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# Brain-Derived Neurotrophic Factor is Upregulated in the Cervical Dorsal Root Ganglia and Spinal Cord and Contributes to the Maintenance of Pain From Facet Joint Injury in the Rat

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The facet joint is commonly associated with neck and low back pain and is susceptible to loading-induced injury. Although tensile loading of the cervical facet joint has been associated with inflammation and neuronal hyperexcitability, the mechanisms of joint loading-induced pain remain unknown. Altered brain-derived neurotrophic factor (BDNF) levels are associated with a host of painful conditions, but the role of BDNF in loading-induced joint pain remains undefined. Separate groups of rats underwent a painful cervical facet joint distraction or a sham procedure. Bilateral forepaw mechanical hypersensitivity was assessed and BDNF mRNA and protein levels were quantified in the dorsal root ganglion (DRG) and spinal cord at days 1 and 7. Facet joint distraction induced significant ($P < 0.001$) mechanical hypersensitivity at both time points. Painful joint distraction did not alter BDNF mRNA in the DRG compared with sham levels but did significantly increase ($P < 0.016$) BDNF protein expression over sham in the DRG at day 7. Painful distraction also significantly increased BDNF mRNA ($P = 0.031$) and protein expression ($P = 0.047$) over sham responses in the spinal cord at day 7. In a separate study, intrathecal administration of the BDNF-sequestering molecule trkB-Fc on day 5 after injury partially attenuated behavioral sensitivity after joint distraction and reduced pERK in the spinal cord at day 7 ($P < 0.045$). Changes in BDNF after painful facet joint injury and the effect of spinal BDNF sequestration in partially reducing pain suggest that BDNF signaling contributes to the maintenance of loading-induced facet pain but that additional cellular responses are also likely involved. 

**Key words:** facet joint; pain; BDNF; trkB; ERK

Over one-third of adults in the United States report back or neck pain (Strine and Hootman, 2007). The facet joint is identified as the source of pain in 65% of neck and 45% of back pain cases (Manchikanti et al., 1999; Eerd et al., 2010). Animal models of facet joint injury support the assertion that mechanical injury to the facet can cause pain and report altered inflammatory mediators in the joint and dorsal root ganglion (DRG) as well as neuronal hyperexcitability associated with behavioral sensitivity (Cavanaugh et al., 1996; Lu et al., 2005; Tachihara et al., 2007; Lee et al., 2008; Quinn et al., 2010; Kras et al., 2013a). Although inflammation and central sensitization have been linked to facet joint pain, the molecular mechanisms involved in facet joint-mediated pain are still unknown.

The facet capsule is innervated by Aδ- and C-fibers that are activated by mechanical stimulation and inflammation (Cavanaugh et al., 1996, 2006; Ohtori et al., 2001; Lu et al., 2005). Both types of stimuli also are associated with upregulation of inflammatory cytokines in the dorsal root ganglion (DRG; Miyagi et al., 2006; Tachihara et al., 2007; Lee et al., 2008) and pain (Dong et al., 2008, 2013; Lee et al., 2008). The behavioral hypersensitivity induced by facet joint distraction is relieved by intra-articular application of an anti-inflammatory agent (Dong et al., 2013), further implicating inflammatory responses in painful mechanical joint injury. Peripheral inflammation increases the neurotrophin brain-derived neurotrophic factor (BDNF) in the DRG (Cho et al., 1997; Mannion et al., 1999). The number of BDNF-expressing afferents innervating the

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facet joint also has been shown to increase with inflammation in that joint (Ohtori et al., 2002). Despite studies suggesting that BDNF may be upregulated after painful facet injury and may contribute to that joint's mechanically induced pain, the relationships among facet joint injury, pain, and BDNF are unclear.

BDNF is involved in nociceptive signaling in many pain states (Cho et al., 1997; Michael et al., 1997; Mannion et al., 1999; Zhou et al., 1999; Li et al., 2006). BDNF released from primary afferents contributes to spinal hyperexcitability by activating its receptor, trkB (Thompson et al., 1999; Pezet and McMahon, 2006; Merighi et al., 2008; Lu et al., 2009), which in turn activates the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) via phosphorylation (Ji et al., 2002; Pezet et al., 2002; Slack et al., 2004). Inhibition of spinal BDNF activity by the sequestering molecule trkB-Fc, which binds endogenously released BDNF and prevents it from activating functional membrane-bound trkB receptors, alleviates sensitivity (Coull et al., 2005; Zhang et al., 2011) and reduces spinal ERK activation (Pezet et al., 2002). BDNF levels in the DRG and spinal cord both increase as soon as 1 day after a painful intraplantar injection of inflammatory complete Freund's adjuvant (CFA) in the rat (Mannion et al., 1999; Duric and McCarson, 2007). Although animal models of joint inflammation and arthritis demonstrate increased BDNF expression in the synovial tissue of those joints and their associated DRGs (Ohtori et al., 2002; Grimsholm et al., 2008), behavioral outcomes have not been evaluated in those studies, so the functional role of BDNF in joint-mediated pain remains unclear.

Given the role of BDNF in nociceptive signaling and its effects on spinal neuronal excitation, we tested the hypothesis that BDNF increases in the DRG and spinal cord after a painful facet joint distraction, contributing to the maintenance of joint-mediated behavioral sensitivity. Accordingly, the BDNF profiles in the DRG and spinal cord were characterized at an early (day 1) and a late (day 7) time point using a rat model of painful facet joint distraction. Based on those findings, trkB-Fc was administered intrathecally in a separate study to evaluate effects of sequestering spinal BDNF on hyperalgesia and spinal ERK activation.

## MATERIALS AND METHODS

Male Holtzman rats (Harlan Sprague-Dawley, Indianapolis, IN; $402 \pm 24$ g) were housed under USDA- and AAALAC-compliant conditions with free access to food and water. All experimental procedures were approved by the University of Pennsylvania IACUC and were carried out under the guidelines of the Committee for Research and Ethical Issues of the IASP (Zimmermann, 1983).

