

Nerve Root Compression Increases Spinal Astrocytic Vimentin in Parallel With Sustained Pain and Endothelial Vimentin in Association With Spinal Vascular Reestablishment

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Study Design. Temporal immunohistochemistry analysis of spinal cord tissue from a rat model of cervical radiculopathy.

Objective. The goal was to measure spinal endothelial and astrocytic vimentin expression after a painful nerve root compression to define spinal cellular expression of vimentin in the context of pain.

Summary of Background Data. The intermediate filament, vimentin, is expressed in a variety of cell types in the spinal cord and is modulated in response to neural pathologies. Early after nerve root compression spinal astrocytes become activated and blood-spinal cord barrier (BSCB) breakdown occurs in parallel with development of pain-related behaviors; these spinal responses remain activated as does the presence of pain. In addition to vimentin, glial fibrillary acidic protein (GFAP) expression is a hallmark of astrocyte activation. In contrast, vascular endothelial cells down-regulate vimentin expression in parallel with vascular breakdown. It is not known whether spinal astrocytes and endothelial cells modulate their expression of vimentin in response to a painful neural injury.

Methods. Mechanical hyperalgesia was measured and spinal cord tissue was harvested at days 1 and 7 after a unilateral nerve root compression in rats. Vimentin was coimmunolabeled with

GFAP to label astrocytes and von Willebrand factor (VWF) for endothelial cells in the spinal cord on the side of injury.

Results. Spinal astrocytic vimentin increases by day 7 after nerve root compression, corresponding to when mechanical hyperalgesia is maintained. Spinal endothelial vimentin increases as early as day 1 after a painful compression and is even more robust at day 7.

Conclusion. The delayed elevation in spinal astrocytic vimentin corresponding to sustained mechanical hyperalgesia supports its having a relationship with pain maintenance. Further, since BSCB integrity has been shown to be reestablished by day 7 after a painful compression, endothelial expressed vimentin may help to fortify spinal vasculature contributing to BSCB stability.

Key words: astrocyte, endothelial cell, nerve root, neuropathic pain, pain, radiculopathy, spinal, vimentin.

Level of Evidence: N/A

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Intermediate filaments, such as vimentin, perform many roles; they maintain cell shape, resist tensile loads, transmit mechanotransductive signals, and act as a substrate for phosphorylation reactions in the cytoplasm and stabilize connectivity among cells within a tissue.^{1–3} Intermediate filaments have a structure rich in α -helices, which facilitates a range of molecular unfolding events with applied stretch.⁴ This structural feature enables intermediate filaments to resist much higher tensile loads than other cytoskeletal filaments.^{1,3,4} One intermediate filament in particular, vimentin, greatly influences the mechanical integrity of the cell cytoplasm. Highlighting this, fibroblasts derived from vimentin deficient (*vim*^{-/-}) mice exhibit half the cytoplasmic stiffness of wild-type fibroblasts.¹ Vimentin is expressed by a variety of cells within the CNS including astrocytes and endothelial cells, and its expression is modulated by pathological insults.^{5–7} As neuronal function and dysfunction at least partially depends on the stiffness of the local environment,^{8–10} changes in the stiffness of astrocytes, which comprise much of the mechanical substrate for

neurons in the CNS, is hypothesized to facilitate aberrant signaling by neurons and other cells in the spinal cord that contribute to nociception.

Although the expression of intermediate filaments, including vimentin and glial fibrillary acidic protein (GFAP), correlates with astrocyte stiffness *in vitro*,⁶ increased astrocytic vimentin expression is more often associated with glial activation.^{7,11} Both vimentin and GFAP are upregulated in the spinal cord by day 7 after chronic constriction of the sciatic nerve and remain elevated for up to 28 days, which parallels persistent pain.¹² However, GFAP and vimentin expression in astrocytes does not always follow the same temporal profile. Ischemic injury induced in the middle cerebral artery increases the number of GFAP-positive and vimentin-positive cells in nearby cortical regions within 48 hours.¹³ However, only GFAP-positive cell populations remain elevated for 30 days, whereas the number of vimentin positive cells returns to normal by that time.¹³ After a nerve root compression, spinal GFAP is upregulated by day 1 when pain-like behaviors develop after that injury; spinal GFAP remains elevated for up to 7 days in parallel with pain-like behaviors.^{14,15} Although both GFAP and vimentin intermediate filaments are established markers of astrocyte activation, it is not known if spinal vimentin expression within astrocytes is associated with pain from neuropathic injury.

