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Original Reports

Ablating Spinal NK1-Bearing Neurons Eliminates the Development of Pain and Reduces Spinal Neuronal Hyperexcitability and Inflammation From Mechanical Joint Injury in the Rat

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Abstract: **The facet joint is a common source of pain, especially from mechanical injury. Although chronic pain is associated with altered spinal glial and neuronal responses, the contribution of specific spinal cells to joint pain is not understood. This study used the neurotoxin [Sar^9,Met(O$_2$)^{11}]-substance P-saporin (SSP-SAP) to selectively eliminate spinal cells expressing neurokinin-1 receptor (NK1R) in a rat model of painful facet joint injury to determine the role of those spinal neurons in pain from facet injury. Following spinal administration of SSP-SAP or its control (blank-SAP), a cervical facet injury was imposed and behavioral sensitivity was assessed. Spinal extracellular recordings were made on day 7 to classify neurons and quantify evoked firing. Spinal glial activation and interleukin 1α (IL1α) expression also were evaluated. SSP-SAP prevented the development of mechanical hyperalgesia that is induced by joint injury and reduced NK1R expression and mechanically evoked neuronal firing in the dorsal horn. SSP-SAP also prevented a shift toward wide dynamic range neurons that is seen after injury. Spinal astrocytic activation and interleukin 1 (IL1α) expression were reduced to sham levels with SSP-SAP treatment. These results suggest that spinal NK1R-bearing cells are critical in initiating spinal nociception and inflammation associated with a painful mechanical joint injury.**

***Perspective:** Results demonstrate that cells expressing NK1R in the spinal cord are critical for the development of joint pain, spinal neuroplasticity, and inflammation after trauma to the joint. These findings have utility for understanding mechanisms of joint pain and developing potential targets to treat pain.*

***Key words:** Pain, joint, saporin, substance P, spinal cord.*

The facet joint has been identified as the most common source of pain, with as many as 62% of chronic neck pain cases being attributed to the facet joint.[1] Neck loading can exceed the physiologic limits of individual spinal tissues such as the facet capsule, which can result in its painful injury during its excessive stretch.[23,37] The facet capsule is innervated by nociceptive afferents that respond to neuropeptides, such as substance P (SP).[12] Tensile loading of the facet capsule activates those afferents and alters neuropeptides, neurotrophins, and neuronal hyperexcitability in association with pain.[17,20,21,24] These reports suggest that joint trauma may be sufficient to activate the spinal neurons that are responsible for pain. However, the role of specific spinal cell types in the development of pain and in modulating spinal nociceptive signaling in facet joint pain remains unknown.

SP has been shown to be differentially modulated in the spinal cord following different severities of facet joint injury,[20] suggesting that its receptor, neurokinin-1 receptor (NK1R), is also important in the development of pain from facet injury. Because spinal neuronal hyperexcitability has also been reported at times after facet injury when behavioral sensitivity persists,[24] it is possible

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that the afferent signals generated by painful joint injury may directly modulate spinal sensitization through SP-NK1R signaling. The same facet capsule stretches that produce pain and alter nociceptive cascades are also associated with increased spinal glial activation and inflammation.[9,18-20] Despite increasing evidence that painful joint injury is associated with spinal neuroimmune responses, the specific role of the spinal SP-NK1 system in joint pain and its relationship to those cascades known to modulate pain is unknown.

The neurotoxin saporin can be conjugated to target molecules and is taken up by cells to lead to cell death via internalization by saporin and its conjugate, thereby inactivating ribosomes, which blocks protein synthesis.[36] The SP conjugate of saporin, [Sar^9,$Met(O_2)^{11}$]-substance P-saporin (SSP-SAP), ablates cells expressing the NK1R.[14,33,36] Selectively ablating NK1-positive cells by SSP-SAP reduces behavioral sensitivity and lowers spinal neuronal hyperexcitability in models of peripheral inflammation (ie, formalin, mustard oil, capsaicin).[14,33,36] However, the role of those cells in the development of joint pain is not defined.

This study used SSP-SAP in a rat model of painful joint injury to eliminate spinal cells expressing NK1R to determine their contribution(s) to the initiation and/or maintenance of joint pain. We hypothesized that eliminating such cells would prevent the development of behavioral hypersensitivity and attenuate or eliminate the neuronal hypersensitivity and spinal inflammation that is normally evident at day 7 after a painful joint injury.[9,18,19,24,25,38]

Methods

Saporin Injections and Injury Procedures

Male Holtzman rats (275-299 g Sprague Dawley; Harlan, Indianapolis, IN) were housed under U.S. Department of Agriculture– and Association for Assessment and Accreditation of Laboratory Animal Care–compliant conditions with free access to food and water. Procedures were approved by the Institutional Animal Care and Use Committee and carried out under the guidelines of the Committee for Research and Ethical Issue of the International Association for the Study of Pain.[39] All injections and surgeries were performed under inhalation isoflurane anesthesia (4% for induction, 2% for maintenance). Rats received either [Sar^9,$Met(O_2)^{11}$] SP conjugated saporin (100 ng in 30 μL phosphate-buffered saline, n = 24; SSP-SAP; Advanced Targeting Systems, San Diego, CA) or nontargeted saporin (100 ng in 30 μL phosphate-buffered saline, n = 12; blank-SAP) (Advanced Targeting Systems) as the control, via lumbar puncture.

