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BASIC SCIENCE

Inflammatory Cytokine and Chemokine Expression Is Differentially Modulated Acutely in the Dorsal Root Ganglion in Response to Different Nerve Root Compressions

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Study Design. Inflammatory proteins were quantified in bilateral dorsal root ganglions (DRGs) at 1 hour and 1 day using a multiplexed assay after 2 different unilateral nerve root compression injuries.

Objective. To quantify cytokines and a chemokine in the DRG after nerve root compression with and without a chemical injury to determine contributing inflammatory factors in the DRG that may mediate radicular nociception in clinically relevant nerve root pathologies.

Summary of Background Data. Inflammatory cytokines are known to relate to the behavioral hypersensitivity induced after injuries to the nerve root. However, the relative expression of these proteins in the DRG after cervical nerve root compression are not known.

Methods. The right C7 nerve root underwent transient compression (*10gf*) or transient compression with a chemical irritation (*10gf* + *chr*). The chemical injury was also given alone (*chr*), and the nerve root was exposed (*sham*), providing 2 types of controls. Mechanical allodynia was measured to assess behavioral outcomes. Interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and macrophage inflammatory protein 3 (MIP3) were quantified in bilateral DRGs at 1 hour and 1 day using a multiplexed assay.

Results. Ipsilateral allodynia at day 1 after *10gf* + *chr* was significantly increased over both *10gf* and *chr* ($P < 0.049$). Cytokines and MIP3 were not statistically increased over *sham* at 1 hour. By day 1 after *10gf* + *chr*, all proteins (IL-1β, IL-6, tumor necrosis factor-α, MIP3) were significantly increased over both normal and *sham* in the ipsilateral DRG ($P < 0.036$), and the cytokines were also significantly increased over *chr* ($P < 0.029$). Despite allodynia at day 1, cytokines at that time were not increased over normal or *sham* after either *10gf* or *chr*.

Conclusion. Nerve root compression alone may not be sufficient to induce early increases in proinflammatory cytokines in the DRG after radiculopathy and this early protein response may not be directly responsible for nociception in this type of injury.

Key words: cytokines, chemokine, interleukins, DRG, allodynia, radiculopathy. **Spine 2011;36:197–202**

Infiltration and activation of immune cells, cytokine upregulation, and growth factor release are all induced in the spinal cord and in the peripheral nerves after nerve root injury that causes behavioral hypersensitivity.[1–3] As part of that response, cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10, are synthesized and released by both resident and infiltrating immune cells and act throughout the periphery and spinal cord in both paracrine and autocrine signaling.[4,5] Inflammatory cytokines produced in the spinal cord contribute to nociception, either indirectly by inducing the expression of a variety of biochemical mediators, including nitric oxide and prostaglandins, or directly by activating glial cells in the central nervous system, leading to spinal sensitization.[1,2,6–14] Application of cytokines to normal neural tissue leads to decreases in neural activity and produces behavioral hypersensitivity,[15,16] implying that cytokine production may be sufficient to initiate nociceptive cascades.

Behavioral hypersensitivity and inflammatory cytokine expression after nerve root injury vary with type of injury that is imposed. A measure of tissue loading severity has been previously shown to be significantly positively correlated with the levels of spinal cytokine mRNA that were induced by a lumbar nerve root ligation injury,[17] suggesting inflammation after nerve root compression depends on injury parameters. In rodent models of disc herniation, behavioral hypersensitivity has similarly been shown to increase when mechanical and chemical injuries are combined compared to application of either injury alone, indicating that behavioral hypersensitivity may also depend on the specific type of nerve root injury.[18,19] Recent work, using a model of cervical disc herniation, showed that allodynia was significantly increased, and also

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induced in the contralateral forepaw, when a chemical injury was applied to the nerve root together with the applied compression.[20] This cervical disc herniation model also demonstrated increased inflammatory cytokine mRNA in both the dorsal root ganglion (DRG) and the spinal cord after nerve root compression with and without chemical irritation, with a further significant increase in IL-1β mRNA in the DRG after the combined injury.[21] However, cytokine protein expression has not been quantified in those injuries or in relation to behavioral outcomes. Although relationships among injury parameters, mechanical allodynia, and cytokine mRNA have been described for cervical nerve root compression, it is not known whether cervical nerve root compression also alters inflammatory cytokine protein in the DRG, and whether these responses exhibit profiles that relate to the mechanical and/or chemical components of the injury.

