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Research paper

Pain from intra-articular NGF or joint injury in the rat requires contributions from peptidergic joint afferents

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HIGHLIGHTS

- NGF injected in the cervical facet induces mechanical and thermal hypersensitivity.
- Spinal neuronal hyperexcitability develops 1 day after intra-articular NGF.
- Intact peptidergic signaling in the joint is necessary for NGF-induced sensitivity.
- Cervical facet injury induces behavioral sensitivity and increases NGF in the DRG.
- Injury-induced sensitivity and NGF require peptidergic signaling in the joint.

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ABSTRACT

Non-physiological stretch of the cervical facet joint's capsular ligament induces persistent behavioral hypersensitivity and spinal neuronal hyperexcitability via an intra-articular NGF-dependent mechanism. Although that ligament is innervated by nociceptors, it is unknown if a subpopulation is exclusively responsible for the behavioral and spinal neuronal responses to intra-articular NGF and/or facet joint injury. This study ablated joint afferents using the neurotoxin saporin targeted to neurons involved in either peptidergic ([Sar^9,Met $(O_2)^{11}$]-substance P-saporin (SSP-Sap)) or non-peptidergic (isolectin B4-saporin (IB4-Sap)) signaling to investigate the contributions of those neuronal populations to facet-mediated pain. SSP-Sap, but not IB4-Sap, injected into the bilateral C6/C7 facet joints 14 days prior to an intra- articular NGF injection prevents NGF-induced mechanical and thermal hypersensitivity in the forepaws. Similarly, only SSP- Sap prevents the increase in mechanical forepaw stimulation- induced firing of spinal neurons after intra-articular NGF. In addition, intra-articular SSP-Sap prevents both behavioral hypersensitivity and upregulation of NGF in the dorsal root ganglion after a facet joint distraction that normally induces pain. These findings collectively suggest that disruption of peptidergic signaling within the joint may be a potential treatment for facet pain, as well as other painful joint conditions associated with elevated NGF, such as osteoarthritis.

1. Introduction

Chronic pain affects nearly 1/3 of adults in the US annually, with neck and back pain being the most common [1]. The facet joint is the source of pain in up to 60% of neck trauma cases [2], with non-physiological capsular ligament stretch a common cause of pain [3]. Proprioceptors and nociceptors innervating the capsular ligament respond to its stretch [4–6]. Both peptidergic (expressing neuropeptides like substance P (SP)) and non-peptidergic (binding isolectin B4 (IB4)) primary afferents innervate the facet joint [7–10]. Clinically and experimentally, anesthetic nerve blocks demonstrate that joint afferents contribute to facet-mediated pain [11–13]. Although excessive facet capsular stretch induces persistent pain and spinal and afferent neuronal hyperexcitability [4,14–16], no study has identified whether a subpopulation of joint afferents mediates injury-induced facet pain.

Peptidergic afferents are sensitive to nerve growth factor (NGF), which is increased in the facet joint by day 1 after its injury [17]. Up to 1/3 of NGF-sensitive afferents bind IB4 [18]. NGF sensitizes adult sensory neurons [7]; intra-articular NGF induces pain and spinal neuronal hyperexcitability within 1 day [17]. Administration of anti-NGF antibodies attenuates experimental and clinical

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joint pain [19,20], and blocking intra-articular NGF after facet injury prevents pain and spinal neuronal dysfunction [17]. Although these findings support NGF's role in joint pain, the relative contributions of peptidergic and non-peptidergic afferents to NGF-induced joint pain is unknown.

This study investigated the separate contributions of peptidergic or non-peptidergic facet joint afferents in the development of behavioral hypersensitivity after intra-articular administration of NGF in the rat using targeted neuronal ablation. Because spinal neuronal hyperexcitability is a hallmark of persistent pain [21], extracellular potentials were recorded from the deep laminae (III–VI) of the dorsal horn, which contain mostly wide dynamic range (WDR) neurons [22], to assess neuronal firing evoked by peripheral mechanical stimuli. Based on those findings and the known role of intra-articular NGF in injury-induced joint pain [17], peptidergic signaling via SP within the facet was eliminated prior to imposing a typically painful facet injury in order to define the role of those joint afferents in pain initiation from joint trauma.

