



## Research article

# Ablation of IB4 non-peptidergic afferents in the rat facet joint prevents injury-induced pain and thalamic hyperexcitability via supraspinal glutamate transporters



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## HIGHLIGHTS

- Ablating non-peptidergic afferents prevents hyperalgesia induced by facet injury.
- Hyperexcitability in the VPL with facet pain is regulated by non-peptidergic afferents.
- Thalamic glutamate transporter expression is altered by painful joint injury.
- Non-peptidergic afferents contribute to the development of central sensitization.

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## ABSTRACT

The facet joint is a common source of neck pain, particularly after excessive stretch of its capsular ligament. Peptidergic afferents have been shown to have an important role in the development and maintenance of mechanical hyperalgesia, dysregulated nociceptive signaling, and spinal hyperexcitability that develop after mechanical injury to the facet joint. However, the role of non-peptidergic isolectin-B4 (IB4) cells in mediating joint pain is unknown. Isolectin-B4 saporin (IB4-SAP) was injected into the facet joint to ablate non-peptidergic cells, and the facet joint later underwent a ligament stretch known to induce pain. Behavioral sensitivity, thalamic glutamate transporter expression, and thalamic hyperexcitability were evaluated up to and at day 7. Administering IB4-SAP prior to a painful injury prevented the development of mechanical hyperalgesia that is typically present. Intra-articular IB4-SAP also prevented the upregulation of the glutamate transporters GLT-1 and EAAC1 in the ventral posterolateral nucleus of the thalamus and reduced thalamic neuronal hyperexcitability at day 7. These findings suggest that a painful facet joint induces changes extending to supraspinal structures and that IB4-positive afferents in the facet joint may be critical for the development and maintenance of sensitization in the thalamus after a painful facet joint injury.

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## 1. Introduction

The facet joint is a common source of neck pain [1], and excessive stretch of that joint's capsular ligament beyond its physiologic limit

can produce painful injury [2–5]. The facet capsule is innervated by both peptidergic and non-peptidergic afferents [6–11], which are activated when it undergoes stretch [3,12], initiating nociceptive signaling and spinal neuronal hyperexcitability [5,13–15]. The involvement of peptidergic neurons in facet joint-mediated pain has been previously been investigated [13,15]. Approximately one third of peptidergic neurons are also positive for isolectin-B4 (IB4) [16–18]. IB4-positive cells have been shown to contribute to inflammatory and neuropathic pain, and their ablation prevents the development of behavioral sensitivity in models of musculoskeletal pain, spinal nerve ligation, and cancer pain [19–21]. Despite the focus on peptidergic afferents in pain, the relationship between

**Abbreviations:** GLT-1, glial glutamate transporter 1; EAAC1, excitatory amino acid carrier 1; IB4, isolectin, B4; IB4-SAP, isolectin-B4 conjugated saporin; VPL, ventral posterolateral nucleus; PAG, periaqueductal gray; ACC, anterior cingulate cortex; S1, primary somatosensory cortex.

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non-peptidergic cells that bind IB4 in the facet joint and its painful injury is unknown.

Dysregulation of the glutamate system is one of the hallmarks of central sensitization in the central nervous system. Glutamate receptors and transporters are highly regulated throughout the spinal cord and thalamus in arthritis [26] and joint pain [23,27,28]. Specifically, the glutamate transporter (GLT-1) and excitatory amino acid carrier (EAAC1) are both downregulated in the spinal cord after painful facet joint injury [23,27,28]. The glutamate system contributes to spinal neuronal hyperexcitability [28,29]. Nociceptive information in the spinal cord is relayed to the thalamus before transmission to other brain structures [30], and altered thalamic neuronal firing has been detected in studies of inflammatory and neuropathic pain [31–33]. Further, many aspects of central sensitization and neuronal hyperexcitability are evident in the spinal cord early and later after painful facet joint injury [5,15,28,34,35]. However, despite documentation of a host of robust peripheral [14,36–39] and spinal changes that may contribute to pain, the supraspinal changes have not yet been evaluated for pain from the facet or traumatic injury to other joints.

