Collagen Organization Regulates Stretch-Initiated Pain-Related Neuronal Signals In Vitro: Implications for Structure–Function Relationships in Innervated Ligaments

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ABSTRACT: Injury to the spinal facet capsule, an innervated ligament with heterogeneous collagen organization, produces pain. Although mechanical facet joint trauma activates embedded afferents, it is unclear if, and how, the varied extracellular microstructure of its ligament affects sensory transduction for pain from mechanical inputs. To investigate the effects of macroscopic deformations on afferents in collagen matrices with different organizations, an in vitro neuron-collagen construct (NCC) model was used. NCCs with either randomly organized or parallel aligned collagen fibers were used to mimic the varied microstructure in the facet capsular ligament. Embryonic rat dorsal root ganglia (DRG) were encapsulated in the NCCs; axonal outgrowth was uniform and in all directions in random NCCs, but parallel in aligned NCCs. NCCs underwent uniaxial stretch (0.25 ± 0.06 strain) corresponding to sub-failure facet capsule strains that induce pain. Macroscopic NCC mechanics were measured and axonal expression of phosphorylated extracellular signal-regulated kinase (pERK) and the neurotransmitter substance P (SP) was assayed at 1 day to assess neuronal activation and nociception. Stretch significantly upregulated pERK expression in both random and aligned gels ($p < 0.001$), with the increase in pERK being significantly higher ($p = 0.013$) in aligned than in random NCCs. That increase likely relates to the higher peak force ($p = 0.025$) and stronger axon alignment ($p < 0.001$) with stretch direction in the aligned NCCs. In contrast, SP expression was greater in stretched NCCs ($p < 0.001$) regardless of collagen organization. These findings suggest that collagen organization differentially modulates pain-related neuronal signaling and support structural heterogeneity of ligament tissue as mediating sensory function. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 36:770–777, 2018.

Keywords: collagen; afferent; ERK signaling; neurotransmitter; painful ligament injury

Ligaments are fibrous tissues that not only provide mechanical support, but can serve also as sensory organs due to their proprioceptive and nociceptive afferents.[1–4] They are increasingly recognized as sources of musculoskeletal pain due to abnormal loading.[5–7] The spinal facet capsular ligament can induce neck and back pain due to its excessive stretch,[8–10] particularly in the cervical spine as a result of whiplash injury and neck trauma.[8,11] The capsular ligament encloses the bilateral facet joints, which facilitate articulations between adjacent vertebrae in the spine. The capsular ligament is primarily comprised of densely packed collagen fibers interspersed with elastin fibers.[1,12] Fiber organization in the capsular ligament is highly heterogeneous; fibers in some regions have parallel orientation but are unaligned in others.[12–14] Afferent nerve fibers, including pain fibers, innervate both regions with parallel collagen bundles and those with irregular organization.[1] Accordingly, afferents, even in the same capsule, can experience very different loading conditions and injury risks during macroscopic tissue stretch.[14] Although supraphysiological strains of the cervical facet capsular ligament are known to activate neurons embedded in it and lead to pain,[9,15–17] it remains unknown if, and how, the microstructure and organization of the capsular ligament affect the sensory function of the neurons in it.

Collagen organization of fibrous tissues affects macro- and micro-scale tissue mechanics,[18–20] which can modulate neuronal activity via their surrounding extracellular matrix.[21,22] The collagen organization before loading has been shown in a host of musculoskeletal tissues, collagen-based tissue-equivalents, and computational models to affect tissue-level stress–strain responses, macroscopic failure forces, and microscale fiber kinematics during loading.[18–20] In neuron-seeded collagen gels with randomly organized fibers, fiber realignment occurs at macroscopic strains that also increase neuronal phosphorylation of the extracellular signal-regulated kinase (ERK), a marker of cellular activation.[21] Further, at macro-scale strains that exceed thresholds for neuronal activation, fibers aligned with the loading direction sustain greater strains than those with other alignment orientations, and undergo fiber stretch that can exceed the applied bulk strain.[21] Failure of load-bearing fibers and subsequent force redistribution can lead to the altered collagen fiber kinematics that have been observed, and taken as microstructural injury, in specific regions of the facet capsular ligament.[14,23] Both local collagen disorganization and persistent afferent activation in facet capsules occur at pain-inducing strains in

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vivo,[9,15,23] further suggesting possible associations between collagen fiber orientation, neuronal signaling, and macroscopic tissue deformation. Accordingly, collagen organization in innervated ligaments is hypothesized to mediate neuronal activation and pain signaling as a result of excessive tissue stretch.

