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The Roles of Mechanical Compression and Chemical Irritation in Regulating Spinal Neuronal Signaling in Painful Cervical Nerve Root Injury

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ABSTRACT – Both traumatic and slow-onset disc herniation can directly compress and/or chemically irritate cervical nerve roots, and both types of root injury elicit pain in animal models of radiculopathy. This study investigated the relative contributions of mechanical compression and chemical irritation of the nerve root to spinal regulation of neuronal activity using several outcomes. Modifications of two proteins known to regulate neurotransmission in the spinal cord, the neuropeptide calcitonin gene-related peptide (CGRP) and glutamate transporter 1 (GLT-1), were assessed in a rat model after painful cervical nerve root injuries using a mechanical compression, chemical irritation or their combination of injury. Only injuries with compression induced sustained behavioral hypersensitivity ($p \leq 0.05$) for two weeks and significant decreases ($p < 0.037$) in CGRP and GLT-1 immunoreactivity to nearly half that of sham levels in the superficial dorsal horn. Because modification of spinal CGRP and GLT-1 is associated with enhanced excitatory signaling in the spinal cord, a second study evaluated the electrophysiological properties of neurons in the superficial and deeper dorsal horn at day 7 after a painful root compression. The evoked firing rate was significantly increased ($p = 0.045$) after compression and only in the deeper lamina. The painful compression also induced a significant ($p = 0.002$) shift in the percentage of neurons in the superficial lamina classified as low-threshold mechanoreceptive (*sham* 38%; *compression* 10%) to those classified as wide dynamic range neurons (*sham* 43%; *compression* 74%). Together, these studies highlight mechanical compression as a key modulator of spinal neuronal signaling in the context of radicular injury and pain.

KEYWORDS – radiculopathy, compression, biomechanics, injury, pain, electrophysiology

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INTRODUCTION

Neck pain has an estimated annual incidence of 20% (Croft et al., 2001) and affects approximately two-thirds of the general population in their lifetime (Côté et al. 1998, 2000). Cervical pain frequently requires medical attention and accounts for more than 1% of patient visits to hospitals and physician offices, contributing to a multi-billion dollar syndrome (Freeman et al., 1999; AAOS, 2008). Cervical injuries are responsible for 28% of neck pain cases (AAOS, 2008). In particular, neck injury has been reported to occur in as many as one-third of motor vehicle collisions (Zuby and Lund, 2010) and constitutes an estimated 30% of traffic-related emergency room visits in the United States (Quinlan et al., 2004). Worldwide, neck injuries and pain are similarly common. Neck injuries were reported in approximately 50% of car-to-car collisions in Japan (Ono and Kanno, 1996). An Australian study reported that at least 69% of vehicle collision occupants sustain neck and back injuries (Littleton et al., 2012). Further, a Canadian study reported that a history of neck injury in motor vehicle collisions enhances the risk of chronic neck pain (Nolet et al., 2010); 62% of individuals who visited hospitals in Britain following road traffic incidents suffered from either acute or chronic neck pain (Deans et al., 1987). Neck injuries can cause tissue injury to a diverse set of spinal tissues (Luan et al., 2000; Siegmund et al.,

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2001), but the cervical nerve root is particularly vulnerable to injury and, when injured, can produce persistent pain across a range of different local tissue loading scenarios (Olmarker et al, 1989; Krivickas and Wilbourn, 2000; Nuckley et al., 2002; Stuber, 2005; Panjabi et al., 2006; Singh et al., 2006; Hubbard et al., 2008a).

Cervical Nerve Root Injury Mechanisms

Cervical radiculopathy, defined as a lesion of the cervical nerve root, is a common source of neck pain (Wainner and Gill, 2000; Abbed and Coumans, 2007; Nicholson et al., 2012). Nerve root injury and associated radicular symptoms are frequently reported in athletes and injured occupants after vehicle collisions (Krivickas and Wilbourn, 2000; Stuber, 2005; Panjabi et al., 2006; Tominaga et al., 2006). Although traumatic spinal injury, foraminal impingement and/or increased hydrostatic pressure due to rapid head and neck motions can all occur from neck injury, nerve root-mediated pain can also arise from a variety of disorders, including disc herniation and cervical spondylosis (Bostrom et al., 1996; Ortengren et al., 1996; Eichberger et al., 2000; Nuckley et al., 2002; Abbed and Coumans, 2007). These different injury modalities present an array of local injury environments to the nerve root and produce varied patterns and extents of nerve-root mediated pain (Olmarker and Myers, 1998; Colburn et al., 1999; Winkelstein et al., 2001; Homma et al., 2002; Robinson and Meert, 2005). As the major cause of radicular pain, disc herniation imposes both mechanical compression to the nerve root and a chemical irritation when the inflammatory material from the disc contacts and compresses the nerve root (Olmarker et al, 1998; Wall and Melzak, 2005; Rothman and Winkelstein, 2010). In addition to root compression via traumatic or degenerative disc herniation, non-physiological neck motions and injurious neck loading from sports and vehicular impacts may also place the nerve root under compression alone due to shape changes of the intervertebral foramen (Carter et al., 2000; Krivickas and Wilbourn, 2000; Ebraheim et al., 2006; Panjabi et al., 2006). It has been previously shown that both mechanical compression and chemical irritation of the nerve root contribute to radicular pain (Kawakami et al., 2000; Rothman and Winkelstein, 2007; Rothman et al., 2009). However, the relative contribution of the mechanical and chemical components of those insults to the cascades in the spinal cord which modulate nociceptive peptides and neuronal excitability and lead to pain remains unknown.

A rat model has been developed and used to understand the effects of nerve root loading parameters on behavioral and cellular outcomes after a nerve root injury. In that model of cervical radiculopathy, both the magnitude and duration of a transient root compression affect the time course and extent of radicular pain symptoms that develop (Hubbard et al., 2008a,b; Rothman et al., 2010). Cervical dorsal root compression with peak loads above 40mN can induce persistent pain symptoms and lead to significantly decreased neurofilament expression in the compressed root and substance P in the dorsal root ganglion (DRG) at day 7 (Hubbard et al., 2008a). Further, a critical duration of applied compression with a 98mN load to the rat nerve root has been identified (6.6 ± 3.0 minutes) as sufficient to decrease the spinal neuronal firing during compression (Nicholson et al., 2011). Transient compressive trauma to the nerve root that is held for longer periods of time than this threshold duration are capable of producing sustained axonal damage in the nerve root, together with sustained mechanical allodynia for 7 days (Rothman et al., 2010; Nicholson et al., 2011). Compressive insults to the rat nerve root with a magnitude of 98mN applied for 15 minutes have been used to investigate pain symptoms and histologic changes in the spinal cord and dorsal root ganglion (DRG) in order to define the inflammatory responses in the peripheral and central nervous systems for different types of painful root injuries (Rothman et al., 2009, 2010; Chang and Winkelstein, 2011). Although the primary afferents in the root are functionally impaired during its compression and exhibit morphology consistent with neurodegeneration and cytoskeletal damage as late as day 14 after painful compression, the synaptic properties of these neurons in the spinal cord have not been defined in the context of chronic radicular pain. Understanding how and where neuronal function, such as neuropeptide expression and excitability, is modified within the spinal cord at times when behavioral sensitivity is maintained is necessary to fully characterize the contribution of ascending circuit(s) to radicular pain.

Chemical irritation of the nerve root, without any mechanical component, has been shown to induce pain in rodent models of radiculopathy and peripheral neuropathy. Chromic gut material is commonly used in animal models of chronic pain to mimic the chemical irritation to the root due to herniated disc material (Maves et al., 1993; Kawakami et al., 1994; Winkelstein and DeLeo, 2004; Rothman et al., 2009, 2010). In a study of lumbar radiculopathy, rats treated with chromic gut alone developed prolonged thermal hyperalgesia that lasted for up to 12 weeks,

peaking at 14 days (Kawakami et al., 1994). Chemical irritation to the lumbar nerve root can also produce bilateral mechanical allodynia that is sustained for at least 2 weeks (Rutkowski et al., 2002); a second exposure of chromic gut to the root at 7 weeks can induce enhanced and longer-lasting behavioral sensitivity for another 6 weeks, along with persistent spinal astrocytic and microglial activation (Hunt et al., 2001). In a rat model of peripheral neuropathy, chemical irritation with chromic gut sutures around the sciatic nerve led to postural alterations and significant ipsilateral thermal hyperalgesia that peaked at day 3; however, no mechanical hyperalgesia was observed (Maves et al., 1993). Chromic gut exposure has also been shown to cause transient mechanical allodynia that lasted for 3 days after its introduction to the cervical nerve root in rats (Rothman and Winkelstein, 2007).

In rodent models of disc herniation that combine the injury components of mechanical compression and chemical irritation, behavioral sensitivity is enhanced compared to the application of either injury element alone (Olmarker and Myers, 1998; Hou et al., 2003; Winkelstein and DeLeo, 2004; Rothman and Winkelstein, 2007; Chang and Winkelstein, 2011). A recent study has reported that compression, chemical irritation or the combination of both of those two injury components together induce immediate and sustained mechanical hyperalgesia on the ipsilateral side that last for up to 2 weeks, while behavioral hypersensitivity on the contralateral side is only observed after the combined injury and for a limited time period (Chang and Winkelstein, 2011). Taken together, this collection of studies suggests that mechanical and chemical nerve root insults induce differential behavioral sensitivity. However, despite studies showing mechanical and chemical insults as inducing behavioral sensitivity and separately modifying pain-related glial activation and neuropeptide release, little remains known about the relative contribution of these different components of nerve root injury to the regulation of neuronal activities in the spinal cord that are associated with their different patterns of pain.