Since IB4-positive neurons may play a role in pain, removing the non-peptidergic afferents that innervate the cervical facet joint is hypothesized to modify aspects of the brain's responses that are involved in the central sensitization that occurs with facet pain. As such, we first evaluated if behavioral sensitivity after a mechanical facet joint injury is modified after targeted chemical ablation of IB4-positive cells with a saporin agent. Based on those findings, a follow up study assessed neuronal hyperexcitability in the thalamus using electrophysiological recordings to examine if ablating IB4-positive cells in the facet joint prior to its injury alters evoked neuronal responses in the thalamus at day 7 after injury. Similarly, GLT-1 and EAAC1 were also quantified by immunohistochemistry in the ventral posterolateral nucleus (VPL) of the thalamus at that same time after injury in groups with and without non-peptidergic joint afferents present.

## 2. Material and methods

### 2.1. Experimental design

Collectively studies evaluated the effects of ablating non-peptidergic neurons on behavioral outcomes, thalamic neuronal hyperexcitability, and glutamatergic responses in the VPL in an established rat model of painful facet joint distraction [13,14,40,41]. All studies used male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, IN) weighing 380–430 g and housed under USDA- and AAALAC-compliant conditions, with a 12–12 h light-dark cycle and free access to food and water. Experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and carried out under the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain [42].

In the first study, behavioral sensitivity was characterized following the ablation of non-peptidergic neurons in the C6/C7 bilateral cervical facet joints prior to imposing a painful facet joint distraction at that same joint level. Rats received a bilateral intra-articular injection in the C6/C7 facet joints of either IB4-saporin (IB4-SAP; n = 16) or vehicle saporin (n = 11) using previously reported methods [15,28,43]. Fourteen days later, rats underwent either a painful facet joint distraction or sham surgical procedure (n = 11 vehicle injury; n = 12 IB4-SAP injury; n = 4 IB4-SAP sham). Forepaw mechanical hyperalgesia was measured at baseline (day 0) before surgery and on days 1, 3, 5, and 7 after surgery.

Neuronal hyperexcitability was measured in the VPL of the thalamus on day 7 after painful facet joint injury (n = 4) or sham (n = 3) surgery, without any joint injection, to define thalamic neural firing in response to the injury or sham surgery alone. Building on the behavioral findings from the first study, thalamic neuronal firing also was measured on day 7 in separate groups of rats receiving IB4-SAP and either injury (n = 6; IB4-SAP injury) or sham (n = 5; IB4-SAP sham) procedures. Brain tissue was harvested from subsets of the rats from the behavioral and electrophysiology studies at day 7 (n = 9 vehicle injury; n = 12 IB4-SAP injury; n = 4 IB4-SAP sham, n = 3 sham) to evaluate changes in glutamate transporter expression in the thalamus using immunohistochemistry.

### 2.2. Intra-articular injections

Neurons that bind IB4 are typically non-peptidergic [44,45] and are involved in pain from nerve injury and inflammation [19,46]. In order to identify the contribution, if any, of that population of sensory neurons in the joint for facet stretch-induced pain, rats received intra-articular injections of 5 µg of saporin conjugated to isolectin-B4 (IB4-SAP; Advanced Targeting Systems; San Diego, CA; n = 27) in 10 µL of PBS to ablate the IB4-binding neurons. That 5 µg dose of IB4-SAP was selected from a previously published report as sufficient to ablate non-peptidergic afferents [19] and is the same as the dose used in a previous study of facet joint-mediated pain [15]. Separate rats received unconjugated saporin (vehicle; Advanced Targeting Systems; San Diego, CA, n = 18) to serve as controls. Either IB4-SAP or vehicle saporin were administered using established methods for intra-articular injection [15,28,43].

All surgical procedures were performed under inhalation isoflurane anesthesia (4% for induction, 2.5% for maintenance). Briefly, the bilateral C6/C7 facet joints were exposed. A 33-gauge beveled needle attached to a 10 µL syringe (Hamilton Company; Reno, NV) was advanced manually into the facet joint using a dorsal approach, and 5 µg of IB4-SAP or vehicle saporin in 10 µL of PBS was slowly injected into the facet joint. Following joint injections, wounds were closed with polyester suture and surgical staples, and rats were recovered in room air.