Because SSP-SAP has been shown to effectively ablate cells within 14 days,[36] injury was imposed 14 days after injection using a bilateral joint distraction across the C6-C7 facet joint. Joint distraction applies a controlled stretch of the capsular ligament that is known to induce sustained behavioral sensitivity.[16,24] Rats were placed in a prone position and the C4-T2 spinal levels were exposed by an incision. The C6 and C7 laminae and bilateral facet capsules were exposed, and those vertebrae were attached to a loading device via microforceps that translates C6 rostrally while C7 remains stationary, imposing a stretch of the C6-C7 bilateral facet capsules.[16] Sham procedures were performed in separate rats as surgical controls, without any joint distraction. Rats receiving SSP-SAP were randomly assigned to undergo either injury (SSP-SAP injury, n = 21) or sham (SSP-SAP sham, n = 13). Rats receiving blank-SAP underwent injury (blank-SAP injury, n = 17) as a positive control. All surgical procedures lasted 90 minutes or less.

During distraction, the magnitude of several biomechanical metrics of tissue trauma was measured to define the severity of joint injury for each rat. Briefly, the joint displacements and capsular ligament strains were quantified.[8,16,19] Each metric describing the joint injury mechanics (vertebral distraction, tensile strain, and peak maximum principal strain) was compared between the injury groups using separate t-tests.

Behavioral Assessments

Forepaw sensitivity to mechanical stimuli was measured prior to surgery (baseline, day 0) and on postoperative days 1, 3, 5, and 7, as previously described.[4,16,24] Response thresholds were measured by a single tester who was blinded to the study groups by stimulating each forepaw with a series of von Frey filaments of increasing strengths ranging from .6 to 26 g (Stoelting, Wood Dale, IL). The lowest-strength filament to provoke a response was taken as the response threshold if the next filament also elicited a positive response. If a rat was unresponsive to all filaments, the maximum filament strength (26 g) was recorded as the threshold. Each testing session consisted of 3 rounds with at least 10 minutes' rest between rounds. The threshold for each rat on each day was determined by averaging the rounds. Because this injury is bilateral across both sides of the C6-C7 facet joint, the right and left forepaw responses were compared using a paired t-test to ensure there were no differences between sides; moreover, because this was the case, the bilateral responses for each rat were averaged. A repeated-measures analysis of variance with Bonferroni correction compared response thresholds between groups.

Spinal Cord Immunohistochemistry

Following behavioral testing on day 7, the C6 spinal cord was harvested from a subset of the rats receiving injections (SSP-SAP injury, n = 8; blank-SAP injury, n = 6; SSP-SAP sham, n = 4) to evaluate if NK1R expression was modified by the ablating agent. Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg) and transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde. Spinal cord at C6 was post-fixed overnight and stored in 30% sucrose for 6 days at 4°C. Samples were axially sectioned (14 μm) and prepared for on-slide immunohistochemical labeling for the NK1R. Slides were blocked in donkey serum (Millipore, Billerica, MA) and .3% triton X-100 and then incubated in a solution containing rabbit anti-NK1R (1:250; Novus, Littleton, CO) overnight at room temperature. The next day, sections were incubated in a secondary antibody solution

containing donkey anti-rabbit Cy3 (1:600; Jackson ImmunoResearch, West Grove, PA).

The degree of astrocytic and microglial activation at day 7 was assessed in the C6 spinal cord using immunohistochemistry to detect glial fibrillary acidic protein (GFAP) and OX-42 (CD11b/c), respectively. Tissue sections were blocked in goat serum (Vector Labs, Burlingame, CA) and incubated in mouse anti-rat CD11b/c primary antibody (1:500; AbD Serotec, Raleigh, NC) overnight at 4°C. The next day, sections were incubated in Alexa488 conjugated F'ab goat anti-mouse secondary antibody (1:1000; Life Technologies, Grand Island, NY), then incubated in the primary antibody mouse anti-GFAP (1:500; Millipore) overnight at 4°C, and then incubated in Alexa568 conjugated F'ab goat anti-mouse secondary antibody.

Spinal expression of the cytokine interleukin 1α (IL1α) was also quantified on day 7 after injury. After blocking in donkey serum, sections were incubated overnight at 4°C in goat anti-IL1α (1:100; Santa Cruz, Dallas, TX) and then in an Alexa488 conjugated donkey anti-goat secondary antibody (1:250; Life Technologies) the next day.