Neurotransmitters & Pain

In response to trauma and/or painful inputs, neurotransmitters are released in both the periphery, in the injured tissue, as well as in the central nervous system (CNS) where they transmit and modulate pain (Cavanaugh, 2000). Noxious stimuli sensed by nociceptors are transmitted to the CNS through small myelinated ($A\delta$) and unmyelinated (C) afferent nerve fibers. After a painful injury, neuropeptides, such as calcitonin gene-related peptide (CGRP) and

substance P (SP), are released in the spinal cord from $A\delta$ and C afferent fibers as nociceptive neurotransmitters in the spinal dorsal horn (Kangrga and Randic, 1990; Smullin et al., 1990). CGRP promotes the secretion of substance P in the superficial dorsal horn and also impedes SP metabolism in the dorsal root ganglia (Allen et al., 1999; Meert et al., 2003). Both CGRP and SP play a role in the initiation and maintenance of neuropathic pain from spinal nerve injury (Lee and Kim, 2007). Although elevated CGRP expression has been reported in association with enhanced behavioral sensitivity and neuronal hyperexcitability in response to noxious stimuli in rodent models (Oku et al., 1987; Neugebauer et al., 1996; Bennett et al., 2000), painful nerve root compression leads to a down-regulation of CGRP expression in the superficial laminae of the spinal cord (Kobayashi et al., 2005; Hubbard et al., 2008a).

CGRP and SP expression, together with behavioral sensitivity, have been reported to depend on the magnitude of the load applied to the nerve root (Hubbard et al., 2008a,b). Bilateral spinal CGRP and SP expression decreases with increasing magnitude of applied nerve root compressive load, while only SP decreases in the DRG with increasing load (Hubbard et al., 2008a,b). In contrast with these findings, SP expression in the DRG has been shown to increase 14 days after painful nerve root injuries that include compression input at injury (Chang and Winkelstein, 2011). The transient decrease in SP in the DRG followed by a later increase in this neuropeptide indicates that the temporal response of neuropeptides from days 7 to 14 may have substantial implications for chronic radicular pain. Even though the spinal expression of CGRP appears to be more robustly modulated by a painful nerve root compression than SP at day 7 (Hubbard et al., 2008a), CGRP levels have not been measured at times after day 7 in this model.

Chemical irritation can also modulate spinal expression of neurotransmitters. After chromic gut exposure to the sciatic nerve, lumbar spinal CGRP and SP were reported to decrease at the late time of day 60 (Xu et al., 1996). Despite the evidence showing reduced CGRP in that model of peripheral neuropathy, CGRP regulation by chemical irritation after CNS injury has not been defined. It is also unclear how nerve root compression, chemical irritation and their combined injury affect the release of spinal CGRP relative to each other. In addition, since second order neurons within individual laminar layers in the spinal cord are innervated by different types of primary afferents (Basbaum et al., 2009),

there may be regional differences in the expression of spinal neurotransmitters following different injury modalities. For example, the deeper dorsal horn (laminae III-V), but not the superficial dorsal horn (laminae I & II) exhibits significantly elevated CGRP expression after joint immobilization in the rat (Nishigami et al., 2009). As such, the spinal regulation of neuropeptides after mechanical and chemical insults to the nerve root must include regional assessments in the superficial and deeper laminae of the dorsal horn.

The Glutamatergic System & Pain

Glutamate is a major excitatory neurotransmitter released by primary afferent fibers at their synapses with second order neurons in the spinal dorsal horn (Basbaum et al., 2009). Excess glutamate can cause excitotoxicity and glutamate homeostasis is required for the survival and normal function of neurons (Anderson and Swanson, 2000). Removal of extracellular glutamate is primarily mediated by glutamate transporters expressed by glial cells, such as astrocytes (Anderson and Swanson, 2000). Glutamate transporter 1 (GLT-1), glutamate-aspartate transporter (GLAST) and excitatory amino-acid carrier 1 (EAAC1) are the three major spinal glutamate transporters in the rat (Gegelashvilia et al., 2000; Tao et al., 2004, 2005). Painful nerve root injury and peripheral nervous tissue injury are known to activate astrocytes in the spinal dorsal horn (Hunt et al., 2001; Weisshaar et al., 2010; Nicholson et al., 2012), which may alter transporter-mediated glutamate uptake that can result in neuronal hyperexcitability. Such plasticity is responsible for the development of nociception and pain (Tao et al., 2005).

GLT-1 is primarily synthesized by astrocytes and is responsible for the clearance of at least 90% of extracellular glutamate (Rothstein et al., 1995; Danbolt, 2001; Mitani and Tanaka, 2003). Growing evidence in animal models has shown that GLT-1 is down-regulated in the spinal dorsal horn after partial sciatic nerve ligation and other painful peripheral nerve injuries (Sung et al., 2003; Xin et al., 2009). In fact, the changes are most robust in the superficial laminae of the dorsal horn, where most glutamate is released by the C and Aδ nociceptors to activate post-synaptic neurons (Basbaum et al., 2009). Disruption of the gene encoding GLT-1 in mice has been shown to result in enhanced extracellular glutamate concentration, neuronal degeneration and neuronal hypersensitivity in the brain (Tanaka et al., 1997), suggesting GLT-1 as essential in preventing neuronal excitotoxicity and hyperexcitability through its

maintenance of low extracellular glutamate concentrations in the CNS. Nevertheless, little remains known about if, and the extent to which, spinal GLT-1 regulates extracellular glutamate in association with neuronal excitability after different neural injuries that cause pain.

Despite the lack of characterization of the relationship between GLT-1 and painful nerve root injuries, the dependency of GLT-1 on astrocytes and the association of astrocytic activation with nerve root loading have been extensively demonstrated (Anderson and Swanson, 2000; Rothman et al., 2010; Wang et al., 2008; Nicholson et al., 2012). Cervical nerve root compression is known to activate spinal astrocytes in parallel with the development of behavioral hypersensitivity (Rothman and Winkelstein, 2007; Nicholson et al., 2012). Nerve injury-induced activation of spinal astrocytes can shift their homeostatic functions to support the re-growth of injured afferent axons, but these alterations also compromise synaptic functions (Aldskogius and Kozlova, 1998; Xin et al., 2009). Among the changes in synaptic function is decreased GLT-1 expression, which may alter neuronal excitability and produce and/or maintain behavioral hypersensitivity. Consistent with this hypothesis, GLT-1 has been shown to be down-regulated at day 7 after a painful nerve root compression and at days 7 and 14 after partial sciatic nerve ligation, when mechanical allodynia is still evident (Xin et al., 2009; Nicholson et al., 2013b). Further, a chemical irritation or a combination of chemical and mechanical insults to the nerve root also induce spinal astrocytic activation and mechanical allodynia (Rothman and Winkelstein, 2007). The combined mechanical-chemical injury induces more spinal astrocytic activation in the ipsilateral spinal cord at day 7 compared to expression in both the contralateral dorsal horn and after the chemical irritation alone; a chemical irritation alone does not induce any astrocytic activation in the ipsilateral spinal cord that is different from the contralateral side (Rothman and Winkelstein, 2007). Based on these reports of spinal astrocytic responses and because there may be direct effects of these activation patterns on the production of GLT-1, spinal GLT-1 is expected to decrease more robustly after painful nerve root compression or the combined injury than after chemical irritation alone. However, this hypothesis has not been explicitly tested.

Overall Goals & Study Design

The goal of this work was to investigate the relative effects of nerve root compression, chemical irritation

and their combined injury on the spinal expression of CGRP and GLT-1 associated with pain symptoms. We hypothesized a down-regulation of both CGRP and GLT-1 expression in the ipsilateral dorsal horn compared to the contralateral dorsal horn, with the most robust changes in the superficial laminae because most neurotransmitters are released by primary afferents in laminae I and II. Based on prior studies using mechanical and chemical insults to the nerve root (Rothman and Winkelstein, 2007; Rothman et al., 2009; Chang and Winkelstein, 2011), a 15 minute compression with 98mN or/and exposure to 3-0 chromic gut suture were applied to induce cervical nerve root injuries in a rat model. Assessment of mechanical hyperalgesia was performed to evaluate each of the spinal cord outcomes in the context of pain for the different nerve root injuries. CGRP and GLT-1 expression in the superficial and deeper laminae of the dorsal horn were evaluated separately using immunohistochemistry at day 14 to determine the relative contribution of each type of injury on regulating these aspects of the spinal nociceptive response.

Based on findings from that characterization study indicating that root compression induces selective down-regulation of both CGRP and GLT-1 in the superficial laminae in parallel with sustained behavioral hypersensitivity, cervical nerve root compression was hypothesized to alter the electrophysiological properties of neurons in the superficial and deeper dorsal horn, where the primary nociceptors and low threshold mechanoreceptors predominantly synapse. A second study was performed to define if, and how, painful compression of the nerve root alters the electrophysiological response of spinal neurons at day 7, measured by extracellular recordings of action potentials evoked by various noxious and non-noxious stimuli to the forepaw. Spinal neuronal electrophysiological responses were measured in the superficial and deeper dorsal horn at day 7 since persistent neuronal hyperexcitability develops by this time point in other painful peripheral tissue injuries and because pain behaviors are fully established in this model by day 7 (Quinn et al., 2010; Nicholson et al., 2012; Crosby et al., 2013). Because the different classes of secondary neurons in the dorsal horn respond differently to nociceptive and non-nociceptive stimuli (Dubner and Bennett, 1983; Hains et al., 2003; Quinn et al., 2010; Zeilhofer et al., 2012), we also categorized each neuron by its response to mechanical stimuli using established methods (Hains et al., 2003; Quinn et al., 2010) and evaluated the relative percentage of neurons with each phenotype.

METHODS

Two complementary studies were performed to collectively define several aspects of cellular regulation of neuronal signaling in the spinal cord associated with pain following cervical nerve root injuries in the rat. Both studies used male Holtzman rats (300-400g; Harlan Sprague-Dawley; Indianapolis, IN), housed in a 12-12 hour light–dark cycle and given free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee and conformed to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmerman, 1983).

In the first study, the relative contribution of different injury modalities (mechanical, chemical, combined) applied to the nerve root were evaluated for their effects on modulating behavioral sensitivity and expression of a spinal neuropeptide and a neurotransmitter transporter. To do this, rats underwent either a mechanical compression insult, an inflammatory insult, or the combination of those two insults, which were separately applied to the right C7 nerve root proximal to the DRG (Figure 1a). An additional sham group was included to control for surgical effects. Behavioral sensitivity was evaluated by measuring the forepaw response threshold to mechanical stimulation for 14 days after surgery and the C7 spinal cord was harvested at day 14 for assessment of CGRP and GLT-1 immunolabeling in the bilateral dorsal horns (Figure 1b). Based on those findings, a second study was performed to further investigate the role mechanical compression that induces pain has on modulating neuronal hyperexcitability in the spinal cord. In that study, following a 98mN compression to the C7 nerve root, behavioral sensitivity was assessed at day 1 and day 7 and electrophysiological recordings were acquired separately in the superficial and deeper dorsal horn at day 7 (Figure 1c). The phenotypic response of, and neuronal firing rate evoked by light brushing, filament stimulations and noxious pinches, were quantified and compared to controls.