To better understand the potential interplay between neurons and their matrix environment, a previously developed in vitro neuron-collagen construct (NCC) model[21] was modified to incorporate collagen fibers with either random or aligned organization. The NCCs encapsulated embryonic rat dorsal root ganglia (DRG) containing the cell bodies of sensory neurons that innervate peripheral tissues like the facet capsular ligament and synapse with spinal cord neurons in vivo.[24,25] The neurotransmitter substance P is important in nociceptive signal transmission and has been shown to be involved in facet join pain[16,25,26] By varying the collagen organization of NCCs, and allowing DRGs to grow axons along collagen fibers, the varied innervation of afferents in sub-regions of the facet capsular ligament with different microstructures[1] was simulated. Collagen fiber alignment and axon orientation were assessed in un-stretched control gels using polarized light imaging and confocal imaging to characterize the structural differences between the NCCs with random and aligned fiber organization. After NCCs were stretched to strains that induce pain in vivo,[9,16] neuronal expression of the phosphorylated ERK (pERK) and the nociceptive neuropeptide SP was measured to evaluate effects of collagen organization on neuronal activation and pain signaling.

METHODS

Studies used an in vitro DRG-collagen model system in which neurons were embedded in collagen, with varied fiber alignment (Zhang et al., Submitted).[21] The NCCs were prepared using rat tail Type I collagen (2 mg/ml; Corning, Inc.; Corning, NY) cast in 12-well plates. Gels with random fiber organization (random NCCs) were made by incubating the collagen solution at 37°C overnight.[21] NCCs with parallel collagen fibers (aligned NCCs) were produced using magnetic alignment[27,28] with plates containing the collagen solution placed in a 4.7T 50 cm horizontal bore magnetic resonance system equipped with a 30 cm inner diameter 3 gauss/cm and a 12 cm inner diameter 25 gauss/cm gradient tube, interfaced to an Agilent DirectDrive console (Agilent Technologies; Santa Clara, CA) at 37°C for 45 min and then incubated at 37°C overnight.

Rat DRGs from all spinal levels were sterilely isolated using fine forceps from embryonic day 18 Sprague-Dawley rats (from the CNS Cell Culture Service Center of the Mahoney Institute of Neuroscience), according to procedures approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. DRG explants (5–10/gel) were plated in the center of the gels and allowed to attach and grow axons on the surface. NCCs were cultured in neurobasal medium supplemented by 1% GlutaMAX, 2% B-27, 1% Fetal Bovine Serum, and 10 ng/ml 2.5S nerve growth factor (all from Thermo Fisher Scientific; Waltham, MA), with addition of 2 mg/ml glucose, 10 mM FdU, and 10 mM uridine (all from Sigma–Aldrich; St. Louis, MO) (Zhang et al., Submitted).[29] Additional collagen was added 3 days after the initial plating to encapsulate the DRGs. NCCs were cultured for another 4 days.

