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Spinal Neuropeptide Responses in Persistent and Transient Pain Following Cervical Nerve Root Injury

Sarah M. Rothman, BSE, Rob A. Kreider, and Beth A. Winkelstein, PhD

Study Design. Behavioral and immunohistochemical analysis in rat models of persistent and transient allodynia.

Objectives. To examine separate cervical nerve root injuries (compression, transection) for producing behavioral hypersensitivity and investigate spinal neuropeptides to understand relationships to pain symptoms.

Summary of Background Data. Mechanical cervical nerve root injury can be a source of neck pain. Painful lumbar radiculopathy models show that different nerve root ligation intensities produce differential allodynia responses. Spinal neuropeptides can mediate pain responses. Yet, little is known about their contributions to pain in the cervical spine.

Methods. Rats underwent separate procedures on the right C7 nerve roots: transection (n = 12), 10-gf compression for 15 minutes (n = 11), or sham (n = 5). Ipsilateral forepaw mechanical allodynia was measured after surgery for 7 days. C7 spinal cord tissue was analyzed by immunohistochemistry for substance P and calcitonin gene-related peptide (CGRP) expression on days 1 and 7 for each injury; densitometry quantified immunoreactivity in lamina I of the ipsilateral dorsal horn.

Results. Both injuries immediately produced significant increases in allodynia. Sensitivity was sustained following root compression, and at day 7, was not different from day 1. By day 7 after transection, allodynia had returned to baseline and sham levels, significantly decreasing from day 1 ($P = 0.0012$). Spinal substance P and CGRP were increased over normal at day 1 for both injuries and decreased with time for CGRP after transection, which paralleled behaviors. For individual rats, substance P was significantly ($P < 0.001$) correlated with CGRP expression for both injuries.

Conclusions. Compression and transection of the cervical nerve root produce different forepaw allodynia responses, with persistent and transient sensitivity, respectively. Spinal neuropeptide expression in these models parallels this sensitivity, suggesting their potential role in pain symptoms.

Key words: neck pain, substance P, calcitonin gene-related peptide, radiculopathy, cervical nerve root. **Spine 2005;30:2491–2496**

From the Department of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania.
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Address correspondence and reprint requests to Beth Winkelstein, PhD, Department of Bioengineering, University of Pennsylvania, 120 Hayden Hall, 3320 Smith Walk, Philadelphia, PA 19104-6392; E-mail: winkelst@seas.upenn.edu

It is estimated that as many as 30% of Americans have chronic pain, and its economic cost is staggering at an estimated $61 billion annually as a result of patient costs, lost workplace productivity, and absenteeism.[1,2] Cervical nerve roots are particularly susceptible to a variety of mechanical injuries from neck motions, because of their mechanical frailty, and may be a source of neck and radiating pain. Despite its high incidence and its tremendous economic and emotional burden, little is known about how mechanical injury is transduced *via* biochemical signaling to contribute either to persistent or transient sensitivity. It may be possible that persistent and transient pain result from varied biochemical pathways, which may diverge following injury. Although a host of physiologic mechanisms have been described for painful lumbar nerve and nerve root injuries,[3–12] the cervical nerve root's response to mechanical injury and its ability to induce pain remains largely uncharacterized at present.

In lumbar pain models, it has been quantitatively shown that the magnitude of nerve root deformation imposed for radicular injury directly relates to the resulting behavioral sensitivity, with higher deformation, producing higher mechanical allodynia.[11,13] Furthermore, qualitatively different lumbar nerve root ligation tightness has also altered hypersensitivity patterns, with loose ligation producing symptoms for only 7 days and tight ligation having longer lasting hypersensitivity.[5] Together, these findings suggest that tissue injury mechanisms may play a role in initiating different cascades contributing to pain symptoms. For example, nerve root compression magnitude alters both amplitude and timing of allodynia responses in low back pain. In studies directly comparing the effects of nerve transection with nerve root compression injury, altered allodynia patterns[4] and responses to pharmacologic treatment[14] further imply that different injuries may initiate separate physiologic mechanisms. Ultimately, different injuries to the same neural structure may also require different therapeutic strategies for alleviating pain. There is currently no clear understanding of how different types of nerve root injury in the cervical spine contribute to either persistent or transient pain symptoms, or the mechanisms contributing to behavioral hypersensitivity.

