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Development of a Duration Threshold for Modulating Evoked Neuronal Responses After Nerve Root Compression Injury

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ABSTRACT – Cervical nerve roots are susceptible to compression injuries of various durations. The duration of an applied compression has been shown to contribute to both the onset of persistent pain and also the degree of spinal cellular and molecular responses related to nociception. This study investigated the relationship between peripherally-evoked activity in spinal cord neurons during a root compression and the resulting development of axonal damage. Electrically-evoked spikes were measured in the spinal cord as a function of time during and after (post-compression) a 15 minute compression of the C7 nerve root. Compression to the root significantly ($p=0.035$) reduced the number of spikes that were evoked over time relative to sham. The critical time for compression to maximally reduce evoked spikes was 6.6±3.0 minutes. A second study measured the post-compression evoked neuronal activity following compression applied for a shorter, sub-threshold time (three minutes). Ten minutes after compression was removed, the discharge rate remained significantly ($p=0.018$) less than baseline by 58±25% relative to sham after the 15 minute compression, but returned to within 3±33% of baseline after the three minute compression. Axonal damage was evident in the nerve root at day seven after nerve root compression only after a 15 minute compression. These studies demonstrate that even a transient mechanical insult to the nerve root is sufficient to induce sustained neuronal dysfunction and axonal pathology associated with pain, and results provide support that such minor neural tissue traumas can actually induce long-lasting functional deficits.

KEYWORDS – nerve root, compression, electrophysiology, axon, injury

INTRODUCTION

Neck pain has an annual incidence of 14-50% and an estimated two-thirds of the population will experience neck pain at least once in their lives (Côté et al. 1998; Côté et al. 2004; Hogg-Johnson et al. 2008). As many as one-half of the reported neck pain cases persist for at least one year (Hill et al. 2004). Neck pain accounts for up to 11% of claims submitted for loss time from work (Côté et al. 2008) and health care costs for individuals with spine-related pain have been reported to be nearly 70% greater than for those individuals without pain (Martin et al. 2008).

Although neck pain can originate from a variety of spinal tissues, the cervical nerve roots have been shown to be vulnerable to injury resulting from foraminal impingement, disc herniation, direct spinal trauma, and/or foraminal stenosis (Cornefjord et al. 1997; Krivickas and Wilbourn 2000; Nuckley et al. 2002; Olmarker et al. 1989; Panjabi et al. 2006; Wainner and Gill 2000). The effect of compression duration is of particular relevance to nerve root injury because the nerve root can be susceptible to both sustained and transient mechanical loading (Abbed and Coumans 2007; Panjabi et al. 2006; Svensson et al. 1993). Sustained nerve root loading is associated with a disc herniation and spondylosis (Abbed and Coumans 2007; Wainner and Gill 2000), while transient loading to the root can result from sports and automotive traumas (Krivickas and Wilbourn 2000; Panjabi et al. 2006; Stuber 2005; Tominaga et al. 2006). The clinical course of symptoms and rate of recovery have been reported to differ based on different modes of loading to the nerve root (Abbed and Coumans 2007). Pain and weakness associated with a transient root insult exhibit varied responses which can resolve within minutes or persist for as long as five months (Krivickas and Wilbourn 2000;

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Wainner and Gill 2000). Although nerve root injuries may result from a variety of loading scenarios and the severity of the symptoms may differ considerably, the relationship between the duration of the nerve root compression, pathophysiology and symptoms remain poorly defined.

Several animal models of radiculopathy demonstrate that compression to the nerve root produces pain symptoms and that the biomechanics of loading (i.e. magnitude, duration, rate) to the nerve root modulate both the extent of the local tissue damage in the root, and the degree and duration of the pain symptoms (Hubbard et al. 2008b; Kobayashi et al. 2005; Olmarker et al. 1989; Rothman et al. 2010; Rydevik et al. 1991; Winkelstein et al. 2002). For example, larger magnitudes of compression to the nerve root increase mechanical sensitivity in behavioral tests and reduce axonal transport in the compressed root (Hubbard et al. 2008b; Kobayashi et al. 2005; Winkelstein et al. 2002). Both rate and magnitude each contribute to increased edema in the nerve root, such that the magnitude of pressure required to produce edema decreases for higher application rates (Olmarker et al. 1989; Rydevik et al. 1991). Although animal models of nerve root compression have shown that sustained behavioral sensitivity (i.e. pain) can develop after a chronic nerve root compression (Colburn et al. 1999; Hashizume et al. 2000; Winkelstein et al. 2002; Winkelstein and DeLeo 2004), mechanical sensitivity is also produced when the nerve root is crushed for as short a time as two seconds (Sekiguchi et al. 2003; Sekiguchi et al. 2009). While these studies did vary the duration of compression, the magnitude of the compressive load applied to the nerve root was not quantified or accounted for. More than likely, nerve root-mediated pain responses are driven by a combination of both the applied load and duration. A recent study using a rat model of painful cervical radiculopathy that did control the magnitude of root compression found that the onset of sustained behavioral sensitivity did indeed depend on the length of time the compression was applied (Rothman et al. 2010). However, despite that study suggesting there may be a duration threshold for applied cervical nerve root compression to elicit pain between three and 15 minutes, it is still unknown which mechanisms signal that behavioral outcome, and how the function of the neurons in the root is altered during compression as a function of the compression time.

Studies using both clinical and in vivo models have shown that compression to the lumbar nerve root produces an immediate change in evoked signal conduction along the fibers of the compressed root (Fumihiko et al. 1996; Morishita et al. 2006; Pedowitz et al. 1992; Rydevik et al. 1991; Takahashi et al. 2003; Takamori et al. 2010). Intraoperative studies in patients with symptomatic lumbar radiculopathy from root impingement associated with disc herniation and spinal stenosis demonstrate decreases in the amplitude of compound muscle action potentials that are evoked by an electrical stimulation to the affected nerve root (Morishita et al. 2006; Takamori et al. 2010). Furthermore, a more-pronounced decrease in the action potentials amplitudes is associated with increased pressure in the intervertebral foramen (Morishita et al. 2006). This finding suggests that neuronal signaling may be mediated by the local mechanical loading profile of the root. In support of this hypothesis, Takamori et al. (2010) demonstrated that the duration of root compression also mediates the response of electrically-evoked neuronal activity. In that study, pre-operative patients were placed in a prone position with their leg slowly raised until the onset of pain and/or numbness. When that position was reproduced during surgery, the amplitude of the evoked action potentials decreased by 41% as early as within 1 minute, and by 63% after three minutes of impingement (Takamori et al. 2010). This change in evoked action potentials that continued to develop throughout the period of nerve root impingement demonstrates that there is a time-dependent response of the nerve root's electrophysiologic properties to deformation and suggests that changes in neuronal signaling during compression may contribute to radiculopathy symptoms. Further, it is likely that specific loading parameters, such as magnitude and duration, may play a role modulating these electrophysiologic responses. Finally, although clinical electrophysiologic studies, such as nerve conduction tests and needle electromyography, are sensitive and specific diagnostic tools for nerve root compression (Abbed and Coumans 2007; Wainner and Gill 2000), a clear understanding of the functional changes that neurons undergo during compressions that result in pain is still limited, which also impairs their prevention.

Many animal models of compression of the nerve roots in the cauda equina demonstrate that evoked neuronal signaling is modulated during and after compression (Fumihiko et al. 1996; Garfin et al. 1990; Rydevik et al. 1991; Pedowitz et al. 1992). In studies compressing the cauda equina of pigs, both 75mmHg and 100mmHg of pressure applied for 120 minutes each decreased the amplitude of electrically-evoked compound nerve action potentials by 41% and 74%, for each pressure magnitude, respectively

(Rydevik et al. 1991). After compression was removed from the cauda equina, the amplitude of the action potentials returned to pre-compression levels in the pigs that received 75mmHg of compression, but not in those receiving 100mmHg of compression (Rydevik et al. 1991). When 100mmHg of compression was applied to the cauda equina for 240 minutes, the decrease in action potential amplitude during compression was even greater and remained more-pronounced even after compression was removed relative to the shorter (120 minute) compression (Pedowitz et al. 1992). Although these electrophysiological studies suggest neuronal function to be related to nerve root loading and that it is mediated by the compression duration, the outcomes in those studies reflect the collective response of all of the nerve roots in the cauda equina and do not delineate the mechanical scenario applied to an individual nerve root. Further, these studies only investigated the acute responses and did not investigate their relationship to the onset or extent of longer-lasting damage in the nerve root.

The acute neuronal responses may likely be related to the longer-term pathophysiology that develops in the nerve root, such as edema, inflammation, and thickening of the connective tissue (Beck et al. 2010; Jancalek and Dubovy 2007; Kobayashi et al. 2004; Mosconi and Kruger 1996). Yet, neuronal dysfunction may be more indicative of pain symptoms due to its central role in pain transmission. Injured axons following mechanical trauma and compression exhibit axonal swelling, loss of cytoskeleton proteins, separation and disorganization of the myelin sheath, loss of axonal transport, Wallerian degeneration, and a decrease in axon packing density (Guertin et al. 2005; Jancalek and Dubovy 2007; Kobayashi et al. 2004; Kobayashi et al. 2005; Mosconi and Kruger 1996; Myers et al. 2003; Serbest et al. 2007). Like the functional changes in neurons during compression, the degenerative changes in axons that develop at later times after a transient compression are also mediated by compression magnitude (Hubbard and Winkelstein 2008; Kobayashi et al. 2005), suggesting that there may be an association between neuronal responses during nerve root compression and neuronal responses that develop after compression. Yet, no study has established whether these acute neuronal outcomes are related to the persistent neuronal injury and/or dysfunction that develop.

