The Effect of Lumbar Spine Osteoarthritis on the Manifestation of Central Sensitization and Neurogenic Inflammation in Musculoskeletal Tissues in Rats

by
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ABSTRACT

THE EFFECTS OF LUMBAR SPINE OSTEOARTHRITIS ON THE MANIFESTATION OF CENTRAL SENSITIZATION AND NEUROGENIC INFLAMMATION IN MUSCULOSKELETAL TISSUES IN RATS

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Symmetrical bilateral spread of neurogenic inflammation (NI) has been shown as a secondary response post experimental primary osteoarthritis (OA) and pelvic organ inflammation. This manifestation requires participation of sensitization of the central nervous system and antidromic release of neuropeptides through peripheral afferents. However, knowledge regarding the presence of neurogenic mechanism in heterologous tissues is limited. The overall aim of this thesis was to investigate the presence of neuropeptides related to NI in heterologous knee and quadriceps muscle tissue as a response of experimentally-induced lumbar facet OA disorder.

Study 1 of this thesis showed greater substance P (SP) immune-expression within heterologous tibiofemoral cartilage following by lumbar spine-induced OA compared to sham and naïve groups. The SP immunoreactivity was higher at superficial cartilage zone and was accompanied by consistent loss of superficial zone chondrocytes, mild roughening of the articular surface and occasional chondrocyte clusters, changes associated with early OA development.
Study 2 investigated the association between naturally occurring lumbar spine OA in elderly rats (L3-L5), SP protein expression at the level of the spinal cord (L4-L5), and the presence of NI within neurosegmentally-linked quadriceps (L2-L5) in elderly rats versus young rats. Elderly OA rats had significantly higher protein expression of SP within the dorsal horn, and higher protein expression of SP and protease-activated receptor 2 (PAR2) within the quadriceps muscle. Similar findings were not seen within the young rat population.

Study 3 aimed to follow up with a rationalization for the associative findings of study two by exploring the causal-effect of experimentally induced spine facet OA and NI and intracellular kinases within a neurosegmentally-linked myotome. The findings of this study provided evidence that lumbar spine OA increases NI and intracellular kinases only within neurosegmentally-related myotomes but not in remote myotomes.

Collectively, these findings support the manifestation of central sensitization and NI within the heterologous knee cartilage and within neurosegmentally-linked myotomes as a secondary response after the induction of a primary lumbar spine OA disorder in rats. These findings may have significant implications to advancing our understanding of the pathophysiology of chronic musculoskeletal disorders such as OA and myofascial pain syndrome.
DEDICATION

I dedicate this thesis to my wife, Vivian, and my lovely kids Pietra and Theodore. I could not have done it without your love and support.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Musculoskeletal</td>
<td>MSK</td>
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<tr>
<td>Osteoarthritis</td>
<td>OA</td>
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<td>Myofascial pain syndrome</td>
<td>MPS</td>
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<td>Myofascial trigger point</td>
<td>MTrP</td>
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<td>Central sensitization</td>
<td>CS</td>
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<td>Neurogenic Inflammation</td>
<td>NI</td>
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<td>Substance P</td>
<td>SP</td>
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<td>Calcitonin gene related-peptide</td>
<td>CGRP</td>
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<td>Protease activated receptor 2</td>
<td>PAR2</td>
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<td>Dorsal root ganglia</td>
<td>DRG</td>
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<td>Calcium</td>
<td>Ca$^{+2}$</td>
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<td>A-delta fibers</td>
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<td>A-alpha fibers</td>
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<td>A-beta fibers</td>
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<td>Micro computed tomography</td>
<td>Micro-CT</td>
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<td>Immunohistochemistry</td>
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<td>Hematoxylin &amp; Eosin</td>
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<td>Western Blot</td>
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<td>Neuromuscular junction</td>
<td>NMJ</td>
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<td>Extracellular signal-regulated kinases</td>
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<td>Term</td>
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<tr>
<td>Calcium/calmodulin-dependent protein kinase II</td>
<td>CaMKII</td>
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<td>Glial fibrillary acidic protein</td>
<td>GFAP</td>
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<tr>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
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<td>N-methyl-D-aspartate receptor</td>
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<td>Magnesium</td>
<td>Mg^{2+}</td>
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<td>Neurokinin 1 receptor</td>
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<td>Tumor Necrosis Factor-alpha</td>
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<td>Interleukin 6</td>
<td>IL-6</td>
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<tr>
<td>Magnetic Resonance Imaging</td>
<td>MRI</td>
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<td>Transforming growth factor beta</td>
<td>TGF-β</td>
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<tr>
<td>Mitogen-activated protein kinase</td>
<td>MAPK</td>
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<tr>
<td>Protein kinase C</td>
<td>PKC</td>
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<tr>
<td>Protein kinase A</td>
<td>PKA</td>
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<tr>
<td>Neurotrophic tyrosine kinase receptor A</td>
<td>TrkA</td>
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1.1 Introduction

1.1.1 Incidence and Burden of MSK Pain

Musculoskeletal (MSK) disorders are one of the world’s most burdening condition (Vos et al. 2012) due to the severity of pain symptoms and long-term physical disability (Woolf et al. 2012). There are various forms of MSK disorders, two of the most common forms including osteoarthritis (OA) and myofascial pain syndrome (MPS) (Cimmino et al. 2011; Gerwin 2001; Storheim and Zwart 2014). The prevalence of OA is about 10-20% worldwide varying according to gender and age (Glyn-Jones et al. 2015). In Canada, OA affects approximately 5 million people (Arthritis Alliance of Canada 2011). The incidence of MPS is not entirely elucidated, but studies suggested MPS may reach 85% of the population during the lifespan (Shah et al. 2005). The prevalence of both disorders has been reported to increase with ageing making the ageing process a critical risk factor for both diseases (Dubin 2016; Gerwin 2001).

OA is a multifactorial disorder characterized by the deterioration of the cartilage and other joint tissues whereas MPS is characterized by regional muscular hypersensitivity associated with the manifestation of palpable muscular tender regions known as myofascial trigger points (MTrP). The commonality between these conditions is the involvement of central sensitization (CS) and neurogenic inflammation (NI), which have been suggested to contribute to the pathogenesis of such diseases (Bajaj et al. 2001; Arendt-Nielsen and Graven-Nielsen 2003; Arendt-Nielsen et al. 2010; Littlejohn and Guymer 2018; Srbely et al. 2010). Evidence indicates that central neuronal...
activation and NI increasing substance P (SP) may underlie the symmetrical nature of bilateral cartilage degeneration (Donaldson 2009). However, no evidence have reported whether similar mechanism may be present between heterologous neurosegmentally-linked joints. Also, MPS is biochemically characterized by the presence of pro-inflammatory cytokines and NI neuropeptides within painful and stiff muscles (Shah et al. 2005). Some studies have shown that MPS and OA may be coexisting conditions (Weiner 2007). Gerwin reported that MPS may be a secondary disorder consequent to an established primary OA (Gerwin 2001). Similarly, Srbely expanded this idea suggesting that any primary pathology (including OA) leading to activity-dependent CS may be driving neurogenic mechanisms to neurosegmentally-linked myotomes (Srbely et al. 2010).

The Neurogenic Hypothesis represents a novel paradigm to explain the pathophysiology of MPS (Srbely et al. 2010). Similar manifestation of NI post CS have been shown in visceral genito-urinary tissues (Ustinova et al. 2006; Pan et al. 2010) and joint musculoskeletal tissues post osteoarthritis (Rees et al. 1996; Decaris et al. 1999). Despite these previous findings, it is unknown whether the manifestation of NI may be increased within neurosegmentally-linked myotomes in the presence of spine OA.

1.1.2 Problem Statement

Despite the attempts in the literature to understand the mechanisms driving the clinical manifestation of MPS and OA some controversies in the field are:
(1) It is unknown if increase of NI proteins are present within neurosegmentally-linked myotomes post spine degenerative disorder such as OA

(2) it is unknown if increase of SP is present within neurosegmentally-linked heterologous ipsilateral cartilage in a similar fashion as occurs between symmetrical joints.

1.1.3 Emerging Research Suggests that Central Sensitization and Neurogenic Inflammation May Play an Important Role in the Pathophysiology of MPS (Neurogenic Hypothesis)

Emerging research suggests that neurogenic mechanisms may play an important underlying role in the pathophysiology of chronic myofascial pain disorders (Srbely et al. 2010). The Neurogenic Hypothesis states that chronic myofascial pain may occur in the absence of local pathology in the muscle (Srbely et al. 2010), in contrast to the prevailing theory (Integrated Hypothesis) which proposes that local mechanical injury within the muscle is the precipitating event in the pathophysiology of chronic myofascial pain (Simons et al. 1999). According to the Neurogenic Hypothesis, the myofascial disorder is secondary to any primary persistent noxious/inflammatory disorder (Srbely et al. 2010). Persistent input leads to activity-dependent central sensitization, resulting in the expression of NI within neurosegmentally-linked myotome due to spine reflex (Srbely et al. 2010, 2013). The most important NI proteins are SP and CGRP (Chiu et al. 2012). Recent evidence has shown that patients with MPS exhibit a unique biochemical milieu within trapezius muscle affected by active myofascial trigger point (MTrP) (Shah et al. 2005, 2008). Elevated SP, CGRP and pro-inflammatory cytokines TNF-α, IL-1β and IL-6, for instance, have been already detected (Shah et al. 2005,
2008). Similarly, highest levels of SP immunoreactivity was shown within biopsied trapezius muscle of women exhibiting MPS in comparison to both fibromyalgic (FM) and healthy controls specimens, although SP was higher in FM compared to controls (De Stefano et al. 2000). Collectively, these findings provide the possibility that neurogenic mechanism may be important mediators underlying the pathogenesis of myofascial pain conditions.

1.1.4 Previous Research Suggests that Primary Pathology in a Tissue Leads to Enhanced Expression of Substance P within Neurosegmentally Linked Joints

Although the higher magnitude effects of Freud's Adjuvant-inducing OA has been shown in the primary affected joint, it also provokes a modulatory impact on the nervous system. Enhanced production of SP and CGRP within the dorsal root ganglia bilaterally (Levine et al. 1986; Mapp et al. 1993) and within the spinal cord (Mapp et al. 1993; Donnerer 1992) have been previously shown. Additionally, enhanced antidromic transport of such neuropeptides through the bilateral sciatic nerve (Donnerer 1992) was also reported, suggesting that bilateral alteration in a variety of nervous system tissues may be presented following experimental monoarthritis. Since articular joints are highly innervated by afferent nerves change in the nervous system may provide support on the symmetric effect of OA seen in clinical and pre-clinical studies (Keenan et al. 2006; Rees et al. 1996).

Evidence suggests that experimentally-induced knee monoarthritis causes a bilateral and symmetrical release of SP with implication on the presence of swelling and hyperalgesia at the contralateral joints (Levine et al. 1985; Kidd et al. 1995). Reflex
neurogenic inflammation has been suggested to supporting such neurogenic inflammatory effects (Levine et al. 1985; Kidd et al. 1995). Levine and Kidd endorsed the involvement of the nervous system when prior depletion of unmyelinated C-fibers with capsaicin or dorsal rhizotomy minimized the effects at the contralateral site indicating that afferents contribute to the contralateral response (Levine et al. 1985; Kidd et al. 1995). Degenerative and cartilaginous catabolic changes consequent to increase of SP have also been shown at ipsilateral (O'Byrne et al. 1990) and contralateral homologous cartilage post-monoarthritis-induced (Decaris et al. 1999). Nevertheless, no information regarding neurogenic mechanism within neurosegmentally-linked heterologous ipsilateral cartilage has been studied.

Similarly, recent evidence showed that rats exposed to L5 nerve root ligation, an experimental model used to mimic clinical neuropathic pain disorder, had higher protein expression of SP and CGRP at neurosegmentally-linked quadriceps muscle (Ota et al. 2014). Nevertheless, little is known about this potential mechanism upon skeletal muscle tissue.
1.1.5 Aging is strongly correlated to spine OA and MPS

Previous studies in the literature have shown that ongoing nociceptive inputs from OA joints lead to activity-dependent central sensitization (Schaible and Grubb 1993; Coderre 1993). OA and MPS may be with coexisting MSK condition (Weiner 2007). A population of farmers with hand OA coexisting with MPS had higher pain disability and functional joint scores as well as lower grip power compared to OA coexisting either to carpal tunnel syndrome or rotator cuff tear population (Kim et al. 2016).

Bajaj 2001 showed that aged-matched population with the presence of knee OA and CS had increased muscle hyperalgesia and referred pain extending to hindlimb muscles after intramuscular hypertonic saline injection in the tibialis anterior (Bajaj et al. 2001). Similar local and referred pain was not seen in healthy controls leading to the conclusion that abnormal referred and muscle pain was due to CS triggered by OA joint (Bajaj et al. 2001). Recently, a systematic review study suggested that myofascial pain may be a pivotal component of pain-associated to OA (Dor and Kalichman 2017). However, a causal-relationship between these two MSK disorders has yet to be studied. MPS similarly has a high prevalence in an elderly population with spine OA and disc herniation (Weiner 2007; Malanga et al. 2010). Despite studies in the literature suggesting the relationship between OA and MPS, it remains unknown whether spine OA may be a common primary pathology underpinning the pathophysiology and clinical manifestation of MPS. The Neurogenic Hypothesis proposed by Srbely suggests that any persistent primary pathology, such as spine OA, may lead to an activity-dependent
CS and the manifestation of dorsal root reflexes within myotomes that are under innervation of sensitized spinal cord segments (Srbely et al. 2010). Dorsal root reflexes lead to the peripheral release of NI peptides (Sluka et al. 1995), a mechanism that may support the biochemical signature of MPS (Shah et al. 2005).

1.2 Nervous System

The nervous system is a complex network of nerve cells that are distributed throughout the body. Nerve cells transmit electrical signals, known as action potentials, from one nerve cell to the next as a form of communication (Paxinos and Mai 2012). The nervous system is capable of receiving and interpreting chemical, thermal, and mechanical stimuli, as well as external environment stimuli and endogenous stimuli (Paxinos and Mai 2012; Abraira and Ginty 2013; Willis 2007). The nervous system is responsible for interpreting various stimuli and determining an appropriate execution response (Dancey et al. 2014; Shaffer and Harrison 2007). The nervous system can be further sub-divided into two main components, the central nervous system and peripheral nervous system (Paxinos and Mai 2012).

1.2.1 Central and Peripheral Nervous System

The central nervous system (CNS) is comprised of the brain and spinal cord, which act collectively to integrate sensory inputs within specific centers of the CNS (Paxinos and Mai 2012; Abraira and Ginty 2013; Takahashi et al. 2003). The CNS interprets afferent sensory information, which is received from several specialized sensory organs that are distributed throughout the body. The CNS deciphers this
information in order to determine the most appropriate response (Paxinos and Mai 2012; Willis 2007).

The peripheral nervous system (PNS) consists of the nerves and ganglia outside the CNS (Paxinos and Mai 2012; Willis 2007). Their primary function is to connect the CNS to the limbs and organs, to carry signals inwards from sensory receptors to the CNS, and outwards from the CNS towards the body and organs (Shaffer and Harrison 2007; Paxinos and Mai 2012; Carlton 2014). Afferent signals consist of action potentials driven from the periphery towards the CNS while nerve impulses going to the opposite direction are efferent signals (Martin et al. 2008; Abraira and Ginty 2013; Bácskai et al. 2013; Paxinos and Mai 2012). The PNS can be further divided into the autonomic and somatic nervous systems (Paxinos and Mai 2012).

1.2.2 Somatosensory System

The somatosensory system consists of afferent neurons that respond to changes that occur at the level of the surface (skin) or deep tissues (joints, muscle, and organs) (Shaffer and Harrison 2007). The somatosensory pathway is organized according to three different sub-types which are referred to as first, second, and third order neurons (Abraira and Ginty 2013; Sengul and Watson 2012).

1.2.3 First Order Neuron

First order neurons are often referred to as primary sensory neurons as they are responsible for detecting, transducing, and transmitting sensory information from peripheral receptors located within the skin, muscles, joints, and organs (Simone et al.
First order neurons project sensory information onto the dorsal horn of the spinal cord, and are the only neurons that have a direct connection with a sensory surface (Willis 2007). Although these specific neurons provide innervations throughout the entire body, the cell-body of these neurons is located within the dorsal root ganglia (DRG) (Willis 2007; Barry et al. 2015). However, the cell body of primary neurons that innervate the facial region reside within the trigeminal ganglia (Harper et al. 2000; Malhotra 2016). DRG neurons are known as pseudounipolar neurons (Basbaum et al. 2009). This name refers to the bifurcation of neuron axons that results in two branches, a peripheral branch innervating peripheral tissues, and a central branch that forms synapses with second order neurons within the dorsal horn of the spinal cord (Sengul and Watson 2012; Paxinos and Mai 2012; Takahashi et al. 2003). This anatomical organization provides DRG neurons with an important advantage as the sensory stimulus is able to reach the spinal cord without passing through the cell body, thus minimizing the conduction time of sensory information (Basbaum et al. 2009; Carlton 2014; Donaldson 2009). The neural coding process allows for the transformation of external stimuli into a language that the nervous system understands. External stimuli, such as heat or pressure, is transformed into an action potential which is perceived by the nervous system (Paxinos and Mai 2012; Abraira and Ginty 2013). This is the first neurophysiological step in transmitting the peripheral stimuli to the CNS, which is mediated by primary peripheral receptors (Mense and Craig 1988; Takahashi et al. 2003).
The primary afferent neurons are functionally divided into two main categories according to their axon diameter and myelination: large diameter myelinated A-fibers, and small diameter unmyelinated C-fibers (Paxinos and Mai 2012; Coward 2013). A-fibers can be further divided into Aα-fibers, Aβ-fibers, and Aδ-fibers (Coward 2013; Paxinos and Mai 2012). In general, larger diameter afferents possess a larger cell body and a faster conduction velocity (Coward 2013; Carlton 2014). Aα-fibers and Aβ-fibers are very rapidly conducting fibers that are involved in the proprioception of muscles and joints, as well as in detecting low-threshold or non-noxious mechanical stimuli such as touch or pressure, respectively (Abraira and Ginty 2013; Shaffer and Harrison 2007; Sluka 2002). In contrast, Aδ-fibers have a smaller axon diameter, a thinner myelin sheath, and smaller cell bodies found in the DRG (Carlton 2014). The majority of the Aδ-fibers respond to intense high-threshold noxious mechanical, thermal, and chemical stimuli, and are therefore classified as nociceptors (Willis 2007; Basbaum et al. 2009). They promote the first phase of a painful stimuli and are characteristic of well localized acute pain (Almeida et al. 2004).

The Aδ-fiber mediated thermal nociceptors are activated by extreme temperatures (> 45 °C or < 5°C). In comparison, the Aδ-fiber mediated mechanical nociceptors are activated by intense pressure (Basbaum et al. 2009; Smith and Lewin 2009). Additionally, a subset of Aδ-fibers transmit non-nociceptive information resulting from the slow movement of hair and are termed D-hair afferents (Smith and Lewin 2009). The other category of primary afferent neurons are the C-fibers which have no myelin sheath surrounding its axon, and are functionally known for their slow-conduction
velocity (Paxinos and Mai 2012; Marieb 2013). C-fibers are found to have small cell bodies within the DRG and are linked to the second phase, or slow pain aspect, of noxious stimuli (Basbaum et al. 2009; Carlton 2014). These types of fibers are considered polymodal nociceptors since they respond equally to high-threshold noxious mechanical, thermal, and chemical stimuli (Almeida et al. 2004; Willis 2007). Furthermore, the cell bodies of DRG neurons produce neurotransmitters, neuropeptides, neurotrophic factors, and a variety of other substances that are transported to peripheral and central terminals (Almeida et al. 2004; Basbaum et al. 2009; Sperry et al. 2017).

