prior to this study. Elimination of the primary antibody was performed in each run as a negative control. In addition, tissue from normal, unoperated rats was also stained in each run. A monoclonal antibody (OX-42) to CR3/CD11b, a marker of microglial activation, was used at a dilution of 1:2 (provided by Dr. William F. Hickey, Dartmouth College, NH). A polyclonal

antibody to Glial Fibrillary Acidic Protein (GFAP) (Dako Corp., Carpinteria, CA), a marker of astrocytic activation, was used at a dilution of 1:20 000. Assessment of spinal cord staining was performed blinded to injury severity and surgery group. Sections were scored according to the following scale [2]: baseline staining (○), mild response (+), moderate response (++), intense response (+++). Scores were based on the staining of at least three sections for each animal using each antibody.

3. Results

All animals in the ligation group exhibited mechanical allodynia following nerve root injury (Fig. 1). The pattern of behavioral sensitivity was typical of that produced using this lumbar radiculopathy model [6,22–24]. Initially following injury, a robust response was observed, followed by a slight and gradual decrease in response magnitude, for both the 2 and 12 g von Frey filaments (Figs. 1 and 2). Mechanical allodynia for the ligation animals following surgery was elevated over the corresponding baseline values and sham animals for both 2 and 12 g stimulations. In addition, mechanical allodynia observed for sham animals was not significantly different from baseline values.

Qualitative assessments of the degree of glial activation using OX-42 and GFAP staining for each animal's L5 section of the spinal cord are provided in Tables 1 and 2, respectively. Also provided in these tables is the calculated nerve root ligation strain at injury for each animal and the recorded mechanical allodynia scores on the day of euthanization. Immunohistochemistry scoring results are presented for each animal on both the ipsilateral and contralateral side relative to the injury in both the dorsal and ventral horns. There was an increase in OX-42-like immunoreactivity (microglial activation) over baseline levels seen in the ipsilateral side of the spinal cord for all postoperative time points (Table 1). On the contralateral side, similar patterns were observed, while less elevated. Ipsilateral astrocytic activation, as measured by GFAP-like immunoreactivity, was also elevated following injury (Table 2). Contralateral astrocytic activation was only slightly elevated. While GFAP staining was not elevated over baseline in sham animals, these same sham tissue samples exhibited mild and moderate intensity in OX-42 staining (Table 1). The OX-42 and GFAP staining intensity were temporally maintained or increased (Tables 1 and 2).

Analysis of error using this strain analysis technique has been previously published [23,24]; the technique has adequate sensitivity to determine the in vivo ligation strains in this model with the appropriate degree of accuracy. The tightly applied nerve root ligation produced radial strains which were compressive and had a mean value of $30.3 \pm 11.1\%$ for all nerve root injuries in this

