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A Nociceptive Role for Integrin Signaling in Pain After Mechanical Injury to the Spinal Facet Capsular Ligament

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Abstract—Integrins modulate chemically-induced nociception in a variety of inflammatory and neuropathic pain models. Yet, the role of integrins in mechanically-induced pain remains undefined, despite its well-known involvement in cell adhesion and mechanotransduction. Excessive spinal facet capsular ligament stretch is a common injury that induces morphological and functional changes in its innervating afferent neurons and can lead to pain. However, the local mechanisms underlying the translation from tissue deformation to pain signaling are unclear, impeding effective treatment. Therefore, the involvement of the integrin subunit β1 in pain signaling from facet injury was investigated in complementary *in vivo* and *in vitro* studies. An anatomical study in the rat identified expression of the integrin subunit β1 in dorsal root ganglion (DRG) neurons innervating the facet, with greater expression in peptidergic than non-peptidergic DRG neurons. Painful facet capsule stretch in the rat upregulated the integrin subunit β1 in small- and medium-diameter DRG neurons at day 7. Inhibiting the $\alpha 2\beta 1$ integrin in a DRG-collagen culture prior to its stretch injury prevented strain-induced increases in axonal substance P (SP) in a dose-dependent manner. Together, these findings suggest that integrin subunit β1-dependent pathways may contribute to SP-mediated pain from mechanical injury of the facet capsular ligament.

Keywords—Facet joint, Tissue strain, Nociceptors, Integrin subunit β1, Substance P.

INTRODUCTION

Ligaments have been increasingly recognized as pain sensors due to their innervation.[27,50,56,64] The facet capsular ligament enclosing the bilateral spinal facet joints that provide articulation between adjacent vertebrae in the spine is innervated by both mechanoreceptors and pain-detecting nociceptors.[27,35,47] In particular, biomechanical, animal and clinical studies have identified the cervical facet capsular ligament as a source of pain from neck trauma,[5,36,49,63] owing to its susceptibility to injury during its excessive stretch.[23,49] Although supraphysiologic deformation of the facet capsule induces pain,[6,16,37] the local cellular and molecular mechanisms that translate macroscopic tissue strains to nociceptive signals in afferents are still unknown, hampering the development of effective treatments for facet joint-mediated pain.

Painful stretch of the cervical facet capsular ligament is a complex injury involving direct mechanical insults as well as secondary inflammatory cascades. Increased nerve growth factor (NGF) in the facet joint and upregulation of the prostaglandin E_2 (PGE_2) receptor in neurons in the dorsal root ganglion (DRG) accompany behavioral hypersensitivity induced by painful cervical facet joint trauma in the rat.[32,34] Both NGF and PGE_2 are known to mediate inflammatory pain and can induce behavioral sensitivity when injected intradermally.[15,46] Functionally blocking or knocking down certain integrin subunits, such as the integrin subunit β1, has been shown to prevent the pain induced by NGF or PGE_2[15,46] (Fig. 1). Activation of neuronal β1 integrins in inflammatory pain may occur *via* direct regulation by NGF,[46,58,68] or interactions with the activated PGE_2 or NGF receptors through intracellular signaling cascades[12,17,46] (Fig. 1).

Integrins are transmembrane receptors that mediate cell adhesion to the extracellular matrix (ECM) and regulate bidirectional signaling and force balance between a cell and the surrounding ECM (Fig. 1).[10,20] Various α and β subunits comprising integrins are ex-

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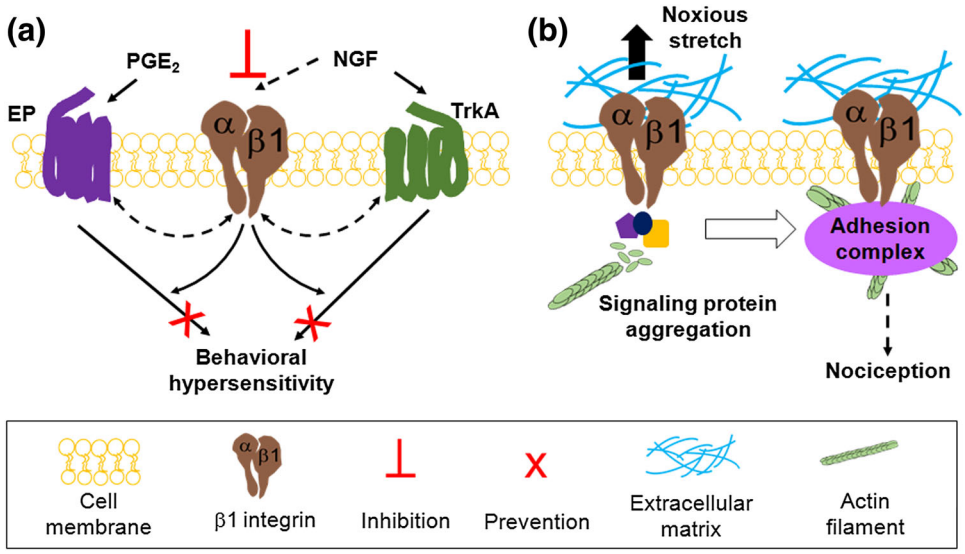

FIGURE 1. Schematic of partial pathways of the β1 integrin involvement in nociception from inflammation or tissue loading. (a) Inhibition of the β1 integrin in sensory neurons can prevent PGE_2 and NGF induced behavioral hypersensitivity (solid arrows), possibly by direct NGF stimulation or *via* interaction with the intracellular signaling cascades mediated by activated the PGE_2 receptor EP or NGF receptor TrkA (dotted arrows). (b) Noxious mechanical stimuli, like a stretch, applied to the ECM may trigger β1 integrin-mediated aggregation of signaling proteins in the sensory neurons embedded in the ECM, which is thought to modulate nociception (dotted arrow).

pressed in a wide range of cells, including primary sensory neurons.[20,60] Integrins, particularly those that contain the subunit β1, play a role not only in nociceptor sensitization by inflammatory mediators,[13,15,46] but also in modulating neuronal excitability and injury after mechanical insults to the surrounding ECM.[19,29] For instance, blocking the $\alpha 2\beta 1$ integrin expressed on peripheral nerve endings has been shown to reduce the reactivity of cutaneous mechanoreceptors to skin stretch.[29,30] Further, recent data suggests that integrin subunit β1-mediated cell-ECM adhesion affects neuronal processing of noxious stretch (nociception)[66] (Fig. 1). Since integrins can be mechanically and chemically coupled to the ECM and the cell cytoskeleton,[10,20] they may be important for the translation of tissue deformation to neuronal loading and intracellular signaling in the facet capsule.