### 2.3. Surgical procedures for facet joint distraction

Because saporin induces cell death within 14 days [13,15,47], the facet joint injury or sham procedure was performed 14 days after the joint injection. Under inhalation isoflurane anesthesia, rats underwent either a facet joint distraction or sham procedure as previously described [13,14,28,40,41]. Briefly, the C6 and C7 laminae were exposed and attached to a loading device via microforceps; the C6 vertebra was displaced 0.7 mm rostrally while the C7 vertebra was held in place. A camera mounted to a surgical scope tracked markers on the C6 and C7 laminae and the capsular ligament during injury to measure the severity of the injury. The relative displacement of the C6 and C7 vertebrae, corresponding capsular ligament distraction and tensile strain across the capsule during distraction were quantified. Displacement and strain values are reported as the mean ± standard deviation and were compared between the IB4-SAP and vehicle injury groups using a Student's *t*-test to compare the injury severity between injury groups.

An additional group of rats underwent the same sham surgical procedures with device attachment only but no joint distraction. Injury or sham only rats in the electrophysiology study underwent the same surgical procedures described but did not have prior joint injection. Following all surgeries, incisions were closed and rats were recovered in room air.

## 2.4. Behavioral assessment of mechanical hyperalgesia

Bilateral forepaw mechanical hyperalgesia was evaluated in all rats using a modified version of Chaplan's thresholding method [5,22,48,49]. Forepaw withdrawal thresholds were measured for each rat at baseline (day 0) before surgery and on days 1, 3, 5 and 7 following facet joint distraction or sham surgery using a series of nine von Frey filaments with increasing strengths (0.4 g, 0.6 g, 1.4 g, 2 g, 4 g, 6 g, 8 g, 15 g, 26.0 g) (Stoelting Co.; Wood Dale, IL). To measure mechanical hyperalgesia, a filament was recorded as the response threshold if the next higher filament also induced a positive response [14,48]. Each testing session consisted of 5 stimulations to each paw over 3 rounds of stimulation, and the average threshold was recorded for each paw. Responses between left and right forepaws were compared using a paired *t*-test and combined to obtain a single average for each rat on each day. A repeated measures ANOVA was used to detect any differences between groups in the overall hyperalgesia; differences on each day were detected using a one-way ANOVA with Bonferroni correction between groups. All threshold data are reported as the mean  $\pm$  standard deviation.

## 2.5. Electrophysiological recordings in the thalamus

In order to quantify thalamic neuronal excitability, extracellular electrophysiological recordings were acquired in the VPL on day 7 after surgery [32]. Rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.). A midline incision was made over the skull, and the soft tissue was resected to reveal the coronal, sagittal, and lambdoid cranial sutures. In order to gain access to the VPL, the cortex overlying the thalamus was exposed via an 8 mm by 8 mm craniotomy over the left hemisphere beginning at bregma and extending caudally and laterally [32]. The mid-cervical trachea was exposed, cannulated, and ventilated (CWE Inc.; Ardmore, PA) with room air at approximately 40 breaths/minute, and the end tidal CO<sub>2</sub> concentration was monitored. The rat was mounted onto a stereotaxic frame with blunt ear bars, and the head was adjusted so bregma and lambda were in the horizontal plane. The dura was resected, and the brain was bathed in 37 °C mineral oil. The core temperature of the rat was maintained at 35–37 °C using a feedback-controlled heating plate with a rectal probe (Physitemp Instruments Inc.; Clifton, NJ). Anesthesia was maintained throughout all procedures with supplemental doses of sodium pentobarbital (5–10 mg/kg, i.p.) as needed based on respiration and toe pinch reflexes.