Vimentin is also an important cytoskeletal filament in endothelial cells and regulates the integrity of individual endothelial cells, and cell monolayers. Vimentin filament networks maintain cellular integrity when expressed as long stable filaments dispersed throughout the cytoplasm.¹⁴ Phosphorylation of vimentin filaments disassembles their intracellular organization and causes nuclear aggregation.¹⁶ That disruption in vimentin stability also corresponds to increased endothelial barrier permeability.^{5,17} A role for endothelial vimentin in vascular stability is further supported by *vim*^{-/-} mice exhibiting increased paracellular transmigration of blood mononuclear cells into the lymph nodes and spleen as an indicator of elevated vascular permeability.¹⁸ The permeability of the blood-spinal cord barrier (BSCB) transiently increases early after a painful nerve root compression and stabilizes by day 7,¹⁹ suggesting that microvascular endothelial function is compromised in response to peripheral neural compression. However, the structural changes that endothelial cells undergo to facilitate that breakdown and subsequent recovery are not fully understood. Modifications in endothelial vimentin expression over time might contribute to the changes in BSCB integrity that are induced by a painful nerve root compression injury.

A peripheral neuropathic injury, applied *via* transient compression of the nerve root, induces BSCB permeability, spinal astrocyte activation, and pain along different time courses.^{19–21} Compressing the nerve root for just 15 minutes induces a substantial breakdown in the BSCB, which occurs at the same time as the development of mechanical hyperalgesia by day 1.²¹ Because of the apparent roles of vimentin in both astrocytic activation and vascular

permeability,^{6,13,19,22–24} this study investigates whether modifications in spinal astrocytic and endothelial vimentin expression are associated with the development and/or maintenance of pain after a nerve root compression injury.

METHODS

Nerve Root Compression and Tissue Harvest Procedures

Surgical procedures were performed as previously described.^{14,15} Briefly, adult male Holtzman rats were anesthetized using inhalation isoflurane anesthesia and the right C6 and C7 vertebrae were cleared of the paraspinal muscle. The right C7 dorsal nerve root was then exposed *via* a C6/C7 hemilaminectomy and partial facetectomy. The nerve root was compressed for 15 minutes (injury, *n* = 10) with a 10 gf microvascular clip.^{14,15,19} Separate rats underwent the same surgical procedures, but did not receive a nerve root compression (sham, *n* = 10) to serve as a surgical control. Spinal cord tissue from the C7 spinal level was harvested from rats on day 1 (injury, *n* = 6; sham *n* = 4) or day 7 (injury, *n* = 4; sham *n* = 6) after surgery, fixed with 4% paraformaldehyde and cryosectioned for immunohistochemical processing and analysis.

Measuring Mechanical Hyperalgesia

Mechanical hyperalgesia is an increased response to a stimulus, which is normally painful.^{25–28} As the C7 nerve root contains afferent sensory neurons that innervate the plantar surface of the forepaw, sensitivity to a mechanical stimulus was measured in the forepaw ipsilateral to the nerve root compression. Mechanical hyperalgesia was measured to determine the mechanical threshold at which a rat responds to an applied mechanical stimulus; a decrease in threshold corresponds to an increase in pain-related behaviors. Briefly, rats were acclimated to the testing apparatus, which consisted of an elevated mesh-floored cage. Mechanical stimuli were applied to the forepaw ipsilateral to injury using a graded series of von Frey filaments of increasing strength (1.4, 2, 4, 6, 8, 10, 15, and 26 g) (Stoelting; Wood Dale, IL). The filament strength eliciting a response, such as shaking or licking of the paw, was recorded as the threshold.^{29–31} Each filament was applied five times and if a rat responded to two consecutive filaments, the lower strength filament was recorded as the paw withdrawal threshold. If no filament elicited a response, then the highest magnitude filament (26 g) was recorded as the threshold. On each testing day, the mechanical threshold was measured three times for each forepaw tested and averaged for each rat across the rounds.

Mechanical hyperalgesia was measured on day 0 (baseline) before surgery and on days 1 and 7 after the root compression or sham surgery. Rats from which tissue was harvested on day 1 only underwent behavioral testing on days 0 and 1. The difference in paw withdrawal threshold was determined between the injury and sham groups at day 1 and day 7 using a two-way ANOVA (group × day) with Tukey's test.