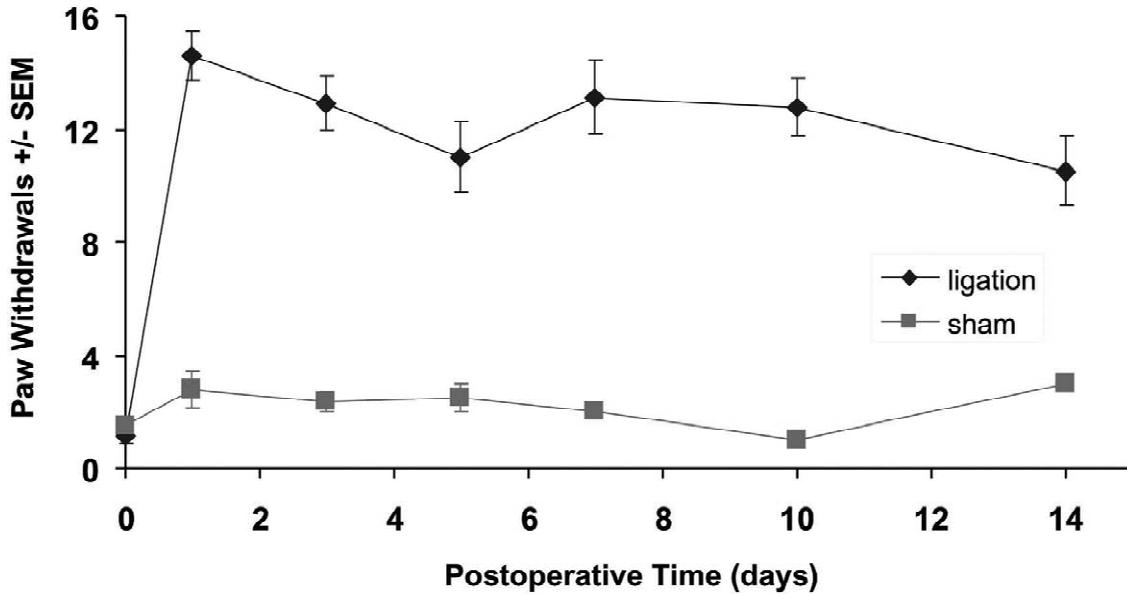


Fig. 1. Mean mechanical sensitivity for ligation and sham groups of animals for stimulation by a 12 g von Frey filament. Foot lift response frequency to stimulation with 12 g von Frey filament is depicted over the 14 days of the study. Stimulation with a 2 g von Frey filament produced similar behavioral sensitivity responses (data not shown). As previously reported [23], more severe deformations at injury produced greater levels of allodynia than in the less severe injuries. The total number of responses resulting from 30 stimulations per animal was recorded and the group average and standard error are reported here.

study. For ease of interpretation of the immunohistochemistry results, the ligated rats were grouped according to the magnitude of applied deformation, for each post-operative time point (Tables 1 and 2). Those rats undergoing ligation strains greater than the overall mean applied strain of all ligation animals had a mean tissue strain of $39.1 \pm 7.3\%$. In contrast, the mean strain for animals

experiencing strains below the overall mean was $21.4 \pm 5.4\%$. Using a Student's *t*-test, the two groups of injury severity were determined to be significantly ($P < 0.0001$) different in strain magnitude. As previously reported [23,24], mechanical allodynia was greater for animals undergoing injuries with greater amounts of applied tissue deformation than for those animals receiving

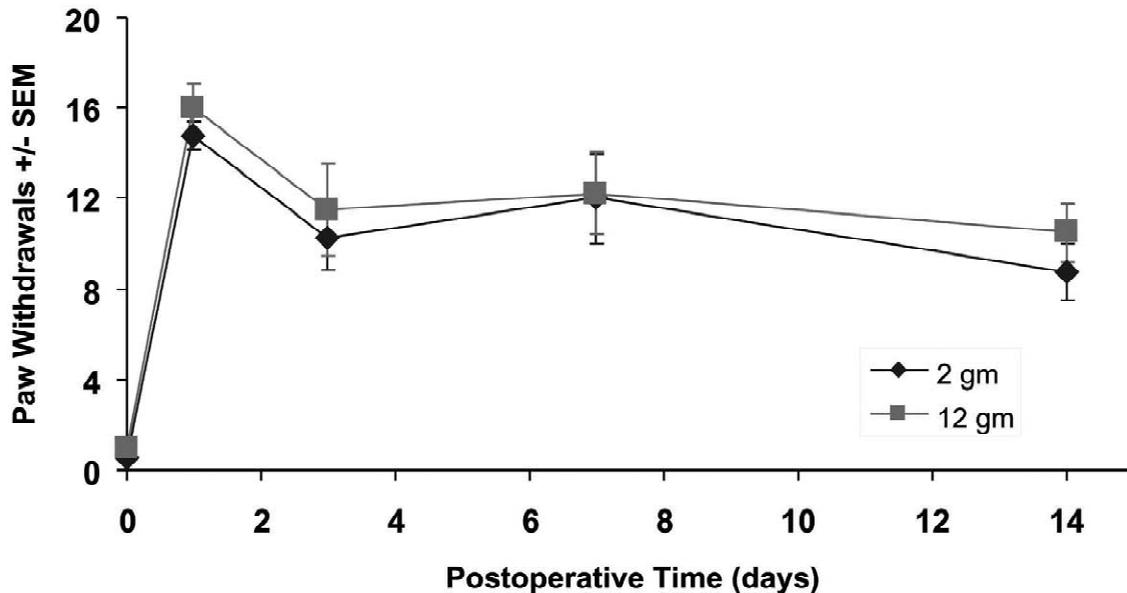


Fig. 2. Mean mechanical allodynia for the ligation animals on the day of euthanization (postoperative days 1, 3, 7, and 14), as shown in Tables 1 and 2 for 2 and 12 g von Frey filaments. Mechanical allodynia is shown to decrease over time despite the maintained glial activation described in Tables 1 and 2. The total number of responses resulting from 30 stimulations per animal was recorded and the group average and standard error are reported here.