Although studies have quantified elements of the inflammatory cascade in the DRG in neuropathy, there has been no characterization for transient, cervical nerve root compression. It remains unknown if inflammatory cytokines are upregulated and/or released in the DRG immediately after transient compression of the cervical nerve root, and whether that expression is influenced by the presence of a chemical injury. The goal of this study was to quantify immediate and early changes in inflammatory cytokines in the DRG after nerve root compression, both with and without a chemical injury, to determine whether local cytokines are associated with the behavioral hypersensitivity that is produced by mechanical and chemical nerve root injuries.

MATERIALS AND METHODS

Inflammatory cytokines IL-1-β IL-6 and TNF-α were quantified in the bilateral DRGs at 1 hour and 1 day after nerve root injuries to characterize cytokine responses in cervical radiculopathy. The chemokine, macrophage inflammatory protein 3 (MIP3), was also quantified since it is shown to be chemotactic for macrophages—the cells that infiltrate in the spinal cord, nerve root, and DRG in models of radiculopathy and neuropathy.[22–24] Two models of transient nerve root compression, which produce different magnitudes of behavioral hypersensitivity were used in the current study.[20] Experiments were performed using male Holtzman rats (Harlan Sprague-Dawley, Indianapolis, ID), weighing 250 to 350 g at the start of the study. All procedures were approved by the Institutional Animal Care and Use Committee.[25] Rats were housed with a 12 to 12 hour light-dark cycle and free access to food and water.

Surgical procedures have been detailed previously, with all procedures performed under inhalation anesthesia (4% isoflurane for induction, 2% for maintenance).[20,21,26] Rats were prone, and an incision was made in the skin from the base of the skull to the second thoracic vertebra. Muscle and soft tissue were cleared to expose the C6 and C7 laminae and a C6/C7 hemilaminectomy, and partial facetectomy were performed on the right side to expose the spinal cord and right C7 dorsal nerve root. Transient compression and exposure to chromic gut suture were used to model mechanical and chemical components of injury, respectively. The chromic salts and pyrogallol, which are used to produce chromic suture are known to be inflammatory to nervous tissue[27]; chromic gut suture has been used extensively in radiculopathy models to simulate the inflammatory effects of disc material.[2,6,21,27–29] Nerve root compression (*10gf*) involved transient compression of the right C7 dorsal nerve root for 15 minutes using a 10*gf* (10 g-force) microvascular clip (World Precision Instruments, Inc., Sarasota, FL). Procedures for the compression with chromic gut material (*10gf + chr*) were the same as those for *10gf* with the addition of 4 pieces (2 mm in length) of 3–0 chromic gut suture (Surgical Specialties, Reading, PA) placed on the right C7 dorsal nerve root proximal to the DRG. Procedures for the chromic exposure (*chr*) control group involved placing 4 pieces of 3-0 chromic gut suture on the right C7 dorsal nerve root proximal to the DRG. The chromic suture pieces were left in place for the duration of the postoperative period of the study. In addition, a *sham* group involved only exposure of the C7 dorsal root to provide a control group for the surgical procedures.

DRGs from a subset of rats (n = 6 *10gf*; n = 6 *10gf + chr*; and n = 4 *sham*) were harvested at 1 hour after injury to quantify cytokine and chemokine levels as described later in the text. A separate subset of rats (n = 6 *10gf*; n = 6 *10gf + chr*; and n = 6 *chr*) was tested for mechanical allodynia at day 1 after surgery, as described later in the text, followed by harvesting the bilateral DRGs at day 1 after behavioral testing to quantify cytokine and chemokine levels.