In addition, C-fibers are also divided between peptidergic and non-peptidergic according to the chemicals that are manufactured in their respective DRG neurons (Basbaum et al. 2009). Peptidergic fibers are the largest group of nociceptors and contain the neuropeptides SP and/or CGRP (Basbaum et al. 2009). Non-peptidergic cells lack these neuropeptides but express the binding site for lectin IB4 (Julius and Basbaum 2001). Nociceptors innervating deep structures, such as muscles and joints, are named group III or IV nociceptors (Mense 1994; Littlejohn and Guymmer 2018). Group III nociceptors have thinly myelinated axons, whereas group IV nociceptors have unmyelinated axons (C-fibers) (Mense 1994; Driscoll et al. 2016; Littlejohn and Guymmer 2018). Groups III and IV afferents correspond to skin A-δ and C-fibres nociceptors (Driscoll et al. 2016; Sluka 2002).
1.2.4 Projection Neurons

Once central terminals of the DRG neurons enter the spinal cord they form synapses with second-order neurons located in the spinal grey matter (Ossipov et al. 2010). The spinal cord is organized into ten different layers, from dorsal to ventral, based on neuronal cytoarchitecture differences (Willis 2007; Naeini et al. 2005). In addition, the spinal cord can also be divided into three distinct functional regions: the dorsal horn, the intermediate zone, and the ventral horn (Basbaum et al. 2009). Some non-nociceptive afferents, such as Aα-fibers and Aβ-fibers, project onto inner parts of lamina II but the majority project onto lamina V (Julius and Basbaum 2001; Abraira and Ginty 2013). Primary nociceptive Aδ-fibers from skin project onto lamina I as well as to lamina V of the dorsal horn (Julius and Basbaum 2001; Latremoliere and Woolf 2009). Similarly, C-fiber peptidergic nociceptors project onto lamina I, the outer part of lamina II, and the nonpeptidergic afferents terminate in the mid-region of lamina II in the dorsal horn of the spinal cord (Julius and Basbaum 2001; Latremoliere and Woolf 2009).

Primary afferents conducting nociceptive information from trunk muscles, limb muscles, joints, and visceral tissues project onto lamina I and V with no projections to lamina II of the dorsal horn (Mense and Craig 1988; Sluka 2002). These central terminals synapse with interneurons that are involved in local circuitry and/or descending information during a fast response without the delay of routing signals through supraspinal circuits (i.e. arch reflex). Moreover, central terminals synapse onto projection neurons that are engaged in the ascending information to supraspinal centers (Basbaum et al. 2009; Woolf 2011; Latremoliere and Woolf 2009). The projection fibers
that carry nociceptive information ascend onto supraspinal centers through the contralateral spinothalamic tract (Julius and Basbaum 2001; Ossipov et al. 2010). These projection fibers synapse with third-order neurons targeting thalamic centers (Julius and Basbaum 2001; Ossipov et al. 2010). Additional targets of collateral projections of the spinothalamic tract include: the brain stem nucleus (dorsal reticular nuclei at the medulla oblongata and rostral ventromedial medulla), as well as to the periaqueductal grey region in the midbrain (Fields and Basbaum 1978; Basbaum et al. 2009; Almeida et al. 2004).

Subsequently, neurons involved in the conduction of nociceptive information project onto different cortical areas such as the somatosensory cortex, which is responsible for the discriminative features of noxious stimuli (Willis 2007). These neurons also target affective subcortical areas including the thalamus, hypothalamus, amygdala, pre-frontal, and insular cortices which are involved in the cognitive and emotional experience of noxious stimuli (Gwilym et al. 2009; Latremoliere and Woolf 2009; Almeida et al. 2004). In this stage, the nociceptive stimulus will be interpreted as a painful stimulus (Almeida et al. 2004).

1.2.5 Segmental Division of the Spinal Cord

The spinal cord is a cylindrical structure of nervous tissue composed of white and gray matter (Paxinos and Mai 2012; Marieb 2013). The grey matter is composed of unmyelinated axons and neuronal body cells supported by glial cells and is surrounded by the white matter composed by ascending and descending myelinated axons and glial cells (Paxinos and Mai 2012; Marieb 2013). The spinal cord is organized into various
spinal segments (Liguori et al. 1992). In humans, there are 31 spinal segments, each comprised of a ventral root and dorsal root, resulting in 31 pairs of nerves named spinal nerves exiting the spinal cord (McCulloch and Waddell 1980; Liguori et al. 1992). The spinal cord is segmentally divided into 8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 1 coccygeal nerve (McCulloch and Waddell 1980; Mense and Craig 1988; Liguori et al. 1992). In rodents, the spinal cord is formed by 34 segments: 8 cervical (C1 to C8), 13 thoracic (T1 to T13), 6 lumbar (L1 to L6), 4 sacral (S1 to S4), and 3 coccygeal (Co1 to Co3) (Paxinos and Mai 2012).

Importantly, each spinal nerve from spinal segments contains a mixed of sensory and motor nerves carrying impulses from the brain to periphery and from periphery to the brain (Ivanusic 2007; Imamura et al. 2008; Gunn 1997). Spinal nerves are made up of dorsal root and ventral root (Paxinos and Mai 2012; Marieb 2013). Both dorsal and ventral roots are attached in the dorsal and ventral aspect of the spinal cord respectively, by 15 dorsal and 15 ventral rootlets (in rodents), forming then the dorsal and ventral roots of each spinal nerve (Paxinos and Mai 2012). The dorsal root is the afferent component containing sensory fibers from the skin, subcutaneous and deep tissues, and viscera (Mense and Craig 1988; Paxinos and Mai 2012). The ventral roots consist predominantly of efferent somatic motor fibers (Peyronnard et al. 1986; Marieb 2013).

Interestingly, the segmental distribution that presents in the spinal cord is maintained peripherally (Ivanusic 2007; Imamura et al. 2008; Mense and Craig 1988). For example, the peripheral skin area supplied with sensory fibers from one spinal
segment is named dermatome (Liguori et al. 1992). Comparatively, muscles and bones supplied with sensory fibers from one spinal segment are named myotome and sclerotome, respectively (Peyronnard et al. 1986; McCulloch and Waddell 1980; Ota et al. 2014; Ivanusic 2007). Knowledge and a proper understanding of spinal segmentation has important clinical relevance for an injury/lesion occurring within a given spinal segment, which may be a reflection of the peripherally affected sensory or motor function of a tissue (Srbely et al. 2008, 2010; Chan Gunn 1997; Paxinos and Mai 2012).

1.3 Pain and Nociception

The most appropriate definitions of pain and nociception were determined at the annual meeting of the International Association for the Study of Pain (IASP) in 2007. This meeting defined pain as: “the unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (Loeser and Treede 2008). Nociception was defined as: “the neural processes of encoding and processing noxious stimuli” (Loeser and Treede 2008). These terms should not be confused with one another, such that pain involves a cognitive and emotional aspect, whereas nociception involves the neurophysiological encoding process and the transmission of the nociceptive information (Loeser and Treede 2008).

Pain and nociception contribute greatly to individual survival, alarming the organism of a real or a potential tissue damage (Coderre et al. 1993). An interesting example is from individuals with congenital insensitivity to pain as they do not present the capability of recognizing noxious stimuli (Chen et al. 2015). In such a condition,
noxious stimuli (i.e., heat, cold, sharp) cannot be distinguished from non-noxious stimuli (Chen et al. 2015). Lack of this protective mechanism may lead to self-injury, therefore increasing the risk of premature death due to infection or excessive bleeding (Basbaum et al. 2009; Chen et al. 2015). Pain is an essential mechanism for survival, protecting organisms from injury, however maladaptive pain is a disease affecting the nervous system that is associated with chronic pain and poor quality of life (Cimmino et al. 2011; Latremoliere and Woolf 2009).

Currently, chronic MSK pain is the leading societal cause of pain and disability, affecting more than 40 million Americans (Cimmino et al. 2011). It is estimated that the cost of chronic MSK pain to society is $47 billion dollars USD per year in the United States (Brooks 2006). In summary, a better understanding of the mechanisms behind chronic pain will provide a promising therapeutic avenue to explore with chronic MSK disorders.

1.3.1 Nociceptive Pain and Sustained Pain

Acute nociceptive pain plays a key role in protecting organisms from potential tissue damage, whereas sustained pain is pathological and very often involves a malfunction of the central nervous system (Latremoliere and Woolf 2009; Dib-Hajj et al. 2010). Nociceptive pain is triggered by an event that stimulates small-diameter unmyelinated C-fibers or thinly myelinated Aδ sensory neurons in the periphery (Winkelstein 2011). Sustained pathological pain may evoke noxious and non-noxious sensory fibers, as well as neuroplasticity at the spinal cord and supraspinal centers. Additionally, several other aspects are affected including, functional, chemical, and
structural factors. This leads to the concept of central neuroplastic sensitization, which is a result of persistent increases in peripheral sensitivity of nociceptors (Dib-Hajj et al. 2010; Latremoliere and Woolf 2009).

Nociception, the process of encoding the noxious stimuli, helps to protect the organisms in three different forms: driving the noxious stimulus to supraspinal areas generating an unpleasant sensation; generating a reflex withdrawal from the stimulus to avoid long-term contact; sensitization of the nociceptive system increasing the firing frequency and lowering the threshold for firing when exposed to a previously non-noxious stimulus (Loeser and Treede 2008; Latremoliere and Woolf 2009; Winkelstein 2011).

Peripheral sensitization and neurogenic responses are thought to be important factors contributing to avoidance/protection since they ensure that inflammation, edema formation, and consequently a proper healing tissue process occurs while the nociceptive stimulus is present (Coderre et al. 1993). Peripheral sensitization of the primary neurons may be subsequent to the inflammatory healing process, whereby pro-inflammatory molecules such as prostaglandins, cytokines, chemokines, and neuropeptides are increased at the site of injury (Latremoliere and Woolf 2009; Miller et al. 2015).

These molecules act on their corresponding receptors located on nociceptor afferent endings, resulting in a large intracellular calcium influx, thus lowering the receptor firing threshold (Basbaum et al. 2009; Winkelstein 2011). Ion-channels and receptors play a crucial role in the transduction of nociceptive signals and are
temporally influenced by short and/or long-term modulation (Winkelstein 2004; Basbaum et al. 2009). For example, exposing peripheral afferents to an ongoing tissue injury result in a pro-inflammatory milieu and have been shown to produce long-term effects in the excitability of peripheral nociceptors. Long term effects have included an increase in the gene expression of NGF (nerve growth factor) and its receptor TrkA in afferent peripheral terminals and the DRG (Donnerer et al. 1992; Miller et al. 2015; Sagar et al. 2015).

NGF and TrkA are key features that lead to an increased sodium ion-channel expression in peripheral nerve terminals, therefore facilitating the transduction of action potentials (Sagar et al. 2015; Reinert et al. 1998; Sluka 2002). In addition, NGF amplifies the synthesis and transportation of neuropeptides SP and calcitonin gene-related peptide (CGRP), thus increasing the neurogenic inflammation over the affected areas as well as facilitating the nociceptive transmission centrally (Donnerer et al. 1992; Galeazza et al. 1995; Dib-Hajj et al. 2010; Miller et al. 2015; Sagar et al. 2015).

Other factors such as increased sodium gated-channels at the level of peripheral and central nociceptor terminals also lead to infiltration of inflammatory cells. For instance, macrophages have been observed within the DRG and assist in sensitizing the nociceptive system (Dib-Hajj et al. 2010; Smith et al. 2008; Donaldson et al. 1995).

1.3.2 Central Sensitization and Clinical Presentation

Changes in both peripheral and central terminals of primary nociceptor afferents occur during continuous peripheral noxious stimuli, contributing to the state of central sensitization (CS) which was first suggested by Woolf in 1983 (Woolf 1983). CS is an
abnormal phenomenon associated with chronic pain disorders that is characterized by decreased thresholds of noxious and non-noxious afferents, enhancing and enlarging the function of the nociceptive system (Coward 2013; Sengul and Watson 2012; Woolf 2011; Latremoliere and Woolf 2009).

CS clinically manifests as allodynia (pain elicited by normally innocuous stimuli), and hyperalgesia (exaggerated and prolonged response to noxious stimuli) (Loeser and Treede 2008). Both allodynia and hyperalgesia spread beyond the original site of inflammation/injury leading to secondary hyperalgesia. (Latremoliere and Woolf 2009). Secondary hyperalgesia is a key factor in distinguishing CS from primary nociceptive pain. Hence, the largest contrast between acute/primary pain and chronic pain/CS is that CS involves the recruitment of non-noxious fibers, such as those involved in light touch/heat sensation. The expansion of pain sensations to non-affected areas is a key constituent of the CS notion (Abraira and Ginty 2013; Woolf 2011). In some cases, CS is present even when obvious noxious stimuli is no longer present (Winkelstein 2004). Evidence suggests that in a subpopulation of arthritic patients presenting with multiple painful joints, surgical joint replacement did not improve the widespread symptoms post-surgery as compared to pre-surgery (Perruccio et al. 2012). Thus, even with the removal of main source of nociceptive stimulus, spontaneous pain remains present and suggests that neuroplastic changes may be supporting such abnormal perception.

A substantial body of research suggests that several chronic pain disorders are characterized by abnormal pain modulation (Woolf et al. 2012). In chronic states such as in osteoarthritis (Schaible and Grubb 1993), fibromyalgia (Arendt-Nielsen and Graven-
Nielsen 2003), MPS (Niraj 2018), and chronic back pain (Lidbeck 2002), constant activation of C-fibers overwhelms the release of neuromodulators into the spinal cord causing an amplification and facilitation of the synaptic communication between primary central afferents terminals to second order neurons at the dorsal horn of the spinal cord (Basbaum 2001; Basbaum et al. 2009; Ji et al. 2003; Latremoliere and Woolf 2009).

A facilitatory component from supraspinal descending pathways projecting onto neurons in the spinal cord has been suggested to increase CS (Li et al. 1998; Latremoliere and Woolf 2009). Ongoing noxious inputs result in phenotypical changes within the dorsal horn of the spinal cord, such as increased expression of the G-protein coupled receptor neurokinin-1 (NK1), its neuropeptide ligand SP, glutamate and glutamate receptors, resulting in the phenomenon of CS (Basbaum et al. 2009; Latremoliere and Woolf 2009).

### 1.3.3 Mechanisms Leading to Central Sensitization

SP is an 11-amino acid tachykinin peptide neurotransmitter that binds preferentially to the NK1 receptor (neurokinin receptor 1) (Basbaum et al. 2009). SP is produced in the cell body of DRG neurons and functions in the normal transmission of nociceptive and inflammatory stimuli towards the dorsal horn of the spinal cord (Pernow 1983). SP is carried to both peripheral and central afferent terminals by axonal transport (Pernow 1983; Carlton 2014). After peripheral noxious stimulation, central terminals of A-δ and C-fibers release SP pre-synaptically (Basbaum et al. 2009). Subsequently, SP will bind to its high affinity NK-1 G-protein–coupled receptor that is present on the post-synaptic neuron (Basbaum et al. 2009; Latremoliere and Woolf 2009). In contrast, extensive research suggests that the increase of SP and NK1 at the dorsal horn initiates
CS during ongoing noxious stimulation (Gao and Ji 2009; Latremoliere and Woolf 2009; Julius and Basbaum 2001). It has been demonstrated that SP coupling with its receptor NK-1 increases the sensitivity of post-synaptic neurons centrally, thus facilitating subsequent subthreshold stimuli like non-noxious stimuli triggering action potentials in the post-synaptic neuron (Arendt-Nielsen et al. 2010; Woolf 2011).

In addition, increased SP release in the dorsal horn of the spinal cord has been shown to enlarge neuronal receptive fields peripherally, thus contributing to the spread of pain (Simone et al. 1994; Xu et al. 1992; Ahmed et al. 1995). Similarly, the binding between SP and NK1 has demonstrated a great implication in the behavioral pain modulation. Experimentally induced CS by injection of Complete Freund Adjuvant or capsaicin provokes the presence of secondary mechanical and heat hyperalgesia, behaviors that were significantly diminished when NK1 receptors in the dorsal horn of the spinal cord were chemically or genetically abolished (Sluka 2002; Levine et al. 1984; Pertovaara 1998; Weisshaar and Winkelstein 2014; Donaldson et al. 2001).

Furthermore, SP and NK-1 have been thought to be enhanced in the contralateral spinal cord and DRG after the experimental induction of OA (Donnerer et al. 1992; Kidd et al. 1995; Donaldson 2009).

CGRP, a 37 amino-acid peptide also produced in the DRG cells, is transported towards afferent central and peripheral terminals (Fernandez et al. 1999; Carlton 2014). Centrally, CGRP participates in the establishment of CS by enhancing the co-release of SP by afferent terminals (Latremoliere and Woolf 2009; Malhotra 2016). Furthermore, CGRP binds to its receptor CRP1 in the post-synaptic neuron triggering further
depolarization (Basbaum et al. 2009; Latremoliere and Woolf 2009). In contrast, a key mechanism involving glutamate and the ligation of its N-methyl-D-aspartic acid (NMDA) G-protein–coupled receptor is essential for development and maintenance of CS (Basbaum et al. 2009; Latremoliere and Woolf 2009). Under normal nociceptive transmission, glutamate binds to its ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor but not with G-coupled NMDA since the latter is blocked by Mg²⁺ (Basbaum et al. 2009; Woolf 2011; Latremoliere and Woolf 2009).

However, during sustained and ongoing noxious stimulation augmented pre-synaptic release of SP and CGRP reinforce the depolarization of post-synaptic neurons unblocking the NMDA receptors from Mg²⁺, thus allowing the coupling of glutamate to NMDA (Basbaum et al. 2009; Latremoliere and Woolf 2009; Woolf 2011; Winkelstein 2004). The binding between glutamate to its metabotropic receptor is determinant to triggering long-term neuroplasticity within the CNS (Latremoliere and Woolf 2009).

Glutamate activates protein kinase A and protein kinase C (PKA and PKC) intracellular signaling pathways and extracellular signal-regulated kinase (ERK), which are the suggested intracellular cascade mechanisms underlying the initiation and maintenance of CS in the spinothalamic tract (Gao and Ji 2009; Kawasaki 2004; Latremoliere and Woolf 2009).

ERK 1/2 are activated as a consequence of extreme peripheral pain, functioning as a preferred marker for neuronal plasticity CS (Latremoliere and Woolf 2009; Cruz and Cruz 2007; Gao and Ji 2009). ERK is a component of the mitogen-activated protein kinases (MAPK) superfamily of signaling pathways and is activated by phosphorylation
of upstream kinases (Gao and Ji 2009; Cruz and Cruz 2007). Once stimulated, phosphorylated ERK (pERK) can be translocated into the nucleus to trigger transcriptional factors, such as cAMP-response element binding protein (CREB), which are necessary for the transcription of many neuronal genes, receptors and neuropeptides, strengthening synapse connections and supporting the long-term synaptic plasticity required for CS (Cruz and Cruz 2007; Gao and Ji 2009). On the other hand, glial cells have demonstrated their importance in the development of CS. Astrocytes are the most common type of glial cells in the CNS and are activated through the release of neuropeptides and pro-inflammatory proteins in the dorsal horn during normal and abnormal nociceptive transmission (Weisshaar and Winkelstein 2014). They are thought to provide nutritional and metabolic support to neurons and synapses (Ren and Dubner 2008). Astrocyte hyperactivation has been shown to participate in the neuroplastic events in chronic pain conditions leading to CS providing for instance metabolic support to the amplified nociceptive transmission signaling in the CNS (Kumabe et al. 2017; Hald 2009; Raghavendra, Tanga, and DeLeo 2004; Sun et al. 2006). Despite changes in the spinal neurons are thought to contribute to CS, biomolecular studies investigating supraspinal brain changes after experimental induction of CS are not sufficiently explored (Fields and Basbaum 1978; Lam et al. 2005; Park et al. 2006; Scheid et al. 2013).

1.3.4 Brainstem and Spinal Pain Transmission

The spinal cord is a fundamental component of the central nervous system and is responsible for processing and transmitting nociceptive inputs (Paxinos and Mai 2012).
There is evidence to suggest that following vertebral facet joint injury neuroplastic changes occur within the spinal cord (Latremoliere and Woolf 2009; Woolf 2018). Alterations include enhanced protein expression of glutamate, SP, CGRP, and ERK, promoting the influx of calcium ions to support the initiation and maintenance of CS (Latremoliere and Woolf 2009; Crosby et al. 2014; Henry et al. 2012; Weisshaar and Winkelstein 2014).

Importantly, at the spinal cord level, nociceptive information received from the periphery crosses the midline and ascends onto supraspinal regions, such as brainstem and thalamus, through a contralateral route (spinothalamic tract) (Paxinos and Mai 2012; Basbaum et al. 2009; Latremoliere and Woolf 2009). Finally, the nociceptive information is modulated and interpreted within the sensorial cortex to produce the appropriate pain behavior response (Ossipov et al. 2010; Marieb 2013).

The brainstem represents an important center of pain modulation in the CNS (Sickle et al. 2005; Park et al. 2006). It functions as a modulatory center of nociceptive information and pain-related responses (Cruz and Cruz 2007; Fields and Basbaum 1978). The brainstem will inhibit or facilitate nociception through ascending projections, targeting other areas of the brain such as sensorial cortex, limbic cortex, and amygdala, as well as through descending projections targeting the spinal cord (Fields and Basbaum 1978; Henderson and Keay 2018; Park et al. 2006).