Spinal CGRP & GLT-1 Responses to Mechanical & Chemical Nerve Root Insults

Surgical Procedures. Surgical procedures were carried out using methods and protocols that have been previously detailed (Rothman and Winkelstein, 2007; Rothman et al., 2009; Chang and Winkelstein, 2011). Rats were placed in a prone position and anesthetized with inhalation isoflurane (4% for induction, 2% for maintenance). An incision was
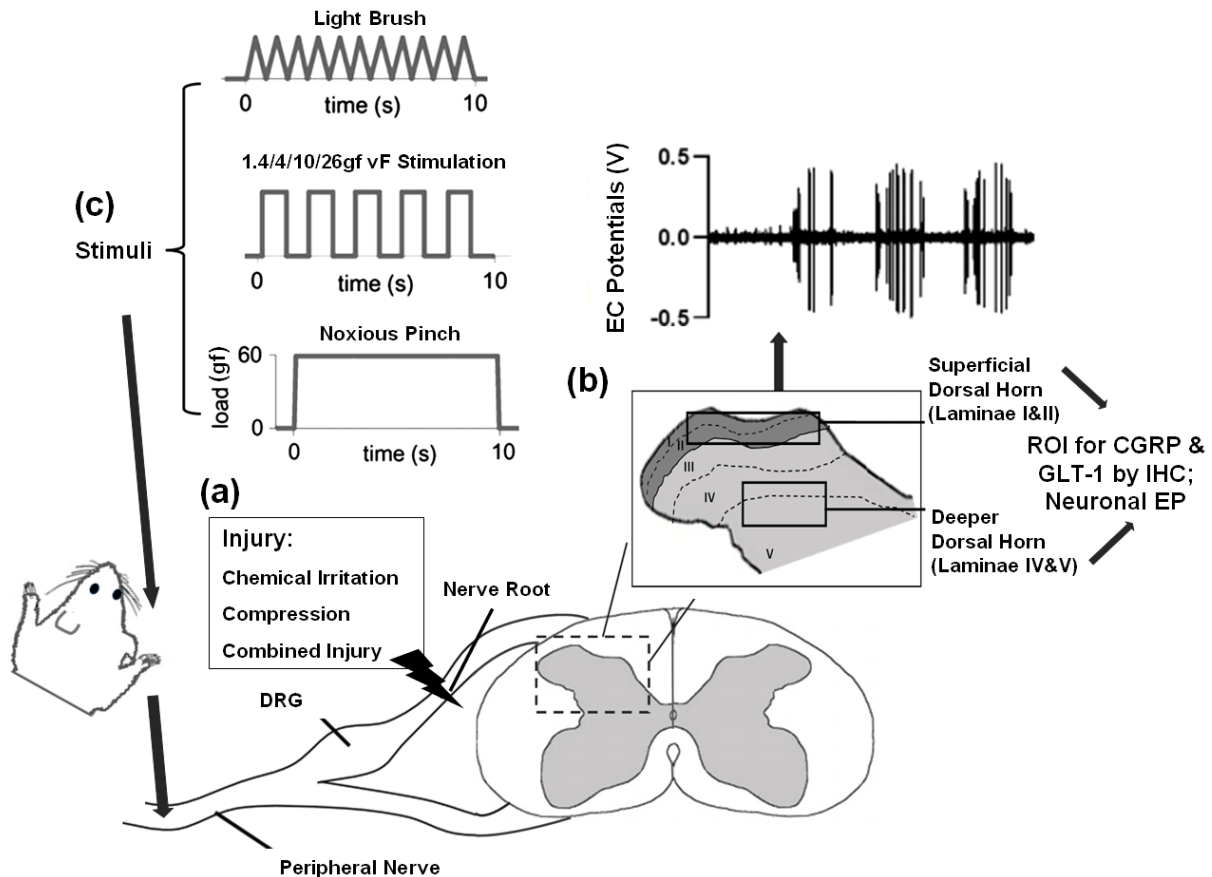
Figure 1. Schematic showing the study design for the nerve root injury modalities and behavioral, immunohistochemistry (IHC) and electrophysiological (EP) outcomes. **(a)** Either chemical irritation, compression, or their combination of injury was applied to the C7 dorsal nerve root in the rat. **(b)** At day 7 after injury, expression of CGRP and GLT-1 was fluorescently labeled and quantified in uniform-sized separate regions of interest (ROI) in each of the superficial (laminae I & II) and deeper (laminae IV & V) dorsal horns. Neuronal electrophysiological outcomes were also measured in the superficial and deeper dorsal horns (150-350μm & 450-1000μm below the pial surface, respectively). **(c)** The electrophysiological response of neurons in the spinal cord was measured in response to a sequence of mechanical stimuli applied to the rat's forepaw, including a series of 10 light brushes, 5 stimulations by each of four von Frey filaments (1.4, 4, 10, 26 gf), and a noxious pinch. Evoked extracellular (EC) potentials for individual neurons were recorded in both regions of the dorsal horn during stimulation.

made in the posterior skin along the cervical spine, and the musculature overlying the C6 and C7 vertebrae was carefully separated. The right C7 dorsal root was exposed by a C6/C7 hemilaminectomy and partial facetectomy on the right side. For the mechanical injury, the right C7 nerve root was compressed using a 98mN (10 gram-force) microvascular clip (World Precision Instruments, Inc; Sarasota, FL) applied for 15 minutes (*compression*; n=8). In a separate group (*chemical irritation*; n=4), 4 pieces (2mm long each) of 3-0 chromic gut suture (Surgical Specialties; Reading, PA) were placed on the right C7 nerve root to induce chemical irritation (Rothman and Winkelstein, 2007; Chang and Winkelstein, 2011). For the combined mechanical and chemical injury

(*combined injury*; n=5), a 98mN compression was applied for 15 minutes followed by the chromic gut exposure that was placed on the C7 nerve root. A sham group (*sham*; n=6), in which no mechanical compression or chemical irritation was performed after nerve root exposure, was included as a surgical control. Wounds were closed using 3-0 polyester sutures and surgical staples. Rats were closely monitored while on a heating pad until they were fully recovered from anesthesia.

Behavioral Assessment. Mechanical hyperalgesia was measured in the bilateral forepaws to evaluate behavioral sensitivity, at baseline, before surgery, and on days 1, 3, 5, 7 and 14 following surgical procedures. A modified Chaplan's up-down method

was used to determine the withdrawal threshold for tactile sensitivity to von Frey filaments applied to the plantar surface of the forepaws (Chaplan et al., 1994; Hubbard and Winkelstein, 2005; Chang and Winkelstein, 2011). Rats were acclimated to the test chamber for 20 minutes prior to testing. The thresholds for eliciting a withdrawal response for the left (contralateral) and right (ipsilateral) forepaws were quantified by stimulating each forepaw using a series of von Frey filaments (Stoelting; Wood Dale, IL) with increasing strengths (0.4, 0.6, 1.4, 2, 4, 6, 8, 15, 26gf). Each filament was applied five times before advancing to the next highest strength. If two consecutive filaments both induced a withdrawal, the lower strength filament was recorded as the threshold. This procedure was repeated three times, with at least 10 minutes of rest between each round for each rat. The average withdrawal threshold determined across the rounds was calculated as the threshold for each forepaw of each rat on each test day. Behavioral sensitivity is reported as the ipsilateral threshold divided by the contralateral threshold for each rat to enable comparison between rats (Kras et al., 2013b) A two-way ANOVA with repeated measures was performed using JMP10 (SAS Institute Inc.; Cary, NC) to compare changes in sensitivity between groups over time. If differences were detected, a one-way ANOVA with Bonferroni correction was used to test for differences between groups at each time point. Significance was achieved at a p-value of less than 0.05.

Immunohistochemistry & Analysis of Spinal CGRP & GLT-1. The C7 spinal cord was harvested en bloc at day 14 after behavioral testing to evaluate expression of CGRP and GLT-1 in the sides ipsilateral and contralateral to the applied injury. Rats were anesthetized by an intraperitoneal injection of 65mg/kg pentobarbital and transcardially perfused with 4% paraformaldehyde in PBS. The C7 spinal cord was harvested with the contralateral side marked for identification, post-fixed, cryoprotected in 30% sucrose, and embedded in OCT medium (Triangle Biomedical Sciences; Durham, NC). Six serial axial sections (12μm) were collected from each tissue sample and thaw-mounted onto Superfrost plus slides. Sections were blocked in 10% normal goat serum (Vector Laboratories; Burlingame, CA) with 0.3% Triton-X100 (Bio-Rad Laboratories; Hercules, CA) for 2 hours and incubated in rabbit anti-CGRP antibody (1:1000; Peninsula Laboratories; Sancarlos, CA) or rabbit anti-GLT-1 antibody (1;1000; Abcam; Cambridge, MA) overnight at 4°C. The next day, sections were washed with PBS and incubated for 2 hours at room temperature in the secondary antibodies, goat anti-rabbit Alexa Fluor 488 (1:1000;

Invitrogen; Carlsbad, CA) for CGRP or goat anti-rabbit Alexa Fluor 546 (1:1000; Invitrogen; Carlsbad, CA) for GLT-1 labeling. After rinsing off the secondary antibodies, spinal cord sections were cover-slipped using Fluoro-Gel (Electron Microscopy Sciences; Hatfield, PA).

The ipsilateral and contralateral spinal dorsal horns of each section were digitally imaged at 200X magnification (1360x1024 pixels). Uniform-sized regions of interest (ROIs) of the superficial dorsal horn (laminae I-II; 150-250μm below the pial surface; 750x150 pixels) and deeper dorsal horn (laminae IV-V; 550-750μm below the pial surface; 600x320 pixels) were cropped from each image for standard analysis (Figure 1b). Quantitative densitometry was performed using a customized MATLAB script (R2012b; Mathworks, Inc.; Natick, MA) to measure the percentage of pixels in the ROIs labeled positively for CGRP or GLT-1 (Chang and Winkelstein, 2011; Dong et al., 2012; Nicholson et al., 2012, 2013a). In order to account for the unilateral nature of the injuries, the percentage of positive labeling determined on the ipsilateral dorsal horn in each region was normalized by the average percentage of positive pixels quantified on the contralateral side for each rat and in the superficial and deeper laminae, separately. One-way ANOVAs with post-hoc Tukey HSD tests were used to compare differences in CGRP and GLT-1 expression between groups for superficial and deeper dorsal horn, separately.