For all immunohistochemical analyses, spinal cords from normal unoperated rats and samples with no primary antibody were included as controls and to verify specificity of each antibody. Sections were imaged at 200× using a digital camera and stereomicroscope with DP2-BSW software (Olympus, Center Valley, PA), and each image was cropped to include only the dorsal horn for densitometric analysis. The percentage of pixels above the threshold expression in normal samples was quantified for each sample and compared between groups using an analysis of variance with Bonferroni test. Because of the bilateral nature of the injury, the right and left sides of the spinal cord were pooled together and analyzed. All immunohistochemical procedures and analyses were performed in a blinded fashion.

Electrophysiology Procedures

Electrophysiology recordings were acquired from the spinal cord in 2 separate studies to define the effects of SSP-SAP on neuronal excitability in the dorsal horn. The first study characterized the effects of administering SSP-SAP; separate groups of rats were given either SSP-SAP (SSP-SAP D0, n = 5) or blank-SAP (blank-SAP D0, n = 5) only, and spinal neuronal activity was measured 14 days later. A separate set of rats received injection and then underwent either injury (SSP-SAP injury, n = 5; blank-SAP injury, n = 5) or sham surgery (SSP-SAP sham, n = 5) as above. In those groups, behavioral sensitivity was assessed on days 0 (baseline), 1, and 7 to confirm that the injuries and behavioral outcomes were comparable across studies.

Electrophysiological measurements of neuronal voltages in the spinal dorsal horn were made on day 0 in the injection-only groups or day 7 after the surgical procedures. Rats were anesthetized via intraperitoneal injection of sodium pentobarbital (45 mg/kg) with supplementary doses (5–10 mg/kg) given as needed according to the hind paw pinch reflex. The C6-C8 spinal cord was exposed via laminectomy and dural resection, and 40°C mineral oil was applied to prevent dehydration. Core temperature was maintained at 35 to 37°C using a temperature-controlled heating pad equipped with a rectal probe (Physitemp, Clifton, NJ). Rats were attached to a stereotaxic frame with earbars and a vertebral clamp at T2 to stabilize the spine (David Kopf Instruments, Tujunga, CA). A tracheotomy provided mechanical ventilation, and CO_2 was continuously monitored. A thoracotomy was performed to minimize respiratory movement during neuronal recordings.

Extracellular voltage potentials were recorded using a glass-insulated tungsten electrode (125 μm shank, 20° taper to <1 μm tip; FHC, Bowdoin, ME), amplified with a gain of 10^3 and conditioned using a passband filter between .3 and 3 kHz (World Precision Instruments, Sarasota, FL). The signal was processed with a 60-Hz noise eliminator (HumBug; Quest Scientific, North Vancouver, British Columbia, Canada), digitally sampled at 25 kHz (Micro1401; CED, Cambridge, United Kingdom), and monitored with a speaker for audio feedback (A-M Systems, Carlsborg, WA). Neurons were located by lowering the electrode using a micropositioner (Narishge, East Meadow, NJ) to depths between 50 and 1000 μm below the pial surface, which corresponds to both the superficial laminae (I-II) and deep laminae (III-VI). Neurons were identified by light brushing of the plantar surface of the forepaw. Once an evoked response was established, the location of maximal response was marked on the forepaw and the mechanical stimulation protocol was performed: 10 nonnoxious brush strokes, five 1-second stimulations with a 1-second rest period using von Frey filaments (1.4 g, 4 g, 10 g, 26 g), and a 10-second noxious pinch by using a 60-g vascular clip.[4,24] Filaments were selected to match those used in the behavioral testing; their application was synchronized with the recording measurements.

To determine the frequency of neuronal firing, voltage recordings from each neuron were filtered with a high-pass fourth-order filter and spike-sorted using Spike2 software (CED, Cambridge, United Kingdom). The total number of spikes evoked during the brush and pinch were summed over each stimulation period for each neuron. The number of spikes evoked by each von Frey stimulation and during the rest period were counted and summed. Baseline firing prior to each stimulus was counted and subtracted from the total spike counts in order to evaluate evoked responses.[4,24] Neurons were classified as either wide dynamic range (WDR), nociceptive specific, or a low-threshold mechanoreceptor, based on their response to von Frey stimulation.[14,28] WDR neurons displayed a graded response to increasing von Frey filament strengths, whereas nociceptive-specific neurons responded only to noxious stimuli (10 g, 26 g, pinch) and low-threshold mechanoreceptor neurons responded to nonnoxious stimuli.[14,28] The percentage of neurons in each classification for each injury group was determined by summing the number neurons for each classification type from all rats in each group and determining the percentage based on the total number of neurons in each group. The proportion of neurons classified as each type was

compared between groups using Pearson chi-square tests. Electrophysiological data were log-transformed to adjust for a positive skew. A mixed-effect nested analysis of variance with post hoc Tukey test identified differences in evoked firing responses between groups.