Mechanical loading to the nerve root initiates a cascade of neuronal, inflammatory, and degenerative responses by producing an acute insult to the axonal, connective, and vascular tissues of the nerve root (Kobayashi et al. 2004; Rydevik et al. 1984; Winkelstein et al. 2002). For example, severe axonal injury can induce degeneration of the axonal process distal to the cell body via Wallerian degeneration (Park et al. 2004; Stoll and Müller 1999). For the central axons of primary afferents, which make up the dorsal nerve root, this degeneration can occur proximal to the site of injury, towards the spinal cord (Hubbard and Winkelstein 2008; Kobayashi et al. 2008). Axonal degeneration, marked by neurofilament degradation and loss of axonal integrity, is evident as early as 15 minutes after spinal cord injury, but may also be present as late as three weeks after injury to the peripheral nerves (Kobayashi et al. 2008; Park et al. 2004; Ramer et al. 2000; Schumacher et al. 1999). The degree of degeneration is modulated by the local mechanics of the insult and is also associated with persistent pain after a transient compression to the cervical nerve root (Dyck et al. 1990; Kobayashi et al. 2005; Hubbard and Winkelstein 2008). Disruption to the axonal structure at both of these time points has been found to be more pronounced for greater loads and longer durations of their application (Dyck 1990; Hubbard and Winkelstein 2008; Kobayashi et al. 2005; Kobayashi et al. 2008). Previous studies also have demonstrated that the presence of axonal degeneration of the myelinated axons in the nerve root is associated with sustained behavioral sensitivity after a compression injury, making axonal integrity an important tissue-injury marker of nerve-root mediated pain and suggesting that neuronal dysfunction and pathology may be related to pain (Hubbard and Winkelstein 2008; Hubbard et al. 2008a). Despite the fact that both myelinated and unmyelinated axons are important for normal sensation and also for detecting the location and intensity of a noxious stimulus (Ramer et al. 2000; Wall and Melzack 1994), no study has evaluated the response of unmyelinated fibers to a transient nerve root compression. Furthermore, although there is an association between degeneration of the axons and the magnitude of the applied load to the root (Hubbard and Winkelstein 2008), it is not known how compression duration affects axonal injury or whether axonal degeneration is associated with the duration-mediated neuronal responses during compression. Characterizing the role of compression duration in altering the morphology of unmyelinated and myelinated axons in the nerve root at a time-point relevant to pain provides added insight into how neuronal dysfunction and/or injury following mechanical root loading relates to symptoms of radiculopathy.

We developed a rat model of cervical nerve root compression in order to study pain responses and relationships to tissue loading; painful transient nerve root compression produces an early inflammatory response in the affected tissues, as well as persistent behavioral sensitivity, spinal inflammation, and tissue damage in the compressed root (Hubbard and Winkelstein 2005; Hubbard and Winkelstein 2008; Hubbard et al. 2008a; Hubbard et al. 2008b; Rothman et al. 2009a; Rothman et al. 2009b; Rothman et al. 2010). In the context of this model, a transient injury to the nerve root is one that is temporary and not sustained, but can last for up to 15 minutes. Although this duration can be longer than those associated with most traumatic events, it is considerably shorter than a sustained compression that can last for one day to several weeks (Colburn et al. 1999; Hashizume et al. 2000; Kobayashi et al. 2004). From that prior work it has been found that within one hour following a compression of the C7 nerve root that is sufficient to produce persistent behavioral sensitivity, the mRNA of the inflammatory cytokines, IL-6 and TNF-α, are elevated in the ipsilateral dorsal root ganglion and also in the cervical spinal cord (Rothman et al. 2009b). This almost immediate increase in proinflammatory cytokine mRNA levels provides evidence that the early response of afferents to a transient compression may play a role in driving the persistent pain that develops following nerve root trauma. Similarly, as early as one day after that same nerve root injury, behavioral sensitivity develops in the affected forepaw along with hallmarks of spinal inflammation, including activation and proliferation of microglia (Rothman et al. 2009a). Further, by seven days after transient injury, the axons of the injured nerve root show signs of degeneration and the spinal inflammation becomes even more pronounced with both activated astrocytes and microglia (Hubbard and Winkelstein 2005; Hubbard and Winkelstein 2008). Together with the decrease in neuropeptides in the spinal cord at this same time point (Hubbard et al. 2008a), there is a suggestion that neuronal signaling may be altered in the spinal cord following a painful injury. Although, collectively, these prior studies demonstrate that transient cervical nerve root compression produces a host of pain-related responses, they do not address how the nervous tissue responds during the applied compression. Understanding the immediate, physiologic response of the nerve root to a compression known to produce pain, will give insight into the early mechanisms of tissue injury that contribute to the downstream sequelae associated with persistent pain.

In this study we hypothesized that when nerve roots undergo compression, there is an associated change in the number of electrically-evoked action potentials of the compressed axons. We further hypothesized that there is a period of compression longer than which the applied compression can produce prolonged changes in the function of the affected axons; those modifications can manifest as sustained changes in electrophysiologic properties and axonal injury after the initial insult. As such, the goal of this study was to identify if there is a critical duration of applied compression to the nerve root that alters the electrically-evoked discharge rate of the neurons from that root during and after compression, and to also evaluate if that neuronal activity relates to sustained changes in function and the extent of neuronal damage in the root after such compression.

In order to evaluate the electrophysiologic response of the afferents in the compressed nerve root, extracellular recordings were made in the superficial laminae of the dorsal horn during and after compression was applied to the C7 nerve root for 15 minutes, a period previously determined to produce immediate and sustained pain (Hubbard et al. 2008a). Extracellular recordings were made while action potentials from the afferents were evoked using an electrical stimulus applied to the area of the forepaw that corresponds to the dermatome innervated by the C7 root (Figure 1). Neuronal activity was quantified by measuring the number of electrically-evoked action potentials during the painful compression in order to define the time at which the conduction of peripherally-evoked spikes is significantly altered (i.e. reduced) (Figure 2). Based on that critical compression duration, a separate electrophysiological study was also performed with nerve root compression applied for a period of time that was shorter than that duration. The goal of that study was to define the neuronal activation patterns for a sub-critical duration of compression and to provide comparisons of the prolonged, post-compression response in these patterns to the response for the longer duration painful compression (Figure 2). In addition, in order to evaluate if that duration threshold for inducing immediate changes in neuronal discharge patterns relates to the development of local tissue damage, axonal pathology in the root was separately evaluated at day seven after a sub-threshold compression was applied.

METHODS

Complementary studies were performed to assess neuronal responses to an applied nerve root compression using both electrophysiology to quantify peripherally-evoked action potentials in the spinal

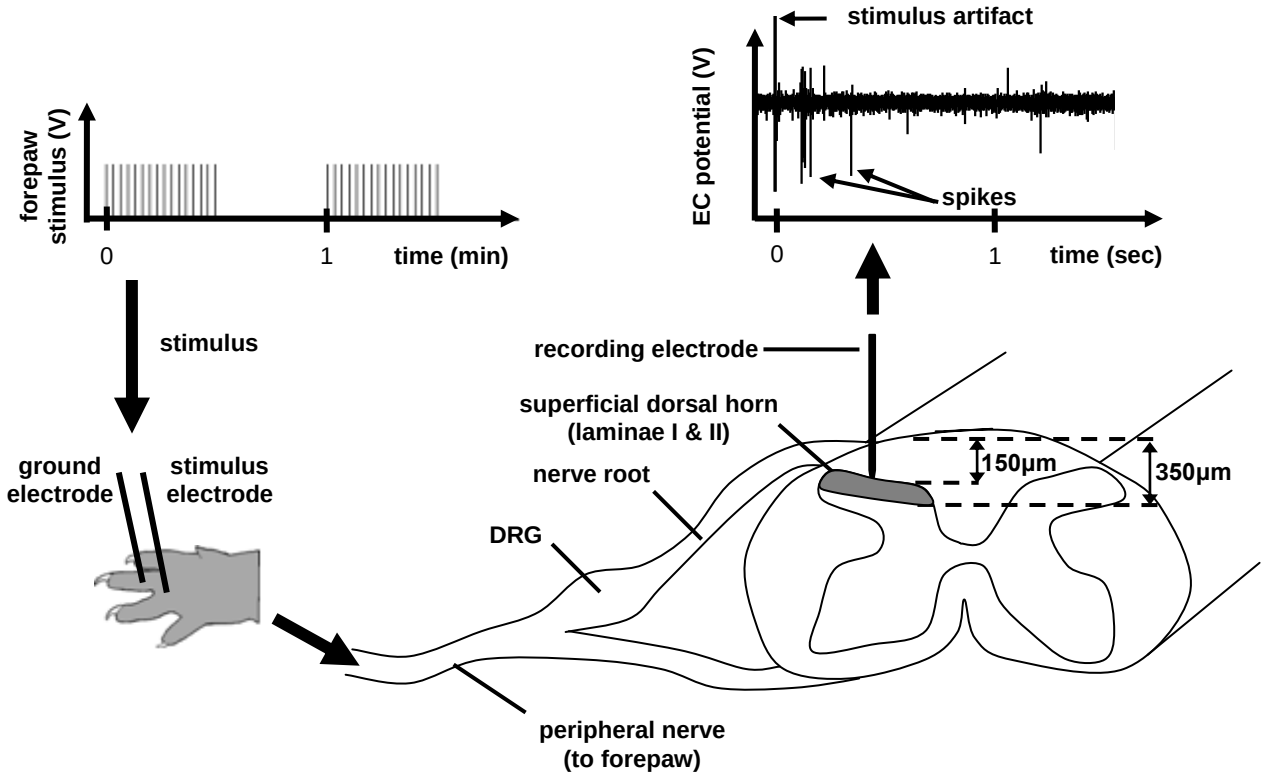

Figure 1. Schematic showing experimental test set-up for recording peripherally-evoked spikes in the superficial dorsal horn of the spinal cord. An electrical stimulus (16-train pulse) was applied to the forepaw at one-minute intervals using a pair of stainless steel electrodes to stimulate neurons in the forepaw. Extracellular (EC) recordings of the evoked action potentials were made in the superficial dorsal horn by initially inserting the tungsten recording probe at a depth of 150µm below the pial surface and searching through the depth of 350µm. The number of action potentials evoked by each stimulus train was quantified during and after a transient compression to the C7 nerve root.

cord and immunohistochemistry to evaluate axonal damage in the nerve root. In the first study, peripherally-evoked neuronal activity in the spinal cord was evaluated before, during and after the application of compression to the C7 nerve root. The number of spikes detected in the spinal cord in response to a peripheral stimulus was measured as a function of the compression duration *during* and *after* a 15 minute compression and compared to baseline activity measured before any compression was applied (Figure 2). The compression period of 15 minutes was selected in order to evaluate neuronal discharge rates during a compression that is known to produce behavioral sensitivity (Hubbard and Winkelstein 2008; Rothman et al. 2010). These electrophysiological data recorded during the applied compression were used to define a duration threshold at which the number of spikes evoked by the peripheral stimulus was significantly reduced relative to the number of spikes evoked by the peripheral stimulus during baseline (i.e. before any compression was applied). Using that threshold, electrically-evoked spikes in the spinal cord were recorded from a separate group of rats to quantify the number of spikes generated during and after a shorter, sub-threshold compression duration (Figure 2). In order to provide an additional measure of the effect of compression duration on neuronal function, the evoked responses were also measured and compared after compression was removed from the nerve root to evaluate if and the extent to which the firing responses recover to pre-compression levels for the two compression groups (Figure 2). In addition, axonal injury was also assessed in the compressed neurons using a separate group of rats that also underwent a nerve root compression applied for this shorter sub-threshold duration or for 15 minutes and the compressed nerve root tissue was assayed at day seven for the development of local tissue damage using immunohistochemistry techniques to assess axonal injury.

