

The Role of Graded Nerve Root Compression on Axonal Damage, Neuropeptide Changes, and Pain-Related Behaviors

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ABSTRACT – Rapid neck motions can load cervical nerve roots and produce persistent pain. This study investigated the cellular basis of radicular pain and mechanical implications of tissue loading rate. A range of peak loads was applied in an *in vivo* rat model of dorsal root compression, and mechanical allodynia (i.e. pain) was measured. Axonal damage and nociceptive mediators were assessed in the axons and cell bodies of compressed dorsal roots in separate groups of rats at days 1 and 7 after injury. In the day 7 group, damage in the compressed axons, evaluated by decreased heavy chain neurofilament immunoreactivity, was increased for compressions above a load of 34.08mN, which is similar to the load-threshold for producing persistent pain in that model. Also, the neuropeptide substance P and glial cell line-derived neurotrophic factor and its receptor significantly decreased ($p < 0.02$) with increasing load in the small nociceptive neurons of the dorsal root ganglion, suggesting that axonal damage may also decrease neurotrophic support in injured nociceptive afferent fibers. In a separate study, roots were compressed at 2mm/s, and held, to develop a quasi-linear viscoelastic model that was validated through comparisons to quasistatic loading. The model demonstrated that nearly 23% less displacement was required to reach the axonal injury load threshold during dynamic loading than for quasistatic rates. Together, these studies demonstrate that nerve root compressions that produce pain symptoms are sufficient to mediate nociceptive cellular changes, and that thresholds for pain and nociceptive pathophysiology may be lower for dynamic loading scenarios.

KEYWORDS – nerve root; compression; biomechanics; injury; pain; neurotrophin, neuropeptide, axon

INTRODUCTION

Neck pain affects up to 71% of individuals in their lifetime (Côté et al. 1998, 2000), and the annual incidence among adults is between 14 and 50% (Côté et al. 2004; Fejer et al. 2005). As many as 45% of those cases are from whiplash injuries, resulting in large societal and economic costs reaching over \$29 billion annually (Freeman et al. 1999). Neck pain may arise from a variety of spinal tissues, including facet joints, ligaments, and surrounding muscles, (Luan et al. 2000; Sanderson 2002; Siegmund et al. 2001; Sundararajan et al. 2004; Wilmlink & Patijn 2001; Winkelstein et al. 2002; Yoganandan et al. 1998), but the cervical nerve roots can be at particular risk for injury due to foraminal impingement and/or increased hydrostatic pressure

during rapid head and neck motions (Aldman 1986; Bostrom et al. 1996; Eichberger et al. 2000; Ortengren et al. 1996; Nuckley et al. 2002). Coupling the mechanical risk for transient nerve root compression in the cervical spine with the known capacity of lumbar nerve roots to elicit low back pain following compressive traumas (Colburn et al. 1999; Hashizume et al. 2000; Kawakami et al. 2003; Tabo et al. 1999; Winkelstein et al. 2001), nerve root compression in the cervical spine is a potential mechanism for producing persistent pain in the neck following a variety of spinal loading conditions.

There is the potential for loading of the cervical nerve root during neck injury. The neural foramen is subject to shape changes and decreases in its diameter during extreme neck motions (Carter et al. 2000; Ebraheim et al. 2006; Krivickas & Wilbourn 2000; Torg et al. 2002; Yoo et al. 1992), which can compress the nerve root within the intervertebral foramen. Injurious, nonphysiologic neck loading may result from axial impacts which occur in recreational accidents and contact sports (Krivickas

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& Wilbourn 2000; Torg et al. 2002) or from neck retraction and extension during whiplash (Grauer et al. 1997; Ito et al. 2004; Kaneoka et al. 1999; Luan et al. 2000; Ono et al. 1997; Pearson et al. 2004; Yoganandan et al. 2002). Nuckley et al. (2004) reported a 20% decrease in area for the C4-C7 intervertebral foramina of cadaveric cervical spines in extension, and the foramen was reduced by nearly 40% during extension imposed after destabilizing lesions (Nuckley et al. 2004). Simulated 8G accelerations of isolated cadaveric cervical spines produce a narrowing of the C5-C6 intervertebral foramen by as much as up to 1.8mm, suggesting that the nerve roots and ganglia in the lower cervical spine may be at risk for compression due to foraminal narrowing in whiplash (Panjabi et al. 2006; Tominaga et al. 2006). Similarly, the spinal canal has been reported to be transiently occluded by as much as 75% during axial compression (Carter et al. 2000). While these cadaveric studies document bony motions for injury conditions in the cervical spine, they do not provide direct measures of neural tissue loading or address the physiologic consequences of such tissue loading.

Based on the results of *in vivo* porcine studies, it was hypothesized that rapid acceleration of the lower cervical spine during whiplash can increase pressure transients within the neural foramen (Aldman 1986; Bostrom et al. 1996; Eichberger et al. 2000; Ortengren et al. 1996; Svensson et al. 1993, 1998). In those studies, a head extension of 8.4Nm applied over 100ms produced transients of increased pressure in the spinal canal and intervertebral foraminal as large as 85mmHg (Aldman 1986; Bostrom et al. 1996; Ortengren et al. 1996; Svensson et al. 1993, 1998). Evans Blue Albumin staining in the dorsal root ganglia (DRG) suggested the pressure gradients produced increased membrane permeability in DRG neurons and neuronal dysfunction. For static neck extension, the cell membranes were not compromised, suggesting to those authors that a pressure pulse in the neural foramen greater than a dynamic pressure criterion of 60mmHg directly elicited neuronal dysfunction regardless of the extent of foraminal narrowing (Bostrom et al. 1996; Svensson et al. 1998). That neck injury criterion (NIC) accounted for the relative acceleration and velocity of the head and torso in order to estimate pressure for predicting neural injury (Bostrom et al. 1996; Eichberger et al. 2000; Svensson et al. 1998). While studies of foraminal occlusion indicate that motions beyond the normal physiologic range directly load nerve roots, the NIC suggests that injurious pressure transients in the neural foramen can also be produced by rapid whiplash motions near

the physiologic range. These studies, however, did not directly investigate the physiologic and symptomatic outcomes for direct loading to the cervical nerve root.

Several hallmarks of neuronal injury such as edema, membrane leakage, Wallerian degeneration, neuropeptide regulation, and decreased nerve conduction velocity are sensitive to the magnitude, duration, and rate of nerve root loading (Kobayashi et al. 1993, 2005a,b; Olmarker et al. 1989; Pedowitz et al. 1992). A study of transient compression of the porcine cauda equina applied pressures from 50-200mmHg for durations ranging from 2 minutes to 2 hours (Olmarker et al. 1989). While 50mmHg of pressure applied slowly and for only 2 minutes did not produce neuronal damage, rapidly applied pressure of 200mmHg for 2 hours was sufficient to produce endoneurial edema. However, edema was also produced after 50-100ms durations of pressures at that same magnitude (200mmHg), suggesting that pressure magnitude may dictate the establishment of neural damage but that loading duration may modulate the severity of physiologic injury. Kobayashi and colleagues applied compression to canine lumbar nerve roots using precalibrated microvascular clips for 1 hour and reported markedly increased edema after compression with a 147mN clip compared to that for the 74mN clip group (Kobayashi et al. 1993; Kobayashi & Yoshizawa 2002). That work suggested there may be a load threshold for producing endoneurial edema in loaded neurons, and further supported the hypothesis that transient nerve root compression load may directly modulate neuronal injury. However, none of these studies incorporated behavioral measures to relate the neuronal pathology to symptoms of persistent pain for nerve root tissue compression.

Nerve roots can also undergo tensile injury caused by nonphysiologic neck motions or disc herniation (Rydevik et al. 2001; Singh et al. 2006a). Singh et al. (2006b) reported accumulation of β -amyloid precursor protein (β APP) in axons after nerve root tension as a measure of injury and impaired axonal transport. The presence of β APP accumulations in the nerve root increased significantly with the strain rate of applied tension (Singh et al. 2006b). In that same study, axonal conduction velocity significantly decreased with applied strain and strain rate; 9% tensile strain produced complete axonal conduction loss for a loading rate of 15mm/s, whereas 16% strain was required to produce complete conduction loss for tension applied at 0.01mm/s. Based on the literature, in both tension and compression, axonal dysfunction and neuronal damage are significantly affected by the

magnitude and rate of loading, yet no study has investigated those indicators of neuronal injury and resulting pain behavioral symptoms.

Compression of nerves and nerve roots is strongly implicated in pain. Lumbar nerve root compression can occur from disc herniation or foraminal stenosis, and animal models demonstrate that sustained lumbar nerve and nerve root compression produce persistent pain (Bennett & Xie 1988; Colburn et al. 1999; Hashizume et al. 2000; Kim & Chung 1992; Rutkowski et al. 2002; Tabo et al. 1999), and depend on the compression mechanics (Winkelstein et al. 2002; Rutkowski et al. 2002; Winkelstein & DeLeo 2004). Studies of lumbar nerve root ligation in the rat using silk suture to apply variable radial compression indicate that increased compression by tighter ligation significantly increases mechanical allodynia (a painful response to stimulus that does not normally produce pain; see Appendix A for a glossary of terms) for at least 7 days (Winkelstein et al. 2002). Additional studies have defined a direct relationship between the magnitude of radial tissue compression and increases in allodynia, and have used those relationships to identify mechanical thresholds to produce acute and persistent allodynia following lumbar root ligation (Winkelstein et al. 2001, 2002; Winkelstein & DeLeo 2002, 2004). However, estimates of injury mechanics in those studies assumed that radial compression measured at the initial suturing were sustained over the entire post-operative period, limiting the capability to investigate precise injury mechanics or thresholds for non-sustained tissue insults.

More recently, the effect of transient loading magnitude on mechanical allodynia has been studied using a cervical nerve root compression model. Dorsal root compression for 15 minutes with a load of 98mN produces significantly elevated mechanical allodynia for 7 days (Hubbard & Winkelstein 2005; Hubbard et al. 2008a; Rothman et al. 2005). Dorsal root loading with magnitudes between 0 and 110mN produces mechanical allodynia only for larger loads, and a compressive load threshold for persistent mechanical allodynia has been defined at 38mN (Hubbard et al. 2008a). Although those studies determined relationships between root compression and mechanical allodynia, they did not investigate local cellular changes in the context of loading mechanics and mechanical allodynia, to define the pathways contributing to persistent radicular pain.