Table 1

Immunohistochemical microglial scoring[§] of L5 spinal cord: sham and radiculopathy injuries according to postoperative time point and applied strain

Day	Strain(%)	Microglial (OX42) Staining				Mechanical Allodynia	
		Ipsilateral		Contralateral		2 gm	12 gm
		DH	VH	DH	VH		
NORMAL							
		o	o	o	o	0.5	1
SHAM							
Day 1		+	o	+	o	3	3
Day 3		++	+	+	o	3	2
Day 7		+	+	+	o	2	2
Day 14		o	+	o	o	1	3
RADICULOPATHY							
Day 1	19.3	+	+	o	o	15	18
	23.8	++	+	+	+	13	13
	30.5	++	+	+	+	16	17
	39.9	++	++	+	+	15	16
Day 3	14.7	+	+	o	o	14	17
	22.3	++	+	+	+	8	8
	37.1	++	++	+	o	8	9
	40.2	++	+++	++	+	11	12
Day 7	27.2	+	++	+	+	14	12
	27.6	+	++	o	+	11	12
	36.8	++	+++	++	+	7	17
	42.3	++	+++	++	+	16	8
Day 14	12.9	+	+	+	+	9	10
	23.2	++	+	+	+	12	14
	32.0	+++	++	++	+	6	8
	54.3	+++	++	+	o	8	10

[§] Staining key: baseline, o; mild response, +; moderate response, ++; intense response, +++ . DH, dorsal horn; VH, ventral horn.

* Strain measurement represents the calculated radial strain (in %) at the time of ligation. No values are reported for sham rats since no ligation is imposed. Shading indicates those animals undergoing a more severe injury (mean strain of $39.1 \pm 7.3\%$), while the unshaded animals are in the less severe injury group (mean strain of $21.4 \pm 5.4\%$).

less severe injuries (data not shown). With this same grouping based on deformation severity at the time of injury, the degree of microglial activation exhibited a graded response depending on strain magnitude (Table 1, Fig. 3). The more severe injury strain group consistently exhibited a more intense response of OX-42 immunoreactivity than was seen in the lower strain group. The gradation of OX-42 expression by microglia according to injury severity was observed at each postoperative time point and this pattern did not change over time. In contrast, GFAP immunoreactivity did not exhibit graded expression according to injury intensity at any time point (Table 2, Fig. 3).

4. Discussion

Behavioral patterns observed in this study are similar to those previously reported for this model [6,23,24]. Overall, these animals also exhibited greater mechanical allodynia for more severe injury compared to responses of less severe injuries. Such graded cumulative behavioral sensitivity, depending on injury intensity is also consistent with previous reports [23,24]. Similarly, the graded expression of spinal OX-42 intensity based on the severity of applied deformation (strain) at the time of injury indicates

a direct relationship between deformation magnitude and one marker of injury in the CNS. While rats experiencing larger compressive ligation strains displayed greater expression of this marker of microglial activation which was sustained at all time points following injury (Table 1, Fig. 3), OX-42 expression intensity did not correlate with the behavioral patterns observed temporally (Table 1). In other words, on a point-by-point basis, while OX-42 intensity showed a graded response based on injury intensity, the individual mechanical allodynia responses measured at the day of euthanization for these animals did not correlate with initial injury magnitude (Table 1).

Table 1 provides a comprehensive summary of the temporal nature of OX-42 staining and allodynia in the context of injury severity on a point-wise and individual basis. In summary, OX-42 staining is dependent on the magnitude of tissue insult and this is maintained for all postoperative time points. In addition, it is observed that the intensity of staining in the ipsilateral dorsal horn is maintained throughout the study and does not decrease for later time points. Allodynia responses were temporally maintained overall yet did not show an individual relationship to the OX-42 staining or injury strain. For example, for day 14, the rat with 32% strain exhibited intense dorsal horn staining while having relatively low mechanical allodynia responses on this particular day.