All surgical procedures were performed with animals under inhalation isoflurane anesthesia (4% induction; 2.5% maintenance). A midline incision was made along the back of the neck, and the laminae and facet joints were exposed and cleared of inserting muscles. The C5–T1 interspinous ligaments were resected, and the C6 and C7 laminae were attached to a loading device via microforceps. Bilateral joint distraction across the C6/C7 facet joints sufficient to induce sustained mechanical hypersensitivity was applied by displacing the C6 vertebra rostrally while C7 was held fixed ($n = 25$; Dong et al., 2008, 2013; Lee et al., 2008). The displacement of the C6 microforceps was continuously recorded during applied distraction to quantify the injury severity. Separate groups of rats underwent joint distraction and were followed for 1 day ($n = 13$) and 7 days ($n = 12$), when DRG and spinal cord tissues were harvested. Sham procedures that included device attachment with no joint distraction were performed in separate groups of rats ($n = 7$ day 1; $n = 10$ day 7). All wounds were closed using polyester suture and surgical staples, and rats were allowed to recover in room air.

The response to mechanical stimulation was quantified in the forepaws of each rat to measure behavioral hypersensitivity after surgical procedures, using previously validated methods (Hubbard and Winkelstein, 2005; Dong et al., 2008; Lee and Winkelstein, 2009). On each testing day, rats were acclimated to the testing environment for 15 min, followed by three rounds of mechanical stimulations to each forepaw using an ascending series of von Frey filaments (Stoelting, Wood Dale, IL). Positive responses to a given filament were recorded for each rat. Each stimulation round was separated by 10 min, and the responses for all three rounds were averaged to determine the mechanical sensitivity for each forepaw. Responses from the bilateral forepaws were averaged to obtain a single measure of mechanical sensitivity for each rat. Sensitivity was determined for 3 days prior to surgery to establish baseline values, as well as postoperatively until the time of tissue harvest. Sensitivity after the surgical procedures was normalized to baseline for each rat and expressed as the fold increase relative to baseline. A two-way ANOVA with Tukey's HSD test compared normalized mechanical sensitivity between distraction and sham groups with group and time point as factors and significance set at $P < 0.05$.

To define the BDNF mRNA levels in the DRG and spinal cord, tissue was harvested at C6 and C7 from the sham and distraction groups at day 1 or day 7 for RT-PCR analysis. After behavioral testing on day 1 ($n = 7$ distraction; $n = 3$ sham) or day 7 ($n = 6$ distraction; $n = 6$ sham), rats were given an overdose of sodium pentobarbital (65 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS). Because the injury is bilateral, only the left DRGs and spinal cord hemisections at the C6 and C7 levels were harvested, immediately frozen on dry ice, and stored at $-80^{\circ}$C until further use. Tissue samples were homogenized in Trizol (Invitrogen, Carlsbad, CA). Total RNA was extracted and reverse transcribed into single-stranded cDNA according to the manufacturer's instructions (Invitrogen). Taqman real-time RT-PCR was carried out using an ABI-7300 system (Applied Biosystems, Foster City, CA) with primers specific to BDNF (forward 5′-GGA-CAT-ATC-CAT-GAC-CAG-AAA-GAA-A-3′, reverse 5′-GCA-ACA-AAC-CAC-AAC–ATT-ATC-GAG-3′, probe 5′-AGT-CAT-TTG-CGC-ACA-ACT-TTA-AAA-GTC-TGC-ATT-3′) and the internal housekeeping gene 18S (forward 5′-CGG-CTA-CCA–CAT-CCA-AGG-AA-3′, reverse 5′-GCT-GGA–ATT-ACC-GCG-GCT-3′, probe 5′-CAC-CAG-ACT-TGC-CCT-

C-3′; Gomez-Pinilla et al., 2002; Lossos et al., 2003). Cycle conditions were 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 60 sec. Samples were run in duplicate, and target gene expression was normalized to the internal housekeeping gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The mRNA level for the distraction group was calculated as the fold change relative to the average levels for sham. BDNF mRNA levels were separately compared between distraction and sham groups for each tissue (DRG; spinal cord) at each time point (day 1, day 7) by *t*-tests.

BDNF is normally expressed in small- and medium-diameter neurons in the DRG, with less expression in the large-diameter neurons (Zhou et al., 1999). Because injury or inflammation shifts the neuronal phenotype that expresses BDNF toward larger diameter neurons (Zhou et al., 1999; Ohtori et al., 2002), we hypothesized that painful facet joint injury may cause a similar phenotypic shift in BDNF expression. To identify any such phenotypic change, immunohistochemistry was used to evaluate BDNF protein in DRG neurons and in the superficial laminae of the spinal dorsal horn in additional separate groups of rats. After behavioral testing on day 1 (n = 6 distraction; n = 4 sham) or day 7 (n = 6 distraction; n = 4 sham), rats were deeply anesthetized with an overdose of sodium pentobarbital (65 mg/kg) and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Cervical spinal cords with bilateral DRGs were harvested en bloc, and the C6–C7 levels were postfixed overnight at 4°C. Tissue was transferred to 30% sucrose in PBS for at least 4 days at 4°C and embedded in Tissue-Tek OCT Compound (Sakura Finetek). Serial DRG and spinal axial sections (14 μm) were taken for immunofluorescent histochemistry and thaw-mounted onto slides, with three to five sections per slide. Tissue sections were washed and blocked with normal donkey serum (Chemicon, Temecula, CA) for 2 hr. DRG sections were incubated at 4°C overnight with a rabbit polyclonal antibody specific to BDNF (1:100; sc-20981; Santa Cruz Biotechnology, Santa Cruz, CA). On the following day, slides were washed and incubated with a Cy3 donkey anti-rabbit secondary antibody (1:500; Jackson Immunoresearch, West Grove, PA) for 2 hr at room temperature. After additional washes with PBS and $dH_2O$, slides were coverslipped with Fluoro-Gel antifade medium (Electron Microscopy Sciences, Fort Washington, PA). Spinal cord sections were incubated at 4°C overnight with the rabbit antibody to BDNF and one of the following: mouse monoclonal antibody to glial fibrillary acidic protein (GFAP; as a marker of astrocytes; 1:500; Millipore, Bedford, MA), mouse polyclonal antibody to microtubule-associated protein-2 (MAP2; as a marker of neurons; 1:200; SMI 52; Covance, Berkeley, CA), or mouse monoclonal antibody to OX-42 (CD11b; as a marker of microglia; 1:300; Serotec, Bicester, United Kingdom). On the following day, the slides were washed and incubated at room temperature for 2 hr with Cy3 donkey anti-rabbit (1:500; Jackson Immunoresearch) and Alexa488 donkey anti-mouse (1:500; Invitrogen) secondary antibodies. Tissue sections that were not incubated with primary antibodies were included as negative controls.