Following a painful injury, nociceptive neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) transmit nociceptive signals through

small myelinated (A δ) and unmyelinated (C) afferent nerve fibers. Those neuropeptides are specifically produced in the small neurons of the DRG for pain signaling and not in the large, mechanoreceptive neurons. SP and CGRP are transported to the superficial laminae (I and II) of the spinal dorsal horn where they are released into the synapse. The release of SP and CGRP in the dorsal horn of the spinal cord has been strongly implicated in both the onset and maintenance of pain (Allen et al. 1999; Aoki et al. 2005; Bennett et al. 2000a; Cavanaugh 2000; Cornefjord et al. 1995; Cridland & Henry 1989; Kawamura et al. 1989; Kobayashi et al. 2004b, 2005a,b; Levine et al. 1993; Ma & Eisenach 2003; Malcangio et al. 2000; Malmberg & Basbaum 1998; Meert et al. 2003; Millan 1999; Munglani et al. 1996; Nichols et al. 1999; Oku et al. 1987; Swamydas et al. 2004). C-fiber stimulation increases SP release from axon terminals in the superficial laminae (Malcangio et al. 2000), and allodynia is reduced following selective C-fiber ablation using SP saporin (Levine et al. 1993; Nichols et al. 1999). These findings imply that SP release is directly mediated by painful stimuli and has direct effects on pain symptoms. CGRP regulates nociception by promoting the release of SP in the superficial laminae of the spinal cord and by slowing the metabolism of SP in the DRG (Allen et al. 1999; Meert et al. 2003). Mechanical allodynia is produced by intrathecal CGRP administration and decreased following administration of a CGRP antagonist (Bennett et al. 2000a; Cridland & Henry 1989; Oku et al. 1987). These studies indicate a strong relationship between nociceptive signaling and the release of SP and CGRP by primary sensory neurons.

Despite a defined role of neuropeptides in nociception, the effect of neural injury on SP and CGRP expression in the DRG and spinal cord remains poorly defined. Sciatic nerve and lumbar nerve root injury decrease SP-immunoreactivity in the superficial laminae of the spinal dorsal horn (Allen et al. 1999; Malmberg & Basbaum 1998; Munglani et al. 1996). Dorsal horn SP-immunoreactivity also decreases in conjunction with decreased SP and CGRP in the small neurons of the DRG (Kobayashi et al. 2004b, 2005a,b). However, other studies report increased SP in the DRG after partial sciatic nerve injury (Ma & Bisby 1998); administration of a SP antagonist reduces allodynia (Cahill & Coderre 2002). Furthermore, SP and CGRP in the spinal dorsal horn are unchanged or increased one day after nerve root injury (Kobayashi et al. 2005a; Rothman et al. 2005). Taken together, these data suggest that changes in DRG and spinal SP- and CGRP-immunoreactivity following nerve

root compression involve a complicated temporal relationship between neuropeptide production, transport, and metabolism. Although injury to the nerve root may initially enhance production of these neuropeptides in the DRG, prolonged nociceptive transmission, in conjunction with progressive axonal degeneration, may also increase utilization, and decrease transport, of neuropeptides to the dorsal horn of the spinal cord (Allen et al. 1999; Cahill & Coderre, 2002; Kobayashi et al. 2005a,b; Schicho et al. 2005; Zheng et al. 2008). Despite numerous investigations of the temporal regulation of SP and CGRP in models of nerve and nerve root compression (Cahill & Coderre 2002; Jang et al. 2004; Kobayashi et al. 2005a,b; Rothman et al. 2005), the contributions of SP and CGRP to the onset and maintenance of pain after nerve root compression in the context of injury mechanics remains unclear. In addition, no study has specifically investigated the effect of nerve root compression magnitude on nociceptive neuropeptide expression in small DRG neurons.

Neurotrophic factors, such as nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor, are primarily expressed by neurons and glial cells to promote neuronal viability, differentiation, and regeneration (Boucher et al. 2000; Dong et al. 2005; Mendell et al. 1999; Ramer et al. 2000; Takahashi et al. 2003; Wilson-Gerwing & Verge 2006). While neural injury enhances the release of neurotrophins, which has been linked to increased SP and CGRP expression and pain sensitivity (Fukuoka et al. 2001; Mendell et al. 1999; Ruiz & Banos 2005; Skoff & Adler 2006; Zhou et al. 2000), glial cell line-derived neurotrophic factor (GDNF) decreases ectopic firing and enhances somatostatin expression which opposes the actions of SP (Boucher et al. 2000; Malcangio et al. 2002). This suggests a potential role for GDNF in pain modulation. After painful sciatic nerve compression, GDNF expression in the DRG has been demonstrated to decrease, thereby decreasing its availability to oppose the nociceptive action of SP release, leading to mechanical hyperalgesia in rats (Takahashi et al. 2003). Studies also show that continuous application of GDNF to the site of neural injury attenuates pain symptoms produced by neural compression, suggesting that preventing a loss of GDNF after neural injury is sufficient to prevent the establishment of persistent pain (Boucher et al. 2000; Hubbard et al. 2008b). While GDNF expression decreases in response to a variety of neural injury modalities, no studies have investigated GDNF expression in the DRG specifically in the context of injury mechanics to determine relationships between mechanical

parameters related to tissue injury, GDNF expression, and mechanical allodynia (i.e. pain).

Studies to determine the mechanical response of neural tissue have largely focused on tension, and often do not account for loading rate (Beel et al. 1984; Chang et al. 1988; Denny-Brown & Doherty 1945; Haftek 1970; Hoen & Brackett 1956; Liu et al. 1948; Okamoto 1955; Rydevik et al. 1990; Sunderland & Bradley 1961; Yoshimura et al. 1989). Studies of *in situ* peripheral nerve stretching report elastic or "pseudo-elastic" moduli ranging from 30KPa to 600KPa and histologic damage being produced for strains ranging from 6% in human cadaveric nerves to nearly 100% in cat sciatic nerves, depending on the strain rates at which the studies were performed (Denny-Brown & Doherty 1945; Hoen & Brackett 1956). This wide range of values highlights the potential influence of rate in affecting tissue responses. Viscoelastic studies of brain, spinal cord, and nerve tissue in tension report stress relaxation as much as 30% for small strains, with a non-linear toe region up to 11% strain (Chang et al. 1988; Kendall et al. 1979; Kwan et al. 1992; Miller & Chinzei 2002). Rat nerve roots also demonstrate viscoelasticity in tension, with a 2.4-fold increase in both maximum load and stress for loading at a rate of 15mm/s compared to the quasistatic rate of 0.01mm/s (Singh et al. 2006a). Those studies highlight the strain rate-dependent nature of neural soft tissues, and suggest that dynamic loading may increase the severity of injury for nerve root loading by increasing the maximum stress in the tissue.

Studies which investigate the compression response of brain tissue demonstrate its time-dependent mechanical properties (Estes & McElhaney 1970; Gefen & Margulies 2004; Miller & Chinzei 1997). Compressed brain tissue stiffens 10-fold for strain rates ranging from $0.64 \times 10^{-3} \% / s$ to $64 \% / s$ (Estes & McElhaney 1970; Miller & Chinzei 1997). Peak loads increase by 50% for *in vivo* indentation of porcine brain tissue from 1mm/s to 3mm/s (Gefen & Margulies 2004). Also in that study, load relaxed over 50% within 90 seconds for both displacement rates, indicating that neural tissue stiffening and relaxation contribute to the forces experienced during traumatic compression (Gefen & Margulies 2004). These studies emphasize the importance of defining the load response of the nerve root to dynamic compression in order to place the behavioral and physiological outcomes of nerve root compression in the context of traumatic injury.

In this study we hypothesize that cervical dorsal root compression elicits the onset and maintenance of

pain-related cellular changes in the axons and cell bodies of dorsal root neurons for specific mechanical loading scenarios. Assessment of mechanical allodynia (i.e. behavioral sensitivity) in rats provides context for the compression mechanics that produce pain symptoms. The effect of compression load on neuronal cellular responses, the load-dependent changes in axonal damage, nociceptive neuropeptide expression, and neurotrophic responses in the DRG are evaluated following root compression with a range of loads up to and including those previously shown to produce persistent pain (Hubbard et al. 2008a). We further evaluate the time-dependence of compressive nerve root loading by establishing a quasi-linear viscoelastic model for nerve root compression to predict the displacement necessary to achieve painful compression for loading rates relevant to those experienced during impact, and provide insight into high-rate loading scenarios necessary to produce nociceptive neuronal responses.

The methods and results of these studies represent new data and/or analyses of previously published experiments. In particular, the viscoelastic model study has not been previously reported; in addition, all assessment and analyses of neuropeptide and neurotrophic responses in the DRG samples have not been previously reported. However, these investigations do assess cellular responses in tissue samples from rats that have been previously reported (Hubbard et al. 2008a; Hubbard and Winkelstein 2008). We also present behavioral data on the day of tissue harvest (that is a subset of data previously reported; Hubbard et al. 2008a) to provide symptom context for this report. Novel analyses of axonal damage data are also presented through a refined method for threshold prediction using the peak load

applied to the nerve root, rather than the previously published steady state (or fully relaxed) load threshold (Hubbard and Winkelstein 2008). We report and analyze peak loads as they have more direct relevance to a dynamic injury. Together, these studies offer a more complete picture of axonal, DRG and behavioral consequences for painful nerve root compression that has not been previously reported.

