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Experimentally induced spine osteoarthritis in rats leads to neurogenic inflammation within neurosegmentally linked myotomes

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ABSTRACT

Naturally occurring spine osteoarthritis is clinically associated with the manifestation of chronic inflammatory muscle (myofascial) disease. The purpose of this study was to investigate the causal association between experimentally induced spine osteoarthritis and neurogenic inflammatory responses within neurosegmentally linked myotomes. Wistar Kyoto rats were randomly assigned to spine facet compression surgery (L4-L6) or sham surgery. Animals exposed to facet compression surgery demonstrated radiographic signs of facet-osteoarthritis (L4-L6 spinal levels) and sensory changes (allodynia, thermal hyperalgesia) at 7, 14 and 21 days post-intervention, consistent with the induction of central sensitization; no radiologic or sensory changes were observed after sham surgery. Increased levels of proinflammatory biomarkers including substance P (SP), calcitonin gene related peptide (CGRP), protease-activated receptor-2 (PAR2) and calcium/calmodulin dependent protein kinase II (CaMKII) were observed post-surgery within neurosegmentally-linked rectus femoris (L2-L5) muscle when compared to the non-segmentally linked biceps brachii (C4-C7) muscle; no differences were observed between muscles in the sham surgery group. These findings offer novel insight into the potential role of spine osteoarthritis and neurogenic inflammatory mechanisms in the pathophysiology of chronic inflammatory muscle (myofascial) disease.

1. Introduction

Chronic musculoskeletal pain disorders are a leading cause of pain and long-term disability (Hoy et al., 2014). Age and sex adjusted prevalence for chronic musculoskeletal pain has been reported as high as 35% in the general population, 38% in females and 31% males (Bergman et al., 2001). Chronic myofascial pain syndrome (MPS) is the most common form of non-articular chronic musculoskeletal pain (Skootsky et al., 1989). The prevalence of MPS has been reported to range from 30% within a general internal medicine practice (Skootsky et al., 1989) to 93% of patients presenting with non-specific neck pain (Cerezo-Tellez et al., 2016), and an estimated lifetime prevalence of 85% (Malanga and Cruz Colon, 2010). Age is an established significant risk factor for chronic MPS, with prevalence peaking at 50% in the over-60 population (Bergman et al., 2001). Estimates project that the senior population will reach 25% of Canada's total population by 2036 (2011); given this rapidly aging demographic, chronic MPS is poised to becoming one of healthcare's greatest challenges. Despite its growing prevalence, however, the mechanisms driving the pathophysiology and clinical manifestation of MPS are still poorly understood.

The current prevailing consensus on the pathophysiology of chronic myofascial pain syndrome (Integrated Hypothesis) is founded on the theory that chronic myofascial pain is initiated by chronic mechanical or overload injury to the affected muscle. Local injury triggers a cascade of events eventually producing the characteristic clinical features of MPS including local inflammation, dysfunctional motor endplates and painful contractures known as myofascial trigger points (Hong and Simons, 1998). Despite the prevalence of this theory, MPS is commonly observed clinically in the absence of local injury to the affected muscle. Myofascial trigger points have been previously reported in a broad profile of non-musculoskeletal conditions such as chronic pelvic pain (Stratton et al., 2015), irritable bowel syndrome (Stahlberg et al., 2018), cystitis (Fitzgerald et al., 2013), prostatitis (Anderson et al., 2009), breast cancer (Torres Lacomba et al., 2010) and psychological stress (McNulty

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et al., 1994), suggesting that alternative, non-injury physiologic mechanisms may play an important role in the pathophysiology of MPS.

Emerging evidence points to the potential for neurogenic inflammation to be an important underlying mechanism driving the initiation, pathophysiology and clinical manifestation of chronic myofascial pain. Neurogenic inflammation is an inflammatory response characterized by antidromic release of proinflammatory neuropeptides, including substance P (SP) and calcitonin gene related peptide (CGRP), into peripheral tissues via sensory fibers (Carlton, 2014; Sorkin et al., 2018). Microdialysis studies of skeletal muscle in patients presenting with MPS have reported higher concentrations of SP and CGRP in addition to a broader profile of inflammatory mediators (Shah et al., 2008; Shah et al., 2005). Similarly, increased levels of SP immunoreactivity have been observed within trapezius muscle of women presenting with MPS and fibromyalgia (De Stefano et al., 2000). These collective observations support the potentially important contribution of neurogenic inflammation in the pathophysiology and/or clinical manifestation of MPS.

The Neurogenic Hypothesis of MPS (Srbely et al., 2010; Srbely et al., 2008) suggests that the pathophysiology and clinical manifestation of MPS is initiated and facilitated by neurogenic inflammatory mechanisms within the affected muscle. These neurogenic responses are mediated via central sensitization arising from a discrete primary pathology residing within heterologous tissues (either somatic or visceral) that are neurosegmentally linked to the affected muscle. A corollary to the Neurogenic Hypothesis states that degenerative spine disease and osteoarthritis may be a common primary pathology driving the pathophysiology of MPS. Previous studies have highlighted the comorbidity between MPS and osteoarthritis (Bajaj et al., 2001; R. Henry et al., 2012; Imamura et al., 2014; Roos et al., 2011; Sanchez-Romero et al., 2019). Moreover, recently published work from our lab using a rat model has demonstrated strong associations between naturally occurring spine osteoarthritis and expression of neurogenic inflammation within neurosegmentally linked muscle, however, the mechanisms are still poorly understood (Duarte et al., 2019). No study to date has explored these mechanisms by directly investigating the causal association between spine osteoarthritis and neurogenic inflammation within neurosegmentally linked myotomes.