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Rats were housed under USDA- and AAALAC-compliant conditions with a 12-12 hour light-dark cycle and free access to food and water. Studies were performed using only male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, IN), weighing 300-480g at the start of the study.

Electrophysiologic Study of Neuronal Activity

Surgical Preparation. Anesthesia was induced via intraperitoneal injection of sodium pentobarbital (50mg/kg). To maintain proper levels of anesthesia, supplementary doses of sodium pentobarbital were given as needed throughout the procedures. With the rat placed in a prone position, a dorsal incision was made along the midline from the base of the skull to the spinous process of T2. The C5-T1 vertebrae were exposed by removing the paraspinal muscle and soft tissue. The cervical spinal cord from C6-C8 and the C7 nerve root on the right side were exposed via a bilateral dorsal laminectomy and partial facetectomy. The overlying dura was also removed and warm mineral oil (Fisher Scientific; Pittsburgh, PA) was used to cover the exposed spinal cord in order to prevent the cord from dehydrating. Following the surgical exposure, the rat was immobilized on a

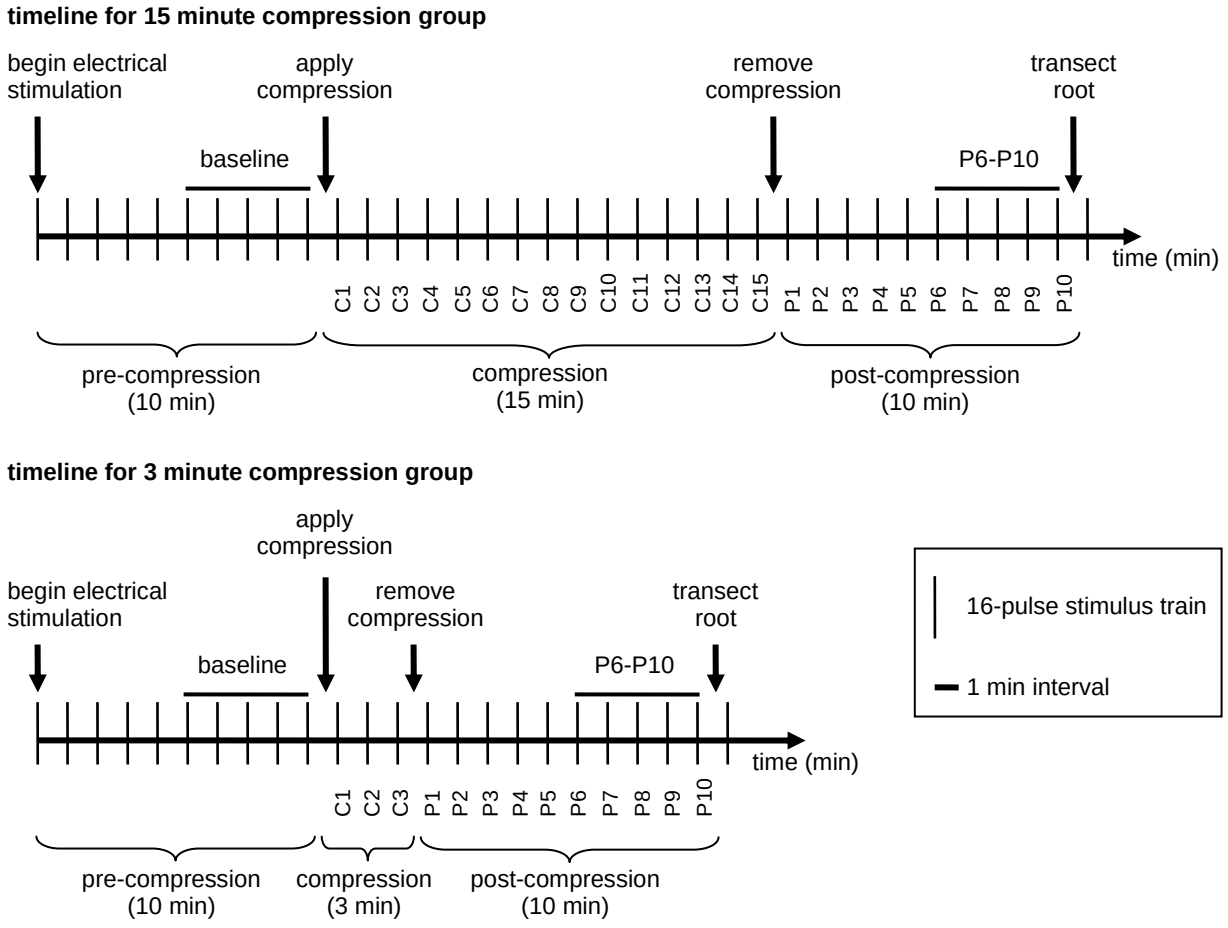

Figure 2. Study design to measure the number of spikes evoked by electrical stimulation (a 16-pulse stimulus) to the forepaw at one-minute intervals in both compression scenarios. Prior to compression, evoked activity was measured for a period of 10 minutes, followed by a single compression to the C7 nerve root of either 15 minutes or three minutes. Post-compression activity was recorded for a period of 10 minutes. Following each study, the nerve root was transected. Each neuron's response was measured as a percent change from its average response measured during the last five minutes before compression (baseline). The post-compression response of the neurons in each group was compared using the neuronal responses at P10 and also during the last five minutes of the post-compression period (P6-P10).

stereotaxic frame (Kopf Instruments; Tujunga, CA) using bilateral ear bars and a vertebral clamp at the T2 vertebra (Figure 3). The core temperature of the rat was maintained between 36–37° during all procedures using a heating plate with a temperature controller and isolated rectal probe (Physitemp Instruments, Inc.; Clifton, NJ).

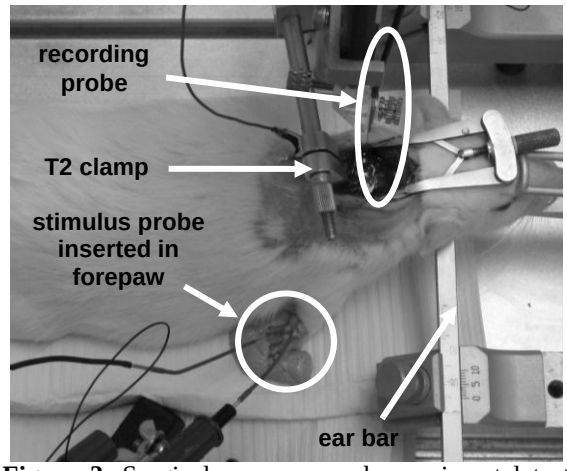

Figure 3. Surgical exposure and experimental test set-up for recording extracellular potentials in the dorsal horn while applying an electrical stimulus to the forepaw.

Extracellular Spinal Recordings to Identify a Duration Threshold. For each rat in which neuronal activity was measured, extracellular voltage potentials were continuously recorded using a 127µm diameter tungsten electrode (A-M Systems; Sequim, WA) that was inserted into the superficial dorsal horn medial to where the C7 right dorsal root enters the spinal cord (Figures 1 & 3). The recording probe was inserted to depths of between 150–350µm below the pial surface in order to measure extracellular potentials in the superficial dorsal horn, where the primary afferent neurons that are contained in the dorsal root synapse with the spinal neurons. The extracellular signal from the recording electrode was amplified with a gain of 1000 (World Precision Instruments; Sarasota, FL), processed with a 60Hz noise eliminator (Quest Scientific; North Vancouver, BC), and then digitized and stored at 25kHz (CED; Cambridge, UK), using methods previously reported (Pezet et al. 2008; Quinn et al. 2010).

To identify the afferent neurons that are associated with the C7 dermatome, sensory fields in the right forepaw were located at the start of each study using a light brush stroke applied to the plantar surface of the forepaw with a cotton swab followed by a series of 10 stimuli using a noxious (98mN) von Frey filament (Hubbard and Winkelstein 2005) (Figure 4). A load cell (5N capacity; SMT S-Type Model; Interface, Inc., Scottsdale, AZ) was attached to the von Frey filament and was used to synchronize the application of the mechanical stimulus with the extracellular recordings that were made in the spinal cord (Figure 4). Each von Frey stimulus was applied for one second at a time. A stainless steel electrode (A-M Systems; Sequim, WA) was then inserted into the forepaw in the location of the sensory field and a train of 16, 2msec-wide pulses was delivered at 0.5Hz with an amplitude of 1V at 1-minute intervals (Lapirot et al. 2009; Pezet et al. 2008; Yu et al. 2009) (Figures 1 & 2). A ground electrode similar to the stimulus probe (A-M Systems; Sequim, WA) was also inserted into the forepaw, near the stimulus electrode (Figures 1 & 3). The electrical stimulation protocol was repeated throughout the duration of each experiment (Figure 2). The stimulus strength of 1V was selected such that the stimulus itself did not create any muscle contractions or twitches, but was sufficiently strong to evoke neuronal responses.