We tested the hypothesis that integrin-dependent pathways, especially those involving the subunit β1, contribute to nociception from facet capsule injury by performing complementary *in vivo* and *in vitro* studies. First, constitutive expression of the integrin subunit β1 in naïve normal rats was confirmed and assessed in the different sub-populations of DRG neurons, including the peptidergic and non-peptidergic neurons involved in the transmission of pain signals[2,8,33] and in the afferents that innervate the facet joint. Expression of the integrin subunit β1 was also assessed in the DRG 7 days after facet capsule stretch when pain is still present, in order to evaluate the association between regulation of integrins and mechanically-induced pain *in vivo*. The integrin subunit β1 can form heterodimers with several different α subunits and lead to varied responses to mechanical and chemical cues.[20] Since the $\alpha 2\beta 1$ integrin is the primary receptor for Type I collagen, which is a major component of the facet capsular ligament,[23–25] effects of inhibiting activation of the $\alpha 2\beta 1$ integrin on mechanically-induced nociceptive signaling in neurons were evaluated. To do this, a DRG-collagen *in vitro* model mimicking the innervated facet capsular ligament was used to investigate if, and how, tissue matrix deformations modulate local axonal responses *via* integrin-dependent pathways in the context of pain. A previously developed three-dimensional neuron-collagen construct (NCC) system[65–67] was used to evaluate relationships between tissue mechanics and neuron activation. This culture model was employed in the current study to measure NCC stretch-induced expression of substance P (SP), a neuropeptide that transmits pain messages, with the presence and absence of $\alpha 2\beta 1$ integrin inhibitors. Peripheral tissue injury can lead to pain signaling in the central nervous system *via* the release of neurotransmitters, such as SP, from the primary afferents into the spinal cord.[2] SP has been reported to be involved in a variety of pain models including facet joint pain.[18,31,40] Therefore, axonal expression of SP was measured as a marker for pain in the NCC model to test whether inhibition of the $\alpha 2\beta 1$ integrin attenuates neuronal nociception in response to tissue loading.

MATERIALS AND METHODS

All experimental procedures involving animals 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.[69] In order to assess whether the integrin subunit $\beta1$ is involved in facet joint pain from mechanical loading, two complementary studies were performed combining *in vivo* and *in vitro* experiments. In the *in vivo* experiments, immunohistochemistry (IHC) was used to measure the expression of the integrin subunit $\beta1$ in the DRG in normal uninjured rats to define constitutive levels; in addition, its expression in DRG neurons 7 days after painful stretch of the C6/C7 facet capsular ligament was also measured. All *in vivo* experiments were performed using adult male Holtzman rats (300–450g; Envigo; Indianapolis, IN). Rats were housed with 12–12 h light–dark cycle and environmental enrichment and given free access to food and water. All surgical procedures were performed under inhalation isoflurane anesthesia (4% induction, 2.5% maintenance). In the complementary *in vitro* study, three-dimensional DRG explant cultures were treated with an $\alpha2\beta1$ integrin inhibitor at different concentrations and underwent stretch simulating the multiaxial strains that are associated with pain *in vivo*.[16,36,39] The effects of integrin inhibition on neurite density before loading and on SP expression after loading were assessed by immunocytochemistry (ICC). For all *in vitro* experiments, DRGs were sterilely isolated in Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific; Waltham, MA) from embryonic day 18 Sprague-Dawley rats that were provided by the Neuron Culture Service Center of the Mahoney Institute of Neurological Sciences at the University of Pennsylvania.

Characterization of the Integrin Subunit β1 in the DRG

Two groups of naïve rats were used to characterize the integrin subunit $\beta1$ expression in different DRG neurons. In the first group ($n = 4$ rats), the expression of the integrin subunit $\beta1$ was measured in peptidergic and non-peptidergic neurons, both of which have been shown to innervate the facet joint and to mediate pain after its injury.[33,35] To specifically evaluate if DRG neurons that innervate the facet joint capsule express the integrin subunit $\beta1$, a separate group of rats ($n = 4$) received intra-articular injections in the bilateral C6/C7 of the retrograde neuronal tracing molecule, cholera toxin subunit B (CTb) conjugated to the fluorescent dye Alexa Fluor 488 (20 μg in 5 μL of PBS; Life Technologies; Carlsbad, CA) as described previously.[35] Briefly, the bilateral C6/C7 facet joints were exposed and the CTb solution was injected into the bilateral facet joints using a 10μL syringe with a 33-gauge beveled needle. Wounds were closed using 3-0 polyester sutures and surgical staples after the injection. On day 10 after CTb injection, rats were anesthetized with sodium pentobarbital (65 mg/kg) and perfused transcardially with PBS (pH7.4) and 4% paraformaldehyde (PFA) in PBS. The C7 DRGs were harvested, post-fixed in 4% PFA in PBS at 4°C for 1 day, incubated in 30% sucrose at 4°C for 1 week and freeze-mounted in Tissue-Tek OCT Compound (Sakura Finetek; Torrance, CA).

To analyze the neuronal expression, each DRG was sectioned (14 μm thickness) and mounted on Superfrost Plus slides (Thermal Fisher Scientific; Waltham, MA) for IHC. DRG sections were blocked in 10% normal goat serum with 0.3% Triton-X PBS for 2 hours at room temperature. Sections were then incubated overnight at 4°C with guinea pig anti-substance P (1:500; Neuromics; Bloomington, MN), biotinylated Isolectin B4 (IB4) (5μg/mL; Sigma-Aldrich Corp.; St. Louis, MO) and rabbit anti-integrin subunit $\beta1$ (1:100; Santa Cruz Biotechnology; Santa Cruz, CA). The next day, sections were fluorescently labeled with secondary antibodies for goat anti–guinea pig 633 (1:1000, Invitrogen; Carlsbad, CA), fluorescein (DTAF)–conjugated streptavidin (1:500, Jackson ImmunoResearch; West Grove, PA) and goat anti–rabbit Alexa Fluor 546 (1:1000, Invitrogen; Carlsbad, CA). Tissue sections were imaged using a Zeiss 710 confocal microscope (Carl Zeiss Microscopy, LLC; Thornwood, NY) with settings that differentiated the positive labeling from the background. For the naïve DRGs, the percentage of SP-positive neurons and the percentage of IB4-positive neurons exhibiting expression of the integrin subunit $\beta1$ were measured by visually comparing the amount of positive labeling to the levels of background fluorescence in each image.[16] The paired expression of the integrin subunit $\beta1$ in peptidergic and non-peptidergic DRG neurons was compared using a non-parametric Wilcoxon singed-rank test. For the rats that received intra-articular CTb injection, the existence of neurons displaying both CTb uptake and positive integrin subunit $\beta1$ expression in the DRG was visually evaluated.