To characterize collagen alignment and axonal orientation, the directions of collagen fibers and axons were measured in NCCs with randomly oriented fibers ($n = 8$) and with magnetically aligned fibers ($n = 9$). At day 7 in vitro, those unloaded control NCCs were fixed with 4% paraformaldehyde. A previously developed polarized light imaging technique[21,23,30] was used to acquire pixel-wise (20 pixels/mm) collagen alignment angles over the entire gel. Circular variance quantified the spread of fiber orientation angles, with a lower circular variance indicating a tighter clustering and a higher degree of fiber alignment.[31] The microstructure of the collagen gel in both aligned and random NCCs also was visualized by immunolabeling of Type I collagen. Gels were blocked in 10% goat serum with 0.1% Triton-X 100 in phosphate-buffered saline (PBS) solution and incubated overnight with a mouse anti-collagen I antibody (1:250; Abcam; Cambridge, MA) followed by incubation with a fluorescent secondary goat anti-mouse Alexa Fluor 488 antibody (1:1,000; Invitrogen; Carlsbad, CA). Labeled gels were imaged with a Zeiss 710 confocal microscope (Carl Zeiss Microscopy; Thornwood, NY). In order to evaluate neuronal morphology, the NCCs were blocked in 1% donkey serum with 0.3% Triton-X 100 in PBS, and fluorescently labeled using a chicken anti-βIII tubulin antibody (1:200; Abcam) and a secondary donkey anti-chicken Alexa Fluor 647 antibody (1:1000; Invitrogen). A Fourier transform method was used to analyze the confocal images and measure the direction of axon outgrowth by computing the axon orientation distribution in polar coordinates based on image intensity values, as described previously.[32,33] This method defines the degree of fiber alignment along the principal orientation axes, the ratio of which indicates the extent of anisotropy.[32,33] As such, the ratio of the axon alignment strength along the minor orientation axis to that along the major orientation axis was computed. Differences in collagen fiber alignment (circular variance) and axonal direction (minor-to-major-axis ratio) were compared between random and aligned NCCs using Student's *t*-tests.

At day 7 in vitro, separate groups of aligned and random NCCs ($n = 8$/organization group) underwent uniaxial stretch using a planar testing machine (574LE2; TestResources; Shakopee, MN) (Fig. 1a). Circular NCCs cast in 12-well plates were cut into vertical strips (21 mm × 8 mm) and a grid of markers was drawn on each gel surface covering the area of the gel containing the DRGs (Fig. 1b). Together with visible DRGs, the markers were used to designate sub-regions of each gel for measuring local strains during mechanical loading. Gels were clamped in the test system, which was equipped with a bio-bath filled with PBS maintained at 37°C and a high-speed camera (Phantom-v9.1; Vision Research, Inc.; Wayne, NJ) to track the movements of the markers and the visible DRGs (Fig. 1a). Uniaxial stretch was applied to distract the NCCs by 4 mm (~25% strain) at 3.7 mm/s to simulate the subfailure strains shown to induce pain in vivo.[9,16] Aligned gels were stretched along the major direction of axonal outgrowth, as determined using a light microscope before the gels were positioned in the test system.

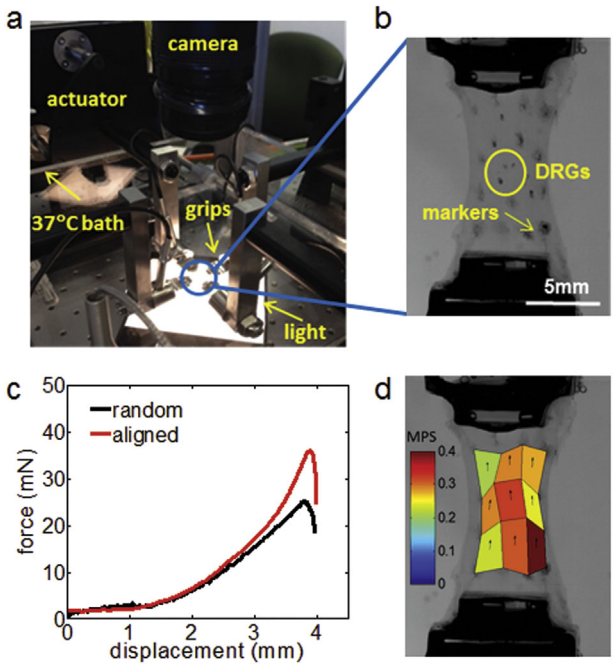

Figure 1. Overview of the experimental test set-up showing (a) the mechanical test system, (b) the high-speed image of a stretched NCC at maximum stretch of 4 mm, (c) representative force–displacement responses from a random gel (R7) and an aligned gel (A8), and (d) a representative strain map showing both the magnitude and direction of MPS for the NCC in (b) at its maximum stretch.