METHODS

Two studies were performed in the rat to define relevant functional and nociceptive cellular outcomes for painful nerve root compression injury and to characterize nerve root tissue loading mechanics for different compression rates. In the first study, the right C7 cervical dorsal root was compressed at a quasistatic rate of 0.004mm/s to enable precise application of load and to match loading conditions that demonstrate a reliable relationship between applied load and behavioral hypersensitivity (Hubbard et al. 2008a). After compression, dorsal root and DRG tissue were separately analyzed by immunohistochemistry in two groups of rats: those with tissue harvested 1 day after injury (day 1 group rats) and those harvested 7 days after injury (day 7 group rats). Analyses defined acute and chronic load-based changes in peptides involved in axonal damage (neurofilament 200), nociception (substance P and calcitonin gene-related peptide), and neuronal survival and regeneration (glial cell line-derived neurotrophic factor and its receptor) (Figure 1). Recognizing that any physiologic outcomes from those studies utilizing quasistatic nerve root compression reflect cellular responses only for that mechanical injury paradigm, the viscoelastic response of the rat dorsal nerve root was defined in a

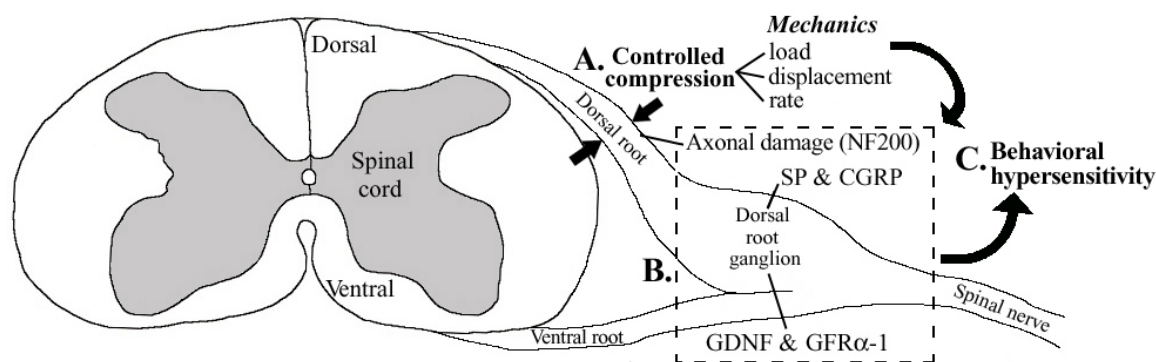


Figure 1. (A) Dorsal root compression at a quasistatic rate of displacement was applied for peak loads between 0 and 120mN. Compression load was investigated for its effects on (B) axonal damage, changes in nociceptive neuropeptides in DRG neurons, and the population of DRG neurons expressing GDNF and its receptor GFR α -1, as well as behavioral hypersensitivity (C). In the second study, (A) loading rate was altered to investigate the tissue mechanical responses and to develop a quasi-linear viscoelasticity model for predicting load responses for tissue displacement at other rates.

second study using dynamic loading. In that study, elastic and relaxation responses of lumbar roots were defined to create a quasi-linear viscoelastic (QLV) model to estimate nerve root tissue forces during dynamic loading. The predictive ability of that model was validated and used to predict mechanical tissue responses at dynamic rates and to place the load-based neuronal findings in the context of displacement rates comparable to traumatic injury. These two studies begin to define relationships between load magnitude, loading rate, and behavioral and cellular indicators of pain, and extrapolate such neuronal pathological outcomes to dynamic injuries.

All procedures were performed using male Holtzman rats (250-400g) (Harlan Sprague-Dawley, Indianapolis, IN) under inhalation anesthesia (4% for induction, 2% for maintenance). Rats were housed with a 12-12 hour light-dark cycle and free access to food and water, and all methods were approved by the Institutional Animal Care and Use Committee.

Load Magnitude & Neuronal Responses

Surgical Procedures & Behavioral Assessment. A C6/C7 hemilaminectomy and facetectomy exposed the right C7 nerve roots, and a customized loading device with microcompression platens (0.7mm width) was used to apply compression to the right C7 dorsal root proximal to the DRG (Figure 2). The loading device is equipped with a piezoelectric motor (resolution 0.5 μ m; Edo Ceramics, Salt Lake City, UT) mounted to a two-axis rotational platform to precisely control displacement of one of the platens; the second platen remains stationary. A 500mN load cell (750mN overload capacity, 0.15mN resolution; Omega, Stamford, CT) is mounted to the platform and quantifies the load applied to the dorsal root by measuring the reaction load on the stationary platen. A non-resistant, single-axis linear voltage displacement transducer (LVDT; 5mm travel distance, 0.25% sensitivity; RDP, Pottstown, PA) is mounted to the platform in contact with the piezoelectric motor and measures the displacement of the moving compression platen, defined in this study as the applied compression of the root. A custom LabView (National Instruments, Austin, TX) virtual interface synchronizes and records the applied loads and displacements. Load cell and LVDT data are acquired at 10Hz. Digital video (Qimaging, Burnaby, British Columbia) acquired at 40Hz monitors tissue compression and is used to verify loading of the nerve root.

For this study, the C7 dorsal root was compressed at a rate of 0.004mm/s to different peak loads ranging

up to 120mN (n=28) (Table 1). Compression was held for 15 minutes and then released. Sham procedures were separately performed with the same surgical technique but without applied tissue compression (n=8); for sham studies, peak load was taken as 0mN. After surgery, wounds were closed using 3-0 polyester suture and surgical staples. Rats were recovered in room air and monitored throughout recovery.

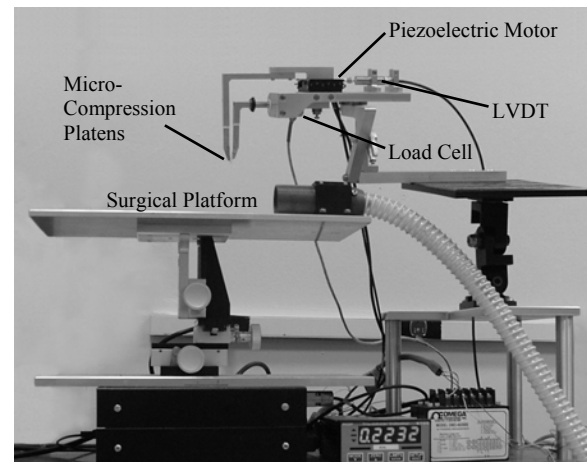


Figure 2. Nerve root compression device. The device consists of two compression platens, a piezoelectric motor, a load cell, and a linear voltage displacement transducer (LVDT), that are mounted atop a 5 degree-of-freedom rotational stage. Dorsal root compression is produced by lowering the compression platens into a dorsal surgical exposure of either the cervical or lumbar spine.

Bilateral forepaw mechanical allodynia was assessed prior to compression (pre-operative baseline) and on the day of tissue harvest for each group of rats (day 1 or day 7), to define behavioral changes both local and remote from the injured side using a frequency-based testing method (Bourquin et al. 2006; Hubbard et al. 2005; Kirita et al. 2007; Rothman et al. 2005; Song et al. 1999). For each testing session, 30 stimulations were applied to each forepaw of the rats using a 4g von Frey filament (Stoelting, Wood Dale, IL). A single tester, blinded to the injury and treatment of each rat, performed all allodynia testing. The total number of paw withdrawals on each day was recorded with respect to the applied dorsal root load. Separate linear regressions were fit between compressive load and mechanical allodynia responses for the day 1 and day 7 responses, to determine load-based changes in the number of paw withdrawal responses at each time point. The correlation coefficient (r) for each linear regression was determined and taken as significant for $p < 0.05$.

Tissue Assessments. The right C7 dorsal root and DRG were harvested *en bloc* from rats on day 1 (n=12) or day 7 (n=16) and prepared for immunohistochemical labeling. Dorsal roots were immunostained against heavy chain neurofilament (NF200) as a marker of axonal damage resulting from platen compression. DRGs were immunostained against the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) and the neurotrophic factor glial cell line-derived neurotrophic factor (GDNF) and its receptor GFR α -1; these assessments were performed to define changes in the expression of nociceptive neuropeptides and a peptide previously shown to increase neuronal viability and decrease ectopic firing in a rat model of chronic nerve compression (Boucher et al. 2000). All primary and secondary antibody dilutions were previously optimized, and exposure to 3,3-diaminobenzidine was used for color development of all immunostaining.

NF200 in the Dorsal Root. On the day of tissue harvest, an overdose of sodium pentobarbital (40mg/kg) was delivered intraperitoneally and rats were transcardially perfused with 150ml of phosphate buffered saline (PBS) and 150ml of 4% paraformaldehyde in PBS. The compressed C7 dorsal roots were harvested from separate groups of rats at day 1 and day 7 after compression and paraffin embedded. C7 nerve root tissue from sham operated rats was also harvested on the ipsilateral side to provide comparison tissue for the day 1 (n=4) and day 7 (n=4) group rats. Three longitudinal sections (10 μ m) were taken from the middle of each nerve root sample and were collected on gelatin-coated slides. Sections were immunolabeled for heavy chain neurofilament using a monoclonal antibody against NF200 (N52, 1:400; Sigma, St. Louis, MO) and a secondary horse anti-mouse antibody (Vector, Burlingame, CA) at a dilution of 1:1000.

Immunolabeled tissue sections were digitally imaged and analyzed for changes in NF200-immunoreactivity. Longitudinal sections were digitally imaged at 100X magnification, and dorsal roots were visually evaluated for the presence of axonal swellings (Chen et al. 1999) or an excessive decrease in fiber staining relative to uninjured nerve roots. Axonal swellings and decreased NF200-immunoreactivity were taken as indications of damage to large myelinated axons. For tissue sections that displayed those abnormalities in NF200, a grading of (+) was assigned to that nerve root. Sections not different from uncompressed roots were assigned a grading of (-). All evaluations were

performed blinded to the imposed compression magnitude.

Using this tissue grading for all compressed roots, a load threshold for axonal damage in the nerve root was defined by determining the applied load that had a greater than 50% probability of producing changes in NF200 immunostaining. To determine the load at which the probability of altered NF200-immunoreactivity surpassed the 50th percentile, load was plotted against the binary outcomes of (+) or (-) and fit using the "turnbull.r" function, a non-parametric empirical distribution function created in the open-source statistical platform R (version 2.7.0, The R Foundation for Statistical Computing, www.r-project.org). Separate load thresholds were determined for producing changes for the day 1 and day 7 group rats. The Turnbull function calculates a distribution of left and right censored data to determine the probability of decreased NF200-immunoreactivity with the application of increasing peak loads (Di Domenico & Nusholtz 2005; Turnbull, 1976). For an applied load that did not produce NF200-immunoreactivity different from that in uncompressed roots (-), the data point was defined as right censored with a probability of 0 on the y-axis. Right censored data indicated that a change in NF200-immunoreactivity would have occurred at some load greater than the load applied to that root. For a dorsal root assigned a grade of (+), the data were defined as left censored with a probability of 1 on the y-axis. Left censored data indicated that changes in NF200-immunoreactivity occurred as a result of some load less than or equal to the load applied to that root. Since the actual load at which axonal damage occurred was unknown at the time injury, all data points included in the analysis were treated as censored data. The load at which the probability for changes in NF200-immunoreactivity crossed 0.5 was taken as the 50th-percentile load for producing axonal pathology in the nerve root.