Expression of the β1 Integrin Subunit in the DRG After Painful Facet Joint Injury

To assess any pain- or injury-related changes in the expression of the integrin subunit $\beta1$ in DRG neurons, separate groups of rats received either a dynamic stretch injury applied to the C6/C7 facet joints ($n = 4$) or a sham control surgery with no stretch ($n = 5$), as

described previously.[35] After exposing the bilateral C6/C7 facet joints, the interspinous ligaments and ligamentum flavum from the C5 to T1 levels were transected so the C6 and C7 laminae could be rigidly attached to microforceps and distracted on a customized loading device.[16] Painful tensile facet capsule stretch injury was imposed by translating the C6 vertebra rostrally (2.5 mm to impose 0.47 mm of vertebral distraction and ~13% capsule strain) while holding the C7 vertebra fixed.[16,35] The magnitude of the imposed strain was computed based on fiducial markers of the joint capsular ligament; strains of the C6/C7 facet capsular ligament were targeted to exceed its maximum physiologic strain (~6%) which is non-painful[16,49] and be below the peak ligament strain (~35%) experienced during painful traumatic loading.[21,22,49] A grid of bead markers was placed on the facet capsule before stretched and was tracked during stretch by a high speed camera (Phantom-v5.1; Vision Research Inc.; Wayne, NJ). The positions of the markers before and at the maximum capsule stretch were used to calculate the applied maximum principal strain (MPS) in LS-DYNA (Livermore Software Technology Corp.; Livermore, CA).[16] A separate group received a sham surgery with attachment to the loading device but no joint distraction.

Pain was assessed before (day 0) and on days 1 and 7 after the injury or sham surgeries by measuring the withdrawal threshold of the forepaws to stimulation by von Frey filaments of increasing strengths from 0.6–26g (Stoelting Co., Wood Dale, IL).[9,35] Each filament was applied five times to the plantar surface of the forepaw; if two consecutive filament strengths evoked withdrawal, licking, or shaking of the forepaw, the lower of those filament strengths was recorded as the withdrawal threshold. Three rounds of testing were performed on the left and right forepaws separately on each testing day; the average of the left and right forepaw response thresholds from all rounds in a session was taken as the forepaw withdrawal threshold for each rat. A two-way repeated-measures analysis of variance (ANOVA) with Tukey HSD test (JMP11 software; SAS Institute; Cary, NC) identified differences in withdrawal thresholds between groups over time.

On day 7 after behavior testing, rats were anesthetized with sodium pentobarbital (65 mg/kg) and perfused transcardially with PBS and 4% PFA as described above. DRGs at C7 were harvested from rats undergoing surgery, as well as from naïve un-operated rats ($n = 3$) that served as negative controls for IHC and analyses. DRGs were sectioned and mounted on slides, as described above. Tissue sections were blocked in 1% normal donkey serum with 0.3% Triton-X PBS, immunolabeled for the integrin subunit $\beta1$ (1:100; Santa Cruz Biotechnology; Santa Cruz, CA) and the neuronal marker microtubule-associated protein 2 (MAP2; 1:500; Aves Labs; Tigard, OR), and imaged using a Zeiss 710 confocal microscope. Imaging settings were selected to ensure that positive integrin labeling was not saturated for intensity quantification and comparison between different groups. Since the small-diameter (4–21 μm) and medium-diameter (21–40 μm) sized DRG neurons are primarily nociceptors and exhibit different expression profiles after painful facet joint distraction,[16,26,62] the average intensity of the integrin subunit $\beta1$ labeling was quantified in each of those populations of neurons separately using ImageJ software (National Institutes of Health, Bethesda, MD) and normalized to the expression in size-matched neurons from naïve rats. Differences in the $\beta1$ integrin expression between the injury and sham groups were compared in small- and medium-diameter DRG neurons using separate t-tests.

Evaluating SP Expression In Vitro with α2β1 Integrin Inhibition and Loading

To test whether mechanical loading of peripheral tissues modulates nociceptive signaling in sensory axons *via* the integrin subunit $\beta1$-dependent mechanisms, an *in vitro* DRG-collagen model that enables integrated mechanical loading, mechanical measurements, and cellular imaging was used.[65,67] NCCs were made using rat tail Type I collagen (2 mg/mL; Corning Inc., Corning, NY) cast in 12-well plates at 37°C overnight. DRGs from all spinal levels were dissected using fine forceps from embryonic day 18 Sprague-Dawley rats after exposing the rat's spine and removing the spinal cord. Approximately 5–10 DRG explants were plated on each collagen gel and allowed to attach and grow for 3 days before the addition of supplemental collagen solution to encapsulate the DRGs in the NCC construct. NCCs were cultured in neurobasal medium supplemented by 1% GlutaMAX, 2% B-27, and 1% fetal bovine serum (FBS) (all from Thermo Fisher Scientific; Waltham, MA). In addition, 2 mg/mL glucose (Sigma-Aldrich Corp.; St. Louis, MO), 10 ng/mL 2.5 S nerve growth factor (Thermo Fisher Scientific; Waltham, MA), 10 mM FdU (Sigma-Aldrich Corp.; St. Louis, MO) and 10 mM uridine (Sigma-Aldrich Corp.; St. Louis, MO) were suppemented.[11,67] The culture media was changed every 2–3 days.

The $\alpha2\beta1$ integrin is the primary functional receptor mediating cell adhesion to Type I collagen,[25] a major component of the facet capsular ligament[23] and our NCC system. The presence of the $\alpha2\beta1$ integrin in the DRG explant culture was confirmed by immunolabeling against the integrin subunits $\alpha2$ and $\beta1$ (both at 1:100; Santa Cruz Biotechnology; Santa Cruz, CA)

and visualizing the co-localization of the two subunits. All ICC for NCCs was performed by blocking the gels with 1% donkey serum in 0.3% Triton-X PBS for 2 hours at room temperature, incubating them with primary antibodies overnight at 4°C and fluorescent labeling with corresponding secondary antibodies for 2 hours at room temperature. After confirming the expression of the $\alpha2\beta1$ integrin in the NCCs, the activation of the $\alpha2\beta1$ integrin was inhibited *in vitro* to evaluate whether neurotransmitter expression induced by loading depends on the integrin subunit $\beta1$. The allosteric inhibitor, TC-I15 (Tocris Bioscience; Minneapolis, MN) that blocks interactions between the integrin subunits $\alpha2$ and $\beta1$ was used to prevent the activation of the $\alpha2\beta1$ integrin.[7,48] Separate groups of NCCs were treated with 10 μM ($n = 4$), 100 μM ($n = 5$), and 1000 μM ($n = 5$) TC-I15 at 36 hours before mechanical loading. Additional NCCs underwent treatment with TC-I15 but no mechanical loading ($n = 3$/concentration) and untreated NCCs ($n = 5$ loaded; $n = 6$ unloaded) also were included as controls; all sets of controls were evaluated for neuronal morphology and neuropeptide expression.