During loading of all gels, acquisition of force and displacement data (200 Hz) was synchronized with that of the high-speed images (200 frames/s). Force–displacement data were filtered with a 10-point moving average filter and the peak force was extracted (Fig. 1c). Based on the positions of fiducial markers and visible DRGs shown in the high-speed images, the maximum principal strain (MPS) in each of the four-node sub-regions of the NCCs was calculated using LS-DYNA (Livermore Software Technology Corp.; Livermore, CA) (Fig. 1d). Each of the peak force and average MPS across each gel's surface were compared between random and aligned NCCs with separate Student's *t*-tests. After tension, the gels that were held in their stretched position were immediately released from the testing machine, and returned to an unloaded state and washed with fresh PBS supplemented by 1% Pen-Strep (Thermo Fisher Scientific). They were then transferred into pre-warmed culture media supplemented by 1% Pen-Strep and incubated for 24 h to allow time for transcriptional and/or translational changes before undergoing fixation with 4% paraformaldehyde. Un-stretched NCCs having each of random and aligned collagen organization ($n = 7$/organization group) were included as controls for defining neuronal regulation of activation and nociceptive signals.

Control and stretched NCCs were immunolabeled for βIII tubulin to visualize neuronal morphology, pERK as a marker of neuronal activation and substance P as a measure of neuropeptide-mediated nociception. NCCs were blocked in 1% normal donkey serum with 0.3% Triton-X PBS for 2 h at room temperature. Then they were incubated overnight at 4°C with chicken anti-βIII tubulin (1:200; Abcam), rabbit anti-pERK (1:200; Cell Signaling Technology; Danvers, MA), and guinea pig anti-substance P (1:500; Neuromics; Bloomington, MN) primary antibodies. The next day, NCCs were fluorescently labeled with secondary antibodies for donkey anti-chicken Alexa Fluor 647 (1:1,000; Invitrogen), donkey anti-rabbit Alexa Fluor 555 (1:1,000, Invitrogen), and donkey anti-guinea pig Alexa Fluor 488 (1:1,000; Jackson ImunoResearch Labs; West Grove, PA). Images were taken using a Zeiss 710 confocal microscope (Carl Zeiss Microscopy) and processed in ImageJ (National Institutes of Health; Bethesda, MD) for background subtraction and intensity measurement. Three regions of interest (ROIs; 1,803 μm × 1,300 μm) containing only axons were randomly selected in each gel, in order to measure axonal expression of pERK and SP throughout the gel and corresponding the regions in which strains were quantified. Axons were outlined based on the positive βIII tubulin labeling; the average intensities of each of pERK and SP labeling were measured in those axons and normalized to their respective unloaded control values to ensure appropriate comparison between different experimental runs. The normalized pERK and SP intensity values were compared between groups by separate two-way analysis of variances (ANOVAs) and post-hoc Tukey HSD tests, with the collagen organization and loading group as the two factors.