The overall aim of this study was to investigate the causal association between spine osteoarthritis and neurogenic inflammatory responses within neurosegmentally linked myotomes. Our specific objective was to study the effect of experimentally induced lumbar spine osteoarthritis on the protein levels of neurogenically mediated neuropeptides (SP, CGRP) within neurosegmentally linked myotomes. In addition to these neuropeptides, we also aimed to examine the effects of lumbar spine osteoarthritis on the protein levels of intramuscular protease activated receptor-2 (PAR2), a well-known marker of inflammation, as well as calcium/calmodulin-dependent kinase II (CaMKII), an intracellular kinase strongly linked to neurogenic inflammatory mechanisms. We set out to test our primary hypothesis that experimental facet compression injury causing lumbar facet osteoarthritis (L4-L6) leads to significantly increased levels of SP, CGRP, PAR2 and CaMKII within the neurosegmentally linked rectus femoris (L2-L5) muscle, when compared to the non-segmentally linked biceps brachii muscle (C4-C7). Our secondary hypothesis states that the levels of biomarkers will be greater in the ipsilateral rectus femoris muscle versus contralateral. The findings of this study aim to build on our previous work by specifically investigating the causal relationship between degenerative spine disease and neurogenic inflammatory mechanisms. This line of research will add important insight into neurogenic mechanisms contributing to the clinical manifestation of chronic inflammatory musculoskeletal (myofascial) pain.

2. Methods

2.1. Animal preparation

All animal procedures were approved by the Animal Care Committee of the University of Guelph and carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals. A total of 37 adult male Kyoto Wistar rats, 13 ± 5 months old (455 ± 42), were housed (2–3 per cage) in a room with a 12-hour alternating light-dark cycle and stable temperature (23.0 ± 1.0 °C) and fed a regular pellet diet ad libitum. Animals were randomly divided into three groups: naïve control (no intervention $n = 5$) and two intervention groups: experimental facet (L4-L5 and L5-L6) compression injury ($n = 16$) or sham intervention (L4-L5 and L5-L6 facets surgically exposed but not submitted to facet compression injury) (n = 16); each animal was assigned a numbered code by a research assistant for blinding purposes. Facet injury and sham animals were further divided in 3 sub-categories according to euthanasia time point post-intervention: 7 days (surgery: n = 5; sham: n = 5), 14 days (surgery: n = 5; sham: n = 5) or 21 days (surgery: $n = 6$; sham: n = 6). Allocation of rats for this study is illustrated in Fig. 1.

2.2. Lumbar facet compression surgery technique

We employed a previously published facet compression injury model to experimentally induce spine osteoarthritis at the L4-L6 levels (Duarte et al., 2020; J.L. Henry et al., 2012; Zwambag et al., 2018). A nonsteroidal anti-inflammatory Carprofen (5 mg/kg) was administered subcutaneously 30 min prior to surgery. Both surgery and sham animals were anesthetized using isoflurane (4%) and local anesthesia was applied over the incision site (2–5 mg/kg using 50/50 lidocaine/bupivacaine). A posterior midline incision was made from L2 to L6 spinous processes through the skin and subcutaneous tissue. Unilateral left side lumbar muscle at the L3-L4-L5 spinous process (multifidus) was resected to expose the left lumbar facet capsule at lumbar segments L4-L5 and L5-L6. The left L4-L5 and L5-L6 facet joints of the facet compression group were compressed to failure by one operator (MH) using micro-rongeurs. Similarly, the left L4-L5 and L5-L6 facet joints were surgically exposed in the sham surgery group but were not subjected to the facet compression intervention. All muscles were sutured (braided 4–0 coated Vicryl) and the skin sutured using stainless-steel skin staples. After regaining consciousness, rats were returned to their cages and maintained in the same conditions as described above. All animals exposed to intervention were weighed weekly from pre-intervention (initial mass) to 3 weeks (21 days) post-intervention.

2.3. Behavioral assessment: mechanical and thermal sensitivity

In order to validate the induction of central sensitization, mechanical and thermal sensitivity tests were administered to all animals at pre-intervention baseline (Day 0), and at days 3, 5, 7, 14 and 21 post-surgery ($n = 6$ surgery; n = 6 sham). Mechanical threshold was assessed by electronic von Frey (EVF) apparatus (IITC Life Science Inc., #2390 series, Woodland Hills, CA, USA). A non-noxious stimulus was applied with the probe, first onto the ipsilateral and then contralateral hindpaws using the EVF, with a positive response defined as the reflex withdrawal of the paw. The average force (grams) required to elicit a withdrawal reflex was calculated from five separate and consecutive readings for each hindpaw (Horst et al., 2014).

Thermal sensitivity was assessed using a hot plate apparatus (Model 58725, Stoelting Co., Wood Dale, IL, USA). Rats were placed on a heated surface (22 × 22 cm) maintained at 50C (±2C) and the withdrawal latency was recorded. The withdrawal latency was defined as the length of time (seconds) it took animals to present heat-avoidance responses such as jumping or licking their hind paws, regardless of laterality (ipsi or contralateral limb). If no response was observed after 30 s, the animal

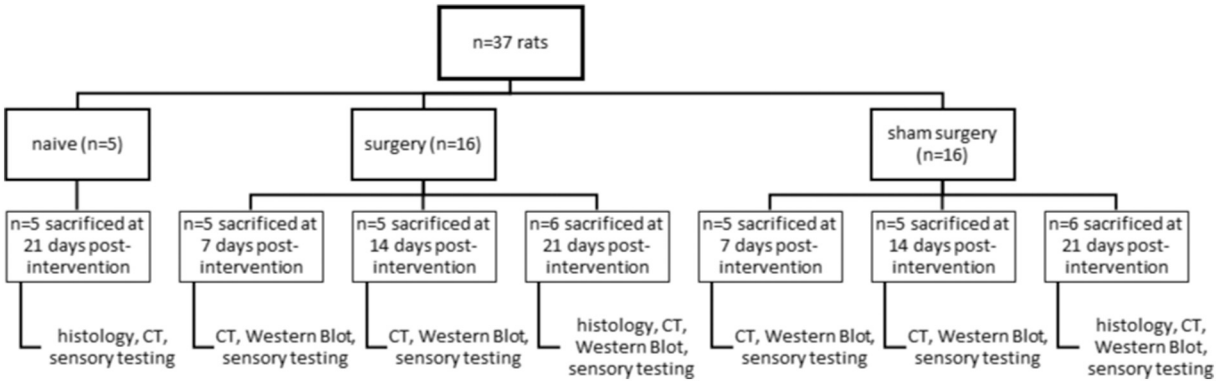

Fig. 1. Group and intervention allocation of rats. A total of 37 rats were allocated to three groups: naïve controls ($n = 5$), facet surgical compression intervention ($n = 16$) and sham surgery intervention (n = 16). All animals received baseline sensory testing. Animals from the compression surgery and sham surgery groups were sacrificed at 7, 14 and 21 days post-intervention; naïve rats (n = 5) were sacrificed at 21 days post-intervention. Western blot and sensory testing were performed at 7, 14 and 21 days post-intervention for both surgery and sham surgery groups. All animals received CT spine imaging after sacrifice.