Extracellular voltage potentials were recorded in the VPL using a glass-insulated tungsten electrode (FHC; Bowdoin, ME). Signals were processed with a 60 Hz noise eliminator (HumBug, Quest Scientific; North Vancouver, Canada) and digitally sampled and stored at 25 kHz (Micro1401, CED; Cambridge, England). Beginning at –2.5 mm from bregma and 2.2 mm left lateral, the electrode was lowered 5–7 mm below the pial surface. Subsequent locations were probed at 0.2 mm intervals in the anterior-posterior and medial-lateral planes, based on known coordinates and somatotopy of the rat VPL [50,51]. Neurons were identified by light brushing of the plantar surface of the right forepaw using a cotton swab. A stimulus train was applied to the forepaw consisting of ten light brushes, a subset of non-noxious and noxious von Frey filaments (1.4 g, 4 g, 10 g, 26 g), and a 10 s noxious pinch using a 60 g vascular clip (WPI; Sarasota, FL) [14,32,52]. Each filament was applied for five consecutive 1-s stimulations. The different stimuli were applied at intervals of at least 60 s, and the number of evoked spikes was summed for each stimulus.

To analyze individual neurons, recordings during the stimulus train were spike-sorted using Spike2 (CED; Cambridge, England). Evoked spikes were summed over the continuous 10-s stimulus

period for both the brush and pinch stimuli. The number of spikes evoked from the initial application of a von Frey filament until 1-s after the 5th application of the filament were counted for each neuron [5,14,32]. The duration of each stimulus was identified, and baseline firing was determined by counting the number of spikes over an equivalent time period (10 s) prior to each stimulus. Baseline spikes were subtracted from the total spike count for each stimulus in order to evaluate the evoked responses. Because the distribution of spike totals for each stimulus exhibited a positive skew, spike counts were log-transformed to obtain a normal distribution for statistical analyses. Evoked firing for each stimulus was compared between groups using a mixed-effect nested ANOVA with post hoc Tukey's HSD test, since there was equal variance in the data from the different groups. As is conventional with reporting spike counts, data are presented as the mean  $\pm$  standard error of the mean.

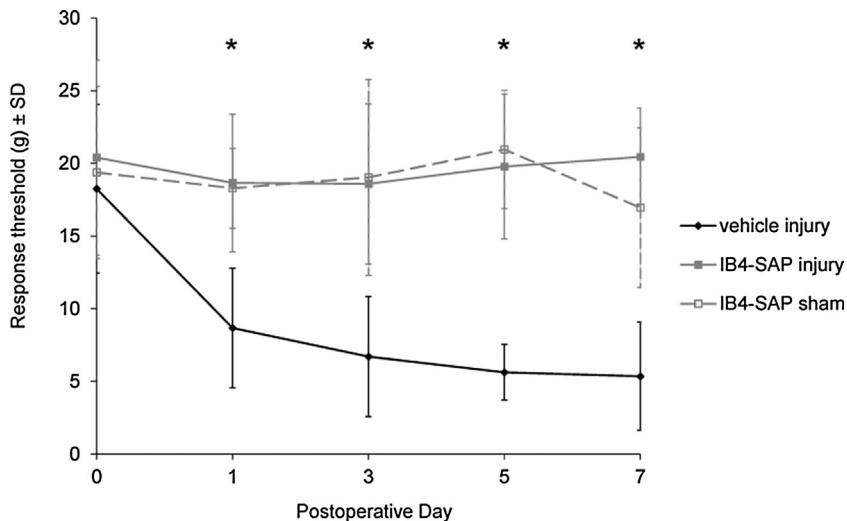
## 2.6. Immunohistochemistry of VPL tissue

Brains were harvested at day 7 to measure expression of the glutamate transporters, EAAC1 and GLT-1, in the VPL (vehicle injury n=9, sham n=3, IB4-SAP injury n=12, IB4-SAP sham n=4). Brains from age-matched un-operated normal rats (n=2) were also included as controls. Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg) and transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde. Samples were post-fixed in 4% paraformaldehyde overnight and stored in 30% sucrose for 6 days at 4 °C. Whole brains were dissected coronally to include only the brain region (–2.28 to –2.64 mm from bregma) containing the VPL. Samples were embedded, frozen, sectioned (16 µm) and mounted onto slides. Sections were blocked for two hours at room temperature with 10% normal goat serum (Vector Labs; Burlingame, CA) and 0.3% Triton-X and incubated overnight at 4 °C with primary antibodies for rabbit anti-GLT-1 (1:500; Abcam; Cambridge, MA) or rabbit anti-EAAC1 (1:500; Alpha Diagnostic; San Antonio, TX). Slides were washed with PBS, labeled with goat anti-rabbit Alexa-Fluor 568 secondary antibody (1:1000; Life Technologies; Carlsbad, CA) and cover-slipped using Fluorogel medium (EMS; Hatfield, PA). VPL sections were imaged at 200x, and the percent of GLT-1-positive and EAAC1-positive pixels within each image was quantified. The percent positive pixels in each group (reported as the mean  $\pm$  standard deviation) was normalized to normal values and compared via ANOVA with post-hoc Tukey's HSD test, since there was equal variance in the data from the different groups.