2. Materials and methods

Male Holtzman rats (Harlan Sprague–Dawley) weighing 393 ± 30 g were housed under USDA- and AAALAC-compliant conditions with free access to food and water. All procedures were approved by our institutional IACUC and carried out under the guidelines of the Committee for Research and Ethical Issues of the IASP [23]. Rats were doubly-housed with 12-h light/dark cycles and randomly assigned to groups before surgical or behavioral procedures. Quantitative analyses were performed without group identification to eliminate bias.

2.1. Joint injection of saporin followed by NGF

Neurons expressing the NK1 receptor (NK1R) were ablated using a targeted SP conjugate of the neurotoxin saporin, [Sar^9,$Met(O_2)^{11}$]-substance P-saporin (SSP-Sap), via intra- articular injection as described previously [10]: 100 ng of SSP-Sap dissolved in PBS (5 μL) and injected into the bilateral C6/C7 facet joints (n = 20). Non-targeted saporin (100 ng) was injected in separate rats as controls (Blank-Sap n = 16). Additional rats received intra-articular injections of 5 μg of saporin conjugated to isolectin B4 (IB4-Sap n = 14) in 5 μL of PBS to ablate non-peptidergic neurons, with similar control injections of 5 μg of unconjugated saporin (Saporin n = 9) in separate rats.

Fourteen days after saporin injections, rats were given an intra-articular injection of 3 μg of rat β-NGF (R&D Systems) in 5 μL of PBS (SSP-Sap + NGF n = 12; Blank-Sap + NGF n = 12; IB4-Sap + NGF n = 8; Saporin + NGF n = 9) or PBS (5 μL) as vehicle (SSP-Sap + veh n = 8; Blank-Sap + veh n = 4; IB4-Sap + veh n = 6), using the same procedures. That NGF dose induces behavioral hypersensitivity and spinal neuronal hyperexcitability within 1 day [17]. Weight gain was monitored regularly after all procedures until each rat's study endpoint.

2.1.1. Behavioral assessments

All behavioral tests were performed between 8 a.m. and noon. Forepaw mechanical withdrawal thresholds were quantified for all rats using customary methods [10,24]. Rats were acclimated to the testing environment for 20 min prior to application of an ascending series of von Frey filaments to each forepaw; the lower of two consecutive filaments eliciting emphatic lifting of the forepaw was taken as the threshold. Thresholds in the bilateral forepaws were quantified and averaged over three testing rounds prior to NGF or vehicle injection (baseline) and on day 1 following it. Baseline responses were quantified and averaged over two days before injections.

A subset of rats in each group also was evaluated for thermal sensitivity after NGF or vehicle injection (SSP-Sap + NGF n = 8; SSP-Sap + veh n = 8; Blank-Sap + NGF n = 4; Blank-Sap + veh n = 4; IB4-Sap + NGF n = 8; IB4-Sap + veh n = 6; Saporin + NGF n = 9). Thermal hypersensitivity was measured following mechanical assessment using a commercially available device (UC San Diego) and customary methods [25]. After acclimation to the apparatus for 30 min, the withdrawal latency for each rat was averaged across forepaws over three testing rounds on each day; baseline measurements were averaged over two days before injections.

2.1.2. Spinal electrophysiological recordings

After behavioral testing on day 1, spinal neuronal excitability was quantified in a subset of rats (SSP-Sap + NGF n = 4; SSP-Sap + veh n = 4; Blank-Sap + NGF n = 4; IB4-Sap + NGF n = 5; IB4-Sap + veh n = 6; Saporin + NGF n = 4) using customary methods [14,17]. Rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.) and maintained with supplementary doses (5–10 mg/kg, i.p.). The C6–C8 spinal cord was exposed and bathed in 37 °C mineral oil. Rats were mounted onto a stereotaxic frame; core temperature was maintained at 35–37 °C.

Extracellular potentials were recorded using tungsten electrodes lowered into laminae III–VI via a micropositioner [14,17]. Sensory neurons were selected for recording if brushing the forepaw plantar surface induced firing. A stimulus train, consisting of light brush (for 10-s), a series of von Frey filaments (1.4 g, 4 g, 10 g, 26 g) each applied for 5 stimulations of 1-s followed by 1-s of recovery, and a noxious pinch (60 g, for 10- s), was applied to the forepaw. Stimuli were applied at 30-s intervals. This stimulation protocol was repeated for each identified mechanosensitive neuron.