was removed and a 30-s latency was recorded as the withdrawal latency (Leri et al., 2007). The researcher performing sensory testing was blinded to group allocation.

2.4. Spinal column preparation for micro-CT and histology

All animals were euthanized by carbon dioxide in accordance with the guidelines set by the Animal Care Committee at the University of Guelph. Spinal lumbar columns were harvested from each animal ($n = 37$) for micro-computerized tomography (micro-CT) scanning to assess for the presence of anatomical and structural changes related to facet joint compression injury and osteoarthritis (Kushchayev et al., 2018; Wachsmuth et al., 2003).

2.5. Micro-CT

Spinal samples were fixed for 48 h in 10% formalin for micro-CT scanning. Fixed spinal columns were micro-CT scanned at 45-micron isotropic voxel resolution (GE Medical Systems eXplore Locus Micro-CT Scanner, GE Medical Systems London, ON, Canada). Image stacks were reconstructed in 3D and visualized with Microview (version 2.5.0-rc21, Parallax Innovations Inc., ON, Canada). Images were submitted to a qualitative dichotomized analysis to assess for the presence of osteoarthritis-like changes at the affected levels (L4-L6) of the spine (Duarte et al., 2019). The researcher performing these assessments was blinded to group allocation. Isosurface projections were used to identify facet injury sites and verify the presence of osteoarthritis-like changes including bone remodeling, osteophytes, facet joint space narrowing, subchondral sclerosis and articular hypertrophy (Kim et al., 2011; Kushchayev et al., 2018).

2.6. Histology

Spinal columns of animals euthanized on day 21 were fixed with 10% formalin for 48 h and decalcified (Cal-X II, Fisher Scientific, Nepean, Canada) for five days. L4-L5, L5-L6 segments were cut in the transverse plane by using surgical stainless-steel blade. Lumbar facet joints were further dehydrated and embedded in paraffin-wax for sectioning in the coronal plane. Sections of 5 μm were cut and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Nepean, ON). Hematoxylin and Eosin (H&E) staining was performed for descriptive analysis of L4-L5 and L5-L6 facet histological lesions.

2.7. Western blotting (WB)

Bilateral rectus femoris (L2-L5) and ipsilateral biceps brachii muscles (C4-C7) were immediately collected after euthanasia. These muscles were specifically chosen owing to their similar type II fibre composition (Fuentes et al., 1998; Kohn and Myburgh, 2007) and distinct innervation, representing myotomes that are both neurosegmentally linked (rectus femoris) and non-segmentally linked (biceps brachii) to the experimentally induced primary spine pathology at L4-L6. Muscles were snap-frozen in liquid nitrogen and stored at −80 °C. Western blot analysis was performed on muscle homogenates using methods previously described (Duarte et al., 2019; Musumeci et al., 2014).

Protein extracts from muscle lysates were equally loaded (15 μg) per lane onto a dodecyl sulfate-polyacrylamide gel (15%), separated by electrophoresis and transferred to a polyvinylidene difluoride membrane to be processed for Western blotting with primary antibodies for substance P, CGRP, PAR2, phosphorylated-CaMKII, total CaMKII antibodies and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (anti-rabbit- 1:2000, A0545-Sigma-Aldrich-Oakville, ON). The primary antibodies used include: anti-Substance P 1:500, (orb215527), anti-CGRP 1:250, (orb182870) and anti-PAR2 1:500, (orb385619) - Biorbyt Ltd., San Franciso, CA; anti-phospho-CaMKII (Thr286) antibody- 1:1000, (#12716) and anti CamKII (Thr286) antibody- 1:1000, (#4436) - Cell Signaling Technology, Denver, MA.

Protein bands were quantified by optical densitometry using a chemiluminescence detection system (Alpha Innotech Fluorchem HD2, Fisher Scientific, Hampton, NH). The raw optical density of the target-protein bands from each individual sample was normalized against its corresponding total protein loading control obtained by Ponceau S (Fisher BioReagents) membrane staining (Cervone and Dyck, 2017). For each biomarker (SP, CGRP, PAR2, pCaMKII and tCaMKII), we obtained a total of 16 measures of protein levels distributed evenly across three time points (7, 14 and 21 days post-intervention) from both surgery and sham surgery groups. As we were not interested in changes in protein levels across time, protein levels were pooled for each biomarker across time for statistical analysis. A total of 5 measures of protein levels for all biomarkers was obtained from naïve control animals at day 21 post-intervention. The researcher performing the histology and Western blotting was not blinded to group allocation.

2.8. Statistical analysis

We conducted a two-way repeated measures ANOVA to test for differences in mechanical and thermal thresholds using intervention (facet injury, sham) and time (0, 3, 5, 7, 14 and 21 days post-intervention) as fixed effects. We followed up on significant findings using post-hoc Tukey's multiple comparisons to inspect for differences within and between groups across time.