Results

Injection of SSP-SAP prior to a facet joint distraction prevented the development of mechanical sensitivity in the forepaw that is typically seen following this injury (Fig 1A). Paw withdrawal thresholds for blank-SAP injury were significantly lower than those for SSP-SAP injury as early as 1 day ($P < .0001$), and this was sustained until day 7 ($P < .0001$) (Fig 1A). Likewise, the response thresholds for blank-SAP injury were significantly lower than those of SSP-SAP sham for all postsurgical time points ($P < .0001$) (Fig 1A). There was no difference in response between the SSP-SAP injury and SSP-SAP sham groups on any day and no change from baseline values.

Despite exhibiting different behavioral responses, the joint injury severity for each injury group was not different (Fig 1B). The mean vertebral distraction was .47 ± .16 mm in the SSP-SAP injury group and .45 ± .18 mm in the blank-SAP injury group. In the SSP-SAP injury group, the capsular ligament underwent tensile strains of 16.40 ± 12.21% and a peak maximum principal strain of 25.27 ± 11.55%. Capsule strains were similar in the blank-SAP injury group, with a tensile strain of 15.01 ± 6.31% and a peak maximum principal strain of 25.98 ± 14.72% (Fig 1B).

NK1R expression in the C6 dorsal horn was reduced following administration of SSP-SAP (Fig 2). There were no differences between NK1R expression in the SSP-SAP injury and SSP-SAP sham groups (Fig 2). Injection of SSP-SAP significantly reduced ($P < .028$) spinal NK1R expression compared to expression after injection of the blank-SAP (Fig 2).

The severity of the applied joint injury and the behavioral responses produced in the rats used in the electrophysiology study were the same as those used for the behavioral and immunohistochemical assessments. The applied vertebral distractions were .51 ± .13 mm and .52 ± .15 mm for the SSP-SAP injury and blank-SAP injury groups, respectively, with corresponding tensile strains of 18.52 ± 12.19% and 22.70 ± 8.87% and maximum principal strains of 27.22 ± 10.65% and 43.41 ± 24.94%. Indeed, there were no differences in any of the biomechanical metrics between the injury groups for any of the studies. Similarly, in the electrophysiological study, the response thresholds for the blank-SAP injury group were significantly lower than the SSP-SAP injury ($P < .003$) and SSP-SAP sham ($P < .001$) groups on both days 1 and 7 (Fig 3A).

A total of 205 neurons were identified at an average depth of 377.42 ± 197.42 μm in the dorsal horn. SSP-SAP attenuated the spinal hyperexcitability that is typically observed on day 7 following injury (Fig 3A). Blank-SAP injury exhibited significantly more spikes in response to the 10-g ($P < .015$) and 26-g ($P < .003$) filaments than either SSP-SAP injury or SSP-SAP sham did, and also compared to the SSP-SAP D0 and blank-SAP D0 groups that received no surgical procedures (Fig 3B and D). Evoked neuronal firing also was increased significantly in response to light brush ($P < .02$) and noxious pinch ($P < .02$) for the blank-SAP injury group compared to all other groups (Fig 3B and D). In addition, the number of neurons classified as WDR neurons was significantly greater for blank-SAP injury compared to SSP-SAP D0 ($P < .006$), SSP-SAP injury ($P < .012$), and SSP-SAP sham ($P < .0002$) (Fig 3C). There also were more WDR neurons identified in the blank-SAP D0 group compared to SSP-SAP sham ($P < .020$) (Fig 3C).

Spinal expression of GFAP and IL1α at day 7 paralleled the behavioral and electrophysiological responses. GFAP expression was significantly increased in blank-SAP injury over both SSP-SAP injury ($P < .001$) and SSP-SAP sham ($P < .001$) (Fig 4). OX-42 reactivity also significantly increased ($P < .020$) in blank-SAP injury compared to SSP-SAP sham (Fig 4). Although SSP-SAP injury exhibited lower OX-42 expression than blank-SAP injury it was not at SSP-SAP sham levels. Expression of IL1α in the

