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Repeated High Rate Facet Capsular Stretch at Strains That are Below the Pain Threshold Induces Pain and Spinal Inflammation With Decreased Ligament Strength in the Rat

Repeated loading of ligamentous tissues during repetitive occupational and physical tasks even within physiological ranges of motion has been implicated in the development of pain and joint instability. The pathophysiological mechanisms of pain after repetitive joint loading are not understood. Within the cervical spine, excessive stretch of the facet joint and its capsular ligament has been implicated in the development of pain. Although a single facet joint distraction (FJD) at magnitudes simulating physiologic strains is insufficient to induce pain, it is unknown whether repeated stretching of the facet joint and ligament may produce pain. This study evaluated if repeated loading of the facet at physiologic nonpainful strains alters the capsular ligament's mechanical response and induces pain. Male rats underwent either two subthreshold facet joint distractions (STFJDs) or sham surgeries each separated by 2 days. Pain was measured before the procedure and for 7 days; capsular mechanics were measured during each distraction and under tension at tissue failure. Spinal glial activation was also assessed to probe potential pathophysiologic mechanisms responsible for pain. Capsular displacement significantly increased ($p = 0.019$) and capsular stiffness decreased ($p = 0.008$) during the second distraction compared to the first. Pain was also induced after the second distraction and was sustained at day 7 ($p < 0.048$). Repeated loading weakened the capsular ligament with lower vertebral displacement ($p = 0.041$) and peak force ($p = 0.014$) at tissue rupture. Spinal glial activation was also induced after repeated loading. Together, these mechanical, physiological, and neurological findings demonstrate that repeated loading of the facet joint even within physiologic ranges of motion can be sufficient to induce pain, spinal inflammation, and alter capsular mechanics similar to a more injurious loading exposure. [DOI: 10.1115/1.4040023]

Keywords: facet capsular ligament, repetitive loading, pain, inflammation, failure

Introduction

Loading to joints and their constituent tissues, particularly ligaments, during high demand repetitive activities like contact sports and long-term occupational tasks can result in limited ranges of motion, joint weakness, and even persistent pain [1,2]. Such injuries due to repetitive loading of joint tissues and surrounding musculature have been collectively termed repetitive strain injuries [3] and have been reported to affect up to 40% of the adult population [4]. Repetitive strain injuries are also attributed to several musculoskeletal disorders including work-related stress syndrome [5] and cumulative trauma disorder [6,7]. Although the loading paradigms in many of these repetitive injuries are well within the physiologic ranges of motion of the tissues, repetitive loading has been hypothesized to induce cumulative microtraumas that over time exceed the physiologic limit of the tissues [8]. For example, repetitive loading of the inferior glenohumeral ligament during physiologic motions has been reported to result in the gradual stretching of the ligament that induces laxity and other structural damage [1]. Although repeated loading at physiologic levels has been attributed to joint instability and pain onset [1,2,8], the biomechanical and pathological mechanisms that are responsible for such modifications to joint tissues remain largely unstudied, particularly in the context of pain.

Both the frequency of cyclic loading and the number of repetitions have been reported to have effects on the accumulation of tissue damage and pain following repeated loading within physiologic ranges [1]. Several studies examining repetitive loading of lumbar supraspinous ligaments have hypothesized that exposure to cyclic loading at rates and forces at the higher range of that tissue's physiologic limits without insufficient rest can result in increased ligament creep and laxity that corresponds to tissue damage [1,7,9,10]. Within the cervical spine, the facet capsular ligaments (FCLs) can exceed their physiologic limits during high acceleration spine loading [11–13]. For example, for isolated cervical spines undergoing 8*g* acceleration, the C6/C7 facet capsular ligament sustained strains that peaked at as high as 39.9±26.3% exceeding those strains during physiologic spine flexion/extension (10.7±9.3%) [11]. A similarly high rate of loading to the facet capsule, corresponding to 500%/s, at magnitudes comparable to those during such an injurious acceleration have been found to induce pain in the rat [14–19]. To date, the influence of strain rate on injury risk has been contested, with sports-related injuries estimated to occur at both lower (1%/s) [20,21] and higher (500%/s) [22,23] strain rates. Further, studies of isolated human cervical spine ligaments support the notion that the mechanical properties

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of those ligamentous tissues exhibit strain-rate dependence [24]. Together, these reports suggest that loading at strain rates beyond those sustained during physiologic scenarios may alter the tissue's mechanical response. In addition to high strain rate loading of the facet capsule being sufficient to alter joint laxity and induce pain [25], the rabbit anterior cruciate ligament has been shown to exhibit ligament laxity and lowered stiffness after loading at a rate of 100%/s [26]. Although these studies suggest that a higher loading rate may be sufficient to alter the overall structure of a ligament, it is not known whether that same effect is induced by repeated high rate loading of the capsule at physiologic strains is sufficient to alter the capsular ligament and/or induce pain.