In order to address our primary and secondary hypotheses, we also

performed multiple planned comparisons for levels of each biomarker using Fisher's Least Significant Difference. Our primary aim was to investigate the presence of neurogenic inflammatory responses within neurosegmentally linked myotomes after surgically induced spine facet compression injury. To assess for these neurosegmental effects (primary hypothesis), we planned specific comparisons between the neurosegmentally linked ipsilateral rectus femoris surgery versus the non-segmentally related biceps brachii surgery groups, as well as the ipsilateral rectus femoris sham versus biceps brachii surgery groups. In order to assess for the ipsilateral effect of surgery (secondary hypothesis), we planned specific comparisons for ipsilateral rectus femoris surgery vs contralateral rectus femoris surgery groups, as well as ipsilateral rectus femoris sham and contralateral rectus femoris sham. Finally, to assess for the effect of surgery, we planned specific comparisons between the ipsilateral rectus femoris surgery and ipsilateral rectus femoris sham groups. Statistical analyses were performed in Prism (V 8.1.2). All data are presented as mean ± SD. Alpha was set at 0.05.

3. Results

All animals maintained their body mass at every time point after sham or experimental surgery interventions (Supplementary Table 1).

Assessment of micro CT imaging confirmed the presence of phenotypic features of spine facet bone injury and osteoarthritis at the L4-L6 levels in all 16 animals exposed to surgically induced facet compression injury; no degenerative changes were observed in sham animals. Compression injury to the most lateral aspect of the left lumbar facet joint at L4-L5 and L5-L6 segments led to notable structural changes on micro-CT images such as bone irregularity and remodeling, osteophytes, facet joint space narrowing, subchondral sclerosis and articular cartilage hypertrophy seen 7, 14 and 21 days (micro-CT and histology) after facet compression injury, but not in sham or controls (Supplementary Fig. 1).

3.1. Mechanical and thermal sensitivity

Animals exposed to spine facet compression injury exhibited behavioral sensitivity consistent with the induction of central sensitization in response to non-noxious mechanical (allodynia) and thermal (thermal hyperalgesia) stimuli applied to bilateral hindpaws. We observed significant effects of intervention [$F(1, 5) = 36.17$, $p = .001$], time [$F(5, 25) = 23.64$, $p < .001$] and intervention * time interaction [$F(5, 25) = 7.45$, $p < .001$] for mechanical allodynia at the ipsilateral hindpaw. The surgery group demonstrated early onset increase in mechanical sensitivity at 3 days post-intervention (37.96 ± 2.18 g, $p = .013$) when compared to pre-intervention baseline values (56.33 ± 2.51 g) (Fig. 2A). Mechanical thresholds remained below baseline levels for the full 21 days, reaching their lowest value at 21 days (19.98 ± 2.18 g, $p < .001$) (Fig. 2A). In contrast, the sham animals demonstrated transient decreases in mechanical threshold, reaching its lowest value 7 days after sham surgery (41.51 ± 1.65 g, $p = .025$) and resolving to baseline by day 14 (Fig. 2A). Significant decrease in mechanical thresholds was observed in the surgery group when compared to sham surgery at 5 ($p = .011$), 14 ($p < .001$) and 21 ($p < .001$) days post-intervention; in contrast, no difference was seen between groups at pre-intervention baseline ($p > .999$). An effect of time [$F(5, 25) = 4.034$, $p = .008$] and time * intervention [$F(5, 25) = 3.274$, $p = .020$] was also observed at the contralateral hindpaw [$F(1, 5) = 5.92$, $p = .590$], however, no significant differences between intervention groups was noted at any timepoint ($p > .05$) (Fig. 2B).

We also observed a significant effect of intervention [$F(1, 5) = 22.28$, $p = .005$], time [$F(5, 25) = 4.91$, $p = .002$] and intervention*time interaction [$F(5, 25) = 3.72$, $p = .011$] for thermal latency, with significant decreases in withdrawal latency of the surgery group at 14 ($p = .001$) and 21 ($p = .002$) days post-surgery, when compared to sham surgery (Fig. 2C).

Mechanical and thermal threshold values in rats exposed to left

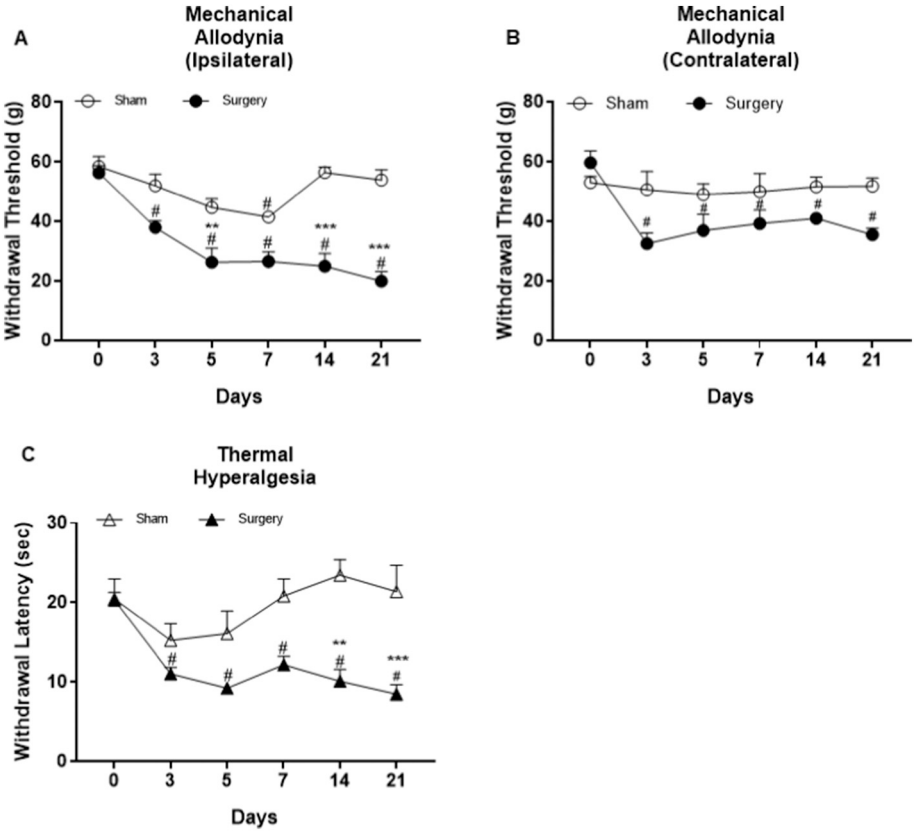

Fig. 2. Mechanical and Thermal Threshold Assessment.

lumbar spine facet compression injury (L4-L6) versus sham surgery. Facet compression injury led to reduced mechanical threshold (increased mechanical sensitivity) at the ipsilateral (A) and contralateral hind paws (B); no significant reduction in mechanical threshold from baseline was observed with sham surgery in either limb. Facet compression surgery also led to reduced thermal threshold (increased thermal sensitivity) (C) while no effect of sham surgery was seen on thermal threshold. Data are represented as mean ± SEM. Significance was set at $\alpha = 0.05$. # denotes significance from pre-intervention (baseline) within each group. Between groups significance is denoted by ** ($p < .05$) and *** ($p \leq .001$).