A host of nociceptive responses are initiated following a painful neural tissue injury.[3,10,14,15] Neuropeptides are transported anterograde and released where they can cause neurogenic inflammation. One such potent pronociceptive neurotransmitter, substance P, can cause a release of calcium from intracellular stores and, in turn,

lead to nitric oxide production and neuronal excitability and long-term sensitization.[16,17] Sciatic nerve injury induces profound changes in substance P expression, even at assay times following the resolution of pain.[18] Also contributing to sensitization, calcitonin gene-related peptide (CGRP), a neuropeptide often colocalized with substance P in the spinal cord, regulates nociception by further promoting the release of substance P, as well as promoting release of glutamate from primary afferents and retarding the metabolism of substance P.[8,19] Antibodies to substance P and CGRP can attenuate pain symptoms in inflammatory models of carrageenan-induced hyperalgesia and painful nerve injury.[20,21]

In addition, application of antagonists to the substance P receptor, NK-1, has induced antinociception in the central nervous system after chronic nerve constriction,[22–24] as well as locally in rodent models of inflammatory pancreatitis[25] and arthritis.[26] Finally, pretreatment with a toxin that selectively eliminates cells containing the substance P receptor attenuates behavioral sensitivity after both inflammatory and mechanical injury.[27–29] These results strongly implicate both of these neuropeptides in the transmission of pain. However, despite research suggesting potent roles for substance P and CGRP in many types of pain, little is known about their relative contributions to the onset and/or maintenance of pain in the cervical spine.

Therefore, the purpose of this study was to define the temporal profiles of cervical spinal substance P and CGRP following cervical nerve root injuries, to begin to assess their roles in persistent and transient pain. Expression of these spinal neuropeptides was studied in the context of allodynia. Nerve root compression and transection were used as painful models inducing persistent and transient allodynia, respectively. Ipsilateral forepaw mechanical allodynia was monitored to establish these as models of persistent and acute behavioral hypersensitivity. Immunohistochemical analysis of spinal substance P and CGRP was performed at 2 postoperative times (days 1 and 7) for insight into spinal mechanisms contributing to pain. This study explores how persistent and transient allodynia mechanisms diverge, as well as begins to investigate mechanisms of chronic neck pain.

■ Materials and Methods

Experiments were performed using male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, ID), weighing 200–350 g at the start of the study, housed under US Department of Agriculture and Association for Assessment and Accreditation of Laboratory Animal Care approved conditions, with a 12– −12 hour light-dark cycle, and free access to food and water. All experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee, and performed according to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.[30]

Surgical Procedures. All procedures were performed with rats under inhalation anesthesia (4% halothane for induction, 2% for maintenance). Rats underwent 1 of 3 surgical procedures: nerve root compression (n = 11), complete nerve root transection (n = 12), or sham exposure (n = 5). Briefly, rats were placed in a prone position, and an incision was made in the skin from the base of the skull to the bony prominence of the second thoracic vertebra. Muscle and soft tissue were cleared, exposing the C6 and C7 laminae, under a surgical microscope (Carl Zeiss, Inc.; Thornwood, NY). A C6/C7 hemilaminectomy and partial facetectomy were performed on the right side to expose the spinal cord and right C7 dorsal nerve root. For nerve root compression, a 10-gf microvascular clip (World Precision Instruments, Inc.; Sarasota, FL) was applied to the right C7 dorsal nerve root, proximal to the dorsal root ganglion (Figure 1). Compression was imposed for 15 minutes before careful clip removal. For nerve root transection, the right C7 dorsal nerve root was transected using microdissecting scissors, and the cut ends were fully separated. Sham procedures involved the same surgical procedures as described with root exposure only and no further manipulation. Following surgery, all wounds were closed using 3-0 polyester suture and surgical staples. Rats were recovered in room air and monitored throughout their recovery.