The C7 DRGs ipsilateral and contralateral to the injury were harvested at 1 hour (n = 6 *10gf*; n = 6 *10gf + chr*; n = 4 *sham*) and 1 day (n = 6 *10gf*; n = 6 *10gf + chr*; n = 6 *chr*) following surgery. DRGs from naïve, normal, unoperated rats were used as negative controls (n = 2). Rats were deeply anesthetized followed by transcardiac perfusion with 200 mL of phosphate buffered saline (pH 7.4). The C7 DRGs were harvested and placed in 300 μL of ice-cold cell lysis buffer (Bio-Rad, Hercules, CA) containing 12 μL of a stock solution of 82.6% phenylmethylsulfonyl fluoride in dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO). Tissue samples were vortexed, homogenized using a sonic dismembrator (Model 100; Fisher Scientific, Pittsburgh, PA), and centrifuged. Supernatant was collected and total protein concentration was determined using a DCA Protein Assay Kit (Bio-Rad, Hercules, CA) and Synergy HT spectrophotometer (Bio-Tek Instruments, Winooski, VT). Homogenates were assayed using a multiplexed 96-well MSD multispot assay (Meso Scale Discovery, Gaithersburg, MD) that quantified IL-1β, IL-6, TNF-α, and MIP3 simultaneously. The assay employs a sandwich immunoassay format in which capture antibodies are coated on a single spot on the bottom of each well. Samples were incubated in each well followed by a wash and the addition of a detection antibody. The plate was washed once more and read on a Sector Imager (Meso Scale Discovery, Gaithersburg, MD). All samples were assayed in duplicate and expressed as the amount of target cytokine in the amount of total protein (pg/mg). A one-way analysis of variance with *post hoc* Bonferroni correction tested for significant differences between *10gf*, *10gf + chr*, and *sham* at 1 hour and at 1 day after

surgery for each protein for ipsilateral and contralateral DRGs, separately.

Mechanical allodynia was measured before surgery and on day 1 for all rats from which tissue was harvested at day 1. Mechanical allodynia was not measured in the rats for which DRGs were harvested at 1 hour after surgery due to residual effects of the anesthesia at that time point. Methods for quantifying forepaw allodynia have been previously validated.[26,30] The rats were stimulated on the plantar surface of the ipsilateral and contralateral forepaws, separately, using 3 von Frey filaments (1.4, 2, 4 g) (Stoelting Co., Wood Dale, IL). Each testing session consisted of 3 rounds of 10 stimulations each applied to each forepaw, with each round separated by 10 minutes. The total number of withdrawals was counted for each paw and averages for each injury group were determined. A one-way analysis of variance with *post hoc* Bonferroni correction tested for differences in the number of paw withdrawals between groups for each forepaw and filament. Paired *t* tests compared the number of paw withdrawals at baseline and day 1, separately for each group.

RESULTS

Mechanical allodynia in the ipsilateral forepaw was significantly increased over baseline values at day 1 after *10gf*, *10gf + chr*, and *chr* for testing with all filaments ($P < 0.013$) (Figure 1A). However, mechanical allodynia in the ipsilateral forepaw after *10gf* was not significantly different from that of *chr*. Ipsilateral mechanical allodynia at day 1 after *10gf + chr* was significantly elevated over both *10gf* and *chr* for testing with all filaments ($P < 0.049$) (Figure 1A). In the contralateral forepaw, allodynia at day 1 after either *10gf* or *chr* was significantly elevated over corresponding baseline paw withdrawals for testing with the 1.4 and 4 g filaments ($P < 0.021$) (Figure 1B). Further, contralateral allodynia after *10gf + chr* was significantly elevated over baseline for all filaments ($P < 0.013$). Contralateral allodynia after *10gf + chr* was also significantly elevated only over *chr*, for all filaments ($P < 0.027$) (Figure 1B). In addition, contralateral allodynia after *10gf + chr* was significantly elevated over *10gf* for testing with the 2 g filament ($P = 0.035$) (data not shown). Previously published work from our laboratory with these procedures has shown that *sham* procedures do not induce mechanical allodynia in either the ipsilateral or contralateral forepaw for up to 7 days after surgery.[20,26,30]