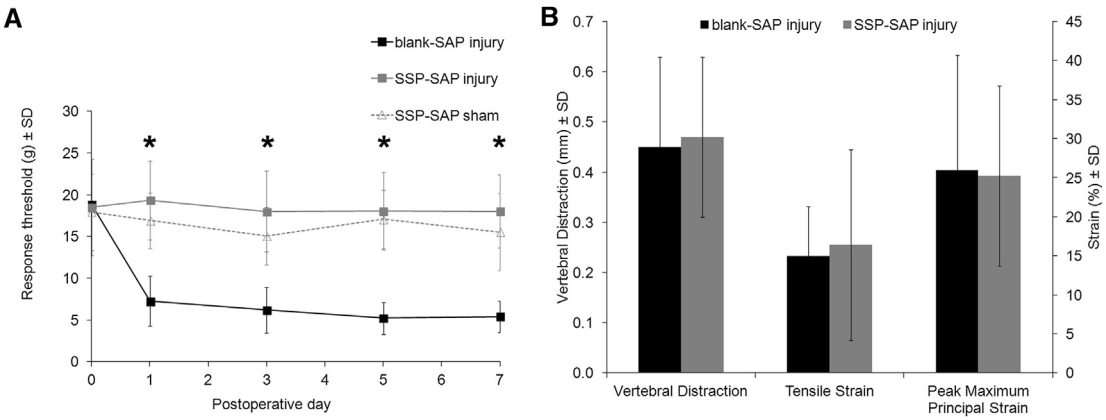

Figure 1. Forepaw withdrawal threshold to mechanical stimulus and injury mechanics. (**A**) Mechanical hyperalgesia was measured on days 0 (baseline), 1, 3, 5, and 7 after surgery. An asterisk (*) indicates significance between blank-SAP injury, SSP-SAP injury, and SSP-SAP sham. (**B**) Measures of vertebral and capsule mechanics were compared to confirm that both injury groups received similar magnitude of injury.

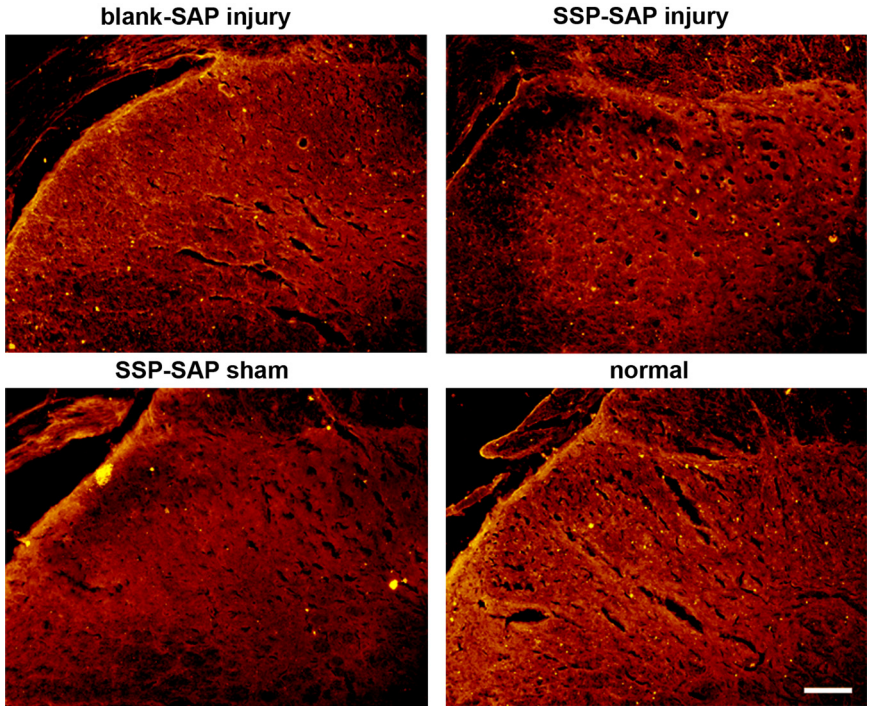

Figure 2. Representative images of NK1R expression in the superficial dorsal horn for each group. Scale (100 μm) applies to all.

superficial laminae of the spinal dorsal horn was significantly lower than blank-SAP injury levels after both an SSP-SAP injury ($P < .020$) and an SSP-SAP sham ($P < .038$) (Fig 4). There were no differences detected between SSP-SAP injury and SSP-SAP sham.

Discussion

This study demonstrates that the spinal NK1 receptor has a pivotal role in the development of pain after a mechanical joint injury. Intrathecal SSP-SAP eliminated spinal NK1-positive cells (Fig 2) and prevented the onset and maintenance of mechanical hyperalgesia (Fig 1), associated with reduced evoked spinal hyperexcitability and prevention of the shift in neuronal phenotype that are evident at day 7 after painful joint injury (Fig 3). Together with the fact that SP is released during C-fiber activation and potentiates *N*-methyl-D-aspartate receptors in the dorsal horn,[15] this study suggests that the SP-NK1R interaction is pivotal for the initiation and/or maintenance of spinal hyperexcitability in joint pain and that removing NK1R-bearing cells prevents the neuronal plasticity and central sensitization that contribute to its persistence. Previous work with this joint pain model shows that joint injury increases neuronal firing as early as 1 day after injury and induces a shift in the neuronal phenotype, with a larger percentage of WDR neurons after painful joint injury.[4,24] When NK1 antagonists are administered in a primate model of peripheral inflammation, spinal hyperexcitability is reduced.[10] Similarly, spinal GFAP, OX-42, and IL1α also decrease after administration of SSP-SAP (Fig 4), which is consistent with findings using NK1 antagonists in models of painful joint inflammation.[6,32] Despite those prior reports, this study is the first to provide evidence that NK1-bearing cells in the spinal cord are integral to the central sensitization and inflammatory cascades that initiate and maintain pain from a transient mechanical joint injury.

