An Anatomical and Immunohistochemical Characterization of Afferents Innervating the C6–C7 Facet Joint After Painful Joint Loading in the Rat

Jeffrey V. Kras, BS,* Kosuke Tanaka, DDS,† Taylor M. Gilliland, * and Beth A. Winkelstein, PhD‡

Study Design. This study used retrograde neuronal tracing and immunohistochemistry to identify neurons innervating the C6–C7 facet joint and those expressing calcitonin gene-related peptide (CGRP) in the dorsal root ganglion (DRG) of rats after painful cervical facet joint injury.

Objective. The objective of this study was to characterize the innervation of the C6–C7 facet joint after painful joint injury in the rat.

Summary of Background Data. The cervical facet joint is a source of neck pain, and its loading can initiate persistent pain. CGRP is a nociceptive neurotransmitter; peptidergic afferents have been identified in the facet joint’s capsule. Although studies suggest that facet joint injury alters CGRP expression in joint afferents, the distribution of neurons innervating the C6–C7 facet joint and their expression of CGRP after a painful joint injury have not been investigated.

Methods. Holtzman rats (Harlan Sprague-Dawley, Indianapolis, IN) received an intra-articular injection of cholera toxin subunit B in the C6–C7 facet joints. After injection, subgroups underwent either a painful joint distraction or sham procedure. Mechanical sensitivity was assessed, and immunohistochemical techniques were used to quantify CGRP expression and cholera toxin subunit B labeling in the C5–C8 DRGs.

Results. Facet joint distraction-induced (P ≤ 0.0002) hypersensitivity. Neurons labeled by the joint injection were identified in the C5–C8 DRGs. Significantly, more (P ≤ 0.0001) cholera toxin subunit B-positive neurons were identified in the C7 DRG than any other level. At C7, 54.4% ± 15.3% of those neurons were also CGRP-positive, whereas only 41.5% ± 5.4% of all neurons were CGRP-positive; this difference was significant (P = 0.0084).

Conclusion. The greatest number of afferents from the C6–C7 facet joint has cell bodies in the C7 DRG, implicating this level as the most relevant for pain from this joint. In addition, peptidergic afferents seem to have an important role in facet joint-mediated pain.