At day 7 *in vitro*, the DRG cultures underwent mechanical loading. NCCs were cut into a cruciform shape with each arm having the dimensions of 6.25 mm × 8 mm,[55,67] and loaded onto a planar biaxial testing machine (574LE2; TestResources; Shakopee, MN) equipped with a bio-bath filled with PBS maintained at 37°C. NCCs were stretched equibiaxially to 1.5 mm (to induce ~20% strain) at 0.3 mm/s (~4%/s) to simulate sub-failure strains that induce pain *in vivo*.[16,38,39,67] The MPS in sub-regions of the NCCs were measured by tracking fiducial markers drawn on the NCC surface and the embedded visible DRGs using a high-speed camera (Phantom-v9.1; Vision Research Inc.; Wayne, NJ). Based on the marker and DRG positions before and after stretch, the average MPS in all non-overlapping four-node sub-regions across the surface of the NCC was computed in LS-DYNA[67] and compared between treated and untreated groups using a one-way ANOVA to ensure consistent loading. Immediately after mechanical loading, NCCs were released from the grips, washed with fresh PBS with 1% Pen-Strep (Thermo Fisher Scientific; Waltham, MA) and transferred to pre-warmed culture media supplemented by 1% Pen-Strep for 1 day, after which time they were fixed with 4% PFA.[67]

To evaluate any morphological changes in axons that may be induced by integrin inhibition before mechanical testing, axonal expression of βIII-tubulin was visualized using ICC and confocal microscopy. Three randomly selected regions of interest were evaluated from each unloaded control NCCs ($n = 3$ gels/group). Two blinded assessors scored the images using a 3-point scale based on neurite density, with scores of 0, 1 and 2 indicating mild, moderate and robust axon outgrowth and branching, respectively.[28] The average score for each group was compared using a non-parametric Kruskal–Wallis rank-sum test in JMP11. The axonal expression of SP was measured using a guinea pig SP antibody (1:500; Neuromics; Bloomington, MN) and confocal microscopy in NCCs with and without integrin inhibition at day 1 after loading, as described above.[65] The average intensity of SP labeling in axons was quantified using ImageJ software (National Institutes of Health; Bethesda, MD) and normalized to levels in untreated unloaded control gels to ensure appropriate comparison between different experimental runs. The normalized SP expression was compared across different groups using a two-way ANOVA, with the loading group and treatment condition as the two factors. To evaluate the relationship between SP expression and the applied NCC strain in untreated NCCs and those pre-incubated with various concentrations of the $\alpha2\beta1$ inhibitor, local SP expression was mapped to the regional strain measured in the same sub-region of the gel.[65] The MPS for unloaded control gels was taken as 0. The associations between SP expression and MPS were evaluated using linear regression models,[65] with F-tests assessing the significance of each of the regressions in JMP11.

RESULTS

The integrin subunit $\beta1$ is highly expressed in SP-positive peptidergic neurons of the uninjured DRG (Fig. 2). From the 4 un-operated naïve rats, 490 SP-positive (peptidergic) neurons and 636 IB4-positive (non-peptidergic) neurons were evaluated. Among those SP-positive neurons, 81 ± 15% exhibit expression of integrin subunit $\beta1$ (Fig. 2b). In contrast, only 50 ± 6% of the neurons that express IB4 are positive for the integrin $\beta1$ subunit, which is significantly lower ($p < 0.001$) than the percentage in neurons expressing SP (Fig. 2b). Although not all CTb-positive neurons show a detectable level of integrin subunit $\beta1$, co-localization of the $\beta1$ subunit and the CTb tracer is observed in the C7 DRG (Fig. 2c), suggesting that some of the afferents that innervate the C6/C7 facet capsule express the integrin subunit $\beta1$.

A painful facet joint injury appears to increase the expression of the integrin subunit $\beta1$ in nociceptive DRG neurons compared to expression in sham-operated rats (Fig. 3). Indeed, facet joint distraction imposing a capsular MPS of 13.3 ± 3.8% induces behavioral hypersensitivity that develops as early as 1 day and lasts for at least 7 days, as evidenced by a