Nociceptive Neuropeptides in the DRG. Changes in the number of DRG neurons positive for the neuropeptides substance P and calcitonin gene-related peptide were assessed for the day 1 and day 7 group rats as a percentage of the total neuron population in the DRGs ipsilateral and contralateral to the side of compression. Neuron size was not characterized for these assessments, as SP and CGRP were expressed exclusively in small, nociceptive neurons. DRG tissue was harvested from the left and right C7 levels on either day 1 or 7, paraffin embedded, and sectioned longitudinally (10 μ m) onto gelatin-coated slides. Sections were immunolabeled for nociceptive neuropeptides by

immunohistochemistry using polyclonal antibodies against either SP (1:1000; Chemicon, Temecula, CA) or CGRP (1:2000; Bachem, San Carlos, CA). Donkey anti-rabbit (1:1250; Chemicon, Temecula, CA) or goat anti-rabbit (1:1000; Vector, Burlingame, CA) secondary antibodies were applied for SP or CGRP immunostaining, respectively. To identify changes in the number of neurons labeled for SP or CGRP, one representative section from each rat was digitally imaged at 100X magnification. From those images, the number of neurons positive for each of the SP or CGRP neuropeptides was counted and reported as a percentage of the number of total neurons in each image. In order to relate nociceptive responses to the peak load applied to the dorsal root, the percent of positive immunoreactive neurons for each neuropeptide was plotted against the corresponding applied peak load, with sham procedures corresponding to an applied load of 0mN. The data were fit by linear regressions on each day to determine the relationships between applied load and SP- or CGRP-immunoreactivity. Correlation coefficients were determined for each regression, and significance was defined at $p < 0.05$.

GDNF & GFR α -1 in the DRG. GDNF and GFR α -1 were assessed in the DRG after dorsal root compression at either day 1 or 7 and evaluated for the number and size of DRG neurons identified as positive for these peptides. Accordingly, the percentage of small and large DRG neurons expressing GDNF and GFR α -1 was evaluated for each time point to identify the relative expression of GDNF and its receptor in nociceptive (small) or mechanoreceptive (large) neurons. Longitudinal DRG sections (10 μ m) from the nerve roots harvested as described in the previous section were separately immunolabeled using polyclonal antibodies against GDNF (1:100; Santa Cruz, CA) and GFR α -1 (1:100; Neuromics, Bloomington, MN). Goat anti-rabbit or horse anti-goat secondary antibodies (Vector, Burlingame, CA) were used at a dilution of 1:200 for GDNF or GFR α -1 immunolabeling, respectively. For detailed analysis of changes in expression for specific neuron size populations, representative GDNF and GFR α -1 immunostained DRG sections from each rat were digitally imaged at 200X magnification. Neuron size was defined by cross sectional area of each cell body: small, nociceptive neurons were defined with cross sectional areas less than 600 μ m², and large neurons were taken as mechanoreceptive for cross sectional areas greater than 600 μ m² (Tachihara et al. 2007). The number of small and large neurons positive for each of GDNF or GFR α -1 was determined as a percentage of the

number of small or large neurons in the image. The percent of positive immunoreactive small or large neurons was linearly regressed against peak load to determine the relationships between load and GDNF- or GFR α -1-immunoreactivity in the cell bodies of the compressed axons.

Characterization of Nerve Root Tissue Viscoelasticity

To define the effect of displacement rate on dorsal root compressive loads, the same customized loading device as described above (Figure 2) was used to apply dynamic compression to lumbar nerve roots of male Holtzman rats (n=8). The lumbar nerve root was used for this study because of its size and ease of exposure in the rat, allowing for more reliable testing. Based on pilot studies, the mechanical properties of rat lumbar and cervical nerve roots in compression were assumed to be similar. The microcompression platens applied load to the lumbar nerve roots on the right or left side just proximal to the DRG. Based on the similar anatomical sizes of the L4 and L6 nerve roots, only roots at these two lumbar levels were compressed.

The nerve root was compressed 0.6mm; this amount of displacement was determined in pilot studies to provide adequate resolution to reliably and sensitively capture the relaxation response. It was also determined that nerve root relaxation reaches steady state within approximately 5 minutes of peak loading. As such, the protocol for relaxation tests included a 0.6mm nerve root compression applied at a rate of 2mm/s and held for 6 minutes. This rate of compression had a typical rise time of approximately 300ms. Both load and displacement data were acquired at 180Hz using the experimental and data acquisition systems described above. Compression of the root was verified by digital video recorded at 40Hz for the duration of testing.

Load and displacement data acquired over the compression tests were filtered by a 41st order generalized Butterworth filter with a cutoff frequency of 9Hz. A quasi-linear viscoelastic (QLV) model was used to fit the load and displacement data for each of the lumbar nerve roots. The QLV model used in this study expressed load as a convolution of a time-dependent relaxation component and a displacement-dependent elastic component (equation 1) (Abramowitch & Woo 2004; Best et al. 1994; Fung 1972):

$$F(t) = \int_0^t G(t-\tau) \frac{\partial F^e(\delta)}{\partial \delta} \frac{\partial \delta}{\partial \tau} d\tau \quad (1)$$

The relaxation function, $G(t)$, and elastic function, $F^e(\delta)$, were expressed as:

$$G(t) = \frac{1 + C[E_1(t/\tau_2) - E_1(t/\tau_1)]}{1 + C \ln(\tau_2/\tau_1)} \quad (2)$$

$$F^e(\delta) = A(e^{B\delta} - 1) \quad (3)$$

where E_1 is the exponential integral function, and A , B , C , τ_1 , and τ_2 are the QLV model parameters describing the shape of the elastic (A , B) and relaxation (C , τ_1 , τ_2) responses. To determine the instantaneous loading rate ($\partial\delta/\partial\tau$), the displacement-time data were differentiated using a centered finite difference approximation in Matlab (Mathworks, Natick, MA). Using a Levenberg-Marquardt least-squares algorithm and customized code in Matlab, the convolved instantaneous elastic, F^e , and the relaxation, $G(t)$, functions were simultaneously fit to the experimental data for each compression test, with the data weighted logarithmically (Abramowitch & Woo 2004). The goodness-of-fit for each relaxation test was assessed by computing the r^2 value of each curve fit.

To define average model parameters, a geometric mean was calculated for each of the parameters to account for any non-normal distributions in the dataset and nonlinear relationships between parameters. To assess the predictive ability of the QLV model, additional lumbar nerve roots ($n=3$) were compressed at a constant rate of 0.004mm/s to displacements of 0.6mm, and load and displacement were recorded as described above. The mean experimental force-displacement response for compression at 0.004mm/s was compared to the force-displacement response predicted by the QLV model for compression applied at 0.004mm/s. For every displacement value, the force-displacement response predicted by the QLV model was evaluated relative to the mean experimental force response. The lumbar roots compressed at a rate of 0.004mm/s used for the model validation had not undergone prior compression in order to prevent any effects of tissue damage from previous loading.

RESULTS

Load Magnitude & Behavioral Responses

For both the day 1 and day 7 group rats, the amount of mechanical allodynia increased with the peak load applied to the dorsal root. The number of paw withdrawal responses measured at day 1 (2.5 ± 1.3 paw withdrawals) following sham procedures was not significantly greater than the pre-operative baseline responses (1.5 ± 1.3 paw withdrawals) ($p > 0.21$) for those rats; similarly, the number of paw withdrawals at day 7 following sham procedures (1.0 ± 0.8 withdrawals) was also not greater ($p > 0.5$) than the corresponding pre-operative responses (1.0 ± 0.0 paw withdrawals). These findings indicate that pain symptoms were not produced by the surgical exposure of nerve roots. As early as one day after dorsal root compression, the number of paw withdrawals elicited in the ipsilateral forepaw was significantly linearly correlated with peak compression load ($p < 0.02$) (Table 1, Figure 3). The same relationship between load and allodynia was preserved over time. Mechanical allodynia on day 7 also increased with applied load, and the linear correlation between load and paw withdrawals was also significant at this time point ($p < 0.003$) (Table 1, Figure 3). However, contralateral allodynia in the left forepaw at day 1 did not vary with the magnitude of applied compression load (Table 1). By day 7, the number of paw withdrawals elicited in the contralateral forepaw significantly increased with load applied to the dorsal root on the right side ($p < 0.002$) (Table 1).

Load Magnitude & Neuronal Responses

Load Threshold for Altered NF200-Immunoreactivity. No differences in axonal staining were observed at either time point in nerve roots after sham procedures compared to normal staining. NF200 immunostaining in the normal, naïve C7 dorsal root demonstrated long, myelinated axons with an even distribution of staining along their lengths (Figure 4A). There were no changes in NF200-immunoreactivity in the dorsal root at day 1 for compressed roots relative to sham responses. As such, an empirical distribution of decreased NF200-immunoreactivity could not determine a 50th-percentile load value (data not shown). However, at day 7 after compression, decreased neurofilament immunoreactivity and the presence of axonal swellings were observed in the ipsilateral dorsal root with the probability of a pathologic grade increasing with the magnitude of applied load (Figures 4 & 5). On day 7, compression by loads less than 35mN

generally produced only minimal decreases in NF200-immunoreactivity in the compressed region of roots (Figure 4B). However, compressions above 70mN produced a dramatic decrease in NF200-Immunoreactivity that was consistently apparent along the full length of the dorsal root (Figure 4C). In those cases, axons formed swellings both proximal and distal to the site of compression (Figure 4C); these swellings were not observed in the dorsal roots compressed by lower loads. The 50th-percentile load for decreased NF200-immunoreactivity was identified at 34.08mN (Figure 5).

Table 1. Summary of Applied Nerve Root Loads and Mechanical Allodynia

	Rat ID	Peak Load (mN)	Paw Withdrawals (count)	
			Ipsilateral	Contralateral
	230	sham	3	2
	231	sham	2	3
	232	sham	1	1
	233	sham	4	2
	234	13.73	0	1
	235	13.73	4	0
	236	18.54	2	0
	237	20.70	3	2
Day 1	263	50.33	6	2
	262	51.80	9	1
	261	53.96	3	1
	264	55.33	5	1
	248	92.90	7	0
	238	98.98	4	1
	239	99.87	7	3
	240	101.93	4	2
	242	sham	2	2
	243	sham	1	3
244	sham	0	0	
245	sham	1	0	
Day 7	220	14.24	2	1
	219	16.88	3	2
	221	19.82	4	0
	217	26.87	2	1
	195	31.20	1	0
	218	34.08	1	1
	200	61.41	3	2
	214	71.26	5	3
	199	74.97	4	4
	196	88.28	3	3
	197	90.64	4	0
	194	98.79	2	3
	216	101.61	1	4
	215	105.19	5	9
	212	106.07	4	3
213	108.75	6	7	
246	normal	1	0	
247	normal	0	1	

Nociceptive Neuropeptide Expression in the DRG. For all DRG sections analyzed, there was no significant change in the total number of neurons at any time point or for any magnitude of dorsal root compression. On average, the DRG contained 270±60 neurons, and neuronal cross sectional area was not a factor in the assessment of positive immunolabeling.