3.2. Biomarker analyses-primary hypothesis

Mean and 95% CI are presented for all planned comparisons across all biomarkers (SP, CGRP, PAR2 and pCaMKII/tCaMKII) (Table 2).

Mean difference and Confidence Intervals (mean [CI]) for planned comparisons of all biomarkers. Planned comparisons were conducted to assess for neurosegmental effects of facet compression injury, ipsilateral effects of surgery and overall effect of surgery. Specific comparisons are listed in the left column.

Significant increases in all biomarkers were observed after facet compression injury in the ipsilateral rectus femoris muscle when compared to the biceps brachii, including SP ($p < .001$), CGRP ($p < .001$), PAR2 ($p < .001$) and pCaMKII/tCaMKII ($p = .001$). Significant increases in biomarker levels were also observed within the ipsilateral rectus femoris sham group versus biceps brachii surgery group for SP ($p < .001$) and PAR2 ($p < .001$), but not pCaMKII/tCaMKII ($p = .861$) or CGRP ($p = .112$). Similar increases were observed in the ipsilateral rectus femoris surgery vs sham groups for SP ($p < .001$), PAR2 ($p = .002$) and pCaMKII/tCaMKII ($p = .001$); differences were not significant for CGRP ($p = .115$) (Fig. 3A–D).

3.3. Biomarker analyses-secondary hypothesis

Biomarker levels in the ipsilateral rectus femoris muscle were also increased after facet compression surgery in comparison to the contralateral rectus femoris, including SP ($p < .001$), PAR2 ($p < .001$) and pCaMKII/tCaMKII ($p = .002$), but not CGRP ($p = .517$). Similarly, increased biomarker levels were found in the ipsilateral rectus femoris sham group versus contralateral sham group for SP ($p < .001$), CGRP ($p = .065$) and PAR2 ($p < .001$), but not pCaMKII/tCaMKII ($p = .928$). No difference in levels for any of the biomarkers was observed within the biceps brachii muscle between any of the three interventions (control, sham, surgery) ($p > .05$). Optical density (AU) values (mean, SD) are listed for each muscle*intervention in Table 3.

Table 2
Planned Comparisons of Biomarker Levels between Interventions.

Comparisons	SP	CGRP	PAR2	pCaMKII/ tCaMKII
ipsi RF surgery-ipsi BB surgery	2.32 [1.94, 2.70]	0.94 [0.52, 1.36]	0.45 [0.32, 0.58]	2.64 [1.05, 4.24]
ipsi RF sham-ipsi BB surgery	1.19 [0.81, 1.57]	0.61 [0.19, 1.02]	0.24 [0.12, 0.37]	−0.14 [−1.74, 1.45]
ipsi RF surgery-ipsi RF sham	1.13 [0.76, 1.51]	0.34 [−0.76, 0.08]	0.21 [0.08, 0.33]	2.78 [1.19, 4.38]
ipsi RF surgery-contra RF surgery	2.09 [1.71, 2.47]	0.14 [−0.28, 0.56]	0.54 [0.41, 0.66]	2.57 [0.97, 4.16]
ipsi RF sham-contra RF sham	1.20 [0.81, 1.59]	0.41 [−0.03, 0.84]	0.43 [0.30, 0.56]	0.08 [−1.57, 1.73]

4. Discussion

The findings of our study support our primary hypothesis that experimental lumbar (L4-L6) facet compression injury leading to lumbar facet osteoarthritis results in significantly increased levels of neurogenically mediated inflammation within neurosegmentally linked myotomes. Our experimental design addresses our hypothesis on the basis of the neurosegmental architecture governing the neuroanatomic relationship between the experimentally induced primary pathology (L4-L6 spine osteoarthritis) and hip/thigh muscles (L2-L5) in rats. We observed significantly increased levels of neurogenically mediated neuropeptides (SP and CGRP), PAR2 and pCaMKII/tCaMKII ratio within the neurosegmentally linked ipsilateral rectus femoris muscle when compared to the non-segmentally linked biceps brachii muscle post-surgery. These observations support the conclusion that a robust neurogenic inflammatory response was evoked within the neurosegmentally linked rectus femoris muscle, ipsilaterally. Furthermore, levels of all biomarkers except pCaMKII/tCaMKII were similarly increased within the ipsilateral rectus femoris after sham surgery when compared to levels within the biceps brachii muscle after surgery. These findings, coupled with the fact that no differences in any biomarker was observed between intervention groups within the biceps brachii, provide novel evidence supporting the presence of a causal relationship between the experimental induction of spine facet osteoarthritis and neurogenic inflammatory responses within neurosegmentally linked myotomes. Except for the pCaMKII/tCaMKII, our findings confirm that the neurogenic responses for all biomarkers were greater ipsilaterally, consistent with the underlying neurophysiologic mechanisms of neurogenic inflammation. These data support our secondary hypothesis and offer novel perspectives into the potential role of neurogenic inflammation in the pathophysiology and clinical manifestation of chronic inflammatory muscle disease associated with degenerative spine disease.

