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Physiologic facet capsule stretch can induce pain & upregulate matrix metalloproteinase-3 in the dorsal root ganglia when preceded by a physiological mechanical or nonpainful chemical exposure

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Abstract

Background—Neck pain from cervical facet loading is common and induces inflammation and upregulation of nerve growth factor (NGF) that can sensitize the joint afferents. Yet, the mechanisms by which these occur and whether afferents can be pre-conditioned by certain nonpainful stimuli are unknown. This study tested the hypothesis that a nonpainful mechanical or chemical insult predisposes a facet joint to generate pain after a later exposure to typically nonpainful distraction.

Methods—Rats were exposed to either a nonpainful distraction or an intra-articular subthreshold dose of NGF followed by a nonpainful distraction two days later. Mechanical hyperalgesia was measured daily and C6 dorsal root ganglia (DRG) tissue was assayed for NGF and matrix metalloproteinase-3 (MMP-3) expression on day 7.

Findings—The second distraction increased joint displacement and strains compared to its first application ($p<0.048$). None of the initial exposures altered behavioral sensitivity in either of the groups being pre-conditioned or in controls; but, sensitivity was established in both groups receiving a second distraction within one day that lasted until day 7 ($p<0.024$). NGF expression in the DRG was increased in both groups undergoing a pre-conditioning exposure ($p<0.0232$). Similar findings were observed for MMP-3 expression, with a pre-conditioning exposure increasing levels after an otherwise nonpainful facet distraction.

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Contributors: The study plan and design was joint conceived of by SS, SK, and BAW. The experiments were performed by BAB, SK and SS and SS, SK and NSS analyzed the data with guidance from BAW. The manuscript was primarily written by SS and SK, with contributions and heavy editing by BAW. All authors reviewed the manuscript prior to submission.

Interpretation—These findings suggest that nonpainful insults to the facet joint, when combined, can generate painful outcomes, possibly mediated by upregulation of MMP-3 and mature NGF.

Keywords

neck pain; injury; facet joint; biomechanics; matrix metalloproteinase; nerve growth factor

1. Introduction

Neck pain affects 30-50% of people in their lifetime with annual costs on the order of $30 billion when considering the expenditures on surgical treatments and lost income (Cohen, 2015; Côté et al., 2004; Hoy et al., 2010). Neck pain commonly results from supraphysiological loading of the cervical facet joints and their tissues (Cavanaugh, 2006; Dong et al., 2012; Ivancic et al., 2008; Lee et al., 2004; Quinn and Winkelstein, 2007; Steilen et al., 2014; Stemper et al., 2005). Yet, physiological loading of the joint does mediate neuropeptides and glutamatergic signaling (Dong et al., 2012; Lee and Winkelstein, 2009) and induces capsule strains below those that cause pain or tissue rupture (Dong et al., 2012; Lu et al., 2005; Panjabi et al., 1996; Siegmund et al., 2001). Painful facet capsule loading induces inflammatory cascades in the joint (Dong et al., 2011; Dong et al., 2013; Lee et al., 2008) and dorsal root ganglia (DRG) (Kras et al., 2013), upregulates nerve growth factor (NGF) in the joint (Kras et al., 2015), and activates nociceptive pain fibers (Chen et al., 2006; Gangadhrana and Kuner, 2013, Inami et al., 2001). Those responses also sensitize the afferents, and produce pain (Dong et al., 2012; Lee et al., 2004). However, joint afferents can also be activated in response to subfailure loading or loading during innocuous physiologic movements, either inducing only transient or no pain (Cavanaugh et al., 2006; Lu et al., 2005; Quinn et al., 2010; Schaible and Grubb, 1993). Although afferent activation is graded with extent of capsule loading (Chen et al., 2006; Lu et al., 2005; Quinn et al., 2009), it is unclear whether tissue loading or other stimuli can predispose joint afferents' responding to otherwise nonpainful facet loading.

The cell bodies of afferent nerve fibers residing in the DRG relay sensory information from the periphery to the central nervous system (Krames, 2014; Lee et al., 1998; Sapunar et al., 2012). DRG neurons regulate a host of nociceptive and inflammatory cascades in response to facet joint loading, many of which are specific to pain (Dong et al., 2008; Dong et al., 2011; Kras et al., 2013; Lee et al., 2009; Weisshaar et al., 2010). In association with pain, DRG neurons undergo transcriptional changes induced by NGF that mediate sensitization of the joint afferents (Mantyh et al., 2011, Norman and McDermott, 2016). Along with those changes, substance P (Woolf and Costigan, 1999), glutamate (Grace et al., 2011; Notcutt, 1997), and glutamatergic receptors increase in the DRG and spinal cord (Dong et al., 2012). Because NGF sensitizes peripheral nociceptors (McMahon et al., 2006; Svensson et al., 2003) and increases in the facet joint after its painful injury (Kras et al., 2015), it is likely altered in afferents after painful joint loading, but has not been probed.

NGF is an important contributor to inflammatory and neuropathic pain (Cheng and Ji, 2008; Khan and Smith, 2015; Kras et al., 2014; McMahon, 1996; Schaible, 2015). It preferentially

binds to the tropomyosin receptor kinase A (TrkA), which induces a number of nociceptive modulators, including substance P, and calcitonin gene-related peptide (CGRP) (McMahon, 1996). NGF induces behavioral sensitivity when injected intradermally into the rat hindpaw, intramuscularly, and into the knee (Ashraf et al., 2014; Hoheisel et al., 2007; Malik-Hall et al., 2005). When injected into the facet, NGF produces behavioral sensitivity in a dose-dependent way: a 3μg dose induces pain within one day that persists for at least one week, whereas a 1μg dose has no effect (Kras et al., 2015), suggesting there is a threshold NGF exposure that activates nociceptors. The effects of such a subthreshold dose of NGF on afferent sensitization and whether it may pre-condition the joint to be more susceptible to develop pain are unknown.