Spinal Cord Immunohistochemistry

Astrocytic and endothelial vimentin were measured by coimmunolabeling for vimentin with either GFAP, as a marker for astrocytes, or von Willebrand factor (VWF), to label endothelial cells, in the spinal cord at days 1 and 7 after a painful nerve root compression. Fixed spinal cord tissue was cryosectioned axially at 14 μm and mounted on slides. Spinal cord sections were incubated in a blocking solution consisting of 5% normal goat serum (Vector Laboratories; Burlingame, CA) with 0.3% Triton-X100 (Bio-Rad Laboratories; Hercules, CA) in PBS for 1 hour at room temperature. Slides were then incubated with goat-anti-vimentin raised in rabbit (1 $\mu\text{g}/\text{mL}$; Abcam; Cambridge, MA) and goat-anti-GFAP raised in mouse (1:750; Millipore; Billerica, MA) overnight at 4°C. Tissue sections were incubated in goat-anti-rabbit Alexa Fluor 488 and goat-anti-mouse Alexa Fluor 568 (1:1000; Life Technologies; Carlsbad, CA) for 2 hours at room temperature. Slides were washed with PBS and cover slipped with TRIS buffer (Electron Microscopy Sciences; Hatfield, PA) for imaging.

The ipsilateral dorsal horn was imaged at 20x for 2 to 6 spinal sections for each rat (day 1: injury, $n = 6$; sham $n = 4$, day 7: injury, $n = 4$; sham $n = 6$). Total spinal vimentin was quantified using densitometry methods^{15,32} to measure the percent of the tissue pixels positively labeled for vimentin. The percent of pixels labeling vimentin in tissue sections was then normalized to the percent labeling in spinal cord tissue from normal naïve rats ($n = 2$). Spinal astrocytic vimentin was measured in the same tissue sections by quantifying the percent of colocalized pixels positively labeled for both vimentin and GFAP.³³ The percent of colocalized vimentin and GFAP pixels was then compared with the percent of colocalized pixels in spinal tissue from normal rats.

To quantify endothelial vimentin, spinal cord sections were coimmunolabeled for vimentin with VWF. Slides were blocked in a solution consisting of 5% normal goat serum (Vector Laboratories; Burlingame, CA) with 0.3% Triton-X100 (Bio-Rad Laboratories) in PBS for 1 hour at room temperature and then incubated with goat-anti-vimentin raised in rabbit (1 $\mu\text{g}/\text{mL}$; Abcam) and goat-anti-VWF raised in mouse (1:75; Abcam) overnight at 4°C. Tissue sections were then incubated in goat-anti-rabbit Alexa Fluor 488 (1:1000; Life Technologies) and goat-anti-mouse Alexa Fluor 568 (1:500; Life Technologies) for 2 hours at room temperature. The ipsilateral dorsal horn was imaged at 20x in 2 to 6 spinal sections from each rat. Spinal endothelial vimentin expression was quantified by normalizing the percent of colocalized vimentin and VWF pixels to the percent of colocalized pixels in spinal tissue from naïve rats.³³

Statistical differences in the quantified percent vimentin, percent pixels for vimentin colocalized with GFAP, and percent pixels for vimentin colocalized with VWF in the spinal cord were compared between injury and sham at day 1 and day 7 using two-way ANOVAs (group \times day) with Tukey's honestly significant difference test.

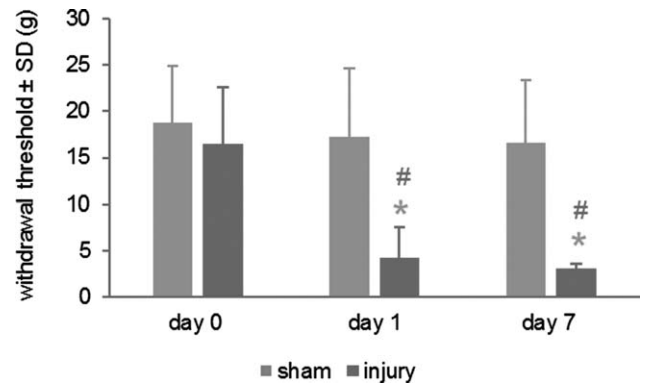


Figure 1. Transient root injury increases mechanical hyperalgesia. C7 nerve root compression significantly decreases the withdrawal threshold of the forepaw ipsilateral to injury by day 1 ($\#P = 0.004$), for at least 7 days ($\#P < 0.001$) compared with baseline (day 0) thresholds. Compression also induces a significant drop ($*P < 0.010$) in withdrawal threshold compared with sham on days 1 and 7. Data are represented as mean \pm standard deviation (SD).