Electrically-evoked neuronal responses for a nerve root compression were recorded in the spinal cord before, during, and after the applied compression for all rats in this study. The baseline response of each rat to the electrical stimulation of the right forepaw was first established by delivering the stimulation train once every minute for a period of 10 minutes, prior to any additional stimulation or manipulation (Figure 2) (Fumihiko et al. 1996; DeLaTorre et al. 2009; Martindale et al. 2001; Rydevik et al. 1991). After the baseline period, compression was applied to the nerve root using a calibrated 98mN microvascular clip (World Precision Instruments; Sarasota, FL) to apply a compressive strain of 81.7±4.7% of an area of 4.0mm^2 (Hubbard et al. 2004) and the 16-pulse stimulation train was applied at one-minute intervals during the compression period (Figure 2). In the first series of rats (n=3 rats; 10 neurons), nerve root compression was applied for 15 minutes. Each rat received only one, single compression to the right C7 nerve root. After that time, the clip was removed from the nerve root and the electrical stimulation to the right forepaw continued at one-minute intervals for a post-compression period of 10 minutes (Figure 2). To account for effects of the surgical exposure and the repeated stimulations, a separate rat (four neurons) underwent sham procedures that included the same experimental and stimulation protocols without application of nerve root compression. Regardless of the surgical procedure, at the end of the 10 minute post-compression recording period, the C7 nerve root was fully transected and an additional electrical stimulus train was delivered in order to determine whether the evoked responses that were

recorded previously during the protocol actually represented signaling from the C7 root (Figure 2). Any neurons that were detected to continue to respond to forepaw stimulation after the root had been transected were removed from subsequent analysis and not included in this study (Figure 4). In this way, neurons that are not associated with the C7 root that was being compressed were not erroneously included.

All extracellular voltage recordings measured during the stimulation protocol were spike-sorted using Spike2 software (CED; Cambridge, UK) to separate the action potentials associated with individual neurons. In order to focus on the inputs of the sensory neurons in the forepaw, only those neurons that were identified to respond to the von Frey filament stimulus were analyzed in Spike2 (Figures 4 & 5). On average, for every three mechanoreceptors that were identified during the von Frey filament stimulus, only one neuron would also be evoked by the electrical stimulus (Figure 4). Thus, only that one neuron that was evoked by both the mechanical and electrical stimulus would be included in the analysis for that rat. For each sensory neuron included in the study, the baseline firing rate was determined by counting the number of spikes that occurred during one second prior to the first application of the von Frey filament (Quinn et al. 2010) (Figure 5). A sensory neuron was defined as any neuron whose firing rate increased over its baseline firing rate during at least one of the 10 one-second von Frey stimulations. Neurons that did not respond to any of the applied von Frey stimuli before the start of the electrical stimulation were removed from subsequent data analysis. Activity that was evoked by electrical stimulation of the forepaw was quantified at each one-minute interval throughout the protocol. Specifically, this was done by summing the number of spikes for each neuron that were elicited within the 10-40msec window immediately after each of the 16 stimulation pulses in order to exclude stimulus artifact and spontaneous activity (DeLaTorre et al. 2009; Ramer et al. 2000; Yu et al. 2009) (Figure 6). In order to account for each neuron's individual discharge rate, the number of spikes measured at each one-minute interval of the compression and post-compression periods was each normalized against the average number of spikes that occurred during the last five stimulations of the baseline period (Figure 2). The normalized number of spikes was then averaged across neurons in the compression group and also across neurons recorded in the sham group. The number of action potentials recorded at each time period was represented as the percentage change from baseline±SEM for that

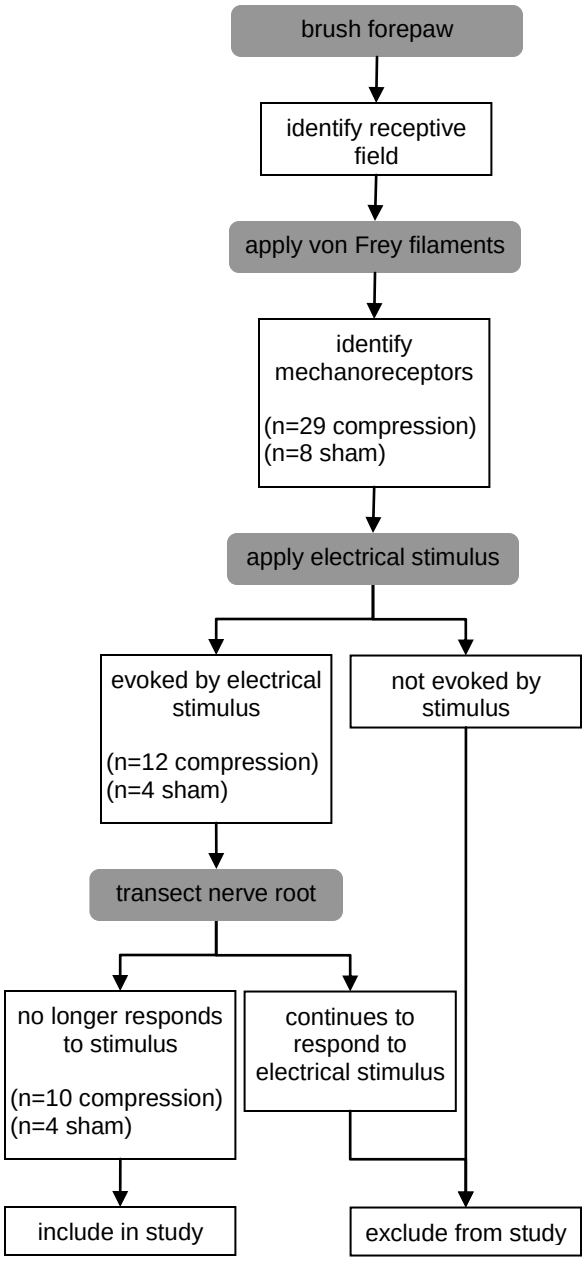

Figure 4: Flow chart illustrating the experimental procedures for identifying (shaded ovals) and selecting neurons (white boxes) to include in the electrophysiologic studies. The number of neurons that were selected at each procedure in the 15 minute compression and sham groups are shown.

group. Differences in the number of evoked spikes between the compression and sham groups during the applied root compression were detected using a two-way, repeated measures analysis of variance (ANOVA) for injury group (sham, compression) and time (baseline through C15). All statistical analyses were performed using the raw data (i.e. the number of

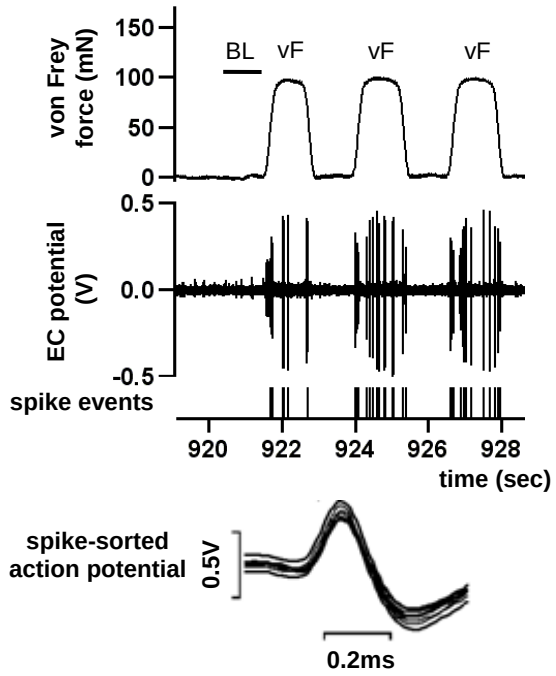

Figure 5: A representative extracellular (EC) recording during the application of a von Frey (vF) filament. The extracellular data were spike-sorted to identify neurons evoked by the mechanical stimulus to the forepaw. Neurons were determined to be evoked by the stimulus if the number of spike events during any application with the von Frey filament (vF) was greater than the number of spike events recorded one second prior to (BL=baseline) the first stimulus.

spikes; Tables A1 & A2); the percent change from baseline is reported in order to show trends in discharge rates that would otherwise be unclear due to the variation in baseline discharge rates between individual neurons.

For each of the neurons from which recordings were made in the 15 minute compression groups, post hoc analysis was performed to determine the time during the compression at which the evoked neuronal activity was substantially modulated. This critical duration was defined as the average time during compression when changes in the evoked activity relative to baseline reached a maximum, using all of the neurons (10 neurons total from three rats) that were recorded from during the 15 minute compressions.

Based on the duration threshold identified for modulating evoked neuronal activity during the 15 minute compression, a second group of rats underwent a nerve root compression that was applied for a sub-threshold period of three minutes (n=6 rats; 9 neurons) (Figure 2). The duration period of three minutes was selected because it is more than one standard deviation shorter than the threshold duration that was identified to modify peripherally-evoked action potentials in the spinal cord. All other surgical and electrophysiological procedures were the same as described for the 15 minute compressions. Similarly, for the sub-threshold compression studies, the evoked activity was recorded during an initial 10 minute baseline period and during the entire period of applied compression to the nerve root (i.e. three minutes) (Figure 2). Recordings were also made for 10 minutes after the compression was removed from the root, analogous to the protocol for the 15 minute compression studies (Figure 2). In order to account for changes in neuronal activity due to the surgical exposure and repeated electrical stimulus, one additional rat (three neurons) also underwent sham procedures with the electrical stimulus protocol matching that used for the three minute compression group.

Comparisons during the first three minutes of compression between the 15 minute compression group and the three minute compression group were made using a two-way, repeated measures ANOVA for group (3 minutes, 15 minutes) and time (baseline through C3). The post-compression data for each of the two duration groups were separately compared using a two-way, repeated measures ANOVA for group (compression, sham) and time (baseline, P1 through P10). In addition, to specifically evaluate the effect of compression duration on discharge rates at the end of the 10 minute recovery period (Pedowitz et al., 1992), a separate two-way, repeated measures ANOVA for group (compression, sham) and time (baseline, P10) was performed for each compression duration study. In addition, to make a more direct comparison between the pre-compression discharge rate (baseline), and the post-compression discharge rates, comparisons were made between baseline and the average number of spikes measured over last five minutes post-compression (P6-P10) using a two-way ANOVA for group (compression, sham) and time (baseline, average of P6-P10) for each duration group, separately. Post-hoc, pairwise comparisons with Bonferroni correction tested for differences in the main effects, where applicable. Significance for all comparisons was defined at α=0.05.

Immunohistochemistry Study of Axonal Injury

In a separate study, immunohistochemistry was used to evaluate the extent of axonal injury in the C7 nerve root at day seven after compression was applied for three minutes (sub-threshold) (n=6 rats) or 15

minutes (n=4 rats). A sham group (n=5 rats) that received the same nerve root exposure without compression was also included to account for the effects of anesthesia, surgical exposure and any tissue manipulation. Surgical procedures were performed under anesthesia induced with inhalation isoflurane (4% for induction, 2% for maintenance). With the rat in a prone position, the C6 and C7 vertebrae were exposed via a dorsal incision from the base of the skull to the spinous process of T2 and removal of the overlying muscle and soft tissue. A C6-C7 hemilaminectomy was performed on the right side to expose the C7 dorsal nerve root (Hubbard et al. 2008a). Compression was applied to the nerve root using the same procedures described in the electrophysiologic study. After surgery, the wound was closed using 3-0 polyester suture and rats were allowed to recover.