After a mechanical or chemical injury, mast cells are the primary source of NGF in the periphery (Leon et al., 1994), where they migrate to the injury site and express proNGF, an inactive form of NGF (Leon et al., 1994; Peleshok and Riberio-da-Silva, 2012). Schwann cells also express proNGF after peripheral nerve injury (Chattopadhyay et al., 2007, Peleshok and Ribiero-da-Silva, 2012); microglia express proNGF after injury to central nervous system tissue (Krenz and Weaver, 2000; Loane and Byrnes, 2010). The conversion of proNGF to its active form depends on cleavage of the pro-domain by matrix metalloproteinases (MMPs) (Hove et al., 2012; Pardo and Selman, 2005). MMPs are involved in tissue and extracellular matrix remodeling and are constitutively expressed in most somatic cells (Alge-Priglinger et al., 2009; Fanjul-Fernandez et al., 2010). MMPs increase after nerve injury (Shubayev et al., 2006), and some MMPs catalyze the conversion of pro-neurotrophins to active conformations (Lee et al., 2001). MMP-3, which targets type II collagen and proteoglycans (Nagase, 2001; Visse and Nagase, 2003) in the extracellular matrix, also cleaves the pro-domain in proNGF, converting it to its active, mature conformation (Ferrari et al., 2010; Ji et al., 2009; Lee et al., 2001). MMP-3 is upregulated after joint trauma (Maksymowych et al., 2007), injurious cyclic loading of cartilage explants (Lin et al., 2004; Steinmeyer and Ackermann, 1999), and physiologic bending of spinal segments (Hartman et al., 2015). Although physiological loading of the facet may upregulate MMPs via changes in the joint environment (Goldring and Goldring, 2007; Hartman et al., 2015), it is unclear to what extent MMP-3 and its catalysis of proNGF to NGF is modulated in DRG afferents by repeated exposures to otherwise nonpainful stimuli.

The objective of this study was to determine whether a nonpainful stimulus to the facet joint pre-conditions that joint, such that a subsequent nonpainful facet joint distraction (FJD) produces pain. Using an established rat model of FJD, the facet joint was exposed to a mechanical stimulus with a biomechanical severity that is known to be nonpainful (Crosby et al., 2015; Dong et al., 2012; Lee and Winkelstein, 2009). Two days prior to that, either a similar nonpainful FJD or nonpainful NGF injection was given intra-articularly (Kras et al., 2015). To evaluate whether such pre-exposure alters pain responses, mechanical hyperalgesia was assessed during the study period until day 7, when DRGs were assayed for NGF expression as a proxy for peripheral sensitization (Calvo and Bennett, 2011; Chang et al., 2016). MMP-3 expression was also quantified in neurons as a precursor for mature NGF that participates in afferent sensitization (Ferrari et al., 2010; Ji et al., 2009; Lee et al., 2001) and matrix remodeling in the DRG (La Fleur et al., 1996; Nishida et al., 2008).

2. Methods

2.1. Study approach

All procedures were approved by the IACUC and carried out under the guidelines of the Committee for Research and Ethical Issue of the International Association of Pain (Zimmerman et al., 1983). Rats were exposed to either a mechanical or chemical stimulus on day 0 followed by a nonpainful FJD on day 2 (FJD/FJD; n=6), imposing facet capsular ligament strains that do not induce any behavioral sensitivity (Lee et al., 2004a) (Fig. 1A). Separate rats received a subthreshold intra-articular dose of NGF dissolved in sterile PBS (Kras et al., 2015) on day 0 followed by a nonpainful FJD on day 2 (stNGF/FJD; n=6) (Fig. 1A). To control for the effects of the surgical procedures in the FJD and the intra-articular injection, separate control groups were included that underwent two separate sham surgeries on days 0 and 2 (sh/sh; n=6), a single sham surgery only on day 0 (sh/-; n=4), and a single injection of PBS vehicle on day 0 (veh/-; n=6) (Fig. 1A). The bilateral forepaw mechanical hyperalgesia (Crosby et al., 2014; Kras et al., 2015) was evaluated before the first procedure (baseline; day 0), before the second surgical procedure (days 1 and 2) and on days 3, 5, and day 7, when DRG tissue was harvested for quantification of NGF and MMP-3 expression (Fig. 1A).

2.2. Surgical procedures, biomechanical measurements and behavioral assessment

All procedures used male Holtzman rats (Envigo; Indianapolis, IN; 350-425g at start of study) under isoflurane inhalation anesthesia (4% induction; 2.5% maintenance). A nonpainful FJD or sham surgery was performed (Crosby et al., 2015; Dong et al., 2012; Lee et al., 2009). Briefly, a midline incision was made along the back of the neck and the bilateral C6/C7 joints were exposed. The paraspinal musculature was cleared from the C5-T1 laminae and the interspinous ligaments from C5-T1 were resected to facilitate attachment of the C6 and C7 vertebrae onto a customized loading device by microforceps (Fig. 1B). A nonpainful distraction (0.2mm) was applied across the bilateral C6/C7 facet joints by displacing the C6 vertebra rostrally with the C7 vertebra stationary (Crosby et al., 2015; Dong et al., 2012; Lee et al., 2009). Polystyrene microspheres (Spherotech, Inc.) were placed on the C6 and C7 vertebrae, and as in a grid on the right C6/C7 facet capsule, and were tracked during distraction using a Phantom v.4.3 CCD camera (Vision Research) to measure the full-field capsule displacement during loading, with a resolution of 20μm/pixel (Fig. 1C). Sham procedures included only muscle clearance and attachment to the distraction device but no FJD. After FJD or sham procedures, wounds were closed with 3-0 polyester sutures and surgical staples, and rats were allowed to recover in room air; their weight was monitored continuously.