Spinal Neuronal Electrophysiology in Response to Painful Nerve Root Compression

Surgical Procedures & Behavioral Assessment. Two separate groups of rats (n=13/group) were used to investigate the effects of a painful mechanical compression on neuronal excitability and phenotype distribution in the spinal cord. Either a nerve root compression was applied for 15 minutes with a 98mN (10gram-force) clip to C7 (*compression*) or surgical *sham* procedures were applied, both as described above (Rothman and Winkelstein, 2007; Rothman et al., 2009; Chang and Winkelstein, 2011). Behavioral sensitivity was assessed at baseline and on days 1 and 7 after injury by measuring the number of withdrawals elicited by stimulation to the plantar surface of the bilateral forepaws with a 4gf von Frey filament (Nicholson et al., 2012; Kras et al., 2013a; Nicholson et al., 2013a). This method of assessing sensitivity to mechanical stimulation was chosen in order to match the methods of forepaw stimulation applied during the protocol for measuring the electrophysiological responses, which evaluated

neuronal firing evoked by a range of mechanical stimuli with the 4gf filament being an intermediate strength. Briefly, for behavioral assessments in this study, three rounds of 10 stimulations, with 10 minutes of rest between each round, were conducted in a given testing session. The total number of forepaw withdrawals in all three rounds was recorded for each forepaw of each rat on each day and the average in each of the *compression* and *sham* group was compared separately for ipsilateral and contralateral sides using two-way ANOVAs with t tests at each time point.

Electrophysiological Recordings & Data Analysis. Extracellular recordings were acquired in the superficial (n=8 rats/group) and deeper (n=5 rats/group) laminae of the ipsilateral dorsal horn at day 7 in order to assess the extent of neuronal excitability that may develop after a painful root compression. Recordings were made only on the ipsilateral side of the spinal cord because no changes have been detected in neuronal firing or phenotype in the contralateral side after painful nerve root compression (Syré et al., 2013). At the time the recordings were made, rats were anesthetized with sodium pentobarbital (45mg/kg) via i.p. injection with supplementary doses (5-10mg/kg) administrated as needed (Quinn et al., 2010; Crosby et al., 2013). The bilateral C6/C7 spinal cord was exposed by removing the overlaying tissue and bones using a laminectomy; the spinal cord was hydrated with 37°C mineral oil. Rats were immobilized on a stereotaxic frame using ear bars and a clamp attached to the T2 spinous process. Core body temperature was maintained at 35-37°C using a heat plate and a rectal probe (TCAT-2DF; Physitemp Instruments Inc.; Clifton, NJ). To minimize the amount of spinal cord movement due to respiration, a thoracotomy was performed and mechanical ventilation was provided by a mid-cervical tracheotomy (40-50 cycles/min; Harvard Small Animal Ventilator Model 683; Harvard Apparatus; Holliston, MA).

Extracellular potentials were recorded using a glass-insulated tungsten electrode (FHC; Bowdoin, ME) inserted vertically in the superficial (150-350μm below the pial surface) and deeper (450-1000μm below the pial surface) laminae of the spinal cord close to the C7 nerve root (Nicholson et al., 2011). Once a mechanosensitive neuron was identified by lightly brushing the plantar surface of the ipsilateral forepaw, a series of non-noxious and noxious mechanical stimuli was applied to the forepaw (Quinn et al., 2010; Crosby et al., 2013). The stimuli included 10 light brushes over a 10-second period, 5 repeated 1-second stimulations by a series of von Frey filaments (1.4, 4, 10, 26gf) spaced 1-second apart, and a 10-second noxious pinch by a 588mN (60gram-force) vascular clip (Roboz, Inc.; Gaithersburg, MD) (Figure 1c). The applications of the different stimuli were separated by 60 seconds and synchronized with a load cell to ensure consistent rate and duration of application. Signals recorded by the electrode were digitally sampled at 25kHz (MK1401; CED; Cambridge, UK), amplified with a gain of 3000 (ExAmp-20KB; Kation Scientific, Inc.; Minneapolis, MN), and processed by a 60Hz noise eliminator (Hum Bug; Quest Scientific; North Vancouver, BC) (Crosby et al., 2013). The depths of the electrode from which recordings were made were compared between the *compression* group and *sham* using a t-test in order to ensure consistent recording locations between groups.

The signals recorded during the stimulation of each neuron were spike-sorted using Spike2 software (CED; Cambridge, UK) to count the number of action potentials and ensure that firing from a single afferent was measured at a time (Quinn et al., 2010; Nicholson et al., 2012; Crosby et al., 2013). For the brushing stimulus, the number of evoked spikes was calculated as the sum of action potentials over the 10-second period of stimulation minus the baseline level of action potentials that were counted during the 10-second baseline period prior to any brushing. For the von Frey filament stimuli, the number of extracellular potentials was summed over the full 10-second period, including 5 pairs of a 1-second applied stimulation followed by a 1-second rest, and the number of spikes from the 10-second baseline period was subtracted from the spike count to determine the spikes evoked by these stimuli (Quinn et al., 2010; Nicholson et al., 2013a,b; Crosby et al., 2013). For the noxious pinch, the number of spikes was summed between 3-8 seconds after the clip was applied to exclude counting action potentials induced in the processes of applying and removing the clip (Quinn et al., 2010; Crosby et al., 2013; Nicholson et al., 2013a,b;). The number of pinch-evoked spikes was reported as the difference between the sum of action potentials during the stimulation period and the baseline firing that occurred within 5-seconds prior to the first stimulation in the spike count. All spike count data were log-transformed for statistical analysis due to a positively skewed distribution. To compare the differences in the number of action potentials evoked by each filament between *compression* and *sham* in each of the superficial and deeper laminae, separate two-way repeated measures ANOVA with Tukey's HSD post-hoc test were performed for each region of the dorsal horn, with group and filament strength as the factors.

Neurons were classified as either low-threshold mechanoreceptive (LTM), wide dynamic range (WDR) or nociceptive specific (NS), based on their response to the light brushing and the noxious pinch (Laird and Bennett, 1993; Hains et al., 2003; Quinn et al., 2010; Nicholson et al., 2013a). LTM neurons were taken as those that responded maximally to the light brushing; neurons that had a graded response were classified as WDR neurons and neurons responding only to noxious pinch were NS neurons (Woolf and Fitzgerald, 1983; Hains et al., 2003). Pearson's Chi-Squared test was used to compare the number of the different types of neurons between *compression* and *sham* identified in each of the superficial and deeper laminae during recordings, using separate tests.

RESULTS

Nerve root injuries with a compression component (*compression & combined injury*) induced immediate and sustained ipsilateral behavioral hypersensitivity that was not evident in *sham* or after a chemical irritation alone (Figure 2). The forepaw withdrawal threshold was not changed after *sham* compared to baseline for any day during the study period. In contrast, root compression induced a significant decrease in response threshold on the ipsilateral side for all 14 days relative to corresponding baseline responses ($p<0.011$) and *sham* ($p<0.005$) (Figure 2). Similarly, the *combined injury* produced behavioral

sensitivity that was significantly more than the *sham* ($p\leq0.05$) responses on all days, but not different from the compression alone. In contrast, *chemical irritation* caused only a transient behavioral hypersensitivity relative to *sham* that was significant ($p<0.026$) only at day 1 and day 3 (Figure 2).

CGRP expression was altered only in the superficial dorsal horn and not in the deeper laminae, and only after the insults to the nerve root that include a compressive component. At day 14, CGRP expression in the ipsilateral superficial dorsal horn was lower than expression levels on the contralateral side, but *only* after injuries that include a compressive component (*compression & combined injury*) and not after the *chemical irritation* alone (Figures 3 & 4). Compression alone (0.554 ± 0.464 fold change) and the combination of compression and chemical irritation (0.560 ± 0.403 fold change) induced similar decreases in CGRP expression in the ipsilateral C7 superficial laminae, which were significantly lower than the expression levels in *sham* (1.140 ± 0.761 fold change) ($p<0.005$) or after *chemical irritation* (1.167 ± 0.729 fold change) ($p<0.005$) (Figure 4a). In contrast, CGRP expression levels in the superficial dorsal horn did not differ between a *chemical irritation* and *sham* (Figure 4a). There were no differences in CGRP expression in the deeper laminae of the dorsal horn between any of the injury groups (Figures 3 & 4b).
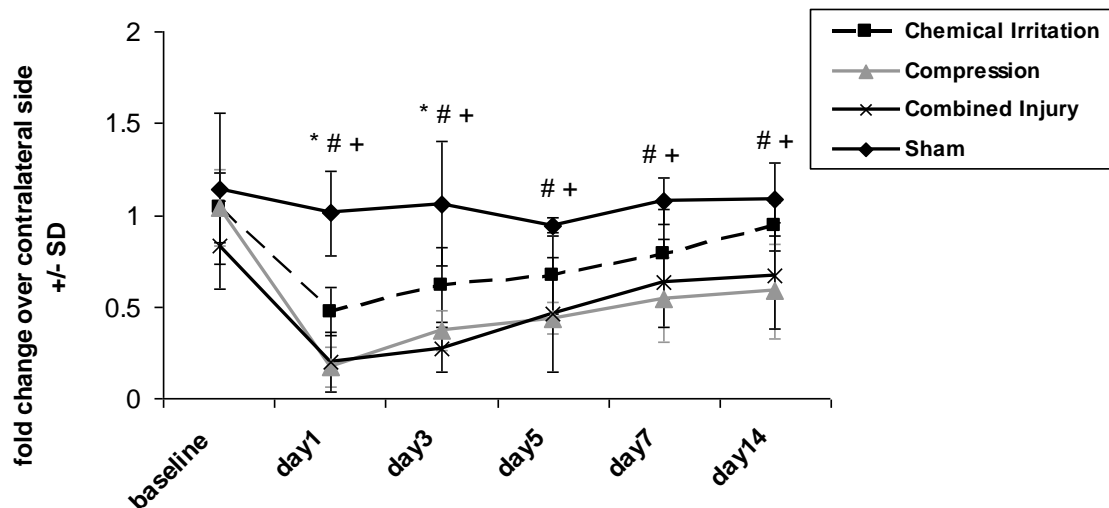