Facet joint distraction (FJD) in the rat has been shown to induce capsular injury and macrostructural damage and even pain, but the extent of which is dependent on the magnitude of the facet joint stretch [27–30]. The facet joint and its capsular ligament are innervated by Aδ and C-fiber afferent fibers that have been shown to act as low-threshold mechanoreceptors during physiologic joint motion and as active nociceptors at tissue strains greater than 25–47% [31–34]. Only a magnitude of capsular stretch above $57\pm0.11\%$ strain produces both ligament laxity [27,35] and activation of the afferent fibers [27,33,36] together with pain and spinal inflammation [27,36]. Spinal inflammation, including the activation of ionized calcium binding adaptor molecule 1 (Iba1)-positive microglia and glial fibrillary acidic protein (GFAP)-positive astrocytes in the spinal cord, is involved in the development of pain [27,37,38] and has been reported in association with facet stretches imposing capsular strains of $27.9\pm11.9\%$ but not physiologic strains of $8.1\pm2.4\%$ [14,27]. In addition to macrostructural tissue strains, joint afferents have been shown to be activated in response to microstructural changes in the local collagen organization, which surrounds them in the ligament [25,39–41]. Although exposure to a single facet stretch at physiologic strain does not change the microstructure of the capsular ligament [40] and is not sufficient to activate high-threshold nociceptive afferents [34], it is possible that exposure of the facet capsule to repeated loading could induce microstructural damage accumulation that could alter the local environment of the joint afferents and lower their threshold for activation. Whether repeated high-rate facet joint loading at physiologic strains is sufficient to induce pain has not been investigated.

The objective of this study was to determine if repetitive loading of the facet capsular ligament at physiological strains is sufficient to induce modifications in the biomechanical properties of the capsular ligament, pain, and/or spinal inflammation. This study utilized an established rodent model of FJD, which imposes a facet capsular stretch to physiologic capsular strains (10%) that has previously been characterized and found to be below the threshold to elicit pain (subthreshold) [14,16]. A repeated loading paradigm consisting of a subthreshold FJD (STFJD) or sham procedure performed first on day 0 and then again on day 2 was used to simulate repetitive strain injuries of repeated physiologic motions with insufficient recovery. The in vivo biomechanical response of the capsular ligament was measured during each distraction to quantify the severity of tissue loading and the effects of a single nonpainful FJD on the ligament's response during subsequent loading. Additionally, pain responses were measured using mechanical hyperalgesia before the first distraction and for up to 7 days thereafter. At day 7, the isolated cervical facet joints underwent tensile loading to failure in order to characterize their failure properties after repeated exposure to loading in order to test the hypothesis that repeated physiologic loading is sufficient to alter the ligament's mechanical response leading to joint instability. Since increased spinal expression of the microglia marker Iba1 and the astrocyte marker GFAP are hallmarks of glial activity related to pain onset and maintenance [27,37,38], spinal cord tissue was also harvested at day 7 to quantify spinal glial activation in order to begin to probe potential pathophysiological mechanisms responsible for pain onset following repetitive facet capsular loading.

Materials and Methods

Study Approach. All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and carried out according to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain [42]. Separate groups of rats underwent repeated exposures, separated by 2 days (Fig. 1). One group of rats (STFJDX2; $n=12$) received a subthreshold FJD (STFJD1) on day 0 and a second subthreshold FJD (STFJD2) again on day 2 (Fig. 1(*a*)). In addition, surgical control groups were included to account for the effects of exposing the facet capsule for distraction. Following a similar paradigm to the repeated subthreshold FJD exposures, rats (SHAMX2; $n=12$) underwent two separate sham surgeries on day 0 (SHAM1) and day 2 (SHAM2). Pain responses were quantified by measuring mechanical hyperalgesia in the bilateral forepaws before the first STFJD1 or SHAM1 exposures (baseline; day 0), after those but before the STFDJ2 or SHAM2 second exposures (days 1 and 2), and subsequently on postoperative days 3 and 7 (Fig. 1(*a*)). To quantify the severity of the biomechanical insult to the capsular ligament during each of the single subthreshold FJD (STFJD1) and second subthreshold FJD (STFJD2), the vertebral and capsular displacements, average and peak maximum principal strain, peak force, and stiffness were determined for each FJD and separately compared (Fig. 1(*b*)). At day 7, in a subset of rats from each group (STFJDX2, SHAMX2; $n=6$/group) the spinal column from the C4 level to the T2 level was removed en bloc and underwent tensile failure testing, with the same mechanical metrics measured for the isolated right C6/C7 facet capsule as described for the in vivo measurements (Fig. 1(*d*)). In the remaining subset of rats (STFJDX2, SHAMX2; $n=6$/group), spinal cord tissue from the C6 level was harvested and labeled for Iba1 and GFAP to determine the extent of microglial and astrocytic activation following repeated loading (Fig. 1(*c*)).

Repeated In Vivo Facet Joint Distraction and In Vivo Biomechanical Analyzes. All surgical procedures were performed using male Holtzman rats (Envigo, Indianapolis, IN) weighing 350–425 g at the start of the study. Rats underwent either a FJD of the C6/C7 facet joint with a magnitude (STFJD1) that has been previously found to be nonpainful and having not effects on the subsequent ligament structure or collagen organization [25,40] or sham surgery (SHAM1) procedure on day 0 followed by a second similar subthreshold FJD (STFJD2) or sham (SHAM2) procedure on day 2. Surgical procedures were performed as previously described [14,16,43,44]. Under inhalation isoflurane anesthesia (4% for induction; 2.5% for maintenance), the dorsal aspect of the cervical spine was cleared of all paraspinal musculature to expose the bilateral C6/C7 laminae, spinous processes, and facet joints. To attach the cervical spine to the custom-built loading device [14,17,19,43,44], the interspinous ligaments, and ligamenta flava from C5-T1 were transected. To ensure repeatability between the first and second distraction, the interlaminar distance of the C6/C7 joint was adjusted to 2.54 mm for each rat upon being placed on the loading device, which corresponds to the average interlaminar distance in naïve rats of this size [25] and is the customary initial position used previously for applying FJD [27,36]. The bilateral C6/C7 facet joints were distracted by holding the C7 vertebrae in place while translating the C6 vertebrae rostrally by 0.2 mm at 500%/s [14,19,43–45]. Sham rats underwent the same surgical procedures with attachment to the loading device, but no joint distraction was applied. After each surgery, incisions were closed using 3-0 polyester suture and surgical staples; rats were monitored during recovery in room air.