For each FJD, marker positions before distraction and at the peak of capsule stretch were used to quantify the displacements of the vertebrae and facet capsule; vector displacements were calculated as the change in Euclidean distance between markers. Capsular displacement was also used to calculate the mean maximum principal capsular strain (MPS) during each FJD using LS-DYNA (Livermore Software Technology Corp.) to provide a mechanical metric for injury severity of the capsule (Crosby et al., 2015; Dong et al., 2013; Lee et al. 2008 Weisshaar and Winkelstein, 2014;). Separate paired t-tests compared the

vertebral displacement, capsular displacement, and mean MPS between the first (D0FJD1) and second FJD (D2FJD2) for the group (NP/NP) receiving two nonpainful injuries. Those same mechanical metrics of injury severity were compared between the second distraction (D2FJD2; D2FJD) applied on day 2 (FJD/FJD, stNGF/FJD) using a Student's t-test.

In a separate group of rats (stNGF/FJD, n=6), an intra-articular injection of NGF was administered to the bilateral C6/C7 facet joints on day 0 before the nonpainful FJD on day 2 (Fig. 1). A midline incision along the back of the neck exposed the bilateral C6/C7 capsules (Kras et al., 2014; Kras et al., 2015). A 10μL syringe (Hamilton Company) with a 33G beveled needle was advanced into each facet joint to administer 1μg of rat-β NGF (R&D Systems) diluted in 5μL of sterile PBS. That dose of NGF has been previously found to be below the dose (3μg/5μL) that induces mechanical hyperalgesia (Kras et al., 2015). Following injection, the exposure was closed in layers using 3-0 polyester sutures and surgical staples. Two days later, rats were re-anesthetized and the nonpainful FJD was applied to the bilateral C6/C7 facet joints (stNGF/FJD), as described above.

Additional control groups were included to account for the surgical and joint injection procedures, respectively. In one group, a sham surgery was performed only on day 0 (sh/-; n=4) to assess the behavioral and tissue responses for a single surgical procedure. A separate group of rats (veh/-; n=4) received only an injection of 5μL of PBS intra-articularly into the bilateral C6/C7 joints to control for the effect of an injection on day 0.

Behavioral sensitivity was assessed by measuring bilateral mechanical hyperalgesia in the forepaws for each rat (Chaplan, 1994; Dong et al., 2011; Kras et al., 2015; Smith et al., 2016; Weisshaar and Winkelstein, 2014). Forepaw hyperalgesia was quantified before surgery (baseline; day 0) and on days 1, 2, 3, 5, and 7 for the FJD/FJD, stNGF/FJD, and sh/sh groups. For the control groups (sh/-, veh/-), forepaw hyperalgesia was measured only on days 0, 1, and 7 since those groups do not develop sensitivity (Crosby et al., 2014; Kras et al., 2015) and were used to provide control tissue for the DRG assays. On each day of behavioral assessment, rats were acclimated to the testing environment for 20 minutes before stimulation with an ascending series of von Frey filaments (Stoelting) from 0.6 to 26g (Crosby et al., 2014; Kras et al., 2015; Lee and Winkelstein, 2009). Each filament was applied five times before moving to the next higher strength filament, and the lower of the two consecutive filaments eliciting a positive response of either licking or lifting the paw was taken as the threshold for that paw. Each testing session consisted of three rounds, separated by a 10-minute recovery period. Testing was performed on each forepaw separately, and the average of all rounds in a testing session was taken as the response threshold. Since all procedures were bilateral, the bilateral forepaw responses were averaged on each test day for each rat. Responses at each day were compared to baseline for each group using paired t-tests. To assess if there is a statistical effect between days for the groups receiving two procedures (FJD/FJD, stNGF/FJD, sh/sh) differences between days were separately assessed using a repeated measures ANOVA. A one-way ANOVA with post-hoc Tukey's test compared differences in withdrawal thresholds between all groups at day 7, corresponding to the day tissue was assessed.

2.3. DRG harvest, immunohistochemical labeling and analysis

Following behavioral testing on day 7, rats were anesthetized using sodium pentobarbital (65mg/kg) and perfused with 300mL of PBS (Gibco) followed by 250mL of 4% paraformaldehyde. DRGs at C6 were harvested and post-fixed with 4% paraformaldehyde overnight at 4°C before being transferred to 30% sucrose in PBS solution at 4°C. Tissue samples were then embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek) and were transversely sectioned into 14μm thick slices and thaw-mounted onto electrostatically-charged slides (Fisher Scientific).

Sections were labeled for NGF and MMP-3 to visualize expression in the DRG neuron cell bodies and extracellular matrix. Slides were washed, and incubated in a blocking solution of 10% goat serum (Vector Laboratories) with 0.3% Triton-X PBS for 2 hours at room temperature. This was followed by incubation overnight at 4°C in rabbit anti-NGF (1:500; Abcam), and mouse anti-MMP-3 (1:100; Thermo Fisher Scientific) primary antibodies diluted with blocking solution. The following day, slides were washed and fluorescently labeled with secondary antibodies for goat anti-rabbit Alexa Fluor 488 (1:1000; Invitrogen) and goat anti-mouse Alexa Fluor 568 (1:1000; Invitrogen) at room temperature. Sections were washed once more, air-dried for 20 minutes, mounted with fluoro-gel (Electron Microscopy Sciences), and coverslipped. Images of the C6 DRGs (3 images from 6 DRGs for each rat) were taken using an Olympus BX53 fluorescence wide field microscope (Olympus) with a 20× objective (1360×1024 pixels).

Expression of each of NGF and MMP-3 in the DRG was quantified using densitometry. Briefly, the total expression of NGF and MMP-3 was separately determined by measuring the percentage of positive pixels in DRG images above a pre-defined pixel intensity threshold for each channel using in-house scripted MATLAB code (Mathworks) (Dong et al., 2011; Rothman et al., 2010). That threshold pixel intensity was defined using validated labeling and expression levels for each antibody. Densitometry results were normalized to the levels observed in the C6 DRGs from naïve rats (n=4) that did not undergo surgery or manipulation on either days. Separate one-way analysis of variance with the Tukey post hoc test compared the normalized percent of positive pixel values between different groups.