Remarkably, it has been demonstrated that a tonic descending inhibition on spinal cord neurons is normally present under resting conditions; however, facilitation of
nociceptive transmission in the spinal cord via contralateral projections targeting the brainstem post noxious stimuli on peripheral afferents have been evidenced (Fields and Basbaum 1978; Li et al. 1998; Kumabe et al. 2017; Scheid et al. 2013) It is likely that facilitatory or inhibitory mechanisms rely on the pro/anti-inflammatory balance located within the central nervous system (Cruz and Cruz 2007; Scheid et al. 2013; Bendtsen 2000).

In humans, the role of the brainstem in conditions associated with CS is commonly determined through neuroimaging (Zambreanu et al. 2005; Henderson and Keay 2018). Despite the importance of the brainstem on pain modulation, few studies have examined the effects of peripheral joint injuries on the brainstem. Most evidence has been obtained from temporomandibular joint (TMJ) inflammation studies. For example, Park et al. (2006) demonstrated that an injection of capsaicin into the TMJ induces spontaneous electrophysiological activity and sensitization in several brainstem neuronal nuclei involved the nociceptive-pain pathway (Park et al. 2006). Furthermore, Lam et al. (2005) demonstrated that brainstem neurons become spontaneously activated after injection of a glutamate agonist into the masseter muscle or TMJ (Lam et al. 2005). However, there is no evidence to support supraspinal changes at the brainstem level following lumbar spine facet OA.

1.4 Neurogenic Inflammation

By definition, NI is the inflammation triggered by neuropeptides that are released from sensory nerve terminals (Willis 1999). The main neuropeptides triggering NI are
SP and CGRP (McDougall 2006; Carlton 2014). The release of these neuropeptides induces arteriolar vasodilation and plasma extravasation (Berczi 2009; Szolcsányi 1988; Basbaum et al. 2009). NI has been relatively well explored in the skin, in comparison to other tissues (Birklein and Schmelz 2008; Carlton 2014). However, NI has also been shown in several other tissues such as muscle (Eccles et al. 1961; Sluka 2002), articular joints (Decaris et al. 1999; McDougall 2006), visceral organs (Ustinova et al. 2006; Pan et al. 2010), lungs (Barnes 1986), and kidneys (Steinhoff et al. 2000). Thus, emerging therapeutics to control NI are of great interest.

1.4.1 **Clinical presentation of NI and release of neuropeptides**

The pathophysiology of several peripheral and central disorders have been characterized by the contribution of NI such as with migraines (Malhotra 2016), fibromyalgia (Littlejohn and Guymet 2018), osteoarthritis (Schaible and Grubb 1993), air-way diseases (Barnes 1986), genito-urinary pelvic organ diseases (Malykhina 2007; Lian et al. 2017), complex regional pain syndrome (Birklein and Schmelz 2008), and stroke and brain injury. The mechanisms of NI initiation have been based on the antidromic release of specific neuropeptides onto peripheral post-afferent circulation (Sluka et al. 1995; McDougall 2006; Birklein and Schmelz 2008). Activation of afferent fibers triggers an action potential which produces a classic orthodromic signal towards the CNS (Abraira and Ginty 2013; Carlton 2014). However, due to the arrangement of primary afferents with multiple axon branches (axon collaterals), such an action potential will reach collateral axons evoking a neuropeptide release (Willis 1999; Sluka et al. 1995). This arrangement allows for NI to spread to a broader area under the
sensory innervation following tissue injury (Chiu et al. 2012). Originally proposed by Lewis in 1927, this particular NI organization was first recognized in the skin and was referred to as an “axon reflex” (Willis 1999; Sluka et al. 1995).

Due to the close proximity between peripheral nerve endings and blood vessels, the release of neuropeptides binding their receptors will consequently act on vascular endothelial and smooth muscle cells in the blood vessels (Willis 1999; Chiu et al. 2012). For instance, CGRP has been functionally associated to vasodilation and SP produces plasma extravasation and edema (Chiu et al. 2012). Centrally, NI has been found to modulate the blood-brain and spinal cord barrier, resulting in an increased permeability of several larger pro-inflammatory molecules that usually do not cross the barrier (Lewis et al. 2013) and contributing to edema and inflammation post-brain trauma and stroke (Lewis et al. 2013). In addition, experimentally, the involvement of mast cell has been demonstrated to influence the enhancement of NI in deep tissues. Ustinova (Ustinova et al. 2006) showed that the release of SP by peripheral nerves increased mast cell degranulation within the bladder, an effect that was depleted following inhibition of peripheral release of SP.

A key target for SP is the mast cell, which releases histamine and other pro-inflammatory mediators via degranulation in the presence of SP (Lotz et al. 1988). SP also evokes immunoregulatory and chemotactic effects on macrophages, as evidenced by enhanced expression of prostaglandin E2, thromboxane B2, and leukotriene C4, which are all indicative markers of macrophage activation (Lotz et al. 1988). Additionally, SP induces robust proliferation of human and murine T-lymphocytes,
effects which are mitigated by exposure to SP antagonists (Lotz et al. 1988). With mast cell degranulation, an autocrine stimulation may also produce the release of SP (Okamura et al. 2017; Basbaum and Levine 1991; Steinhoff et al. 2000).

Likewise, mast cell degranulation releases tryptase, the most abundant enzyme that cleaves peptide bonds in proteins (Fitzgerald et al. 2013; Steinhoff et al. 2000). Several proteins are subjected to cleavage by tryptases, one of which is protease activated-receptor 2 (PAR2) (Steinhoff et al. 2000; Russell and McDougall 2009). Tryptase is released from degranulated mast cells which then cleaves PAR2 at the membrane of afferent nerve endings to expose a tethered ligand domain that binds and activates its cleaved receptor (Steinhoff et al. 2000; Vergnolle et al. 2003). Activation of PAR2 stimulates the release of SP and CGRP from sensory nerve endings, reinforcing the neurogenic inflammation milieu (Steinhoff et al. 2000; Vergnolle et al. 2001). Overall, this cascade has been suggested to strongly contribute to disorders where NI is present, such as in OA (Russell and McDougall 2009).

1.4.2 Role of SP and CGRP in Mediating Inflammation

SP and CGRP have shown the ability to upregulate proinflammatory cytokines that play a crucial role in mediating the inflammatory response (Delgado et al. 2003). Exposure to SP triggers the production of TNFα, IL1-β, IL-2 and IL-6 in three different populations of white blood cells including T-lymphocytes, macrophages, and neutrophils (Delgado et al. 2003; Payan et al. 1983). Also, the presence of SP and CGRP at the neuromuscular junction were shown to increase the release of acetylcholine (ACh) and increase calcium mobilization within the muscle (Wali 1985; Gaydukov et al. 2016;
Malomouzh 2012). These findings may have implications for regulating muscle pain, inflammation, and MTrP formation in MPS. Consequently, it may be of interest to investigate whether the presence of neuropeptides and NI in musculoskeletal tissues are modulated after the development of CS.

1.4.3 Dorsal root reflex and Neurogenic Inflammation

In addition to the axonal reflex, accumulating evidence supports the involvement of the CNS in mediating NI, a mechanism that is titled the dorsal root reflex (DRR) (Sluka et al. 1995; Rees et al. 1996). A determinant factor of the DRR cascade is the involvement of signals that are being propagated centrally in the spinal cord as nociceptive information, and peripherally as SP is released from neuronal terminals (Sluka et al. 1995; Pan et al. 2010; Malykhina et al. 2013). Activation of the nociceptive system in the spinal cord produces an antidromic (central to peripheral) response in the same afferent nerve fibers releasing SP and CGRP (Eccles et al. 1961; Rees et al. 1996; Sluka et al. 1995; Lin et al. 2000). The DRR have been demonstrated in all fiber types including highly myelinated A-α/β, type IA sensory afferents in muscles, and A-δ and C-fibers (Sluka et al. 1995; Rees et al. 1996; Eccles et al. 1961). However, low myelinated A-δ and C-fibers terminals store a large amount of SP and CGRP within vesicles, and therefore these nociceptive fibers produce larger NI effects (Reinert et al. 1998; Hughes et al. 2006). Supporting this assumption is the fact that DRR triggers NI only under noxious condition (Eccles et al. 1961; Willis 1999)

Sluka et al. (1995) and Rees et al. (1996) studied the effects of articular
inflammation using carrageenan in rats (Sluka et al. 1995; Rees et al. 1996). Both of these studies showed that augmented NI responses occur peripherally in the hind paw of rats (Sluka et al. 1995; Rees et al. 1996). Such effects were eliminated by dorsal rhizotomy (Sluka et al. 1995; Rees et al. 1996), and by central administration of inhibitors of non-NMDA glutamatergic receptors CNQX (Sluka et al. 1995) and NMDA glutamatergic receptors (AP7) (Lin et al. 2000) in the spinal cord. Similarly, DRR was abolished after intrathecal application of the GABA\textsubscript{A} receptor agonist bicuculline (Sluka et al. 1995; Willis 1999). Glutamatergic receptors play a dual role in the nociceptive circuit such that they play a role in establishing the start of CS and are important for influencing the DRR (Latremoliere and Woolf 2009; Lin et al. 2000). These studies supported that conditions leading to central neuroplastic modulation may present with an antidromic release of neuropeptides by sensory afferents (Sluka et al. 1995; Rees et al. 1996; Lin et al. 2000).

Therefore, the current accepted model of NI involves a stimulating event at the neuronal terminal, an axon reflex spreading to collaterals, followed by a resultant antidromic depolarization from the CNS (Sluka et al. 1995; Willis 1999; Donaldson 2009). Although the presence of antidromic action potentials (efferent) (DRR) have been shown in the dorsal root of myelinated type 1 afferents of skeletal muscle (Eccles et al. 1961) it is unknown if the release of SP and CGRP occur in skeletal muscle following spine OA with presence of CS.
1.4.4 Osteoarthritis and Neurogenic Inflammation

OA is one of the most common musculoskeletal diseases that affects millions of patients per year (van Saase et al. 1989; Dubin 2016; Zhang and Jordan 2010). OA is characterized as a progressively painful inflammatory condition affecting multiple articular joint structures including cartilage, synovia, subchondral bone, and adjacent tissues (Hurtig et al. 2009; Ayral et al. 2005; Walsh et al. 2010; Rice et al. 2011). The clinical presentation of OA exhibits an asymptomatic phase that often goes undiagnosed and undertreated until the appearance of primary symptoms including pain and joint dysfunction (Martin et al. 2004; Ayral et al. 2005). The persistent and continuous peripheral stimuli of an OA joint is believed to play a key role in the transition between a local and acute disorder, to a chronic and widespread disease (Winkelstein 2011; Arendt-Nielsen et al. 2010).

OA often presents with spontaneous pain, allodynia, and hyperalgesia in multiple body parts which supports the role of central mechanisms such as CS in experimental and human studies (Perruccio et al. 2012; Niu et al. 2003; Ferland et al. 2011; Ohmichi et al. 2012; Bove et al. 2006; Carlesso et al. 2017; Rakel et al. 2015). Additionally, OA is difficult to manage due to its complexity and multifactorial etiology (Cimmino et al. 2011). Studies have suggested that the pathophysiology of OA is a result of a biomechanical impairment and a crossover between the nervous and immune systems (Sperry et al. 2017; Winkelstein 2011; Donaldson 2009; McDougall 2006). OA has largely been considered as peripheral disease; however evidence suggests that maladaptive central neural mechanisms are an important underlying factor in OA.
presentation and prognosis (Rees et al. 1996; Levine et al. 1984; Basbaum and Levine 1991; Schaible and Grubb 1993). The disease profile in a subset of OA patients, where OA progresses beyond the primary arthritic tissue/joint to secondary joints, supports the role of a central mechanism in the pathophysiology of this disorder (Decaris et al. 1999; Perruccio et al. 2012; Arendt-Nielsen et al. 2010)

Some authors have demonstrated that contralateral homologous joints are also commonly affected by pain and degeneration (Levine et al. 1986; Kidd et al. 1995; Mapp et al. 1993; Kelly, Dunham, and Donaldson 2007). Donaldson (2009) suggested that a vicious cycle is initiated by stimulating nociceptors in the primary articular OA joint, which activates joint afferents that act to maintain a local/regional inflammatory process through neurogenic inflammation (Donaldson 2009). In a similar manner, activation of peripheral afferents in the primary OA joint lead to sensory changes and initiation of inflammatory and catabolic changes in the cartilage at the contralateral joint (Kidd et al. 1995; Mapp et al. 1993; Kelly et al. 2007). Although the mechanisms which spread OA to remote sites are not completely understood, CS influences on the antidromic release of SP and CGRP by DRR triggering neurogenic inflammation have been previously established in the literature (Sluka et al. 1995; Rees et al. 1996; Lin et al. 2000; Donaldson 2009).

Interestingly, Levine et al. (1986) demonstrated vasodilation, plasma extravasation, and hyperalgesia in rats at the homologous contralateral joint after a unilateral series of saline injections under the paw (Levine et al. 1986). Kidd et al. (1995) reported similar findings, adding that neuropeptide infiltration in the synovium membrane
was linked to changes at the vascular tissue and leukocyte infiltration, thus creating an favorable inflammatory microenvironment within this tissue (Kidd et al. 1995). Many additional studies have further highlighted the ability of SP to enhance inflammation and progression of intraarticular degeneration by stimulating synovial cell production of prostaglandins and inflammatory cytokines (Decaris et al. 1999; Mapp et al. 1993; Schaible 2000). Similarly, SP antagonists have been shown to effectively reduce intraarticular inflammation (Decaris et al. 1999; Bileviciute et al. 1993).

It was previously shown that an elevated expression of SP in synovial tissue was related to cartilage degeneration in models where monoarthritis was experimentally induced (Galeazza et al. 1995; Sutton et al. 2009; Scott, Lam, and Ferrell 1992). Indeed, injection of SP into the hind paw of rats was seen to be associated with patellar metabolism changes inducing degeneration (Decaris et al. 1999). Additionally, previous research has shown that deafferentation of the unaffected joint via capsaicin application (Donaldson et al. 1995), eliminates the neurogenic response observed in the unaffected contralateral joint (Gouze-Decaris et al. 2001). Moreover, it has been widely shown that SP is augmented in the dorsal horn of the spinal cord in arthritic conditions (Bove et al. 2006; Schaible et al. 1990; Abaadie et al. 1996).

Saxler et al. (2007) demonstrated that there is a higher density A-δ and C-fibers expressing CGRP and SP within an OA joint versus a non-OA joint (Saxler et al. 2007). Mapp et al (1993) and Bileviciute et al. (1993) both reported that there was an increase in the SP neuropeptide profile within knee joint terminals, mainly those localized close to synovium cartilage in rats (Mapp et al. 1993; Bileviciute et al. 1993). These two studies
also showed an augmented transport and release of the SP neuropeptide on bilateral spinal cord axon terminals, demonstrating a relationship between the affected joint and spinal concentration of SP (Mapp et al. 1993; Bileviciute et al. 1993). Importantly, Gouze-Decaris et al. (2001) reported on the neurophysiology of articular cartilage injury and concluded that the signals that trigger articular cartilage damage induced distant and local inflammation, which is transmitted through the afferent C-fibers (NI) and involves spinal and supraspinal sensitization (Gouze-Decaris et al. 2001).

Similar mechanisms have also been observed in visceral tissues. Experimentally induced colitis was shown to enhance the release of SP in the neurosegmentally linked bladder, leading to enhanced mechanical sensitivity of bladder afferents and chronic cystitis (Ustinova et al. 2006). Moreover, C-fibers were demonstrated to mediate this neurogenic response (Malykhina 2007). However, it is unknown if similar responses could occur in heterologous skeletal tissues post primary spine OA.

1.5 Cartilage

1.5.1 Structure and Function of Cartilage

Cartilage is a connective tissue found in many areas in the body including joints between bones, rib cage, ear, nose and intervertebral discs (Millward-Sadler et al. 2003; Martin and Buckwalter 2001). Articular cartilage is highly specialized tissue designed to absorb and disperse the forces experienced during joint loading (Usmani et al. 2016). It is composed of a dense extracellular matrix (ECM) with a limited distribution of chondrocytes embedded within the ECM which protects chondrocytes (Fox et al. 2009;
Poole et al. 2001). Chondrocytes are the only cell type located in cartilage (Poole et al. 2001). Chondrocytes are mechanosensitive cells and significant contributors to ECM production, maintenance, and repair (Fox et al. 2009; Usmani et al. 2016). The ECM is predominantly composed of water, collagen, and proteoglycans (Fox et al. 2009; Martin and Buckwalter 2001). The ECM contains mainly type II collagen fibres representing 90% to 95% of the collagen in ECM and forms fibrils and fibres tangled with proteoglycans (Dowthwaite 2004; Fox et al. 2009). Together, these components help to retain water within the ECM, which is critical to maintaining its unique mechanical properties as well as providing a smooth and lubricated articular surface (Fox et al. 2009; Glyn-Jones et al. 2015).

The articular cartilage is organized in different cartilage zones presenting morphological and functional variations within each of them (Dowthwaite 2004). The superficial zone comprises 10-20% of the cartilage; contains a high number of flattened and dissipated chondrocytes (Fox et al. 2009). This zone is in contact with the synovial fluid and is responsible for most of the tensile properties of cartilage, which enable it to resist the sheer, tensile, and compressive forces imposed by articulation (Fox et al. 2009; Poole et al. 2001). The integrity of this layer provides protection and maintenance of deeper layers of the cartilage (Martin and Buckwalter 2001). The middle zone represents 40% to 60% of the total cartilage volume (Fox et al. 2009; Martin and Buckwalter 2001). The chondrocytes are spherical and at lower density compared to superficial zone (Fox et al. 2009; Dowthwaite 2004). The collagen fibres in the ECM are organized obliquely but more sparse compared to superficial zone (Martin and
Buckwalter 2001; Fox et al. 2009). Functionally, the middle zone is the first line of resistance to compressive forces (Fox et al. 2009).

The deep zone represents approximately 30%-35% of articular cartilage volume and is responsible for providing the highest resistance to compressive forces (Martin and Buckwalter 2001; Fox et al. 2009; Poole et al. 2001). Its unique function is due to the arrangement of the chondrocytes, collagen fibres, highest proteoglycan content, and the lowest water concentration within this zone compared to other cartilaginous zones (Martin and Buckwalter 2001; Fox et al. 2009; Poole et al. 2001). Chondrocytes are arranged in columnar orientation, yet collagen fibres are arranged perpendicular to the articular surface (Fox et al. 2009). The calcified zone plays an integral role in securing the cartilage to bone, by anchoring the collagen fibrils of the deep zone to subchondral bone (Fox et al. 2009). Chondrocytes are scarce and hypertrophic at this zone (Fox et al. 2009). Hypertrophic chondrocytes may synthesize Type X collagen and can calcify the extracellular matrix, providing an ideal structural integration with subchondral bone (Poole et al. 2001).

1.5.2 Pathophysiology of Cartilage

Cartilage architecture and its biochemical composition are strictly regulated by chondrocyte cells (Glyn-Jones et al. 2015). In healthy cartilage, the normal turnover of ECM components is mediated by chondrocytes which synthesize collagen and proteoglycan as well as the proteolytic enzymes responsible for their breakdown (Man and Mologhianu 2014). Failure of chondrocytes to maintain homeostasis between
synthesis and degradation of the ECM components due to for instance trauma, biochemical stress and inflammation may result in pathological joint disorders as OA (Man and Mologhianu 2014; Glyn-Jones et al. 2015; Thudium et al. 2018).

Chemical and mechanical alterations in the joint environment impact the metabolic activities of chondrocytes and consequently the homeostasis of the articular joint (Akkiraju and Nohe 2015; Glyn-Jones et al. 2015). Activated chondrocytes may produce several inflammatory responses such as IL-1β, IL-6, and TNFα, and matrix-degrading enzymes including the metalloproteinases and a disintegrin that have anabolic and catabolic effects upon ECM (Akkiraju and Nohe 2015; Glyn-Jones et al. 2015). Some of these compounds, such as the metalloproteinases 1, 3, and 13 and aggrecan-degrading enzymes (ADAMTS 4 and 5), may impact the cartilaginous tissue contributing to the joint pathogenesis negatively (Glyn-Jones et al. 2015).

1.5.3 Role of Cartilage in Pathophysiology of OA

Articular cartilage joint degradation and loss of cartilage has been thought to be the primary change occurring in OA (Man and Mologhianu 2014). Inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, are recognized to be upregulated during OA progression (Sutton et al. 2009; Glyn-Jones et al. 2015). These inflammatory cytokines are very often secreted by chondrocytes and synovial cells (synoviocytes) (Glyn-Jones et al. 2015; Remst et al. 2015; Akkiraju and Nohe 2015). Initial stages of OA lead to cartilage softening, fibrillation and erosions of the articular surface (Glyn-Jones et al. 2015; Akkiraju and Nohe 2015). Breakdown of proteoglycans leads to a reduction in the
compressive stiffness of the tissue that accelerates the rate of collagen loss (Akkiraju and Nohe 2015).