RESULTS

The withdrawal threshold in the forepaw ipsilateral to injury significantly decreases ($P < 0.004$), corresponding to increased mechanical hyperalgesia, on days 1 and 7 after a nerve root compression injury compared with baseline (Figure 1). Nerve root injury also induces a significant decrease in the withdrawal threshold compared with sham overall ($P < 0.001$), and on each of days 1 and 7 ($P < 0.010$) (Figure 1).

Vimentin is expressed in the spinal cord of normal rats, and those undergoing a nerve root injury or sham operation (Figure 2). A nerve root compression injury induces a robust increase in total vimentin expression in the spinal cord ipsilateral to injury by day 7 (Figure 2). That injury-induced increase in spinal vimentin is significantly elevated ($P < 0.001$) over sham overall and on day 7 (Figure 2). At day 1, both a nerve root injury and sham operation increase spinal vimentin to approximately two-fold over normal expression levels.

Spinal astrocytic expression of vimentin is visually increased over normal levels for both groups at both days 1 and 7 (Figure 3). Vimentin expression in spinal astrocytes also increases by day 7 after a root compression over sham at that time point and either procedure at day 1 (Figure 3). Indeed, spinal astrocytic vimentin is significantly elevated after a compression injury compared with sham overall ($P = 0.001$) and on day 7 ($P = 0.036$) (Figure 3).

Spinal vimentin expression also localizes to vascular endothelial cells (Figure 4). Endothelial vimentin expression in the ipsilateral spinal cord is greater at day 1 and day 7 after injury compared with its expression in sham tissue at the same times and normal tissue. When quantified, spinal endothelial vimentin is significantly greater after injury than sham overall ($P < 0.001$) (Figure 4). Spinal endothelial vimentin is significantly elevated over sham levels by day 1 ($P = 0.030$) and that injury-induced increase is maintained until day 7 ($P = 0.036$) (Figure 4).

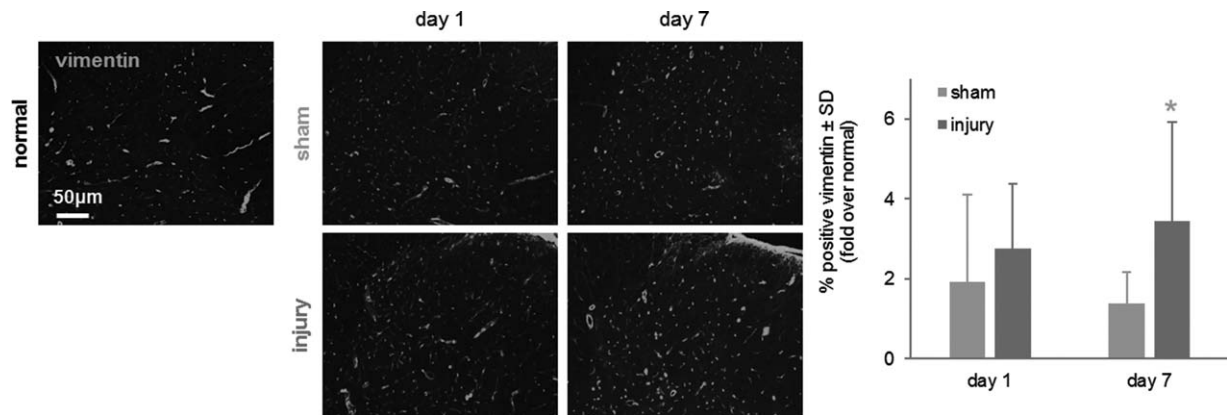


Figure 2. Root injury increases spinal vimentin. Vimentin labeling (green) is elevated in the ipsilateral spinal dorsal horn by day 1 relative to normal levels and is more robustly increased by day 7 compared with normal and sham levels at either time. The percent of spinal vimentin expression, normalized to normal expression, significantly increases after root injury compared with sham overall ($P < 0.001$) and on day 7 ($*P < 0.001$). Data are represented as mean \pm standard deviation (SD).

DISCUSSION

Spinal endothelial vimentin increases after mechanical trauma to the nerve root (Figure 4), paralleling the development and maintenance of mechanical hyperalgesia evident after a compressive nerve root injury (Figure 1).¹⁴ A transient nerve root compression induces mechanical hyperalgesia and mechanical allodynia by day 1, both of which are maintained for at least 7 days (Figure 1).^{15,19,29} A painful root compression also increases spinal astrocytic vimentin; however, that increase is not observed until day 7 (Figure 3), when pain persists (Figure 1).