Figure 2. Behavioral sensitivity in the ipsilateral forepaw measured as a fold-change relative to the contralateral forepaw for 14 days after each of the unilateral nerve root injuries. A value of 1 indicates a response that is unchanged from contralateral values and a decrease below 1 indicates an increase in sensitivity. For all days, the fold-change significantly decreases after *compression* relative to its baseline and *sham* (#$p<0.011$), and after *combined injury* compared to *sham* (+$p\leq0.05$). In contrast, *chemical irritation* alone only induces significant changes from *sham* on days 1 and 3 (*$p<0.026$). Error bars represent the standard deviations.
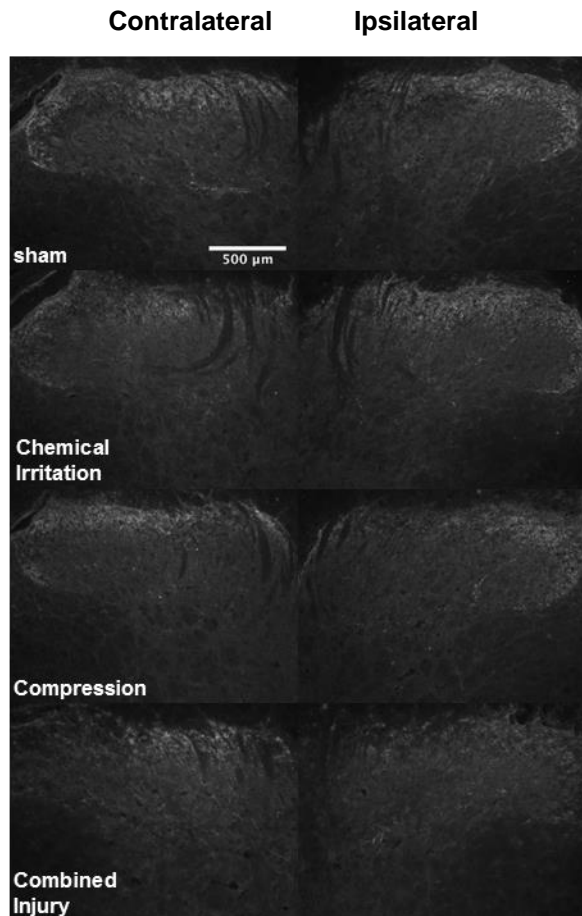
Figure 3. Representative images showing CGRP expression in the ipsilateral and contralateral C7 spinal dorsal horns at day 14 after the different nerve root injuries. Representative images show ipsilateral CGRP labeling is lower than the contralateral levels in the superficial but not deeper dorsal horn after *compression* and *combined injury*. Ipsilateral and contralateral spinal CGRP is similar after *sham* and *chemical irritation*. The scale bar (500μm) applies to all panels.

Similar to the selective decrease in expression of spinal CGRP after compression alone and the combined injury (Figure 4), the expression of GLT-1 in the superficial laminae was changed *only* by injuries having a compressive component (Figures 5 & 6). Expression of GLT-1 on the ipsilateral side was reduced relative to the contralateral levels after *compression* and the *combined injury*, and only in the superficial dorsal horn and not in the deeper dorsal horn (Figures 5 & 6). Rats undergoing a nerve root injury with *compression* alone (0.562±0.416 fold change) or the *combined injury* (0.565±0.480 fold change) exhibited significantly less GLT-1 expression in the ipsilateral superficial laminae than
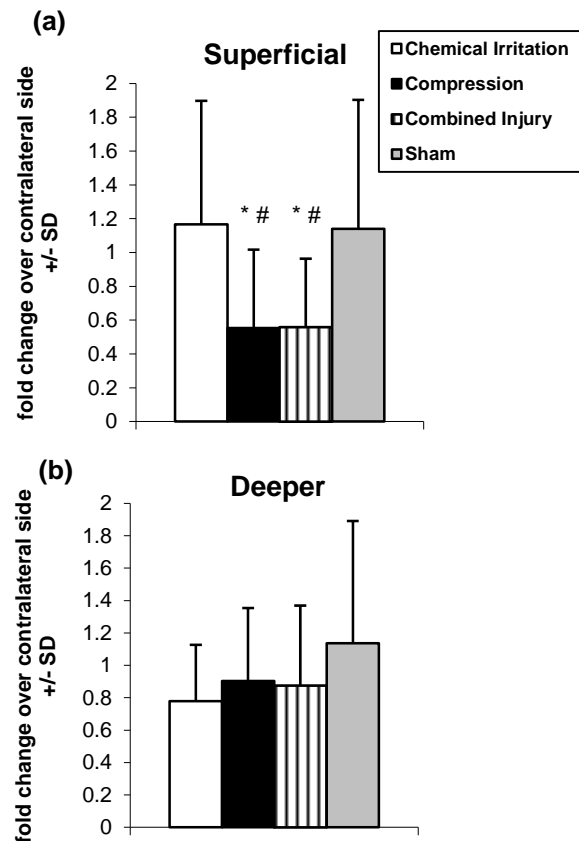
Figure 4. Quantification of CGRP expression in the superficial and deeper dorsal horns at day 14 after the different root injuries. CGRP expression is quantified as the ratio of percent-positively labeled pixels in the ROI on the ipsilateral side normalized to the contralateral side. **(a)** CGRP expression levels in the superficial dorsal horn are significantly lower after *compression* and *combined injury* compared to levels after *sham* (*$p<0.005$) and *chemical irritation* (#$p<0.005$). **(b)** There are no differences in CGRP expression between any of the groups in the deeper dorsal horn. Error bars represent the standard deviations.

sham (1.114±1.109 fold change) ($p<0.037$) (Figure 6a). Although GLT-1 expression after a *chemical insult* (0.828±0.444 fold change) was elevated above the other injury groups, it was not statistically different from any group. As with CGRP expression (Figure 4b), there were no differences in GLT-1 expression in the deeper dorsal horn detected between any of the groups (Figure 6b).

In the electrophysiology study, nerve root compression was found to elevate behavioral sensitivity at day 7 (Figure 7). But, this increase in

Contralateral   Ipsilateral
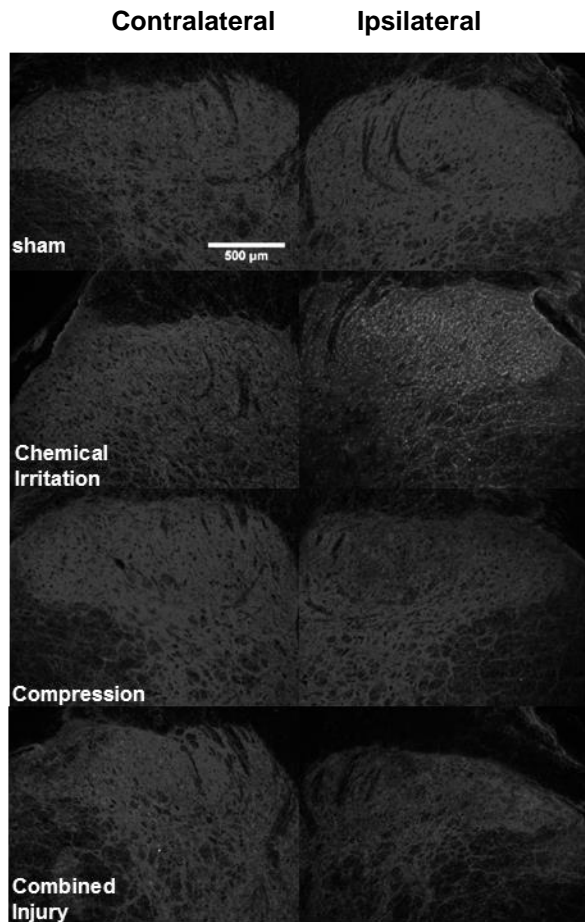
Figure 5. Representative images showing GLT-1 expression in the bilateral C7 spinal dorsal horns at day 14 after *sham*, *chemical irritation*, *compression* and *combined injury*. GLT-1 labeling on the ipsilateral side is lower than the contralateral side in the superficial, but not deeper, dorsal horn after *compression* and *combined injury*. Ipsilateral and contralateral spinal GLT-1 are similar after *sham* and *chemical irritation*. The scale bar is 500μm and applies to all panels.

sensitivity was not paralleled by a mechanically-evoked increase in firing rate of neurons in the superficial laminae at this same time point (Figure 8); in contrast, it did correspond to an increase in such evoked neuronal firing rate in the deeper laminae of induced tactile sensitivity at day 1 that also was evident at day 7 (Figure 7). No significant differences were detected between the baseline response and those at day 1 and day 7 in the *sham* group. However, rats receiving the compression injury developed ipsilateral behavioral sensitivity, quantified by the number of forepaw withdrawals, that was significant when compared to their corresponding baseline responses (0.6±0.9 withdrawals) (p<0.0001) and the *sham* (p<0.0001) group, at days 1 (*compression*
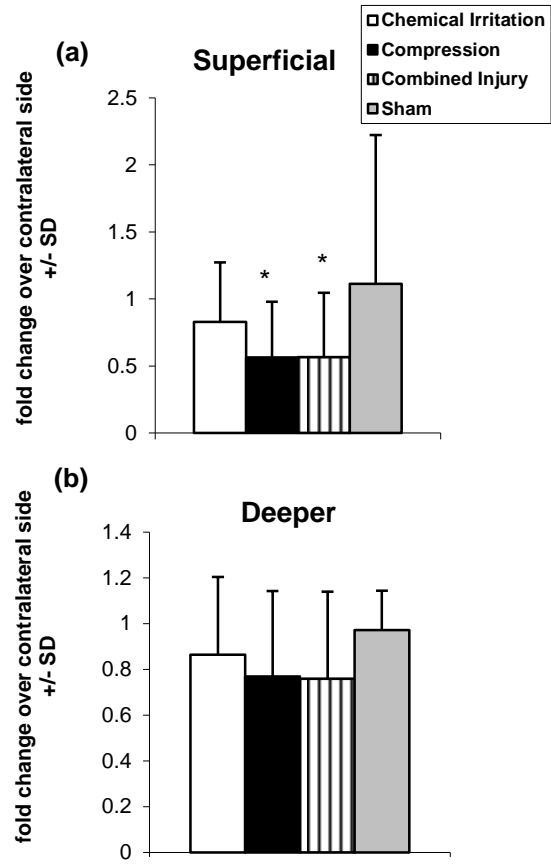
Figure 6. Quantification of GLT-1 labeling in the superficial and deeper dorsal horns of the ipsilateral spinal cord relative to expression in the contralateral side. **(a)** GLT-1 labeling in the superficial dorsal horn decreases significantly after *compression* and the *combined injury* relative to *sham* (*p<0.037). **(b)** No difference in GLT-1 expression is observed between any groups in the deeper dorsal horn. Error bars represent the standard deviations

5.3±1.5 withdrawals; *sham* 1.6±0.7 withdrawals) and 7 (*compression* 4.9±1.4 withdrawals; *sham* 1.0±0.8 withdrawals) (Figure 7). There were no differences in the mechanical sensitivity on the contralateral side between *sham* and *compression* (data not shown), consistent with the first study (Figure 2).