Chondrocytes identify the loss of ECM and dynamically produce collagen type II and proteoglycans (Akkiraju and Nohe 2015; Man and Mologhianu 2014). However, over time, the ratio between the ECM production to proteolytic enzyme production is imbalanced and more pronounced damage can be observed culminating in total cartilage destruction and exposure of underlying subchondral bone plate (Man and Mologhianu 2014; Akkiraju and Nohe 2015). The cartilage damage is irreversible despite chondrocytes attempting to repair the cartilaginous tissue (Glyn-Jones et al. 2015). Fibrosis formation, osteophytes and joint stiffness are examples of the reparative effect chondrocytes may employ on fibroblastic and osteoblast cells in OA (Findlay and Atkins 2014; Remst et al. 2015).

1.6 Ostearthritis (OA)

1.6.1 Prevalence and Burden of OA

OA is one of the most common musculoskeletal diseases that affect millions of patients per year (Cimmino et al. 2011; Zhang and Jordan 2010). It is the most common painful and disabling joint disease worldwide affecting an estimated 10% of men and 18% of women over 60 years of age (Glyn-Jones et al. 2015). The most commonly affected joints include the hands and weight-bearing joints (hips, knees, feet and spine)(Liva et al. 2017; Gellhorn, Katz, and Suri 2013; Arthritis Alliance of Canada 2011). This prevalence may increase according to criteria of diagnosis and principles of
inclusion in epidemiological studies and may reach 45% when radiographic signs, symptoms and lifetime risk are combined (Murphy et al. 2008). It is estimated that 15% of the Canadian population suffer from OA (Arthritis Alliance of Canada 2011) with age being the strongest risk factor for osteoarthritis (McDaniel and Rozanova 2011).

Due to increased longevity, it is projected that 1 in 4 people in Canada will present with OA by the year of 2040 (Arthritis Alliance of Canada 2011). The costs involved with OA diagnosis, treatment, pain management and eventually joint replacement in advanced stages of OA are enormous reaching hundreds of billions of dollars per year (Zhang and Jordan 2010; McDaniel and Rozanova 2011; Cimmino et al. 2011; Arthritis Alliance of Canada 2011).

1.6.2 Clinical Features of OA

OA is a painful and disabling multifactorial disease, which may affect the primary structural components of the articular joint including cartilage, synovia, subchondral bone, bone marrow and adjacent tissues (Fox et al. 2009; Glyn-Jones et al. 2015). The main clinical symptoms are joint pain, stiffness, diminished physical function (Thudium et al. 2018). OA exhibits an asymptomatic phase and often goes undiagnosed until primary symptoms appear including pain and joint stiffness (Woolf et al. 2012). The most traditional method of clinical diagnosis of OA is the presence of joint structural changes on radiographic imaging including narrowing of the joint space width, osteophyte formation, and the development of subchondral sclerosis at radiography (Glyn-Jones et al. 2015; Guermazi et al. 2012). However, such structural changes may only appear in the radiographic exams after the establishment of joint changes in a
molecular level. Thereby, detectable structural changes in radiographic exams may be preceded by early molecular alterations in OA that are undetectable in radiographic exams (Conaghan 2013; Glyn-Jones et al. 2015). The lack of sensitivity of such exams to molecular anomalies in OA joint may delay the clinical diagnosis and management of osteoarthritic patients (Conaghan 2013; Glyn-Jones et al. 2015).

OA may have several disease phenotypes, for instance, OA could be an isolated cartilage disease, may extend to inflammatory disorder at synovium tissue and indeed may affect the bone marrow (Conaghan 2013). Understanding whether OA affects one or more joint tissues may have a direct impact on therapeutic effectiveness (Conaghan 2013). Although the etiology of OA is still unknown ageing and previous joint injury are two of the most common risk factor to develop OA (Usmani et al. 2016). Animal studies have provided important awareness when studying different forms of OA. Opposite therapeutic effects on post-traumatic experimental models compared to spontaneous ageing OA have been shown (Usmani et al. 2016; Pest et al. 2014). Despite strong phenotypic similarities in the osteoarthritic joints exist between post-traumatic and spontaneous OA (due to ageing), these subtypes of OA seem to involve different molecular events in the progression of OA (Usmani et al. 2016; Pest et al. 2014). Such differences may have significative implication on the pathophysiology of OA as well as in the therapeutic method of choice for their management (Usmani et al. 2016; Pest et al. 2014).

The presence of chronic pain and central sensitization in a subset of OA patients may impact the therapeutics negatively OA disorder as evidenced in a study conducted
with patients with advanced OA (Perruccio et al. 2012). In addition, patients with joint OA have higher probability of presenting with painful symptoms involving other joint-associated tissues, such as muscles and other joints (Carlesso et al. 2017; Bajaj et al. 2001). A greater understanding of the central nervous system’s role in the pathophysiology of OA is needed.

1.6.3 Association Between Age and OA

Age is recognized as a significant risk factor for degenerative spine diseases such as OA, and is the most common cause of chronic disability in older adults (Lotz and Loeser 2012; Musumeci et al. 2015). The most commonly affected joints of OA are the knee, hip, spine, and hands (Greene and Loeser 2015). Although elderly individuals are at a higher risk for developing OA, the etiology is unclear. It has been widely argued that a low-grade, continuous, inflammatory process is a contributing factor of OA pathophysiology (Franceschi et al. 2007), with a combination of local and systemic inflammatory age-related changes supporting the development of OA in elderly populations (Greene and Loeser 2015; Mobasheri et al. 2015).

Gibson and Farrell (2004) suggest that plastic changes in the structure and function of the nociceptive system may also contribute to a higher risk of OA development (Gibson and Farrell 2004). Some of the functional changes that occur in the nervous system are thought to be related to NI (Gibson and Farrell 2004) as NI has been shown to be a significant contributor to the ongoing inflammatory process observed in OA joints (Schaible and Grubb 1993; McDougall 2006).
In addition, chondrocyte senescence has been described as normal component of the natural aging process, typically observed after 40 years of age (Mobasher et al. 2015). Chondrocyte senescence is characterized by features such as chondrocyte loss at the superficial layer, and loss of cartilage function (Martin et al. 2004). The decrease in the proteoglycan aggregate within the ECM is a feature of OA (Akkiraju and Nohe 2015). Also, the occasional presence of chondrocytes in the superficial zone is followed by an increase in the number of chondrocytes in deep layers, increasing the stiffness of cartilage (Akkiraju and Nohe 2015).

Several other factors may contribute for the loss of the chondrocytes and cartilage function including overload, inflammation, and oxidative stress, which increase until the final stage of chondrocyte death is reached (Martin and Buckwalter 2001; Martin et al. 2004; Hardy et al. 2002; Mobasher et al. 2015). However, the primary factor responsible for increasing the incidence of OA in an elderly population remains unclear.

1.6.4 Current Theories of OA Pathophysiology

Osteoarthritis was once viewed as a disease of purely mechanical cartilage degradation, but it is now accepted to be a multifactorial condition affecting the whole joint (Glyn-Jones et al. 2015; Fox et al. 2009). Trauma, mechanical alterations and inflammation may lead to deterioration of cartilage, increase subchondral bone remodelling and bone sclerosis which individually or collectively may have a crucial role in OA symptom generation and OA pathogenesis (Glyn-Jones et al. 2015; Fox et al. 2009). While the exact etiology of OA remains unclear, numerous risk factors have been
identified. Associations with systemic inflammation and metabolic disease have shown importance on OA progression (Greene and Loeser 2015; Gandhi et al. 2014).

Chondrocytes may be activated by inflammatory signals originating from other joint structures such as synovium or subchondral bone as well as by systemic low-grade inflammatory process in patients with metabolic syndrome justifying its relationship with OA disorder (Gandhi et al. 2014; Thudium et al. 2018; Sutton et al. 2009). Also, joint injury and advanced age are two crucial risk factors related to OA development (Usmani et al. 2016). It has been well recognized that a history of joint injury including ligament tears, meniscal injuries, and cartilage micro-trauma is a crucial risk factor for the development of post-traumatic OA (Muthuri et al. 2011). The ageing population, cumulative effects of repetitive loading and microdamage at the joint over time, increased collagen network formation in the ECM of the cartilage, decreased in proteoglycan deposition and increased oxidative stress are some of the possible theories associated to joint stiffness and consequently loss of joint function in OA (Martin et al. 2004; Aigner and Richter 2012; Usmani et al. 2016). Other theories relate to specific age-related changes may be loss of cartilage cells due to chondrocyte senescence, autophagy, and apoptosis (Martin and Buckwalter 2001; Martin et al. 2004). Despite the prevalence of OA, its pathophysiology is still poorly understood.

1.6.4.1 Symmetry and Spread of OA

In contrast to rheumatoid arthritis, OA has been historically considered an asymmetric disease and the majority of research continues to focus on the individual joint as a single unit (Brooks 2006; Glyn-Jones et al. 2015). Accumulating cross-
sectional studies and longitudinal studies have revealed that OA preferentially progresses to the contralateral homologous joint. Keenan, in a cross-section study, showed that individuals over fifty presenting bilateral painful/disabling joint represented four times greater the number of individuals with a single joint disorder (Keenan et al. 2006). Perruccio (Perruccio et al. 2012) observed that individuals with advanced knee OA presented with an average of 5 different painful joints, in addition to the primary OA joint. The authors concluded that multiple symptomatic joints in advanced OA may negatively impact any therapeutic/surgical intervention these individuals are exposed (Perruccio et al. 2012).

Carlesso, assessing bilateral structural radiographic changes of knee joints observed that although evidence of bilateral structural changes was not perceived over radiographic images, the presence of bilateral painful knee was high incident (Carlesso et al. 2017). A possible mechanism involving central sensitization was speculated to underpin bilateral joint pain (Carlesso et al. 2017). Also, in a longitudinal study, Metcalfe revealed that 80% of patients with unilateral OA disease at baseline developed bilateral OA after 12 years (Metcalfe et al. 2012). Similarly, in another longitudinal study performed in elderly women population, 34% of the population assessed presented bilateral OA after two years of follow up (Spector, Hart, and Doyle 1994). Thereby, bilateral knee osteoarthritis is prevalent with time and is a frequent problem in the ageing population (Metcalfe et al. 2012; Spector et al. 1994). It is possible that changes in the biomechanics of load bearing-joints may contribute to the progression of OA to the contralateral side (Spector et al. 1994; Metcalfe et al. 2012), however the nervous
Experimental animal studies have shown that a neurogenic mechanism may have an essential influence on the symmetrical development of OA. Levine, back in 1985, suggested that peripheral afferent nerves mediating neurogenic inflammation may underpin the symmetric inflammation and catabolic effects on both single and contralateral joints (Levine et al. 1985). Kelly elegantly demonstrated this using an electrophysiologic study which highlights the manifestation of antidromic action potentials in neuronal cells in knee joint afferents bilaterally (Kelly et al. 2007). These central to peripheral action potentials or antidromic impulses are known to result in the release of neurogenic inflammatory and vasoactive neuropeptides as SP and CGRP (Kelly et al. 2007; Donaldson 2009). In fact, several studies have supported the involvement of nervous system and neurogenic inflammation in the release of SP and CGRP in symmetrical OA joints (Donaldson et al. 1995; Bileviciute et al. 1993; Mapp et al. 1993; Kidd et al. 1995; Sluka et al. 1995; Rees et al. 1996). While these segmental pathways form an anatomic basis rationalizing the spread of inflammation to neurosegmentally-linked contralateral homologous joints, no studies have investigated similar mechanisms between neurosegmentally-linked heterologous joint tissues.

1.6.4.2 Neurogenic Inflammation and OA pathophysiology

Despite the fact that bilateral spread of OA has been identified in the pathophysiology of individuals presenting with primary OA, the underlying mechanisms
mediating the spread of OA has not yet been elucidated (Keenan et al. 2006; Spector 1994). Some studies have proposed a role of the nervous system mediating the symmetric and bilateral abnormalities related to OA (Kidd et al. 1995; Rees et al. 1996; Decaris et al. 1999; Kelly et al. 2007). Since joints are richly innervated by neuropeptide-containing primary afferent, it paves the possibility that neurogenic mechanism may mediate symmetric inflammation and metabolic alterations within joint tissues (Rees et al. 1996; Decaris et al. 1999; Kelly et al. 2007). It is widely recognized that synovium tissue is an innervated tissue and cartilage, instead, is a non-innervated and non-vascular tissue (Fox et al. 2009; McDougall 2006). Studies have already shown the role of neurogenic inflammation in the bilateral synovitis (Bileviciute et al. 1993; Ayral et al. 2005; Sutton et al. 2009). Both human and pre-clinical studies have raised the possibility that changes in the peptidergic joint innervation in the articular cartilage aligned with presence of neurogenic inflammation may underline the worsening of cartilage damage and progression of OA (Walsh et al. 2010; Decaris et al. 1999; Miller et al. 2015; Thudium et al. 2018).

Existing animal models suggest that neurogenic inflammation is mediated by the Dorsal Root Reflex (DRR), characterized by central terminal depolarization of primary low-myelinated and non-myelinated afferents and leading to antidromic efferent release of neuropeptides (SP and CGRP) through these same afferents into peripheral tissues (Sluka et al. 1995; Rees et al. 1996). In pre-clinical studies of OA the increase of SP and glutamate content within the dorsal horn of the spinal cord have been observed, mediating the manifestation of CS, increasing the antidromic transport of neuropeptides
related to NI in the afferents innervating peripheral knee joint (Sluka et al. 1994; Lin et al. 2000). These peripheral effects were reversed by spinal cord infusion of NMDA and non-NMDA glutamate receptor antagonists (Sluka et al. 1994; Lin et al. 2000). Thus, these studies evidenced the connection between activity-dependent CS and DRR facilitating the efferent release of inflammatory neuropeptides.

Experimental evidence also suggests that the DRR is mediated through neurosegmentally innervated pathways, both ipsilaterally and contralaterally (Sluka et al. 1994; Sluka et al. 1995; Rees et al. 1996; Kelly et al. 2007). The contralateral spread of inflammation has been abolished by selective denervation of small afferents of either limb (Donaldson et al. 1995), the contralateral limb (Rees et al. 1996) as well as by dorsal rhizotomy (Sluka et al. 1994) and pretreatment with SP receptor antagonist (NK-1) (Decaris et al. 1999) suggesting that the DRR is strongly mediated through neurosegmental pathways. Donaldson (1995) highlighted the unique importance of a class of vanilloid receptor (TRPV1) expressing fibers in mediating contralateral joint inflammation by reporting decreases in the expression of pre-protachykinin (PPT) and calcitonin gene-related peptide (CGRP) mRNA in contralateral dorsal root ganglia (DRG) after lesioning of small unmyelinated afferents with capsaicin of either hindlimb (Donaldson et al. 1995). The protective effect of deafferentation has also been observed clinically. Thompson and Bywaters (Thompson and Bywaters 1962) reported on 4 cases in which hemiplegic joints have been spared following the subsequent development of rheumatoid arthritis (RA). Glick (Glick 1967) published similar findings in a series of 12 case studies of RA where strong associations between the degree of paralysis and joint
sparing in the affected limb were reported, while Veale (Veale et al. 1993) also said similar joint sparing in a patient with left-sided hemiplegia that subsequently developed psoriatic arthritis. These collective observations underscore the role of sensory afferent pathways in mediating neurogenic effects and facilitating the neurogenic responses influencing the spread of inflammation to contralateral homologous tissues. However, it is still unclear if a similar mechanism may be present between the neurosegmentally-linked heterologous joint.

1.6.5 Structural Organization of Spine Facet Joints

Low back pain (LBP) has been identified as a common occurrence that affects a significant proportion of the population (Patrick et al. 2014; Golob and Wipf 2014). Diverse innervated tissues such as lumbar facet joints are a potential key source of LBP, thus suggesting the involvement of the CNS in the pathogenesis of chronic LBP (Tessitore et al. 2015)

The facet joints or zygapophysial joints are anatomically composed by superior and inferior articular processes projecting posterolaterally from the vertebral body (Cohen and Raja 2007). Coupling between the adjacent superior vertebrae and inferior vertebrae form the facet joint (Cohen and Raja 2007). Facet joints are responsible for providing support and stabilizing the spine, then preventing injury by limiting motion in all planes of action (Cohen and Raja 2007; Sperry et al. 2017). In addition, the facet joint is highly innervated by nociceptors that are fired in the presence of capsule facet joint stretch, mechanical spine overloading and spine degenerative diseases (Iita et al.
2017; Cohen and Raja 2007) Notably, facet joints are innervated by segmental and suprasegmental DRG afferent axons (Cohen and Raja 2007).

1.6.6 **Spine Osteoarthritis, Facet Injury and Central Sensitization**

Facet joint OA is characterized by joint-space narrowing, osteophytes, and articular-process hypertrophy (Gellhorn, Katz, and Suri 2013). Localized pain is the most common side effect of facet joint OA (Tachihara et al. 2007); however referred pain has also be reported. For example, patients with lumbar facet OA on segments L3-S1 reported pain that spread to anterior thigh regions (Gellhorn et al. 2013; Sperry et al. 2017). In contrast, OA located at L1-L4 evoked pain that remained more localized in the lumbar and gluteal region (Gellhorn et al. 2013; Huang et al. 2014). Henry et al. (2012) presented a new model for experimentally inducing facet joint OA at the lumbar spine by applying mechanical facet compression on the L4-L5 facet join in rats. This new model effectively demonstrated the presence of local nociceptive pain as well as referred secondary mechanical allodynia (Henry et al. 2012). In addition, histological examinations of the facet cartilage demonstrated severe cartilage degeneration, proteoglycan loss, and surface irregularities, confirming the presence of OA (Henry et al. 2012).

Neuroplastic changes related to CS, and OA-like changes at the level of the spinal cord, have been shown to develop post facet injury (Sperry et al. 2017). For instance, Henry et al. (2012) demonstrated an increase in phosphorylation of ERK 28 days after spine OA-like changes had occurred (Henry et al. 2012). In addition, an increase in SP concentration in the dorsal horn of the spinal cord was presented.
Increases in SP in the spinal cord have been reported as early as 24 hours post spine facet injury (Crosby et al. 2013). Interestingly, ablation of neurons expressing the SP receptor in the dorsal horn of the spinal cord, and a combination of SP conjugated with neurotoxin saporin, resulted in an elimination of the abnormal sensory behaviors associated with CS post facet injury (Weisshaar and Winkelstein 2014). Spine OA is found to trigger CS as a consequence of the continuous release of peripheral neuropeptide and inflammatory biochemicals onto peripheral afferents located at the facet joints (Niissalo et al. 2002; Latremoliere and Woolf 2009; Sperry et al. 2017).

In a similar manner, when expression of neuroinflammation was evaluated, rats exposed to experimentally induced facet joint OA showed an increase in several pro-inflammatory markers in the spinal cord such as cytokines, SP, and ERK (Henry et al. 2012). The authors inferred from their findings that this novel facet compression injury leading to articular joint OA heightened the presence of mechanical allodynia and CS in the spinal cord (Henry et al. 2012). Moreover, recent evidence has supported the role of facet joint OA in chronic myofascial pain (Tachihara et al. 2007; Huang et al. 2014; Quintner and Cohen 1994; Gunn 1997). Research suggests that the underlying presence of MPS as a comorbid disorder of spine facet OA, is a combination of: a malfunction of the CNS, the presence of segmental sensitization due to spine OA, and neurogenic inflammation within the associated myotome (Littlejohn and Guymet 2018; Srbely et al. 2008; Gunn 1997).

Also, an increase in SP expression in the spinal cord parallels to its decrease in the DRG in early stages post facet injury suggesting that the central transport of SP, as
well as its utilization, is highly prominent centrally (Weisshaar and Winkelstein 2014). Studies have identified that 90% of SP is manufactured within the DRG and it is transported towards peripheral terminals of A-δ and C-fibers (Pernow 1983). However, no studies assessing SP release in secondary peripheral tissues post facet joint injury have been identified.

1.7 Myofascial Pain Syndrome

1.7.1 Prevalence and Characteristics

Myofascial pain syndrome (MPS) is the leading form of non-articular musculoskeletal pain and is highly prevalent, affecting up to 95% of individuals with other chronic pain disorders (Shah et al. 2008). MPS is a form of neuromuscular dysfunction characterized by the presence of local and referred muscle pain, pressure pain hypersensitivity, muscle stiffness, limited range of motion, and palpable taut nodules within the muscle (Rivers et al. 2015; Bourgaize and Newton 2018). These defining features are referred to as the clinical presentation of the myofascial trigger point (MTrP), the most commonly agreed upon source of MPS diagnosis (Bron and Dommerholt 2012; Srbely et al. 2010). Emerging research has reported that there is a disruption of the neurophysiological and biochemical activity found within the MTrP interstitial milieu (Partanen et al. 2010; Shah et al. 2005).