The proinflammatory proteins IL-1β, IL-6, TNF-α, and MIP3 were detected in the ipsilateral C7 DRG for all groups at 1 hour, but to varying degrees (Figure 2). *Sham* procedures did not induce significant increases over levels measured in naïve, nonsurgical rats for any of the proteins quantified in the ipsilateral DRG at 1 hour (Figure 2). IL-10 was significantly ($P < 0.049$) elevated over normal at 1 hour following *10gf* and *10gf + chr*, and IL-6 was significantly ($P = 0.017$) elevated over normal following *10gf + chr* (Figure 2). However, no significant increases in TNF-α or MIP3 were detected in the ipsilateral DRG at 1 hour following *10gf*, *10gf + chr*, or *chr* (Figure 2). In contrast, cytokine levels in the ipsilateral DRG at day 1 were only significantly increased for the *10gf + chr* group (Figure 2). At day 1 after *10gf + chr*, all of the proteins were significantly increased ($P < 0.036$) over normal and *sham*, and also over *chr* (Figure 2). Expression of both IL-1β and IL-6 at day 1 after the *10gf + chr* injury were also significantly ($P < 0.036$) greater than levels for *10gf* compression (Figure 2). Exposure of the nerve root to chromic gut suture (*chr*) produced only a slight nonsignificant increase in IL-1β, IL-6, and TNF-α at day 1 in the DRG (Figure 2). No significant differences were detected in the contralateral DRGs for any cytokine probed at 1 hour or 1 day after either compression injury (*10gf*, *10gf + chr*) (Figure 3). In the contralateral DRG, only MIP3 increased; at day 1 MIP3 was significantly increased after *chr* compared to *sham* and to *10gf* ($P < 0.047$) (Figure 3D).

DISCUSSION

IL-1β, IL-6, TNF-α, and MIP3 protein expression in the DRG were unchanged relative to *sham* responses at 1 hour after a compression either with or without a chemical injury (Figures 2, 3). However, these proteins increased at day 1 after the combination of mechanical compression and chemical irritation. The presence of a chemical injury in conjunction with a transient nerve root compression induced a rapid and significant increase in mechanical allodynia over a compression alone (Figure 1). Although neither mechanical compression nor chemical irritation alone appear sufficient to alter cytokine protein in the DRG at day 1 after injury,

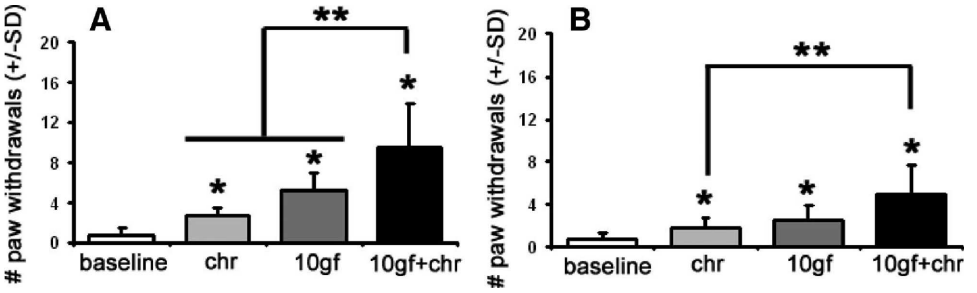

Figure 1. Average mechanical allodynia in the (**A**) ipsilateral and (**B**) contralateral forepaws following *chr, 10gf,* and *10gf + chr* for testing with the 4 g von Frey filament. Allodynia was measured as the number of paw withdrawals for 30 stimulations with von Frey filaments. Baseline values represent the number of paw withdrawals measured before surgery and are shown as an overall average for all rats. An asterisk (*) indicates significant differences from baseline values and the double asterisk (**) represents a significant difference between the indicated groups. Trends and statistical outcomes were similar for testing with the 1.4 g and 2 g von Frey filaments (data not shown).