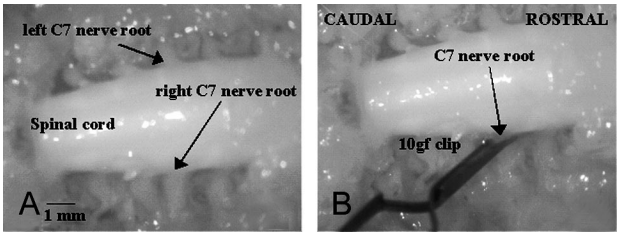

Figure 1. Representative *in vivo* images illustrating surgical procedures. Nerve roots were fully exposed (**A**) and underwent either compression (**B**) or transection (not shown). A 10-gf clip was used to compress the C7 nerve roots for 15 minutes (**B**). Separately, transection of the C7 nerve root was followed by separation of nerve root ends; this separation prevented axons from communicating with the spinal cord. For illustrative purposes, here the laminectomy has been extended to include more spinal levels to reveal fully the cord, neural anatomy, and surgical techniques.

Mechanical Allodynia. Following surgery, mechanical allodynia (pain caused by a stimulus that does not normally provoke pain) was measured. Rats were evaluated for allodynia in the ipsilateral forepaw on postoperative days 1, 3, 5, and 7, or until the designated time of tissue harvest. Methods for quantifying forepaw allodynia used in this study have been adapted from those commonly used to measure hind paw allodynia in lumbar models of low back pain[3–5,10,11,14,16] and have been previously validated for the forepaw.[31,32] Briefly, before surgery, animals were acclimated to the tester and environment, and baseline measurements were recorded as a matched control to compare to responses after injury. For testing, after 20 minutes of acclimation, rats were stimulated on the plantar surface of the ipsilateral forepaw using von Frey filaments (2, 4 g) (Stoelting Co.; Wood Dale, IL). Each testing session consisted of 3 rounds of 10 stimulations each, separated by 10 minutes. For each session with a given filament, the total number of withdrawals was counted for each rat, and averages for procedural groups were determined. A single tester performed all testing blinded to surgical procedures.

Tissue Harvest, Immunohistochemistry, and Densitometry. Cervical spinal cord tissue was harvested at 2 times after surgery to determine the temporal profile of substance P and CGRP expression in the C7 spinal cord following injury. In separate investigations, subsets of rats were studied on each of day 1 (n = 6 compression; n = 6 transection; n = 2 sham) or day 7 (n = 5 compression; n = 6 transection; n = 3 sham) following injury. In addition, cervical spinal cord from naïve (unoperated) rats (n = 4) was also processed for comparison. For tissue harvest, rats were deeply anesthetized, followed by transcardiac perfusion with 200 mL of phosphate buffered saline (PBS) and 300 mL of 4% paraformaldehyde in PBS (pH 7.4). Following perfusion, C7 spinal cord was exposed by laminectomy, harvested, and postfixed in 4% paraformaldehyde for 20 minutes. Samples were transferred to 30% sucrose/PBS and stored for 3 days at 4°C. Tissue was freeze-mounted with Histoprep (Fisher Chemical, NJ) medium for axial sectioning.

Free-floating C7 spinal cord sections (20 μm) were collected in PBS for immunohistochemical analysis of substance P and CGRP reactivity. For substance P analysis, slices were blocked with normal goat serum (Vector Labs; Burlingame, CA) for 20 minutes before incubating for 24 hours in a rabbit polyclonal antibody to substance P (1:2000) (Chemicon; Temecula, CA) diluted in PBS-triton. For analysis of CGRP reactivity, sections were blocked with normal goat serum (Vector Labs) and incubated with a primary rabbit anti-CGRP antibody diluted 1:4000 (Bachem; San Carlos, CA) for 36 hours. For both antibodies, after incubation, sections were treated with biotinylated goat antirabbit immunoglobulin G, quenched in 0.3% peroxide solution, and developed using 3,3-diaminobenzidine (Vector Labs). For CGRP analysis, chromogen was enhanced by glucose oxidase-nickel-3c3-diaminobenzidine. Negative controls (omission of primary antibody) and naïve tissue samples were included for comparison. Sections were mounted on gelatin-coated slides, dehydrated in an ethanol series, and coverslipped using Permount (Fisher Chemical; Fairlawn, NJ).