1.7.2 Gap

Despite these descriptions and findings, the pathophysiology of MPS and MTrP is still unresolved (Gerwin 2001; Shah et al. 2005; Simons et al. 2002; Srbely et al. 2008; Moraska et al. 2013).
1.7.3 Integrated Hypothesis

The current prevailing thought suggests that local mechanical injury within the muscle is the precipitating event in the pathophysiology of chronic myofascial pain (Gerwin et al. 2004). This theory, known as the Integrated Hypothesis, proposes that local pathophysiological changes manifest within the muscle at the site of injury, developing local, hyperirritable contraction knots known as MTrPs (Gerwin 2008; Gerwin et al. 2004). The ongoing release of acetylcholine is suggested to be the main contributor to this muscle knot (Margalef et al. 2018). The Integrated Hypothesis, however, does not adequately account for several other features of MPS. For example, the MTrP is a commonly employed therapeutic target in the management of MPS (Srbely et al. 2008). Intramuscular dry needle therapy and/or direct digital pressure applied to the MTrP site often elicits a beneficial therapeutic/physiologic response, where patients often intuitively express a soothing sensation described as ‘good pain’ (Giamberardino et al. 2011; Moraska et al. 2013). Furthermore, MTrPs have been observed in a number of somatic disorders such as spine OA (Huang et al. 2014), radiculopathy (Gunn 1997), facet joint injury (Rivers et al. 2015), and visceral conditions (Niraj 2018), all in the absence of muscular local injury.

1.7.4 Role of CS and NI in the Pathophysiology of MPS

Several proposals have attempted to elucidate the relationship between MTrPs and MPS. Emerging evidence suggests that MPS and MTrP may develop following previous pathology that leads to altered central nociception processing (Srbely et al. 2010). To this end, several proinflammatory cytokines have been identified in the MTrP
milieu of patients suffering from MPS in increased quantities including TNF-α and IL-1β (Shah et al. 2005). Neurogenic inflammatory peptides SP and CGRP have also been reported as highly prevalent within the MTrP milieu (Shah et al. 2005, 2008). However, observations from distant and remote muscles found only baseline levels of SP and CGRP (Shah et al. 2008). As such, a robust segmental influence mediating such changes have been proposed (Srbely et al. 2010; Sluka et al. 1995).

1.8 The Role of Calcium/Calmodulin-Dependent Protein Kinase II in Skeletal Muscle

Calcium/CaM-dependent protein kinases (CaMKs) belongs to a family of protein kinases that are sensitive to intracellular calcium (Ca$^{2+}$) fluctuations (Sacchetto, Bovo, and Damiani 2005). The CaMKII family is composed of 4 different types of kinases, each with a different gene mediating their expression in various tissues (Rose and Hargreaves 2003; Tavi et al. 2003). The major CaMKII expressed in skeletal muscle is CaMKII$^{thr286}$ (Cervone and Dyck 2017). At basal levels, skeletal muscle CaMKII is inactive, however an increase in Ca$^{2+}$ concentrations (i.e., following a muscle twitch) causes it to quickly become phosphorylated (Sacchetto, Bovo, and Damiani 2005).

Previous research has demonstrated a dose-relationship between phosphorylation of CaMKII and submaximal exercise in biopsied human quadriceps muscle (Rose and Hargreaves 2003). Similarly, studies conducted on rats have confirmed that a repeated series of muscle contractions induced by electrical stimulation leads to an increase in the expression of CaMKII (Sacchetto et al. 2005). The role of
CaMKII in skeletal muscle is still poorly understood, but evidence suggests that it may be involved in modulating muscle function during a twitch or exercise in the context of force production, metabolism, inflammation, and activation of transcription factors (Rose and Hargreaves 2003; Gaydukov and Balezina 2018; Cervone and Dyck 2017). The effect of CS on the expression of CaMKII in skeletal muscle has not previously been studied.

1.9 Extracellular Signalling Regulated Kinase (ERK) 1/2 in Skeletal Muscle Post Central Sensitization

The Mitogen-Activated Protein Kinase (MAPK) pathway is a signal transduction pathway that transduces extracellular signals into intracellular responses (Wojtaszewski et al. 1999). MAPKs are involved in several aspects of skeletal muscle including muscle mass maintenance, damage induced muscle regeneration, and muscle diseases (Yu et al. 2001; Widegren et al. 2001). Extracellular signal-regulated kinases or ERK 1/2 belongs to the family of MAPKs (Wang et al. 2011). This is a cytosolic signaling protein that requires dual phosphorylation on threonine and tyrosine residues for their activation (Aronson et al. 1997; Wojtaszewski et al. 1999).

Muscle contraction, exercise, and inflammatory stress are potent activators of skeletal muscle ERK 1/2 in humans and rodents (Aronson et al. 1997; Wojtaszewski et al. 1999). When ERK becomes phosphorylated, it activates downstream signaling that exerts control enabling transcription factors that modulate muscle cell survival, proliferation, differentiation and adaptation (Yu et al. 2001; Wojtaszewski et al. 1999). In
ageing and muscle dystrophic diseases, ERK activation is decreased which has negative implications on muscle cell survival (Parkington et al. 2004). Despite the importance of ERK in skeletal muscle, no known studies have examined the effect of CS on the expression of this kinase in skeletal muscle.

CHAPTER 2: AIMS OF THE THESIS

2.1 Overall Aim of the Thesis

The current body of literature demonstrates that experimentally induced primary monoarthritis leads to increased expression of neurogenic inflammation markers in homologous contralateral joints.

Similar neurogenic mechanisms have also been observed within visceral tissues, whereby experimentally induced primary pathology has been shown to evoke neurogenic inflammation within neurosegmentally linked visceral tissues.

No studies to date, however, have investigated the expression of neurogenic inflammatory markers within neurosegmentally-linked heterologous tissues.

In particular, given the growing prevalence of spine OA, the overall aim of this thesis was to specifically investigate the expression of neuropeptides related to neurogenic inflammation in the heterologous knee cartilage and quadriceps muscle tissue as a response to primary lumbar osteoarthritis, both naturally occurring and experimentally induced, in rats.
2.1.1 Study 1

Purpose:

(1) Investigate whether experimentally induced lumbar spine facet OA-like pathology increases the expression of substance P (SP) within the ipsilateral neurosegmentally-linked tibiofemoral cartilage.

(2) Investigate whether experimentally induced lumbar spine facet OA-like pathology leads to degenerative changes within the ipsilateral neurosegmentally-linked tibiofemoral cartilage.

Hypothesis:

(1) SP will demonstrate increased expression within neurosegmentally linked joint cartilage after the induction of experimentally-induced lumbar spine facet OA-like pathology when compared to sham surgery and naive animals.

(2) Experimentally induced lumbar spine OA-like pathology will lead to degenerative changes within the ipsilateral neurosegmentally-linked tibiofemoral cartilage compared to sham surgery and naive animals.

2.1.2 Study 2

Purpose:

(1) Investigate the association between the clinical manifestation of naturally occurring lumbar spine OA and SP expression within the neurosegmentally linked dorsal horn of the spinal cord (L4-L5) as well as the neurosegmentally...
linked ipsilateral quadriceps muscle (common myotome) in an elderly rat population.

(2) Investigate the expression of Proteinase-Activated Receptor 2 (PAR2), an established marker of neurogenic inflammation, within the neurosegmentally linked ipsilateral quadriceps muscle (common myotome) in an elderly rat presenting with naturally occurring lumbar spine OA.

**Hypothesis:**

(1) Aging rats presenting with naturally occurring lumbar spine OA (L3-L5) will exhibit greater concentrations of SP within the neurosegmentally linked dorsal horn (L4-L5) and within neurosegmentally linked quadriceps muscle tissue (common myotome) compared to young healthy controls.

(2) Aging rats presenting with naturally occurring lumbar spine OA (L3-L5) will exhibit greater protein expression of PAR2 within neurosegmentally linked quadriceps muscle tissue (common myotome) compared to young healthy controls.

**2.1.3 Study 3**

**Purpose:**

(1) Investigate the causal-relationship between lumbar spine facet OA-like pathology and the expression of neurogenic inflammatory markers (SP, CGRP and PAR2) within neurosegmentally-linked myotome
(2) Investigate the causal-relationship between lumbar spine facet OA-like pathology and the expression of intracellular kinases associated with inflammation and muscle twitch (extracellular signal-regulated protein kinase-ERK 1/2 and calcium/calmodulin-dependent protein kinase II – CaMKII) within neurosegmentally-linked myotome.

(3) Contrast whether the lumbar spine facet OA-like pathology affects the expression of neurogenic inflammation proteins and intracellular kinases within neurosegmentally-linked myotome and non-neurosegmentally remote myotome.

**Hypothesis**

(1) L4-L6 spine-induced facet OA-like pathology will enhance the protein expression of neurogenic inflammation markers (SP, CGRP and PAR2) within bilateral neurosegmentally-linked rectus femoris (L2-L5 innervation) compared to sham intervention

(2) L4-L6 spine-induced facet OA-like pathology will increase the protein expression of ERK 1/2 and CaMKII within bilateral neurosegmentally-linked rectus femoris (L2-L5 innervation) compared to sham intervention

(3) L4-L6 spine-induced facet OA-like pathology will increase the protein expression of SP, CGRP, PAR2, ERK 1/2 and CaMKII only within bilateral neurosegmentally-linked rectus femoris (L2-L5 innervation) contrasting to the non-neurosegmentally-linked biceps brachii (C4-C7 innervation)
2.2 Statement of Ethics

All animal procedures employed in the present thesis were approved by the Animal Care Committee of the University of Guelph (Guelph, Ontario, Canada).
CHAPTER 3 - Study 1: Increased Substance P Immunoreactivity in Ipsilateral Knee Cartilage of Rats Exposed to Lumbar Spine Injury

Felipe C.K. Duarte, Derek P Zwambag, Stephen H.M. Brown, Andrea Clark, Mark Hurtig, John Z. Srbely

3.1 Abstract

Augmented expression of Substance P (SP) in the cartilaginous tissue has been shown to promote degenerative changes in the cartilaginous matrix of distal contralateral articular joints via neurogenic inflammation post monoarthritis-induction contributing to the symmetrical spread of osteoarthritis (OA). However, no studies have explored whether similar changes are also present within neurosegmentally-linked ipsilateral heterologous cartilage. **Objective:** The present study aimed to investigate whether experimentally induced lumbar facet-joint OA lead to degenerative changes and enhanced SP expression within the ipsilateral neurosegmentally-linked tibiofemoral cartilage. **Methods:** Adult male Sprague-Dawley rats were assigned to left side L5-L6 facet mechanical compression injury (surgery) (n=6), L5-L6 facet exposure with no compression (sham) (n=5), or naïve (no surgery) (n=4) groups. The morphology of the tibiofemoral articular cartilage was assessed using a modified Mankin scoring system. Immunohistochemistry was used to examine the density of chondrocytes stained positive for SP (cells/cm²) in the ipsilateral tibiofemoral cartilage at 28 days post-intervention. **Results:** Tibiofemoral cartilage in the surgery group showed consistent loss of superficial zone chondrocytes, mild roughening of the articular surface and occasional chondrocyte clusters as well as a greater density of SP mainly in the superficial cartilage zone compared to sham and naïve groups, although they also had a basic SP-expression. **Conclusion:** Our results support the hypothesis that neurogenic mechanisms may mediate the spread of SP to neurosegmentally-linked heterologous joints impacting the distal cartilage homeostasis. These findings contribute additional
insight into the potential role of neurogenic inflammation with implications in the pathophysiology of chronic inflammatory joint disease and OA.

3.2 Introduction

Osteoarthritis (OA) is a prevalent, painful and progressive musculoskeletal condition that is characterized by the disruption of the joint microenvironment, affecting the cartilage and surrounding tissues including subchondral bone, synovium, ligaments and muscles (Zhang and Jordan 2010; Hurtig et al. 2009). OA affects synovial articular joints, with the highest prevalence observed in the knee, hand, hip and spine (Van Saase et al. 1989). A number of systemic risk factors for OA have been described including age, gender, ethnicity, hormones, genetics, obesity and nutrition, as well as biomechanical factors including prior injury, muscle weakness, joint deformity and ligament laxity (Zhang and Jordan 2010; Decaris et al. 1999; Sperry et al. 2017; Martin and Buckwalter 2001). Despite the growing awareness of these risk factors, the pathophysiology of OA is still unresolved. Emerging clinical and experimental research suggests that the nervous system may play an essential role in mediating the pathophysiology and symmetrical manifestation of OA (Decaris et al. 1999; Sperry et al. 2017; Arendt-Nielsen et al. 2010; Miller et al. 2015).

OA has been traditionally considered an asymmetrical disease, however, studies have shown a predilection over time in humans towards symmetrical involvement, especially in knees (Metcalfe et al. 2012) and hands (Niu et al. 2003). Research shows that experimentally induced unilateral knee OA in animals results in symmetrical spread of neurogenically-mediated inflammation and articular degeneration to contralateral
homologous joints (Donaldson et al. 1993; Donaldson 2009; Mapp et al. 1993). Spine facet joints are regularly associated with back pain radiating to lower extremities such as knee and foot (Tachihara et al. 2007), however no animal studies of lumbar facet joint injury exist addressing similar mechanisms of spread of neurogenically-mediated inflammation to these joints. Neurogenic inflammation is an acute inflammatory response triggered by the nervous system (Sluka et al. 1995) which manifests in neurosegmental innervation patterns to release substance P (SP) into peripheral joints and tissues (Sluka et al. 1995; Sluka 2002). SP is a potent vasodilator and proinflammatory neuropeptide (Willis 1999; Suvas 2017) which may influence the pathophysiology of OA through its dose-dependent effect on intraarticular inflammation (Levine et al. 1984; Sutton et al. 2009; Murakami et al. 2015).

The specific role of neurogenic inflammation in the pathophysiology of OA is unknown, however, the accumulating research suggests it may be an important mechanism in mediating, and possibly initiating, the systematic spread of OA via neurosegmental patterns (Donaldson et al. 1995; Kelly et al. 2007). The clinical and experimental literature consistently demonstrates that SP is released via peripheral nerve terminals mediating neurogenic inflammation within neurosegmentally innervated homologous joints (Levine et al. 1986; Sutton et al. 2009; Decaris et al. 1999; Mapp et al. 1993). In addition other neuropeptides, via nerve sprouting within cartilaginous tissue (superficial and deep cartilage zone), may be released by peripheral nerves and subsequently worsen OA progression (Mapp and Walsh 2012; Walsh et al. 2010). In contrast, SP expression has been found within non-neuronal tissues such as in
osteoclasts, macrophages and chondrocytes (Millward-Sadler et al. 2003; Delgado, McManus, and Chambers 2003). Chondrocytes are cartilage cells organized in well-defined tissue zones that maintain the cartilage integrity and cartilage function protecting bones from shearing stress and compressive forces between bone to bone within synovial joints (Halliday et al. 1993; Hurtig et al. 2009). Interestingly, it was previously showed that SP stimulates the production of prostaglandin and collagenases in superficial chondrocytes (Halliday et al. 1993). Prostaglandins play an important homeostatic role in chondrocyte remodeling however in OA its abnormal increase leads to cartilage degradation (Hardy et al. 2002). Although previous research demonstrated that SP participates in the joint and cartilage inflammation, no studies have investigated whether its expression is observed within ipsilateral neurosegmentally linked heterologous cartilage joint tissue following induction of spine OA.

The primary purpose of our study was to investigate whether experimentally induced spine OA modulates proinflammatory SP responses within neurosegmentally linked heterologous joint cartilage tissue and if it leads to cartilaginous OA-like changes. In addition, this study aimed to investigate whether a difference in the SP expression between cartilage zone is presented. We set out to test the hypothesis that the concentration of SP expressed within the knee articular cartilage of rats exposed to surgically induced L5-L6 spinal facet joint OA will be greater than rats exposed to either sham intervention (L5-L6) or naive controls changing the cartilage homeostasis. We also hypothesized that the concentration of SP expressed within the superficial zone of rats exposed to surgically induced L5-L6 spinal facet joint OA will be larger than sham
intervention (L5-L6) or naive controls. The findings of this study will address a significant gap in our understanding of the underlying mechanism of SP spread by exploring whether neurogenic mechanisms facilitate the expression of proinflammatory neuropeptides within neurosegmentally linked heterologous tissues. These findings will inform future research investigating mechanisms potentially contributing to the pathophysiology and clinical manifestation of OA.

3.3 Methods

3.3.1 Experimental groups

All animal procedures in this study were approved by the Animal Care Committee of the University of Guelph. Fifteen male Sprague Dawley rats (303.2±38.3 g) were housed in a room with a stable temperature (23.0±1.0 °C) and fed a regular pellet diet ad libitum. Animals were then randomized into one of three groups including surgery (n = 6), sham (n = 5) and control (n = 4).

3.3.2 Induction of Facet Injury

A single injection of Carprofen (5 mg/kg body mass) was administered subcutaneously 30 minutes before surgery. Surgery and sham groups were anesthetized using isofluorane and injected subcutaneously over the incision site with a 50/50 lidocaine/maracaine mixture (2 mg/kg body mass). Once animals were anesthetized, a posterior midline incision was made from the spinous processes of L3 to S1 through the skin and subcutaneous tissue. An incision was then made through the thoracolumbar fascia and the erector spinae aponeurosis lateral to the left multifidus muscle. Blunt dissection was used to separate the erector spinae and multifidus

65
muscles. The multifidus tendon attaching to the L5-L6 facet was cut to expose the facet joint. In surgery animals only, the left L5-L6 facet joint was then injured by compressing the L5/L6 facet joint (~1 mm gap in locked position) for three minutes with modified Kelly forceps. This procedure has previously been shown to evoke spine facet cartilage degeneration, tactile allodynia, and spinal cord sensitization 28 days post surgery\(^1\). All muscles of surgery and sham animals were then sutured (braided 4-0 coated Vicryl) and the skin was closed using stainless steel skin staples. After regaining consciousness, the rats were returned to their cages and were maintained in the same conditions described above. Naïve control animals did not undergo any surgical interventions and were freely maintained in their cages under the same conditions as surgery and sham animals.

3.3.3 Tissue Preparation, Histology and Immunohistochemistry (IHC)

Rats were euthanized by carbon dioxide and their L5-L6 lumbar vertebral segments and left knees were harvested 28 days post surgery. Lumbar vertebral segments were fixed (10% formalin) for 48 hours and decalcified (Cal-X II, Fisher Scientific, Nepean, ON) for five days. L5-L6 was then cut in the transverse plane using a surgical stainless-steel blade and then dehydrated and embedded in paraffin wax for sectioning in the coronal plane. Five microns thick sections were sliced and mounted on microscope slides (Superfrost™ Gold Adhesion slides, Fisher Scientific, Nepean, ON). Safranin-O staining was performed for visualization of the L5-L6 facet morphology and cartilage degeneration.
Knee tissues collected from the rats were fixed (10% formalin) for 48 hours, decalcified (Cal-X II, Fisher Scientific, Nepean, ON) for five days and cut in half in the sagittal plane by a surgical stainless-steel blade. These halves were then dehydrated and embedded in paraffin-wax for sectioning in the coronal plane. Only the medial half was considered for additional analysis since the medial aspect of the tibiofemoral joint has been more associated to cartilage loss and OA development (Van Ginckel et al. 2016). Five-micron thick sections were cut and mounted on microscope slides (Superfrost™ Gold Adhesion slides, Fisher Scientific, Nepean, ON) and left unstained for immunostaining or stained with safranin-O/fast green and hematoxylin and eosin (H&E). Tissue immunohistochemistry was conducted using the Avidin Biotin Complex (ABC) method to determine the expression of SP in knee cartilage. The slides were exposed to a series of xylene and alcohol changes to deparaffinize and rehydrate the sections. Sections then underwent antigen retrieval (proteinase K, cat#P6556- 20 µg/ml-Sigma-Aldrich, St. Louis, MO-USA) to unmask antigens and epitopes in order to enhance the staining intensity of the antibody. Blocking solutions (peroxidase blocking solution and DAKO protein block) were applied prior to primary antibody incubation for 45 minutes (1:100 dilution, polyclonal rabbit Anti-Substance P, cat# 20064, Immunostar, Hudson, WI-USA). A secondary antibody was then applied for 30 minutes (EnVision+TM, Peroxidase, Anti-Rabbit, DAKO Laboratories, Carpinteria, CA) and a DAB Peroxidase Substrate (DAKO Laboratories, Carpinteria, CA) was used for colour detection. All steps were applied to negative control slides except the incubation of the primary antibody which was replaced with PBS. Slides were visualized by light
microscopy (200X magnification) and imaged with an Olympus BX 60 camera (Olympus-PA-USA). Images were captured and positively stained (brown) cells were manually counted at 200x magnification using Northern Eclipse software (Northern Eclipse v8.0, Empix Imaging, Mississauga, ON).