Each DRG section was imaged using a fluorescent microscope equipped with a digital camera (Olympus) with standardized exposure times. A blinded experimenter analyzed BDNF expression in at least three sections from each rat for a subset of distraction and sham rats at each time point (n = 3). For each section, at least 50 neurons were classified as being either positive or negative for BDNF immunoreactivity, and only those neurons with a visible nucleus were included in the count (Weisshaar et al., 2010). Mean signal intensity and cross-sectional area of the neurons were determined by manually outlining each neuron in ImageJ (NIH). For each section, the BDNF intensity was normalized to the average background intensity calculated from the intensities of the BDNF-negative neurons to determine the BDNF intensity ratio. BDNF-positive neurons were classified into seven neuronal sizes (in 100-$\mu m^2$ bins) based on their soma area (Zhou et al., 1999). Average BDNF intensity ratio was compared between distraction and sham groups by a two-way ANOVA, with group and neuronal size as the factors, at day 1 and day 7. Total and BDNF-positive neuron cell counts were also made for each section, and the percentage of neurons positive for BDNF was compared between distraction and sham groups by two-tailed *t*-tests.

At least six images of the spinal dorsal horn were taken from each rat for analysis by immunolabeling, with all exposure times standardized. Automated densitometry was performed by using Matlab code to quantify the BDNF expression in a standardized pixel area (1,360 × 510) corresponding to laminae I–IV (Weisshaar et al., 2010). Because BDNF expression data were positively skewed, the raw data were log-transformed to achieve an approximately normal distribution prior to statistical analysis. All statistical analyses were performed on the transformed data, but the means and 95% confidence intervals are presented. BDNF expression in the dorsal horn was compared between groups at each time point by *t*-test.

To evaluate the role of spinal BDNF in behavioral sensitivity and phosphorylated ERK expression after pain has been established, a separate study administered trkB-Fc intrathecally after injury in order to sequester spinal BDNF. Rats underwent a C6/C7 facet joint distraction as described above. Immediately after behavioral testing on day 5, under inhalation isoflurane anesthesia, rats received a single injection of either the BDNF sequestering molecule trkB-Fc (trkB-Fc; n = 6) or IgG-Fc as the matched vehicle control (vehicle; n = 7) via lumbar puncture, which has previously been shown to be capable of delivering agents to the cervical spinal cord in the rat (Rothman and Winkelstein, 2010). Both treatments (R&D Systems, Minneapolis, MN) were given at 5 μg in 20 μl sterile PBS, a dose that was based on reports of its being effective for preventing or alleviating behavioral sensitivity after nerve injury (Coull et al., 2005; Zhang et al., 2011). Sensitivity to mechanical stimulation was quantified in the bilateral forepaws as described above before (day 0; baseline) and after joint distraction on days 1, 3, 5, 6, and 7. Mechanical response thresholds were compared with baseline responses by using a repeated-measures two-way ANOVA with Tukey's HSD and group and time as factors.

Cervical spinal cord hemisections were harvested from the left side from both treatment groups at day 7 as described above to quantify ERK activation by Western blot. Tissue was homogenized in lysis buffer (150 mM NaCl, 50 mM Tris Cl, pH 8.0, 1 mM EDTA, 1% Triton X-100) in the presence of protease and phosphatase inhibitors and centrifuged at 12,000*g* for 15 min at 4°C. Supernatants were collected and stored at

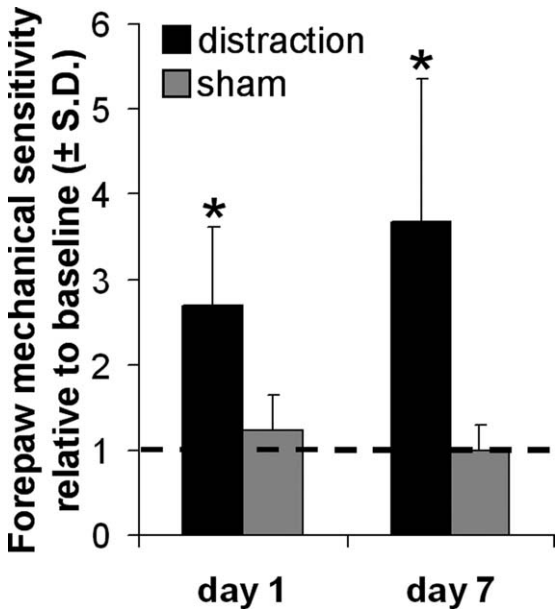


Fig. 1. Mechanical sensitivity in the forepaw as measured by a fold change from baseline sensitivity in response to von Frey filament stimulation. Sensitivity significantly increases ($\star P < 0.001$) after joint distraction (n = 13 day 1; n = 12 day 7) compared with sham (n = 7 day 1; n = 10 day 7) on the days when tissue was harvested. The dashed line indicates corresponding average baseline sensitivity, and the asterisk indicates significant difference between joint distraction and sham.