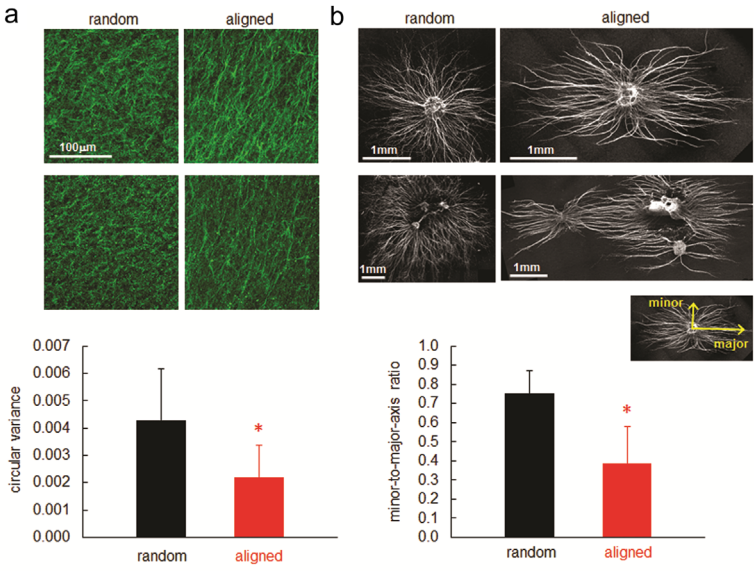

Figure 2. The orientation of collagen fibers and alignment of axons are more homogeneous in aligned NCCs than in random NCCs. (a) Representative confocal images demonstrate that the collagen fibers are oriented more parallel to each other in aligned NCCs as compared to the random NCCs. The scale bar is 100 μm and applies to both images. The circular variance obtained using polarized light imaging for the entire gel area is significantly lower ($^*p = 0.007$) in the aligned than random NCCs, indicating higher similarity in fiber angles in aligned gels. (b) Representative images show the isotropic axon orientation in random NCCs and directed axon outgrowth in aligned NCCs (scare bar = 1 mm). The ratio of axon alignment strength in the minor and the minor axes (axes of a representative DRG shown in the inset) is significantly lower ($^*p < 0.001$) in aligned NCCs than in random NCCs.

RESULTS

Magnetic alignment of collagen produces NCCs with more homogeneous collagen fiber orientation, accompanied by directed axon outgrowth (Fig. 2). Specifically, the circular variance of fiber angles measured over the entire gel area is significantly lower ($p = 0.007$) in the aligned NCCs than in the random NCCs (Fig. 2a). In addition, randomly distributed collagen fibers and parallel fibers are observed in unaligned and aligned NCCs, respectively (Fig. 2a), demonstrating higher structural anisotropy of the magnetically aligned collagen matrices. Immunolabeling of βIII tubulin indicates that axons in the aligned, but not random, NCCs exhibit a preferred outgrowth direction, which is apparent by visual assessment of the confocal images and is quantified by a significantly lower ratio ($p < 0.001$) of alignment strength between the minor and major axon orientation axes; aligned NCCs have a ratio of 0.39 ± 0.19 which is almost ½ that of the random NCCs which have a ratio of 0.71 ± 0.19 (Fig. 2b).

Varying the collagen organization in an NCC alters the peak force, but not the strain, of the loaded NCCs. The peak force of aligned gels (32.7 ± 13.9 mN) is significantly greater ($p = 0.025$) than the peak force sustained by random gels (21.1 ± 6.5 mN) at the same displacement (Table 1). However, there is no difference ($p = 0.886$) between the average MPS sustained by the NCCs with randomly organized collagen fibers (0.25 ± 0.06) and those with aligned fibers (0.26 ± 0.07) (Table 1).

Collagen organization also differentially upregulates pERK and SP expression at 24 h after NCC stretch (Table 1; Figs. 3 and 4). Immunolabeling of pERK in neuronal axons is greater ($p < 0.001$) than control levels after NCC stretch, regardless of whether the collagen fibers are randomly organized or aligned (Table 1; Fig. 3). The normalized intensity of pERK labeling in aligned gels is also greater ($p = 0.013$) than the level in random gels after equivalent stretches (Table 1; Fig. 3). Similar to pERK, SP labeling is significantly greater in both random and aligned NCCs relative to their respective un-stretched control levels ($p < 0.001$) (Table 1; Fig. 4). In contrast to pERK, the normalized expression of SP after stretch is not different between the two collagen organization groups ($p = 0.760$), despite the peak force being higher in the aligned gels (Table 1; Fig. 4).

DISCUSSION

Although ligaments, particularly spinal facet capsular ligaments, are increasingly recognized as pain

Table 1. Summary of Mechanics and Normalized Protein Levels for Random and Aligned NCCs

Random				
Sample	Peak Force (mN)	Average MPS[a]	Average pERK[b]	Average SP[b]
R1	30.8	0.313	3.09	1.22
R2	14.7	0.32	2.98	3.48
R3	14.0	0.282	3.05	3.13
R4	22.8	0.18	1.11	1.01
R5	23.4	0.226	0.95	1.18
R6	14.3	0.21	1.00	1.22
R7	28.2	0.189	1.36	1.67
R8	20.3	0.32	1.19	1.96
Mean	**21.1**	**0.255**	**1.84**	**1.86**
SD	**6.5**	**0.06**	**1.00**	**0.95**
Aligned				
Sample	Peak Force (mN)	Average MPS	Average pERK	Average SP
A1	39.9	0.213	2.59	1.59
A2	22.8	0.22	3.21	2.92
A3	13.7	0.203	3.56	1.57
A4	29.3	0.216	2.43	2.02
A5	54.8	0.402	1.93	2.41
A6	45.5	0.337	1.86	2.54
A7	19.5	0.258	2.33	2.38
A8	36.0	0.231	2.41	1.74
Mean	**32.7**[c]	**0.26**	**2.54**[c]	**2.15**
SD	**13.9**	**0.072**	**0.58**	**0.49**