To quantify facet capsule deformation during facet joint distraction, fiducial markers were placed on the right C6/C7 laminae and vertebrae before loading and tracked at 500 Hz during loading using high-speed video recording (Phantom v4.3 CCD Camera, Vision Research Inc., Wayne, NJ) during each distraction.

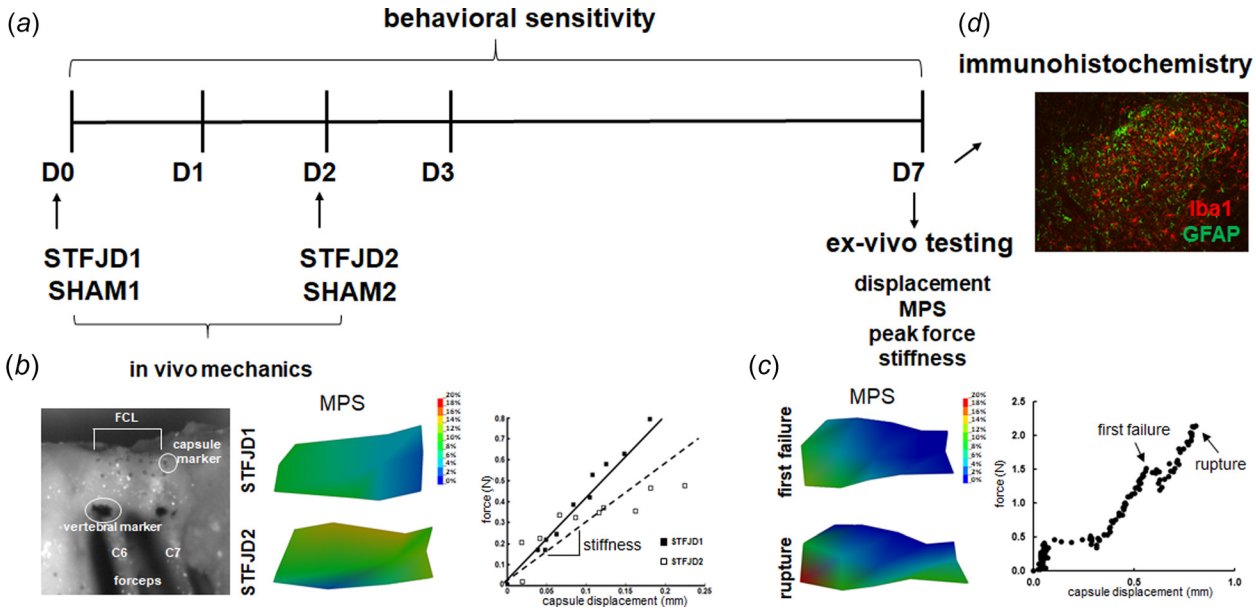

Fig. 1 (*a*) **A STFJD1 or sham control (SHAM1) procedure was performed on day 0 and repeated 2 days later day 2 (STFJD2, SHAM2). Behavioral sensitivity was measured on day 0 before procedures and on days 1, 2, 3, and 7. (*b*) For the FJD, microforceps were affixed to the C6 and C7 vertebrae to distract the C6/C7 FCL. Markers on the FCL were used to measure the capsule's displacement, MPS, peak force, and stiffness during FJD. (*c*) In a separate set of rats, spinal columns were harvested to measure mechanical properties under tensile failure of the right FCL; displacements, MPS, peak force, and stiffness were measured in the right FCL at first failure and rupture. (*d*) On day 7 in the remaining subset of rats, spinal cord tissue was harvested for immunohistochemistry labeling for the glial markers Iba1 and GFAP.**

Similarly, load and displacement data were simultaneously recorded at 500 Hz using a load cell (WMC Series 5N, Interface Inc., Scottsdale, AZ) and linear variable differential transformer (S-DVRT-24, Microstrain Inc., Williston, VT). The centroid of each fiducial marker was tracked and quantified using PROANALYST software (3D Professional Edition 1.5.7.9, Xcitex Inc., Woburn, MA). Vertebral displacement for each FJD was defined as the relative displacement of the centroid of the C6 and C7 vertebral markers and the corresponding capsule displacement was defined as the average resultant displacement of the fiducial markers from the rostral edge of the capsule to the caudal edge [16,30,44]. Maximum principal strain (MPS) of the facet capsular ligament at 0.2 mm was calculated for each distraction, using the capsule marker positions and displacements using LS-DYNA (Livermore Software Technology Corp., Livermore, CA) as previously described [27,30,46]. Both the average and maximum MPS across the capsule were calculated for each FJD. The force data from the load cells were used to generate force-capsule displacement curves for each FJD and the maximum force was during each distraction was calculated as the peak force [25]. Using those same force-displacement curves, the facet capsular stiffness was defined from the linear fit through that curve (Fig. 1), using a customary script in MATLAB (Mathworks, Natick, MA) [25,35]. The vertebral and capsular displacements, average and maximum MPS, peak force and stiffness were separately compared between the STFJD1 and STFJD2 exposures for each rat using separate paired t-tests for each outcome.