## 3. Results

The biomechanical loading to the joint and facet capsule were the same for both groups of rats undergoing facet joint distraction (IB4-SAP and vehicle) when considering the metrics that capture the injury severity. The mean vertebral distractions ( $\pm$ standard deviation) for the IB4-SAP injury and vehicle injury groups were  $0.39 \pm 0.15$  mm and  $0.45 \pm 0.17$  mm, respectively. The mean capsule distraction ( $\pm$ standard deviation) was  $0.22 \pm 0.09$  mm for the IB4-SAP injury group and was  $0.19 \pm 0.08$  mm for the vehicle injury group. Maximum tensile strains (mean  $\pm$  standard deviation) were  $17.34 \pm 11.43\%$  and  $13.57 \pm 10.19\%$  for the IB4-SAP and vehicle injury groups, respectively. There is no difference in any of the mechanical metrics between the injury groups.

Injection of IB4-SAP prevented the development of forepaw mechanical hyperalgesia that is typically induced after painful facet joint injury. Overall, the response thresholds for the injury group with a vehicle injection were lower than the thresholds for both the IB4-SAP injury ( $p < 0.00003$ ; ANOVA main effect) and IB4-SAP sham ( $p < 0.00003$ ; ANOVA main effect) groups (Fig. 1). In fact, the



**Fig. 1.** Forepaw mechanical hyperalgesia in response to von Frey filament stimulation. Injury with vehicle injection (vehicle injury) exhibits significantly reduced response thresholds starting at day 1 and persisting until day 7 compared to both IB4-SAP injury and IB4-SAP sham (\* $p < 0.00003$ ). IB4-SAP injury is not different from IB4-SAP sham on any day. Data are reported as the mean  $\pm$  standard deviation.

thresholds for the IB4-SAP and IB4-sham groups were not different from their baseline pre-injury levels (Fig. 1). As early as 1 day after surgery, the response thresholds in the vehicle injury group were lower than both IB4-SAP injury ( $p < 0.00001$ ; post hoc) and IB4-SAP sham ( $p < 0.023$ ; post hoc) (Fig. 1). This difference persisted until day 7 when response thresholds for IB4-SAP injury ( $p < 0.00001$ ; post hoc) and IB4-SAP sham ( $p < 0.0001$ ; post hoc) remained significantly greater than the thresholds for the rats undergoing injury with vehicle injection (Fig. 1). The responses of the IB4-SAP injury group were not different from those receiving the IB4-SAP with sham surgery for any day (Fig. 1).

At day 7, facet joint injury induced hyperexcitability in thalamic neurons, which was attenuated by ablating non-peptidergic afferents in the facet joint (Fig. 2). A total of 135 neurons were recorded in the VPL at an average depth ( $\pm$ standard deviation) of  $6.33 \pm 0.39$  mm,  $2.65 \pm 0.69$  mm lateral, and  $-3.27 \pm 0.29$  mm caudal from bregma. The total number of evoked spikes was significantly increased following facet joint injury for paw stimulation with all von Frey magnitudes compared to the number of spikes evoked after the IB4-SAP injury ( $p < 0.0054$ ; post hoc), IB4-SAP sham ( $p < 0.0037$ ; post hoc), and sham ( $p < 0.0170$ ; post hoc) procedures (Fig. 2A). Evoked neuronal firing also increased after facet joint injury compared to all other groups for stimulation by both the brush ( $p < 0.0020$ ; post hoc) and the pinch ( $p < 0.0097$ ; post hoc) (Fig. 2B).