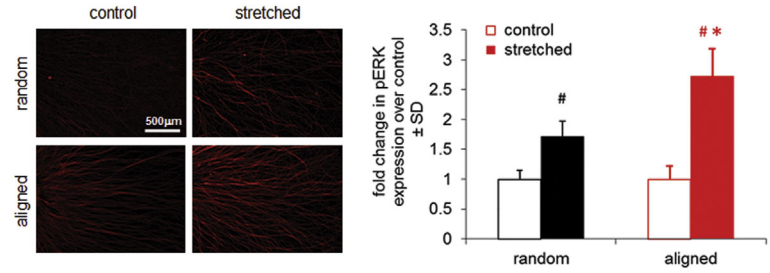

Figure 3. Stretch-induced increases in pERK expression differs between random and aligned NCCs. Representative images and quantification of normalized intensity of pERK exhibit significant increases in axonal pERK after NCC stretch in both random and aligned NCCs ($^{\#}p < 0.001$). That increase in pERK expression is significantly higher ($^{*}p = 0.013$) in aligned gels after stretch than in random NCCs after stretch.

sensors,[1,6–8,34] the structure–function relationships that underlie mechanically induced ligament pain are not well defined. This is partially due to the complex hierarchical organization of such tissues and their heterogeneous fibrous structure. This is the first study to show that collagen organization appears to regulate stretch-induced, pain-related signaling in neurons embedded in an extracellular matrix. Regional differences in collagen organization have been well-documented in human cervical facet capsular ligaments.[13,14] Recently, spatial correlation analysis suggests that this ligament contains sub-regions in which collagen fiber orientation is highly similar[13]; those sub-regions are approximately $^{1}/_{10}$ the size of the overall tissue domain and about 400 times larger than the diameter of the nociceptive free nerve endings that innervate the cervical facet capsular ligament.[13,35] The in vitro NCC model used here simulated two types of sub-feature domains in the cervical facet capsule—regions with irregularly organized fibers and regions with parallel collagen fibers. The width and gauge length of the stretched NCCs are ~2 orders of magnitude larger than the diameter of the embedded axon bundles (Figs. 1 and 2), which is similar to in vivo conditions.[13,35]

By applying a strong magnetic field around the collagen solution during gelation and embedding DRG explants in that gel, NCCs with collagen fibers preferentially oriented in one direction were created (Fig. 2a). To avoid exposing live DRGs to a strong magnetic field, the top collagen layer was added to encapsulate the DRGs in the aligned NCCs but it was not magnetically oriented. However, that collagen layer was 10 times thinner than the aligned substrate underneath the DRGs and was added 3 days after the initial DRG plating. While that collagen layer did not alter the parallel axonal outgrowth directed by the aligned collagen fibers (Fig. 2b), it had direct contact with the axons and may have affected the micromechanical environment of the neurons. Nevertheless, in the aligned NCCs, the axonal outgrowth resembles nerve fibers in the facet capsule that run along parallel collagen fibers.[1] In contrast, random NCCs simulating the irregular fibrous structure in that ligament exhibit uniform axonal outgrowth towards all directions (Fig. 2b). Although the in vitro NCC system only included Type I collagen and no other minor extracellular components, it did model the innervation of SP-positive afferent nociceptors (Fig. 4) that have been reported in sub-regions of the human cervical facet capsular ligaments that have both parallel collagen bundles and irregularly organized fibers.[1] By enabling the integrated assessment of macroscopic mechanics and neuronal responses, this work links tissue structural heterogeneity to the varied mechanical environment and signaling of embedded neurons in the context of facet joint pain.[9,14,15]