Although SP and NK1 are believed to be involved in pain and joint inflammation, their roles in joint-mediated nociception are conflicting. NK1 antagonists reduced mechanical hyperalgesia and joint swelling in an arthritis model[32] but prevented cytokine upregulation without affecting behavioral sensitivity in a model of temporomandibular joint inflammation.[6] The same mechanical joint injury used here also induces sustained upregulation of SP in both the spinal cord and dorsal root ganglion,[20] lending further support to the assertion that widespread SP regulation contributes to the development and maintenance of hyperalgesia in mechanical joint pain. It is possible that the behavioral effects of blocking NK1 depend on the specific mode of joint tissue injury, as well as whether local inflammatory or mechanical factors are involved. Additionally, neurotrophins involved in peptidergic signaling contribute to behavioral sensitivity and modulate spinal activity in this same joint injury.[17] Collectively, the early response of the peptidergic system via NK1R-positive cells may initiate behavioral sensitivity and/or regulate responses to inflammation in the joint.

Ablating NK1R-positive cells reduces spinal hyperexcitability and prevents a shift in neuron phenotype after joint injury (Fig 3). In particular, removing NK1R cells prior to joint injury prevents an increase in evoked firing to noxious/supra-threshold mechanical stimuli and blocks a shift toward WDR neurons similar to the outcomes observed in the SSP-SAP sham group (Fig 3B and C), suggesting that NK1R cells are critical to the development of neuronal sensitivity after a painful joint

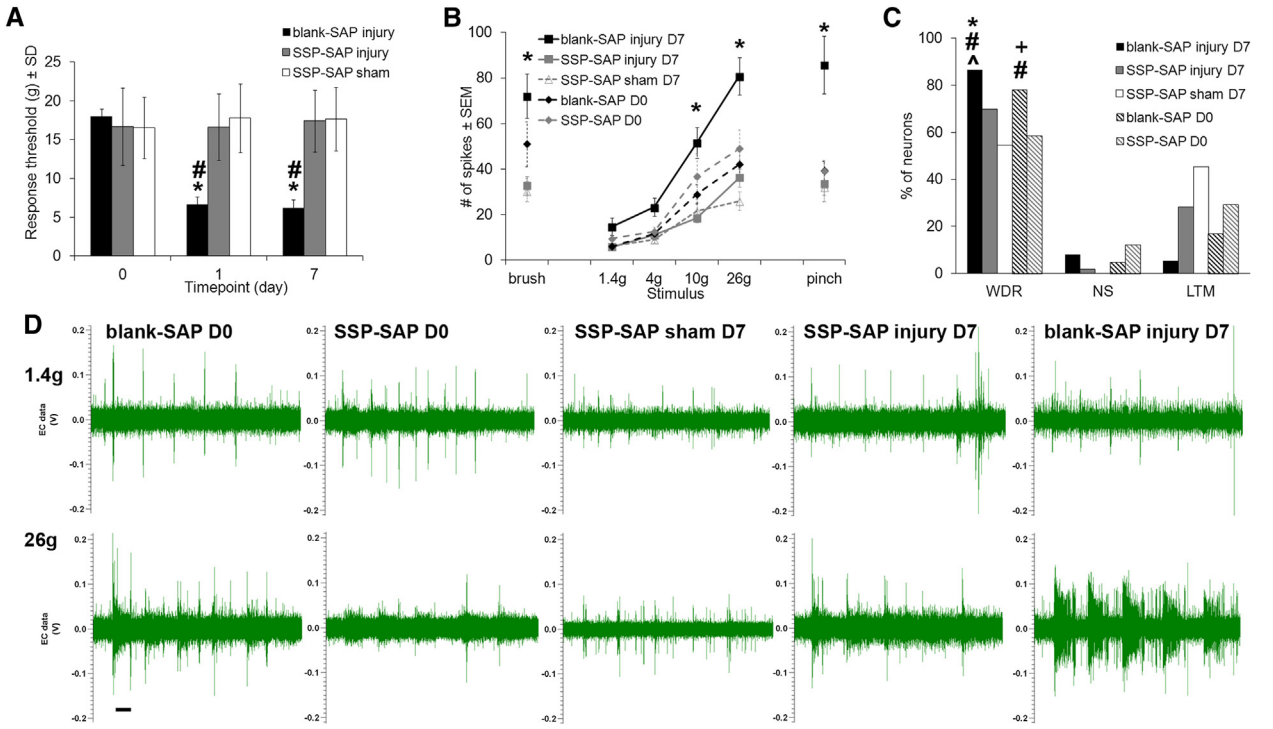

Figure 3. Electrophysiological outcomes. (**A**) Forepaw hyperalgesia was measured on days 0 (baseline), 1, and 7 after surgery. An asterisk (*) indicates significance between blank-SAP injury and SSP-SAP injury and a hash sign (#) indicates significance between blank-SAP injury and SSP-SAP sham. (**B**) The number of spikes evoked by paw stimulation. Significance (*) was detected between blank-SAP injury compared to all other groups for brush, 10-g von Frey, 26-g von Frey, and pinch. (**C**) The proportion of each neuron phenotype for each group. A caret (^) denotes significance between blank-SAP injury and SSP-SAP D0, an asterisk (*) denotes significance between blank-SAP injury and SSP-SAP injury, and a hash sign (#) denotes significance between blank-SAP injury and SSP-SAP sham. The plus sign (+) indicates significance between blank-SAP D0 and SSP-SAP sham. (**D**) Representative extracellular recordings during application of 1.4-g and 26-g filaments, with scale representing 1 second.