3. Results

3.1. Biomechanical injury of facet joint distractions

The joint distraction applied on day 2 after either a prior FJD (D2FJD2) or subthreshold NGF injection (D2FJD) exposure is consistent (Fig. 2A). The vertebral displacement applied on day 2 (D2FJD2; mean 0.39mm (SD 0.11mm) is significantly greater ($p=0.043$) than the corresponding vertebral displacement for that group on day 0 (D0FJD1; mean 0.27mm (SD 0.14mm) (Fig. 2A). In addition, there is also a significant difference ($p=0.031$) between the vertebral displacement between the FJDs that were applied on day 2 (D2FJD; mean 0.25mm (SD 0.10mm) (Fig. 2A). Similarly, although the corresponding capsular displacement for D2FJD2 is significantly higher than that of D0FJD1 ($p=0.0011$), capsular displacements on day 2 are not different between the two groups (D2FJD; D2FJD2), with the mean capsular displacement of mean 0.17mm (SD 0.04mm) for D2FJD and mean 0.20mm (SD 0.03mm)

for D2FJD2 (Fig. 2B). In contrast, the corresponding mean MPS induced by a second mechanical exposure (D2FJD2; mean 17.5% (SD 4.9%) is significantly ($p=0.021$) greater than the MPS generated by the first exposure (D0FJD1; mean 9.2% (SD 6.1%) (Fig. 2C), with greater strains specifically in the lateral and caudal regions of the facet capsule. In addition, the mean MPS in the group undergoing a prior distraction (D2FJD2) is significantly higher ($p=0.042$) than that of the group receiving a subthreshold NGF dose followed by FJD on day 2 (D2FJD).

3.2. Behavioral sensitivity

Overall, the forepaw withdrawal thresholds are significantly different across days for two consecutive nonpainful FJDs (FJD/FJD) ($p=0.024$) and subthreshold NGF followed by a FJD on day 2 (stNGF/FJD) ($p<0.0001$) (Fig. 3). Although a single nonpainful FJD for the FJD/FJD group does not change the withdrawal threshold on days 1 and 2 from its corresponding baseline thresholds, thresholds on day 3 are significantly lower after a second FJD ($p=0.008$) and remain significantly lower than baseline on both day 5 ($p=0.014$) and day 7 ($p=0.019$) (Fig. 3). Similarly, an intra-articular injection of NGF in the facet joint does not lower thresholds from baseline on either day 1 or 2 (Fig. 3). Yet, starting on day 3 after that injection and the nonpainful FJD, the withdrawal thresholds for the stNFG/FJD group are significantly lower than baseline as soon as day 3 ($p<0.0005$) and remain decreased until day 7 ($p<0.0001$) (Fig. 3). The withdrawal thresholds are not different from baseline for any day after a single sham exposure on day 0 or after repeated sham surgeries (sh/sh). Also, there are no differences in withdrawal thresholds from baseline on days 1 or 7 for either of the groups receiving only a single procedure (sh/-; veh/-) on day 0 (Fig. 3). At day 7, there is a significant difference in the group effect ($p=0.0270$) detected among all of the groups; the withdrawal thresholds are significantly lower ($p=0.0141$) for the stNGF/FJD group than the sh/sh group (Fig. 3).

3.3. NGF and MMP-3 expression in the DRG

Positive NGF labeling is observed in the DRGs of each group receiving two exposures (Fig. 4). However, NGF expression after two sham exposures (sh/sh) is not different ($p>0.8904$) from naïve normal tissue levels or after a single sham surgery or joint injection (sh/-; veh/-) (Fig. 4). NGF expression is greatest after a subthreshold NGF joint injection followed by a nonpainful FJD (stNGF/FJD) and is almost an order of magnitude (mean 8.9 (SD 2.3) fold) greater than normal expression. NGF expression in the DRG is similarly increased in both of the groups undergoing a nonpainful FJD on day 2 (FJD/FJD; stNGF/FJD) ($p<0.0232$) compared to the other control groups, and is not different from each other ($p=0.1807$).

MMP-3 labeling follows similar trends as NGF in the DRG (Figs. 4 and 5). Neither of the single exposures (sh/-; inj/-) or double sham exposure (sh/sh) alter MMP-3 from naïve levels (Fig. 5). Although MMP-3 expression does increase in both the groups undergoing a nonpainful FJD on day 2 (NP/NP; stNGF/FJD), it is less than a 4-fold increase (mean 3.4 (SD 1.0) for stNGF/FJD; mean 2.6 (SD 0.9) for FJD/FJD) (Fig. 5). Expression of MMP-3 in both the FJD/FJD and stNGF/FJD groups is similar and not different from each other ($p=0.232$), but significantly greater ($p<0.0024$) than the other groups (Fig. 5).

4. Discussion

A nonpainful facet stretch can induce pain when preceded by an otherwise subthreshold mechanical or chemical insult to that spinal joint two days earlier (Fig. 1A). In fact, neither insult induced any behavioral sensitivity on its own (day 1 and 2) (Fig. 3), which is consistent with this same degree of facet distraction (mean 0.27mm (SD 0.14mm); Fig. 2B) not altering behavioral sensitivity for up to 14 days (Lee et al., 2004). Similarly, intra-articular NGF at this dose (1μg) did not induce sensitivity for 7 days (Kras et al., 2015). Yet, after a subsequent nonpainful FJD, rats developed unresolving mechanical hyperalgesia for 5 days. The concomitant upregulation of both NGF and MMP-3 as a consequence of chemically or mechanically-mediated pre-conditioning (Figs. 4 and 5) may explain that pain onset from otherwise non-injurious exposure (Jull et al., 2011) and support their involvement in unresolving pain (Chattopadhyay et al., 2007; Van Hove et al., 2012).