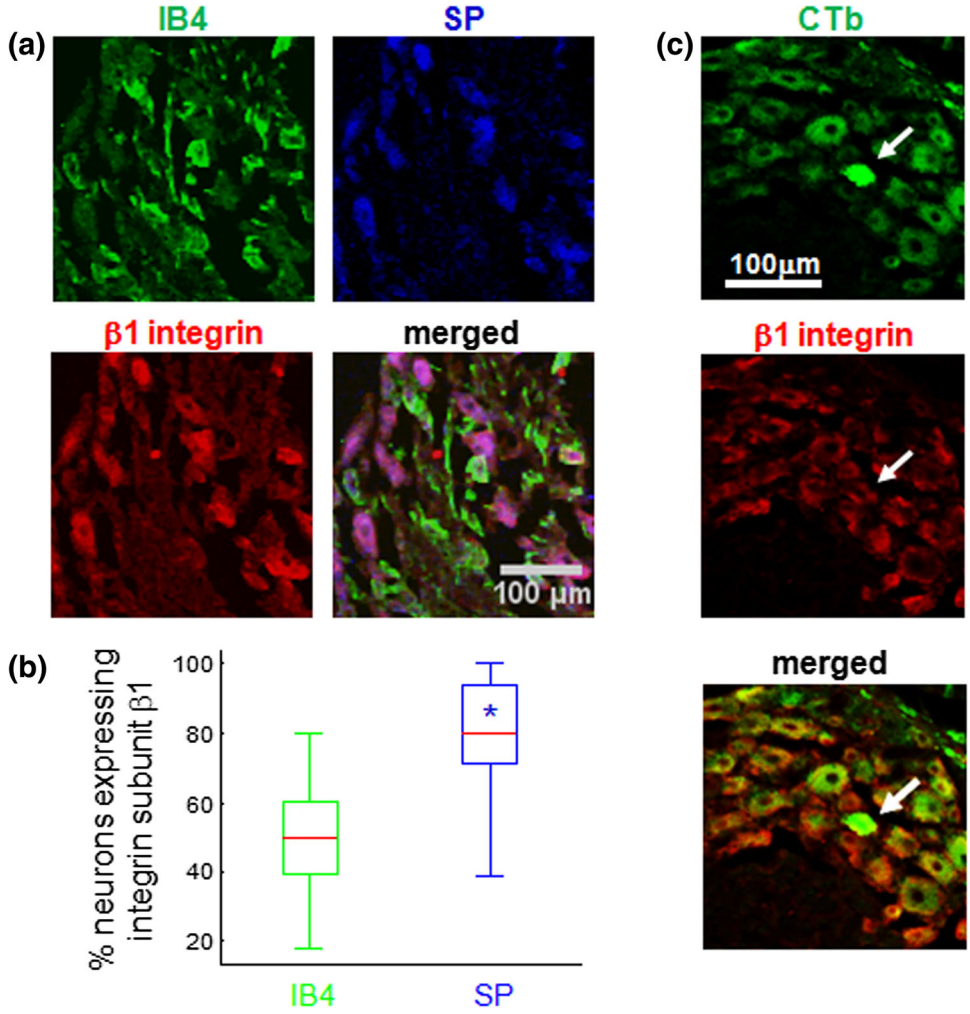

FIGURE 2. Expression of the integrin subunit $\beta 1$ in neurons in the C7 rat DRG. (a) Representative images and (b) quantification show localization of the integrin subunit $\beta 1$ in both IB4-positive non-peptidergic neurons (co-localization shown in yellow) and SP-positive peptidergic neurons (co-localization shown in pink), and that expression is significantly higher (*$p = 0.004$) in SP-positive neurons (quantification performed on 8–11 images/rat from 4 rats). The box plot shows the interquartile range (IQR; box height), sample median (red line), and maximum and minimum values (whiskers). (c) Some CTb-positive neurons that innervate the facet joint also exhibit the integrin subunit $\beta 1$. Arrows point to an afferent neuron with co-localized CTb tracer and the integrin subunit $\beta 1$. The scale bar in (a) and (c) is 100 μm and applies to all images in the same panel.

significantly decreased forepaw withdrawal threshold from baseline (day 0) at day 1 ($p = 0.014$) and day 7 ($p = 0.020$) (Fig. 3a). At day 7, the expression of the integrin subunit $\beta 1$ is significantly greater in the DRG in the injury group than in shams for both small-diameter ($p < 0.001$; 129 neurons for injury; 204 neurons for sham) and medium-diameter ($p = 0.005$; 247 for injury; 311 neurons for sham) neurons (Figs. 3b, 3c).

Inhibiting the activation of the $\alpha 2\beta 1$ integrins *in vitro* prior to stretch prevents loading-induced upregulation of axonal SP expression. Co-localization of the integrin subunits $\alpha 2$ and $\beta 1$ in axons is observed in the neuronal NCC cultures, confirming the existence of $\alpha 2\beta 1$ integrins (Fig. 4a). The density score describing neurite density on the day of mechanical loading is not different between NCCs regardless of whether they received inhibition or not; in addition, the inhibitor concentration does not affect the neurite density score (Fig. 4b, 4c). Of note, the average strain across the gel surface is not different between any loaded group (MPS = 20.5 ± 3.5%), indicating that all loaded groups underwent the same degree of macroscopic deformation. There is no difference in the level of SP expression in unloaded control NCCs, with and without TC-I15 treatment (Fig. 5). In untreated NCCs, SP is significantly upregulated after stretch relative to unloaded controls ($p = 0.003$) (Fig. 5). Although treated and untreated NCCs exhibit similar neurite density and undergo comparable strain severity, inhibition of $\alpha 2\beta 1$ integrins with 100 or 1000 μM TC-I15 significantly lowers SP expression after loading ($p < 0.03$) compared to untreated loaded NCCs (Fig. 5). The expression of SP

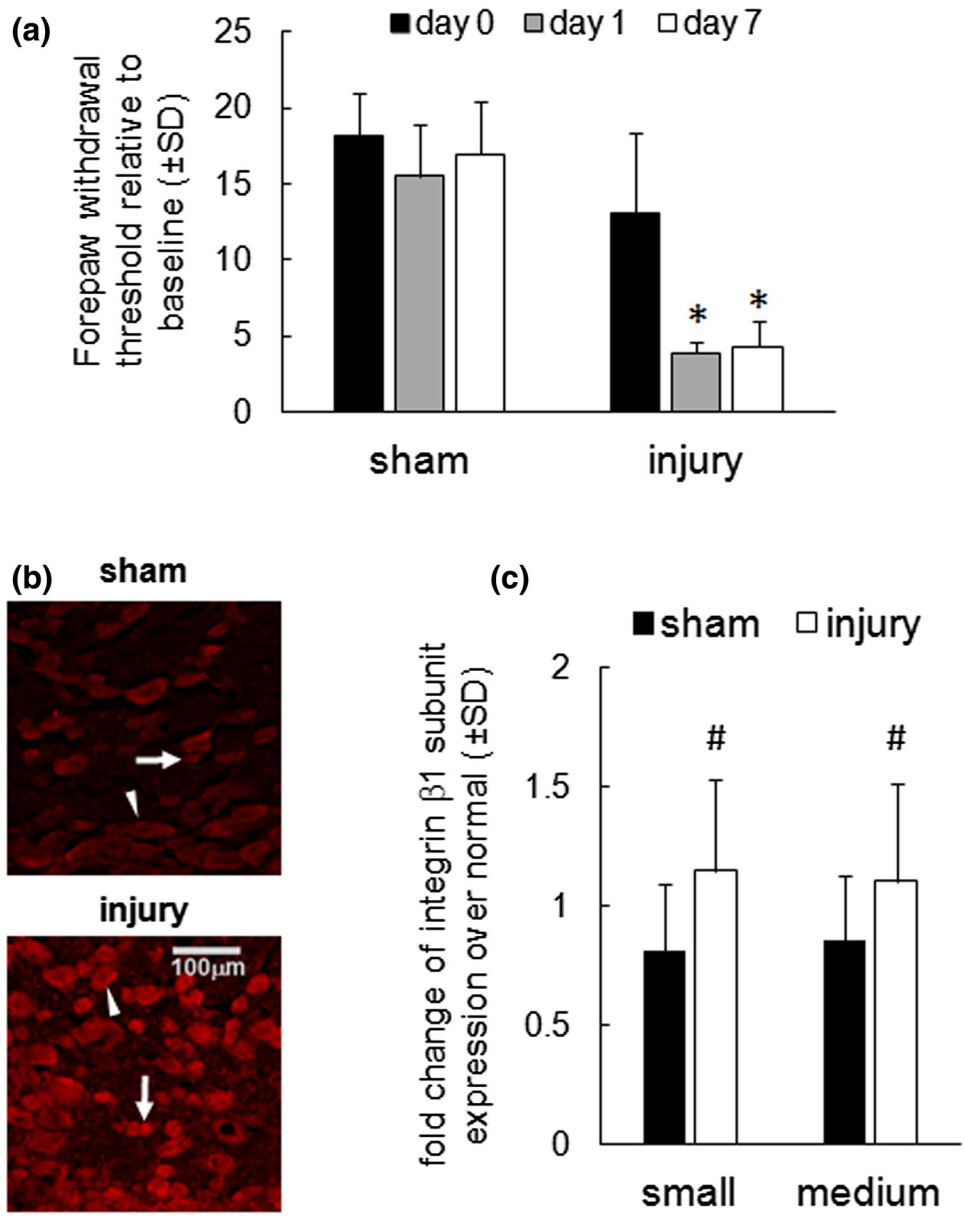

FIGURE 3. Expression of the integrin subunit $\beta 1$ after painful facet capsule injury. (a) The forepaw withdrawal threshold is significantly decreased ($^{*}p \leq 0.020$) at days 1 and 7 from baseline pre-injury levels (at day 0) only in rats receiving facet capsule injury ($n = 4$ rats), but not in those with sham control ($n = 5$ rats) surgeries. (b) Representative images and (c) intensity quantification ($n = 3$ images/rat) reveal significantly more ($^{\#}p \leq 0.005$) expression of the integrin subunit $\beta 1$ in small-diameter (arrows) and medium-diameter (arrow heads) DRG neurons after a painful facet capsule injury ($n = 4$ rats) than after sham ($n = 5$ rats) surgery.

in TC-I15-treated NCCs is not different from the corresponding unloaded controls regardless of the inhibitor concentration (Fig. 5).