Key words: facet, joint, pain, innervation, DRG, neuron, CGRP, retrograde labeling. Spine 2013;38:E325–E331
underwent either a painful cervical facet joint distraction and 5
Company, Reno, NV) with a 33-gauge beveled needle was
and their capsules were exposed. A 10-
Fluor 488 (Life Technologies, Carlsbad, CA) and dissolved in
 toxin subunit B (CTb) conjugated to the fluorescent dye Alexa
of 20
fl urane anesthesia (4% induction, 2.5% maintenance). All rats
in the rat. 12–14,30,31 Specifically, painful joint distraction upregu-
traces with a 12 to 12 hour light-dark cycle and free access to
food and water. Experimental procedures were approved by
ore AA accreditation of Laboratory Animal Care-compliant condi-
tions with a 12 to 12 hour light-dark cycle and free access to
food and water. Experimental procedures were approved by
our Institutional Animal Care and Use Committee and carried
out under the guidelines of the Committee for Research and
Ethical Issues of the International Association for the Study of
Pain. 35
All surgical procedures were performed under inhalation iso-
flurane anesthesia (4% induction, 2.5% maintenance). All rats
received a bilateral intra-articular C6–C7 facet joint injection
of 20 μg of the retrograde neuronal tracing molecule cholera
toxin subunit B (CTb) conjugated to the fluorescent dye Alexa
Fluor 488 (Life Technologies, Carlsbad, CA) and dissolved in
sterile phosphate buffered saline (PBS). A midline incision was
made along the back of the neck, and the C6–C7 facet joints
and their capsules were exposed. A 10-μL syringe (Hamilton
Company, Reno, NV) with a 33-gauge bevelled needle was
advanced into the facet joint, and 5 μL of the CTb solution
was slowly injected. After injection, the exposure was closed
in layers using 3–0 polyester sutures and surgical staples.
Three days after the CTb injection, a subset of rats
underwent either a painful cervical facet joint distraction
injury (n = 4) or sham procedure (n = 5), as described
previously.12,16,36 Under inhalation anesthesia, the surgical
staples and suture were removed, and the C6–C7 facet joints
were re-exposed. The interspinous ligaments and ligamentum
flavum from C5 to T1 were transected, and the C6 and C7
laminae were rigidly attached to a customized loading device
via microforceps. For the painful injury group, the bilateral
C6–C7 facet joints were distracted by displacing the C6 verte-
bra rostrally, while holding the C7 vertebra fixed.14,16,17,37 A
camera mounted to a surgical dissecting scope tracked mark-
ers on the C6 and C7 laminae during injury to quantify the
distraction. An additional group of rats underwent sham
surgical procedures with device attachment, but no applied
joint distraction. After surgery, the incision was closed and
rats recovered. The remaining group of rats (normal, n = 4)
received no surgical procedures after the initial CTb injection.
Bilateral forepaw mechanical hyperalgesia was evaluated in
those rats undergoing the painful joint injury or sham
control procedure using previously validated methods.14,31,37
Baseline measurements were recorded for 2 days after the
CTb injection. Hyperalgesia was measured on days 1, 3, 5,
and 7 after the injury or sham procedure. In each testing ses-
sion the rats were placed in elevated cages with a wire mesh
floor and allowed to acclimate to the testing environment.
Testing consisted of 3 rounds of mechanical stimulation to
each forepaw using an ascending series of von Frey filaments
(Stoelting, Wood Dale, IL). Each filament was applied 5 times
with at least 10 minutes separating each round of stimulation.
Positive responses, defined as emphatic lifting of the forepaw,
were used to determine the mechanical response threshold. A
given filament was recorded as the response threshold if the
next higher filament also induced a positive response. Because
the applied joint distraction is a bilateral injury, response
thresholds were averaged between the right and left fore-
paws for each rat. At each time point, response thresholds
were compared between groups and to the respective baseline
values using a 2-way repeated measures analysis of vari-
ance (ANOVA) with the Tukey honestly significant difference
(HSD) test, with time and group as the factors.
On day 7 after the injury or sham procedures, rats were
given an overdose of sodium pentobarbital (65 mg/kg) and
perfused transcardially with 300 mL of PBS and 250 mL of
4% paraformaldehyde in PBS (pH7.4). The DRGs on the
right side were harvested and postfixed in the same fixative
solution for 2.5 hours at 4°C. DRGs were then transferred
to 30% sucrose for 5 days at 4°C before being embedded in
Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA).
Each DRG was axially sectioned at a 14 μm thickness through
its entire length, and sections were thaw-mounted onto slides.
All sections were washed and blocked with normal donkey
serum (Chemicon, Temecula, CA) for 2 hours before incuba-
tion with a polyclonal rabbit anti-CGRP antibody (1:5000; T-4032; Peninsula Laboratories; San Carlos, CA) overnight
at 4°C. The following day, sections were washed and incu-
bated with a Cy3-conjugated donkey-anti-rabbit secondary
antibody (1:500, Jackson ImmunoResearch, West Grove,
PA) for 2 hours at room temperature and coverslipped using
Fluoro-Gel anti-fade medium (Electron Microscopy Sciences,
Hatfield, PA).
A fluorescent microscope equipped with a digital camera (Olympus, Center Valley, PA) was used to image each DRG section that contained at least one neuron positively labeled with CTb. The total number of neurons that were positive for CTb was counted for each DRG; care was taken to avoid double-counting neurons that appeared in multiple consecutive sections. The total number of CTb-positive neurons was summed for each group at each DRG level. Also, for those CTb-positive neurons, both the cross-sectional area and the intensity of CGRP labeling were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Each neuron was identified as being either CGRP-positive or CGRP-negative on the basis of its intensity of CGRP labeling. The number of CTb-positive neurons also positively labeled for CGRP was counted at each level for each rat; the total number of those double-labeled neurons at each level was computed for each group. The average percentage of all CTb-positive neurons that were also positive for CGRP also was determined for each group in each DRG. The average cross-sectional area of CTb-positive neurons expressing CGRP was determined. The number of CTb-positive neurons at each level was compared using a 2-way ANOVA with the Tukey HSD test, with group and level as the factors. A 2-way ANOVA with the Tukey HSD test (with group and level as factors) compared the average cross-sectional area of neurons positive for both CGRP and CTb; a separate 2-way ANOVA with the Tukey HSD test compared the ratio of CTb-positive neurons that were CGRP-positive to the total number of CTb-positive neurons. All statistical analyses were performed using JMP 8 (SAS Institute; Cary, NC) software.