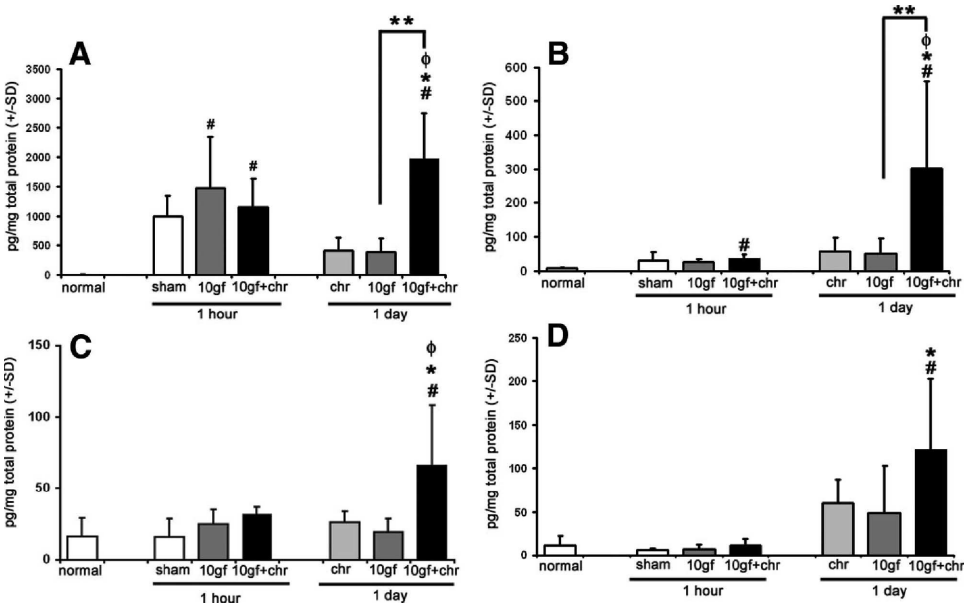

Figure 2. Quantification of (**A**) IL-1β, (**B**) IL-6, (**C**) TNF-α, and (**D**) MIP3 protein in the ipsilateral C7 DRG at 1 hour and 1 day after *sham*, *chr*, *10gf*, and *10gf + chr*, as well as in normal tissue. The pound sign (#) indicates a significant increase over normal, the asterisk (*) indicates a significant increase over *sham*, and the phi (φ) indicates a significant increase over *chr*. The double asterisk (**) labels a significant difference between the indicated groups.

the combination of these injuries induces a significant increase in proinflammatory cytokines in the DRG (Figure 2). This finding implies an additive effect for the combination of these injuries on cytokine expression in the DRG when behavioral sensitivity is also increased as compared to either injury alone (Figures 1, 2). Cytokine protein was not significantly increased in the DRG at day 1 after a compression alone, despite the presence of behavioral hypersensitivity after a compression alone at that time point and the fact that a compressive injury is sufficient to induce sustained behavioral hypersensitivity.[20] This suggests that although expression of proinflammatory mediators within the DRG may increase in association with behavioral hypersensitivity after nerve root injury, it may not be required for the onset of sustained hypersensitivity.