Recordings were spike-sorted using Spike2 (CED). Evoked spikes were summed over the continuous 10-s stimulus period for both the brush and pinch. Neurons were classified as low threshold mechanoreceptive (LTM) or WDR, based on pinch-evoked firing [16]. The number of spikes evoked from the initial application of a von Frey filament until 1- second after its 5th application was summed. For each filament, the baseline spikes 1-second prior to its initial application were subtracted from the total spike count.

2.1.3. Facet joint injury and assessment of NGF in the dorsal root ganglion (DRG)

Separate groups of rats received SSP-Sap (SSP-Sap n = 11) or non-targeted saporin (Blank-Sap n = 7) as described above. Fourteen days later under inhalation isoflurane anesthesia, rats underwent either a facet joint distraction (FJD) (SSP-Sap + FJD n = 7; Blank-Sap + FJD n = 7) or sham (SSP-Sap + sham n = 4), as previously described [10,24]. A loading device distracted the bilateral C6/C7 facet joints by displacing the C6 vertebra rostrally and holding C7 fixed. Sham procedures included mounting onto the device with no applied distraction. Forepaw mechanical withdrawal thresholds were measured at baseline (day 0) and days 1, 3, 5, and 7 after injury or sham procedures.

On day 7, rats were given an overdose of sodium pentobarbital and transcardially perfused with PBS and 4% paraformaldehyde. Bilateral C7 DRGs were harvested, post-fixed, and cryo-protected. Serial axial DRG sections (14 μm thick) were thaw-mounted onto slides and incubated in DeCal Antigen Retrieval (BioGenex) solution for 2 hrs. Slides were washed, blocked with goat serum, and incubated overnight with rabbit- anti-NGF antibody (1:100; Santa Cruz) at 4 °C. Sections were incubated in an Alexa488-conjugated goat-anti-rabbit secondary antibody (Life Technologies) for 2 hrs at room temperature, washed, and coverslipped. Sections from naïve rats and with no primary antibody incubation were included as controls. NGF labeling was quantified using densitometry in four sections per rat [24]. The percentage of neurons expressing NGF