The severity of facet capsular ligament distraction drives the onset and pattern of pain (Lee and Winkelstein, 2009; Lu et al., 2005). The imposed capsular strains for the first nonpainful FJD (mean 10.5% (SD 5.8%)) (Fig. 2) was similar to the 6% capsular strain reported during physiological sagittal bending (Panjabi et al., 1998; Pearson et al., 2004). In contrast, in the FJD/FJD group, the strains generated in the second exposure (mean 17.5% (SD 5.0%)) are in the range reported with a single *painful* cervical facet injury (16%) (Dong et al., 2012; Lu et al., 2005). As such, it may be possible that the pain in the FJD/FJD group is solely due to the increased magnitude of the displacements and strains experienced by the capsule due to the second FJD that may also induce ligament laxity (Ivancic et al., 2008; Quinn & Winkelstein., 2011) and not due to any pre-conditioning effect of the afferents from the first FJD. Further, it is possible that the changes in NGF and MMP-3 (Figs. 4 and 5) observed in that group may be related as much to the presence of pain as the double FJD exposure. Although repeated loading of ligaments has been shown to induce laxity and tissue injury (Ivancic et al., 2008; Quinn & Winkelstein, 2007; Quinn & Winkelstein., 2011), the mechanical response of the ligament was not evaluated here. Defining the DRG expression of MMP-3 and NGF after a single FJD would strengthen interpretation of those findings. Yet, a single nonpainful distraction does not alter mRNA of inflammatory cytokines in this model nor is NGF increased in the facet joint (Kras et al., 2015; Lee et al. 2009). Nevertheless, since the stNGF/FJD group is more sensitive than the sh/sh group at day 7, NGF and MMP-3 may reflect the NGF priming due to pre-treatment by the injection and not parallel pain (Figs. 3-5). Although neither NGF nor MMP-3 expression in the DRG were evaluated as a result of either a single subthreshold injection of FJD, each condition was selected as conditions not inducing pain and since the DRG responses follow pain (Figs. 4 and 5), they are not expected to be elevated at day 7 after a single exposure alone.

Painful joint loading and aging initiates repair and remodeling of the tissue ECM (Igrashi et al., 2007). Since MMPs mediate ECM remodeling, their increase is a hallmark of early joint degeneration (Bobacz et al., 2003; Chen et al., 2014; Chu et al., 2015; Fujita et al., 2009). Elevated MMP-3 in the DRG has also been documented in painful neuropathy (Nishida et al., 2008), which is consistent with our finding of its increase in the groups with pain. The similar levels of NGF and MMP-3 in both chemical and mechanical pre-conditioned joints (Figs. 4 and 5) support the interactivity of their pathways (Ji et al., 2009; Takano et al.,

2016) (Fig. 6). NGF directly contributes to afferent sensitization (Ferrari et al., 2010); since MMP-3 is upstream in the injury cascade (Fig. 6), inhibiting it would more clearly define the unique pathways driving pain.

Although the current findings support pain onset from innocuous joint loading involving NGF and MMP-3, they only characterize responses at a single time point. Since the NGF antibody targets all of proNGF, free NGF, and NGF that is already bound to TrkA and $p75^{NGF}$ membrane-bound receptors, it does not distinguish the specific effects of NGF in this context. NGF bound to the TrkA receptor dictates growth, survival, and modulates the PKCε pathway that can increase neuronal hypersensitivity (Ferrari et al., 2015; Kandasamy and Price, 2015; Reichling and Levine, 2009). In contrast, NGF bound to p75 receptors activates the apoptotic pathway (Beattie et al., 2002; Ioannou and Fahnestock, 2017). It is also likely that changes in the spinal dorsal horn where DRG neurons synapse may sensitize spinal neurons for pain maintenance (Khan and Smith, 2015). Although pilot studies found no changes in either spinal NGF or MMP-3 in this study, other neuropeptides would be expected to exhibit more robust changes since they are exocytosed from the DRG (Fig. 6).

NGF is important in the generation and maintenance of pain (Fig. 6) (Kumar and Mahal, 2012; Rukwied et al., 2010). In fact, intra-articular administration of anti-NGF in this same FJD model prevents pain onset (Kras et al., 2015). After joint injury, mast cells degranulate (Birklein and Schmelz, 2008; Mckelvey et al., 2013) and upregulate NGF (Leon et al., 1994; Nicol et al., 2007) (Fig. 6), which binds to TrkA receptors on the neuronal cell body membrane, increasing expression and translocation of TRPV1 to the afferent cell membranes (Amaya et al., 2004; Jen-Kun Chenga, 2008; Ji et al., 2014) (Fig. 6). NGF binding to TrkA upregulates TrkA receptors (Kumar and Mahal, 2012), glutamatergic receptors (Pezet and McMahon, 2006), and substance P/CGRP through the PKCε pathway (Kallakuri et al., 2004; Kobayashi et al., 2004; Kumar and Mahal, 2012). Accordingly, a nonpainful subthreshold injection of NGF of sufficiently high concentration would be taken up by the afferents and retrogradely transported to the DRG (Kumar and Mahal, 2012; Richardson and Riopelle, 1984). NGF at increasing doses has a graded response on hypersensitivity (Apfel et al., 1998; Woolf et al., 1994), supporting the hypothesis that NGF at levels below those for initiating pain or intracellular cytokine cascades (Kuner, 2010; Mifflin and Kerr, 2014) may enhance, but not trigger, nociceptive cascades.

NGF also catalyzes proNGF after mechanical injury (Lu et al., 2005; Peleshok and Ribeiroda-Silva, 2012). Conversion of proNGF to NGF is facilitated by MMP-3 (Lee et al., 2001; VanHove et al., 2012), which is constitutively expressed (Ra and Parks, 2007) and upregulated by neurons after traumatic brain and spinal cord injury (Giraudon et al., 1997; Kim et al., 2005). MMP-3 is induced by inflammation (Tsuzaki et al., 2003), and implicated in traumatic osteoarthtritis (Fujita et al., 2009; Kerin et al., 2002) since it is observed in the synovia early on following joint injury (Furman et al., 2014; Kurz et al., 2005; Stevens et al., 2009; Tchetverikov et al., 2005). While it is possible that non-injurious joint loading can stimulate afferents to upregulate NGF and MMP-3 even marginally (Kim et al., 2005), proNGF catalysis could be initiated but not enough to exceed the threshold for pain.