Assessment of Behavioral Sensitivity. Behavioral sensitivity was measured by assessing mechanical hyperalgesia in the bilateral forepaws for each rat using previously described methods [44,47,48]. The withdrawal threshold to a mechanical stimulus to the forepaw was quantified before surgery (baseline; day 0) and on days 0, 1, 2, 3, and 7 for the STFJDX2 and SHAMX2 groups (Fig. 1). On each day of behavioral assessment, rats were acclimated to the testing environment for 20 min prior to stimulation with an ascending series of von Frey filaments (Stoelting, Wood Dale, IL) from 0.6 g to 26 g [19,44,48]. Each forepaw was stimulated with an individual filament five times before moving to the next filament of a higher strength; the lower of two consecutive filaments eliciting a positive withdrawal response of either paw licking or lifting was taken as the threshold for the paw. Thresholds were recorded in three separate testing rounds separated by a 10-min recovery period between rounds. Withdrawal thresholds from the bilateral paws were recorded and averaged on each test day for each rat given since the procedures are imposed to the bilateral C6/C7 facet joints. Differences in averaged withdrawal thresholds between the first subthreshold FJD or sham procedure (STFJD1 or SHAM1) and the second subthreshold FJD or sham procedure (STFJD2 or SHAM2) were compared using a two-way repeated measures analysis of variance (group × day) with Tukey's honest significant difference test (JMP Prov15.0.2, SAS Institute Inc., Cary, NC).

Ex Vivo Facet Failure Testing and Biomechanical Analyzes. After behavioral testing on day 7, spinal columns were harvested from a subset of rats (STFJDX2, SHAM2; $n = 6$/group) to undergo ex vivo failure testing. Rats were given an overdose of sodium pentobarbital (65 mg/kg) and transcardially perfused with phosphate buffered saline. The spinal column from C4-T2 was removed en bloc and stored at −20 °C. For mechanical testing, frozen spinal columns were thawed, rehydrated in saline and cleared of the paraspinal musculature surrounding the C6 and C7 laminae. Both the C6/C7 intervertebral disc and the left facet capsular ligament were transected in order to isolate the right C6/C7 facet capsular ligament. Mechanical testing was performed using the same custom facet joint loading device that was used for the in vivo FJDs and has been used previously for facet failure testing [16,25,35,49]. Fiducial markers were placed on the right C6/C7 capsular ligament and the interlaminar distance was adjusted to 2.54 mm to match the same interlaminar distance as used as the reference position in the in vivo distractions and for the joint in situ [25]. Each specimen underwent 60 cycles of preconditioning from 0 to 0.2 mm of distraction at 500%/s. Following preconditioning, specimens underwent tensile loading at 500%/s until gross ligament failure occurred, as confirmed by visible rupture of

Table 1 Summary of in vivo FCL mechanics during the first and second FJD and pain responses (withdrawal threshold) on the following day (mean ± S.D.)

	Rat #	Vertebral displacement (mm)	Capsule displacement (mm)	Average MPS (%)	Max MPS (%)	Peak force (N)	Stiffness (N/mm)	Withdrawal thresholds (gf)
STFJD1	20	0.22	0.06	5.30	7.02	1.55	27.83	8.83
	31	0.22	0.08	8.46	14.04	0.72	10.09	17.73
	33	0.22	0.08	10.09	17.50	0.72	4.389	10.67
	38	0.17	0.13	6.51	8.66	1.70	12.21	18.67
	41	0.64	0.29	19.60	22.92	1.01	4.005	17.16
	44	0.21	0.16	12.55	25.63	0.56	4.426	20.50
	59	0.29	0.12	22.2	40.42	1.23	12.34	15.00
	61	0.08	0.08	0.06	0.13	0.58	17.39	21.50
	62	0.49	0.19	13.01	17.81	1.22	5.651	8.00
	65	0.31	0.10	5.74	11.62	1.55	3.953	17.83
	66	0.2	0.10	6.47	10.68	1.56	5.116	13.16
	67	0.26	0.12	7.95	16.27	1.57	6.357	12.83
	Average ± S.D.	0.28 ± 0.15	0.13 ± 0.06	9.83 ± 6.22	16.06 ± 0.35	1.16 ± 0.43	9.48 ± 7.2	15.3 ± 4.45
STFJD2	20	0.14	0.08	5.17	9.07	1.77	25.45	3.67
	31	0.30	0.08	9.43	19.56	0.71	7.178	6.50
	33	0.33	0.22	10.05	15.14	0.6	2.602	6.33
	38	0.21	0.13	10.18	16.95	0.92	6.306	9.16
	41	0.47	0.22	10.48	21.19	0.65	2.889	12.50
	44	0.22	0.15	7.79	10.56	0.58	4.907	15.33
	59	0.30	0.22	25.64	46.67	1.62	9.516	6.667
	61	0.37	0.17	19.14	54.36	1.31	6.631	11.00
	62	0.52	0.23	16.71	30.46	1.31	5.847	6.00
	65	0.42	0.18	15.81	21.58	1.52	2.673	10.50
	66	0.30	0.25	10.36	18.53	1.50	3.293	9.50
	67	0.35	0.21	17.16	23.89	1.43	2.716	9.33
	Average ± S.D.	0.33 ± 0.11	0.18 ± 0.06	13.16 ± 5.77	24.01 ± 13.72	1.16 ± 0.44	6.67 ± 6.32	8.87 ± 3.23
	p-value	0.1394	***0.0194**	0.1397	0.1208	0.9697	***0.0082**	***0.0007**

Note: Bold p-values (*) denote mechanical parameters with significant differences between STFJD1 and STFJD2.

the capsule [16,25,35]. Vertebral displacement, capsular displacement, average MPS, maximum MPS, peak force, and stiffness at both the first occurrence of tissue failure and also at complete tissue rupture were quantified for tensile loading to the right C6/C7 facet capsule using described for the in vivo mechanical analyzes [14,19,43–45]. The first failure was defined as the first drop in force of the force-displacement curve and rupture was defined as the maximal drop in force after which there is no recovery of force with increasing capsule displacement. Each of the metrics describing the mechanical properties of the isolated facet capsule were separately compared between the STFJDX2 and SHAMX2 groups at first failure and rupture using Student's t-tests.