Table 2

Immunohistochemical astrocytic scoring[§] of L5 spinal cord: sham and radiculopathy injuries according to postoperative time point and applied strain

Day	Strain(%) [†]	Astrocytic (GFAP) Staining				Mechanical Allodynia	
		Ipsilateral		Contralateral		2 gm	12 gm
		DH	VH	DH	VH		
NORMAL							
		o	o	o	o	0.5	1
SHAM							
Day 1		o	+	+	+	3	3
Day 3		o	+	+	o	3	2
Day 7		o	o	o	o	2	2
Day 14		o	o	+	o	1	3
RADICULOPATHY							
Day 1	19.3	+	+	o	+	15	18
	23.8	++	+	o	+	13	13
	30.5	+	o	o	o	16	17
	39.9	++	++	+	+	15	16
Day 3	14.7	+	+	o	o	14	17
	22.3	++	+	o	+	8	8
	37.1	++	+	++	+	8	9
	40.2	++	++	+	+	11	12
Day 7	27.2	++	++	+	+	14	12
	27.6	+	++	+	+	11	12
	36.8	++	+	o	o	7	17
	42.3	++	++	+	+	16	8
Day 14	12.9	+	o	+	+	9	10
	23.2	++	++	+	+	12	14
	32.0	++	+++	++	++	6	8
	54.3	+	++	o	o	8	10

[§] Staining key: baseline, ○; mild response, +; moderate response, ++; intense response, ++++. DH, dorsal horn; VH, ventral horn.

[†] Strain measurement represents the calculated radial strain (in %) at the time of ligation. No values are reported for sham rats since no ligation is imposed. Shading indicates those animals undergoing a more severe injury (mean strain of $39.1 \pm 7.3\%$), while the unshaded animals are in the less severe injury group (mean strain of $21.4 \pm 5.4\%$).

While qualitative assessment of OX-42 immunoreactivity showed initial injury severity to have a graded effect, the sham animals demonstrated some OX-42 staining (Table 1), similar to previous work [6], suggesting some physiological CNS response in sham exposures. This finding further demonstrates the sensitivity of this cell marker for detecting injury while not necessarily serving as a marker specific for nociception. Together with other work dissociating OX-42 expression and behaviors in a peripheral pain model [1], these findings suggest that OX-42, while serving as a sensitive marker for perceived insult to the CNS, is not a specific indicator of nociceptive changes of cellular functioning. Also, the specific relationship of initial injury and astrocytic activation remains unknown. While GFAP staining was increased in injured animals compared to shams, the lack of a clear relationship to initial injury magnitude may suggest a role for astrocytes in maintaining behavioral sensitivity following initiation of a central response.

The relationship demonstrated between the magnitude of compression and spinal microglial activation as assessed by OX-42 reactivity is not surprising. Compression of neural tissue in pain models has resulted in changes in behavioral sensitivity, edema formation, altered blood flow, electrical activity, ambulation, dorsal horn neuronal

firing, and spinal cytokine expression [5,6,12,13,15,22–26]. These findings, together with the work reported herein, strongly implicate mechanical injury as a significant *initiator* of a CNS nociceptive response which may be associated with persistent pain. However, the initial injury event may not be responsible for the *maintenance* of behavioral sensitivity (allodynia) and other sequelae of persistent pain. Therefore, other neuroimmune markers of cellular activation or processing may better explain and more closely follow the temporal mechanical allodynia responses. Simply, microglial activation (as measured by OX-42 staining) may not explain behavioral patterns because it may not be directly responsible for them. The evidence of microglial activation in shams suggests that OX-42 may actually serve as a sensitive marker of the existence of a general insult to the CNS or its perceived insult due to general tissue trauma. Moreover, this work suggests that there may be a threshold for mechanical injury above which microglia and/or astrocytes become activated. It may be the resulting actions of different subpopulations of activated microglia, releasing inflammatory cytokines and expressing proteins specifically involved in neuroinflammation, which, in turn, affect differential CNS responses and may be responsible for varying degrees of pain, some more severe than others. It