CGRP-immunoreactivity in both the ipsilateral and contralateral DRGs did not significantly increase or decrease with increased load on either day 1 or 7 (data not shown). In normal rats, 13.0±4.4% of the ipsilateral DRG neurons were positive for CGRP staining; after sham procedures 11.7±1.1% and 16.2±3.2% of neurons in the ipsilateral DRG were CGRP-positive for day 1 and 7 group rats, respectively. For compression by loads above the threshold determined for axonal damage (34.08mN), CGRP-immunoreactivity for day 1 and 7 group rats remained at 15.7±1.8% and 16.8±7.0% of DRG neurons, respectively. No significant change in CGRP-immunoreactivity following painful loading was observed between day 1 and day 7 groups.

SP-immunoreactivity was significantly decreased in both the ipsilateral and contralateral DRGs in the day 7 group, but not the day 1 group, after dorsal root compression. SP-immunoreactivity in the ipsilateral DRG of the day 1 group did not decrease significantly as a function of dorsal root compression load (Figure 6A). On day 7, the average percentage of neurons positive for SP staining decreased relative to sham for applied loads that were greater than 40mN (Figure 6B). SP-immunoreactivity in the ipsilateral DRG in the day 7 group was significantly negatively correlated with applied load ($p<0.005$). In addition, considering all applied loads together, there was also a significant decrease in SP-immunoreactivity between day 1 group and day 7 group rats ($p<0.01$). In the contralateral DRG containing the neuron cell bodies of uncompressed neurons, SP-immunoreactivity was not significantly altered at either time point for sham procedures. Dorsal root loading produced a slight, but insignificant, decrease in contralateral SP-immunoreactivity in the day 1 group (Figure 6C). For the day 7 group, SP-immunoreactivity in the contralateral DRG decreased significantly ($p<0.004$) with increasing magnitude of applied load to the opposing C7 dorsal root ($p<0.004$) (Figure 6D). A significant decrease in the percentage of SP-positive neurons in the contralateral DRG was also determined between day 1 and day 7 group rats for all compression loads ($p<0.03$).

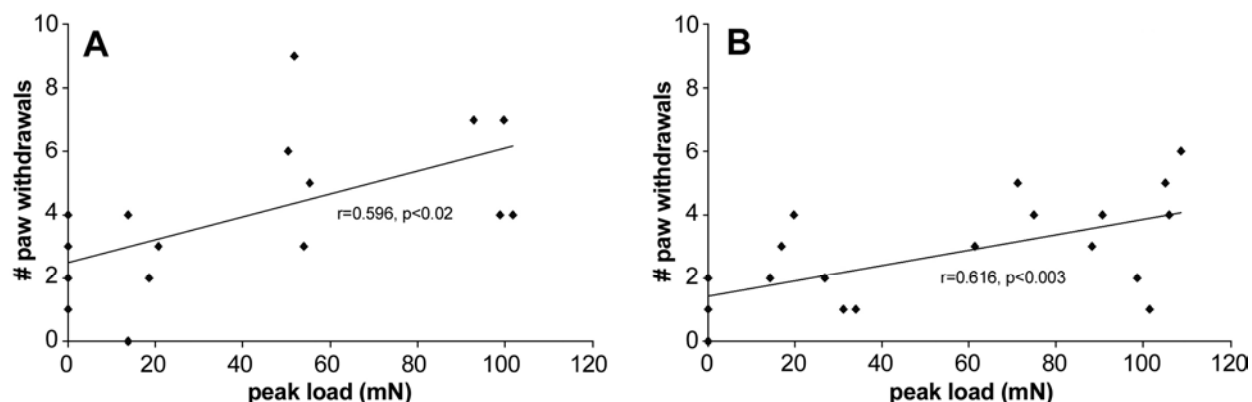


Figure 3. Ipsilateral mechanical allodynia measured on days (A) 1 and (B) 7 using a 4g von Frey filament. Responses are reported as the number of forepaw withdrawals plotted against the applied peak compression of the dorsal root. Peak load and mechanical allodynia are significantly correlated on both days 1 ($r=0.596$, $p<0.02$) and 7 ($r=0.616$, $p<0.003$).

GDNF & GFR α -1 in the DRG. GDNF-immunoreactivity was observed in both small and large neurons of bilateral DRGs in all rats including shams, at both time points. The percentage of small and large neurons positive for GDNF did not differ between sham and naïve rats. Load did not significantly affect the total number of small or large neurons present in the DRGs at either time point, indicating that no overall cell loss was produced as a result of compression of the dorsal root. DRG sections contained an average of 153 ± 33 neurons with a cross sectional area less than $600\mu\text{m}^2$ and 128 ± 25 neurons with a cross sectional area greater

than $600\mu\text{m}^2$. GDNF-immunoreactivity in small neurons of the bilateral DRGs in the day 1 group significantly decreased with increasing compression load ($p<0.006$; Figure 7A & 7C). No significant correlation between GDNF-immunoreactivity and load was observed in large DRG neurons on either side for the day 1 group of rats. Also, for the day 7 group, GDNF-immunoreactivity in small neurons of both DRGs returned to normal, such that the percentage of GDNF-positive neurons did not significantly depend on applied dorsal root compression load (Figure 7B & 7D).

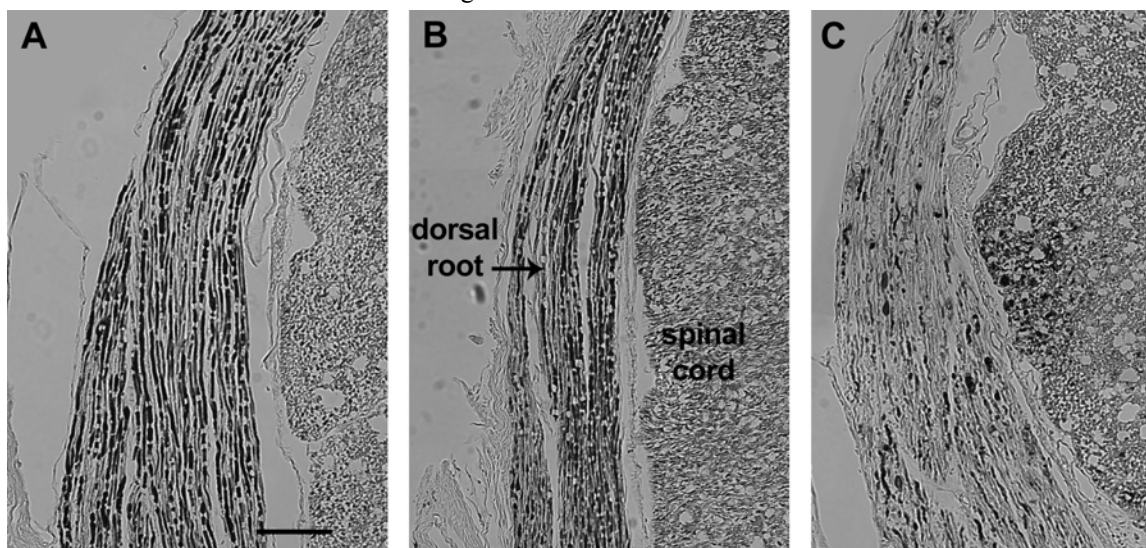


Figure 4. Representative images within the compressed region of the C7 dorsal root at day 7 immunostained against NF200. (A) Uncompressed dorsal root and (B) a root compressed with a load of 26.87mN, exhibit long, unbroken axonal staining. (C) A dorsal root compressed by a load of 74.97mN, exhibits substantial decreases in NF200-immunoreactivity and the presence of axonal swellings. Scale bar in (A) represents $100\mu\text{m}$ and applies to all.

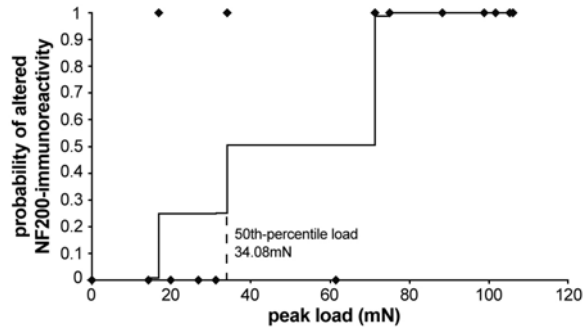


Figure 5. Non-parametric, empirical distribution function for NF200- in the context of peak dorsal root compression load. The load at which the probability surpasses 0.5 for altered NF200-immunoreactivity is indicated by a dashed vertical line.

GFR α -1-immunoreactivity in the ipsilateral DRG was significantly ($p < 0.02$) correlated with load only for the day 7 group (Figure 8). GFR α -1-immunoreactivity was not different from that observed in normal rats at either time point after sham procedures. In contrast to GDNF, GFR α -1-immunoreactivity in the ipsilateral and contralateral

DRGs was not significantly correlated with load in the day 1 group (data not shown). Yet, for the day 7 group, the percentage of GFR α -1-immunopositive small neurons in the ipsilateral DRG decreased with applied load ($p < 0.02$; Figure 8A). In that same group of rats, the percentage of large DRG neurons positive for GFR α -1 increased with load ($p < 0.02$; Figure 8B). This shift towards GFR α -1-immunoreactivity in large neurons of the ipsilateral DRG observed in the day 7 group was most pronounced in DRGs from roots that were compressed by loads above 60mN (Figure 9). No changes in GFR α -1 were detected in the contralateral DRG for either the day 1 or 7 groups (data not shown).

Characterization of Nerve Root Tissue Viscoelasticity

Lumbar nerve roots compressed at 2mm/s exhibited considerable force relaxation over time (Figure 10). The average peak nerve root displacement for all compressions was 0.651 ± 0.085 mm, and the average rate of displacement was 1.72 ± 0.09 mm/s (Table 2).