Spinal Cord Immunohistochemistry. After behavioral testing on day 7, the spinal cords from the remaining rats were collected to evaluate the extent of glial activation using immunohistochemistry. Rats were deeply anesthetized with an overdose of sodium pentobarbital (65 mg/kg) and transcardially perfused with phosphate buffered saline and 4% paraformaldehyde. Following perfusion, the C6 segment of the cervical spinal cord was harvested and postfixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose for 1 week at 4 °C and embedded in optimal cutting temperature Medium (Sakura Finetek, Torrance, CA) for cryosectioning. That fixed spinal cord tissue was sectioned axially at a 14 μm thickness and thaw-mounted directly onto slides. Spinal cord tissue was also harvested from the C6 level of normal naïve rats ($n = 2$) and included in tissue processing for comparison.

Spinal cord sections were fluorescently co-immunolabeled for both Iba1 and GFAP to label positively microglia and astrocytes, respectively. Slide-mounted tissue sections were blocked in 5% normal donkey serum (Vector Laboratories, Burlingame, CA) with 0.3% Triton X-100 (Bio-Rad Laboratories, Hercules, CA) for 1 h at room temperature. Slides were then incubated overnight at 4 °C with rabbit anti-Iba1 (1:1000; Wako USA, Richmond, VA) and mouse anti-GFAP (1:500; Millipore, Billerica, MA), followed by a 2-h incubation at room temperature with donkey anti-rabbit 546 and donkey anti-mouse 488 secondary antibodies (1:500; Invitrogen, Waltham, MA). The superficial dorsal horns of 4–6 spinal cord sections from each rat were imaged at 20× using an Olympus 5 × 51 microscope (Olympus, Center Valley, PA). Each of Iba1 and GFAP expression was separately quantified in images that were uniformly cropped to include the superficial laminae of the spinal dorsal horn (750 × 250 pixels) using a custom densitometry MATLAB script (Mathworks, Natick, MA) as previously described [14,50]. In order to quantify the extent of positive spinal Iba1 and GFAP expression, a separate pixel intensity threshold was set to include each of positive Iba1 or GFAP labeling in the C6 tissue sections of naïve rats, and kept constant for analyses in the STFJDX2 and SHAMX2 groups. The percentage of total pixels in each tissue section that was above the respective threshold for positive labeling in naïve tissue was taken as the percentage of positive pixels of that label [14,27,43,44]. The percentage of positive pixels for each label was averaged separately across tissue sections for each rat in each of the STFJDX2 and SHAMX2 groups. The percentage of positive pixels of Iba1 and GFAP was separately compared between groups using a one-way analysis of variance with Tukey's test.

Results

Although the vertebral displacement was not different between STFJD1 and STFJD2, the capsular displacement was significantly ($p = 0.019$) higher during the second distraction (STFJD2) compared to the first (STFJD1) (Table 1). There were no significant differences between the average MPS, maximum MPS, and peak force between the first (STFJD1) and second (STFJD2) applications of distraction. However, the stiffness during the second application of distraction (STFJD2) was significantly ($p = 0.008$) lower at 6.67±6.32 N/mm than during the first distraction (STFJD1), which was 9.48±7.2 N/mm. The withdrawal thresholds or pain responses measured on the day after each STFJD

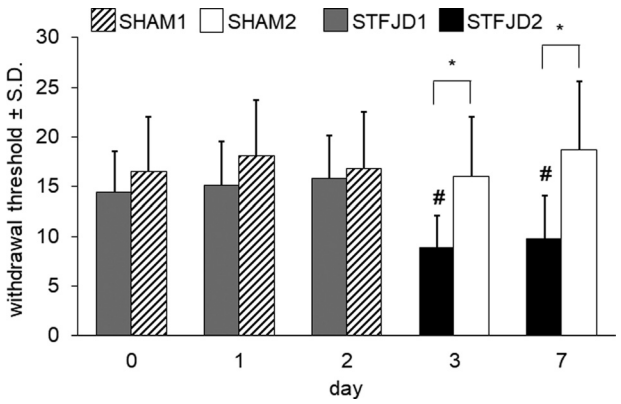

Fig. 2 After a single STFJD1, withdrawal thresholds were not different from baseline for either day 1 or day 2. However, after the second STFJD2, the threshold measured on day 3 was significantly lower than baseline and days 1 and 2 (#$p < 0.035$) and remained lower also at day 7, which was significantly different than baseline, day 1 and day 2 (#$p < 0.048$). Although on days 0, 1, and 2 there was no difference between thresholds for the STFJD1 and SHAM1 groups, thresholds after STFJD2 were significantly lower than after a second sham procedure (SHAM2) on both days 3 and 7 (*$p < 0.001$).

were also significantly different ($p = 0.0007$) between the STFJD1 and STFJD2 groups.

Neither a single subthreshold FJD (STFJD1) nor a sham procedure (SHAM1) on day 0 altered the withdrawal threshold from baseline on days 1 or 2 (Fig. 2). However, on day 2 after the second application of the subthreshold FJD (STFJD2), the withdrawal threshold was significantly lower compared to thresholds at baseline ($p < 0.035$) and on days 1 and 2 ($p < 0.001$) (Fig. 2). Those thresholds remained significantly ($p < 0.048$) lower than baseline and days 1 and 2 through day 7. In addition, the average withdrawal threshold after the second distraction (STFJD2) was also significantly lower on both days 3 ($p < 0.0001$) and day 7 ($p < 0.0001$) than the threshold of rats that received a second sham procedure (SHAM2) (Fig. 2). There were no differences detected between the first (SHAM1) and second (SHAM2) control procedures on any day tested.