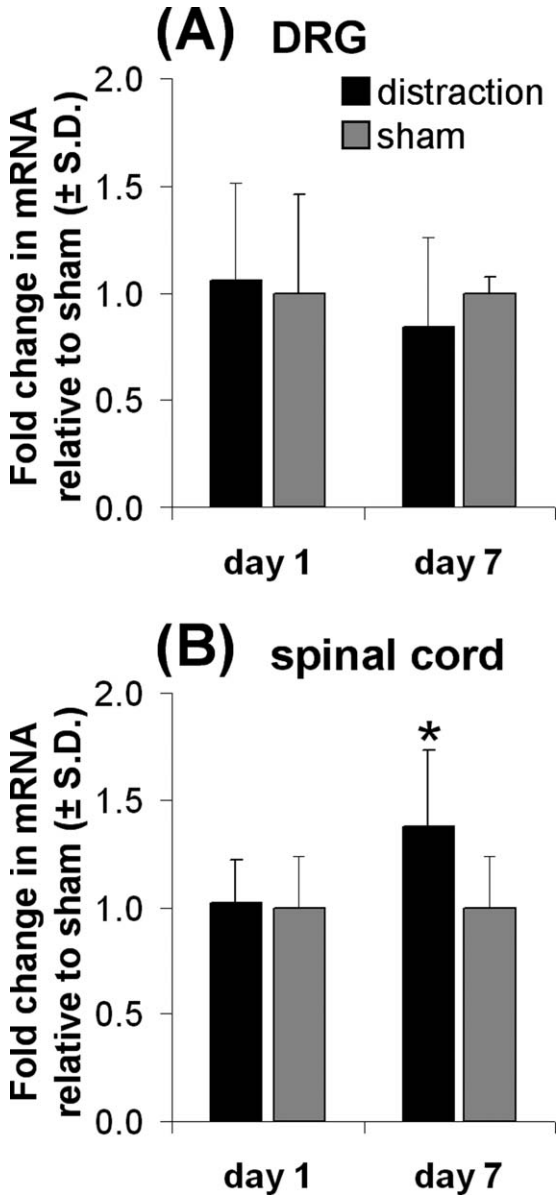


Fig. 2. Quantification of BDNF mRNA in the DRG (**A**) and spinal cord (**B**) at days 1 and 7. **A**: Painful joint distraction does not alter BDNF mRNA levels in the DRG (distraction: n = 7 day 1, n = 6 day 7; sham: n = 3 day 1, n = 6 day 7). **B**: Spinal BDNF mRNA increases ($\star P = 0.031$) at day 7 after a painful joint distraction compared with sham (distraction: n = 7 day 1, n = 6 day 7; sham: n = 3 day 1, n = 6 day 7). BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglion; the asterisk indicates significant difference between joint distraction and sham.

−80°C. Protein samples were combined with NuPAGE LDS Sample Buffer, NuPAGE Reducing Agent, and NuPAGE Antioxidant (Invitrogen) and heated at 95°C for 5 min before loading onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen). Proteins were separated and transferred to an Immobilon-FL transfer membrane (Millipore) and blocked for 2 hr with 5% nonfat dry milk in 0.1% Tween-20 Tris-buffered saline. Membranes were incubated overnight at 4°C with rabbit anti-phospho-ERK1/2 (pERK; 4370; Cell Signaling Technology, Beverly, MA) and mouse anti-ERK1/2 (ERK; 4696; Cell Signaling Technology) antibodies diluted at 1:500 and 1:2,000, respectively. On the following day, membranes were incubated at room temperature for 2 hr with goat anti-rabbit IRDye 800CW and goat anti-mouse IRDye 680 secondary antibodies (1:15,000; LI-COR). Membranes were imaged using the Odyssey Infrared Imaging System (LI-COR). Quantitative analysis of pERK and ERK fluorescent band intensity was performed in Odyssey Application Software v2.1. Expression of pERK1 and pERK2 was normalized to ERK1 and ERK2, respectively, as the loading control for each sample. pERK protein expression relative to ERK protein expression was compared between the trkB-Fc and vehicle groups by two-way ANOVA with Tukey's HSD test, with group and molecular weight (44 kDa pERK1/ERK1, 42 kDa pERK2/ERK2) as factors.

## RESULTS

All rats undergoing joint distraction received the same severity of mechanical injury. The mean displacement of the C6 forceps for the day 1 distraction group is 2.32 ± 0.17 mm, which is not different from that for the day 7 group (2.65 ± 1.45 mm). Similarly, the joint distractions applied in the trkB study are also not different from each other (2.53 ± 0.01 mm trkB-Fc; 2.49 ± 0.03 mm vehicle) or when compared with the injured rats used to define BDNF responses. For all studies, because the