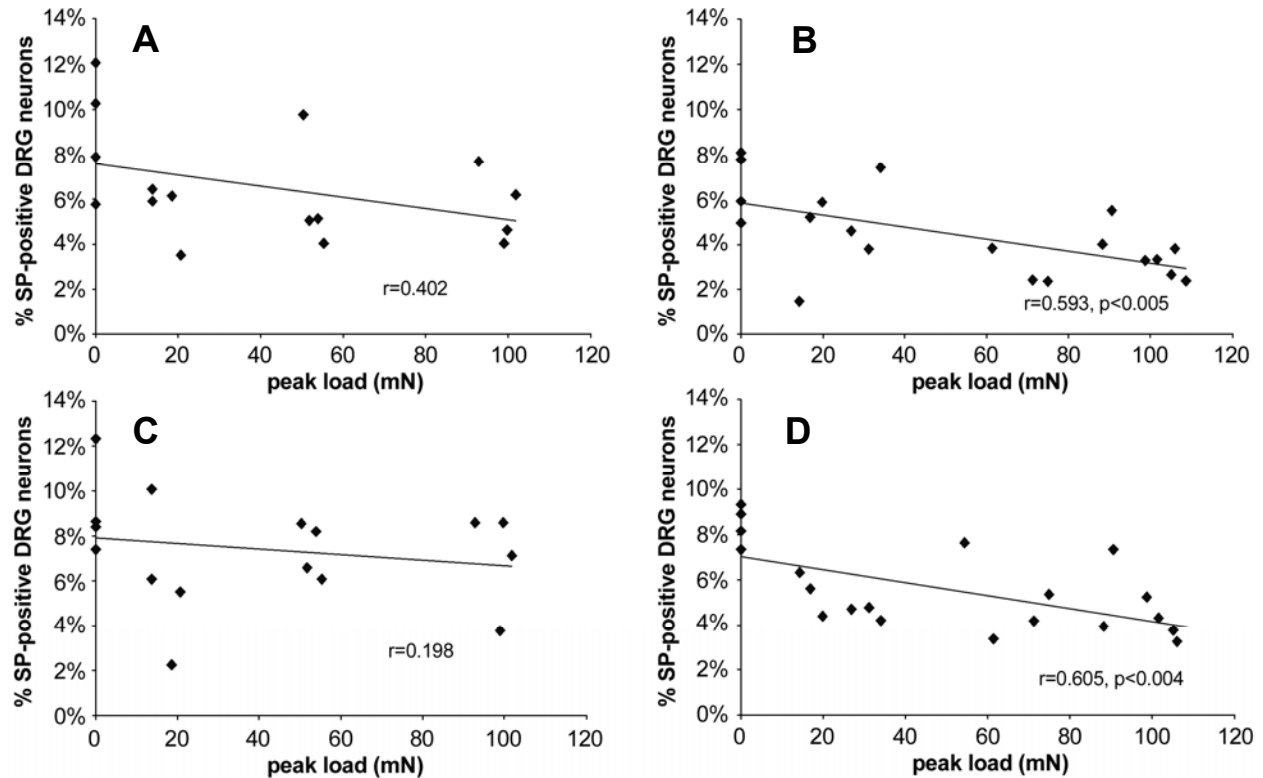


Figure 6. Percent SP-immunopositive neurons in the DRG (A,B) ipsilateral and (C,D) contralateral to the side of dorsal root compression on (A,C) day 1 and (B,D) day 7. There was no significant correlation between load and SP-immunoreactivity on day 1. However, on day 7, fewer neurons were positive for SP than on day 1 and SP-immunoreactivity significantly decreased with increasing load for both the ipsilateral ($r = 0.593$, $p < 0.005$) and contralateral ($r = 0.605$, $p < 0.004$) DRGs.

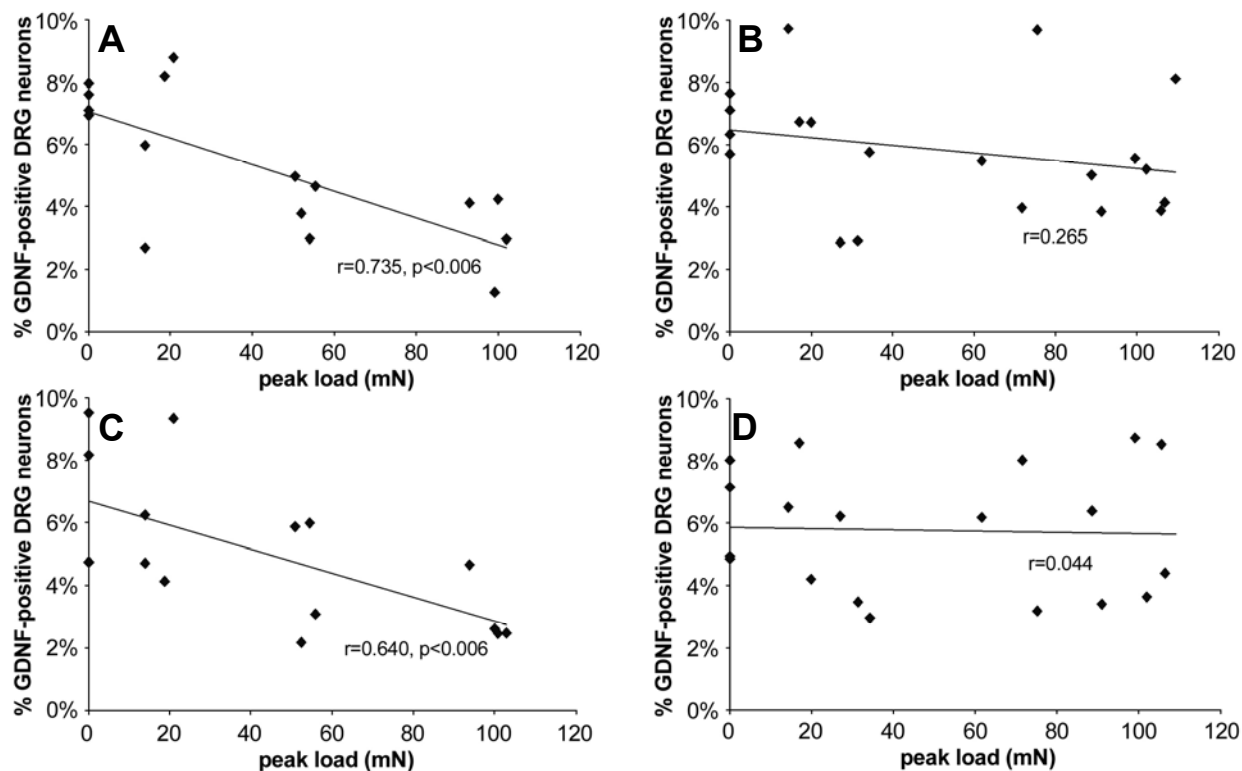


Figure 7. GDNF immunohistochemistry assessed at (A,C) day 1 and (B,D) day 7 in small neurons of the DRG (A,B) ipsilateral and (C,D) contralateral to dorsal root compression, shown as a function of peak compression load. GDNF-immunoreactivity in small neurons of the (A) ipsilateral and (C) contralateral DRG was significantly ($p<0.006$) correlated with peak load ($r=0.735, r=0.640$, respectively).

The mean time to reach peak load was 0.378 ± 0.052 seconds for these studies. The corresponding mean peak force for all tests was 177.95 ± 63.45 mN (Table 2). During the 6 minute hold time, load decreased rapidly from its initial peak load, reaching an average load of 46.17 ± 29.39 mN at 6 minutes.

Individual QLV model curve fits were well-fit ($r^2>0.999$) to the force-time data for each of the eight trials performed at 2mm/s (Figure 10). Table 3 summarizes the corresponding QLV model parameters, A, B, C, τ_1 , and τ_2 for each nerve root and their means. Because the five model parameters were not normally distributed, the geometric mean of each model parameter was used to assess the predictive capability of the model in the quasistatic experimental validation tests (Table 3). The QLV model constructed using the geometric mean parameters in Table 3 predicted the mean experimental force-displacement response within one standard deviation (Figure 11).

For the compression validation studies on 3 additional nerve roots targeting the average peak nerve root displacement from dynamic testing (0.651 ± 0.085 mm), the average displacement rate was

0.0045 ± 0.0005 mm/s to reach a final displacement of 0.643 ± 0.065 . Those quasistatic compressions produced a mean peak force of 102.26 ± 31.83 mN at that displacement. The QLV model prediction for quasistatic loading between 0 and 0.6 mm fell within one standard deviation of the average force response of the experimental validation tests (Figure 11). The peak load for quasistatic compression was considerably less than the comparable peak load for dynamic loading (177.95 ± 63.45 mN). The QLV model predicted a displacement of 0.555 mm to produce a load of 34.08 mN (the 50th percentile load for changes in NF200-immunoreactivity: see Figure 5) at 0.004 mm/s. That predicted displacement was considerably larger than the predicted displacement of 0.429 mm needed to produce that same load at a displacement rate of 2 mm/s.

DISCUSSION

These studies quantitatively define the effect of transient nerve root compression load on modulating nociceptive and neurotrophic cellular changes in the context of pain. Furthermore, dorsal root compression at a dynamic loading rate considerably increases the peak force generated across the nerve

root over those generated by slower compression. Together, these studies identify relationships between pain symptoms and the nerve root compression mechanics which contribute to symptomatic responses. Mechanical allodynia significantly increased with load for 7 days after dorsal root compression (Figure 3). Peak loads greater than 34.08mN decreased NF200-immunoreactivity (Figure 5). The decrease in NF200, taken as an indicator of axonal damage, likely contributed to persistent mechanical allodynia by reducing the transport of neuropeptides and neurotrophic factors between neurons in the DRG and the spinal cord. SP also decreased in DRG neurons with increasing applied peak load (Figure 6), consistent with observations in models of lumbar nerve root compression (Kobayashi et al. 2004b, 2005a). At the same time, GDNF expression in the small nociceptive neurons was reduced for large compression loads (Figure 7), which may have decreased the ability of that neurotrophic factor to reduce SP-mediated nociceptive signaling (Boucher et al. 2000; Malcangio et al. 2002), thereby maintaining the presence of pain. Large loads

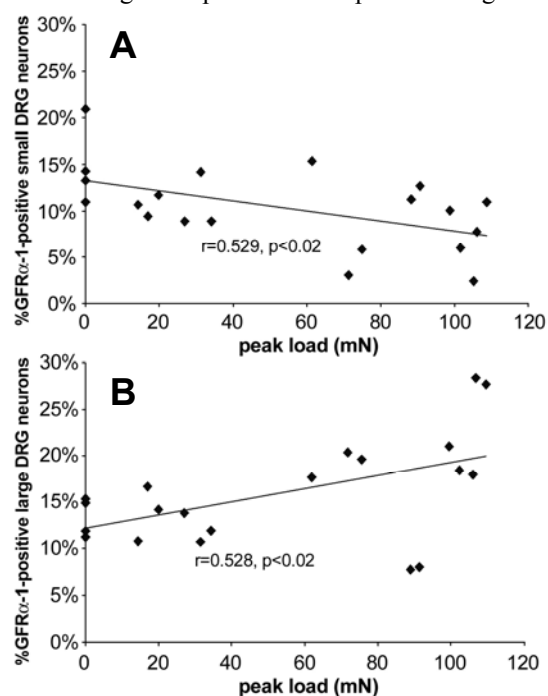


Figure 8. GFR α -1 immunohistochemistry assessed at day 7 in (A) small and (B) large neurons of the DRG on the side of dorsal root compression, as a function of peak compression load. (A) GFR α -1-immunoreactivity in small neurons significantly decreased with load ($r=0.529, p<0.02$), (B) while GFR α -1-immunoreactivity in large neurons of the ipsilateral DRG significantly increased with peak compression load ($r=0.528, p<0.02$).

reduced GDNF receptor expression in small DRG neurons at day 7 while increasing expression in large neurons for the same insult (Figure 9). These changes in neurotrophic support likely contribute to the maintained behavioral hypersensitivity observed in those rats (Figure 1). The cellular changes manifested in this injury model were significantly correlated with the applied peak load as was mechanical allodynia, suggesting a relationship between those cellular changes and painful dorsal root loading.