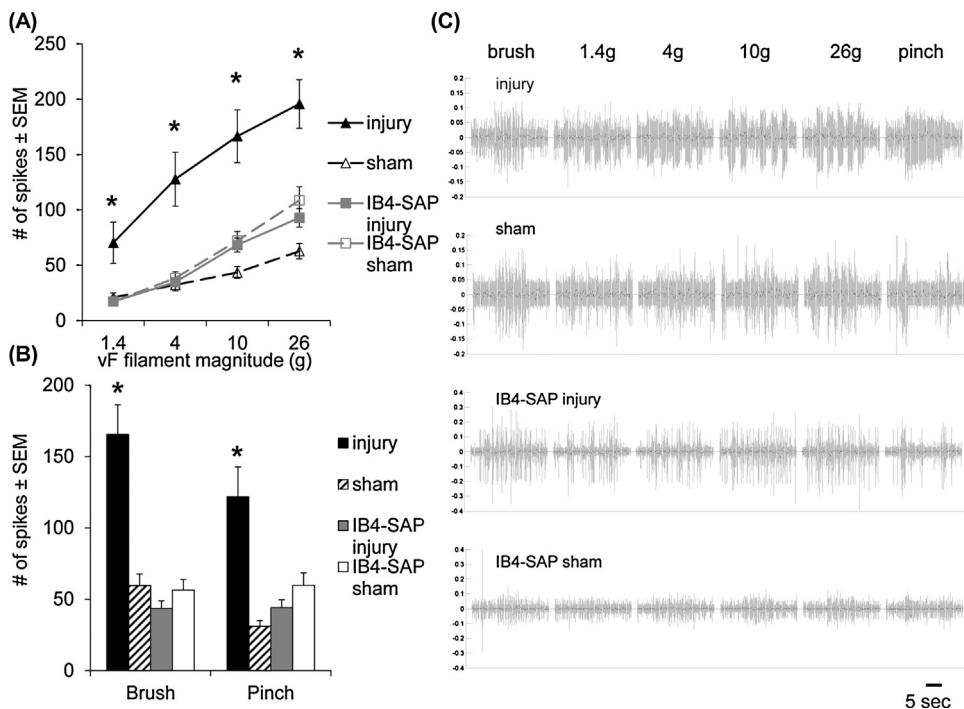
Ablating IB4-positive afferents in the C6/C7 facet prevented the increased expression of GLT-1 and EAAC1 that is induced in the brain at day 7 after injury. Thalamic expression of the glutamate transporters, GLT-1 and EAAC1, at day 7 followed the behavioral response patterns (Figs. 1 and 3). After injury, GLT-1 expression in the VPL increased over sham ( $p < 0.0198$ ; post hoc), IB4-SAP sham ( $p < 0.0001$ ; post hoc), and normal ( $p < 0.0001$ ; post hoc) levels (Fig. 3B). GLT-1 reactivity in the vehicle injury group also was elevated over that observed in the IB4-SAP injury group ( $p < 0.0072$ ; post hoc) (Fig. 3B). Likewise, EAAC1 expression in the vehicle injury group also was significantly increased over all of the groups: sham ( $p < 0.0001$ ; post hoc), IB4-SAP injury ( $p < 0.0001$ ; post hoc), and IB4-SAP sham ( $p < 0.0006$ ; post hoc) and normal ( $p < 0.0001$ ; post hoc) (Fig. 3C). There were no differences between the IB4-SAP injury and IB4-SAP sham groups in the expression of either glutamate transporter in the VPL.