To assess the frequency of peptidergic neurons among joint afferents compared with all neurons in the DRG at the C7 level, 3 sections were chosen from C7 at random from each rat by an evaluator who was blinded to the rat identifications and tissue samples. The cross-sectional area and CGRP labeling intensity of all neurons were quantified. All neurons in the C7 DRG were classified as either CGRP-positive or CGRP-negative. Both the ratio of CGRP-positive neurons to all neurons in each section and the average cross-sectional area of all CGRP-positive neurons in each section were determined. Separate comparisons of the ratio and the average cross-sectional area of CGRP-positive neurons were made between 2 populations of neurons in the C7 DRG: (1) joint afferents (those identified as CTb-positive neurons) and (2) all other neurons. Comparisons were tested using a 2-way ANOVA with the Tukey HSD and group (injury, sham, normal) and neuron population (CTb-positive neurons, all other neurons) as factors.

RESULTS
All rats undergoing a facet joint injury received the same magnitude of distraction, and no macroscopic injuries to the facet joint capsular ligament were observed during any of the applied distractions. The average applied distraction was 0.47 ± 0.05 mm. There were no differences in baseline withdrawal threshold between the injury and sham groups. Behavioral sensitivity was induced in all rats undergoing a joint distraction (Figure 1). The withdrawal threshold was significantly reduced (P ≤ 0.001) compared with baseline responses in the injury group at all time points after distraction, but sham procedures did not change responses from baseline at any time point (Figure 1). The withdrawal threshold was significantly reduced (P ≤ 0.0002) after injury compared with sham at all postoperative time points (Figure 1).

Neurons positive for CTb labeling were detected in all of the DRG levels that were assayed (Figures 2 and 3, Table 1). Significantly more (P ≤ 0.0001) CTb-positive neurons were identified in the C7 DRG than any other DRG (Table 1). The C8 DRG contained significantly more (P ≤ 0.0202) CTb-positive neurons than either of the C6 or C5 DRGs. Although C6 contained more CTb-positive neurons than C5 (Table 1), that difference was not significant. Although these trends were observed within each of the injury, sham, and normal groups, statistical significance was not achieved within each group individually; significance was only achieved when considering all groups together. Further, there were no differences in the number of CTb-positive neurons between groups at any of the cervical levels evaluated (Table 1). There were no significant differences detected in the ratio of CTb-positive neurons that were positive for CGRP to the total number of CTb-positive neurons between any groups at any level (Table 1). Similarly, there were no differences in the average cross-sectional area of the neurons positive for both CTb and CGRP between any groups at any level (Table 2).