After behavioral testing on day 7, a total of 145 neurons were recorded at average depths of 264±90μm (superficial dorsal horn recordings) and 635±120μm (deeper dorsal horn recordings) in both the *compression* and *sham* groups. The recording depths in each region were not different between *sham* and *compression* for either the superficial (*sham* 271±85μm; *compression* 258±95μm) or deeper (*sham* 630±143μml *compression* 640±95μm)

recordings. Although the number of evoked spikes was increased after *compression* compared to *sham* for forepaw stimulation by 10 and 26gf filaments in the superficial laminae (Figure 8a) and by all filaments in the deeper laminae (Figure 9a), this increase in the *compression* group was only significantly greater (p=0.045) than *sham* in the deeper laminae.

Interestingly, while increased neuronal hyperexcitability was detected only in the deeper dorsal horn after painful nerve root compression (Figure 9a), a significant shift in the distribution of neuronal phenotypes was only detected in the superficial dorsal horn (Figures 8b & 9b). Specifically, the relative number of neurons in the superficial dorsal horn classified as LTM neurons

after *sham* decreased after *compression*, with an associated increase in the number of neurons identified as WDR neurons (Figure 8b). The percentage of WDR neurons increased significantly (p=0.002) from 43% after *sham* to 74% after nerve root *compression*. Only 10% of the neurons were classified as LTM neurons in the *compression* group, which was significantly lower than the percentage after *sham* (38%; p=0.002). The proportion of NS neurons in the superficial dorsal horn remained unchanged after *compression* compared to *sham*. Although there was a slight increase in the percentage of neurons in the deeper laminae that were identified as WDR after painful *compression*, this was not significant compared to *sham* (Figure 9b).
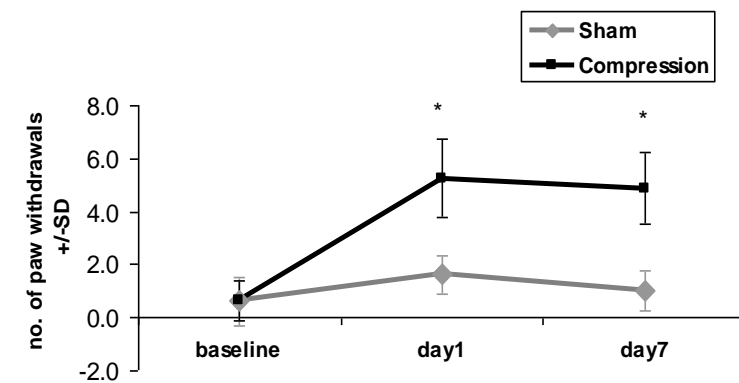
Figure 7. Behavioral sensitivity in the ipsilateral forepaw after nerve root *compression* or *sham* for 7 days. The number of paw withdrawals elicited by stimulation with a 4gf filament is shown; the greater the number of withdrawals, the more sensitivity is present. *Compression* induces significantly more paw withdrawals than at baseline (p<0.0001) and compared to *sham* (*p<0.0001) on both day 1 and day 7. Error bars represent the standard deviations.
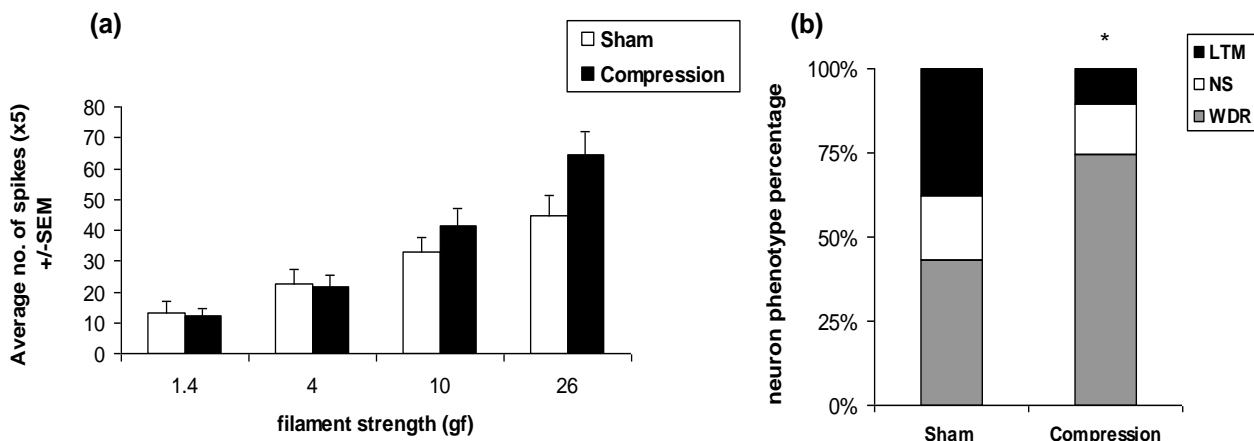
Figure 8. Electrophysiological responses in the superficial dorsal horn at day 7. **(a)** Evoked firing, quantified as the number of spikes, in the ipsilateral superficial dorsal horn after *compression* and *sham*. No difference in evoked firing is evident between the *compression* and *sham*. Error bars represent the standard error of the mean. **(b)** The distribution of neurons classified by phenotype (LTM, NS, WDR) at day 7 after *compression* and *sham*. After a painful compression, the proportion of neurons classified as LTM or WDR differs significantly (*p=0.002) from that in *sham*. There is no difference in the distribution of NS neurons.
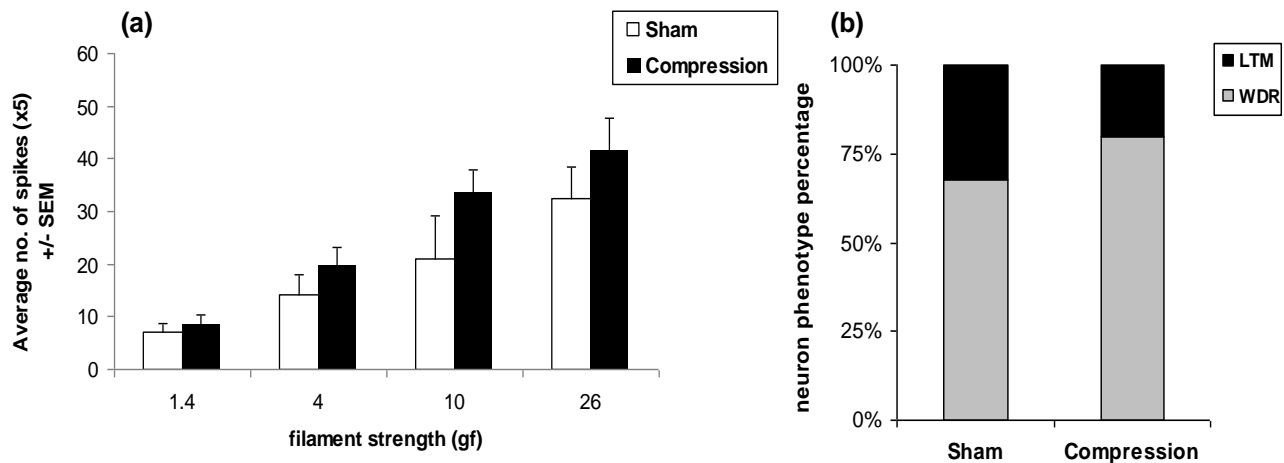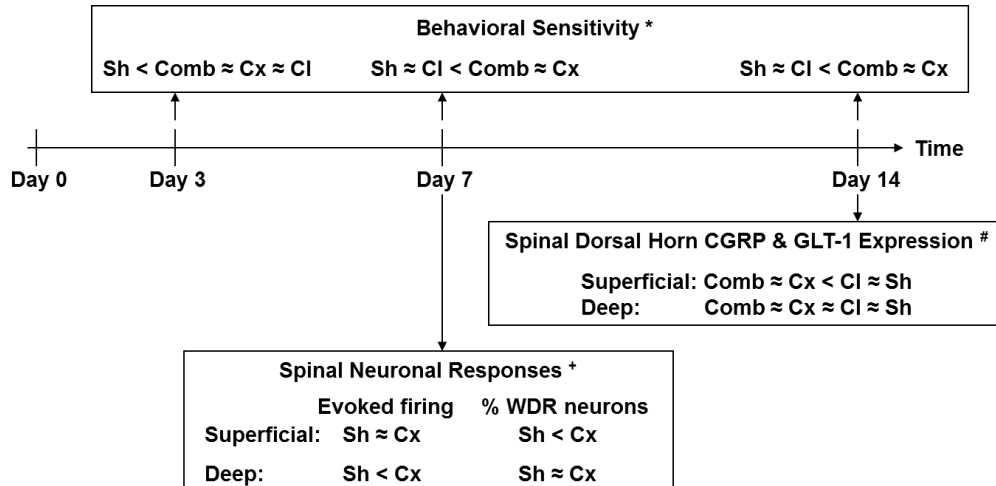
Figure 9. Electrophysiological responses in the deeper dorsal horn at day 7. **(a)** Overall, significantly more (p=0.045) spikes are evoked in the *compression* group than *sham*. Error bars represent the standard error of the mean. **(b)** The distribution of neurons classified by phenotype is not different between groups; no NS neurons were found in the deeper dorsal horn of the spinal cord.