At day seven after the surgical procedure, rats were given an overdose of sodium pentobarbital (65mg/kg) via intraperitoneal injection and then transcardially perfused with 200ml of Dulbecco's Phosphate-Buffered Saline (PBS; Mediatech, Inc.; Manassas, VA) followed by 300ml of 4% paraformaldehyde (Sigma; St. Louis, MO). The C7 ipsilateral nerve root was exposed via a bilateral C6-C7 laminectomy and facetectomy and harvested en bloc with the adjacent spinal cord and dorsal root ganglion attached at the proximal and distal ends of the root, respectively (Figure 1). Tissues were post-fixed in 4% paraformaldehyde overnight and then transferred to 30% sucrose at 4°C for five days and embedded in OCT medium (Sakura Finetek USA, Inc.; Torrance, CA) for cryo-sectioning. The nerve roots were sectioned (14μm) along the long-axis, near the centerline of the root and then thaw-mounted directly onto slides. Matched nerve roots were also harvested from naïve uncompressed rats (n=2) and were also included in tissue processing for comparison.

Slides were immunofluorescently labeled for neurofilament-200 (NF200) and isolectin-B4 (IB4) to identify myelinated and unmyelinated fibers, respectively. Slides were blocked in 10% normal donkey serum (Millipore; Billerica, MA) with 0.3% Triton X-100 (Bio-Rad Laboratories; Hercules, CA) for two hours and incubated overnight at 4°C in mouse anti-NF200 (1:500; Sigma; St. Louis, MO) and biotinylated IB4 (5μg/ml; Sigma; St. Louis, MO). Slides were then incubated for two hours at room temperature in donkey anti-mouse Alexa Fluor 546 (1:1000; Invitrogen; Carlsbad, CA) and streptavidin conjugated with dichlorotriazinyl amino fluorescein (DTAF) (1:500; Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA).

Three tissue sections from each rat were analyzed for axonal damage. Digital images were taken of the nerve root proximal to the site of compression at 200X magnification. Each axonal marker was evaluated, separately, for signs of axonal swelling and loss of immunoreactivity as indicators of axonal damage (Hubbard et al. 2008b; Serbest et al. 2007; Singh et al. 2006). Tissue sections that displayed any signs of these abnormalities were assigned a positive score (+) and those tissues sections that were not different from normal uncompressed roots were assigned a score indicating the absence (–) of changes. Evaluations were performed blinded to the group.

RESULTS

A total of 10 neurons that responded to the von Frey and electrical stimuli were recorded at a mean depth of 221±63μm in the dorsal horn of the spinal cord in the studies with 15 minutes of applied compression. In the corresponding sham group, recordings were made from a total of four neurons at a depth of 150μm. Compression of the C7 nerve root significantly decreased the discharge rate over time compared to sham procedures (p=0.035, two-way interaction group x time). As early as two minutes after the start of the applied compression, evoked activity was reduced by 37±24% relative to baseline. The number of action potentials that were evoked by the electrical stimulus continued to decrease over time during the compression and was 76±13% lower than baseline at seven minutes into the compression, while the discharge rate during sham procedures was only 9±9% less than baseline (Figure 7A). After seven minutes of compression, neuronal discharge rates remained 50-80% lower than the comparable baseline activity for the remaining period of the compression and post-compression periods (Figure 7A). On average, the maximum decrease in neuronal activity occurred at 6.6±3.0 minutes into the compression period. As such, this time was taken as the duration-threshold for modulating evoked neuronal activity during a 98mN compression to the nerve root. Moreover, for the subsequent electrophysiologic and immunohistochemistry studies, a sub-threshold duration of three minutes of compression was applied because it was a time less than the average minus one standard deviation (i.e. 3.6 minutes); experimentally, it was not feasible to apply a compression for 3.6 minutes and to synchronize the electrical stimulation and measurement protocols across studies.

In the sub-threshold compression study, recordings were made from nine neurons at a depth of 251±79μm in rats that received compression for three

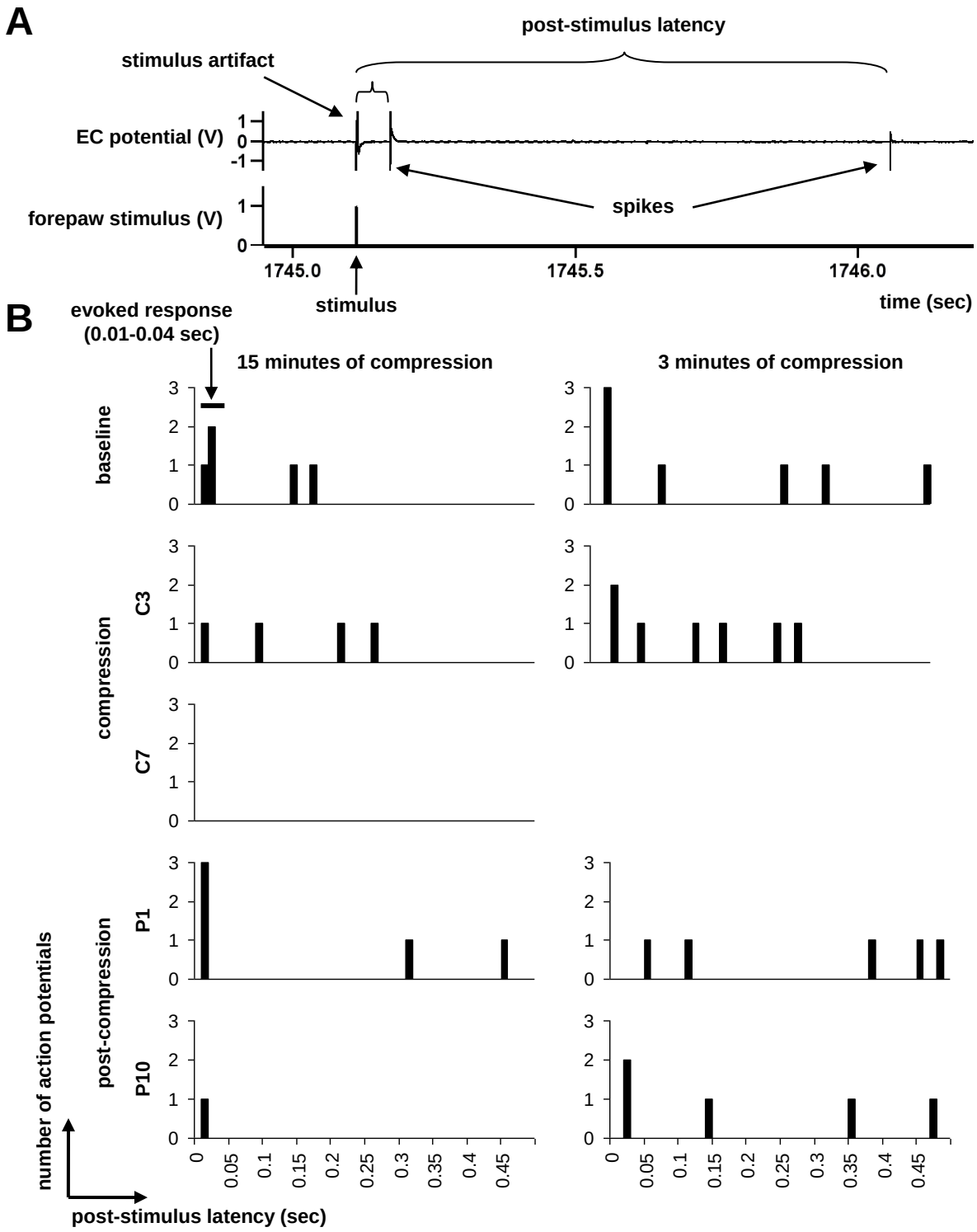

Figure 6. (A) A representative extracellular (EC) recording made in the spinal cord after a single electrical stimulus to the forepaw during compression. For each spike, the length of time that occurred between application of the stimulus and the evoked spike (0.01-0.04sec) was quantified as the post-stimulus latency. **(B)** Representative post-stimulus histograms (bin-width of 10ms) quantifying the number of action potentials evoked from a neuron in the 15 minute compression group and a neuron in the three minute compression group before compression (baseline), during compression (C3, C7), and post-compression (P1, P10).

minutes (Figure 7B). There was no significant difference in the depth of the neurons from which recordings were made in the 15 minute and three minute compression groups (p=0.370), as evaluated by a t-test. For the corresponding sham group for the three minute study, recordings were made from three neurons at a depth of 160μm. No significant differences were detected during the first three minutes of compression between the group that received 15 minutes of compression and the group that received three minutes of compression (p=0.373, group; p=0.543, group x time). Significance was only detected over time (p=0.032) for the two injury groups; there was a significant difference in the discharge rates measured during compression from baseline at C2 (p=0.005) and C3 (p=0.035). At two minutes into the compression, the discharge rate was reduced by 42±21% relative to baseline in the three minute group (Figure 7B). This decrease in evoked action potentials at two minutes of compression in the sub-threshold duration group was similar to that observed in the 15 minute compression group (Figure 7A)

The electrically-evoked discharge rate that was measured after the compression was removed from the nerve root exhibited differences based on the duration of the applied compression (Figures 6-8). Although there was a significant difference (p=0.001) between the post-compression discharge rates (BL, P1-P10) after 15 minutes of compression compared to its sham, no significant differences were detected in post-compression discharge rates after three minutes of compression compared to its corresponding sham group. At the end of the post-compression period (P10), the number of evoked spikes in the 15 minute compression group remained lower than baseline by 58±25% (Figure 7A) and was