In the C7 DRG, 41.5% ± 5.4% of all of the neurons were CGRP-positive. However, 54.4% ± 15.3% of CTb-positive neurons at that level expressed CGRP (Table 1), and this difference in the ratios of CGRP-positive neurons between these two populations of neurons was significant (P = 0.0084). This trend was also observed in each of the groups, but was not significant for any of the groups. Interestingly, the average

Figure 1. Mechanical hyperalgesia in the forepaw as measured by the average ± standard deviation withdrawal threshold (gram) elicited by von Frey filament stimulation. Forepaw hyperalgesia is induced after facet joint injury compared with baseline (P ≤ 0.001) on all days, but sham responses are unchanged from baseline. Withdrawal threshold in the injury group is significantly reduced (*P ≤ 0.0002) compared with sham at each postoperative time point.
cross-sectional area of neurons positive for both CTb and CGRP at the C7 level (724 ± 133 μm²) was significantly smaller ($P = 0.0005$) than the average area of all the CGRP-positive neurons in the C7 DRG (892 ± 116 μm²). Although this relationship was consistent for all of the experimental groups, the differences within each group were not significant.

**DISCUSSION**

These data characterize a multisegmental innervation of the C6–C7 facet joint in the rat and demonstrate that the joint innervation is unchanged at day 7 after painful mechanical joint loading (Tables 1 and 2). The applied distraction of 0.47 ± 0.05 mm in this study is in close agreement with a previously identified distraction magnitude (0.49 ± 0.09 mm) that was found to be sufficient to induce sustained behavioral sensitivity, while a lower magnitude of distraction (0.19 ± 0.03 mm) does not induce even transient mechanical sensitivity. In that context, it is not likely that the joint distractions used in this study (≈0.5 mm) are induced by the normal head movements in the rat, though this has not been studied explicitly. Of the spinal levels analyzed, the greatest number of neurons with projections to the C6–C7 joint had cell bodies in the C7 DRG, followed by the C8, C6, and C5 DRGs (Table 1). This trend in the segmental joint innervation is maintained despite an injury-induced increase in sensitivity to mechanical stimulation of the forepaw (Table 1, Figure 1). Although painful injury does not alter the percentage of joint afferents expressing CGRP in the C7 DRG, greater than one-half of the joint afferents are peptidergic (Table 1), but only slightly more than 40% of all neurons in the C7 DRG are peptidergic. Further, the average cell body is smaller for the peptidergic joint afferents ($724 ± 133 \text{ μm}^2$, Table 2) than for all of the peptidergic neurons ($892 ± 116 \text{ μm}^2$). Although this relationship was consistent for all of the experimental groups, the differences within each group were not significant.

The distribution pattern of neurons innervating the C6–C7 facet joint identified here is consistent with the studies
characterizing innervation of other cervical facet joints in that joint afferents originate from multiple spinal levels with one level having a dominant number of neurons.\textsuperscript{15,18} Indeed, multisegmental innervation of facet joints is also evident in humans in which the lower cervical facets receive fibers from the medial branches of the dorsal rami above and below the joint.\textsuperscript{39} The finding that most C6–C7 joint afferents originate in C7 and C8 (Table 1) supports the observation of forepaw hypersensitivity (Figure 1) because the C7 and C8 dermatomes in the rat extend from the neck to the forepaw.\textsuperscript{40} Further, neurons innervating lumbar facet joints have been identified with dichotomizing axons projecting to peripheral targets,\textsuperscript{34,41} suggesting that some neurons innervating the C6–C7 facet joint may also possess dichotomizing axons extending into the forelimb and contributing to referred pain. Studies using multiple retrograde tracers are necessary to determine the incidence of dichotomizing axons projecting to the C6–C7 facet joint and forepaw. Nevertheless, these data indicate that the C7 spinal level is likely to be a major contributor to C6–C7 facet joint-mediated pain.

Surprisingly, both the ratio of CGRP-positive joint afferents and their phenotype are unchanged by injury (Tables 1 and 2, Figure 2). This is surprising because several studies have identified a shift in the phenotypic expression of pain-associated proteins such as CGRP and brain-derived neurotrophic factor toward larger-diameter afferents in response to facet inflammation or traumatic injury.\textsuperscript{15,18} Despite the lack of change in the phenotype of joint afferents, injury-induced behavioral sensitivity may still result from afferent sensitization. While it is unlikely that the discs and other spinal ligaments contribute to pain in this model, previous work with this same injury model demonstrated that intra-articular injection of a non-steroidal anti-inflammatory drug abolishes facet joint injury-induced pain.\textsuperscript{16} Combining that observation with the findings of this study supports the contribution of joint afferents in pain after facet joint distraction. Yet, the subpopulations of joint afferents contributing to injury-induced pain still remain unknown. CGRP- and substance P–containing fibers have been identified in human cervical facet capsular ligaments,\textsuperscript{32,33} supporting the assertion that peptidergic afferents likely mediate pain in this joint. At C7 in the rat, CGRP-positive afferents account for a greater percentage of neurons innervating the C6–C7 joint than they do among all neurons in the C7 DRG.