Nociceptive neuropeptide immunoreactivity decreased in DRG neurons 7 days after dorsal root compression without an associated decrease in DRG cell count. One day after injury, neither SP- nor CGRP-immunoreactivity was affected by the magnitude of the applied load. However, 7 days after root compression, SP, but not CGRP, significantly decreased with load bilaterally in the DRG (Figure 6). The depletion of this nociceptive neuropeptide in DRG neurons likely suggests increased transport, synaptic release, and utilization of SP following painful injury, which depletes intracellular stores of SP in the DRG (Cahill & Coderre 2002; Jang et al. 2004; Kobayashi et al. 2005a; Schicho et al. 2005; Skoff et al. 2003; Weissner et al. 2006). In fact, while the number of SP-positive neurons in the DRG has been shown to decrease over time (Lee et al. 2001), the number of neurons expressing preprotachykinin A mRNA (the precursor to SP) goes up, suggesting that utilization of SP is faster than its

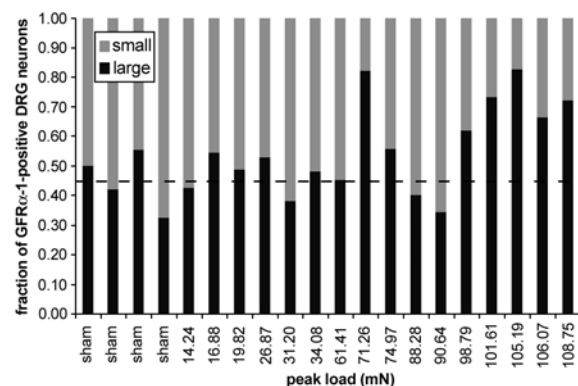


Figure 9. Relative fraction of GFR α -1-immunoreactivity in large and small neuron populations of the DRG ipsilateral to dorsal root compression at day 7. For larger compression loads, GFR α -1-immunoreactivity is shifted towards large neurons relative to GFR α -1-immunoreactivity following sham procedures (average value indicated by horizontal dashed line). The relative decrease in GFR α -1-immunoreactivity in small, nociceptive neurons may underlie the maintenance of nerve root-mediated pain.

production (Marchand et al. 1994; Noguchi et al. 1994). Previous studies of nerve and nerve root injury have reported a similar decrease in spinal SP, that is absent at early time points (Hughes et al. 2007; Kuraishi et al. 1985; Lee et al. 2001; Malmberg & Basbaum 1998; Rothman et al. 2005). In the present model, the decrease in SP-positive neurons with increasing load may result from increased SP utilization in the spinal cord. Although no significant changes in CGRP were identified at day 7, we have previously reported a significant reduction in spinal CGRP for loads greater than 24.03mN (Hubbard et al. 2008). Furthermore, in a canine model of nerve root compression, both SP and CGRP expression were reduced for 1 week following 147mN compression (Kobayashi et al. 2005a,b). Taken together, findings suggest that the rates of utilization for both SP and CGRP increase after painful dorsal root loading, and the lack of significant changes for CGRP in the present study may simply reflect insufficient power in the immunohistochemical technique used to identify those changes. Additional studies of neuropeptides and electrophysiological responses are needed to more fully understand the relationship between mechanical loading of the nerve root, axonal damage, and pain regulation.

The decrease in SP-immunoreactivity in the DRG with increasing load at day 7 (Figure 6) suggests that axonal compression depletes intracellular SP stores in DRG neurons due to increased spinal utilization. This suggests that, despite the application of painful axonal compression, axonal transport may be sustained in undamaged axons and/or surviving dysfunctional axons. Axonal damage was assessed in this study by a decrease or localized accumulation of NF200-immunoreactivity in the dorsal root (Figure

4). The 50th-percentile load threshold for decreased NF200-immunoreactivity at day 7 was defined at 34.08mN (Figure 5), within 4mN of that threshold previously reported to produce persistent behavioral hypersensitivity (38mN) for this injury (Hubbard & Winkelstein 2008). This agreement in thresholds to within 4% of the overall applied load range for pain and injury implicates damage to the dorsal root axons as potentially contributing to persistent pain or initiating those cellular cascades which are responsible for pain. Of note, NF200-immunoreactivity was the only marker used to evaluate axonal damage in this study. It is possible that afferent C-fibers are also injured; additional studies are needed to fully define axonal damage in this tissue after its injury and to further evaluate the possibility of putative unstained swellings (Figure 4). Axonal damage has been proposed to contribute to pain via involvement in ectopic firing, axonal degeneration, altered ion channel expression, decreased axonal flow, and regrowth of A β fibers into the superficial laminae of the spinal cord (Boucher et al. 2000; Cahill & Coderre 2002; Iwata et al. 2004; Kirita et al. 2007; Lee et al. 2001; Nakamura & Myers 1999, 2000; Ramer et al. 1997; Watanabe et al. 2007; Waxman et al. 1999; Zhang et al. 2004). Although the number of DRG cells did not decrease following compression in this study, cell death has also been documented in the DRG following severe nerve root crush (Sekiguchi et al. 2003). From the present study and previous observation of Wallerian degeneration in myelinated axons at day 7 in this model (Hubbard & Winkelstein 2008), axonal damage and degeneration generally followed the trend of decreased NF200-immunoreactivity with increasing load. This further

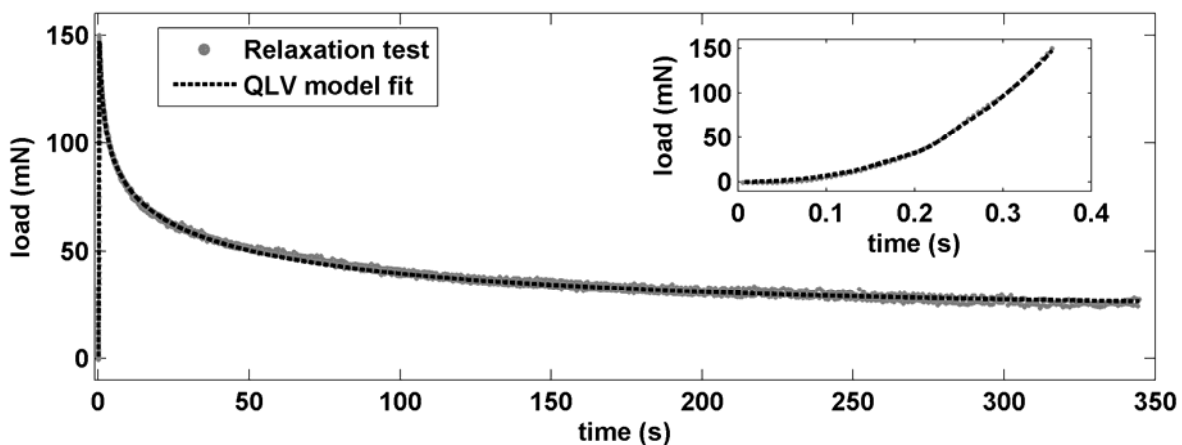


Figure 10. Experimental data and curve-fit of the ramp and hold for a representative test (specimen 166-L6R). (Inset) The ramp loading occurs within the first 0.4 seconds at a rate of 1.67mm/s.

Table 2. Summary of Nerve Root Compression Mechanics

Specimen	Peak load (mN)	Displacement (mm)	Loading rate (mm/s)
166-L6R	150.00	0.595	1.67
167-L6R	167.40	0.577	1.65
168-L6R	103.18	0.527	1.82
174-L6L	233.54	0.685	1.81
166-L6L	95.06	0.608	1.68
167-L4R	166.56	0.753	1.70
168-L4L	248.11	0.714	1.61
174-L4R	259.70	0.747	1.84
Mean	177.95	0.651	1.72
S.D.	63.45	0.085	0.09

suggests that axonal damage may be specifically produced by painful compression loads. Axonal degeneration can induce infiltration of inflammatory mediators, which alter the electrical properties, and increase spontaneous firing, of intact axons (Li et al. 2000; Obata et al. 2003). After the clearance of myelin debris by macrophages, aberrant regeneration of mechanoreceptive A β fibers can remap to the superficial laminae of the spinal cord, forming permanent synapses with spinal neurons, which normally convey painful sensory signals (Nakamura & Myers 1999, 2000; Ramer et al. 1997; Watanabe et al. 2007). While dorsal root compression may produce these neuropathic sequelae, this model is the first to specifically demonstrate that axonal pathology in myelinated axons occurs following controlled loads applied to the nerve root that produce persistent mechanical allodynia.

Axonal viability in nociceptive neurons is regulated by neurotrophic factors (Boucher et al. 2000; Dong et al. 2005; Mendell et al. 1999; Ramer et al. 2000; Takahashi et al. 2003; Wilson-Gerwing & Verge 2006). One day after dorsal root compression by loads that also produced persistent mechanical allodynia, GDNF-immunoreactivity decreased in small, nociceptive neurons (Figure 7). At that time point, GFR α -1 receptor immunoreactivity was unchanged. Seven days after injury, GDNF-immunoreactivity was restored to normal; however,

GFR α -1 receptor immunoreactivity had shifted towards expression in the large neurons of the DRG for painful compressions (Figures 8 & 9). In addition to promoting neuronal viability, GDNF has been shown to reduce behavioral hypersensitivity by decreasing ectopic firing in small myelinated axons (Boucher et al. 2000). Yet, the early decrease in GDNF-immunoreactivity and late decrease in GFR α -1-immunoreactivity in nociceptive neurons following painful compressions in our study suggest that reduced GDNF expression prevented its analgesic effects following painful dorsal root compression. With decreased GDNF signaling through the GFR α -1-RET receptor complex, nociceptive neurons have enhanced ectopic activity and are susceptible to degeneration, leading to increased behavioral hypersensitivity (Boucher et al. 2000; Hubbard et al. 2008b). With the loss of neurotrophic activity in nociceptive neurons after large, pain-producing compressions it is probable that altered GDNF and GFR α -1 expression together contribute to persistent behavioral hypersensitivity.