Elevated spinal endothelial vimentin paralleling the development of pain-like behaviors after a nerve root compression (Figures 1, 4) suggests that endothelial vimentin may contribute to pain. The same painful injury used in this study induces BSCB breakdown early after injury, which is restored to normal permeability by day 7 after injury.¹⁹ Because increased endothelial vimentin expression has been associated with a strengthened endothelial barrier integrity *in vitro*,^{5,19} the compression-induced increase in endothelial vimentin that is even more robust at day 7 than day 1 might

contribute to restoring BSCB stability, which is evident at day 7 after painful compression.¹⁹ Although spinal endothelial vimentin is also upregulated after a compression at day 1 when the BSCB is disrupted,¹⁹ it may not control the increased vascular permeability at that early time point. Instead, endothelial tight junction disruption may contribute to the decreased integrity of the spinal vasculature at day 1 after a compression.^{34–36}

The fact that vimentin expression increases in both spinal endothelial cells and astrocytes after painful root compression suggests that spinal cells may respond by changing their mechanical properties after a neural injury, even when that trauma is remote from the spinal cord itself. Increases in astrocytic and endothelial vimentin independently correlate with increases in cytoplasmic stiffness.^{1,6} Astrocytes comprise a majority of the cellular makeup of the spinal cord and significantly contribute to the tensile stiffness of the spinal cord.^{37,38} The elevated astrocytic vimentin observed at day 7 (Figure 3) may contribute to an increase in the stiffness of the spinal cord after a painful nerve root compression. In contrast, compressive stiffness of the spinal cord decreases for

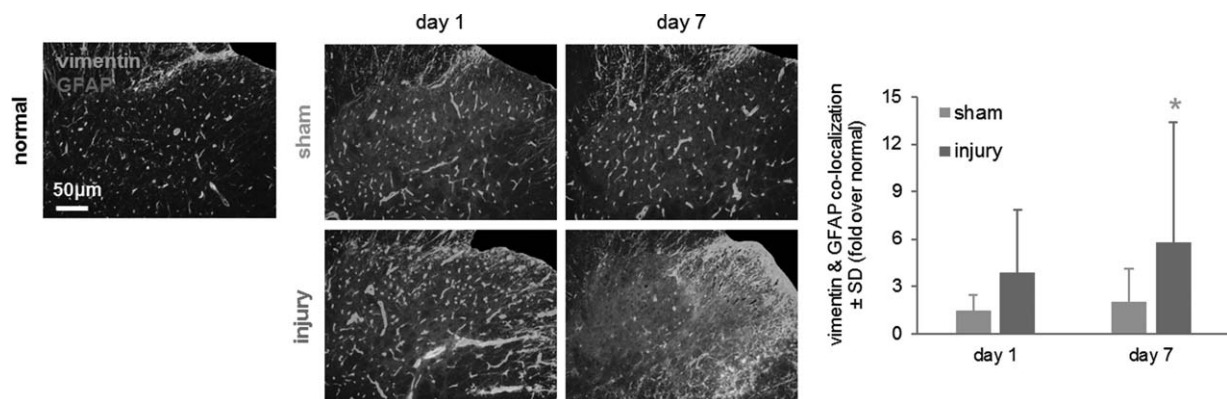


Figure 3. Spinal astrocytic vimentin expression is elevated after root compression. Spinal vimentin (green) labeling colocalizes (yellow) with GFAP (red) for all groups at all time points. The quantified colocalization of vimentin with GFAP labeling normalized to expression in tissue from normal rats is significantly increased compared with sham overall ($P = 0.001$) and on day 7 ($*P = 0.036$). Data are represented as mean \pm standard deviation (SD).