Infiltrating macrophages have not been reported around the nerve root until 7 days after its compression.[24] That finding, together with the cytokine data presented here, suggests that resident cells within the DRG may be responsible for the production of cytokines observed at day 1 (Figure 2D). Ligation of the sciatic nerve with chromic gut suture induces an immediate (day 1) increase in mRNA for IL-1β, IL-6, and TNF-α in the DRG[31]; RT-polymerase chain reaction data demonstrate a significant increase in TNF-α mRNA in the DRG immediately after the combined mechanical and chemical injury used in the current study.[21] Those results further corroborate the hypothesis that DRG cells may produce these cytokines, since there is an increase in cytokine mRNA in the DRG before the detection of increased protein.[31] However, in that work, IL-6 mRNA was not significantly increased over *sham* in the DRG after either nerve root compression or compression with a chemical irritation,[21] which suggests that the increase in IL-6 observed in the current study at day 1 after a combined injury may be due to the retrograde transport of

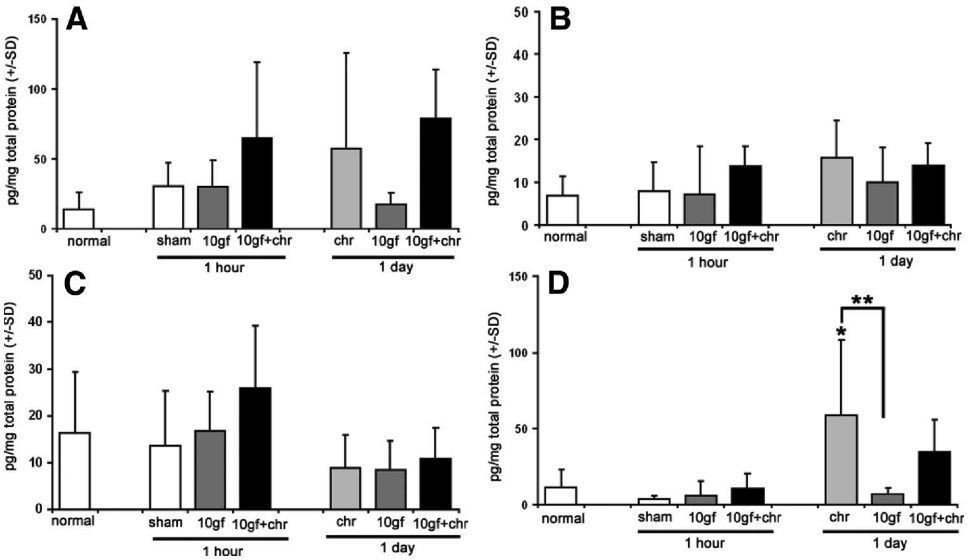

Figure 3. Quantification of (**A**) IL-1β,(**B**) IL-6, (**C**) TNF-α, and (**D**) MIP3 protein in the contralateral C7 DRG at 1 hour and 1 day after *sham, chr, 10gf*, and *10gf + chr*, as well as in normal tissue. The asterisk (*) indicates a significant increase over *sham* and the double asterisk (**) indicates a significant difference between groups.

IL-6 from the injured nerve root. Both IL-1β and TNF-α are produced in the injured nerve root after its compression.[32] There is also evidence that cytokines are transported along axons in the periphery in both anterograde and retrograde directions; after peripheral injury, biotintagged TNF-α delivered to peripheral nerves has been detected in the DRG.[33,34] The short distance between the cervical nerve root and the DRG in the rat can further increase the likelihood that cytokines may be transported to the DRG as early as 1 day after an injury when a chemical injury is present. It is likely that after a combined mechanical and chemical injury (*10gf + chr*), cytokines are produced in both the DRG and the nerve root, since studies show cytokines are present in both the nerve root and DRG after root compression or nerve ligation.[32,35] Cytokine data presented here (Figure 2) and mRNA data from previously published work[21] indicate that IL-6 protein, but not mRNA, is increased in the DRG which suggests that IL-6, and maybe other cytokines, may be transported from the nerve root to the DRG where they act on those neurons to induce behavioral hypersensitivity.