injury. These same hallmarks of central sensitization are established in the spinal cord within 6 to 24 hours after joint injury[4] and are still present at day 7,[24] suggesting that abnormal neuronal activity is established *very early* after joint injury and is primarily driven by the NK1R-expressing cells in the spinal cord. Removal of spinal NK1R-positive cells, leading to blocking or reducing central sensitization following joint injury, is consistent with NK1R antagonists reducing sensitization in spinothalamic tract neurons in response to capsaicin.[10] We recently reported that the nonsteroidal anti-inflammatory drug ketorolac attenuates pain and spinal astrocytic activation if given intra-articularly after pain develops,[9] supporting a similar establishment of inflammatory contributions in the joint following its trauma. Interestingly, *spinal* ablation that blocks peptidergic signaling appears to similarly interrupt spinal inflammation (Fig 4). Aδ- and C-fibers become sensitized after joint inflammation and release glutamate and neuropeptides that activate postsynaptic *N*-methyl-D-aspartate receptors, inducing long-term neuroplastic changes contributing to sensitization.[29] Although the interaction of NK1R and glutamate was not studied here, glutamate release may also be altered. This painful injury upregulates the ε-isozyme of protein kinase C in the dorsal root ganglion,[8,35] further supporting the role of an altered glutamatergic system in contributing to hyperalgesia and neuronal excitability.

Ablating spinal NK1R cells has been reported to reduce neuronal excitability to mechanical and thermal stimuli and neuronal windup after peripheral capsaicin.[14] Our findings suggest that these cells also appear to be involved in increasing the number of neurons classified as WDR, which is also evident after painful facet joint injury,[24] because removing them either with an injury (SSP-SAP) or without an injury (SSP-SAP D0) prevents any increase and also maintains the relative distribution of other neuronal phenotypes (Fig 3C). This observation after facet joint injury is different from that of the Khasabov et al study, which showed that ablating spinal NK1R cells reduces the number of nociceptive-specific, high-threshold neurons and increases the proportion of WDR neurons following the administration of intrathecal SP-SAP.[14] This difference in observations between the 2 studies may be due to the fact that Khasabov's group classified neurons prior to administering capsaicin, whereas our studies classified neurons *after* the mechanical joint injury. Additionally, the searching protocols were different. For example, the use of a paw pinch in that study may elicit more nociceptive-specific neurons than the brush stimulus used in this study. In fact, only 11 of the 205 neurons identified here were nociceptive specific (Fig 3C). Others have investigated the effects of SP-SAP *only* in WDR neurons and found reduced evoked responses in those neurons, suggesting that adaptations occur, at