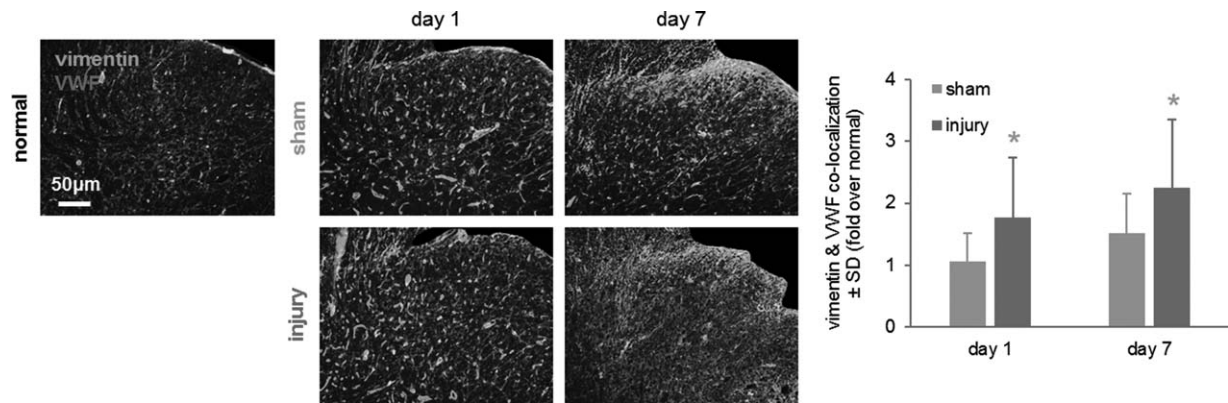


Figure 4. Spinal endothelial vimentin increases early and remains elevated after nerve root compression. Spinal vimentin (green) colocalizes with von Willebrand factor (VWF; green) and is shown (yellow) in the representative images. When quantified, normalized spinal endothelial vimentin expression is significantly elevated after root injury compared with a sham procedure overall ($P < 0.001$) and on days 1 and 7 ($*P < 0.036$). Data are represented as mean \pm standard deviation (SD).

up to 8 weeks after a hemisection injury.³⁹ A hemisection injury compromises the local cellular and extracellular integrity of the spinal cord itself and the lesion is repopulated by activated astrocytes, oligodendrocytes, immune cells, and connective tissue elements that collectively form a glial scar.^{40,41} However, the macroscale changes in mechanical integrity of the spinal parenchyma after spinal cord injury are robust and likely dominate any contributions from changes in the mechanical properties of the resident cells. As a nerve root compression is remote from the spinal cord and does not produce bulk structural changes to the spinal cord, any mechanical changes in the spinal parenchyma must be because of changes of the mechanical responses of the cells, or of the ECM proteins, in the spinal cord.

There are several limitations in the current study. Measuring vimentin expression alone does not provide information about the functionality of this intermediate filament. For example, the subcellular organization of vimentin, and the state of its phosphorylation, both contribute to its control over cytoplasmic stiffness.^{4,18,42} However, as the mechanical properties of the spinal parenchyma were not measured directly, the hypothesis that changes in spinal vimentin expression modulated spinal parenchymal stiffness was not tested. Microscopic techniques to measure compressive stiffness, such as microindentation or atomic force microscopy (AFM), allow measurement of regional mechanical properties of biological tissues.^{43,44} Measuring the micromechanics of the spinal cord in regions that exhibit increased vimentin at day 7 after painful nerve root compression (Figure 2), would determine whether the spinal parenchyma undergoes stiffness changes that correspond to changes in cell mechanics. It is important to note that the studies presented here only begin to investigate the complex responses of spinal vimentin in the context of a neuropathic injury. Cause and effect relationships and the specific upstream and downstream cellular pathways that are involved in vimentin modifications are necessary to define before the full influence of vimentin on pain is elucidated. Moreover, although the findings of this study point to an

increase in spinal vimentin after painful neural trauma, they may be associated with the repair of the BSCB rather than modulating pain. Additional studies looking at these outcomes and at later time points will particularly add utility in the context of translating these findings to human patients.

Nevertheless, this is the first study to investigate the response of the intermediate filament, vimentin, within spinal astrocytes and endothelial cells after a peripheral neural injury. Astrocytic vimentin undergoes a delayed elevation at day 7 after a painful nerve root compression (Figure 3), which occurs along the same temporal profile as spinal GFAP elevation^{14,15} and the production of mechanical hyperalgesia (Figure 1). Endothelial vimentin also increases in the spinal cord at day 7 after a nerve root compression (Figure 4). The increase in endothelial vimentin at day 7 after painful injury corresponds with the delayed recovery of the BSCB stability,¹⁹ suggesting that an increase in endothelial vimentin might even be protective after neuropathic injury.

➤ Key Points

- ❑ Spinal vimentin exhibits a delayed upregulation following a painful nerve root compression.
- ❑ Astrocytic expression of vimentin in the spinal cord is elevated at day 7 after root compression when pain-like behaviors are maintained.
- ❑ Spinal endothelial vimentin expression parallels the time course of behavioral hypersensitivity after a nerve root compression.

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