#### 4. Discussion

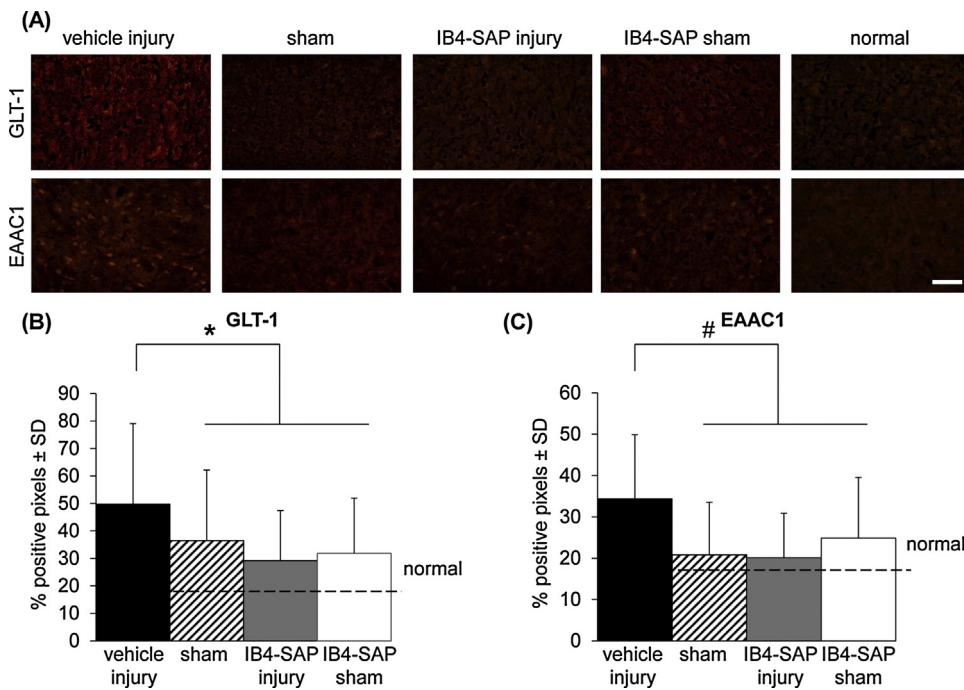
The non-peptidergic, IB4-positive neurons that innervate the cervical facet joint are critical for the establishment of pain and central sensitization after a mechanical joint injury. Eliminating these cells using the IB4-conjugated saporin prevents the development of the mechanical hyperalgesia that is typically present after facet joint injury (Fig. 1) and alters supraspinal neuronal and glutamatergic responses (Figs. 2 and 3). Together, these behavioral and thalamic data strongly suggest that input from non-peptidergic cells in the facet joint is requisite for initiating pain from its injury.

Glutamate is a major neurotransmitter in the central nervous system, and contributes to nociceptor sensitization, which has been shown to be an important regulator for maintaining behavioral hypersensitivity after a painful joint injury. In particular, the metabotropic glutamate receptor 5 (mGluR5) increases in models of peripheral joint pain [22,23,53]. Accordingly, the current findings suggest that removing IB4-positive joint afferents prior to a painful injury prevents any modification to glutamate transporter expression, and so, disrupts central sensitization in the thalamus (Figs. 2 and 3). Both GLT-1 and EAAC1 are *upregulated* in the VPL (Fig. 3). In contrast, *spinal* GLT-1 decreases in models of chronic pain [28,54,55] and glutamate levels in the VPL increase [56–58]. The elevated expression of glutamate transporters in the thalamus (Fig. 3) is consistent with an increase in glutamate in the brain. These results are also consistent with the increased hyperexcitability in the brain that is only evident after a painful facet joint injury (Fig. 2) since prolonged exposure to glutamate is correlated with increased neuronal hyperexcitability [59,60].

Although IB4-positive cells are critical components in the pain response, there is conflicting evidence for their mechanistic regulatory effects. Many studies report that ablating IB4-positive cells reduces pain [19,20,61,62]; but others assert that removing those cells can increase hyperalgesia, suggesting they may contribute to anti-nociceptive cascades [63,64]. Since ablating those afferents prevents the pain that is typically observed after the capsular ligament stretch used in this study (Fig. 1) the notion that IB4-positive cells have a role in nociception is supported. It is possible that the different behavioral findings reported in the literature after IB4-SAP administration could be attributed to differences in the testing methodology or the model used. In a study of formalin injection in



**Fig. 2.** Neuronal hyperexcitability in the VPL of the thalamus at day 7. **(A)** Facet joint injury significantly increases the number of spikes evoked in the VPL in response to forepaw stimulation by 1.4, 4, 10, and 26 g von Frey filaments, compared to all other groups (\* $p < 0.017$ ). **(B)** Neuronal hyperexcitability also increases after facet joint injury compared to all other groups (\* $p < 0.0097$ ) in response to both the brush and pinch stimuli. **(C)** Representative extracellular recordings during stimulation for each group; the scale bar represents 5 s. Data in **(A)** and **(B)** are reported as the mean  $\pm$  standard error of the mean.