3.3.4 Data Analysis

To examine the effect of intervention (surgery, sham, control) on the overall SP expression within the tibiofemoral joint cartilage, the density of SP-positive chondrocytes within each of the tibial and femoral cartilage surfaces was calculated (Fig. 1 A, B and C) by averaging the density from three different regions within each articular surface (tibial, femoral). In order to determine the density of SP-positive chondrocytes, a grid comprised of 100 x 100 microns squares (1 cm$^2$) per grid window (Fig.1D) was superimposed onto the cartilage image (200x field)(Kamisan et al. 2013) (Fig. 1 C). The number of SP-positive (brown) chondrocytes was manually counted with the assistance of the Northern Eclipse software within the tibia and femur. The density of SP-positive cells was then calculated based on the number of SP-positive chondrocytes divided by the total cartilage area ($\mu$m$^2$) in each cartilaginous tissue. Finally, the total density of the tibial and the femoral cartilages was averaged and recorded as the total tibiofemoral joint cartilage density of SP per animal. To find the total cartilage area, cartilage thickness was multiplied by its length. Cartilage thickness was measured from the articular surface to the subchondral bone and the length measure of 500 micrometers along the surface was used as a constant measure through all images (Fig. 1D). All density values are reported per grid window.
(#cells/cm²). Safranin-O and H&E stained knee joint histological sections also underwent histological evaluation for osteoarthritic changes using the modified Mankin scoring system (van der Sluijs et al. 1992). In order to inspect for difference between cartilage zones approximate boundaries of the superficial, middle, and deep zones were identified using a cartilage schematic (Sophia Fox, Bedi, and Rodeo 2009). A grid comprised of 100 x 100 microns squares (1 cm²) per grid window was superimposed onto the cartilage image (200x magnification) (Fig. 1D). The total cartilage thickness was first determined, and then based on the schematic, proportions occupied by each zone were measured using the ruler tool from the Northern Eclipse software. Thus, superficial zone represented 14% of the overall cartilage thickness; middle zone 51% and deep zone 35%. Positively stained SP chondrocytes within each zone were counted with the assistance of the Northern Eclipse software from tibial and femoral cartilages. The density within each zone was further calculated as the number of SP-positive chondrocytes divided by individual zone area (µm²) that was established as the zone thickness (superficial, middle and deep) multiplied by the 500 µm length of surface (Fig. 1D). Finally, the density of SP in the tibia and femur in each individual cartilage zone were pulled together (i.e. matching superficial zone of the femur and tibia per animal), averaged and recorded as the tibiofemoral joint density per zone per animal. All SP density values of the superficial, middle and deep cartilage zones were reported as the number of positive cells per cm² (#cells/cm²).
3.3.5 Statistical Analysis

In order to address our main hypothesis, we conducted a two-way ANOVA using the factors of intervention (surgery, sham, control) and location (tibia, femur) with post-hoc comparisons using Tukey’s multiple comparison’s test to examine for differences in number of positively stained cells between location and intervention. To address our second hypothesis, we conducted a two-way ANOVA using the factors intervention (surgery, sham, control) and cartilage zone (superficial, middle and deep zones) with post-hoc comparisons using Sidak’s multiple comparison’s test to examine for the differences in the number of positively stained cells in each individual zone between groups. Tukey’s multiple comparisons was also used to test for differences in number of positively stained cells in the individual zones within each group.

Mankin scores were compared between the three groups using the Kruskal-Wallis variance analysis. Dunn’s multiple comparisons test was performed when p<0.05. All statistical analyses were performed in Prism (V 7.03). Alpha was set at 0.05.
Figure 1. Immunostaining images from coronal sections of the rat knee from control, sham, and surgery groups. (A) An overview representation of the knee positively stained with substance P (SP). (B) and (C) represent positively stained SP in the tibial and femoral cartilages respectively, followed by the tibial and femoral negative control images of the surgery group (SP primary antibody was replaced with phosphate buffered saline (PBS) for negative control). (D) A grid 100 × 100 µm (1 cm²) was superimposed onto the image. The number of SP-positive chondrocytes was manually counted through the area of interest (500 µm in length × cartilage thickness) and the positively SP chondrocyte density was then established (number of positive SP cells divided by 500 µm length × full cartilage thickness). In addition, (D) shows the 3 cartilage zones: superficial (S), middle (M), and deep (D) representing 14%, 51%, and
35% of the full cartilage thickness, respectively. A = 100× magnification, B and C = 200× magnification. Scale bars A = 125 µm; B and C = 30 µm

### 3.4 Results

The L5-L6 facet joints from surgery and sham groups were analyzed histologically to evaluate the impact of the joint compression on facet morphology (Fig. 2). On surgery animals, the left compressed side showed some alteration on the facet cartilage such as loss of proteoglycan staining, some proliferation of chondrocytes in the superficial and upper mid zone and focal retention of matrix staining around chondrocyte clusters in the superficial zone. However, these changes were not presented on all animals. Sham animals demonstrated intact facet joint with healthy morphological features.
Figure 2: Safranin-O staining on surgery (A,B) and sham (C,D), injured (left) (A,C) and contralateral (right) (B,D) L5-L6 lumbar facet joints. (A) Left injured side showing loss of proteoglycan staining, some articular margin abnormalities, extracellular tissue remodeling and chondrocyte clustering between mid and superficial zone. (B) Contralateral side showing normal cartilage surface with normal articular margins. (C and D) Left and contralateral sham L5-L6 showing smooth and intact cartilage surfaces and normal articular margins. 100 x magnification, scale bars = 200µm.

All femoral cartilage samples were analyzed. Two tibial samples in the surgery group as well as one tibia in the sham group were excluded due to structural damage during dissection. Mankin scores of the ipsilateral (left) knees in the spinal control, sham and surgery groups all showed varying levels of mild safranin-O stain loss and occasional loss of superficial cells. Knee joints in the surgery group had consistent loss of superficial zone chondrocytes, mild roughening of the articular surface and occasional chondrocyte clusters. The modified Mankin scoring of the surgery group (4.4±1.7, n=6) was significantly different to control (0.6±0.8, n=4, p=0.01, Kruskal-Wallis test) and sham (0.6±0.5, n=5, p<0.01, Kruskal-Wallis test) groups (mean ± SD) (Fig. 3). No significant difference was found between sham and control (p=0.99, Kruskal-Wallis test).
Figure 3: Modified Mankin score of the knee as a function of spinal surgical intervention. Modified Mankin scores were used with 0 indicating normal and a maximum of 13 points indicating end-stage osteoarthritis. Values are presented as means with standard deviation bars. Different letters indicate statistically different groups (p≤.01). Significance was set at alpha of 0.05.

The mean and standard deviation of the density of SP positive chondrocytes/grid window (1cm²) by location and intervention are reported in Table 1.
Table 1: Density of SP positively stained knee chondrocytes/grid window (10,000 µm² = 1 cm²) by location and spinal surgical intervention. The number of SP-positive chondrocytes were counted in three separate regions within both femoral and tibial cartilage regions in the left knee of rats. Values are expressed as mean and standard deviation.

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<td>9.10</td>
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Additionally, the mean and standard deviation of the density of SP positive chondrocytes (number of cells/cm²) in the superficial, middle and deep cartilage zones are listed in the Table 2.

Table 2: Density of SP positively stained knee chondrocytes within the superficial, middle and deep cartilage zones as a function of spinal surgical intervention. The density of the tibial and femoral SP-positive chondrocytes within each zone was averaged in the left knee of rats over all groups and was reported as the number of positive cells per cm². Values are expressed as mean and standard deviation.

<table>
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A two-way ANOVA revealed a significant effect of spinal intervention on the SP-positive chondrocytes within the knee cartilage of rats \( F (2, 21) = 5.563, p=0.0115 \). No effect of location \( F (1, 21) = 0.9436, p = 0.3424 \) or interaction between intervention and location was observed \( F (2, 21) = 0.3616, p=0.7008 \). Post-hoc individual comparisons between groups revealed significant increases in SP-positive chondrocytes in the surgery group when compared with both sham surgery \( (p=0.0407) \) and controls \( (p=0.0079) \). No difference was observed between sham and control groups \( (p=0.7025) \) (Fig. 4).

**Figure 4:** Density of SP-positive knee chondrocytes as a function of spinal surgical intervention. A grid with windows 100 x 100 microns was superimposed onto the cartilage image (200x magnification). The number of SP-positive chondrocytes was
manually counted from three separate regions from each cartilage surface (tibial, femoral). The density of SP-positive cells within each of the tibial and femoral cartilage surfaces was determined by averaging the density of three different regions within each surface. Values are presented as means with standard deviation bars. Significance was set at alpha of 0.05 and denoted by *.

Furthermore, a significant difference in SP-positive chondrocytes between different cartilage zones \( F (2, 36) = 67.74, p<.0001 \) as well as an interaction effect between spinal intervention and cartilage zones \( F (4, 36) = 9.48, p<.0001 \) was found. The spine facet injury L4-L5 performed in the surgery groups affected the density of the SP-positive chondrocytes within the superficial cartilage zone compared to sham (MD = -6.614; 95% CI [-10.29 to -2.941]; p<.0001) and control (MD = -5.409; 95% CI [-8.855 to -1.964]; p=.0003) groups. In contrast, no difference within the superficial zone between sham and control (MD = 1.204; 95% CI [-2.613 to 5.021]; p=0.9789) groups was present. A significant difference within the spinal facet joint-OA surgery group between the superficial zone compared to the middle (MD = 8.619; 95% CI [5.334 to 11.9]; p<.0001) and the deep cartilage zones (MD = 12.14; 95% CI [8.854 to 15.42]; p<.0001) was evident. In addition, there was difference between the middle and deep zones (MD = 3.52; 95% CI [0.235 to 6.806]; p<.0280) within this same group. Similarly, significant difference within the control group between the superficial zone compared to the middle (MD = 4.646; 95% CI [1.047 to 8.245]; p=.0040) and the deep cartilage zones (MD = 4.019; 95% CI [4199 to 7.618]; p=.0191) was present. A difference between superficial and deep zones (MD = 5.034; 95% CI [1.011 to 9.058]; p=.0058) within the sham group.
was also present but no difference between superficial and middle zone (MD = 3.366; 95% CI [-0.6572 to 7.39]; p=0.1637) was found (Fig. 5).

**Figure 5:** Density of SP-positive chondrocytes in knee cartilage zones as a function of spinal surgical intervention. Density was determined as the number of positive chondrocytes within each individual zone divided by the corresponding zone area. Data is represented as mean ± SEM. * represents significative difference between groups within the superficial cartilage zone. # represents significative difference between superficial zone compared to corresponding middle and deep zones. “a” denotes a significative difference between the superficial zone to the deep zone within the sham
group. "b" denotes a significative difference between middle and deep zones within the surgery group. Significance was set at alpha of 0.05.

3.5 Discussion

The results of our study support our hypothesis that the expression of SP is increased within the ipsilateral knee cartilage of rats exposed to experimentally induced left L5-L6 spinal facet joint OA injury, compared to sham and control animals. No difference in SP expression was observed between sham surgery and control animals. Similarly, no difference of SP expression between tibial and femoral cartilage surfaces were found. In addition, our results support the second hypothesis that the expression of SP is increased within the superficial cartilage zone of rats exposed to experimentally induced left L5-L6 facet joint OA injury, compared to sham and control animals. These are the first data demonstrating a causal association between the spread of SP to neurosegmentally linked heterologous tissues following experimentally-induced spinal facet joint OA injury. As such our results strengthen our understanding of the physiologic expression of neurogenic inflammatory mechanisms in the clinical manifestation of joint inflammation. These findings may have potential implications to the pathophysiology of OA in that the proinflammatory effects of SP may directly influence cartilage and chondrocyte homeostasis, with potential implications to joint inflammation and OA.

SP is a key neurotransmitter released during neurogenic inflammation and important in the pathophysiology of OA (Kidd et al. 1990; Decaris et al. 1999; Schaible and Grubb 1993). SP is a pro-inflammatory neurotransmitter acting on the neurokinin-1
(NK-1) receptor to increase vascular permeability, promote vasodilation and evoke powerful proinflammatory responses within joints and tissues (Birklein and Schmelz 2008; Lotz et al. Carson 1988; Sutton et al. 2009). Levine was the first to propose that intraarticular SP concentration may be directly correlated to the degree of inflammation observed in OA, and that the origin of intraarticular SP may be neurogenic (Levine et al. 1984; Hildebrand et al. 1991). This has been shown experimentally in several animal studies where intraarticular injection of SP led to dose-dependent synovial plasma extravasation (Sutton et al. 2009; Scott et al. 1992) and increased OA severity (Levine et al. 1984; Mapp et al. 1993).

The existing body of research highlights causal associations between experimentally induced unilateral arthritis and the spread of inflammation and articular degeneration in contralateral, homologous joints. Acute onset inflammatory responses have been reported in the contralateral limb after injections of crystals (monosodium urate, calcium pyrophosphate dihydrate, hydroxyapatite, calcium oxalate) into the footpad (Denko and Petricevic 1978) and hypertonic saline into the hindpaw (Thompson and Bywaters 1962) of rats. Experimental unilateral arthritis models of OA using Complete Freund’s Adjuvant (CFA) injections also exhibit heightened inflammatory responses with significant increases in cartilage degradation (42% decrease in proteoglycan synthesis) (Decaris et al. 1999) in a dose-dependent relationship (Donaldson et al. 1993) in the contralateral limb. A dose-response association has also been observed clinically where bilateral knee OA involvement has been more commonly observed with increased severity of arthritis in one knee joint (Metcalf et al. 2012). On
the other hand, Decaris (1999) showed a significant acute decreased in proteoglycan
anabolism in the ipsilateral knee patella six hours after subcutaneous injection of CFA in
the hindpaw (Decaris et al. 1999). This effect was minimized with a pre-treatment with
NK-1 antagonist thereby suggesting a neurogenic mechanism dependence (Decaris et
al. 1999).

Although most notably expressed in neuronal cells, SP and the NK-1 receptor
have been identified in a growing number of non-neuronal cells. For example, SP is
expressed in immune cells including monocytes, T-lymphocytes and eosinophils (Lotz
et al. 1988; Scott et al. 1992), where it plays a role in regulating cytokine production (IL-
1, 6 and 8, TNFα), inflammation and immune responses. SP expression has also been
reported in the costal cartilage of mice (Millward-Sadler et al. 2003) and in adult human
chondrocytes (Opolka et al. 2012), where its precise role is still unclear. In this context,
SP appears to play a key role in mechano-transduction as evidenced by its ability to
enhance the electrophysiological response of human articular chondrocytes exposed to
mechanical stimulation (Millward-Sadler et al. 2003) through its direct action on the NK-
1 receptor. In addition, studies in vitro have shown that exogenous SP stimulates
chondrocyte proliferation suggesting a role in the maintenance of the articular structure
(Opolka et al. 2012). Interestingly, our findings show that a basic expression of SP is
presented in all groups studied supporting its potential function in the maintenance of
the cartilage structure.

The knee articular cartilage is structurally composed by three important cartilage
zones and their combination provides and important functional advantage absorbing
and transmitting mechanical forces (Fox et al. 2009). Curiously, chondrocytes have a dual role synthetizing key component of the cartilage matrix (collagen and proteoglycan) as well as producing enzymes that degrade matrix components (collagenases and proteinases) (Martin and Buckwalter 2001; Fox et al. 2009; Millward-Sadler et al. 2003).

It is widely described that the superficial zone is the smallest zone containing a relatively high number of flattened chondrocytes and its integrity is crucial to the protection and maintenance of deeper layer homeostasis (Fox et al. 2009). Functionally, the middle and deep zones are responsible for most of the resistance to compressive forces (Sperry et al. 2017; Fox et al. 2009). Karahan (2002) demonstrated that after a series of physical exercise an increased expression of SP in the middle and deep cartilage zones becomes evident in dogs (Karahan et al. 2002). Its upregulation was then suggested to positively contribute to cartilage matrix organization in response to mechanical stimulation (Karahan et al. 2002). In the present study chondrocytes located in the superficial cartilage zones had higher positive-SP chondrocyte density regardless of the group studied. The superficial layer in the control group presented approximately 35% higher density of SP-positive chondrocytes compared to middle and deep zones. The superficial layer in the spinal facet joint-OA surgery group is approximately 50% and 70% higher than middle and deep zone. Yet, the sham intervention group displayed a 30% and 45% larger density at the superficial zone compared to middle and deep zones, although statistical significance was only reached between superficial and deep zones within this group. The interpretation of these findings is equivocal on the basis of the existing body of literature in this field. The
significant difference of the overall SP chondrocyte expression as well as the significant difference in the superficial layer between spinal facet OA-injury to sham intervention (37%) and control (31%) groups could represent an adaptive response of chondrocytes to subsequently altered gait patterns and mechanical joint stresses. It has been previously shown that lumbar spine facet injury significative impacts gait biomechanics in rodents (Fukui et al. 2015). In this context however, given the role of deeper cartilage zones in the resistance of compressive forces compared to superficial zone (Oliva et al. 2005) and the positive influence of SP in cartilage mechano-transduction (Grässel 2014) and maintenance of matrix organization (Karahan et al. 2002) post exercises, we would expect to see a larger density of SP immunoreactivity in deeper zones, which was not apparent. Due to the complexity of the influence of joint loading on cartilage and chondrocyte metabolism, SP may have a different response to the pathological context presented in our work compared to the physiological stress-force loading associated with exercise. Further studies are needed to understand the pathological versus physiological differences in the expression of SP in the cartilage.

In contrast, enhanced expression of SP in chondrocytes could also represent a maladaptive subclinical response to neurogenic responses within the knee as shown by the larger modified Mankin score in the present study. It has been shown that spinal facet joint injury leads to increased sensory inputs within the spinal cord segments leading to chronic pain and distal inflammation (Henry et al. 2012; Sluka et al. 1995; Sluka 2002). Existing animal models suggest that neurogenic inflammation is mediated by the Dorsal Root Reflex (DRR), characterized by proximal (central) terminal
depolarization of primary afferents (A delta and C fibers) and leading to antidromic release of neurotransmitters (i.e. SP and CGRP) into peripheral tissues (Rees et al. 1996). Experimental evidence also suggests that the DRR is mediated through neurosegmental innervated pathways, both ipsilaterally and contralaterally (Sluka et al. 1995; Beloeil et al. 2006; Rees et al. 1996). Interestingly, peripheral A delta and C fibers terminals innervating knee joint tissues present central projections to lumbar segments 4, 5 and 6 (Craig et al. 1988). Although consensus is that cartilage tissue is aneural (Fox et al. 2009; Oliva et al. 2005), nociceptive fibers have been previously reported within osteoarthritic joints and articular cartilage, introducing a potentially important pathway for the mediation of neurogenic inflammation in cartilaginous tissues (McDougall 2006; Ahmed et al. 1995). CGRP producing nerve fibers have been identified in contact with surface chondrocytes of developing cartilaginous tissue (Ahmed et al. 1995; Millward-Sadler et al. 2003). In the context of OA disorder, Im et al (Im et al. 2008) elegantly demonstrated that IL-1 stimulates the expression (mRNA) and the protein secretion of the SP and its receptor NK1 in arthritic human adult articular chondrocytes. Their binding was thought to accelerate cartilage degradation by enhancing the production of cartilage-degrading enzymes (MMP-13) as well as attenuating proteoglycan deposition in adult human cartilage (Im et al. 2008). Substance P-mediating induction of MMP-13 was showed to occur via Raf-ERK MAPKs and NFk-B intracellular pathways in human adult articular chondrocytes(Im et al. 2008). Similarly, cultured bovine chondrocytes with the C-terminal fragment of SP but not SP stimulated the production of prostaglandin, calcium and collagenase in chondrocytes. Since SP
can be cleaved in the synovial fluid chondrocyte-mediated cartilage pathology may also be influenced by SP residual fragments (Halliday et al. 1993). Additionally, expression of the vascular endothelial growth factor (VEGF) as well as the co-expression of nerve growth factor (NGF) and calcitonin gene related-peptide (CGRP) was recently demonstrated in the superficial zone chondrocytes of the human medial tibial plateau OA cartilage but not in a non-arthritic cartilage (Walsh et al. 2010). Since these growth factors, especially NGF, is widely known to stimulate SP and CGRP synthesis, axonal transport and release in arthritic joints (Ahmed et al. 1995; Suvas 2017) their participation in the superficial cartilage breakdown was suggested (Walsh et al. 2010).