4.1. Central sensitization and neurogenic inflammation

Central sensitization is a state of enhanced excitability of neurons within the central nervous system. It has been shown to manifest locally within the dorsal horn, or it can spread to other regions of the nervous system contralaterally and/or supraspinally. We validated the successful induction of central sensitization in our experiment by testing for sensory changes (allodynia, thermal hyperalgesia). Our experimental surgery model evoked unilateral dorsal horn sensitization ipsilateral to the surgical intervention, as evidenced by the fact that we observed significantly increased allodynia within the ipsilateral hindpaw of the surgery group when compared to sham controls; in contrast, no difference between surgery and sham surgery was observed in the contralateral hindpaw. Further evidence that the spine surgery intervention evoked central sensitization was the fact that we observed significantly decreased pain-behaviour latencies to thermal stimulation (thermal hyperalgesia) in the surgery group versus sham controls. Although this test does not enable us to resolve the laterality (ipsi versus contralateral hindlimb) of sensitization effects, it was confirmatory for the presence of sensitization within the affected lumbar spinal segments.

Neurogenic inflammation is a common sequela to central sensitization (Carlton, 2014; Richardson and Vasko, 2002). Dorsal horn sensitization activates GABAergic interneurons to trigger Primary Afferent Depolarization of both myelinated and non-myelinated sensory afferents converging onto the same spinal segment (Peng et al., 2001; Willis, 1999). Primary afferent depolarization, in turn, initiates the Dorsal Root Reflex (DRR) characterized by antidromic (outflow) activity along primary afferent fibers (Willis, 1999). The most common neuropeptides released peripherally during neurogenic inflammation are substance P and CGRP, both of which have powerful vasoactive and proinflammatory properties by way of their direct influence on capillaries and immune cells (Birklein and Schmelz, 2008; Lin et al., 2000).

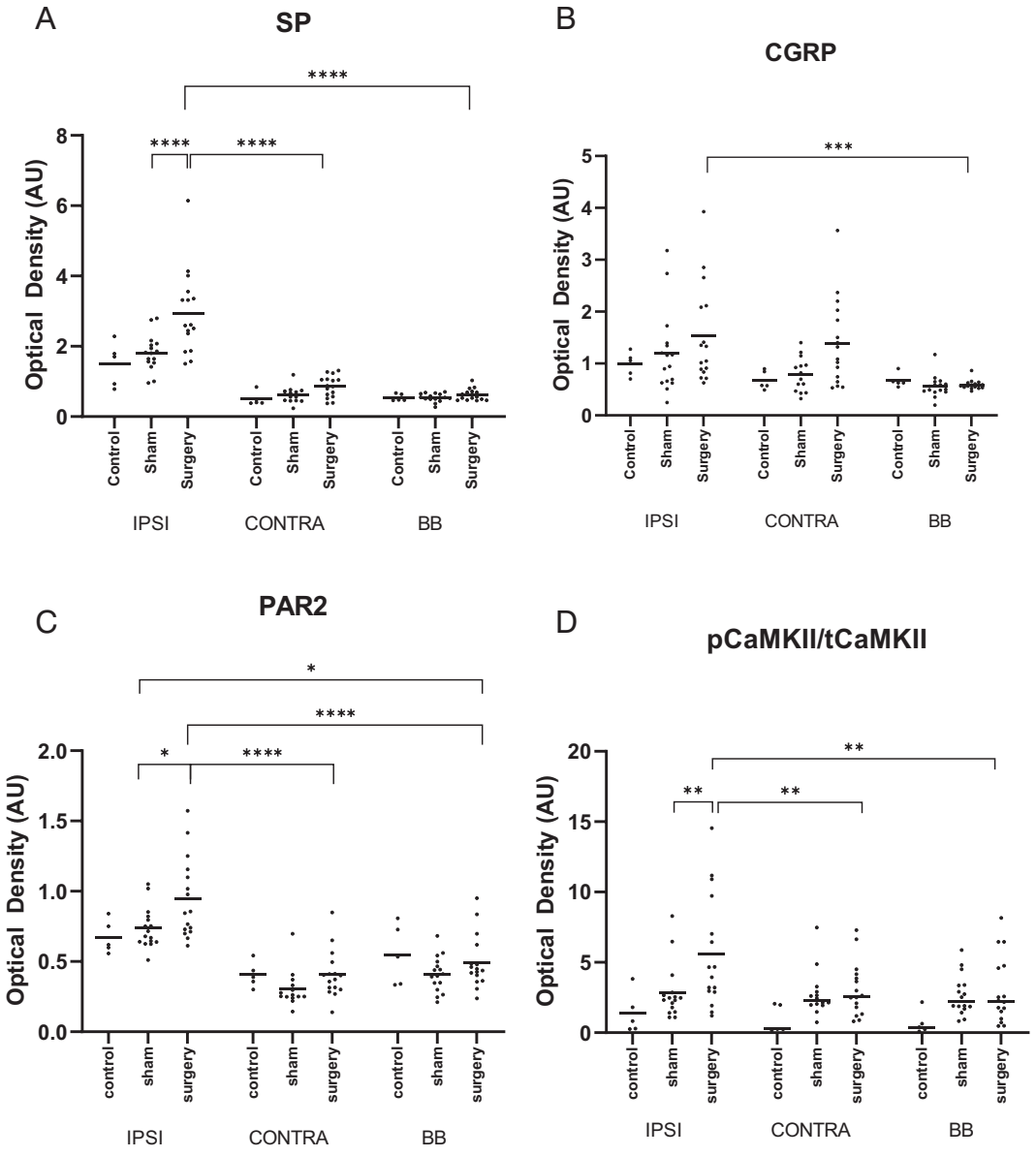

Fig. 3. Biomarker levels between interventions.
(A–D): Levels of neurogenic inflammatory mediators after control, sham, and surgery interventions observed within the ipsilateral rectus femoris (ipsi), contralateral rectus femoris (contra) and ipsilateral biceps brachii (BB) muscles. Panels A, B, C, and D represent biomarker levels of SP, CGRP, PAR2, and pCaMKII/tCaMKII ratio, respectively. Band intensities of each protein in the Western blots were quantified with optical densitometry and normalized to Ponceau staining. Dot plot means are indicated by horizontal line. Significance level denoted by asterisks *[≤0.05], **[≤0.01], ***[≤0.001] and ****[≤0.0001].