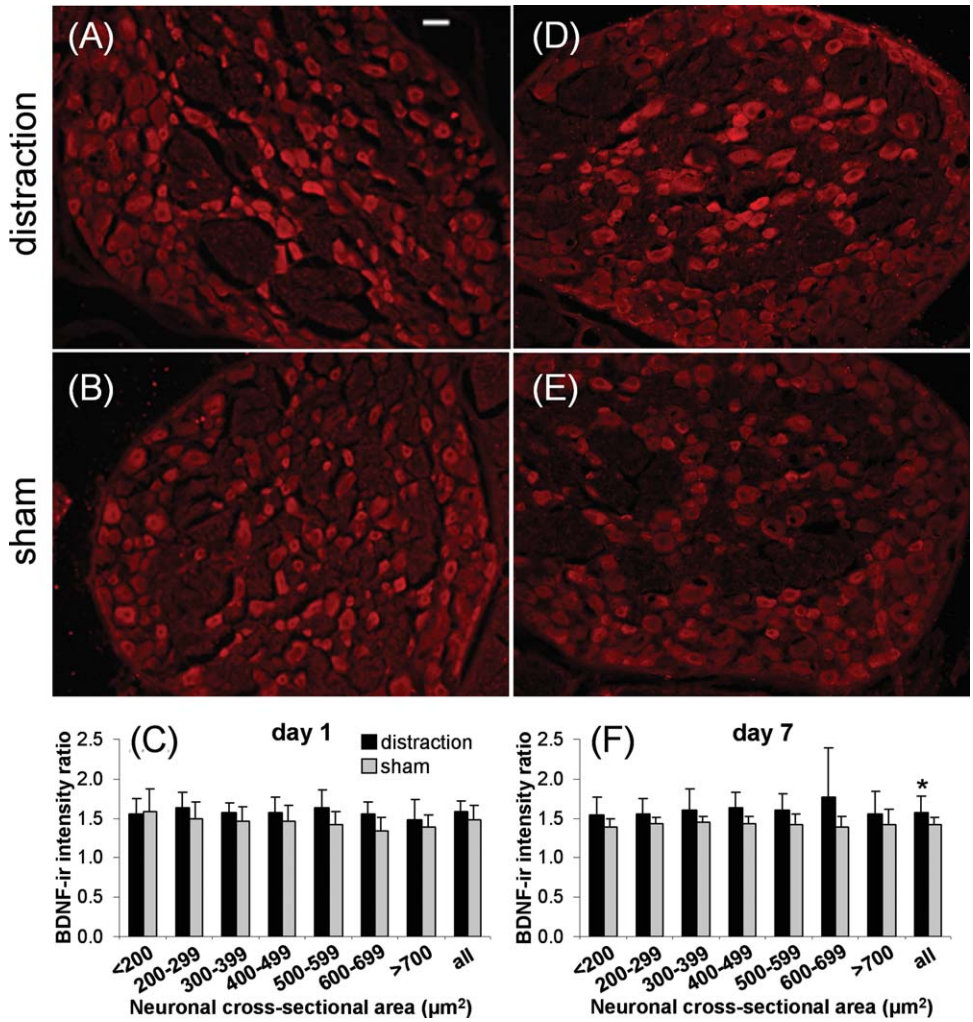


Fig. 3. BDNF-ir intensity in neurons in the C6 DRG on day 1 (**A–C**) and day 7 (**D–F**). Average BDNF-ir intensity ratio is not different between distraction (**A**) and sham (**B**) groups at day 1 (**C**). Average BDNF-ir intensity ratio increases (*$P = 0.016$) after distraction (**D**) relative to sham (**E**) on day 7 (**F**). Quantitative data for BDNF-ir intensity ratios (**C,F**) are average ± standard deviation for three rats/group and at least three sections/rat. BDNF-ir, brain-derived neurotrophic factor immunoreactive; DRG, dorsal root ganglion; the asterisk indicates significant difference between joint distraction and sham. Scale bar = 50 μm in **A**. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

responses to mechanical stimulation are not different between the right and the left forepaws, the responses are averaged between forepaws for each rat. Mechanical sensitivity after sham procedures is not changed from baseline at either time point (Fig. 1). However, facet joint distraction induces an immediate increase in mechanical sensitivity compared with baseline on day 1 ($P < 0.001$) that is also evident at day 7 ($P < 0.001$; Fig. 1). Forepaw mechanical sensitivity in the distraction group also is significantly greater than sham ($P < 0.001$) at both time points and not different between days 1 and 7 ($P = 0.183$).

Transcript levels of BDNF after a painful facet joint distraction differ from sham levels only in the spinal cord at day 7 (Fig. 2). BDNF mRNA is detected in the DRG and spinal cord in the distraction group, but is not different from sham in the DRG at either day 1 or day 7 (Fig. 2A). After painful joint distraction, BDNF mRNA is significantly increased ($P = 0.031$) over sham in the spinal cord at day 7 (Fig. 2B).

Although neurons are identified in the DRG that express BDNF at both days 1 and 7 for all rats, BDNF expression increases only in the DRG at day 7 after painful distraction compared with sham (Fig. 3). There is no change in the percentage of neurons expressing BDNF at either time point. The average BDNF-immunoreactive (BDNF-ir) intensity ratio across all neuron sizes is unchanged after painful distraction compared with sham on day 1 (Fig. 3A–C; $P = 0.078$). At day 7, the average BDNF-ir intensity ratio across all neuron sizes increases ($P = 0.016$) after painful distraction compared with sham, but no change in the response of neurons is detected among the different sizes ($P > 0.338$; Fig. 3D–F).