The superficial zone is in contact with synovial fluid, contains large density of chondrocytes and is responsible for most of the tensile properties of cartilage (Fox et al. 2009). It has been shown that the superficial zone is more susceptible to chondrocyte apoptosis and chondrocyte catabolism (Dowthwaite 2004; Fox et al. 2009). Interestingly, the superficial zone chondrocytes, in the presence of synovium IL-1 was attributed to be more susceptible to catabolic and damage effects than deeper zones (Dowthwaite 2004; Häuselmann et al. 1996). Thereby, given the close relationship that superficial chondrocytes present with innervated synovial tissue it is possible that the L4-L5 facet lesion triggered an antidromic transport of SP or another neuropeptide, which may have initiated catabolic changes in the synovial environment, resulting in the enhanced chondrocytes SP mainly in the superficial zone, early osteoarthritic changes and significantly elevated modified Mankin scores in the ipsilateral knee.
Our findings should be interpreted in light of the fact that the surgically induced primary pathology in our model was mild when assessed histologically and compared to Henry (2012) which showed a completely degenerated facet joint (Henry et al. 2012). Previous research has demonstrated a robust dose-response relationship between the severity of primary pathology and intensity of contralateral neurogenic inflammatory responses (Niu et al. 2003; Donaldson et al. 1993; Metcalfe et al. 2012). Similarly, a strong inverse association has been reported between SP concentration and proteoglycan production in cartilage (Im et al. 2008). These observations point to the importance of future studies in advancing this line of inquiry by employing a more severe injury model. In addition, we did not control for non-segmentally linked tissues in our design as we were primarily interested in the causal association of experimentally induced spinal injury and neurogenic responses within neurosegmentally linked heterologous tissues. Future research should investigate these effects within non-segmentally related heterologous tissues as well as neurosegmentally linked contralateral heterologous tissues to provide greater insight into the segmental and non-segmental (systemic) physiologic mechanisms potentially contributing to these observations. In addition, psychophysical outcomes would add additional insight into potential effects of altered gait and/or joint loading on SP expression.

3.6 Conclusion

While a growing body of literature reports increased expression of SP within the contralateral homologous joint after experimentally induced monoarthritis, our findings are the first to demonstrate similar increases in SP expression ipsilaterally within
neurosegmentally linked heterologous cartilage tissue 28 days post lumbar facet OA injury. Here, we report increased expression of SP within the chondrocytes of knee cartilage after surgically induced facet compression injury at the common L5-L6 spinal level. In addition, a likely enhancement of SP preference in the superficial cartilage zone was also showed at 28 days post lumbar facet OA injury compared to sham and control which may have contributed to early OA development. These findings potentially support our hypothesis that pro-inflammatory molecules may spread via neurogenic mechanisms to neurosegmentally linked heterologous tissues (in addition to homologous tissues) and contributes important insight into the mechanisms facilitating the clinical manifestation of neurogenic inflammation. Future directions should advance the findings of this study by investigating the dose-response relationship between primary pathology and neurogenic inflammatory response within heterologous tissues such as synovial membrane and synovial fluid, as well as assessing histological and radiological changes within the affected knee joint. Given the aging societal demographic and growing burden of OA, this line of research is timely and urgent to informing our understanding of the pathophysiologic mechanisms and clinical manifestation of chronic inflammatory joint disease and OA.
Chapter 4 – Study 2: Association Between Naturally Occurring Spine Osteoarthritis in Geriatric Rats and Neurogenic Inflammation Within Neurosegmentally Linked Skeletal Muscle

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4.1 Abstract

Objective: This study aimed to investigate the association between naturally occurring spinal osteoarthritis (OA) (L3-L5), the expression of substance P (SP) centrally (L4-L5) and the presence of neurogenic inflammation within the neurosegmentally linked quadriceps (L2-L5) in elderly rats versus young controls.

Design: Eight aged (27±3.2 months) and six young (4±0.0 months) male Wistar Kyoto rats were euthanized and submitted to micro-computerized tomography for determination of spine OA. SP expression (% area) at the dorsal horn of the spinal cord as well as the relative expression of SP and protease-activated receptor 2 (PAR2) to alpha-tubulin within quadriceps muscle were determined by immunohistochemistry and Western Blot.

Results: Spine osteoarthritis was confirmed in all aged rats but no young controls. Aged rats expressed significant increase of SP protein expression within the dorsal horn (MD = .086; 95% CI [0.026 to 0.145]; p = 0.0094) and quadriceps (MD = 1.209; 95% CI [0.239 to 2.179]; p = 0.0191) and PAR2 (MD = 0.797; 95% CI [0.160 to 1.435]; p = 0.0187) compared to young controls.

Conclusion: These observations provide novel insight into the potential role of neurogenic inflammation in the pathophysiology of myofascial pain syndrome in the naturally occurring spinal OA in elderly population.
4.2 Introduction

Chronic musculoskeletal pain (MSK) is a leading cause of illness and disability, accounting for over 50% of all chronic conditions in people over the age of 50 in developed countries (Brooks, 2006). Myofascial pain syndrome (MPS) is the leading form of non-articular chronic musculoskeletal pain (Shah et al., 2008), affecting up to 95% of people with chronic pain disorders (Gerwin, 2001; Shah et al., 2008). The prevalence of MPS has been reported to significantly increase with aging reaching up to 85% in the elderly population over 65 years (Gerwin, 2001). Despite its strong association with age, the pathophysiologic mechanisms driving the clinical manifestation of MPS in the elderly are poorly understood.

MPS is a complex clinical condition characterized by chronic regional muscular pain associated with sensory, motor and autonomic dysfunction (Simons et al., 1999). The most commonly accepted clinical feature of MPS is the presence of discrete, palpable, hyperirritable extrafusal-fiber contraction nodules known as myofascial trigger points (MTrP) (Gerwin et al., 2004). Affected skeletal muscle also demonstrates increased concentrations of a broad profile of inflammatory mediators and cytokines (Shah et al., 2005), most notably substance P (SP), a powerful vasodilator and proinflammatory neuropeptide (Shah et al., 2008).

The outstanding gap in the field of MPS is whether the primary pathology driving MPS exists within the symptomatic muscle or whether MPS a clinical syndrome. The Integrated Hypothesis is the current prevailing theory for the pathophysiology of MPS and suggests that the MTrP is the primary pathologic focus mediating the clinical
manifestation of MPS. According to this hypothesis, MPS is initiated by overload injury of the muscle, leading to local contracture as a result of spontaneous release of acetylcholine (ACh) at the dysfunctional motor endplate (Gerwin et al., 2004).

Emerging research, however, suggests that neurogenic mechanisms may play a foundational role in the clinical manifestation of MPS (Partanen et al., 2010). The Neurogenic Hypothesis (Srbely et al., 2008) proposes that the clinical manifestation of MPS is initiated and facilitated by central sensitization leading to neurogenic inflammatory mechanisms within the affected muscle, and not overload injury. Neurogenic inflammation is a neurally-mediated inflammatory response caused by retrograde (antidromic) release of vasoactive and proinflammatory neuropeptides, predominantly SP, into peripheral tissues via sensory nerves (Sluka et al., 1995).

One of the primary drivers of central sensitization and neurogenic inflammation is persistent nociceptive input arising from a primary pathology located within the common neuromeric field of the affected sensory pathways (Srbely et al., 2008). Osteoarthritis (OA) is one of the most common disorders in humans that often leads to persistent pain and inflammation (Arendt-Nielsen et al., 2010; Musumeci et al., 2015; Thudium et al., 2018). It is the most common cause of chronic disability in elderly population (Lotz and Loeser, 2012; Musumeci et al., 2015) affecting primarily the knee, hip, spine and hand joints (Gellhorn et al., 2013; Perruccio et al., 2012; Zhang and Jordan, 2010) and is characterized by the ongoing deterioration of articular cartilage and periarticular tissues (Ahmed et al., 1995; Lotz and Loeser, 2012; Rice et al., 2011). Despite the fact that its etiology remains unresolved, several age-related factors including repetitive loading and
articul
t microdamag
e (Martin et al., 2004; Musumeci et al., 2015), local joint
inflammation (Thudium et al., 2018), loss of cartilage cell function
(chondrosenescence)(Mobasheri et al., 2015), chondrocyte apoptosis (Musumeci et al.,
2011) and systemic age-related inflammation (Gandhi et al., 2014; Giunta et al., 2015)
have been proposed to contribute to the development and the progression of OA. Given
that OA is a common source of chronic persistent nociception in the elderly, and
consistent with the Neurogenic Hypothesis, we postulate that age-related spine OA may
be a common primary pathology driving the neurogenic inflammatory mechanisms
mediating the clinical manifestation of MPS in the elderly population. To this extent, no
one to date has investigated the association between naturally occurring spine OA and
SP expression within neurosegmentally linked skeletal muscle, using an in vivo spine
OA animal model.

The overall purpose of this line of research is to investigate the association
between naturally occurring spine OA and neurogenic inflammatory mechanisms within
neurosegmentally linked skeletal muscle tissues. This study aimed to specifically
investigate the association between the clinical manifestation of naturally occurring
spine OA (L3-L5) and the protein expression of SP within neurosegmentally linked
dorsal horn of the spinal cord (L4-L5) as well as skeletal muscle tissue within the
common myotome (quadriceps, L4-L5) using an in vivo model of naturally occurring
spine OA in an aging rat population. In order to validate the presence of inflammation
within the affected muscle, the secondary aim was to compare the protein expression of
Proteinase-Activated Receptor 2 (PAR2), an established marker of inflammation, within
the neurosegmentally linked muscle of aging rats presenting with naturally occurring spine OA versus young healthy controls. We set out to test the primary hypothesis that aging rats presenting with naturally occurring lumbar spine OA (L3-L5) will exhibit significantly greater protein expression of SP within both the neurosegmentally linked dorsal horn (L4-L5) and quadriceps muscle (L2-L5 myotomes), when compared to young healthy controls free of lumbar spine OA. Also, this study tested the secondary hypothesis that aging rats presenting with naturally occurring spine OA (L3-L5) will exhibit higher protein expression of PAR2 within the neurosegmentally linked quadriceps muscle (L2-L5 myotomes), compared to young healthy controls.

4.3 Methods

4.3.1 Animals

All procedures in this study were approved by the Animal Care Committee of the University of Guelph (Guelph, Ontario, Canada). Eight male Wistar Kyoto rats (27±3.2 months) and six young male Wistar Kyoto rats (4±0 months) were housed (2-3 per cage) in a room with a 12-hour alternating light-dark cycle with a stable temperature (23.0±1.0 °C) and fed a regular pellet diet ad libitum.

4.3.2 Tissue Preparation

Animals were euthanized by carbon dioxide and spinal lumbar columns were harvested and fixed for 48 hours in 10% formalin for micro-computerized tomography (micro-CT) scanning and further immunohistochemistry analysis of the spinal cord (L4-L5). In addition, muscle samples from left side quadriceps (L2-L5 myotome) were collected, snap frozen in liquid nitrogen and stored at -80°C for western blot analysis.
Six animals per group were assigned for micro-CT scanning. After completing the imaging, spine samples were decalcified (Cal-X II, Fisher Scientific, Nepean, ON) for five days. Spinal cord was then collected, cut in the transverse plane using a surgical stainless-steel blade and dehydrated and embedded in paraffin-wax blocks for sectioning in the coronal plane. Five-micron thick sections were sliced and mounted on microscope slides (Superfrost™ Gold Adhesion slides, Fisher Scientific, Nepean, ON).

4.3.3 Micro-CT Analysis

Fixed spine samples were micro-CT scanned at 45-micron isotropic pixel resolution (GE Medical Systems eXplore Locus Micro-CT Scanner, GE Medical Systems London, ON, Canada). The software Microview (MicroView- version 2.5.0-rc21, Parallax Innovations Inc., ON, Canada) was used for image analyses to determine the presence of age-related spine OA features (osteophytes, facet joint space narrowing and/or facet joint sclerosis). The presence of age-related spine OA features were qualitatively assessed in a binary fashion (yes/no) by two different examiners. The inter-rater reliability was expressed as percent agreement between the raters (Mchugh, 2012). Images were evaluated in three different planes (frontal, sagittal and axial) in regular dark background (raw image) and in light background (adjusted image) by employing the “image invert tool” of the software. Adjusting dark background to light background was previously reported to better define the boundaries of the vertebral body and facet joints increasing the reliability of the measurements (Lai et al., 2007).

4.3.4 Immunohistochemistry (IHC)

To determine the SP immunoreactivity in the dorsal horn of the spinal cord,
immunohistochemistry was conducted using the Avidin Biotin Complex (ABC) method as previously described (Musumeci et al., 2014). Histological spinal cord sections were exposed to a series of xylene and alcohol changes to deparaffinize and rehydrate the sections. Sections then underwent antigen retrieval (proteinase K, cat#P6556- 20 µg/ml-Sigma-Aldrich, St. Louis, MO-USA) to unmask antigens and epitopes to enhance the antibody staining intensity. Blocking solutions (peroxidase blocking solution and DAKO protein block) were applied prior to primary antibody incubation for 45 minutes (1:100 dilution, polyclonal rabbit Anti-Substance P, cat# 20064, Immunostar, Hudson, WI-USA). A secondary antibody was then applied for 30 minutes (EnVision+TM, Peroxidase, Anti-Rabbit, DAKO Laboratories, Carpinteria, CA) and a DAB Peroxidase Substrate (DAKO Laboratories, Carpinteria, CA) used for colour detection. All steps were applied to negative control slides except the incubation of the primary antibody which was replaced with PBS. Slides were visualized by light microscopy (200X magnification) and imaged with an Olympus BX 60 camera (Olympus-PA-USA). The SP positively stained cells in Laminae I and II was measured with assistance of a semiautomated computer imaging analysis system (ImageJ 1.45 - NIH Image software).

The percent area of SP immunoreactivity was determined based on the method of Abaadie et al. (1996), calculating the ratio of positively stained pixels to total pixels within lamina I and lamina II of the left dorsal horn of the L4-L5 spinal cord segments, with a slight modification to further define the threshold grayscale value (black 0 to white 255) key for determination of positively stained pixels within lamina I and II (Fig.6) (Abaadie et al., 1996). The results were compared and averaged between two independent
researchers.

4.3.5 Western Blotting (WB)

Western blot analysis was performed on muscle homogenates using methods described previously (Musumeci et al., 2014). A copper-based assay using bicinechonic acid (BCA) protein detection kit (Thermo Scientific, Pierce BCA Protein Assay - Canada) was employed to determine protein content from homogenized samples for subsequent sample preparation (Cervone and Dyck, 2017). Equal amounts of muscle sample proteins (15 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis and transferred for 1 h at 0.2 A to a polyvinylidene difluoride membrane. Membranes were blocked in 5% non-fat skim milk tris-buffered saline containing 0.1% tween-20 (TBS-T) for 1 h at room temperature followed by overnight incubation at 4°C with primary antibody diluted in 5% bovine serum albumin (substance P antibody - 1:500, orb215527-Biorbyt Ltd, San Francisco - CA; PAR2 antibody – 1:500, orb385619 - Biorbyt Ltd, San Francisco - CA). After three washes in TBS-T, membranes were incubated at room temperature for 1 h with the secondary antibody (anti-rabbit - 1:2000, A0545 - Sigma-Aldrich - Oakville, ON). The membranes were detected using enhanced chemiluminescence (PerkinElmer, Waltham, MA), protein bands were quantified by densitometry using a chemiluminescence detection system (Alpha Innotech Fluorchem HD2, Fisher Scientific, Hampton, NH) and the optical density values were recorded. Additionally, the optical density of SP and PAR2 were internally normalized within subject against the loading control alpha-tubulin protein (ab7291, Abcam, Cambridge, MA-USA) to determine the relative protein expression of these target proteins. The relative protein
expression of such target-proteins was recorded for statistical analysis.

Figure 6: Figure illustrating the method for calculating the percent area of SP immunoreactivity within Lamina I and II of the left dorsal horn of the spinal cord in young and aged rats (L4-L5). The image in (B) represents the total number of pixel values in the region of interest (lamina I and II) resulting from capturing and digitizing the section illustrated in (A). The total number of pixels within the lamina I and II was counted and recorded as the total pixel value. (C) Represents the SP positively stained pixels within the Lamina I and II after applying a threshold grey value. The threshold grey value was established as the mean pixel value of the total pixel value found in B. Values below the threshold were considered SP-positively stained ruling out unspecific overstaining. The
ratio of SP positively stained pixels / total number of pixels within the lamina I and II was calculated to find the percent area of SP immunoreactivity within Lamina I and II. (D) Schematic illustration of the Lamina I and II of the dorsal horn of the spinal cord. A = 200x magnification. Scale bars = 60 microns.

4.3.6 Statistical Analysis

The primary outcome measures (dependent variable) used for our analyses were the percentage (%) area of SP-positive immunoreactivity within Lamina I and II of the left dorsal horn of the spinal cord and the relative protein expression of SP and PAR2 (optical density) within neurosegmentally-linked quadriceps muscle. A t-test was performed to investigate differences between the mean of the percentage area of SP-positive immunoreactivity within Lamina I and II of the left dorsal horn of the spinal cord between groups (aging, young), our independent variable. Similarly, we performed a t-test to inspect for significant differences of the relative protein expression of SP and PAR2 (dependent variables) within the left quadriceps. Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed in Prism (V 7.03). Significance was set at α = 0.05.

4.4 Results

Micro-CT images confirmed the presence of naturally occurring spine OA features in aged versus young rats in the sagittal, frontal and axial planes (Fig.7). Based on a binary (yes or no) approach, a total of 6 aged rats (n=6) presented spine
osteophytes in contrast to young rats (n=6) which did not present any. Only one aged rat did not present signs of facet joint space narrowing and/or facet joint sclerosis. All young rats did not present any signs of facet joint space narrowing and/or facet joint sclerosis. The inter-rater agreement of presence of spine osteophytes in aged rats was 100% and facet joint narrowing and/or facet joint sclerosis 67%. In comparison, agreement in young rats was 83% for both presence of osteophytes and facet joint narrowing and/or facet joint sclerosis (Table 3).

**Figure 7:** Comparison of micro-CT lumbar spine images between young and aged rats. Signs of naturally occurring spine OA were binarily assessed in the raw (dark background) and adjusted images (light background) in 3 different planes (I-frontal and sagittal) and (II-axial). (I) Micro-CTs of young lumbar spine rats with thick arrows showing presence of provisional calcification of cartilaginous growth plate at the
vertebral body of young rats which allows continued vertebrae growth (A, C, E and G). Arrows on B, D, F and H depicting presence of diffuse osteophytes on lumbar vertebral bodies. (II) Arrows in B and D showing bilateral facet joint narrowing and /or facet joint sclerosis compared to A and C.

Table 3: Binary (yes/no) evaluation of the presence of naturally occurring spine OA features in young and aged rats and percentage of agreement between two different raters.

<table>
<thead>
<tr>
<th>Presence of osteophytes</th>
<th>Presence of facet joint space narrowing and/or facet joint sclerosis</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Young</td>
<td>Yes</td>
</tr>
<tr>
<td>Aged</td>
<td>6</td>
</tr>
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The percent area of SP-positive immunoreactivity was measured in the L4-L5 dorsal horn of the spinal cord from young and aged rats by IHC. Only intact spinal cord sections were considered for SP analysis (IHC); consequently, samples from 5 young and 6 aged rats were considered for analysis. The t-test analysis showed a significant 4.5-fold increased of SP-positive immunoreactivity within the dorsal horn of the spinal cord.
cord (Lamina I and II) in aged rats compared to young (MD = 0.086; 95% CI [0.026 to 0.145]; p = 0.0094) (Fig.8).

Figure 8: Percent area of SP-positive immunoreactivity within the dorsal horn of the spinal cord (Lamina I and II) between young and aged rats. The percent area of SP-positive immunoreactivity was calculated based on the ratio of positively SP-stained pixels to total pixels within lamina I and lamina II of the left dorsal horn of the L4-L5 spinal cord segments with assistance of a semiautomated computer imaging analysis system (ImageJ 1.45). Values were compared and averaged between two independent researchers. Values are presented as means with standard error. Significance was set at alpha of 0.05 and denoted by *.