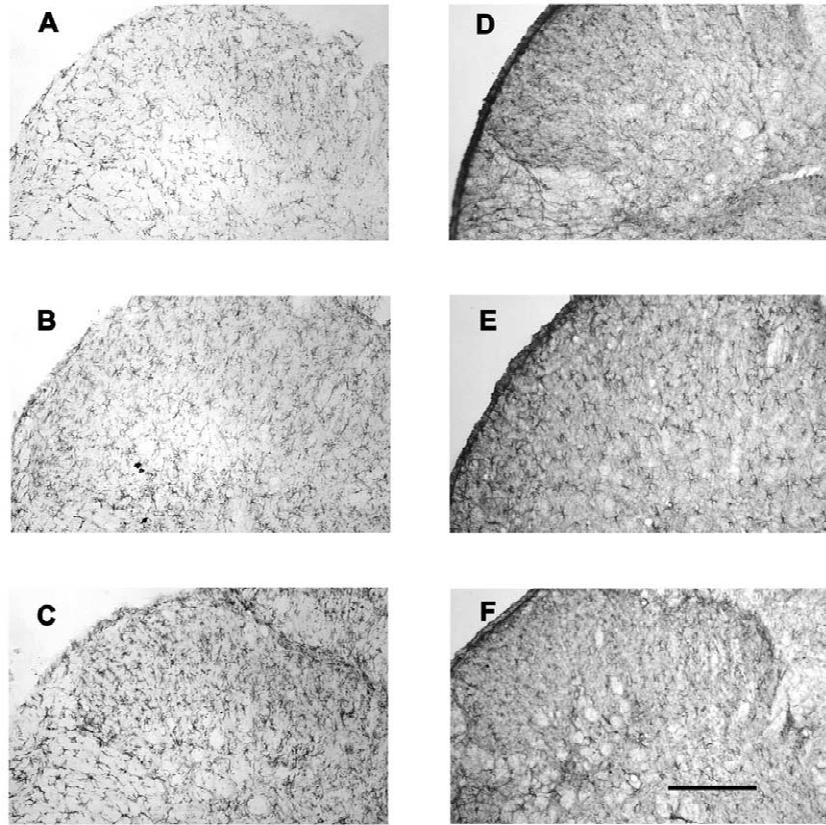


Fig. 3. Photomicrographs depicting glial activation in the L5 lumbar spinal cord dorsal horns of representative rats at postoperative day 7 for both microglial (A–C) and astrocytic activation (D–F). Microglial activation, using OX-42 staining, was more intense in the rats undergoing greater applied ligation strain (C) than those sustaining less severe ligations (B), but was elevated over normal (A) for all injured animals. However, there were no observable trends according to injury intensity in astrocytic activation using GFAP staining: normal (D), less severe injury intensity (E), and more severe injury (F). Scale bar = 250 μ m in F (applies to A–F).

has been shown in this and other pain models that subpopulations of activated glia express membrane proteins (e.g. Major Histocompatibility Complex II, cluster determinant-4) which have been suggested to play a role in CNS neuroinflammation and persistent pain [16,17]. Examination of specific responses in these glial subpopulations in this context would provide a more detailed understanding of the relationship between *nociceptive* responses and injury mechanics. Indeed, the activation patterns of these subpopulations of glia may better explain behavioral patterns and actually serve not only as a more specific, but as a more appropriate, marker of cellular neuroimmune changes in the CNS in association with pain behaviors.

While continued studies are needed to fully delineate the relationship between local biomechanics and the specific physiologic pathways involved with persistent pain, these results indicate that the degree of initial mechanical injury plays an important role in modulating one aspect of the spinal cascade following injury. Immunohistochemical techniques used in this study are limited since they provide only qualitative evaluation of spinal responses, yet they allow in situ examination of cellular responses in regions

of the spinal cord anatomically relevant to nociception. Nonetheless, continued research using more quantitative approaches for examining specific cellular responses, such as RT-PCR and Western Blot analysis, are necessary for mRNA and protein quantification of spinal nociceptive responses in the context of nerve root injury. While quantifying injury strains in individual animals is possible in this model, it is not pragmatic to relate these individual strains to individual glial scores as they may be confounded by many factors other than mechanics, such as genetics. However, the overall microglial responses reported here highlight the need for continued work to decipher the specific role of mechanical deformation in painful radiculopathy and its relationship to the other physiological factors involved in nociception. Also, these findings suggest the need for determining those specific CNS cell populations and their activities which are sensitive and appropriate contributors to pain maintenance. Continued work defining thresholds of mechanical injury leading to nociceptive behaviors and responses of immunologically relevant cell populations of the CNS is undoubtedly important for better understanding persistent pain.

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