Integrin inhibition does alter the relationship between SP expression and regional strains in the NCC (Fig. 6). Mapping local SP expression to MPS in the same sub-region (Fig. 6a), the axonal SP expression is found to be significantly correlated with regional MPS in untreated NCCs ($R^2 = 0.460$; $p < 0.001$). In contrast, no significant linear correlation is detected between SP expression and regional MPS with the presence of 10 μM ($R^2 = 0.294$; $p = 0.055$), 100 μM ($R^2 = 0.036$; $p = 0.372$) or 1000 μM ($R^2 = 0.020$; $p = 578$) of the $\alpha 2\beta 1$ integrin inhibitor (Fig. 6b–6e).

DISCUSSION

Consistent with previous work,[61] the integrin subunit $\beta 1$ is highly expressed in DRG neurons (Fig. 2). Integrin

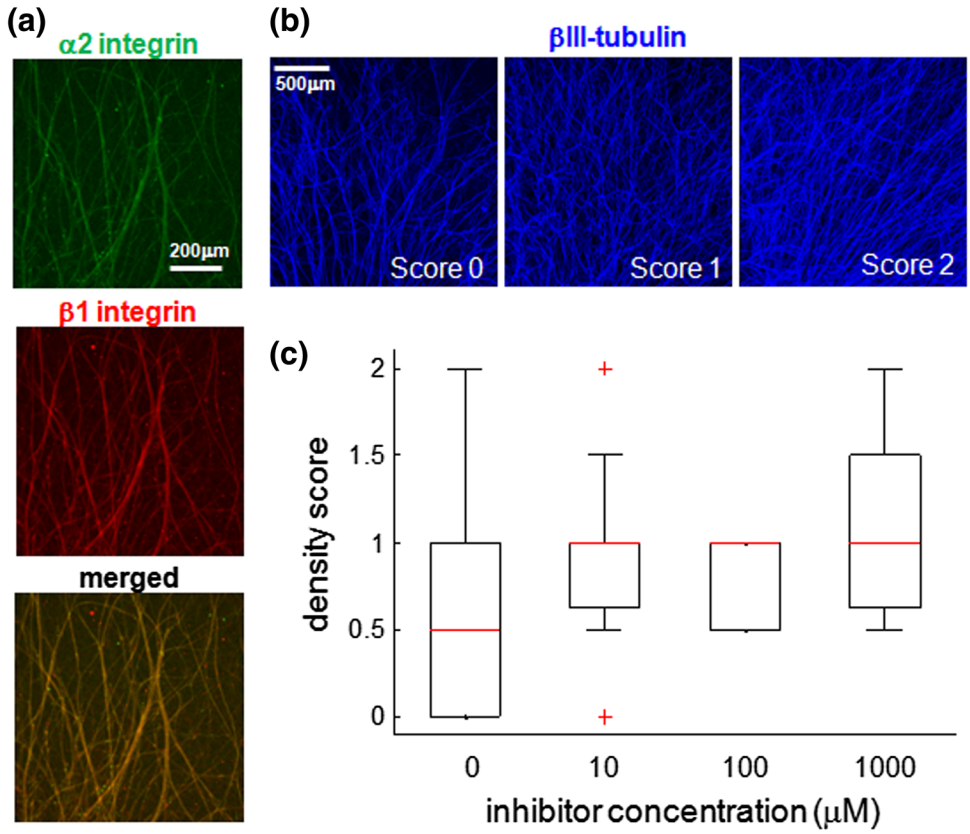

FIGURE 4. Characterization of integrin subunit expression and neurite density in the DRG-collagen gel culture system. (a) Abundant expression of the α2 and β1 integrin subunits and their co-localization is evident in axons in the NCC. The scale bar is 200 μm and applies to all images. (b) Representative images correspond to the 3-point scoring scheme used to evaluate neurite density as visualized by immunolabeling of βIII-tubulin, with Score 0 being low density, and Score 2 being robust axon outgrowth and branching. The scale bar represents 500 μm and applies to all images. (c) The density scores are not different between NCCs with or without the α2β1 integrin inhibitor ($n = 3$ images/NCC with 3 NCCs/concertation), regardless of the concentration used. The box plot shows the interquartile range (IQR; box height), sample median (red line), maximum and minimum values within 1.5 IQR away from the box (whiskers) and outliers (+).

subunit β1 expression is evident in nearly all (81 ± 15%) of the peptidergic afferents (Fig. 2), which themselves account for over half of the afferents innervating the facet joint in rats and are critical for initiating facet joint pain.[33,35] The integrin subunit β1 is expressed on DRG neurons whose peripheral terminals reside in the C6/C7 facet joint and is upregulated in small- and medium-diameter DRG neurons at day 7 after painful facet capsule stretch (Figs. 2, 3). The imposed capsular ligament strains (13.3 ± 3.8%) are comparable to previously reported strains (5–15%) for activating group III and group IV nociceptors in the cervical facet capsule[43,44] and strains (>8–12%) that induce pain.[16] Of note, the facet capsule injury used in the current study produces forepaw withdrawal thresholds that are lower than those in the uninjured sham rats (Fig. 3) and are similar to prior reports.[16,34] Both the presence and regulation of the β1 subunit in DRG neurons suggest its possible involvement in joint pain from mechanical injury.