### TABLE 1. Ratio and Average Percentages of CGRP-Positive Neurons That Are Also CTb-Positive Compared With the Number of CTb-Positive Neurons

<table>
<thead>
<tr>
<th>C5</th>
<th>C6</th>
<th>C7</th>
<th>C8</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury (n = 4 rats)</td>
<td>2/3</td>
<td>6/11</td>
<td>35/64</td>
<td>19/31</td>
</tr>
<tr>
<td>Sham (n = 5 rats)</td>
<td>1/4</td>
<td>16/21</td>
<td>38/61</td>
<td>30/41</td>
</tr>
<tr>
<td>Normal (n = 4 rats)</td>
<td>2/6</td>
<td>15/27</td>
<td>37/83</td>
<td>33/45</td>
</tr>
<tr>
<td>Total (n = 13 rats)</td>
<td>5/13</td>
<td>37/59</td>
<td>110/208</td>
<td>82/117</td>
</tr>
</tbody>
</table>

**Average% of peptidergic CTb-positive neurons**

| Injury (n = 4 rats) | 66.7 ± 57.7% | 45.0 ± 33.2% | 56.2 ± 18.4% | 61.1 ± 13.0% | 56.4 ± 9.4% |
| Sham (n = 5 rats) | 25.0 ± 35.4% | 78.4 ± 25.5% | 59.8 ± 14.7% | 69.4 ± 43.3% | 66.9 ± 18.3% |
| Normal (n = 4 rats) | 41.7 ± 52.0% | 47.8 ± 32.6% | 45.9 ± 12.6% | 75.1 ± 19.8% | 53.1 ± 13.7% |
| Total (n = 13 rats) | 46.9 ± 47.1% | 57.1 ± 31.9% | 54.4 ± 15.3%* | 68.6 ± 28.3% | 59.4 ± 14.8% |

*The denominator is the total number of CTb-positive neurons from all rats in each group for each DRG level; the numerator is the corresponding total number of neurons from all rats in each group that are positive for both CTb and CGRP.

† The percentage values represent the average percentage of CTb-positive neurons that are also CGRP-positive for each group and DRG level.

‡ \( P = 0.0084 \) compared with the percentage of CGRP-positive afferents of all neurons at C7.

CGRP indicates calcitonin gene-related peptide; CTb, cholera toxin subunit B; DRG, dorsal root ganglion.

### TABLE 2. Average Cross-Sectional Area of Neurons Positive for Both CTb and CGRP

<table>
<thead>
<tr>
<th>Cross-sectional Area (( \mu m^2 ) ± S.D.)</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
<th>C8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury</td>
<td>660 ± 353</td>
<td>840 ± 264</td>
<td>757 ± 142</td>
<td>825 ± 71</td>
</tr>
<tr>
<td>Sham</td>
<td>741*</td>
<td>732 ± 220</td>
<td>688 ± 173</td>
<td>802 ± 189</td>
</tr>
<tr>
<td>Normal</td>
<td>599 ± 206</td>
<td>864 ± 139</td>
<td>738 ± 79</td>
<td>754 ± 134</td>
</tr>
<tr>
<td>Total</td>
<td>652 ± 212</td>
<td>804 ± 200</td>
<td>724 ± 133‡</td>
<td>794 ± 130</td>
</tr>
</tbody>
</table>

* Only one neuron found.