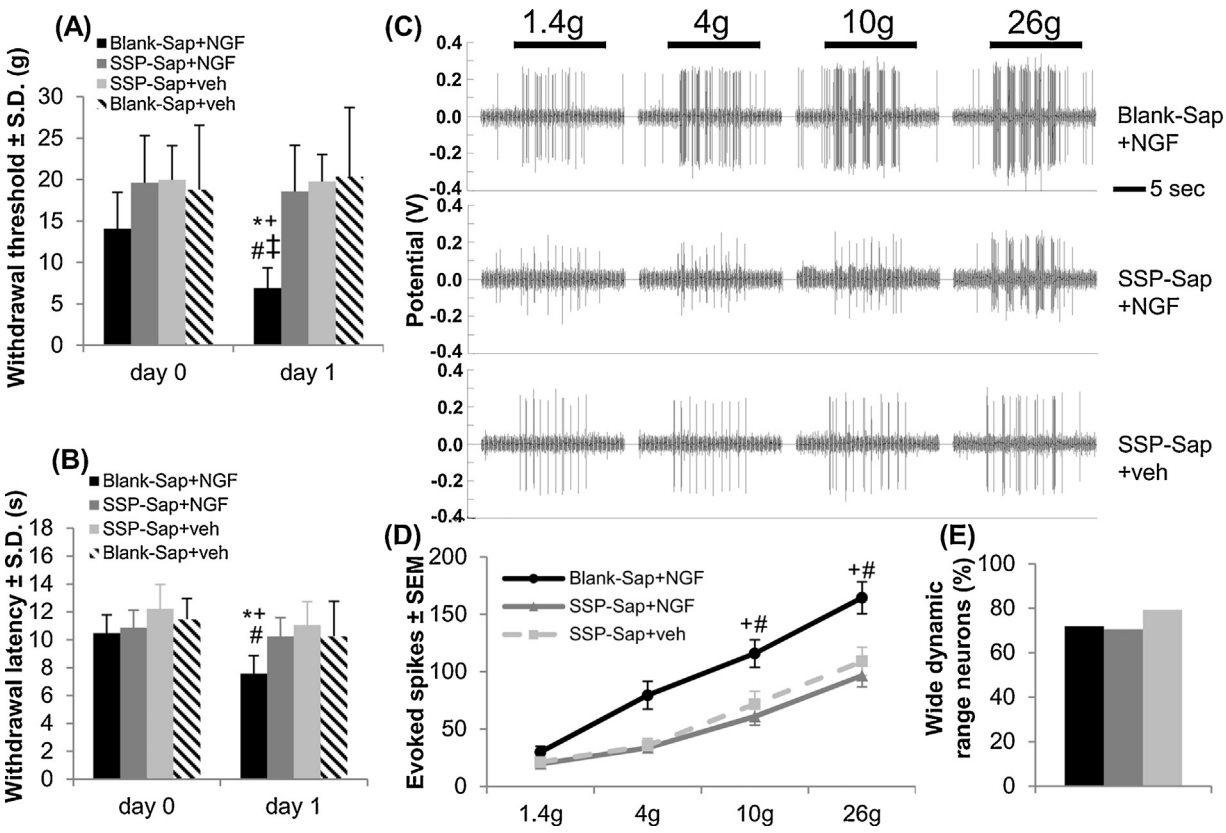

Fig. 1. Intra-articular NGF-induced behavioral hypersensitivity and neuronal hyperexcitability require joint afferents involved in peptidergic signaling. (A) Mechanical withdrawal threshold at day 1 is reduced from baseline for Blank-Sap + NGF (*$p < 0.004$; $n = 12$) and compared to SSP- Sap + NGF (+$p < 0.001$; $n = 12$), SSP-Sap + veh (#$p < 0.001$; $n = 8$), and Blank-Sap + veh (‡$p < 0.001$; $n = 4$). (B) Thermal withdrawal latency is reduced for Blank-Sap + NGF at day 1 compared to baseline (*$p < 0.009$; $n = 4$) and SSP-Sap + NGF (+$p < 0.022$; $n = 8$) or SSP-Sap + veh (#$p < 0.011$; $n = 8$) but not Blank-Sap + veh ($n = 4$). (C) Spinal neuronal extracellular recordings show greater evoked firing for Blank-Sap + NGF ($n = 4$) than for SSP-Sap + NGF (+p <0.008; $n = 4$) and SSP-Sap + veh (#$p < 0.044$; $n = 4$) for 10 g and 26 g filaments (D). The proportion of wide dynamic range neurons (E) is not different between groups.

was quantified by cropping each DRG (472 × 472 pixel area) and counting the number of neurons positive or negative for NGF.

2.1.4. Statistical analyses

Withdrawal thresholds and latencies were separately compared between groups in JMP Prov10.0.2 (SAS Institute Inc.) using univariate two-way repeated measures ANOVAs with group and time, their interaction, and individual rats nested within group as factors and a post hoc Tukey's HSD test. For all behavioral analyses, the number of repeats is the number of days responses were quantified. The total number of spikes evoked for each stimulus was compared between groups using a two-way nested ANOVA with group and stimulus as factors and neurons nested within rats, nested within group, with a post hoc Tukey's HSD test. Differences in the ratio of WDR neurons were compared between groups using Pearson's chi-square test. NGF expression was compared between groups using separate ANOVAs with Bonferroni tests.

3. Results

Mechanical withdrawal thresholds at day 1 for Blank-Sap + NGF are reduced from baseline ($p < 0.004$), with no other groups changed from baseline (Fig. 1A). Further, the withdrawal threshold for Blank-Sap + NGF is lower ($p < 0.001$) than all other groups (Fig. 1A). The thermal withdrawal latency at day 1 after NGF with non-targeted saporin (Blank-Sap + NGF) decreases from baseline ($p < 0.009$) and is lower than both SSP-Sap + NGF ($p < 0.022$) and SSP-Sap + veh ($p < 0.011$) (Fig. 1B). From 95 neurons (average depth 658 ± 19 μm), more spikes are evoked by stimulation with the 10 g and 26 g filaments in the Blank- Sap + NGF group than in either SSP-Sap + NGF ($p < 0.008$) or SSP-Sap + veh ($p < 0.044$) (Fig. 1C and D). There are no differences in the proportion of WDR neurons (Fig. 1E) between groups.