In addition to different axonal morphology in the DRG explants, differences in collagen organization also result in varied NCC mechanics during tensile loading. The higher peak force for the aligned NCCs than the random ones (Table 1) is likely because more fibers are aligned with the loading direction in the aligned gels. Previous studies incorporating fiber- and tissue-level mechanics in simulated collagen networks have shown that during uniaxial tension, fibers that are not initially oriented in the loading direction first rotate and bend under small macroscopic deformations before aligning and elongating along the stretch direction.[21,36] The transition from bending- to stretching-dominated deformation has been associated with strain hardening and ERK-mediated neuronal activation,[21,36] and likely occurs at a lower stretch level in the aligned gels, exerting high loads on the encapsulated neurons, possibly activating them, and for a longer duration. Despite differences in force, both the random and aligned NCCs experience comparable macroscopic strains (Table 1), which has been shown

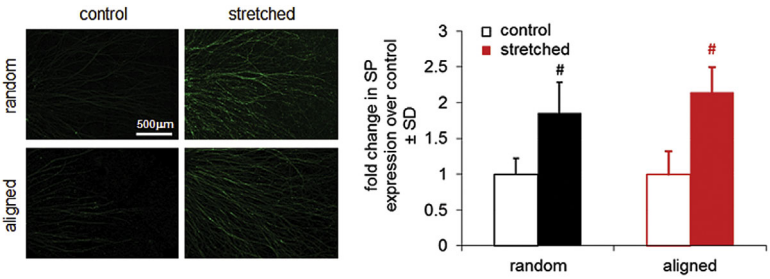

Figure 4. Substance P expression increases in both random and aligned NCCs after stretch. Representative images and quantification of normalized intensity demonstrate significant increases in axonal SP after NCC stretch in gels with both collagen organizations ($^{\#}p < 0.001$) relative to their respective controls.

to mediate neuronal excitability, ERK activation, and SP expression.[15,21,22] Because strains were similar across groups (random 0.25 ± 0.06 strain; aligned 0.26 ± 0.07 strain) (Table 1), any difference in protein expression that is observed here between random and aligned NCCs is not due to the macroscopic strains being different, but may be attributed to altered macroscopic force, axonal orientation or the micromechanical environment due to the varied collagen organization (Fig. 2; Table 1).

The increase in pERK expression in both random and aligned NCCs after loading (Fig. 3) is consistent with prior work finding that strains exceeding 0.11 activate ERK signaling in embedded neurons.[21] However, that prior work used only gels with randomly organized fibers. Since the two different collagen organizations appear to regulate pERK differently (Fig. 3), this work supports the role of initial matrix structure in mediating neuronal activation in response to macroscopic stretch. ERK phosphorylation can be induced by cellular deformation and is involved in nociception in neurons.[37–40] Indeed, pERK levels after stretch simulating painful loading in vivo in similar NCC systems with random fiber orientation is greater than that for physiologic stretch that is "non-painful."[9,21] Although ERK signaling is triggered in both random and aligned NCCs by stretch, the increase is even greater in the aligned gels than in the random gels (Fig. 3). This differential upregulation of pERK dependents on collagen organization is likely due to the fact that local axonal stretch and forces are greater in the aligned NCCs as a result of directed axon outgrowth and the larger tissue-level forces (Fig. 2; Table 1). Fibers aligned along the stretch axis experience higher strains,[21] and the afferents also in that direction can interact with surrounding collagen fibers via cell-collagen adhesion.[41,42] So, axons that are oriented in the loading direction in the aligned NCCs may undergo greater deformations due to higher local collagen fiber strains, leading to more robust ERK activation (Figs. 2 and 3). Further, the axons in aligned NCCs likely undergo greater forces that may directly affect ERK-mediated neuronal responses via integrin-signaling and cytoskeletal tension.[43,44] Although pERK can mediate neuroplasticity and neuronal excitability,[45–47] ERK activation is involved in many different signaling pathways that regulate various cellular cascades in addition to pain transmission.[48] In addition, since a variety of cells regulate ERK, the pERK measured in the current study is not specifically localized to nociceptive neurons. For this reason, it should be noted that the DRG explant culture system used here contains heterogeneous neuronal populations, including various mechanoreceptors and nociceptors, making it difficult to isolate responses in only the nociceptive neurons. Moreover, the heterogeneous cell populations in this primary neuronal culture, in addition to varied strain across the gel, may contribute to the variability observed in protein expression in different regions (Figs. 3 and 4). To better evaluate the effects of collagen organization on nociceptors, the expression of the neuropeptide SP was assessed since it is more specifically involved in pain signaling.