The failure responses of both groups were largely similar at the first occurrence of failure, and slightly different at gross tissue rupture (Fig. 3). Visible tears in the capsule were evident in the lateral aspect of the capsular ligament in 4 of the rats in the STFJDX2 group and 5 in the SHAMX2 group. Similarly, complete tissue rupture was evident predominantly in the lateral aspect of the capsular ligament in 5 rats in each of the STFJDX2 and SHAMX2 groups. Both the vertebral and capsular displacements were not different between groups at the first occurrence of failure (Fig. 3). The same was true for the average MPS and maximum MPS, as well as stiffness at first failure (Fig. 3). However, the peak force at the first failure for the STFJDX2 group (0.92 ± 0.45 N) was significantly ($p = 0.028$) lower than that of the SHAMX2 group (2.44 ± 1.16 N) (Fig. 3). The peak force at rupture was also significantly ($p = 0.014$) decreased in the STFJDX2 group compared to the SHAMX2 group (Fig. 3). The vertebral displacement at tissue rupture for the group undergoing repeated subthreshold distractions was also significantly decreased ($p = 0.041$) compared to a repeated sham exposure despite the capsular displacement being unchanged (Fig. 3). Similar to first failure, no differences were detected at rupture for the average MPS, maximum MPS, and stiffness between the STFJDX2 and SHAMX2 groups.

Repeated subthreshold facet distractions (STFJDX2) that induce pain at day 7 (Fig. 4) also increased glial activation in the spinal cord above those levels observed in the repeated sham surgical group (SHAMX2) (Fig. 4). The increased expression of the astrocytic marker, GFAP, and microglial marker, Iba1, were most evident in the superficial dorsal horn of the spinal cord (Fig. 4). GFAP expression was $35.1 \pm 3.3\%$ greater in the STFJDX2 group than expression levels in the SHAMX2 group ($p = 0.007$) and $56.5 \pm 4.4\%$ times greater than normal expression ($p = 0.0001$) (Fig. 4). Similarly, Iba1 expression was also significantly increased after a repeated subthreshold FJD compared to levels

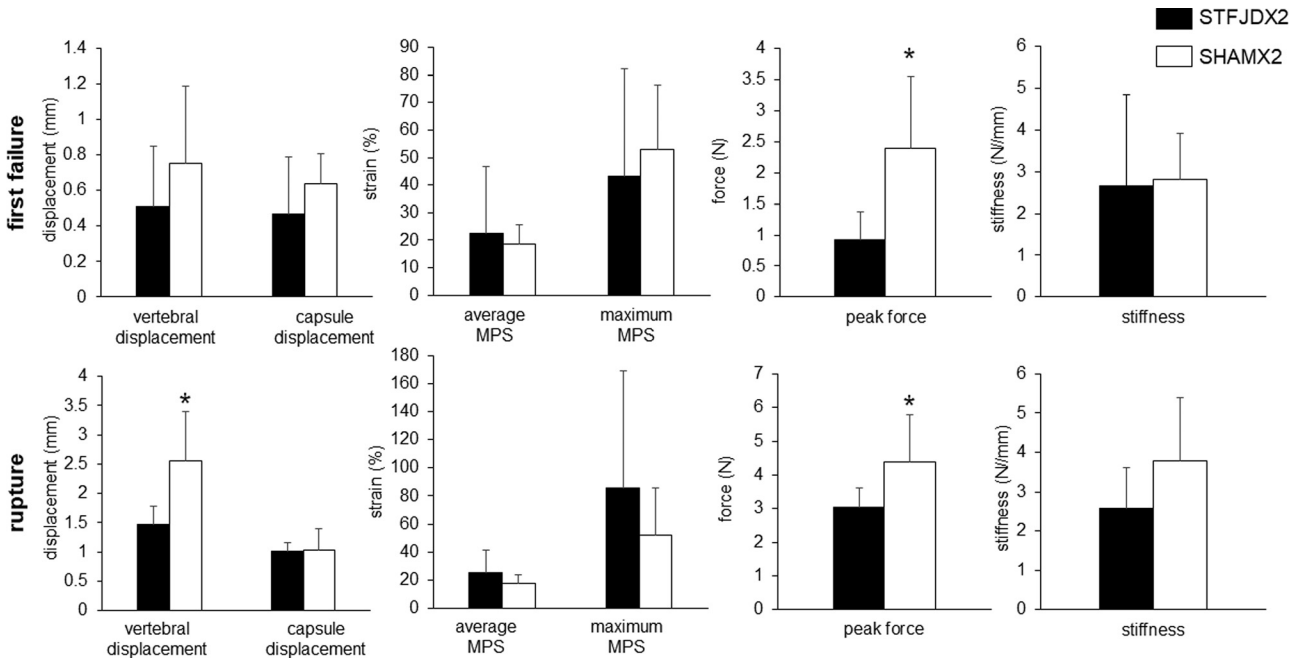

Fig. 3 At the first failure, only the peak force was significantly altered, with a significant reduction in peak force in the STFJDX2 group (*$p = 0.028$) compared to the SHAMX2 group. There were no differences detected in any other biomechanical metric measured at first failure. At tissue rupture, the vertebral displacement was also significantly lower (*$p = 0.041$) in the STFJDX2 group compared to the SHAMX2 group; peak force remained significantly decreased at tissue rupture (*$p = 0.014$) following repeated STFJDs.