DISCUSSION

Many studies have shown the potent roles of mechanical loading and chemical irritation to the nerve root in producing and modulating radicular pain (Maves et al., 1993; Kawakami, et al., 1994; Kajander et al., 1996; Hashizume et al., 2000; Hunt et al., 2001; Rutkowski et al., 2002; Cornefjord et al., 2004; Winkelstein and DeLeo, 2004; Rothman and Winkelstein, 2007; Hubbard et al., 2008a,b; Chang and Winkelstein, 2011; Nicholson et al., 2012). However, this study is the first to specifically delineate the relative contribution(s) of mechanical and chemical nerve root insults on the spinal regulation of neuronal activity in the context of radicular pain. In particular, mechanical compression (*compression*, *combined injury*) induces prolonged effects on mechanical sensitivity, while chemical irritation alone only induces transient behavioral hypersensitivity (Figures 2 & 10). Of note, these differences in behavioral outcomes between the four injury groups used here have been reliably produced using different assessment methods, with consistent trends as those observed here (Rothman and Winkelstein, 2007; Hubbard et al., 2008a; Chang and Winkelstein, 2011). Moreover, all of the assessment methods are objective and were performed by assessors who were blinded to the individual groups. This approach enables unbiased assessment of the behavioral outcomes, as well as the immunohistochemical and neurophysiological responses. CGRP and GLT-1 expression in the superficial dorsal horn (Figures 3-6 & 10) followed the same pattern as behavior with only those injuries

with mechanical loading to the root inducing significant spinal modifications at day 14. This implies that mechanically-induced modulation of CGRP and GLT-1 in the superficial spinal dorsal horn after root compression may partially contribute to the persistence of pain symptoms after this type of injury.

All of the spinal modifications– CGRP expression, GLT-1 expression, neuronal firing and phenotype – exhibit regional variation (Figures 4, 6, & 8-10). CGRP and GLT-1 are down-regulated in the superficial dorsal horn, but remain unchanged from *sham* in the deeper dorsal horn at day 14 (Figures 4 & 6). Similarly, and paralleling the maintenance of mechanical allodynia, the neurons in the superficial laminae show a phenotypic shift at day 7 from LTM to WDR neurons only after mechanical root loading (Figure 8b), suggesting that superficial dorsal horn WDR neurons play a role in nociception that leads to behavioral hypersensitivity after root compression. Although this same phenotypic switch is not observed in the deeper laminae (Figure 9b), the constituent neuronal populations in the deeper laminae are more sensitive to mechanical stimulation, as evidenced by the significant increase in activity in response to stimuli after root compression (Figure 9a). These studies demonstrate that neuronal and glutamatergic responses are differentially mediated across the laminae of the dorsal horn (Figures 3-6 & 8-10). However, because of the interconnections between neurons and astrocytes throughout and across the dorsal horn, the decrease in CGRP, down-regulation of GLT-1 and increase in WDR neurons in

Figure 10. Summary of key temporal findings, including behavioral sensitivity, spinal CGRP and GLT-1 expression, and spinal electrophysiological responses over 14 days after different nerve root insults. CI=chemical irritation; Cx=compression; Comb=combined injury; Sh=sham. Specific data are referenced to other figures: (*) Figures 2 & 7; (#) Figures 3-6; (+) Figures 8 & 9.

the superficial dorsal horn may contribute, or respond to, the neuronal hyperexcitability that is present in the deeper laminae.

Our findings that mechanical, but not chemical, nerve root insults produce sustained mechanical hyperalgesia and down-regulate spinal CGRP and GLT-1 at day 14 (Figures 2 & 4) are consistent with reports that behavioral sensitivity is altered and spinal inflammation is more robust following mechanical deformation of the root or its combined mechanical and chemical insults compared to chemical irritation alone (Olmaker et al., 1997; Olmarker and Myers, 1998; Kawakami et al., 2000; Hou et al., 2003; Rothman and Winkelstein, 2007; Rothman et al., 2009; Chang and Winkelstein, 2011). For example, root compression is required to induce myelin degeneration, up-regulate expression of SP within the nerve root and facilitate the infiltration of phagocytotic macrophages around the root (Chang and Winkelstein, 2011). Similarly, microglial activation in the spinal cord is sensitive to the type of insult, with compressive trauma being required to induce sustained microglial activation at day 7 (Rothman and Winkelstein, 2007). The compression-dependent behavioral, glial and neuronal outcomes (Figures 2-9) (Rothman and Winkelstein, 2007; Rothman et al., 2009; Chang and Winkelstein, 2011) are likely initiated within minutes-to-hours after root injury. Within 1 hour of a painful nerve root compression, the inflammatory cytokine IL-6 is upregulated in the spinal cord, but is unchanged after a painful inflammatory insult (Rothman et al., 2009).

These findings suggest a more critical role of mechanical compression than chemical irritation in the initiation and maintenance of radicular pain in this injury model.

Modulation of spinal CGRP and GLT-1 not only require mechanical compression to the root, but are sensitive to the compression loading profile (i.e. load and duration). Using the same model of cervical radiculopathy as in the current study, spinal CGRP expression has been shown to be sensitive to the magnitude of applied compression, while spinal GLT-1 depends on the compression duration (Kobayashi et al., 2005; Hubbard et al., 2008a; Nicholson et al., 2013b). Spinal CGRP on the ipsilateral side only decreases after a 15 minute compression when the force is above 19.52mN (Hubbard et al., 2008a). Likewise, there is a duration threshold between 3 and 15 minutes for inducing decreased spinal GLT-1 at day 7 after a 98mN compression (Nicholson et al., 2013b). Spinal CGRP and GLT-1 are both modulated, in part, by inputs from primary afferents (Kobayashi et al., 2005; Yang et al., 2009; Ghosh et al., 2011). CGRP is produced in the DRG and transported to the spinal cord by axons in the nerve root (Kobayashi et al., 2005). Astrocytes require neuronal input to express GLT-1 (Yang et al., 2009; Ghosh et al., 2011). Interestingly, damage to these primary afferents is modulated by the magnitude and duration of compression, and the mechanical threshold for initiating such damage is similar to those described above for CGRP and GLT-1 (Hubbard and Winkelstein, 2008; Nicholson et al.,

2011). Above 31.6mN, the cytoskeletal axonal protein, NF200, is reduced in the nerve root after a 15 minute compression (Hubbard and Winkelstein, 2008). Yet, if a suprathreshold (98mN) compression is applied to the root for only 3 minutes, the axons retain their normal morphology (Nicholson et al., 2011), indicating that the magnitude threshold varies with the compression duration. In a porcine model, the magnitude and duration thresholds to produce injury in the nerve roots of the cauda equina decrease with increasing rates of compression (Olmarker et al., 1989). Given that the nerve root axons are sensitive to the combined contribution of the duration, magnitude and rate of compression and that their damage likely precipitates the down-regulation of spinal CGRP and GLT-1 (Olmarker et al., 1989; Hubbard and Winkelstein, 2008; Nicholson et al., 2011), both of these proteins may also be sensitive to these mechanical parameters. The relationship between the primary afferents and spinal neurotransmitters and transporters, may have a central role in mechanotransduction of the nerve root tissue loading into the pain that develops (Olmarker et al., 1989; Hubbard and Winkelstein, 2008; Yang et al., 2009; Ghosh et al., 2011; Nicholson et al., 2011).

The load magnitude of nerve root compression controls the development of persistent behavioral sensitivity (Hubbard et al., 2008a). For the same 15 minute cervical root compression used in this study, load thresholds of 38.16mN and 38.26mN have been defined for producing mechanical allodynia in the ipsilateral and contralateral forepaws, respectively at day 7 (Hubbard et al., 2008a). Although spinal CGRP decreases proportionally to the applied load, the loads necessary to induce a significant decrease in the corresponding superficial dorsal horns are lower than those for producing bilateral mechanical allodynia (Hubbard et al., 2008a). Therefore, down-regulation of spinal CGRP in the superficial dorsal horn may contribute to the development of radicular pain, but likely is not the only regulator of this since a load that is large enough to produce a reduction in CGRP may not also induce pain. The load thresholds and load-dependency necessary to modulate spinal CGRP and induce behavioral sensitivity together imply that loading parameters have an influence on mechanotransduction in the nerve root and directly modulate spinal responses to injury which may directly impact the behavioral outcomes.

Injury-induced changes in spinal CGRP expression exhibit distinct spatiotemporal patterns that differ between the superficial and deeper lamina across different animal models of pain (Sluka and Westlund, 1993; Miki et al., 1997; Kobayashi et al., 2005;

Hubbard et al., 2008a; Zheng et al., 2008; Nishigami et al, 2009; Nicholson, et al., 2013a). Although compression or transection of the nerve root decreases CGRP in the superficial dorsal horn (Villar et al., 1991; Hubbard et al., 2008b), spinal cord and peripheral nerve injuries are associated with an *increase* in this same region (Figures 3 & 4) (Krenz and Weaver, 1998; Zheng et al., 2008). Furthermore, there is no change in spinal CGRP in the deeper laminae at day 14 after any root injury (Figure 4b); yet, CGRP increases in laminae III-V at this same time after a sciatic nerve crush (Zheng et al., 2008). In addition to exhibiting spatial variability, the distribution of CGRP after a nerve root compression varies temporally. A root compression with the same mechanical profile as this study produces a transient increase in CGRP expression in the deeper laminae at day 7 (Nicholson et al. 2013b), suggesting that a painful chemical irritation to the root also may have modified spinal CGRP at earlier time-points than probed here. In fact, spinal CGRP transiently decreases after spinal cord transection and transiently increases after sciatic nerve crush (Krenz and Weaver, 1998; Zheng et al., 2008). It is necessary to assess spinal expression of CGRP at earlier and later time-points in order to fully determine the extent to which it is modified after a painful chemical insult to the nerve root. Although expression of CGRP in the deeper dorsal horn has not been evaluated at day 1 after a painful nerve root compression, CGRP is unchanged in the superficial dorsal horn compared to *sham* at this time point (Rothman et al., 2005); it is likely, therefore, that the redistribution of CGRP in both the superficial and deeper dorsal horns does not develop until sometime between days 1 and 7 after a nerve root compression.