Densitometric image analysis was performed to quantify the amount of spinal substance P and CGRP immunoreactivity in the ipsilateral dorsal horn of each spinal cord sample, based on staining intensity. An analyzer blinded to surgical groups and times performed all analyses. At least 2 representative cord sections for each neuropeptide were imaged for each rat using a Zeiss Axioskop40 microscope (Thornwood, NY) at 50×. Using the Image-Pro Plus5.0 software (Media Cybernetics Inc.; Silver Spring, MD), images were converted to grayscale, and flattened to reduce and normalize variations in intensity across the background pixels. Area of interest (AOI) boxes (101 × 101 pixels) were used to sample regions for image intensity. For each section, 2–3 AOIs were randomly sampled in lamina I of the ipsilateral dorsal horn. Likewise, AOI sampling was also performed to quantify intensity in the general background of the slide. Average intensity of lamina I was determined and subtracted from average background intensity, normalizing each section. For each surgical group and time, average intensity was quantified and compared.

Statistical Analysis. To determine significant differences in allodynia between injury groups, a 1-way analysis of variance (ANOVA) with Bonferroni correction was used, with significance at $P < 0.05$. Differences were tested between groups at baseline (day 0), day 1, and day 7. Two sample *t* tests, assuming equal variances, tested for differences between day 1 and 7 times for a given injury. For densitometry, 1-way ANOVA with Bonferroni correction and significance at $P < 0.05$, tested for differences in intensity between groups at each time. A 2-sample *t* test with equal variances was similarly used to test for differences between times in substance P and CGRP intensity, respectively. The relationship between the intensity of expression of the 2 neuropeptides (substance P and CGRP) was examined by linear regression. Using all animals, a correlation was performed between the substance P and CGRP staining intensity; significance for this correlation was tested using an ANOVA, with $P < 0.05$.

■ Results

For both types of nerve root injury, ipsilateral forepaw mechanical allodynia was immediately increased over sham (Figure 2). In contrast, allodynia following sham procedures remained at baseline levels, with no significant differences at any time for stimulation with either filament. On day 1, root compression produced a significant increase in allodynia over both sham ($P < 0.012$) and baseline ($P < 0.001$) for the 4-g filament (Figure 2). Behavioral trends were similar for stimulation with the 2-g filament, also showing significant increases in allodynia following compression over sham ($P < 0.006$) and

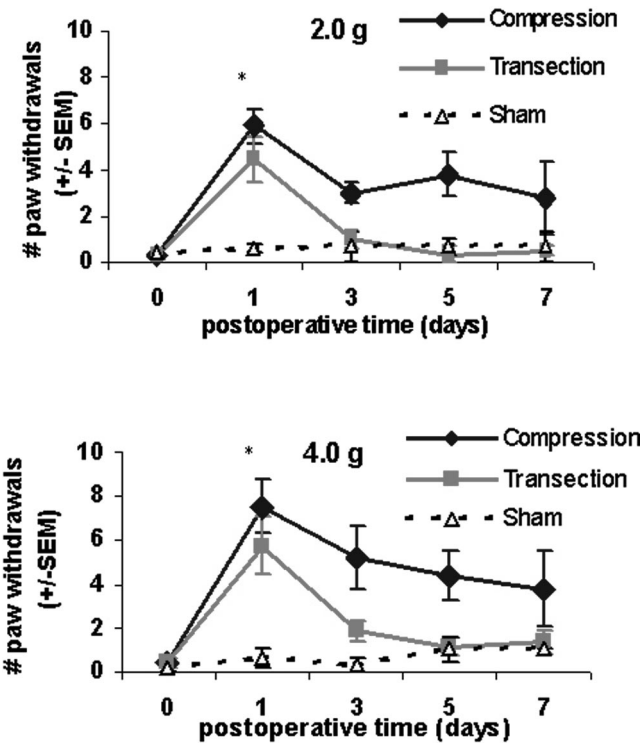

Figure 2. Average ipsilateral forepaw mechanical allodynia as measured by the number of paw withdrawals for compression, transection, and sham injuries. Allodynia was significantly increased for both nerve root injuries compared to sham and baseline at day 1 for both von Frey filaments. However, over time, allodynia following compression injury remained appreciably increased, while allodynia after transection returned to sham levels. Trends were similar for both the 2 and 4-g filaments, indicating verification of these trends. Higher numbers of paw withdrawals correspond to higher allodynia and increased behavioral sensitivity. Asterisk (*) indicates significant increases over sham. SEM, standard error of the mean.