At day 7, the GFR α -1 receptor increased significantly with load in large, non-nociceptive neurons (Figures 8B & 9). Bennett et al. (2000b) reported a similar shift in GFR α -1-immunoreactivity towards large neurons in the DRG following sciatic nerve transection. That study and the findings in our

Table 3. Summary of QLV Model Parameters

Specimen	A (mN)	B	C	τ_1 (sec)	τ_2 (sec)
166-L6R	1.669	7.573	0.839	0.389	314.5
167-L6R	6.919	5.509	0.470	1.394	901.9
168-L6R	4.903	6.058	0.978	0.041	160.8
174-L6L	0.341	9.434	0.316	0.295	350.9
166-L6L	0.103	11.286	1.040	0.165	68.2
167-L4R	6.814	4.282	0.382	0.603	283.8
168-L4L	7.777	4.944	0.800	0.264	204.9
174-L4R	11.584	4.465	0.232	0.010	346.2
Arithmetic Mean	5.014	6.694	0.632	0.395	328.9
S.D.	4.050	2.536	0.318	0.446	251.5
Geometric Mean	2.432	6.319	0.555	0.190	261.7

model indicate that both pre- and post-ganglionic axonal damage appear to produce similar shifts in neuron subtypes expressing GFR α -1. Preferential expression of the GFR α -1 receptor in large neurons suggests that both degeneration of small neurons and aberrant regrowth of myelinated axons mediate persistent pain. Taken together, following compressions large enough to be painful, a cellular response of decreased anti-allodynic neurotrophic support in nociceptive neurons may facilitate acute behavioral hypersensitivity. However, an adaptive response of enhanced utilization of SP and decreased GFR α -1 expression in nociceptive neurons, along with increased GFR α -1 expression in regenerating large neurons, likely mediates the maintenance of chronic, cervical radicular pain.

Dynamic compression produced considerably greater peak loads than quasistatic nerve root displacement (Figure 11). By defining the load response to displacement at both 0.004mm/s and 2mm/s, a QLV model was developed to extrapolate the cellular results here to those for loading at impact velocities. The QLV model estimated the dynamic displacement that was necessary to produce 34.08mN, the load above which quasistatic compression produced altered axonal NF200-immunoreactivity (Figure 4C & 12). At the 0.004mm/s displacement rate, a compressive displacement of 0.555mm was required

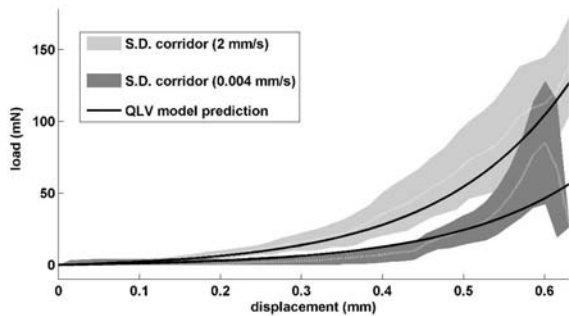


Figure 11. QLV model prediction of the load response to displacement at 2mm/s and 0.004mm/s. The gray areas represent the mean force \pm one standard deviation over 0.643mm of displacement. The 2mm/s corridors (light gray) are taken from the ramp portion of the relaxation tests ($n=8$), while the 0.004mm/s corridors (dark gray) were taken from the validation compressions ($n=3$). The QLV model prediction was based on the geometric means of the parameters derived from the relaxation tests. A sharp decline in the experimental load response to displacement at 0.004mm/s is observed after 0.601 mm when two of the three validation compressions terminate.

to generate a peak load of 34.08mN; however, for a 2mm/s displacement, the QLV model predicted a required displacement of only 0.429mm to produce that same peak load (Figure 12). Assuming that loading in this study is similar to transient compression produced by foraminal occlusion during impact injury (Carter et al. 2000; Nuckley et al. 2002,2004), nearly 23% less foraminal narrowing would be needed for dynamic loading to produce loads that cause axonal damage and subsequent cellular and behavioral changes than for quasistatic loading. Previous studies indicate that the intervertebral foramen narrows significantly with increased spinal extension (Ebraheim et al. 2006; Nuckley et al. 2002; Yoo et al. 1992). Coupled with normal physiologic foraminal narrowing, tissue stiffening and increased dynamic fluid pressure, as well as potential intervertebral disc protrusion and facet dislocation, may be sufficient to apply painful loading to the nerve root during a whiplash event (Bostrom et al. 1996; Giles, 2000; Ortengren et al. 1996; Tanaka et al. 2000). It should be noted that for loading rates greater than 2mm/s, the QLV model predicts that tissue stiffening is not increased as a result of the ramp time decreasing below the time constant for initiation of relaxation (τ_1). Therefore, the viscoelasticity studies performed at 2mm/s are assumed to be valid for modeling nerve root compression at the rapid strain rates observed in whiplash loading. While moderate compressive displacement of the nerve root may not produce axonal damage or pain symptoms at low rates of loading, the QLV model suggests that similar displacement at higher rates may increase the loads applied to the nerve root and cause nociceptive and

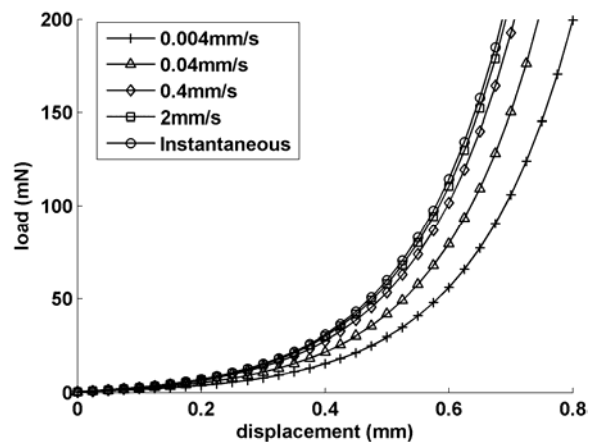


Figure 12. QLV model prediction of the load response to displacement at rates ranging from 0.004mm/s to an instantaneous application of load. Stiffness only marginally increases for force-displacement predictions above 2mm/s.

structural changes that result in persistent pain symptoms.

The duration of transient loading in the study of load-based cellular changes was substantially longer than the duration of a traumatic injury event (Grauer et al. 1997; Kaneoka et al. 1999; Ono et al. 1997; Sundararajan et al. 2004; Yoganandan et al. 1998). The 15-minute duration of loading may have reduced microvascular blood flow, which could also augment the behavioral and cellular changes (Lundborg et al. 1983; Rydevik et al. 1989). However, previous studies suggest that loading rate and magnitude directly modulate physiologic outcomes and pain symptoms for nerve root compression (Hubbard & Winkelstein 2008; Kobayashi et al. 1993; Kobayashi & Yoshizawa 2002; Olmarker et al. 1989). Therefore, the relationships between load magnitude, mechanical allodynia, NF200-immunoreactivity, and peptide expression in the DRG determined in this study can be extended to traumatic insult scenarios, but may include additional ischemic effects related to the increased duration of loading. However, the viscoelasticity study investigated the effect of compression rate on the load response of nerve root tissue. While only three quasistatic load-displacement curves validated the model parameters, additional validation studies at other rates and displacements would prove useful in determining the robustness of the QLV model. Nonetheless, this study provides otherwise unavailable quantitative relationships that lay the initial groundwork for future studies investigating nerve root compression mechanics and physiologic responses for injury. Also, the study of load-based cellular changes represents 28 additional validation load responses at a displacement rate of 0.004mm/s. Despite differences in size of cervical and lumbar nerve roots, the range of peak loads for injurious cervical root loading (Table 1) approximately matches the peak load of 49mN estimated by the QLV model for 0.6mm of compression at 0.004mm/s. Additionally, the force-time relationship determined by the QLV model (Figure 10) compares favorably to a force relaxation experiment performed on porcine brain tissue, in which peak loads for rates of 1-3mm/s range from 100 to 150mN and achieve a final relaxed load of approximately 50mN (Gefen & Margulies 2004). Therefore, the QLV model presented here appropriately captures the response of nerve root tissue in compression for loading rates from 0.004mm/s to 2mm/s and is the first to integrate relationships for tissue loading and pathological responses in axons for this injury.

CONCLUSION

In summary, increasing dorsal root compression magnitude significantly increased behavioral hypersensitivity and altered the expression of several cellular markers of pain. NF200-immunoreactivity in the compressed root significantly decreased and exhibited an increase in the number of axonal swellings for loads above 34.08mN. Utilization of the nociceptive neuropeptide SP in the DRG was also dependent on load magnitude 7 days after dorsal root compression, which, in conjunction with decreased GDNF support in nociceptive neurons and the difference in expression of the GFR α -1 receptor on neuronal populations, may lead to persistent behavioral hypersensitivity. Based on mechanical testing of the nerve roots, dynamic root loading produces tissue stiffening and an increased peak load relative to quasistatic loading for a given displacement, and this increased compression rate may provide the necessary forces during traumatic foraminal narrowing to produce persistent radicular pain. Rapid nerve root loading likely decreases the displacement threshold for painful injury, and increases axonal damage and the severity of associated nociceptive cellular modifications involved in chronic neck pain.

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APPENDIX A: GLOSSARY OF TERMS

Allodynia - Pain due to a stimulus which does not normally provoke pain.

Calcitonin Gene-Related Peptide (CGRP) - A polypeptide encoded by the calcitonin gene that acts as a potent vasodilator and neurotransmitter. It is widely distributed in the central and peripheral nervous systems and is commonly colocalized with the neuropeptide substance P.

Glial Cell Line-Derived Neurotrophic Factor (GDNF) - An astrocyte- and neuron-derived small protein which promotes the survival and differentiation of various types of neurons.

GDNF family receptor α -1 (GFR α -1) - The cell surface receptor for GDNF that mediates activation of a receptor tyrosine kinase encoded by the oncogene RET (REarranged in Transfection).

Immunoreactivity - The level of interaction between an antibody and its antigen in an assay to measure the concentration or presence of a biological substance.

Mechanical allodynia - Pain in response to non-noxious light touch or pressure.

Mechanical hyperalgesia - An increased response to a mechanical stimulus which is normally painful.

NF200 - Also known as heavy chain neurofilament. A 200kDa intermediate filament that is found only in neurons and serves as a cytoskeletal element that supports the axonal cytoplasm.

Neurotrophic factors - A family of proteins which induce the survival and maintenance of neurons.

Nociceptive - Relating to the neural processes associated with the perception of, or response to, pain.

Substance P (SP) - A polypeptide that functions as a neurotransmitter especially in the transmission of pain impulses from peripheral receptors to the central nervous system.