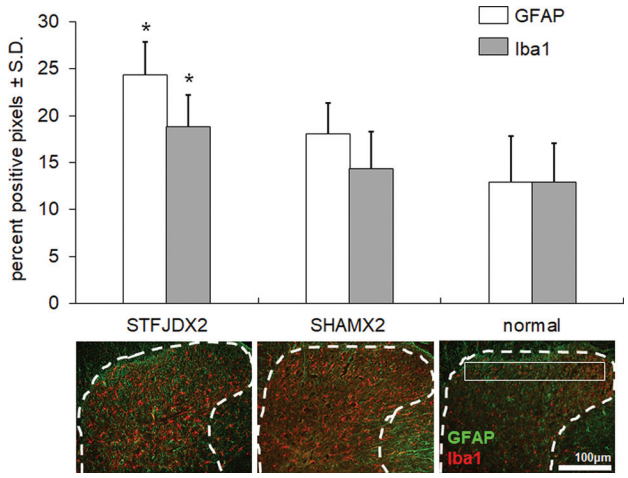

Fig. 4 At day 7, increased expression of both GFAP (green) and Iba1 (red) was evident after repeated subthreshold loading in the spinal cord dorsal horn (dashed line). GFAP significantly increased in the superficial dorsal horn (white box) of the STFJDX2 group over both the SHAMX2 (*$p = 0.007$) and normal (*$p = 0.0001$) groups. Similarly, Iba1 expression significantly increased in the superficial dorsal horn after repeated subthreshold loading over both the SHAMX2 (*$p = 0.013$) and normal (*$p = 0.0004$) groups. Representative images indicate the greatest GFAP and Iba1 labeling occurs in the superficial dorsal horn of the STFJDX2 group.

after repeated sham exposures ($p = 0.013$) and in normal tissue ($p = 0.0004$). In addition, the expression levels of GFAP and Iba1 in the SHAMX2 group were not significantly different from normal levels.

Discussion

A high-rate subthreshold FJD at a magnitude of stretch that ordinarily does not induce pain or any macrostructural changes to the capsular ligament [25,27], when repeated with only 2 days rest was sufficient to induce pain, spinal inflammation, and altered ligament failure properties (Figs. 2–4). Further, it appears that even a single subthreshold FJD at 500%/s alters the ligament's response to subsequent loading 2 days later by increasing the associated joint displacements and exhibiting decreased stiffness (Table 1). At day 7 after repeated joint loading, in the presence of pain (Fig. 2), there was also increased activation of spinal glia (Fig. 4). Increased activation of astrocytic and microglial cells in the spinal cord is a hallmark of the mechanisms of central sensitization that are involved in pain maintenance [37,38] and has been reported after a single exposure to an injurious and more severe loading of the facet joint [27,50,51]. Repeated loading of the facet joint also reduced the force at both first occurrence of tissue failure and at complete ligament rupture (Fig. 3), suggesting that such repetitive loading compromises the ligament's mechanical properties and response to subsequent loading. Together, these data suggest that repeated high rate tissue loading even within physiologic ranges of motion is sufficient to induce pain and may even be pathologically similar to a single more injurious insult.

The magnitude of facet stretch and associated capsular deformation during loading has been shown to relate to the extent of pain [16,31,36]. A single tensile stretch of the facet capsule at low accelerations and at a strain sustained during physiological bending (around 6%) [11,15] has been shown to neither alter ligament stiffness during loading nor induce laxity [25], suggesting it has no effect on the ligament's structure or function. In contrast, in this study, a single subthreshold FJD at 500%/s did lower the capsular ligament's stiffness during a *second* FJD distraction (Table 1), suggesting that the initial high-rate subthreshold stretch modifies its biomechanical response to subsequent loading. Although these data suggest that a single high-rate STFJD is sufficient to alter the capsule's biomechanical response to its subsequent loading, pain responses are *only* detected following STFJD2 (Table 1 and Fig. 2), suggesting that it may develop via the ligament undergoing greater stretch and having a decreased stiffness during STFJD2. Of note, the prior work reporting no changes was an ex vivo test using an isolated facet joint that also calculated the *tangent* stiffness at a rate (0.08 mm/s) 2–3 orders of magnitude lower [25] than that used in this in vivo study (15 mm/s). Given that viscoelastic tissues like ligaments have been shown to exhibit strain rate-dependent stiffness responses [52] that are higher at faster loading rates [53], it is possible that the altered stiffness responses between that study and the current one is more reflective of the difference in rates between the two studies. It is also possible that the time period between the two FJDs used here is insufficient for recovery of the tissue, collagen, and/or neuronal components in the joint to (re)equilibrate of a high-rate loading. Since the second FJD was only 2 days after the initial exposure, the ligament may not have sufficiently recovered, a scenario commonly experienced with repetitive strain injuries [1,8]. The separation time between FJDs was chosen since it is enough time to detect any measurable changes in pain responses after the first exposure (Fig. 1); yet, it may not have been enough time for putative microtraumas in the ligament to recover, compromising the capsular ligament's response and leaving it vulnerable to damage accumulation. Although capsular ligament damage was not directly evaluated in this study, the altered mechanical responses (Table 1 and Fig. 3) do provide proxy data for such speculation. Although the individual in vivo mechanical responses during both distractions suggest that the imposed vertebral displacements were consistent (Table 1), variability in the individual capsular mechanics between rats point to the need for additional examination of the ligament structure and also to more deeply investigate the effects of the time between FJD exposures on the biomechanical and painful responses.