Increased synaptic glutamate in the spinal dorsal horn is associated with pain states (Basbaum et al., 2009). Due to difficulties in direct measurement of synaptic glutamate concentration, modifications of the glutamatergic system were examined here via measurement of GLT-1. Like CGRP, the expression of spinal GLT-1 varied by region and injury type. Decreased expression of GLT-1 in the superficial dorsal horn corresponds to prolonged mechanical hyperalgesia at day 14 and was observed only after nerve root injuries that included a compressive component (Figures 2 & 6). Altered GLT-1 expression has been reported to develop between days 1 and 7 (Nicholson et al., 2013b); GLT-1 in the superficial laminae is unchanged at day 1, but significantly decreases relative to *sham* by day 7. Since nerve root-induced pain is initiated by day 1 after injury (Figures 2 & 7), GLT-1 may play a more

substantial role in the maintenance, but not the induction, of cervical radicular pain.

The reduction of spinal GLT-1 (Figures 5 & 6) may be partially attributed to altered afferent signaling, but may also result from increased utilization of glutamate by glutamate receptors in the superficial laminae. Spinal metabotropic glutamate receptors (mGluRs) are upregulated after both peripheral nerve and cervical nerve root compression injuries (Hudson et al., 2002; Dong and Winkelstein, 2010; Nicholson et al., 2012). Their up-regulation after a nerve root compression may enhance the uptake of glutamate requiring less GLT-1 to maintain the homeostasis of extracellular glutamate in the superficial dorsal horn. Although the signaling cascades that down-regulate spinal GLT-1 after a painful root compression are still undefined, they are likely initiated at the time of injury. Neuronal signaling through the afferents is disrupted almost immediately during a nerve root compression (Nicholson et al., 2011), with mechanically-evoked spikes in the superficial spinal dorsal horn being maximally reduced within 6.6±3.0 minutes into a 98mN compression (Nicholson et al., 2011). A compression applied for 3 minutes (which is below that critical compression threshold) does not induce behavioral sensitivity or changes in spinal GLT-1 at day 7 (Rothman et al., 2010; Nicholson et al., 2013b). Since astrocytic expression of GLT-1 depends on afferent input (Yang et al., 2009; Ghosh et al., 2011), it is possible that the changes in afferent signaling that are observed during the applied compression directly alter neuronal signaling in the spinal cord which leads to the long-term modifications of astrocytic GLT-1 that are associated with persistent nerve root mediated pain (Figures 2 & 6). Additional studies directly measuring glutamate are needed to define the mechanisms that alter GLT-1 expression for this and other neural traumas that cause pain and dysfunction.

Neuronal hyperexcitability and phenotype exhibited regional differences in the spinal dorsal horn after painful root compression (Figures 8-10). In the superficial laminae, there is no overall difference in neuronal firing rates between the *compression* and *sham* groups, but a significant increase in WDR neurons was observed (Figure 8). Our findings are consistent with a report that the frequency of excitatory post-synaptic potentials does not increase after a painful lumbar nerve root ligation (Terashima et al., 2011). That study did demonstrate that the amplitude of the action potentials increases (Terashima et al., 2011), suggesting that nerve root injury changes the properties of synapses in this region of the spinal cord. This synaptic plasticity may

contribute to the overall increase in WDR neurons observed in the superficial dorsal horn (Figure 8) (Kohno et al., 2003; Keller et al., 2007). As we observed (Figure 8b), an increase in WDR neurons is accompanied by a decrease in LTM neurons for cases of chronic allodynia after spinal cord injury (Hao et al., 2004). This supports the growing evidence that WDR neurons mediate neuropathic pain (Hao et al., 2004; Nishigami et al., 2009; Quinn et al., 2010; Liu et al., 2011). An increase in the percentage of WDR neurons in the superficial laminae indicates a potential increase in the number of neurons in that region of the dorsal horn that respond to noxious stimuli, which may augment afferent signaling and facilitate nociception (Hains et al., 2003; Quinn et al., 2010).

In contrast to the neurons in the superficial dorsal horn, the number of spikes evoked by a range of stimulations to the forepaw was significantly increased after *compression* compared to *sham* at day 7 in the deeper dorsal horn (Figure 9a). Although the percentage of WDR neurons increased in those laminae, it was not significant (Figure 9b). However, recent studies report that enhanced post-synaptic firing rates are also accompanied by an increase in the proportion of WDR neurons at day 14 after a painful nerve root compression (Syré et al., 2013). It is possible that the lack of significance detected in phenotypic changes in our study may be due to that time point being too early to capture the entirety of the shift or to the small number of neurons (51 WDR neurons and 18 LTM neurons) recorded in the deeper dorsal horn. Therefore, even though most nociceptors synapse in the superficial dorsal horn, the current study adds to a growing body of literature suggesting that deeper dorsal horn neurons also have a significant contribution to pain sensation (Figure 9) (Palecek et al., 1992; Chang et al., 2009; Quinn et al., 2010; Crosby et al., 2013).

Although the mechanism by which nerve root injuries lead to pain has not been well defined, this study, together with the literature, begins to characterize the integrated axonal, neuronal and inflammatory responses which contribute to the onset and maintenance of radicular pain. At day 1 after painful cervical root compression (with or without a chemical insult), despite elevated behavioral sensitivity (Figures 2, 7, 10), macrophage infiltration and axonal degeneration are not observed at the injury site, nor is there any difference in spinal GLT-1 expression (Hubbard and Winkelstein, 2008; Nicholson et al., 2013b). By day 7, however, when behavioral sensitivity still remains (Figures 2, 7, 10), there is extensive axonal damage in the compressed

nerve root, as well as glial activation and decreased CGRP and GLT-1 expression (Figures 3-6) (Hubbard et al., 2008b; Hubbard and Winkelstein, 2008; Chang and Winkelstein, 2011; Nicholson et al., 2011, 2013a,b). In contrast, chemically-induced root injury does not induce sustained behavioral sensitivity, macrophage infiltration into the root, nor axonal degeneration (Chang and Winkelstein, 2011); that type of injury also does not change spinal CGRP or GLT-1 expression at day 14 (Figures 2-6, 10). It is important to note that conclusions drawn here regarding the behavioral and spinal biochemical outcomes reflect the sample sizes used in our study. Indeed, power analyses (with 0.8 power) indicate 4-5 rats are need to determine behavioral differences and these studies have a power of 0.921, which both substantiate our finding of only transient sensitivity after a chemical insult (Figure 2). In contrast, depending on the spinal region and outcome measure used (CRGP, GLT-1), as many as 15 rats are required, with the greatest number being needed for analyses of GLT-1 the deeper laminae, which exhibits a large variability across all of the groups (Figures 4 & 6). Indeed, the tests reported for those assessments have powers of 0.993 and 0.791 for CGRP and GLT-1 expression, respectively; showing the results reflect meaningful trends. The integration of quantitative and sensitive electrophysiology methods along with the immunohistochemical techniques strengthens the findings related to compression but does not eliminate the caution needed for interpreting the meaning of the lack of differences between chemical insult and sham. Nonetheless, similar lack of differences have been reported for other outcomes at other time points (Rothman and Winkelstein, 2007; Chang and Winkelstein, 2011).

Despite CGRP and GLT-1 expression not changing in the deeper laminae of the dorsal horn (Figures 4b & 6b), neuronal hyperexcitability and phenotypic changes are evident after a painful nerve root compression in the deeper dorsal horn neurons (Figure 9a) (Syré et al., 2013), which may be promoted by changes in neuronal electrophysiology in the superficial dorsal horn via spinal circuits (Suzuki et al., 2002). Inflammatory responses, including macrophage infiltration, are evident at days 7 and 14 after cervical nerve root compression and are known to contribute to the maintenance of radicular pain (Marchand et al., 2005; Thacker et al., 2007; White et al., 2009). The transient behavioral hypersensitivity that is produced after a chemical root insult may result from the early transient increase in pro-inflammatory cytokines in the spinal cord and DRG (Figure 2) (Rothman et al., 2009). Although

myelin degeneration is not produced after that insult (Chang and Winkelstein, 2011), spinal CGRP and GLT-1 are also not altered nor is behavioral hypersensitivity evident at day 14 (Figures 2-6), bilateral increases in macrophages and enhanced support of Schwann cells to axons have been observed in the affected root (Chang and Winkelstein, 2011). Mechanical insults have more robust and prolonged effects on axonal damage, nociceptive neuropeptides and neuronal signaling that contribute to pain. However, the extent of nociceptive signaling and pain may vary based on the loading paradigm, including the load type (compressive or tensile), load magnitude and duration and strain rate (Pedowitz et al., 1992; Singh et al., 2006; Hubbard et al., 2008a,b; Nicholson et al., 2012).

CONCLUSION

Mechanical compression, but not chemical irritation, of the cervical nerve root produces behavioral hypersensitivity that is sustained for 2 weeks and is accompanied by altered neurotransmitter, transporter expression and neuronal activity for up to 2 weeks after the initial injury. Only those injuries with a compressive component down-regulate CGRP and GLT-1 expression in the superficial dorsal horn at day 14 when behavioral sensitivity is still present (Figure 10), indicating that root injuries involving a mechanical compression influence the neurotransmitter systems that may contribute to the maintenance of radicular pain. Notably, the lack of change from sham levels for the chemical insult may reflect the methodology for assessing CGRP and GLT-1 expression, the late time point (day 14) and/or the relatively small sample size. Regardless, a painful nerve root compression differentially modulates CGRP, GLT-1, neuronal excitability, and neuronal phenotype in the superficial and deeper dorsal horns of the spinal cord (Figure 10). In the superficial dorsal horn, where nociceptive neuropeptides are predominantly released by primary afferents to signal post-synaptic neurons, CGRP and GLT-1 expression is decreased and this is accompanied by a phenotypic shift towards WDR neurons. Therefore, neurons in the superficial dorsal horn likely contribute to nociception by increasing the number of neurons that respond to noxious stimuli, as evidenced by the shift from LTM to WDR neurons after nerve root compression injuries. This phenotypic shift may be due to changes in the normal neurotransmitter levels, based on the decrease in CGRP and the glutamate transporter (GLT-1). In contrast, neurons in the deeper dorsal horn may contribute to persistent pain by increasing their firing rate without modifying their expression of CGRP and without any changes in the

glial expression of GLT-1. In conclusion, cervical nerve root compression, but not chemical irritation, is sufficient to modulate prolonged radicular pain via disparate spinal regulations of nociceptive neuropeptides and neuronal excitability in the superficial and deeper dorsal horn.

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