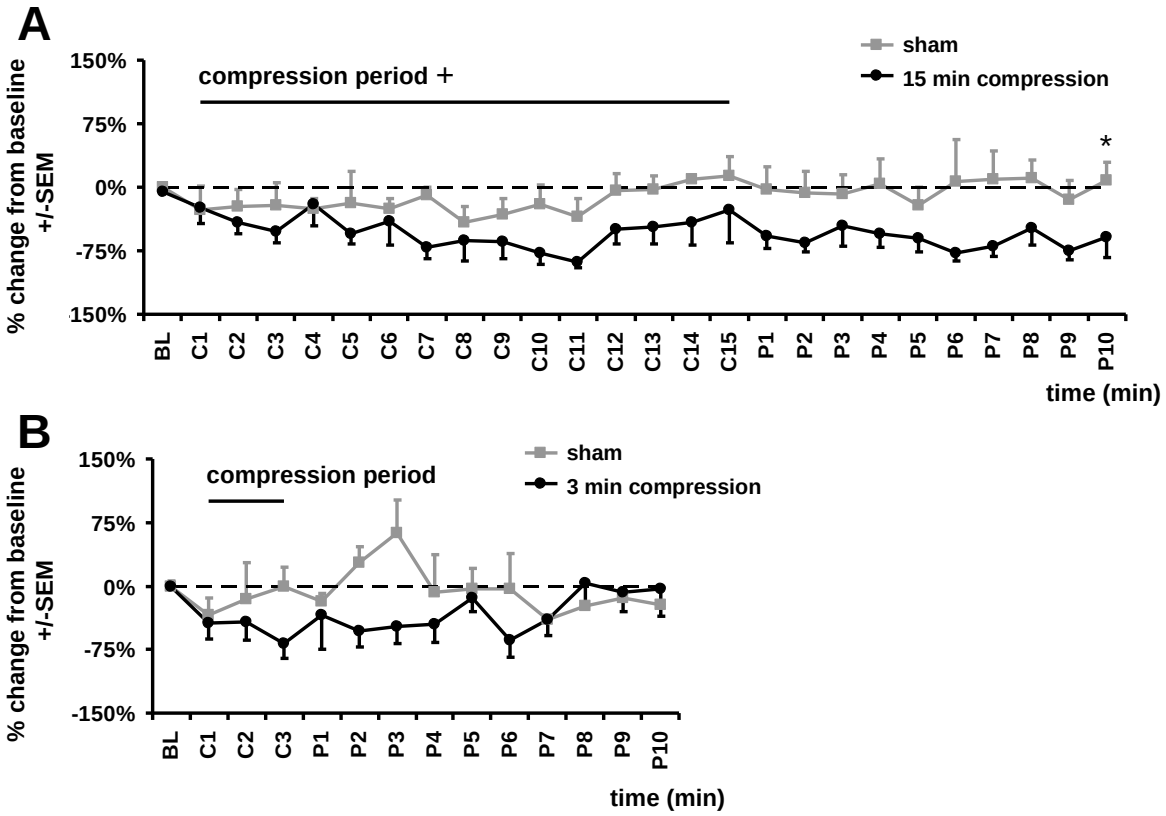

Figure 7. Evoked spikes during and after compression applied to the C7 nerve root and corresponding sham procedures applied for a period of 15 minutes **(A)** or for a period of three minutes **(B)** expressed as a percent change from baseline (BL). The number of spikes was counted at one-minute intervals during a nerve root compression held for 15 minutes (C1-C15) or three minutes (C1-C3). After compression was removed from the nerve root, evoked action potentials were counted at one-minute intervals for a post-compression period of 10 minutes (P1-P10). The number of evoked spikes during compression in the 15 minute group (C1-C15) were significantly decreased over time relative to sham procedures (+p=0.012, group x time). There was not change for the three minute compression. The number of evoked spikes at the end of the post-compression period (P10) were significantly less than baseline and sham (*p=0.018, group x time) after a 15 minute compression, but not after a three minute compression.

significantly different than sham and baseline (p=0.018, two-way interaction group x time). Yet, in the three minute compression group evoked activity had returned to within 3±33% of baseline (Figure 7B) and was not significantly different from baseline or sham (p=0.088, two-way interaction group x time). Similarly, 15 minutes of compression significantly reduced the average discharge rate measured during the last five minutes of post-compression (P6-P10) compared to baseline and sham (p=0.020, two-way interaction group x time), but not a three minute compression (p=0.125, two-way interaction group x time) (Figure 8). On average, the number of spikes measured at P6-P10 after a 15 minute compression was 56±14% lower than baseline, while the number of spikes after a three minute compression was 22±12% lower than baseline during this five-minute period (P6-P10; Figure 8).

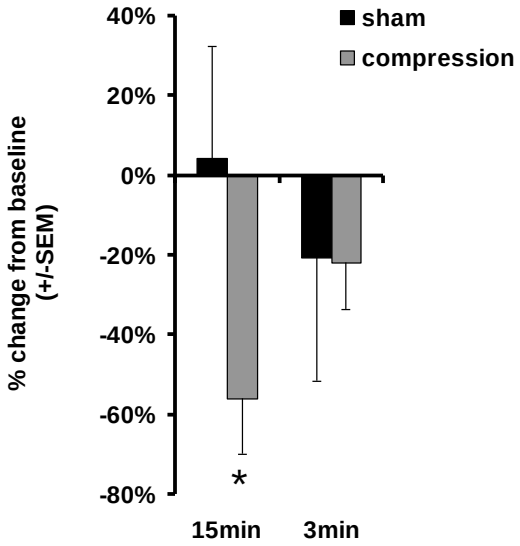

Figure 8. Average change from baseline during the last five-minutes of post-compression (P6-P10) after compression was applied for three minutes or 15 minutes or the corresponding sham procedures. The number of spikes after 15 minutes of compression was significantly (p=0.020, group*time) less than sham and baseline.

Generally, axonal staining for NF200 and IB4 in the C7 right nerve root did not differ from normal tissue for either the sham or three minute compression groups (Table 1). Yet, when the nerve root was compressed 15 minutes, both axonal markers (NF200 and IB4) demonstrated robust changes, including a loss of immunoreactivity and axonal swelling (Figure 9 &Table 1). In all but one of the nerve roots compressed for three minutes (Rat #118), NF200 staining exhibited long, myelinated axons with an even distribution in their staining (Figure 9). IB4 staining labeled long, thin axons that also exhibited an even distribution in their staining along the nerve root for all samples except Rat #118 (Table 1). Neither the myelinated nor the unmyelinated populations of axons exhibited substantial signs of axonal discontinuity or axonal swellings in either the sham or the three minute compression groups. Of the six rats that underwent a nerve root compression for three minutes, only one (Rat 118) exhibited signs of decreased NF200-immunoreactivity and axonal swelling in both the unmyelinated (IB4) and myelinated (NF200) axon populations (Table 1). However, the remaining five rats that received compression for three minutes showed normal pathology. Likewise, no pathology was observed in any of the five roots that that received sham procedures (Figure 9 & Table 1). In contrast, three of the four nerve roots that were compressed for a period of 15 minutes exhibited altered immunoreactivity for both NF200 and IB4, including both a decrease in NF200-immunoractivity and also the presence of axonal swelling (Figure 9 & Table 1).

DISCUSSION

This study provides the first quantitative evaluation of the role of compression duration in altering neuronal signaling during an applied compression to the C7 nerve root. The electrophysiological data demonstrate that 15 minutes of compression of the cervical nerve root induces immediate neuronal dysfunction that is sustained even after the compression is removed and also produces robust axonal injury in the nerve root seven days after the injury (Figures 6-9 & Table 1). Yet, neither neuronal function nor axonal morphology are significantly affected after a nerve root compression that is applied for only three minutes (Figures 6-9 & Table 1). Compression to the nerve root was accompanied by an immediate and continuous decrease in the number of peripherally-evoked action potentials in the spinal cord that reached its peak at 6.6±3.0 minutes (Figure 7A). Compression that was held longer than that time also maintained a decrease in evoked action potentials by between 50-80%, but did not exhibit any further decreases in the evoked activity during compression (Figures 6-8). In addition, for the nerve root compression that was held longer than that duration threshold, evoked action potentials continued to be decreased relative to baseline responses by 56±14% during the last five-minute period after compression was removed (Figures 7A & 8). However, although three minutes of nerve root compression reduced the number of action potentials evoked from peripheral stimulation by as much as

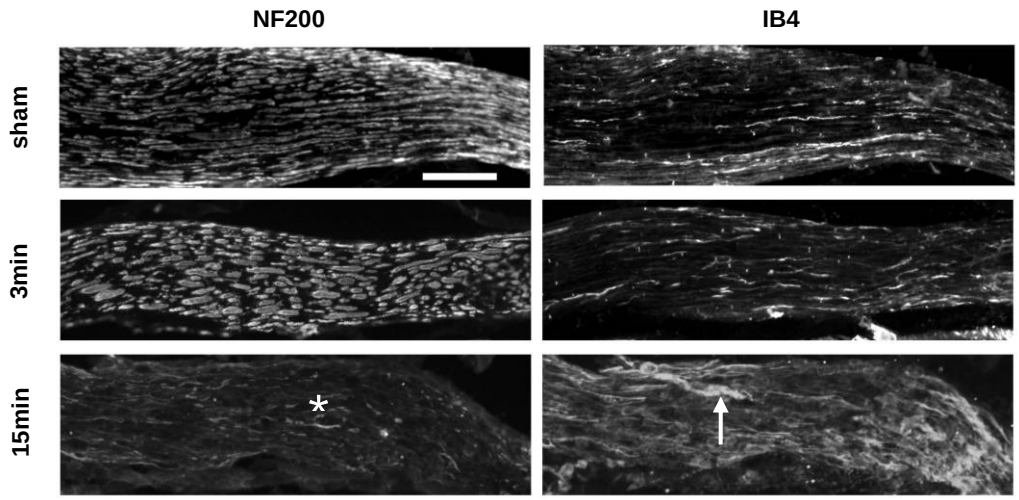

Figure 9. Representative images of C7 nerve roots labeled for myelinated axons (NF200) and unmyelinated axons (IB4) at day seven following sham procedures or a C7 root compression. Sham operated roots show even distribution of immunolabeling for both NF200 and IB4. Both the myelinated and unmyelinated axons appear intact along the length of the nerve root. Most of the nerve roots in the three minute compression group also exhibited this even distribution of NF200 and IB4. However, most of the nerve roots in the 15 minute compression group had evidence of nerve root damage, including a loss of NF200-immunoreactivity (*) and axonal swelling (arrows). Scale bar is 50μm and applies to all.