**Fig. 3.** Representative images and quantification of GLT-1 and EAAC1 expression in the VPL of the thalamus at day 7. **(A)** Representative images of GLT-1 and EAAC1 expression in the VPL. The scale bar is 100  $\mu$ m and applies to all panels. **(B)** Thalamic expression of GLT-1 is significantly increased (\* $p < 0.0198$ ) after injury compared to all of the other groups: sham, IB4-SAP injury, and IB4-SAP sham and normal tissue. **(C)** Similarly, labeling of EAAC1 is elevated (# $p < 0.0006$ ) following vehicle injury compared to all other groups. Data in **(B)** and **(C)** are reported as the mean  $\pm$  standard deviation.

the lip after ablating IB4-positive neurons in the trigeminal nerve, behavioral sensitivity was reported to increase [63]; in that work, face rubbing behavior was measured, which may capture different aspects of the pain experience. Moreover, in those studies showing increased sensitivity after ablation, nerves were injured either by

chemical stimuli or underwent sustained constriction [63,64], both of which are largely neuropathic injuries. The joint pain model used here has been shown to have both neuropathic and inflammatory components [28,43,65].

Similar to the behavioral outcomes, removing non-peptidergic facet joint afferents prevents neuronal changes in the VPL that normally accompany facet joint pain (Fig. 2). Although spinal neuronal hyperexcitability is reported within 6–24 h after this painful facet joint injury [14] and persists until day 7 [5,13], the current data are the first to show that a mechanical joint injury is sufficient to produce *supraspinal* hyperexcitability that is sustained (Fig. 2). Increased activation in the thalamus has been reported in arthritis and joint inflammation in both humans and animals [66–68]; patients with temporomandibular joint pain show functional and structural changes in the thalamus and somatosensory cortex (S1) [69]. Further, although deep brain stimulation in the thalamus offers relief in rats experiencing neuropathic pain [33], the mechanisms by which this is achieved are still unclear, and this technique has yet to be tested in joint pain. However, other brain regions, including the periaqueductal gray (PAG), anterior cingulate cortex (ACC), and primary somatosensory cortex (S1), are also implicated in joint pain [70–72], and a lesion to S1 alleviates neuronal hyperexcitability associated with inflammatory pain [73,74]. Examining if, and how, these other regions are affected by increased thalamic hyperexcitability from facet joint injury could provide further insight to pain perception and higher order sensory processing.

This study used IB4-SAP to identify the role of non-peptidergic joint afferents in mediating facet pain and supraspinal modifications. Although ablating non-peptidergic neurons in the cervical facet joint induced measurable changes in the thalamus, it is possible that other off-target changes occur in other brain regions or elsewhere in the peripheral and central nervous systems. Further work is necessary to evaluate other potential changes both local to, and remote from, the facet joint. Nevertheless, these findings suggest that non-peptidergic afferents innervating the facet joint are important for the development of sustained pain from mechanical joint injury. Ablating those cells using IB4-SAP in the facet joint prior to its painful injury prevented both neuronal hyperexcitability and glutamate transporter dysregulation in the thalamus. These findings indicate that non-peptidergic cells have widespread effects on joint pain and contribute to central sensitization. A better understanding of nociceptive pathways may help to develop more effective therapies for patients experiencing joint pain.

## Disclosures

CL Weisshaar performed joint injections and surgeries, measured and analyzed mechanical hyperalgesia, and wrote the manuscript. JV Kras performed some of the surgeries and also analyzed some of the electrophysiology studies. PS Pall performed the electrophysiology studies and analyzed some of the electrophysiology data. S Kartha labeled and analyzed all of the brain tissue samples. BA Winkelstein obtained funding, designed the study, analyzed and interpreted the data, and wrote and edited the manuscript. All authors have approved the final submission.

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