The mechanical withdrawal thresholds for both IB4-Sap + NGF and Saporin + NGF are reduced at day 1 from their respective baseline responses ($p < 0.001$) and not different from each other (Fig. 2A). IB4-Sap + NGF and Saporin + NGF exhibit lower withdrawal thresholds than IB4-Sap + veh at day 1 ($p < 0.006$) (Fig. 2A). Similarly, the IB4-Sap + NGF ($p < 0.001$) and Saporin + NGF ($p < 0.001$) groups exhibit shorter thermal withdrawal latencies after NGF injection (Fig. 2B). At day 1, only the withdrawal latency for Saporin + NGF is lower than IB4-Sap + veh ($p < 0.046$) (Fig. 2B). Stimulation by the 26 g filament evokes more spikes (108 neurons; depth 741 ± 16 μm) for IB4-Sap + NGF ($p < 0.038$) and Saporin + NGF ($p < 0.020$) than for IB4-Sap + veh (Fig. 2C and D). There is no difference in the number of WDR neurons (Fig. 2E).

For those rats undergoing an FJD after intra-articular Blank-Sap, the withdrawal threshold decreases by day 1 and remains below baseline at all days ($p < 0.001$) (Fig. 3A). The withdrawal threshold for Blank-Sap + FJD is lower than that for SSP-Sap + FJD ($p < 0.001$) and SSP-Sap + sham ($p < 0.001$) for all days (Fig. 3A). Neither of those groups changes from baseline (Fig. 3A). Total NGF expression and the percentage of NGF-expressing neurons in the DRG increases ($p < 0.001$) for Blank-Sap + FJD over both SSP-Sap + FJD and SSP-Sap + sham (Fig. 3B–F).