SP is produced by peptidergic neurons, which are a subset of primary afferents, that are primarily nociceptors and modulate pain after stretch injury of the capsular ligament.[24–26] SP has been previously found to increase in the innervated tissue and/or DRG in models of knee and facet joint pain.[16,34] In contrast to pERK, neuronal regulation of SP depends only on the applied tissue strain, despite gels sustaining significantly different forces (Fig. 4; Table 1). The macroscopic strains that increase axonal SP in both types of NCCs (Fig. 4; Table 1) directly map to facet capsule strains that induce pain in vivo.[9,16] That strain-dependent SP modulation, regardless of collagen organization, is consistent with its differential expression in DRG neurons in vivo in response to different magnitudes of facet injury and extents of pain.[16] Although the current findings suggest SP may be regulated by deformation and be less sensitive to stress inputs, only macroscale measurements were made here, and microstructural mechanics were not assessed. Defining the *local* (on the order of 10-100μm) biomechanical environment of the neurons would provide a more specific measure of the direct mechanical cues that neurons sense. For example, because fiber kinematics have been shown to affect neuronal activation,[21] relating those responses to fiber stresses and strains which can cause stress and strain concentrations on the neuronal cell surface may elucidate the mechanical basis for local axonal deformation and activation of SP-mediated nociception in tissues with varied collagen organization. Since it is challenging to experimentally measure the microscopic tissue stresses and strains during loading, computational models that estimate local mechanics[21,36] can provide insight into potential regulatory inputs and be helpful in evaluating how collagen organization mediates the translation of tissue-level mechanical signals into specific neuronal responses.

Although this study used an idealized simplified model whose structural anisotropy and fiber density are lower than those in sub-regions of the ligaments exhibiting long parallel collagen bundles, that NCC system enabled assessment of the relationships between collagen organization and neuronal responses in the context of facet capsular ligament pain. Indeed, the initial collagen fiber organization was found to not only modulate macroscopic mechanics and axonal outgrowth, but to directly and differentially regulate stretch-initiated *neuronal* signals. The differences in neuronal regulation of pERK and SP are possibly due to the fact that those molecules are involved in *different* cell signaling cascades, with SP being more nociceptive-specific[24–26] and pERK playing a role in a host of cellular responses to external stimuli, including regulation of neuronal activity and excitability.[37–40,48]

Fully understanding the local biomechanical factors that trigger the upregulation of these and other signaling molecules in different structural collagen matrices requires further investigation of the micromechanical environment and intracellular signaling pathways of afferents. In addition, future studies evaluating the effects of varied degrees of fiber alignment and density, which are not uniform within and between facet capsular ligaments, on neuronal responses and pain, would help with the understanding of the responses of the native ligaments and also provide more physiologic parameters for the NCC system to better simulate that tissue. Nevertheless, these findings show that collagen organization differentially modulates pain-related neuronal responses likely through the macro- and micro-scale mechanics. Current findings provide a possible structural basis for regional differences in tissue mechanics,[13,14,23] differential regulation of pain signaling proteins in afferents[9,16] and varied pain symptoms,[49] all observed in the case of facet capsular ligament injury. This study may also shed light more broadly on pain development in other joint ligaments, such as the hip and knee joint capsules.

AUTHORS' CONTRIBUTIONS

SZ, SS, and BAW all contributed to the formation of the study design and writing and editing the manuscript. SZ, assisted by SS, conducted the experiments involving neuronal culture, mechanical testing, and imaging. SZ and SS performed the data analysis and interpreted and integrated findings in collaboration with and with input from BAW. BAW and SZ obtained funding to support this work.

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