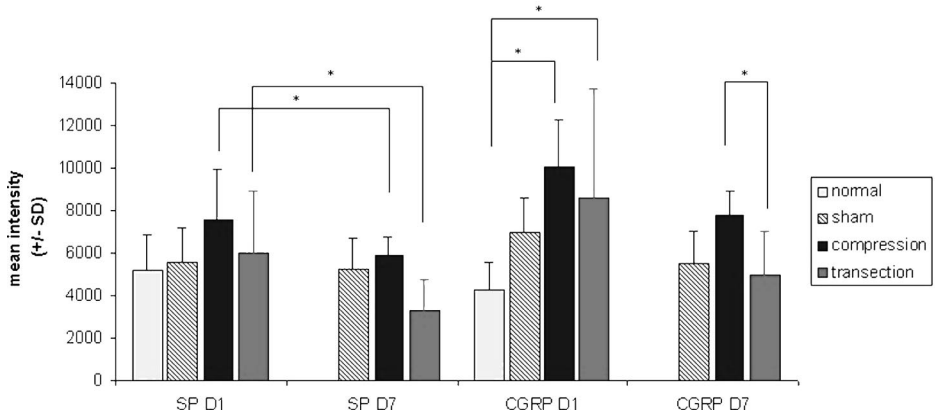

Figure 3. Average staining intensity in the ipsilateral dorsal horn for substance P (SP) and CGRP at days 1 (D1) and 7 (D7) following injury. Higher intensity indicates more neuropeptide expression. CGRP displayed a more robust response to injury than substance P. Substance P was increased over normal in compression and transection, but neither increase was significant; yet, CGRP significantly increased for both injuries at day 1. By day 7, after transection, substance P levels decreased significantly and were below normal, while substance P following compression decreased but remained above normal. Similarly, by day 7, CGRP decreased most notably in transection. Sham procedures did not produce any significant increases in substance P or CGRP reactivity compared to normal at either time. An asterisk (*) indicates statistically significant differences. SD, standard deviation.

baseline ($P < 0.001$). Similarly, at day 1, following transection, allodynia increased significantly ($P < 0.001$; both filaments). On day 1, allodynia was not significantly different between the 2 root injuries for either filament. Behavioral hypersensitivity was sustained after root compression, and was not significantly different between days 1 and 7 (Figure 2). In contrast, following transection, allodynia had fully returned to baseline and sham levels by day 7, showing a significant decrease from day 1 ($P = 0.0012$, 2 g; $P = 0.004$, 4 g). At day 7, allodynia following transection was not different from sham, indicating resolution of behavioral sensitivity.

Densitometric quantification of neuropeptide expression in the ipsilateral dorsal horn of the spinal cord was consistent with direct observation of the intensity of tissue reactivity (Figures 3, 4). Sham procedures did not produce any significant increases in substance P reactivity over normal levels at either postoperative time (Figure 3). Likewise, although spinal CGRP for sham was slightly increased over normal at both times, this was not significant. Neuropeptide expression was not changed over time following sham procedures (Figure 3). Although both neuropeptides were altered following injury, CGRP produced more sensitive changes than substance P, particularly for compression injury (Figures 3, 4). For example, although substance P expression was immediately increased following nerve root injury, these increases were only 1.5-fold over normal for compression and 1.2-fold for transection, and were not significant.

In contrast, CGRP expression on day 1 was 2.4 times higher than normal for compression and 2 times higher for transection (Figure 3). These CGRP increases over normal were significant for both compression ($P = 0.005$) and transection ($P = 0.046$). By day 7, following transection, spinal substance P had significantly ($P = 0.025$) decreased to half its expression at day 1. For compression, substance P decreased to only two thirds its expression on day 1, despite being a significant change ($P = 0.047$). On day 7, after transection, substance P was significantly less than either compression ($P < 0.001$) or sham ($P = 0.007$) levels. In contrast, at day 7, CGRP decreased only slightly compared to day 1 for compression, but this was not significant and remained increased over normal ($P = 0.001$) and root transection ($P = 0.002$) expression. For transection, CGRP robustly decreased by day 7 and was no longer different from normal or sham levels.