Initiation and facilitation of primary afferent depolarization and neurogenic inflammation requires persistent afferent nociceptive input from a primary pathologic source (Sorkin et al., 2018). Primary afferent depolarization and neurogenic inflammation are also predominantly manifest within neurosegmentally linked tissues ipsilateral to the pathologic source; this neurophysiologic architecture forms the foundation for the theoretical framework of our study design. Owing to the chronic and persistent inflammatory nature of osteoarthritis, we hypothesized that degenerative spine disease could be a common pathologic source driving neurogenic inflammation and the pathophysiology of chronic inflammatory musculoskeletal disease in the aging population. This hypothesis offers biologic plausibility for the mechanisms linking the clinical comorbidity of these two conditions in the aging population.

Our main findings are also in line with the neurosegmental mechanisms of NI, which are largely manifest ipsilaterally. We observed significantly increased levels of all biomarkers within the neurosegmentally linked rectus femoris ipsilaterally when compared to the non-segmentally related biceps brachii muscle after spine facet compression surgery (Fig. 3, A–D). Moreover, except for CGRP (Fig. 3B), all biomarker levels were also found to be significantly increased within the ipsilateral rectus femoris when compared to the contralateral rectus femoris. Importantly, our findings further add to the existing body of research by being the first to demonstrate these causally linked mechanisms in heterologous tissues, such as spine and muscle.

Animal research shows that the neurogenic release of neuropeptides can evoke significant inflammatory responses ipsilaterally within both muscle and joints (Bileviciute et al., 1993; Daemen et al., 1998; Sakaguchi et al., 1991). Peripheral release of SP and CGRP has been reported from homologous muscle afferent terminals after local intramuscular injection of nerve growth factor and Freund's adjuvant (Reinert et al., 1998). Experimentally induced L5 nerve ligation injury in rats has also

Table 3
Biomarker levels within each muscle by intervention.

		Control		Sham		Surgery	
		Mean	SD	Mean	SD	Mean	SD
SP	ipsi	1.498	0.627	1.812	0.506	2.946	1.182
	contra	0.501	0.193	0.614	0.222	0.856	0.304
	BB	0.542	0.102	0.542	0.118	0.625	0.163
PAR2	ipsi	0.673	0.118	0.737	0.145	0.945	0.286
	contra	0.405	0.091	0.304	0.130	0.408	0.168
	BB	0.549	0.217	0.409	0.126	0.495	0.191
CGRP	ipsi	0.991	0.231	1.194	0.789	1.531	0.945
	contra	0.675	0.182	0.785	0.330	1.393	0.838
	BB	0.671	0.135	0.558	0.208	0.589	0.088
pCaMKII/ tCaMKII	ipsi	1.404	1.487	2.839	1.952	5.622	3.990
	contra	0.939	0.980	2.763	1.651	3.053	1.907
	BB	0.707	0.840	2.658	1.428	2.980	2.377

Levels of SP, PAR2, CGRP and CaMKII were assessed within each muscle, including the ipsilateral rectus femoris (ipsi), contralateral rectus femoris (contra) and ipsilateral biceps brachii (BB). Biomarker levels are presented as mean and standard deviation (SD) of arbitrary units of Optical Density (AU).

been shown to enhance edema and leukocyte infiltration within the neurosegmentally linked gastrocnemius muscle (L4-L6) (Daemen et al., 1998; Eccles et al., 1961) as well as similarly increasing expression of SP and CGRP within the neurosegmentally linked quadriceps (L4-L5) (Ota et al., 2014). Increased expression of both SP and CGRP have also been reported within contralateral homologous (Bileviciute et al., 1993; Donaldson et al., 1995) and heterologous joints (Duarte et al., 2020) after the experimental induction of arthritis, leading to extensive joint inflammation (Donaldson et al., 1995), metabolic changes in the articular cartilage (Decaris et al., 1999) and reduction of nociceptive mechanical thresholds (Rees et al., 1996). Although predominantly ipsilateral, dorsal root reflex and neurogenic inflammation have also been reported contralateral to experimentally induced monoarthritis (Donaldson, 2009; Kelly et al., 2007), owing to polysynaptic neural connections decussating within the spinal cord (Bagust et al., 1993; Donaldson, 2009). Descending supraspinal pathways originating within brainstem nuclei have also been shown to initiate and facilitate the dorsal root reflex bilaterally (Peng et al., 2001). Our study adds to this collective body of research by being the first to demonstrate a causal association between experimentally induced spine osteoarthritis and neurogenic inflammatory responses within neurosegmentally linked heterologous tissues.

4.2. Protein activated receptor-2 (PAR2)

Our finding of increased PAR2, along with SP, within the rectus femoris post-surgery is not unexpected, given that SP triggers mast cell degranulation and subsequent PAR2 activation (Bileviciute et al., 1998; Nilsson et al., 1990). PAR2 is a G-protein coupled receptor present in nociceptive afferent nerves and immune (T-cells, macrophages, mast cells) cells (Lindner et al., 2000). It is a neurogenically mediated protease (Rothmeier and Ruf, 2012) that is activated by mastocyte degranulation, contributing to the inflammatory process via autocrine and paracrine action to further enhance peripheral release of SP, CGRP, TNF-alpha and bradykinin from afferent terminals or mast cells (Steinhoff et al., 2000). Our finding of significantly increased levels of PAR2 within the rectus femoris, but not biceps brachii, post-surgery provides evidence of a robust neurosegmentally mediated neurogenic inflammatory response (Fig. 3C). Future research should investigate the role of PAR2 pathways in the pathophysiologic mechanisms of chronic musculoskeletal pain.