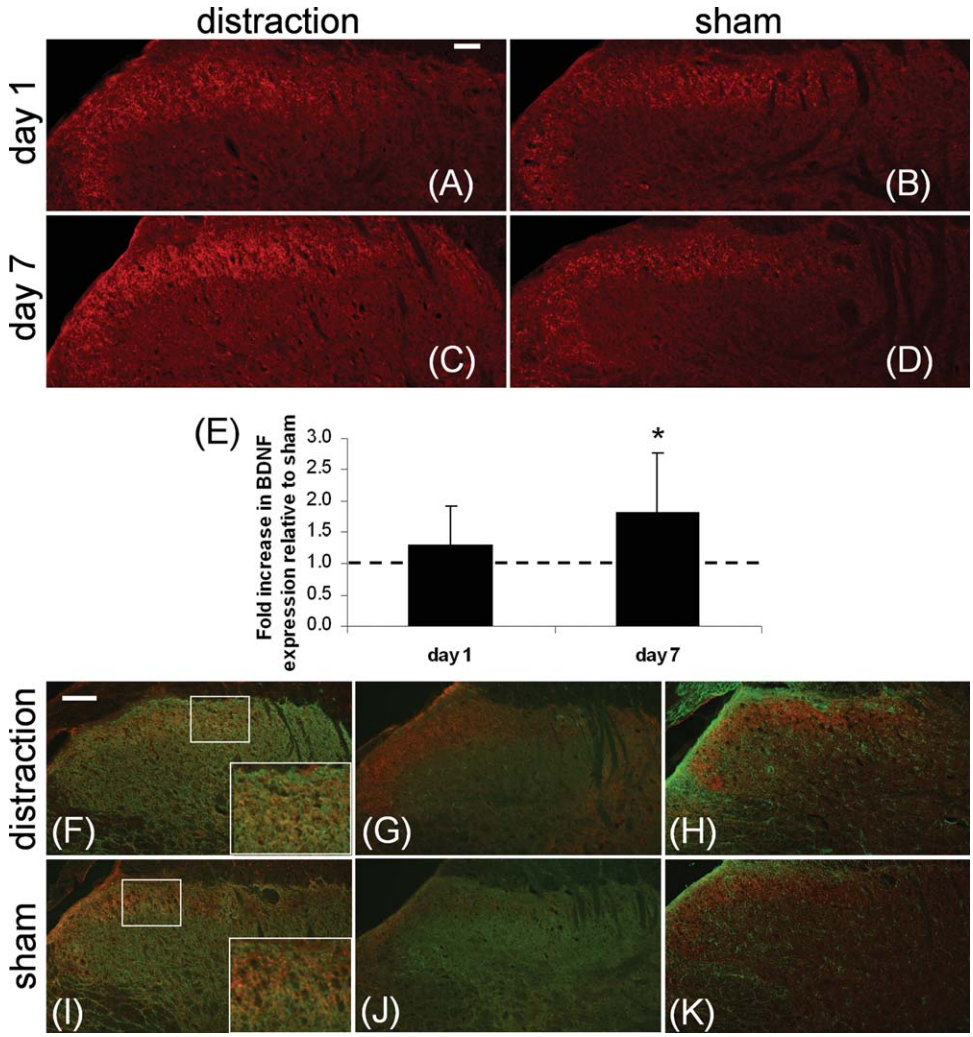


Fig. 4. Representative images of BDNF in the spinal cord at day 1 (**A,B**) and day 7 (**C,D**), its quantification (**E**), and colabeling of BDNF with MAP2 (**F,I**), OX-42 (**G,J**), and GFAP (**H,K**). **A,B**: At day 1, BDNF-ir labeling is not different between joint distraction and sham. **C,D**: Joint distraction (n = 6) significantly (*$P = 0.047$) increases BDNF-ir labeling over sham (n = 4) at day 7. Data in **E** are average ± 95% confidence limit. **F–H**: BDNF immunolabeling (red) colocalizes (yellow) with MAP2 (**F**) but not with OX-42 (**G**) or GFAP (**H**) on day 7 after facet distraction. **I–K**: Similarly, BDNF colocalizes with MAP2 (**I**) but not OX-42 (**J**) or GFAP (**K**) on day 7 after sham. MAP2, OX-42, and GFAP label as green in **F–K**. BDNF-ir, brain-derived neurotrophic factor immunoreactive; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein-2; OX-42, cluster of differentiation molecule 11b; the asterisk indicates significant difference between joint distraction and sham. **Insets** show boxed areas at higher magnification. Scale bars = 50 μm in **A** (applies to **A–D**); 100 μm in **F** (applies to **F–K**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

BDNF protein also is detected in the spinal cords of all the rats receiving a joint distraction or sham procedure. As with the mRNA responses, there is no difference in BDNF expression in the superficial dorsal horn between distraction and sham at day 1 ($P = 0.252$; Fig. 4). However, BDNF expression in the spinal cord at day 7 after painful distraction is significantly increased ($P = 0.047$) over expression in sham at that same time point (Fig. 4). Furthermore, the double immunofluorescent labeling suggests that BDNF colocalizes with the neuronal marker MAP2 (Fig. 4F) but not with the microglial marker OX-42 (CD11b; Fig. 4G) or the astrocytic marker GFAP (Fig. 4H) at day 7. A limited amount of BDNF is also found to colocalize with neurons, but not microglia or astrocytes, in the spinal cord in the sham group at day 7 (Fig. 4I–K). The same patterns are observed at day 1 for both groups (data not shown).

Before treatment, a joint distraction significantly reduces the withdrawal threshold compared with baseline responses in both the trkB-Fc ($P < 0.017$) and vehicle ($P < 0.005$) groups that is evident on all days up to and including day 5 (Fig. 5A). The single intrathecal injection of trkB-Fc increases the withdrawal threshold on both day 6 and day 7, returning it to baseline levels for those rats (Fig. 5A; $P > 0.522$). However, the vehicle injection has no effect on the withdrawal threshold, which remains