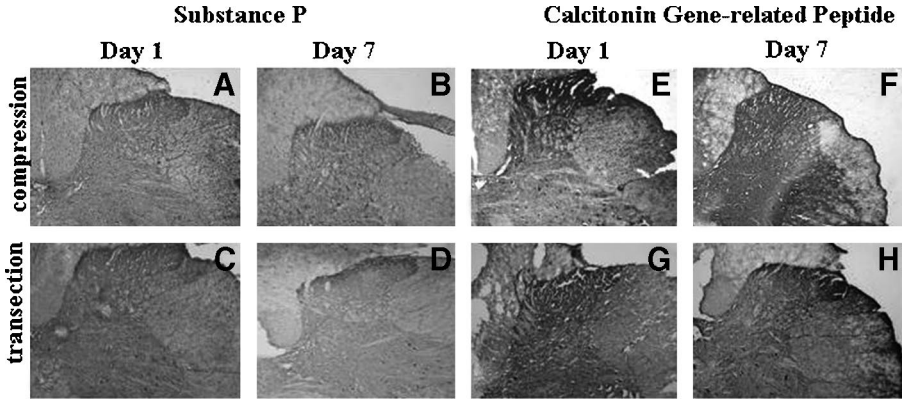

Figure 4. Representative micrographs of the C7 ipsilateral dorsal horn showing immunohistochemical staining for substance P (**A**–**D**) and CGRP (**E**–**H**) at postoperative day 1 (**A**, **C**, **E**, **G**) and day 7 (**B**, **D**, **F**, **H**). For compression, CGRP reactivity at day 1 (**E**) decreased noticeably by day 7 (**F**), whereas substance P reactivity at day 7 (**B**) was only slightly decreased compared at day 1 (**A**). Transection produced a more robust decrease in reactivity between days 1 and 7 in both CGRP (**G**, **H**) and substance P (**C**, **D**). Sham samples (not shown) were not different from normal for either neuropeptide over time. Scale bar (shown in **E**) is 200 μm and applies to all.

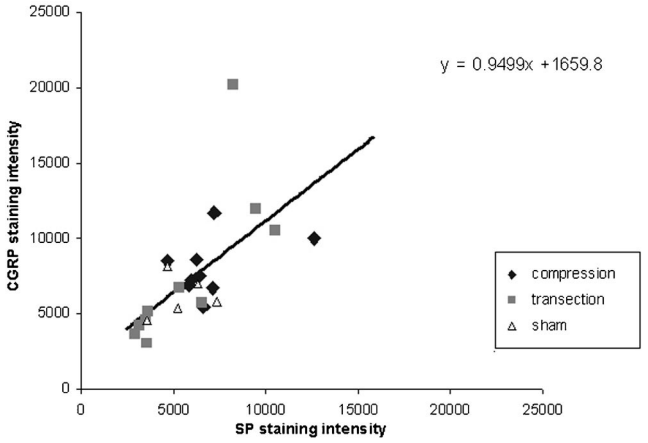

Figure 5. Plot showing individual substance P (SP) and CGRP intensity for each individual rat. Substance P and CGRP staining intensity were significantly correlated ($r = 0.63$; $P = 0.001$). Results suggest a general colocalization of the 2 proteins in lamina I.

Substance P and CGRP intensity in the ipsilateral dorsal horn correlated with each other for individual rats, with those animals showing high substance P reactivity also expressing higher levels of CGRP (Figure 5). Substance P and CGRP were significantly ($P = 0.001$) and positively correlated ($r = 0.63$) with each other. In fact, individual responses indicated that the highest levels of neuropeptide expression corresponded to a transection injury on day 1, whereas the lowest intensity correspond to day 7 transection and sham responses, where allodynia magnitude was lowest (Figure 5).