68±17%, neuronal activity returned to pre-compression levels within 10 minutes after compression was removed from the nerve root (Figures 6-8). In addition, for that same sub-threshold duration of compression (three minutes), there was no evidence of axonal injury in either of the NF200- or IB4-labeled axons at day seven after compression (Figure 9 & Table 1). The lack of widespread axonal injury in that group is in contrast to the substantial decrease in NF200-immunoreactvity, axonal swelling, and myelin degeneration that has been observed at seven days after a compression of the C7 nerve root for 15 minutes (Figure 9 & Table 1) (Hubbard and Winkelstein 2008). Accordingly, the prolonged decrease in evoked action potentials after a 15 minute compression observed in the present study is likely associated with the hallmarks of axonal injury that are observed seven days later (Hubbard and Winkelstein 2008). Taking these data together with the literature, it can be inferred that compressions of the nerve root that are sustained for a period longer than 6.6 minutes not only produce sustained neuronal dysfunction, but may be associated with the cascades associated with nerve root pathophysiology.

The established relationship between altered afferent signaling to the spinal cord and the development of behavioral sensitivity in neuropathy (Hao et al. 1992; Khan et al. 2002; Shim et al. 2005) suggests that 15 minutes of nerve root compression not only produces a sustained decrease in peripherally-evoked action potentials (Figures 6-8), but also induces mechanical sensitivity. In fact, several rat models of inflammatory pain have demonstrated that there is an association between pain-related behaviors and the number of action potentials evoked by a transcutaneous electrical stimulus, and that both the behavioral and electrophysiologic responses are produced as early as 10 minutes after the inflammatory peripheral formalin injection (Asante et al. 2009; DeLaTorre et al. 2009; Martindale et al. 2001; Pezet et al. 2008; Stanfa et al. 1992). Those studies suggest that the significantly reduced number of action potentials that is observed to persist for at least 10 minutes after a 15 minute compression in our study (Figures 6-8) may be a sensitive indicator of enhanced nociception following nerve root compressions of this duration. Moreover, the duration-threshold of 6.6±3.0 minutes that was determined in this study to maximally reduce electrically-evoked neuronal signaling also falls within the range of root compression durations (3-15 minutes) in which the threshold for producing mechanical allodynia in this model of cervical radiculopathy was previously reported (Rothman et al. 2010). In that study, persistent mechanical allodynia of the forepaw developed immediately (within one day) after a 15 minute compression to the C7 nerve root. Yet, when the same compression was applied for a shorter, three minute, period it did not induce allodynia. Although the current study did not

Table 1. Summary of NF200 and IB4 grading

Group	Rat ID	NF200	IB4
Sham	97	–	–
	104	–	–
	105	–	–
	117	–	–
	121	–	–
3min	98	–	–
	99	–	–
	103	–	–
	115	–	–
	118	+	+
	120	–	–
15min	82	–	–
	83	+	+
	84	+	+
	85	+	+

investigate time points beyond 10 minutes after the compression was removed, studies of compression to the cauda equina in the pig report that impaired signaling across the root can continue for at least 90 minutes after the compression is removed (Pedowitz et al. 1992; Rydevik et al. 1991). Taking all of these electrophysiologic and behavioral findings together suggests that compression of the nerve root lasting longer than 6.6±3.0 minutes is sufficient to produce sustained neuronal dysfunction and that this change in the electrophysiologic properties of the compressed neurons may be associated with the development of mechanical allodynia and pain.

Seven days after a 15 minute compression to the nerve root, robust morphological changes were evident in the myelinated and unmyelinated axons of the injured axon, compared to uninjured axons (Figure 9 & Table 1). In contrast, seven days after a three minute compression, both the myelinated and unmyelinated axons appeared intact throughout the length of the nerve root, with morphology consistent with uninjured axons (Figure 9 & Table 1). Previous studies in several animal models of radiculopathy have demonstrated that chronic nerve root compression applied over a period of 1-8 weeks produces substantial pathology in the neurons of the nerve root including axonal swelling, axonal condensation, myelin fragmentation, and loss of axonal transport (Cornefjord et al. 1997; Jancalek and Dubovy 2007; Kobayashi et al. 2004). None of those studies measured pain, but the indicators of axonal injury that were investigated are also associated with changes in pain and functional behavioral outcomes (Chen et al. 1992; Hubbard and Winkelstein 2008). In addition, both the rate and magnitude of compression contribute to the development of axonal injury (Hubbard et al. 2008b; Kobayashi et al. 2005; Olmarker et al. 1990). The 98mN compressive load applied in our study is more than twice the magnitude-threshold of 34.1mN that has been identified as requisite to produce decreased NF200-immunoreactivity at day seven after a 15 minute compression (Figure 9 & Table 1) (Hubbard et al. 2008b). Although that same 98mN compressive load was used for the three minute compression in this study, no such decrease in NF200-immunoreactivity was evident in five of those six nerve roots (Figure 9 & Table 1). Likewise, IB4-immunoreactivity was only altered in the nerve roots that sustained a 15 minute compression and not after a three minute compression (Figure 9 & Table 1). The difference in the response of NF200-labeled and IB4-labeled axons after three and 15 minutes of this compression magnitude suggests that compression duration may play a role in mediating the development of axonal pathology in the nerve root.

Changes in the nerve root structure as a whole that develop over time during its compression, such as compaction of the axons and a decrease in the overall nerve root width, may be among the underlying factors that contribute to the ability of the compressed axons to tolerate three minutes of compression (Dyck et al. 1990; Rothman et al. 2010), despite the supra-threshold pain-provoking load of 98mN. Although pathology was not apparent after a three minute compression to the C7 nerve root in the present study, our evaluation approach did not incorporate more sensitive techniques such as higher magnification, SEM and/or axial views of the nerve root that are capable of detecting subtle pathology in the axons, such as changes in axonal diameter size, axonal splitting, or disorganization in the myelin sheath (Guertin et al. 2005; Jancalek and Dubovy 2007; Myers et al. 1993). However, the presence of those morphologic changes in the axons parallels the presence of decreased NF200-immunoreactivity (Hubbard and Winkelstein 2008). Thus, the normal expression of NF200 that was observed in the nerve root after a three minute compression (Figure 9 & Table 1) is likely a true indication of normal axonal morphology. Also, the absence of axonal injury after a three minute compression is consistent with previous reports suggesting that the development of

axonal pathology after a nerve root compression is associated with behavioral sensitivity, which is also not elicited after a three minute compression to the nerve root (Hubbard and Winkelstein 2008; Rothman et al. 2010).

Although the findings from our study demonstrate that compression duration mediates the electrophysiologic and morphologic responses of the nerve root (Figures 6-9) and provides clinically-relevant insight into its response to compression, these relationships and the specific outcomes for the durations used here are specific to the rat. Additional studies are needed to determine if there are scaling issues in these metrics and outcomes as they relate to the human. Indeed, the nerve root's apparent ability to tolerate a short duration of compression (Figures 6-9 & Table 1), suggests that early intervention alleviating any root compression may be sufficient for improved functional recovery following trauma. In fact, clinical studies have shown that early treatment of nerve root and spinal cord injuries does reduce the severity and number of complications associated with injuries to these tissues (Carlstedt et al. 2000; Fehlings and Perrin 2006). In addition, intraoperative monitoring of nerve root function through the use of electromyography and/or electrophysiology reduces the rate of neurological complications associated with spine surgeries by allowing surgeons to quickly detect and resolve an unintentional compression to the root during surgical manipulation (Bose et al. 2002; Kelleher et al. 2008). Although the nerve root may be able to recover from certain mechanical injuries, the fact remains that nerve root tissues are still susceptible to sustained neuronal damage even for transient compressions (Figures 6-9 & Table 1). Additional studies investigating the tolerance of nerve root tissue to traumatic injuries will provide both clinicians and engineers an improved understanding regarding the need to prevent these injuries, provide rapid treatment and the expected degree of recovery following these types of injuries.

The compressions in the present study were applied to the nerve root transiently; yet, even a brief, 15 minute, compression is sufficient to produce sustained neuronal dysfunction and abnormal axonal morphology (Figures 6-9 & Table 1). Despite being transient, this duration is substantially longer than those associated with tissue loading in the neck during real-world traumatic exposures (Svensson et al. 1993; Panjabi et al. 2006). The physiologic response of the nerve root to compression is likely dependent on a combination of inputs and signaling as a result of both the duration and magnitude of the insult (Garfin et al. 1990; Kobayashi et al. 2005; Olmarker et al. 1989; Olmarker et al. 1990; Pedowitz et al. 1992). Although the compression magnitude (98mN) was held constant in the present study, the axonal damage observed in the 15 minute group (Figure 9 & Table 1) is also evident even when the magnitude of compression is at or near the magnitude threshold of 38.2mN for developing persistent behavioral sensitivity (i.e. pain) in this rat model (Hubbard and Winkelstein 2008; Hubbard et al. 2008a). Furthermore, tissue damage in the nerve root following a transient nerve root compression has been shown to accompany sustained neuronal dysfunction (Rydevik et al. 1992). Thus, it is likely that any 15 minute compression applied using a load above the threshold load (38.2mN) for producing pain would similarly induce sustained neuronal dysfunction and axonal damage. Conversely, because axonal damage has been reported not to develop in the nerve root following compressions below that load threshold, even for compressions that are held as long as 15 minutes (Hubbard and Winkelstein 2008), neuronal function is likely to recover following application of less severe compressions as was observed for the 3 minute group (Figures 6-8). Further, it is likely that for loads below 38.2mN there is a similar duration of compression that would also produce pain and/or neuronal dysfunction. Such a duration would presumably be required to be much longer, and possibly even permanent, as in the case of stenosis. Indeed, currently the relationship defining the "response-space" between load, duration, neuronal dysfunction and symptomatic outcomes is not defined. However, the present study together with studies in the literature begins to establish such a multi-dimensional response.