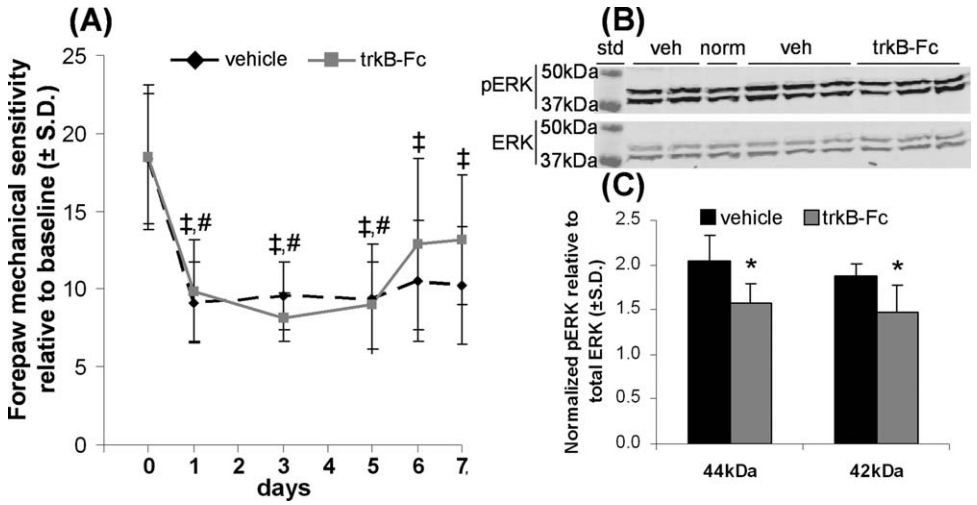


Fig. 5. **A**. Withdrawal threshold for von Frey filament stimulation is significantly reduced from baseline responses after joint distraction in both the trkB-Fc ($^{\#}P < 0.017$; n = 6) and vehicle ($\ddagger P < 0.005$; n = 7) groups until treatment is given on day 5. TrkB-Fc injection removes that decrease in the withdrawal threshold on days 6 and 7; vehicle injection has no effect on the withdrawal threshold, which remains significantly lower ($\ddagger P < 0.022$) than baseline on those days. **B:** Representative immunoblots showing pERK1 (44 kDa), pERK2 (42 kDa), total ERK1 (44 kDa), and total ERK2 (42 kDa) in the spinal cord at day 7. **C:** TrkB-Fc treatment significantly decreases the ratios of both pERK1/ERK1 ($^{\star}P = 0.013$) and pERK2/ERK2 ($^{\star}P = 0.045$). pERK, phosphorylated extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase. The pound sign indicates significant differences between trkB-Fc and the corresponding baseline response; the double dagger indicates significant differences between vehicle and the corresponding baseline response; the asterisk indicates significant differences between trkB-Fc and vehicle.

significantly decreased compared with baseline at both days 6 and 7 ($P < 0.022$; Fig. 5A). Both phosphorylated ERK1/2 and total ERK1/2 are detected at the expected molecular weights in the spinal cords of all rats receiving either intrathecal injection (Fig. 5B). Paralleling the behavioral outcomes, both spinal pERK1/ERK1 and pERK2/ERK2 are significantly decreased ($P < 0.045$) at day 7 after treatment with trkB-Fc.

## DISCUSSION

These findings demonstrate that BDNF is upregulated by day 7 in both the DRG and the spinal cord after a painful facet joint injury (Figs. 2–4). Although increased BDNF is associated with a variety of painful conditions (Cho et al., 1997; Mannion et al., 1999; Zhou et al., 1999; L. Li et al., 2006; Obata et al., 2006; C.Q. Li et al., 2008), this is the first demonstration for joint pain. Synovial BDNF is elevated in arthritic joints, and inflammation of the lumbar facet joint can upregulate BDNF in afferents that innervate the joint (Ohtori et al., 2001, 2002; Rihl et al., 2005; Grimsholm et al., 2008). However, none of those studies defined BDNF in the context of pain. Behavioral sensitivity increased nearly threefold immediately after a painful distraction and was sustained through day 7 (Fig. 1); however, increases in BDNF were not as robust and were evident only at day 7 (Figs. 2–4). Sequestering spinal BDNF only partially attenuated the behavioral hypersensitivity after distraction (Fig. 5). Spinal trkB-Fc significantly reduced spinal ERK activation, which is associated with pain and is known to be involved in BDNF-trkB signaling (Ji et al., 2002; Pezet et al., 2002; Slack et al., 2004). These findings indicate that a mechanical joint injury induces a functionally relevant BDNF response but that it is not the sole mediator of facet joint pain.

Although mRNA levels of BDNF were altered at day 7 in the spinal cord, no changes were detected in the DRG at either day 1 or day 7 (Fig. 2). BDNF mRNA has been reported to increase in the DRG as early as day 1 after a painful CFA-induced inflammatory stimulus not causing mechanical damage to afferents (Mannion et al., 1999; Kobayashi et al., 2008). A previous study using our same rat model found no change in substance P mRNA in the DRG after a *painful* joint injury but an increase over time after *nonpainful* mechanical joint loading (Lee and Winkelstein, 2009). Because substance P and BDNF are produced in the same primary afferent neurons (Michael et al., 1997; Pezet and McMahon, 2006; Merighi et al., 2008) and substance P mRNA is not altered by a painful joint distraction (Lee and Winkelstein, 2009), the lack of change in BDNF mRNA levels observed here is not surprising. Furthermore, because substance P mRNA changes only over time after a nonpainful, but not a painful, joint distraction (Lee and Winkelstein, 2009), it is possible that painful mechanical facet injury may disrupt mRNA transcription, possibly via damage to the afferents in the facet joint capsule. The joint loading applied in our model produces mechanical strains in the capsule tissue that are similar to those (60–82%) that

induce axonal damage in capsule afferents in a goat model of facet distraction (Quinn et al., 2007; Kallakuri et al., 2008). Furthermore, axonal degeneration is not fully developed until day 7 following axonal trauma (Hubbard and Winkelstein, 2008), suggesting that the absence of altered BDNF mRNA in the DRG (Fig. 2) may be due to the disruption of normal mRNA production via axonal damage from painful facet distraction.

BDNF mRNA increased in the spinal cord at day 7 after painful distraction (Fig. 2), which is consistent with observations of other acute and chronic pain models (Li et al., 2006; Duric and McCarson, 2007). Normally, BDNF mRNA is undetected in the dorsal horn, so any amount after injury is believed to be from novel expression (Li et al., 2006). Increased transcription of spinal BDNF after painful joint distraction supports the localized synthesis of BDNF in the spinal cord, although there are several possible cellular sources. Sensory and motor neurons, as well as activated microglia and astrocytes, can upregulate BDNF transcription after injury (Dougherty et al., 2000; Ikeda et al., 2001; Coull et al., 2005; Li et al., 2006; Merighi et al., 2008). Although a 1.3-fold increase in spinal BDNF mRNA is detected by RT-PCR (Fig. 2), that technique prevents the cellular and regional localization of mRNA. The increase may be due to a large increase in a small region of the spinal cord or from a generalized increase throughout or may be in a specific cell type. Identification of the spatiotemporal cellular sources of spinal and DRG BDNF mRNA will further clarify the biochemical mechanisms mediating facet pain.