This study suggests that while pain can be initiated by a single traumatic event (Cavanaugh, 2006; Dong et al., 2012; Ivancic et al., 2008; Lee et al., 2004; Quinn and Winkelstein, 2007; Steilen et al., 2014; Stemper et al., 2005), repeated nonpainful stimuli may cumulatively increase at least one neurotrophin (NGF) in parallel with development of pain (Figs. 3 and 4). Since mechanical hyperalgesia was maintained until day 7 along with robust NGF labeling in the DRG (Figs. 3 and 4), afferent sensitization may be mediated by the NGF-TrkA pathway (Fig. 6) which has a more sustained response and is associated with a positive-feedback loop inducing expression and transport of TrkA and TRPV1 receptors to the afferents (Mifflin and Kerr, 2014; Nicol et al., 2007). The increase in MMP-3 in the DRG may corresponds to catalysis of NGF (Kim et al., 2005) and early stage joint degeneration through the activation of other MMPs (Nagase, 2001; Visse and Nagase, 2003). Nevertheless, a single nonpainful chemical or mechanical insult seems to be sufficient to condition the facet joint to pain that likely involves MMP-3 regulation in afferents.

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Highlights

- It is unknown if a nonpainful facet insult primes it to pain from later nonpainful stretch.
- Physiologic stretch is painful after nonpainful stretch or intra-articular nerve growth factor.
- Nerve growth factor and matrix metalloproteinase-3 increase with repeated nonpainful insults.
- Repeated nonpainful insults have additive physiologic effects in peripheral neurons.

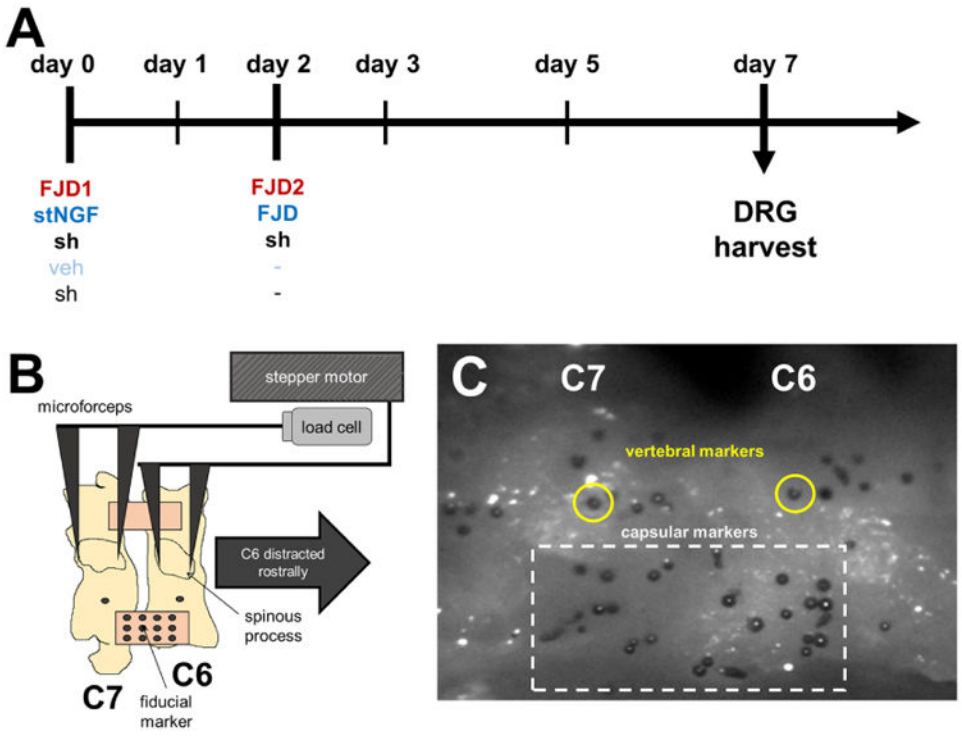

Fig. 1.

(A) At day 0 rats were exposed to a nonpainful facet joint distraction (FJD1), an intra-articular injection of NGF at a subthreshold dose for producing pain (stNGF), sham surgery (sh) or an injection of PBS vehicle (veh). At day 2, three groups of rats were exposed again either to a second nonpainful distraction (FJD) or a sham surgery; remaining rats received nothing (-) on day 2. Mechanical withdrawal thresholds using increasing sizes of von Frey filaments were measured on day 0 (before injury) and days 1, 3, 5, and 7 after which C6 DRG tissue was harvested. **(B)** Schematic and **(C)** image showing anatomy and methods to distract the facet joint, with microforceps affixed to on the C7 and C6 vertebrae and C6/C7 facet capsule.