■ Discussion

To our knowledge, this study is the first to investigate neuropeptide mechanisms contributing to pain symptoms after cervical nerve root injury. Results show that different allodynia patterns are produced for different cervical root injury paradigms. These behavioral data are consistent with other experience with these models.[32] Likewise, this study shows that cervical spinal substance P and CGRP may also be differentially modulated by injury. Nerve root compression produced increased behavioral sensitivity, sustained robust CGRP responses, and increased spinal substance P (Figures 2–4), implying a role for either or both neuropeptides in the onset and maintenance of allodynia. However, neuropeptide expression in the transection model displayed a different temporal profile than for root compression. Resolution of behavioral sensitivity following transection corresponded to decreases in both substance P and CGRP, to below and at-normal levels, respectively (Figures 3, 4). These findings suggest that different injury paradigms may produce differential neuropeptide responses.

The observed difference in spinal neuropeptide responses may be caused by 1 of several physiologic mechanisms. The tempered decrease in substance P could indicate that this neuropeptide plays a role specifically in the initiation, but not maintenance, of pain for injuries in the neck. In contrast, following compression, CGRP did not significantly decrease postoperatively, implying a potential role for CGRP in the maintenance of pain. Further support for this assertion is provided in the literature by evidence that antibodies to CGRP can attenuate hyperalgesia in a model of long-lasting repeated cold stress.[20,21] Also, substance P immunoreactivity has been reported to decrease after sciatic nerve transection and increase after compression, only in those neurons that survive the injury.[7] Because root transection in our model causes complete dissociation of axons from their cell bodies in the dorsal root ganglion, signaling from the periphery to the dorsal horn of the spinal cord is completely eliminated, perhaps reducing the number of viable neurons in the spinal cord. Furthermore, the use of forceps to crush manually lumbar nerve roots has been previously shown as sufficient to induce spinal neuronal apoptosis,[9] implying that the 10-gf compression applied in our study may also cause cell death or neuronal plasticity in the root and/or spinal cord.

Spinal biochemical changes can persist long after pain symptoms have resolved,[18] further implying a dynamic and potent biochemical cascade for nociception. If cervical root injury caused by local compression incites spinal immune responses as in the lumbar spine,[11,13] glial activation may further contribute to the complex nociceptive physiology of neck pain.[32] However, it is noteworthy that although this study shows that cervical nerve root transection produces only transient allodynia, this is in contrast to responses observed in the lumbar spine where root transection can produce persistent and sustained behavioral sensitivity.[4,5] This behavioral finding suggests that a difference may exist in pain transmission for similar injuries in these 2 spinal regions, highlighting the need for further research into mechanisms of pain in the cervical spine.

Our study found a significant positive correlation between substance P and CGRP expression in lamina I for both injuries. These findings show simultaneous robust substance P and CGRP expression (Figures 3–5), and suggest their regional colocalization in lamina I, consistent with previously published work implicating them in pain.[16,17] However, these findings do not provide direct evidence of cellular colocalization of these neuropeptides. From individual analysis (Figure 5), the highest reactivity for substance P and CGRP was observed in tissue from transection injuries sampled on day 1; whereas lowest reactivity was observed in samples from transection injuries on day 7 and sham procedures. These observations parallel behavioral trends for transection in which mechanical allodynia was highest on day 1 and returned to sham levels by day 7, with little-to-no allodynia response in those animals (Figure 2). By examining neuropeptide responses in this way, any effects that may be lost as a result of grouping are recovered. For example, the relatively large variance at day 1 after transection (Figure 3) does not fully indicate the relationship between spinal cord and behavioral responses observed in the individual rats in that group.

Although the root injury paradigms presented here produce differential allodynia and neuropeptide profiles, further studies are needed to understand fully the mechanisms contributing to their differences. For example, information on cell survival and temporal expression of the substance P receptor after both cervical injuries will provide context for the findings presented here. Indeed, antagonist studies in the cervical spine are needed to both verify a role for these neuropeptides and their receptors in pain, and to help define effective treatments for these painful disorders in the neck. The results presented here do provide a valuable foundation for identifying those cellular signaling pathways that may contribute differentially to persistent and transient pain.

■ Key Points

- Different forepaw mechanical allodynia patterns are produced for cervical nerve root compression and transection.
- Nerve root compression and transection produce differential ipsilateral dorsal horn substance P and CGRP temporal profiles.
- Substance P and CGRP are significantly, positively correlated for individual animals according to behavioral hypersensitivity.

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