The same cells that upregulate transcription of BDNF at day 7 may also increase BDNF protein after joint distraction. Spinal BDNF is nearly doubled after a joint distraction (Fig. 4). However, this increase corresponds to a less than 20% increase in BDNF in the afferents in the DRG (Fig. 3). Because neurons in the DRG normally produce and transport BDNF to the spinal cord, the change in BDNF in those neurons after distraction suggests that increased transport from the DRG may contribute to the elevated spinal BDNF at day 7. A previous study has shown that the dorsal nerve roots must be intact for increased BDNF to be evident in *both* the DRG and the spinal cord (Zhou et al., 1999), implying anterograde transport of BDNF from the DRG as the source of spinal BDNF. Although BDNF is elevated in DRG neurons after joint distraction at day 7, the increase is only slight compared with the more robust spinal response (Figs. 3, 4). Together with the increase in BDNF mRNA in the spinal cord, it is likely that sources other than the primary afferents contribute to the increased spinal BDNF at day 7. Although mRNA and protein were assayed in different groups of rats, all rats received the same magnitude of joint distraction and exhibited the same behavioral outcomes; furthermore, because tissue assays used the same spinal levels (C6/C7) for both techniques, they can be taken as the same. Nevertheless, it is possible that variability in these responses may be due to interanimal variation, so future work quantifying both mRNA and protein levels in the same animals will help to define clearly the relationships of BDNF and other responses in painful joint injury. Furthermore, the current study did not identify which DRG neurons specifically innervate the injured facet joint, but retrograde neuronal tracing in this same model has identified over 50% of the DRG neurons innervating the C6/C7 facet joint to be peptidergic (Kras et al., 2013b). The high percentage of peptidergic joint afferents, which are known to express BDNF, supports an important role for BDNF and this class of neurons in joint pain. Also using tracing methods, Ohtori et al. (2002) found that facet joint inflammation increases the number of joint afferents expressing BDNF, providing evidence that stimulation to the facet joint does induce changes in BDNF expression in its afferents. Although the afferents innervating the facet joint were not identified in the current study, such studies would help to determine whether the increased spinal BDNF at day 7 is due to localized synthesis or increased transport from joint afferents.

The increase in spinal BDNF protein is consistent with other models of pain in the periphery (Cho et al., 1997; Mannion et al., 1999; Zhou et al., 2011). The increase in spinal BDNF at day 7 corresponds to a time point when spinal neurons are hyperexcitable and glutamate signaling is altered in this model (Dong and Winkelstein, 2010; Quinn et al., 2010). Elevated spinal BDNF has been associated with both neuronal and behavioral hyperexcitability through release from primary afferents and subsequent activation of glutamate receptors (Slack et al., 2004; Matayoshi et al., 2005; Geng et al., 2010). The colocalization of BDNF with the neuronal marker MAP2 in the dorsal horn (Fig. 4F) suggests that neurons may be responsible for the increase in spinal BDNF and supports a potential contribution of BDNF to the joint loading-induced neuronal hyperexcitability that is evident (Quinn et al., 2010). Sequestering spinal BDNF partially alleviates injury-induced pain together with reducing spinal pERK (Fig. 5), which supports BDNF's contribution to joint pain via an ERK-mediated pathway. In fact, these data are in agreement with previous studies reporting reduced ERK activation resulting from BDNF sequestration (Pezet et al., 2002). ERK is activated when the BDNF receptor trkB is phosphorylated, as occurs during noxious mechanical stimulation of the paw (Pezet et al., 2002; Slack et al., 2004).

Activation of ERK is associated with inflammatory pain, and interference with its activation inhibits the development of mechanical hypersensitivity in the rat after inflammatory stimuli in the paws (Ji et al., 2002; Kawasaki et al. 2004), so ERK activation is a key intracellular signaling mechanism leading to pain. Our findings that both ERK activation and behavioral sensitivity are reduced after BDNF sequestration in the spinal cord are consistent with the notion of ERK as a key contributor to pain and support a role for BDNF-trkB signaling in joint pain. Because BDNF induces long-term potentiation in spinal neurons through an ERK-dependent mechanism (Zhou et al., 2008), elevated spinal BDNF may contribute to the neuronal hyperexcitability after painful joint distraction. Furthermore, glutamate receptor activation

contributes to BDNF-induced mechanical sensitivity (Geng et al., 2010) and ERK activation is associated with phosphorylation of glutamate receptors (Slack et al., 2004). Altered glutamate signaling, which also has been demonstrated to occur after a painful joint distraction (Dong and Winkelstein, 2010), may be a mechanism through which BDNF and ERK contribute to facet joint pain. Additional studies specifically determining the effects of BDNF on spinal neuronal excitability and glutamate signaling after painful and nonpainful joint injuries would further clarify the mechanisms through which spinal BDNF might contribute to facet joint-mediated pain.

This study supports at least a partial role of spinal BDNF in the maintenance of pain from injurious facet joint loading. However, contributions from other neuropeptides and inflammatory mediators likely also contribute at both early and later time points. Although BDNF sequestration attenuates pain after joint distraction, it does not fully abolish it. Further studies clarifying the mechanisms by which spinal BDNF and ERK activation contribute to pain after mechanical joint injury are needed to define the relationship between that neurotrophin and the complex mechanisms underlying facet-mediated pain. Nevertheless, an upregulation of BDNF and partial alleviation of hypersensitivity via BDNF sequestration provides the first evidence of a role for spinal BDNF in joint pain.

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