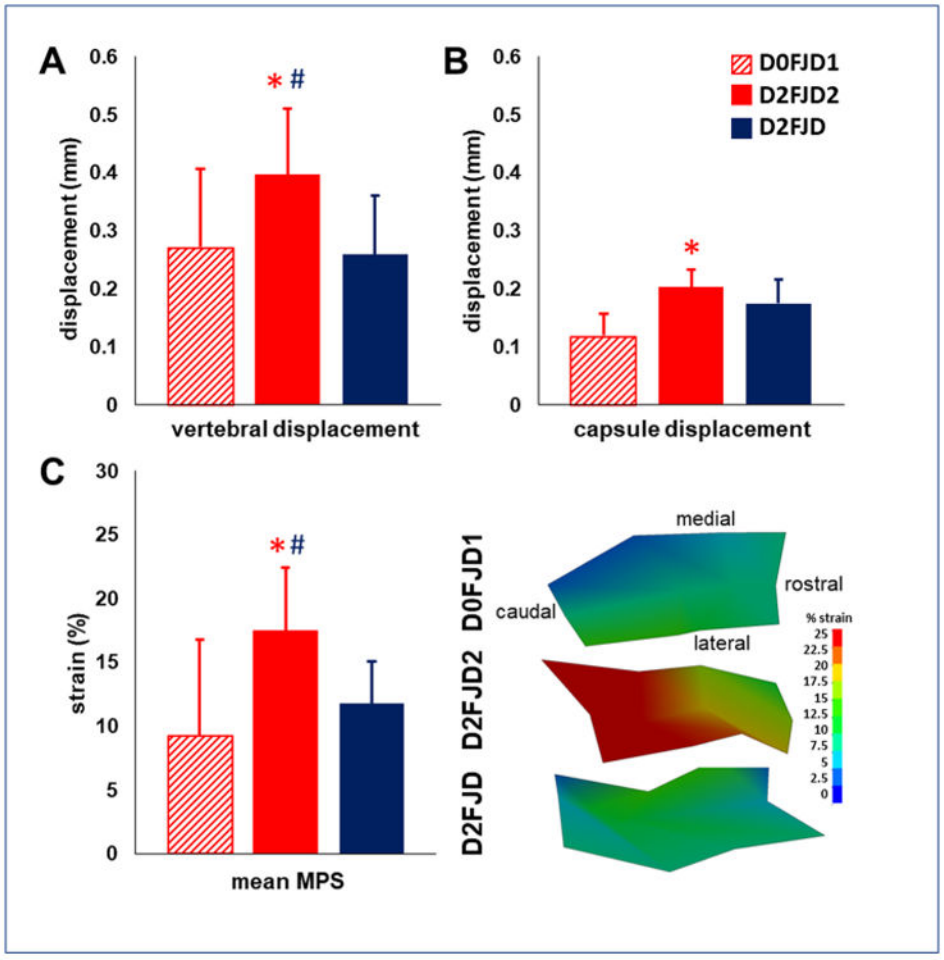

Fig. 2.

(A) Vertebral displacement in the FJD/FJD is significantly greater (*p=0.043) during the second distraction (D2FJD2) than the first distraction (D0FJD1) and from the nonpainful distraction on day 2 after stNGF (D2FJD) (#p=0.031). **(B)** The mean capsular displacement for the second FJD on day 2 (D2FJD2) group was significantly lower than the first FJD on day 0 (D0FJD1) (*p=0.0011), and not different from the single FJD after stNGF (D2FJD). **(C)** The mean MPS for D2FJD2 is significantly higher than the mean MPS of each of D0FJD (*p=0.021) and D2FJD (#p=0.042), primarily in the caudal and lateral regions of the capsule.

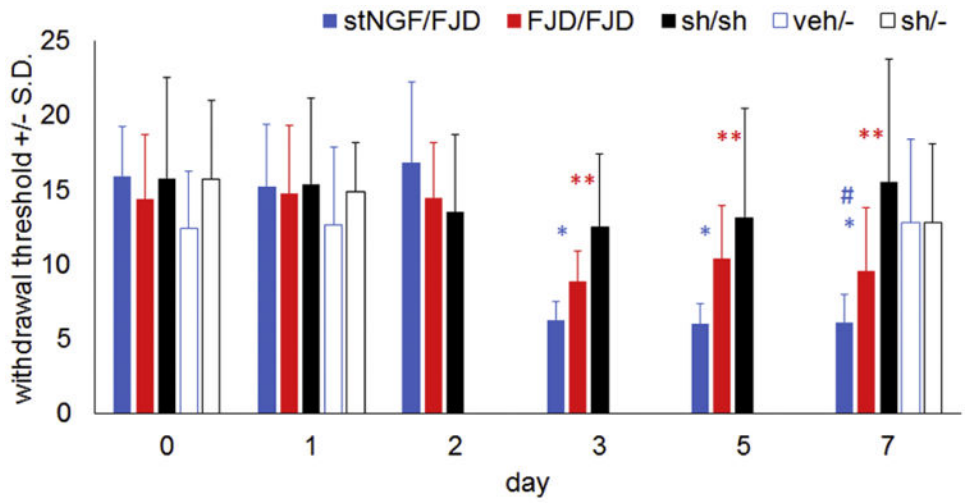

Fig. 3.

After the nonpainful distraction (FJD) at day 1, forepaw withdrawal thresholds are significantly lower than baseline beginning on day 3 and lasting until day 7 for both the stNGF/FJD (*$p<0.0005$) and FJD/FJD (**$p<0.019$) groups. In contrast, there is no change from baseline for the other control groups (sh/sh; veh/-, sh/-). At day 7, forepaw thresholds are significantly different (#$p=0.0141$) only between the stNGF/FJD group and the sh/sh group.

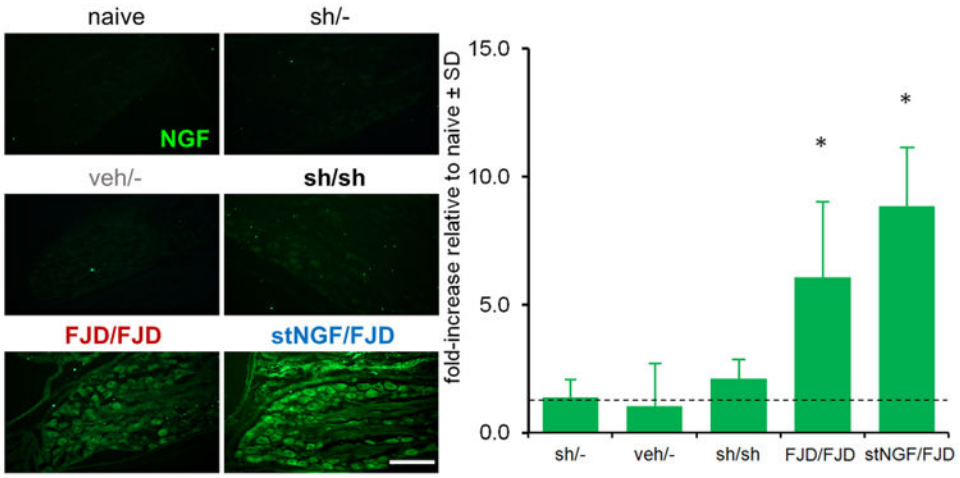

Fig. 4.