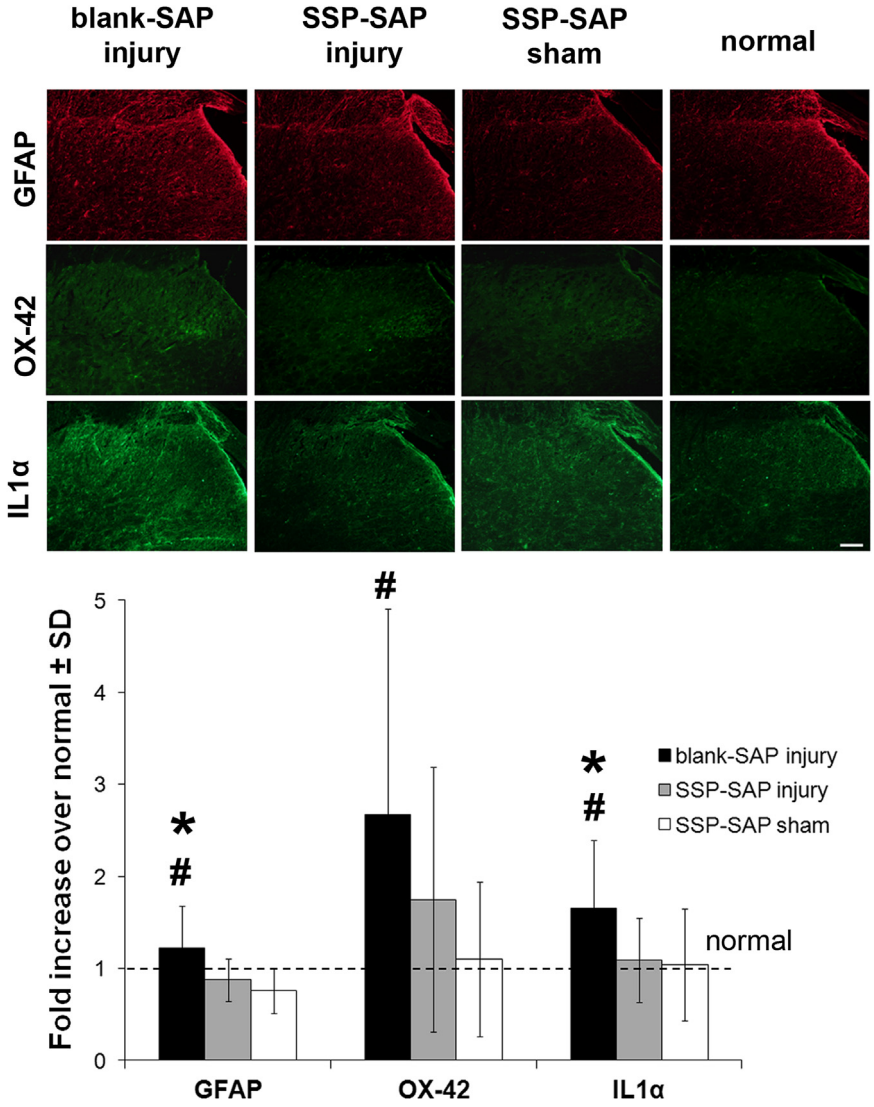

Figure 4. Representative images and quantification of GFAP, OX-42, and IL1α to label for spinal inflammation at day 7. An asterisk (*) indicates significant differences between blank-SAP injury and SSP-SAP injury. Hash sign (#) indicates significance between blank-SAP injury and SSP-SAP sham. Scale (100 μm) applies to all.

least partially, in WDR neurons.[26,30] Of note, uninjected or blank-SAP sham groups were not included in the current study. However, the behavioral responses in the SSP-SAP sham group are not different from previously reported responses for uninjected shams in this model,[4,17,24] suggesting that SSP-SAP alone does not affect behavioral responses. Those studies, together with our current findings, indicate that WDR neurons and altered neuropeptide signaling may be major contributors to the spinal hyperexcitability and central sensitization after painful joint injury.

Astrocytic activity can be dysregulated following inflammation and can activate other inflammatory mediators, such as cytokine release and glial activation.[5,34] Astroglial activation and cytokine release also contribute to neuronal hyperexcitability by changing the chemical environment surrounding neurons,[11,27] which may also contribute to the reduced neuronal firing observed in this study (Fig 3B). In addition to being involved in inflammation and neuronal activity, resident astrocytes, microglia, and macrophages express NK1R and are potential sources of SP release, thereby promoting the production of the proinflammatory cytokines IL1 and tumor necrosis factor α.[22] A study of pain from opioid withdrawal showed that an NK1R antagonist reduced *both* spinal astrocytic and microglial activity while also mitigating hyperalgesia,[31] whereas our findings show that ablating spinal NK1R cells reduces astrocytic activation and IL1α expression but does not reduce microglial activation in the spinal cord (Fig 4). However, spinal microglial activation has been previously reported to be not as robustly modified as astrocytic activation at this time point after injury,[7,18,35] which could explain the differences in microglial activation between these studies. Although there is notable variability in the immunohistochemical data, in particular for OX-42 and IL1α, power analyses confirmed that these sample sizes are adequate. Certainly, additional quantitative assessments of these and other inflammatory responses would strengthen these findings.

This study used SSP-SAP to better understand the involvement of spinal NK1R-expressing cells in producing behavioral hypersensitivity and spinal neuronal excitability and inflammation following painful mechanical facet injury. However, the direct translation to the clinic is limited. Although NK1 antagonists reduce behavioral sensitivity in animal models,[14,32,33,36] they have had little success in clinical pain trials.[22] Moreover, delivering treatment *prior* to an injury is not realistic, and clinical trials using NK1R antagonists show inconsistent results for pain relief.[13,22] Complete anesthetic nerve blocks do alleviate facet pain from injury,[2,3] but such techniques ablate the *entire* innervation of the joint rather than selectively targeting the specific fiber types that are involved in pain.

Nonetheless, ablating NK1R-positive cells by SSP-SAP indicates spinal SP/NK1 signaling as having a major role in the initiation and maintenance of pain from a mechanical joint injury. Targeting these spinal cells also reduced the spinal neuronal hyperexcitability and central sensitization that are normally associated with pain from this joint injury. Further, spinal astrocytic activation and cytokine expression were similarly attenuated. Collectively, these results strongly suggest that pain from mechanical joint injury is produced by the early modulation of SP/NK1 signaling in the spinal cord that initiates neuronal dysfunction and inflammatory cascades that lead to behavioral sensitivity, providing novel information about nociceptive pathways for mechanically induced joint pain.

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