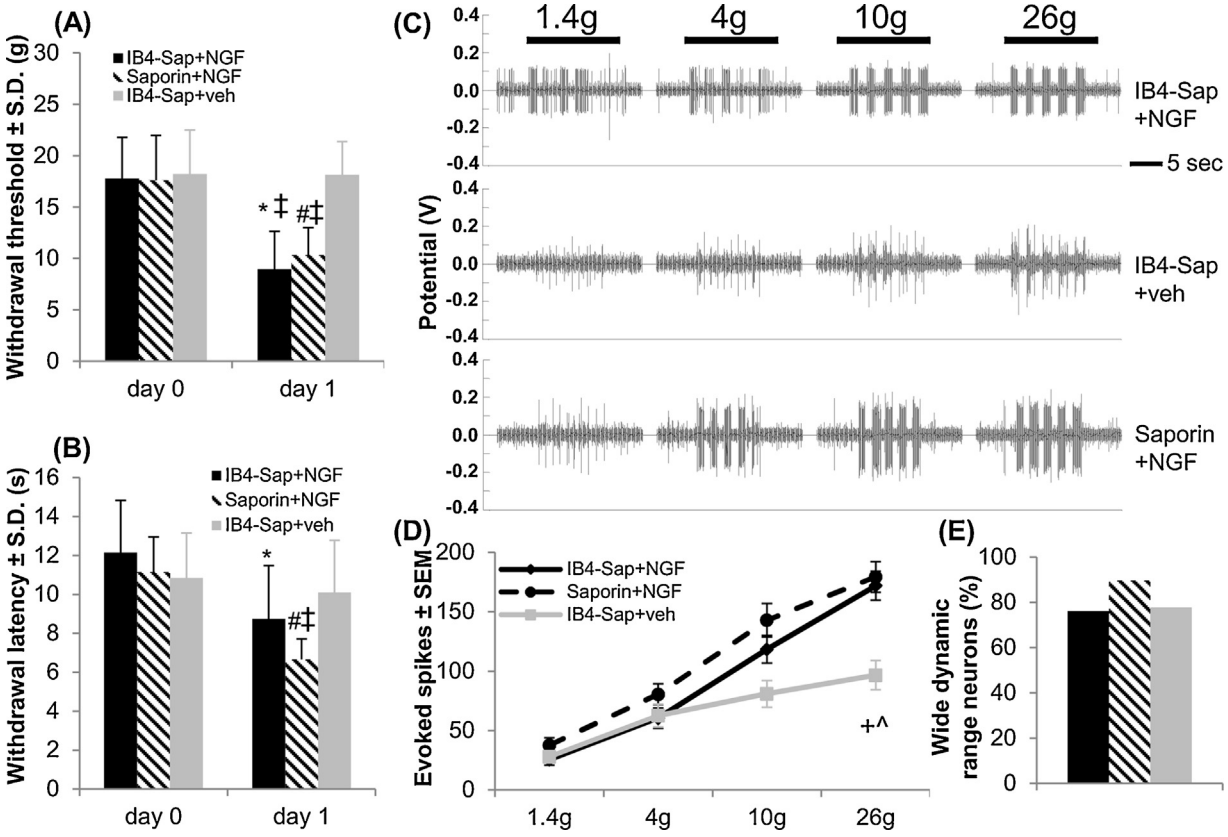

Fig. 2. Ablating non-peptidergic joint afferents does not prevent intra-articular NGF-induced behavioral sensitivity or neuronal hyperexcitability. (A) Mechanical withdrawal threshold at day 1 is reduced compared to baseline for IB4-Sap + NGF (*$p < 0.001$; $n = 8$) and Saporin + NGF (#$p < 0.001$; $n = 9$); both exhibit lower thresholds than IB4-Sap + veh at day 1 (‡$p < 0.006$; $n = 6$). (B) Thermal withdrawal latency is reduced for IB4-Sap + NGF (*$p < 0.001$; $n = 8$) and Saporin + NGF (#$p < 0.001$; $n = 9$) compared to baseline, but only Saporin + NGF is lower than IB4-Sap + veh at day 1 (‡$p < 0.046$; $n = 6$). Spinal neuronal recordings (C) demonstrate the increased evoked firing (26 g stimulation) for IB4-Sap + NGF (+$p < 0.038$; $n = 5$) and Saporin + NGF (^$p < 0.020$; $n = 4$) compared to IB4-Sap + veh ($n = 6$) (D). The ratio of wide dynamic range neurons is not different between groups (E).

4. Discussion

This study defines peptidergic signaling in the facet as a critical initiator of both NGF- and injury-induced pain. Intra-articular NGF induces thermal and mechanical hypersensitivity associated with spinal neuronal hyperexcitability (Figs. 1 and 2). Pharmacologic ablation of joint afferents involved in peptidergic signaling prevents onset of these NGF-induced responses; yet, elimination of non-peptidergic joint afferents prevents only thermal hypersensitivity. Ablating joint afferents that are sensitive to SP also prevents the pain and increased NGF expression in the DRG that are evident after a facet injury (Fig. 3). Considering that intra-articular NGF increases early after a painful facet injury [17], NGF likely acts on the subpopulation of joint afferents involved in peptidergic signaling to initiate injury-induced facet pain.

Intra-articular NGF increases thermal sensitivity, which is abolished when NK1R- bearing afferents are ablated (Fig. 1). When non-peptidergic neurons are removed, thermal sensitivity after NGF injection is also not different from controls (Fig. 2). Since the transient receptor potential vanilloid-1 responds to noxious thermal stimuli and is expressed on peptidergic and non-peptidergic afferents in the rat [26], both afferent populations may contribute to thermal sensitivity, which our findings support. In contrast, ablating joint afferents involved in peptidergic, but not non-peptidergic, signaling prevents mechanical hypersensitivity induced by intra-articular NGF (Figs. 1 and 2). This result is not surprising since the NGF receptor, trkA, is expressed mainly by peptidergic afferents [7,27]. Peptidergic signaling also contributes to mechanical sensitivity after FJD (Fig. 3). Because intra- articular NGF is necessary to initiate mechanical sensitivity after FJD [17] and non- peptidergic joint afferents do not contribute to NGF-induced mechanical sensitivity, those afferents likely do not contribute to FJD-mediated mechanical sensitivity. These findings suggest peptidergic signaling has more influence than non-peptidergic signaling on joint pain from both NGF and injury.

Ablating SP-sensitive spinal neurons prevents pain in the rat after nerve injury, inflammatory stimulus, and this facet joint injury [28,29], suggesting the central release of SP is required for pain maintenance after injury. Yet, the actions of SP are not limited to the spinal cord [30]. Intra-articular injection of SP sensitizes joint afferents [31], and inhibition of NK1R alleviates joint pain [32]. Because expression and release of SP from primary afferent neurons is regulated by NGF [27], the increased intra- articular NGF that initiates pain after FJD [17] likely stimulates SP synthesis and release. In fact, SP increases in the DRG 7 days after painful FJD [33], paralleling the increased NGF expression observed here (Fig. 3). Ablating joint afferents sensitive to SP *before* injury prevents both NGF-induced and injury-induced facet pain (Figs. 1 and 3), similar to blocking intra-articular NGF signaling at injury [17]. Yet, blocking intra-articular NGF even 1 day after injury does not alleviate established pain [17]. Antagonism of spinal NK1R prevents the behavioral effects induced by systemic NGF [34], demonstrating spinal SP signaling as necessary for NGF-induced pain. Although ablating afferents (Fig. 3) or spinal neurons [29] bearing NK1R prevents injury-induced facet pain, assessing the temporal contribution of SP signaling to any form of facet pain would clarify relationships, if any, between NGF, substance P, and pain maintenance. Further, characterizing NK1R-bearing joint afferents and