The β1 integrins likely contribute to nociception after painful loading of the facet capsular ligament *via* modulation of the neuropeptide substance P. This hypothesis is due to the fact that the majority of the SP-positive peptidergic neurons identified in the *in vivo* study express β1 integrins (Fig. 2) and since input from peptidergic facet joint afferents has been shown to be required for pain generation after capsule stretch.[33,35] Inhibiting the primary Type I collagen receptor, the α2β1 integrin, before painful NCC stretch (20.5 ± 3.5% strain) prevents the upregulation of axonal SP due to loading and weakens the positive correlation between local SP expression and strain in a dose-dependent manner (Figs. 5, 6). The correlation between SP expression and the MPS might be stronger or significant for the three integrin inhibition conditions if the strain fields were constructed with finer meshes using more markers, but marker placement was selected in this study to not interfere with the axonal signals in the fluorescent images. Since the treated and untreated NCCs exhibited similar morphology and neurite density (Fig. 4) and underwent the same degree of stretch (i.e., injury severity), any difference in pro-

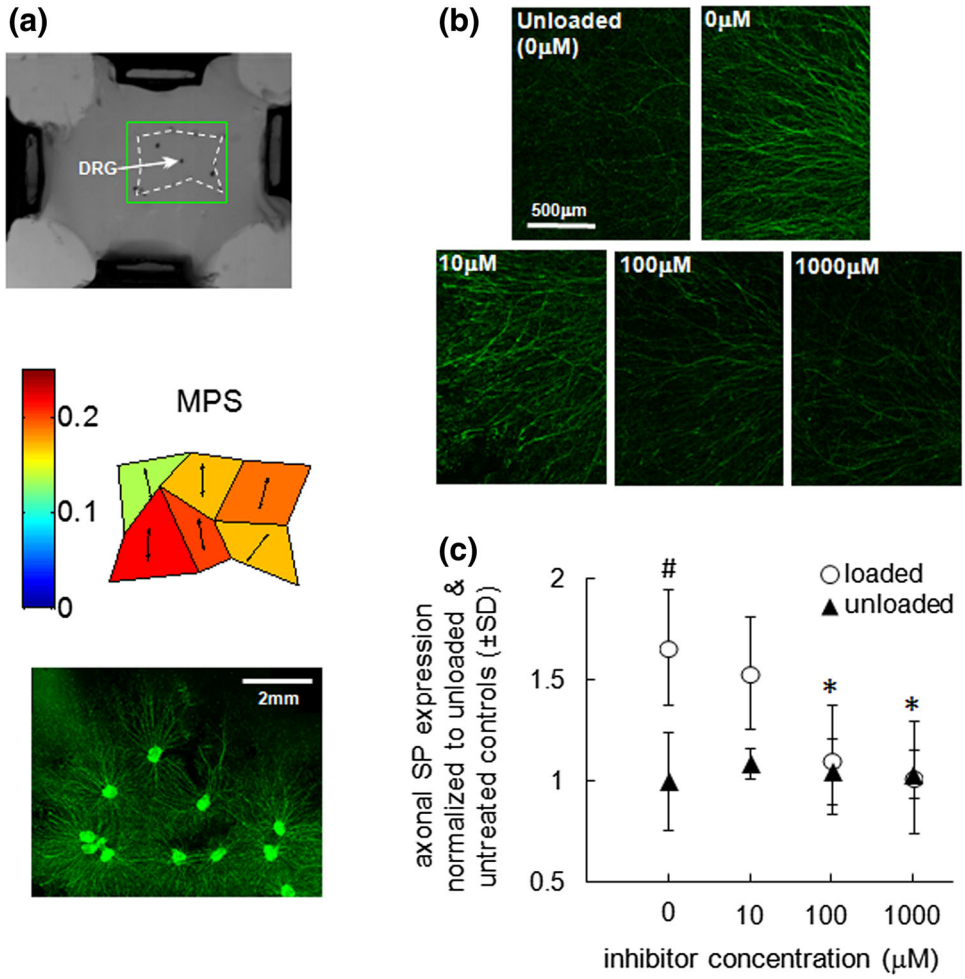

FIGURE 5. Stretch-induced SP expression is decreased after integrin inhibition in NCCs. (a) Sample images show an NCC in a stretched configuration, with the region used for strain calculation enclosed by the dashed-line box and the region used for SP assessment enclosed by the solid-line box. The corresponding strain map and SP expression for that gel are also shown, with both located in the same region of interest. (b) Representative images showing variable axonal SP expression in unloaded untreated controls and in loaded NCCs that received TC-I15 treatment (0, 10, 100 or 1000 μM), with decreased expression in unloaded untreated gels and in loaded gels with increased inhibiter concentration. (c) Quantification of normalized axonal SP showing significantly greater expression level in loaded untreated NCCs compared to unloaded untreated controls ($^{\#}p = 0.003$) and loaded NCCs treated with 100 or 1000 μM of TC-I15 ($^{*}p < 0.03$). Data represent results from 11 untreated NCCs ($n = 5$ loaded; $n = 6$ unloaded), 7 NCCs treated with 10 μM TC-I15 ($n = 4$ loaded; $n = 3$ unloaded), 8 NCCs treated with 100 μM TC-I15 ($n = 5$ loaded; $n = 3$ unloaded), and 8 NCCs treated with 1000 μM TC-I15 ($n = 5$ loaded; $n = 3$ unloaded).

tein expression between loaded groups can be attributed to the inhibition of the $\alpha 2\beta 1$ integrin. Collectively, findings in these integrated *in vivo* and *in vitro* studies point to a potential role of integrins, especially those that contain the $\beta 1$ subunit, in the sensitization of nociceptors from supraphysiologic ligament stretch at the cell-matrix interface.

Substance P is a neuropeptide with pro-inflammatory and neurotransmitter effects in modulating pain. It is elevated in the innervated tissue and/or DRG in models of knee and facet joint pain.[18,31,40] Further, since ablating peptidergic joint afferents using a targeted SP conjugated neurotoxin reduces NGF expression in the DRG, suppresses injury-induced firing of spinal neurons and prevents behavioral hypersensitivity after painful facet joint injury,[33] SP-positive peptidergic afferents are hypothesized to respond directly to deformations in the tissue they innervate and to, at least in part, initiate mechanically-induced facet joint pain by sensitizing secondary neurons in the spinal dorsal horn. Since the $\beta 1$ integrin has a role in mediating cell-ECM interactions[29,57] and neuronal mechanosensing, and it is preferentially expressed in SP-positive peptidergic afferents (Fig. 2), the hypothesis that $\beta 1$ integrin activation occurs in response to an altered microenvironment in joint trauma and mediate SP in neurons is supported. The *in vitro* findings that $\alpha 2\beta 1$ integrin inhibition prevents the axonal SP