This is the first study to our knowledge to detect an increase in MIP3 after nerve root injury sufficient to cause behavioral hypersensitivity. MIP3 was significantly increased in the DRG over *sham* at day 1 after *10gf + chr* (Figure 2D). Although there are little data on the role of MIP3 in nociception, MIP3 has been shown to increase in the synovial fluid of patients with rheumatoid arthritis, implicating it in joint inflammation, and potentially pain, since joint pain is a common symptom of rheumatoid arthritis.[36] MIP3 is a known chemoattractant for macrophages.[37] Infiltrating macrophages have been detected in the spinal cord at day 14 after spinal nerve transection and at day 14 in the DRG after unilateral spinal nerve ligation,[22,23] both of which cause behavioral hypersensitivity, suggesting that macrophage infiltration in this and other neuropathy models may be related to the maintenance of nociception. Data from our laboratory support macrophage infiltration surrounding the nerve root by day 7, but not at day 1.[24] Those results suggest that macrophage infiltration may mediate the sustained sensitivity observed in these models. It is, therefore, possible that the increase in MIP3 detected in the combined model (*10gf + chr*) at day 1 attracts macrophages to the nerve root, which are then later detected at day 7 after injury.[24]

The current study did not investigate cytokine levels in the DRG later than 1 day after either injury. It is likely that the significant increases in IL-1β and IL-6 in the DRG after a combined injury (*10gf + chr*) (Figure 2) are sustained beyond the first day since inflammatory cytokines are upregulated at later time points in the DRG in other models of neuropathy and radiculopathy.[35,38] Proinflammatory cytokines can immediately and directly affect neuronal function; application of TNF-α or IL-1β to the DRG increases basal discharge, mechanosensitivity, and peripheral receptive fields of DRG Aδ fibers in a dose-dependent manner.[39] If the increases in proinflammatory cytokines in the DRG after a combined mechanical and chemical injury are sustained, these cytokines could lead to continuous sensitivity of the Aδ fibers and induce nociceptive responses. This proposed phenomenon could explain why nerve root injuries involving both mechanical and chemical components initiate greater and longer-lasting symptoms of behavioral sensitivity than those involving compression alone.[15,18–20] In the current study, bilateral allodynia was induced for a unilateral injury, a response that has been reported in other models of radiculopathy and neuropathy.[2,19,40–42] The contralateral sensitivity observed here could be due to a population of ipsilateral primary afferents that project to the contralateral side of the spinal cord, a phenomenon that is more common in the cervical region than in the lumbar region of the spinal cord.[43] Yet, it is also possible that contralateral hypersensitivity is not a direct effect of the damage to ipsilateral primary afferent, but rather that either the interneurons that form commissural connections between the ipsilateral and contralateral sides of the spinal cord can become sensitized by events on the ipsilateral side, or that diffusible inflammatory signals reach the contralateral side and directly sensitize neurons.[44]

The data presented here demonstrate an additive effect for the combination of mechanical compression and chemical irritation in proinflammatory cytokines in the DRG after nerve root injury that induces behavioral hypersensitivity. Results support the hypothesis that inflammation in the DRG, while not required for sustained hypersensitivity, likely mediates or contributes to the significant increase in behavioral hypersensitivity that is detected after a combined mechanical and chemical nerve root injury compared to a transient compression alone (Figures 1, 2). Further insight into inflammatory changes in the DRG after compression are necessary to fully outline the contribution of DRG inflammation to radiculopathy, and to determine whether therapeutic intervention for radiculopathy should be adjusted depending on the presence of a chemical injury.

➢ Key Points

- ❑ Both cervical nerve root compression alone and chemical irritation alone induce mechanical allodynia by day 1 and the combination of the 2 injuries significantly increases sensitivity.
- ❑ At 1 hour after nerve root compression, either with or without a chemical injury, the cytokine and chemokine levels in the bilateral DRGs are not significantly increased over *sham* controls.
- ❑ At 1 day after nerve root compression with an additional chemical irritation, inflammatory cytokines and a chemokine (MIP_3) are significantly increased. However, cytokines are not increased after either a cervical nerve root compression alone or a chemical irritation alone, despite both injuries inducing significant allodynia at that time point.

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