4.3. Calcitonin gene related peptide (CGRP)

Significant increases in CGRP were also observed bilaterally post-surgery within the neurosegmentally linked rectus femoris muscle versus the biceps brachii (Fig. 3B), adding further evidence of a neurosegmentally mediated neurogenic inflammatory response within muscle. Interestingly, CGRP was the only biomarker to show elevated levels within the contralateral rectus femoris post-surgery when compared to the biceps brachii postsurgery. This bilateral expression of CGRP within the neurosegmentally linked rectus femoris muscle should be further investigated to highlight the relationship between ipsilateral vs contralateral mechanisms, and their potential role in the pathophysiology of inflammatory muscle disease.

4.4. PCaMKII/CaMKII

The present study is also the first to report increased levels of pCaMKII/CaMKII ratio within neurosegmentally linked myotomes after experimentally induced spine osteoarthritis. CaMKII is a calcium-calmodulin regulated serine-threonine kinase, the activation of which has been shown to promote sustained release of neuropeptides from nerve terminals in drosophila (Shakiryanova et al., 2007), facilitates SP release via central terminals (Zhong et al., 2016), and is a requisite step for the endogenous release of CGRP in mouse motor nerve terminals (Gaydukov and Balezina, 2018). Neurogenic inflammatory-related neuropeptides and proteases may also activate cytosolic intracellular signaling cascades leading to calcium increase and CaMKII activation, promoting further activation of NF-KB, transcription of DNA and cytokine production (Planells-Cases et al., 2005; Rothmeier and Ruf, 2012). We observed significantly enhanced pCaMKII/CaMKII ratio levels in the rectus femoris muscle ipsilaterally post-surgery, suggesting modulation of a signaling calcium-dependent cascade within neurosegmentally linked myotomes. Further studies are needed to elucidate whether these findings could have important mechanistic implications to the formation of local muscle contractures (myofascial trigger points), which are hallmark characteristics of muscles afflicted with chronic myofascial pain syndrome.

4.5. Limitations

The results of this study should be interpreted in light of the following limitations. Both rectus femoris and biceps brachii are predominantly comprised of fast-twitch fibers, however, significant regional variation exists in the distribution of these fibre types in rats (Fuentes et al., 1998; Kohn and Myburgh, 2007). Although we systematically tested samples from the same region of muscle in each animal, we cannot exclude that variation in muscle fibre composition could have influenced the observed results. The present study also sampled neurogenic biomarkers up to 21 days post injury. This time point was chosen based on previous studies employing chemically-induced spine osteoarthritis (Kim et al., 2011; Shuang et al., 2015), however, the longer-term effects of chronic degenerative spine disease on neurogenic inflammatory responses in muscle remains unknown. Elucidating a longer term course of neurogenic inflammation in skeletal muscle may provide greater insight into the physiologic mechanisms contributing to the chronification of muscle pain.

4.6. Novelty and contribution

We have previously reported an association between naturally occurring lumbar spine osteoarthritis in rats and expression of SP and PAR2 bilaterally within the neurosegmentally linked rectus femoris muscle (Duarte et al., 2019). The present study advances those findings by demonstrating a causal association between the experimental induction of lumbar spine osteoarthritis and neurogenic inflammatory mechanisms within neurosegmentally linked myotomes, using a sham controlled experimental design. These findings add novel and important insight into our understanding of the underlying mechanisms of neurogenic inflammation and its potential role in the pathophysiology of chronic inflammatory musculoskeletal (myofascial) pain in the aging

population.

Previous research in humans has shown that a unique intramuscular biochemical milieu exists within the muscles of patients suffering from chronic myofascial pain (Shah et al., 2005), with prevailing consensus that this inflammatory profile is the direct result of local mechanical injury to the affected muscle. Our findings, however, offer novel evidence to suggest that mechanisms other than local injury could potentially initiate and/or mediate inflammatory responses within muscle tissue. Future directions should advance this line of research by investigating the biochemical profile of neurogenically mediated muscle inflammation using this animal model.

Age is the single greatest risk factor for the development of osteoarthritis (Arden and Nevitt, 2006) with several studies reporting progressive increase in prevalence of osteoarthritis with age (Felson et al., 1987; Jordan et al., 2007; Jordan et al., 2009; van Saase et al., 1989). In light of the comorbidity of age-related spine degeneration/osteoarthritis with chronic myofascial pain (Bajaj et al., 2001; R. Henry et al., 2012; Roos et al., 2011), our findings add important evidence to support our hypothesis that similar muscle inflammatory profiles could also be mediated via neurogenic mechanisms evoked from common degenerative spine pathologies, such as osteoarthritis, in the aging population. These collective findings draw attention to the clinical importance of treatment and/or prevention of chronic degenerative spine disease in the long-term management of MPS.

5. Conclusion

Despite the prevalence of chronic MPS, its pathophysiologic mechanisms are still poorly understood. Previous research from our lab has reported an association between naturally occurring spine osteoarthritis and SP within neurosegmentally linked myotomes. The findings of the present study advance our previous work by being the first to demonstrate a causal association between experimentally induced primary spine osteoarthritis and neurogenic inflammation within neurosegmentally linked myotomes. These observations offer empirical evidence to support the Neurogenic Hypothesis for the pathophysiology of chronic myofascial pain and highlight a potentially significant role of spinal degenerative pathology in mediating these mechanisms. Our work informs future research exploring neurogenic mechanisms in the pathophysiology of chronic myofascial pain and suggests that therapeutic management of chronic musculoskeletal (myofascial) pain should address the therapeutic management and prevention of degenerative spine disorders, central sensitization and neurogenic inflammation.

Submission declaration

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CRediT authorship contribution statement

Duarte, Felipe: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft and review/editing, visualization, project administration. **Hurtig, Mark**: conceptualization, methodology, investigation, resources, writing – review/editing, supervision, project administration, funding acquisition. **Clark, Andrea**: conceptualization, methodology, investigation, resources, writing – review/editing, supervision, project administration. **Brown, Stephen**: methodology, investigation, resources, writing – review/editing. **Simpson, Jeremy**: methodology, investigation, resources, writing – review/editing. **Srbely, John**: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft and review/editing, visualization, project administration, supervision, funding acquisition

Declaration of competing interest

The authors declare no actual or potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2021.111311.

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