Representative images and quantification of NGF immunolabeling in the C6 DRG from each group and naïve normal rats. Positive labeling is evident in the cytoplasm of the neuron cell bodies. Expression is most robust in the FJD/FJD and stNGF/FJD groups, with two nonpainful exposures (FJD/FJD) increasing NGF levels significantly (*$p<0.0232$) over sh/-, veh/-, and sh/sh levels. Exposure to a nonpainful FJD after a prior subthreshold NGF injection (stNGF/FJD) also significantly (*$p<0.0001$) elevates NGF expression over each of the control groups (sh/-, veh/-, sh/sh). The NGF levels between FJD/FJD and stNGF/FJD are not different ($p=0.1807$).

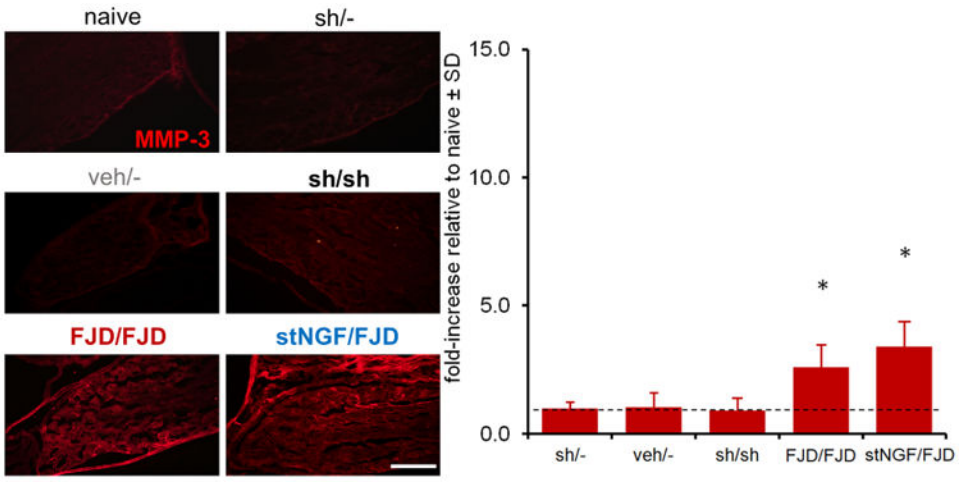

Fig. 5.

Representative images and quantification of MMP-3 immunolabeling in C6 DRGs. Positive labeling is observed in both the neurons cell bodies and surrounding matrix. Expression is increased in the groups with two exposures (FJD/FJD; stNGF/FJD). Two nonpainful FJD exposures (FJD/FJD) increases MMP-3 levels significantly (*$p<0.0035$) over levels in sh/-, veh/-, and sh/sh, which remain at normal levels. Exposure to a subthreshold NGF injection followed by a nonpainful distraction (stNGF/FJD) increases MMP-3 expression to the same level, which is significantly elevated (*$p<0.0001$) over sh/-, veh/-, and sh/sh. MMP-3 is not different between the FJD/FJD and stNGF/FJD groups ($p=0.2320$).

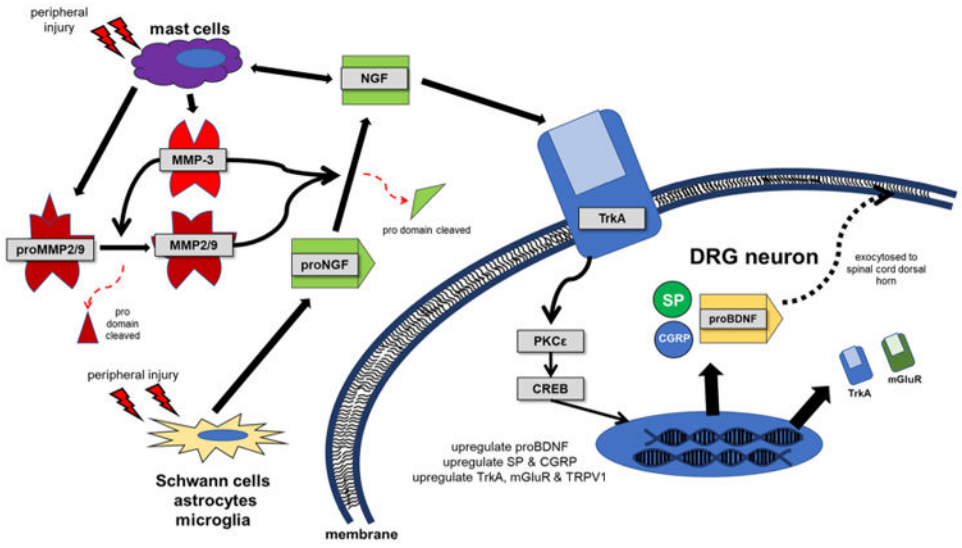

Fig. 6.

Schematic showing interactions between inflammation, MMP-3, and NGF. proNGF is a precursor to NGF, which is regulated by mast cells and Schwann cells in the periphery after a insult. MMP-3 catalyzes the cleavage of the pro-domain in proNGF to active NGF, which can either be retrogradely transported from joint afferents to the DRG soma, or can bind to TrkA receptors directly which activates PKCe and CREB to initiate transcription of TrkA, TRPV1, and neuromodulators like substance P (SP) and calcitonin gene-related peptide (CGRP), which are transported to the membrane (for further NGF-TrkA binding), or exocytosed. MMP-3 also acts to catalyze the active forms of MMP-2 and MMP-9 which can also bind to proNGF, potentially amplifying DRG neuron transcriptional changes.