As observed with repetitive strain injuries, repeated subthreshold loading of the facet joint did alter the failure properties of the ligament (Fig. 3) and could suggest there is facet joint instability. Since repeated exposure did lower both the vertebral displacement and peak force at complete ligament rupture (Fig. 3), it is possible that following repeated loading of the facet joint the ligament is more vulnerable to mechanical injury. Although a single high-rate subthreshold FJD has been previously shown *not* to induce any pain for up to 7 days [16] or altered mechanical properties [25], a single STFJD was not included in this study to provide a control to compare mechanical properties following high-rate repeated STFJDs. Despite this limitation, the force at failure after the repeated high-rate physiological FJD (3.03 ± 0.6 N) was consistent with that measured at failure of a ligament having undergone only a single *painful* FJD (2.96 ± 0.69 N) [35], suggesting that the two loading scenarios, both of which are also painful (Fig. 2) [14,16,27], are comparable. Although, repeated dynamic loading of the facet joint is presumed to impose joint instability and pain, by altering the structure of the capsular ligament, the magnitude and number of exposures that establish cumulative ligament microtraumas are still unknown. Additional mechanical parameters like yield properties of the capsular ligament were not measured in the current study. However, ex vivo measurement of the yield point in the rat facet capsular ligament has been found to correspond to capsular stretch that is sufficient to induce pain [49]. Therefore, measuring additional mechanical parameters may help provide insight into which pathobiomechanical changes are responsible for pain onset during repeated loading of the facet joint.

Although the onset of pain has been hypothesized to occur via macrostructural changes in the joint that activate nociceptive afferents in the capsular ligament [15,25,27,32,35,54], nociceptor activation has been shown to be more relevantly influenced by the local environment [41,55–57]. This study did not explicitly identify the effects of repeated subthreshold exposure on the

microstructure of the ligament. Stiffness of soft tissues has been shown to be positively correlated with collagen fiber realignment [58], suggesting that the decrease in stiffness (Table 1) may be accompanied by collagen fiber disorganization. Disorganized collagen fiber networks in aortic tissue have been shown to produce inhomogeneous local strains [59], As such disorganized local fiber networks in the ligament during a subthreshold loading (in this case during the second FJD) could induce less homogenous strain fields and higher local strains that would directly activate nociceptive fibers in the ligament and could be a mechanism for pain generation. Since collagen realignment is evident in the facet capsule after a single painful exposure but not a single subthreshold exposure [25,40], changes in ligament microstructure may only be induced after a repeated exposure. Despite excessive facet stretch that induces pain initiating afferent activation and neuronal hyperexcitability in electrophysiology studies [19,34], whether afferent activation is induced by *cumulative* activation following repeated exposure or if a single subthreshold FJD possibly lowers the activation threshold of joint nociceptors are open questions. Relating the extent of collagen disorganization and local strains in the facet capsular ligament to the degree of nociceptor activation would elucidate the contribution of the ligament's structural mechanics to the development of pain during repeated loading.

In addition to inducing pain, repeated subthreshold loading also increased spinal astrocytic and microglial activation that was consistent with the patterns of glial activation in the spinal cord that have been reported with a single painful FJD [27,36,50,51]. Since spinal astrocytic activation is not evident after a single subthreshold FJD [36], the increased expression of the astrocyte marker GFAP that was observed after a repeated subthreshold FJD may be due to the cumulative effects of repeated loading. For example, spinal glial activation has been observed with repeated whole body vibration (WBV) to varying degrees [60,61]. Repeated WBV separated by 7 days at a frequency of 8 Hz, but not 15 Hz, produced pain for up to 14 days after the initial exposure and both spinal astrocytic and microglial activation increased at that time [61]. Although this study did implement two distractions, the current study is limited in that it did not extend beyond two exposures. As such, it is unknown if subsequent distractions would be additive or have a more pronounced effect on the capsular mechanics, pain, or inflammatory responses. Daily repeated WBV for 7 days at 15 Hz was sufficient to induce sustained pain for 14 days [62], yet two separate WBV exposures at the same vibration frequency did not induce pain [61], suggesting that the number of exposures *may* contribute to the pain onset. Examining the effects of frequency of exposures would provide insight into the pathobiomechanical mechanisms responsible for pain development. Nevertheless, several studies have suggested that astrocytic activation is necessary for trauma-induced facet-mediated pain [27,36,51] and is a direct result of facet capsule stretch and activation of its nociceptive fibers [51]. Although a subthreshold FJD at a quasi-static rate does not activate nociceptors [11,15,31,34,63], the presence of pain and glial activation in the spinal cord (Figs. 2 and 4) do suggest that repeated subthreshold dynamic FJDs may be sufficient to activate ligament afferents via cumulative factors. Further, it has been shown in a caprine model of FJD that if the tension in the capsular ligament is large enough ($34.1 \pm 5.1\%$ strain), joint afferents can produce discharge even after the joint is unloaded [31,33].

In summary, this is the first study to demonstrate that repeated high-rate loading of the facet joint at exposures that are within physiologic range and strains below those that elicit pain is sufficient to induce pain and spinal inflammation similar to a single more injurious facet loading exposure [14,19,27,43,50]. Although the specific mechanisms responsible for pain are not identified here, the altered mechanical properties of the capsular ligament indicate it is weaker since it has reduced stiffness during the second exposure and after the repeated exposures (Table 1 and Fig. 3). This is a possible mechanism by which repetitive loading may change the macrostructural and even microstructural properties of the ligament, and can alter the environment and activation thresholds of the nociceptive fibers that innervate the capsular ligament leading to pain development. These findings also suggest that although high-rate loading at physiologic strains do not produce pain or obvious macrostructural damage in the ligament, that exposure *does* alter the ligament's mechanical response and possible lower its injury threshold to subsequent loading.

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