Certainly, the magnitude and durations applied to the nerve root in the present study represent only two points along the duration-magnitude response curve of the nerve root. At the two extremes of this spectrum are (1) transient compressive injuries that deliver a brief compression with a high magnitude of force and (2) chronic compressions applied at a low magnitude. In vivo studies of nerve roots crushed with a high, but undetermined, force for a very brief period (2-15 seconds), report that axonal injury and neuronal dysfunction develop in the injured nerve root, as well as pain that persists for at least seven days (Ramer et al. 2000, Sekiguchi et al. 2003). Likewise, chronic compressions also produce substantial axonal damage, the onset of which occurs earlier for higher magnitudes of compression (Kobayashi et al. 2005). That study demonstrated that chronic compression to the canine lumbar nerve root reduces axonal transport within one week for

compressions applied with 73.5mN (7.5gf) load and as early as one day for compressions applied with 147.1mN (15gf) a load (Kobayashi et al. 2005). It is not known whether pain developed for either of those compression magnitudes. Nevertheless, that study does support the notion that for chronic compressions, the rate at which pathology develops is mediated by the magnitude of the force applied to the tissue, with a greater force leading to earlier onset pathology. Based on those findings, the time-dependent decrease in discharge rates observed during compression in the present study (Figures 6-7), also likely varies with the magnitude of the load applied. By extension, for any force greater or less than the 98mN applied in the current study, the duration-threshold for mediating changes in neuronal activity would be expected to develop earlier or later, respectively, than the 6.6 minutes determined during the compression period.

The current study demonstrated that nerve root compression reduces the number of peripherally-evoked action potentials in the spinal cord (Figures 6-8). This outcome of spinal neurons was evaluated as an indicator of the electrophysiologic response of the neurons of the compressed nerve root. Inserting the recording probe in the spinal cord rather than the nerve root itself was selected because it limited the injurious exposure to the nerve root to only the compression-induced injury by the microvascular clip. Previous similar studies of nerve root crush demonstrate that neuronal activity in the spinal cord that is evoked by a peripheral stimulus correlates with the presence of pathology in the nerve root (Ramer et al. 2000; Rydevik et al. 1991; Wang et al. 2008). Furthermore, all neurons in the present study were measured in the superficial dorsal horn, where the afferents from the nerve root synapse with second order neurons (Basbaum et al. 2009, Wall and Melzack 1994). In contrast to our findings of a sustained decrease in the discharge rate after a nerve root compression (Figure 8), chronic compression of the nerve root has been previously reported to increase the duration of neuronal discharge that is evoked by a mechanical stimulus applied directly to the nerve root (Howe et al. 1977). This discrepancy suggests that the changes in the electrophysiologic properties of the neurons after nerve root injury depend on the type and location of the stimulus. While no attempt was made to differentiate between primary or second order neurons from the data in our study, transection of the nerve root at the conclusion of each recording ensured that the evoked responses were associated with the C7 root. It should also be noted that the electrophysiologic properties of neurons that are evoked by a peripheral electrical stimulus do not vary within the 150-350μm range of depths measured in the present study (Wall et al. 1981; Woolf and Fitzgerald 1983). Therefore, despite the fact that the neurons measured in the three minute and 15 minute sham groups were 71μm and 91μm shallower than their corresponding compression groups, respectively, it is unlikely that this experimental condition contributed to differences in the neuronal responses measured in those groups.

Of note, the identification of the duration threshold of 6.6 minutes in this study only considered the response of action potentials that were evoked early (10-40msec) after the electrical stimulus (Figure 6). The neurons associated with such action potentials are the myelinated A-fibers (DeLaTorre et al. 2009; Pezet et al. 2008; Yu et al. 2009), which have a lower electrical threshold for excitation than the unmyelinated C-fibers (Ramer et al. 2000). However, both fiber types have been shown to exhibit a uniform decrease in the amplitude of electrically-evoked compound action potentials in response to axonal stretch (Shi and Whitebone 2006), suggesting that the electrophysiologic results of the present study may extend to the unmyelinated C-fiber population. Conversely, morphologic studies of peripheral nerve compression suggest that the myelinated fibers are more susceptible to mechanical injury (Jancalek and Dubovy 2007; Mosconi and Kruger 1997; Strain and Olson 1975). Thus, the response of myelinated fibers, rather than unmyelinated fibers, may be the more conservative estimate for nerve root compression injuries. Furthermore, several studies suggest that mechanical and thermal sensitivity are transmitted along distinct neuronal subpopulations, with A-fibers transmitting mechanical sensitivity (Cavanaugh et al. 2009; Scherrer et al. 2009). Sensitivity to a mechanical stimulus after a transient C7 nerve root compression has been well-documented (Hubbard et al. 2008a; Rothman et al. 2010). The results of the present study provide the electrophysiologic response of the compressed neurons that are associated with transmitting pain in this model of radiculopathy. As such, not only does the sustained neuronal dysfunction of and axonal injury in this class of neurons that is observed in the present study indicate the presence of tissue injury, but also indicates a potentially pivotal contribution in the mechanisms leading to persistent pain that can also result form a transient nerve root injury that may not demonstrate any observable signs of structural injury.

CONCLUSION

Compression to the nerve root produces an immediate decrease in electrically-evoked neuronal discharge rates from the periphery to the spinal cord.

As the compression period is increased, the discharge rate continues to decrease for the first 6.6±3.0 minutes, at which time this decrease in the rate of signaling plateaues. When the nerve root is compressed for a period shorter than this critical duration (in this case for three minutes), the evoked neuronal signaling returns to pre-compression levels within 10 minutes and no axonal damage is observed seven days after the injury. Conversely, for a root compression that is held for 15 minutes, a period known to produce sustained pain and considerable pathology in the nerve root, the evoked spikes remain significantly less than baseline for at least 10 minutes after compression is removed. The duration of compression to the nerve root appears to be critical in mediating both the acute neuronal functional responses and the subsequent development of axonal pathology. Notably, the findings of this study also demonstrate that compression of the nerve root initiates a pattern of abnormal signaling in the neurons that develops into *lasting* pathophysiology even when the initial mechanical insult is itself only *transient*.

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APPENDIX A

Discharge rates measured by the number of action potentials for each neuron in the electrophysiologic studies for the sham and compression groups in the 15 minute (Table A1) and 3 minute (Table A2) studies. In each table, each neuron that is included is indicated by the rat number (R followed by the number) followed by a letter to distinguish neurons from the same rat.

Table A1. Number of action potentials evoked at each time-point starting with baseline (BL) and running through the compression (C) times and post-compression (P) times for each neuron in the 15 minute study.

	Group	Sham n=4 neurons (1 rat)				Compression n=10 neurons (3 rats)									
	Neuron ID	R3A	R3B	R3C	R3D	R5A	R5B	R5C	R6A	R6B	R7A	R7B	R7C	R7D	R7E
Time-point	BL-1	6	12	2	7	1	4	3	1	2	3	4	8	2	3
	BL-2	4	19	1	12	2	4	5	2	1	5	7	0	4	6
	BL-3	7	9	7	6	2	6	5	0	0	0	6	5	3	4
	BL-4	6	12	6	8	2	6	2	0	1	2	3	3	3	2
	BL-5	7	16	4	7	1	6	3	1	1	1	1	4	3	1
	C1	1	10	2	12	1	4	1	1	2	0	3	4	4	1
	C2	4	14	1	9	1	3	2	2	1	0	3	1	3	0
	C3	6	12	0	10	2	2	1	1	1	0	3	1	1	0
	C4	3	12	4	5	4	4	3	1	2	0	2	1	2	0
	C5	1	15	1	14	1	2	1	2	1	0	2	1	1	0
	C6	6	12	2	5	1	1	2	1	3	0	2	1	1	0
	C7	5	16	3	7	2	1	0	1	0	0	3	1	0	0
	C8	3	15	2	2	4	1	1	1	0	0	1	0	0	0
	C9	5	7	1	9	0	4	0	1	2	0	1	0	0	0
	C10	4	16	1	9	0	1	0	2	0	0	4	0	0	0
	C11	3	8	1	10	0	1	2	0	0	0	2	0	0	0
	C12	7	11	2	11	2	6	4	0	1	0	1	0	1	0
	C13	7	13	2	10	1	4	3	0	2	0	3	0	0	1
	C14	6	15	4	10	1	11	2	0	2	0	1	1	0	0
	C15	9	16	2	11	6	7	6	0	0	0	1	0	1	0
	P1	9	12	1	10	1	6	3	0	1	0	0	0	2	0
	P2	7	13	1	11	1	5	2	1	0	0	2	0	0	1
	P3	6	14	1	11	0	9	2	1	2	0	1	0	1	0
	P4	8	13	1	13	2	1	2	2	1	0	0	0	1	0
	P5	4	15	1	9	2	5	0	1	1	0	1	0	0	0
	P6	7	10	0	19	0	1	1	1	0	0	2	0	2	0
	P7	7	15	1	15	0	3	3	0	1	0	1	0	1	0
	P8	9	14	2	11	0	3	3	1	2	0	1	0	3	0
	P9	5	15	1	10	1	5	1	0	0	0	0	1	1	0
	P10	6	18	2	12	4	2	1	0	1	0	0	0	0	0

Table A2. Number of action potentials evoked at each time-point starting with baseline (BL) and running through the compression (C) times and post-compression (P) times for each neuron in the 3 minute study.

	Group	Sham n=3 neurons (1 rat)			Compression n=9 neurons (6 rats)								
	Neuron ID	R16A	R16B	R16C	R13A	R14A	R15F	R17A	R17B	R18A	R19A	R19C	R19D
Time-point	BL-1	70	4	1	4	3	5	20	2	2	1	1	3
	BL-2	52	6	2	7	2	1	23	0	1	0	1	1
	BL-3	50	3	1	16	4	2	20	1	0	1	1	2
	BL-4	56	10	1	11	0	2	28	0	0	1	0	0
	BL-5	47	10	1	10	3	2	23	0	2	1	1	0
	C1	37	3	1	11	3	2	14	0	0	1	0	0
	C2	43	6	1	8	3	1	23	1	0	0	0	0
	C3	52	8	1	13	2	0	16	0	0	0	0	0
	P1	39	6	1	7	0	0	14	0	0	0	3	1
	P2	53	8	2	10	3	0	16	0	0	1	0	0
	P3	63	8	3	9	0	1	11	0	0	0	1	2
	P4	74	4	1	9	1	1	8	0	2	0	0	1
	P5	64	6	1	6	2	2	15	1	1	0	1	1
	P6	73	5	1	12	1	0	37	0	0	0	0	0
	P7	75	3	0	9	3	0	23	0	1	0	1	0
	P8	85	5	0	11	0	2	17	0	2	1	2	1
	P9	70	3	1	8	1	0	22	1	2	1	1	0
	P10	78	6	0	7	2	0	12	2	0	1	1	1