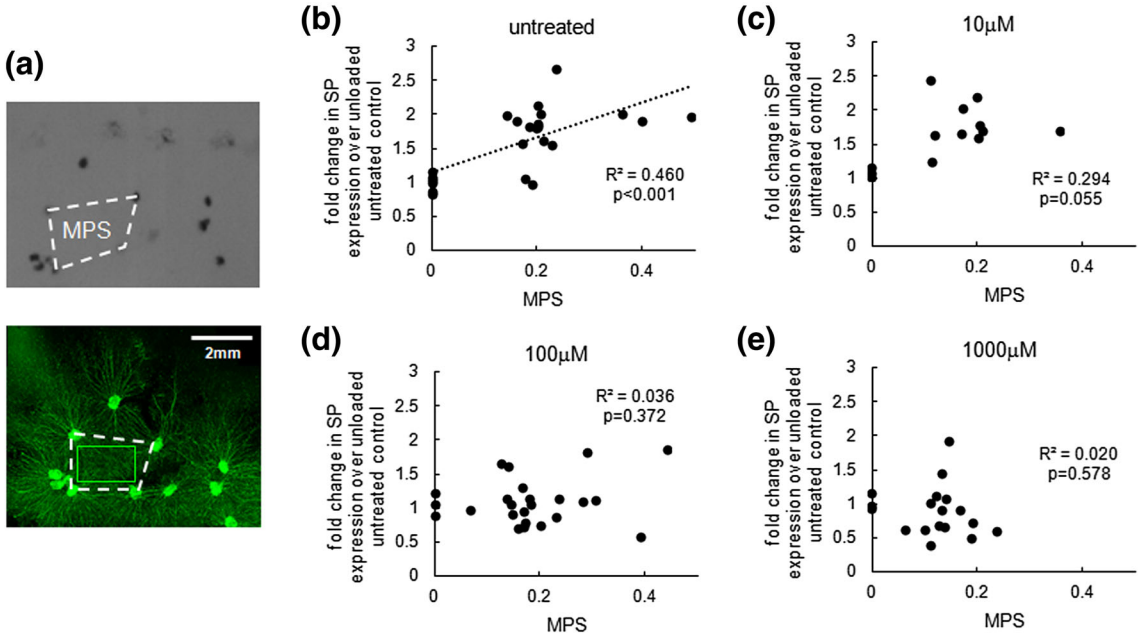

FIGURE 6. Linear correlations between strain and SP expression with and without integrin inhibition. (a) Representative images show the mapping between regional strain (dashed line box) and the local SP assessment (solid line box) from the same sub-region of an NCC. Linear regressions of normalized SP expression against regional strain reveal only a (b) significant correlation in the case without inhibition of the $\alpha 2\beta 1$ integrin; (c)–(e) there is no significant association after treatment with 10, 100 or 1000 μM of the TC-I15 inhibitor. Data points represent 11 untreated NCCs (n = 5 loaded; n = 6 unloaded) in (b), 7 NCCs treated with 10μM TC-I15 (n = 4 loaded; n = 3 unloaded) in (c), 8 NCCs treated with 100μM TC-I15 (n = 5 loaded; n = 3 unloaded) in (d), and 8 NCCs treated with 1000 μM TC-I15 (n = 5 loaded; n = 3 unloaded) in (e).

expression after tensile loading (Figs. 5, 6) suggest that SP may be directly modulated by mechanically-induced integrin activation. Yet, the specific signaling cascades involved in integrin-dependent SP production and release are unknown and require further investigation. It is possible that integrin activation triggers increases in SP *via* cytoskeletal regulation, because the cytoskeleton interacts with integrins,[20,54] can contribute to second messenger signaling for inflammatory pain,[4,14] and modulates N-methyl-D-aspartate receptor activity that alters SP release from the primary afferents.[41,42] In addition, extracellular signal-regulated kinase (ERK) signaling, which can mediate inflammatory pain *via* the integrin subunit $\beta 1$,[13] and SP are both elevated in response to neuronal injury in a strain-dependent manner[65] (Fig. 6) and have direct effects on each other in primary sensory afferents.[45,59] As such, specific second messenger cascades that interact with the integrin subunit $\beta 1$, including the ERK pathway, may play critical roles in altering SP expression *via* integrin activation.

Defining how the integrin subunits regulate the mechanotransduction cascades of afferents to ligament injury is challenging partly due to the difficulty in decoupling the mechanical stimuli from the rapidly developing local inflammatory responses after joint trauma.[34] Even in the NCC DRG explant culture model used here that neurons themselves produce inflammatory mediators; but, the number of glial and immune cells is largely reduced, eliminating a host of inflammatory factors that sensitize neurons in joints.[2] The NCC model does enable mapping local physiological responses (like SP) to the applied macroscopic strain and local regional mechanics (Figs. 5, 6), providing a simplified microenvironment suitable to investigate the role of integrins and other mediators of cell-ECM interactions in neuronal mechanosensing of its surrounding microenvironment.

Although SP is a well-known nociceptive mediator, its expression in sensory neurons is only a proxy for pain and no *in vitro* system can link integrin activation to the integrated responses, like pain, afforded by *in vivo* systems. The effects of integrin inhibition on the development of pain from ligament trauma was not investigated *in vivo*; but, altered integrin subunit $\beta 1$ expression was evaluated in nociceptors in parallel with pain (Fig. 3). At that same time after painful facet joint injury when concurrent increases in the integrin subunit $\beta 1$ expression and pain are detected (Fig. 3), upregulation of both SP and protein kinase C-ε (PKCε) is evident in the DRG.[16,40] Although pain-inducing PKCε signaling in primary afferent nociceptors has been shown to depend on the integrin $\beta 1$,[13] no causal relationships between its increase and that of SP

and PKCε in the DRG after painful facet injury. Nonetheless, these findings support the notion that the integrin subunit β1 may be needed to maintain facet pain *via* well-defined nociceptive signaling cascades involving the SP and PKCε pathways.

The current results, together with prior reports, begin to elucidate possible mechanisms by which integrins, the subunit β1 in particular, mediate nociceptor responses to their altered mechanical and chemical environment that arises during and after painful facet injury. The facet capsular ligament is primarily composed of heterogeneously organized dense collagen fibers,[1,52] which can be abnormally reoriented and exert force on the neurons embedded in them during excessive ligament loading.[51–53] Integrins at the interface between afferents and their surrounding collagen may be activated by the ligament loading, generate stress concentrations and induce changes in electrophysiological signaling, morphological impairment and neurotransmitter regulation.[19,20,29] Tissue inflammation after facet injury may also activate integrins. Inflammatory mediators acting on their cell membrane receptors trigger intracellular mechanisms that regulate both focal adhesions and the cytoskeleton, which initiate second messenger cascades, such as the ERK and the PKC pathways, leading to pain.[3,13,17,20,32,33,46] The increased expression of integrin subunits a few days after a painful facet capsule injury (Fig. 4) points to possible interactions between sustained pain signaling in nociceptors and regulation of integrins in those neurons. Although these studies support an emerging schema for integrin-dependent mechanically-induced facet joint pain, work is needed to fully characterize the temporal expression of different integrin subunits in the peripheral terminal of primary afferents in response to tissue injury. Nevertheless, these findings provide evidence for β1 integrin involvement in nociceptor sensitization from supraphysiologic tissue deformations. Integrin-dependent SP regulation in DRG neurons after stretch points to the α2β1 integrin as a potential mediator of pain from trauma and a potential therapeutic target for facet capsular ligament pain.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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