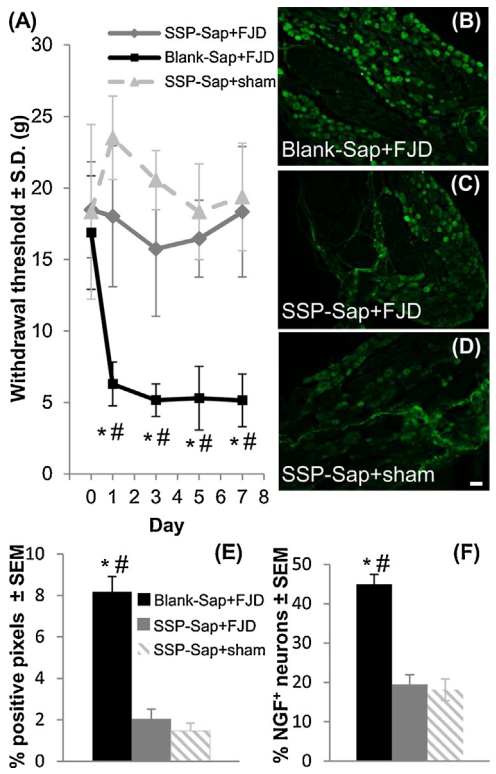

Fig. 3. FJD-induced pain and increased NGF in the DRG utilizes joint afferents involved in peptidergic signaling. (A) Forepaw mechanical withdrawal threshold is reduced by day 1 and remains reduced through day 7 ($p < 0.001$) after injury for Blank-Sap + FJD ($n = 7$) and is lower than that for SSP-Sap + FJD (*$p < 0.001$; $n = 7$) or SSP-Sap + sham (#$p < 0.001$; $n = 4$) at all days. NGF expression and the percentage of neurons expressing NGF (NGF^+) are each increased at day 7 in the DRG after Blank-Sap + FJD ($n = 7$) (B) compared to SSP- Sap + FJD (*$p < 0.001$; $n = 7$) (C) and SSP-Sap + sham (#$p < 0.001$; $n = 4$) (D) (E and F). 50 μm scale bar in (D) applies to (B–D).

their role in development and/or maintenance of NGF- and injury-induced facet pain would identify population-specific receptors and associated signaling pathways underlying joint pain that could be new therapeutic targets.

Tensile loading of the cervical facet activates afferent fibers innervating that joint [4,6], which is hypothesized as initiating pain [21]. Indeed, blocking all joint afferent activity prevents both pain and spinal neuronal hyperexcitability after FJD [12], which is also achieved by blocking intra-articular NGF [17]. Interestingly, intra-articular NGF induces hyperexcitability in dorsal horn neurons even when non-peptidergic joint afferents are eliminated (Fig. 2) but not when intra- articular peptidergic signaling is ablated (Fig. 1). Despite demonstrating that intact peptidergic signaling in the facet is necessary for NGF-induced spinal neuronal dysfunction, these findings do not distinguish whether intra-articular NGF mediates spinal hyperexcitability by increasing pain mediator release or inducing afferent sprouting, hyperinnervating the joint and increasing its sensitivity to motion(s). Accordingly, defining whether intra-articular NGF induces afferent sprouting in the joint is critical for understanding the pathophysiology of this system.

Collectively, intra-articular NGF and peptidergic joint afferents contribute to the development of pain and spinal neuronal hyperexcitability after facet injury. Although peptidergic signaling contributes to the maintenance of arthritic joint pain [30,32], this is the first study demonstrating intact peptidergic signaling within the facet as necessary for pain onset after a joint injury that increases NGF expression [17]. However, additional negative treatment controls and assays quantifying thermal and mechanical sensitivity in rats undergoing FJD and NGF injections would strengthen these results and evaluate if priming effects contribute to NGF- induced sensitivity. Nevertheless, this work provides a basis for both defining the complex mechanism(s) that lead to persistent pain and neuronal hyperexcitability after facet joint trauma and identifying similarities with non-traumatic pain initiators.

Disclosures

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