‡ \( P = 0.0005 \) compared with all CGRP-positive neurons in the C7 DRG.

CGRP indicates calcitonin gene-related peptide; CTb, cholera toxin subunit B; DRG, dorsal root ganglion.

Copyright © 2013 Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.
Taken together, these data indicate that peptidergic joint afferents may make a greater contribution to facet joint pain than other neuronal subpopulations. Future studies specifically investigating the roles of these and other populations of joint afferents in joint injury would determine their relative contributions to facet-mediated pain.

Although these data provide insight into the innervation of the C6–C7 facet joint in the rat from C5 to C8, additional spinal levels also may contain joint afferents. In fact, Ohtori et al.15 found that the C5–C6 facet joint in the rat contains fibers originating in the DRGs from C3–T3, although the vast majority originates in the cervical DRGs. Nevertheless, the C6–C7 facet joint is likely innervated by additional neurons with cell bodies in the upper thoracic DRGs. Only the right DRGs were analyzed in this study, despite the application of a bilateral joint distraction; it is not expected that there would be differences based on sides because this injury is symmetric. Of note, CTb may not label all joint afferents because not all sensory neurons express ganglioside-monosialic acid (GM1), to which CTB binds. Because the majority of sensory neurons (85% of small, 45% of medium, and 60% of large diameter neurons) do express GM1 these findings likely represent the majority of neurons innervating the C6–C7 facet joint. However, it is possible that some neurons, especially among the larger myelinated neurons, may not be able to be labeled by CTb because GM1 is not universally expressed. The use of additional and distinct retrograde neuronal tracing agents would provide a more robust characterization of the full extent of the facet joint’s innervation. However, the majority of the nociceptive afferents are likely captured using this technique. Further, although no visible leakage of the CTb solution from the facet joint was observed immediately after injection, a small amount may have leaked from the joint into the surrounding soft tissues. Nonetheless, any such leakage likely had only a minimal impact on the neuronal counts because the number of labeled neurons innervating the facet joint in our study is consistent with those reported in a study without joint injury in which cyanoacrylate was applied as a joint sealant.15

This study identified no differences in the ratio or cross-sectional area of CGRP-positive joint afferents after injury; however, other peptides such as substance P may be differentially upregulated in these neurons. Previous work using this model identified increased substance P and the prostaglandin E2 receptor EP2 in the DRG after painful joint injury,31,36 supporting that additional targets may be upregulated by afferents after injury. The lack of change in the ratio and cross-sectional area of CGRP-positive joint afferents observed in this study after injury may be due to the small sample sizes. Indeed, a previous study by Ohtori et al.15 required nearly twice as many rats in each group to identify changes in the ratio and size of joint afferents expressing CGRP after a joint capsule transaction compared with controls. Additional studies including larger group sizes are necessary to verify our pilot studies finding that the ratio and cross-sectional area of the peptidergic joint afferents are unchanged by painful facet joint distraction. Despite these known injury-induced changes in the DRG, the specific roles of joint afferents in the generation and maintenance of facet-mediated pain are unknown. Regardless, by characterizing the segmental innervation of the C6–C7 facet joint, this study has identified those spinal levels most likely contributing to facet joint pain and provides direction for future studies investigating the cellular mechanisms underlying joint injury-induced pain.

Key Points

- The C6–C7 facet joint in the rat is innervated by neurons from the C5–C8 DRGs.

- The greatest number of neurons innervating the C6–C7 facet joint has cell bodies in the C7 DRG, followed by the C8, C6, and C5 DRGs, and this distribution is unchanged by painful facet joint injury.

- At C7, the ratio of joint afferents that were peptidergic was significantly greater than the ratio of all neurons that were peptidergic at this level.

- These findings suggest that peptidergic afferents in the C7 DRG play a major role in pain from the C6–C7 facet joint.

References


15. Ohtori S, Takahashi K, Moriya H. Calcitonin gene-related peptide immunoreactive DRG neurons innervating the cervical facet joints...