Western Blot analysis of the relative SP protein expression to the loading control alpha-tubulin within the neurosegmentally linked quadriceps muscle (L2-L5 innervation) showed a significant increase in aged rats compared to young (MD = 1.209; 95% CI
[0.239 to 2.179]; p = 0.0191) (Fig.9A). Furthermore, Western Blot analysis of the relative PAR2 protein expression to the loading control alpha-tubulin within the same muscle also showed a significant increase in aged compared to young rats (MD = 0.797; 95% CI [0.160 to 1.435]; p = 0.0187) (Fig.9B). Due to structural damage during dissection one quadriceps muscle from the young group was excluded.

![Western Blot analysis of relative SP (A) and PAR-2 (B) protein expression to loading control alpha-tubulin within the neurosegmentally linked quadriceps muscle (L2-L5 innervation) of young and aged rats. Alpha-tubulin was used as a loading control protein. Values are presented as means with standard error (n=5 young; n=8 aged). Significance was set at alpha of 0.05 and denoted by *.

**Figure 9:** Western Blot analysis of relative SP (A) and PAR-2 (B) protein expression to loading control alpha-tubulin within the neurosegmentally linked quadriceps muscle (L2-L5 innervation) of young and aged rats. Alpha-tubulin was used as a loading control protein. Values are presented as means with standard error (n=5 young; n=8 aged). Significance was set at alpha of 0.05 and denoted by *.

### 4.5 Discussion

The results of this study support the primary hypothesis that aged rats presenting with naturally occurring spine OA exhibit higher concentrations of SP within
neurosegmentally linked skeletal muscle (quadriceps), in comparison to young healthy controls free of spinal disease. These findings offer support for the role of neurogenic mechanisms in the clinical expression of MPS (Neurogenic Hypothesis) and offer support for the theory that spinal OA may be an important driver of MPS in the elderly.

Our findings align with previous research which demonstrates the neurogenic release of SP in the cross-sensitization of both somatic and visceral tissues (Fitzgerald et al., 2013; Shah et al., 2008); Increased SP concentrations have been observed in contralateral homologous knee joints after experimentally induced monoarthritis (Donaldson et al., 1995). Similarly, experimentally induced colonic inflammation via intracolonic administration of trinitrobenzene sulfonic acid triggered a 3-fold increase in SP within the neurosegmentally linked bladder 5 days post-intervention, and 35-fold increase 30 days post-intervention (Pan et al., 2010); this increase in SP was shown to significantly enhance bladder inflammation via cell-specific mast cell degranulation (Fitzgerald et al., 2013).

We also observed increased of SP immunoreactivity within the dorsal horn of the L4-L5 spinal segment in the aging rat population when compared to healthy young controls. These observations also align with the existing literature in which animal models of chronic inflammatory joint disease show similar increases in SP and excitatory neurotransmitters within the neurosegmentally linked dorsal horn (Donaldson et al., 1995; Staton et al., 2007). Increased levels of these neurotransmitters induce dorsal horn sensitization, leading to the amplification of peripheral nociceptive input (windup) and modulation of descending antinociceptive influences (Latremoliere and
Woolf, 2009; Woolf, 2011). Importantly, dorsal horn sensitization triggers dorsal root reflexes (DRR) which evokes the antidromic release of proinflammatory neurotransmitters (SP) peripherally, ultimately leading to neurogenic inflammation within peripheral tissues (Sluka et al., 1995).

Our findings of increased expressions of SP within neurosegmentally linked muscle has significant implications to our understanding of the pathophysiology of chronic muscle pain and inflammation. SP is the most extensively studied of the bioactive peptides from the tachykinin family (Pan et al., 2010; Suvas, 2017). It is manufactured within the cell body of sensory fibers located within the dorsal root ganglia, and stored within the peripheral terminal (Sluka et al., 1995). SP is released from peripheral sensory terminals to evoke profound proinflammatory effects in peripheral tissues through the direct action on a broad profile of immune cells via SP receptors (Abaadie et al., 1996; Lotz et al., 1988; Sluka et al., 1995). (Delgado et al., 2003), leading to the production of TNFα, IL1-β, IL-2 and IL-6 in three different populations of white blood cells including T-lymphocytes, macrophages and neutrophils (Delgado et al., 2003; Payan et al., 1983). This profile of inflammatory cytokines and neuropeptides downstream of SP is consistent with the biochemical milieu previously observed within human trapezius muscle presenting with MPS (Shah et al., 2008, 2005) and fibromyalgia (De Stefano et al., 2000), suggesting that elevated SP within skeletal muscle may be an important factor driving the pathophysiology and clinical manifestation of muscle pain and inflammation in chronic muscle pain. Our study contributes to this line of research by providing additional novel evidence supporting the
potentially important role of naturally occurring spine OA in driving the neurogenic inflammatory responses within neurosegmentally linked muscle tissues, using an in vivo animal model.

Increased levels of SP within the muscle may also provide a physiologic rationale for the discrete extrafusal muscle fiber contractures, known as myofascial trigger points (MTrP), often observed in association with chronic musculoskeletal pain (Gerwin et al., 2004; Simons et al., 1999). SP and CGRP are amongst the most powerful mediators of smooth muscle contraction (Lotz et al., 1988; Pan et al., 2010). Similar effects have also been reported in skeletal muscle with strong excitatory influences on spinal motoneurons and enhanced transmission reported at both the pre and post junctional membranes of the neuromuscular junction (Akasu et al., 1983; Nicoll, 1978; Wali, 1985). Dose-dependent increases in the twitch and tetanic contraction amplitudes have been reported in skeletal muscle across several species (Nicoll, 1978; Wali, 1985) in addition to increased frequency of mini endplate potentials (MEPP) (Akasu et al., 1983). These collective observations emphasize the potential for enhanced levels of SP to contribute to the pathophysiology of local contractures commonly observed at MTrP sites. Future research should investigate the causal associations using in situ animal models.

The finding of enhanced PAR2 expression within the quadriceps of aging rats provides evidence for a robust inflammatory response within the neurosegmentally linked quadriceps muscle of aged rats compared to controls. PAR2 belongs to a class of G-protein coupled receptors observed within a broad profile of immune cells including T-cells, macrophages, neutrophils and mast cells (Russell and McDougall, 2009) and
peripheral and central neurons (Vergnolle et al., 2001). PAR2 activation is induced primarily by the release of tryptase from local tissue injury, as well as from mast cell degranulation (Russell and McDougall, 2009; Vergnolle et al., 2003). Its activation triggers the release of neurogenic proinflammatory peptides including SP into peripheral tissues to induce pain, leukocyte aggregation, acute inflammation and edema (Steinhoff et al., 2000; Vergnolle et al., 2001). PAR2 also enhances the expression of proinflammatory cytokines within the local biochemical milieu including bradykinin, TNF\(\alpha\) and IL-8 via further mast cell degranulation suggesting that PAR2 is intimately involved in the inflammatory cascade (Russell and McDougall, 2009; Vergnolle et al., 2003). In contrast, PAR2 knockout mice show delayed onset of acute inflammation after tissue injury (Lindner et al., 2000) and significantly less joint swelling and joint damage in a model of chronic adjuvant induced monoarthritis (Russell and McDougall, 2009). The observation of enhanced PAR2 and SP expression within the peripheral muscle of the naturally occurring spine OA group supports the hypothesis that neurogenic inflammatory mechanisms may contribute to the characteristic local biochemical milieu previously reported within the muscle of chronic MPS patients.

The observations from this study support the Neurogenic Hypothesis which postulates that naturally occurring spine OA creates a persistent nociceptive environment conducive to the induction of dorsal horn sensitization and dorsal root reflexes mediating the antidromic release of SP into neurosegmentally linked peripheral muscle tissue with an important implication in the pathophysiology of MPS (Srbely et al., 2010, 2008). Although the findings from this study are associative, they are the first to
highlight the potential for naturally occurring spine OA to drive neurogenic mechanisms in neurosegmentally linked muscle tissue in the elderly. Collectively, this study informs an important and emerging line of research with significant implications to advancing the understanding of the pathophysiology of MPS, as well as its successful diagnosis and management. We are further pursuing this line of research by currently investigating the causal relationships between spine OA and SP expression within neurosegmentally linked muscle using an experimentally-induced OA animal model.

The findings of this study should be interpreted in light of several limitations, the most evident being the fact that age matched controls free of spine OA was not employed in this study. The overall objective of this research thread is to investigate whether neurogenic mechanisms consequent to naturally occurring spine OA may be important underlying contributors to the manifestation of MPS in the aging population. This hypothesis is predicated on the presence of a strong association between the clinical manifestation of age-related spine OA and SP expression in both the neurosegmentally linked dorsal horn and myotome, which we observed. Significant correlations exist between age and spine diseases such as OA (Lotz and Loeser, 2012); for this reason, age matched controls free of OA are rare in a geriatric rat population.

An additional limitation of this study was the absence of a neurosegmental control muscle; consequently, we cannot conclude whether the increases in SP within both the spinal cord and muscle were mediated through neurosegmental mechanisms alone or by synergistic effects of combined supraspinal, spinal and/or other mechanisms. The aim of this seminal study, however, was to first explore associations
between naturally occurring spine OA and SP expression in neurosegmentally linked spinal cord and myotome, using an in vivo animal model; in order to resolve the mechanism of this observation, future research should investigate the causal relationship between spine OA and SP expression using experimental animal OA models.

We chose to study only males for this study so as to avoid potentially confounding effects of sex. Previous studies have reported that female rats are more susceptible to autoimmune chronic arthritis than males, owing to complex dysregulation of neuroendocrine-immune system (Holmdahl et al., 1986; Joe and Wilder, 1999; Vingsbo-Lundberg et al., 1998). Importantly, the next phase of studies should advance this line of research by comparing these effects in females.

In addition, secretion of neuropeptides and inflammatory molecules related to central sensitization have been shown in non-muscular tissues such as cerebrospinal fluid, as well as systemic circulation in several chronic painful disorders including OA, fibromyalgia, migraine and complex regional pain syndrome (Arendt-Nielsen et al., 2010; Birklein and Schmelz, 2008; Littlejohn and Guymer, 2018; Malhotra, 2016). Future research could investigate the causal associations between these conditions and expression of neuropeptides after experimentally induced animal-OA models. Due to the short half-life of neuropeptides such as SP and CGRP (Greene and Loeser, 2015; Steinhoff et al., 2000; Suvas, 2017), it may be difficult to detect changes within the circulating blood, although their metabolites post cleavage/degradation could be investigated. Additionally, although we acknowledge that our study employed a small
sample size, the present study is still in accordance with sample sizes previously published in the literature (Gao et al., 1996; Kras et al., 2013).

4.6 Conclusion

This study is the first to demonstrate an association between the clinical manifestation of naturally occurring spine OA and enhanced SP expression within both the neurosegmentally linked spinal cord and myotome, using an in vivo animal model of naturally occurring OA. The findings from this research add support to the Neurogenic Hypothesis of MPS, which represents a potential paradigm shift in the field of chronic musculoskeletal pain management as it addresses the foundational role that neurogenic inflammation may play in the pathophysiology of chronic MPS. These findings also shed light on the potential importance of OA as a primary pathology driving the clinical manifestation of MPS, further emphasizing the potential importance of advancing therapeutic strategies in the preventative management of spine OA in the elderly. Our observations are associative; future research should advance this thread by exploring the causal relationship between OA and SP expression within the common myotome using animal models of experimental OA. As these findings support the underlying mechanisms proposed by the Neurogenic Hypothesis, they may have important implications to the diagnosis and management of chronic musculoskeletal pain, a significant and growing priority in today’s aging demographic.
Chapter 5 – Study 3: Lumbar Facet Joint Injury Leads to Supraspinal Sensitization and Neurogenic Inflammation in Rats

Felipe C.K. Duarte, Mark Hurtig, Andrea Clark, Derek P Zwambag, Stephen H.M. Brown, Jeremy Simpson, John Z. Srbely

Manuscript in preparation for Trends in Neuroscience
5.1 Abstract

Background: Myofascial pain syndrome (MPS) is the most common form chronic of musculoskeletal pain, however its pathophysiology is poorly understood. The Neurogenic Hypothesis proposes that MPS may be a clinical expression of intramuscular neurogenic inflammation (NI) arising from central sensitization (CS) induced by a primary pathology residing within the common neuromeric field.

Objective: This study aimed to investigate the effect of induced spine facet injury and OA on the evolution of CS by measuring concentrations of neuropeptides (SP, CGRP), receptor (PAR2), and intracellular kinases (ERK1/2, CaMKII) within neurosegmentally linked myotomes as well as remote non-connected myotomes.

Methods: Thirty-two Wistar Kyoto rats were assigned for experimental lumbar spine injury, sham-operation and naïve controls. The presence of secondary mechanical allodynia, thermal hyperalgesia and extracellular signaling kinase (ERK 1/2) and astrocyte activation (GFAP) in the brainstem were assessed by electronic von Frey and hot plate behavioral tests and by immunoblotting respectively. The relative concentration of neuropeptides, ERK 1/2 and CaMKII in muscles were measured by western blot.

Results: Animals exposed to experimental facet injury presented early signs of spine osteoarthritis (OA), CS evidenced by bilateral hind paw allodynia (p<.01), thermal hyperalgesia (p<.01) and increased concentration of p-ERK 1/2 and GFAP in the brainstem (p<.05). Animals presenting early signs of spine OA had increased
concentration of SP, CGRP, PAR2, p-ERK 1/2 and p-CaMKII in neurosegmentally-linked myotomes compared to sham (p<0.05), while facet injury did not alter concentrations at the remote myotome.

**Conclusion:** The results of the present study are the first to provide evidence that primary lumbar spine OA pathology may enhance the concentration of NI within neurosegmentally-linked myotomes. These findings add further support for the Neurogenic Hypothesis which states that MPS is the physiologic expression and clinical manifestation of NI within skeletal muscle that is neurogenically linked to a distinct, heterologous primary pathology (somatic or visceral) residing within the common neuromeric field of the primary pathology.

**5.2 Introduction**

Spine facet joint osteoarthritis (OA) is a common cause of chronic lumbar and neck pain (Sperry et al. 2017; Manchikanti et al. 2004) and can be a significant comorbidity with MPS (Huang et al. 2014; Tachihara et al. 2007). The prevalence of OA is about 10-20% worldwide varying according to gender and age (Glyn-Jones et al. 2015). The incidence of MPS is not entirely elucidated, but studies suggest MPS may affect 85% of the population during the lifespan (Shah et al. 2005). Gerwin reported that MPS may be a secondary disorder consequent to an established primary OA (Gerwin 2001). The commonality between these conditions is the involvement of central sensitization (CS) and neurogenic inflammation (NI), which have been suggested to
contribute to the pathogenesis of such diseases (Bajaj et al. 2001; Arendt-Nielsen and Graven-Nielsen 2003; Arendt-Nielsen et al. 2010; Littlejohn and Guymers 2018; Srbely et al. 2010).

Previous research has demonstrated MPS is characterized by the presence of palpable and hyperirritable taut band(s) myofascial trigger points (MTrP) (Gerwin et al. 2004). Recent evidence showed that MTrP loci exhibit a unique muscle biochemical milieu that includes increased neurogenic inflammatory substance P (SP) and calcitonin gene-related peptide (CGRP) (Shah et al. 2005, 2008). Although the pathophysiology of MPS is still poorly understood, emerging evidence points to a neurogenic inflammatory mechanism secondary to central sensitization (CS) that may play a crucial role in the clinical manifestation of MPS (Srbely et al. 2008, 2010).

Evidence has revealed that centrally mediated antidromic activity conducted by A-δ and C-fiber afferents is a potential underlying mechanism mediating the release of SP and CGRP into neurosegmentally linked tissues (McDougall 2006; Levine et al. 1986; Malykhina 2007; Pan et al. 2010). The current body of literature demonstrates that experimentally induced primary monoarthritis leads to increased expression of neurogenic inflammation markers in homologous contralateral joints (Decaris et al. 1999; Rees et al. 1996) and also in heterologous tissue (Duarte et al. 2018). Similar neurogenic mechanisms have also been observed within visceral tissues, whereby experimentally induced primary pathology has been shown to evoke neurogenic inflammation within neurosegmentally linked visceral tissues (Ustinova et al. 2006; Pan et al. 2010).
The release of SP by peripheral afferents leads to mast cell degranulation and subsequent activation of protease-activated receptor 2 (PAR2). Activation of PAR2 stimulates the release of SP and CGRP from sensory nerve endings, reinforcing the NI response (Steinhoff et al. 2000; Vergnolle et al. 2001). Moreover, extracellular signal-regulated kinase (ERK) is a signal transduction pathway that transduces signals such as muscle contraction and inflammation into intracellular responses (Wojtaszewski et al. 1999) controlling transcription factors engaged in muscle cell adaptation and inflammation (Zimowska et al. 2017; Yu et al. 2001; Wojtaszewski et al. 1999). In skeletal muscle, evidence suggests that Ca\(^{2+}\)-CaM-dependent protein kinases (CaMKs) perform a modulatory role in functional aspects of skeletal muscle such as force production, metabolism, inflammation and transcription factors during muscle twitch or physical exercise (Rose and Hargreaves 2003; Gaydukov and Balezina 2018; Cervone and Dyck 2017).

An increase in ERK and CaMKII levels may inform the presence of inflammatory mediators as SP, CGRP and other cytokines, and the fluctuation of intracellular calcium levels that may underpin the formation/presence of contracted knots seen in MPS patients. No studies to date, however, have investigated the expression of neurogenic inflammatory markers within neurosegmentally-linked heterologous muscle tissue post spine OA-induction. Therefore, an experimentally induced spine facet injury may be particularly informative in the investigation of MPS.

Given the growing prevalence of spine OA and MPS, the overall aim of this study was to specifically investigate the concentration of neuropeptides related to neurogenic
inflammation in the heterologous neurosegmentally-linked quadriceps muscle tissue as a response to primary lumbar spine facet injury in rats in order to follow up with our previous study (Duarte et al. 2019) that showed an association between the manifestation of spine OA and neurogenic inflammatory peptides in elderly rats. We set out to test the hypothesis that experimentally induced spinal facet injury (L4-L6) will enhance concentration of SP, CGRP and PAR2, as well as ERK 1/2 and CaMKII within neurosegmentally linked bilateral quadriceps muscle (rectus femoris) (L2-L5), compared to sham intervention while no alteration will be seen in the non-segmentally linked biceps brachii (C4-C7). The presence of secondary mechanical and thermal allodynia/hyperalgesia as well as modulation of established neuroplastic markers of CS (SP, CGRP, glial fibrillary acidic protein (GFAP) and ERK 1/2), at the brainstem, were used to validate the presence of CS. The findings from this study could provide novel evidence to link CS and NI within the neurosegmental myotome in the pathophysiology of MPS.

5.3 Methods

5.3.1 Animals

All animal procedures were approved by the Animal Care Committee of the University of Guelph. A total of 38 adult male Kyoto Wistar rats, 12±4 months old (461±37g), 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 divided into three groups: experimental surgery (animals underwent facet compression at segments L4-L5 and L5-L6 in the left side) (n=16), sham intervention
(animals with left side facets L4-L5 and L5-L6 exposed but without facet compression) (n = 16) and naïve-controls (no surgery) (n = 6). Surgery and sham animals were further divided in 3 sub-sets of animals according to euthanasia time point post surgery: seven days (surgery: n=5; sham: n=5), 14 days (surgery: n=5; sham: n=5) or 21 days (surgery: n=6; sham: n=6).

5.3.2 Induction of Lumbar Facet Injury

The method of induction of the lumbar facet injury is a model that has previously been used to induce cartilage degeneration and central sensitization evidenced by the presence of both tactile primary and secondary allodynia, as well as spinal cord neuroinflammation and peripheral increase of substance P (Henry et al. 2012; Zwambag et al. 2018; Duarte et al. 2018). Briefly, a nonsteroidal anti-inflammatory drug Carprofen (5 mg/kg body mass) was administered subcutaneously 30 minutes before surgery. Both surgery and sham animals were anesthetized using isoflurane (4%) and had a local anesthesia applied over the incision (2-5 mg/Kg using a 50/50 lidocaine/bupivacaine). Once animals were anesthetized, 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 side lumbar facet capsule from lumbar segments L4-L5 and L5-L6. In the experimental facet surgery intervention group, the left L4-L5 and L5-L6 facet joints were compressed to failure using micro-rongeurs by one operator (MH).

In the sham intervention group, the left L4-L5 and L5-L6 facet joints were exposed but not compressed. All muscles were sutured (braided 4-0 coated Vicryl) and
the skin closed using stainless-steel skin staples. After regaining consciousness, rats were returned to their cages and maintained in the same conditions as described above. Naïve-control animals did not undergo any surgical intervention and were maintained in their cages under the same conditions as surgery and sham animals. All animals were weighed weekly from pre-intervention (day 0) to 3 weeks (21 days) post-intervention.

5.3.3 Behavioural Assessment

To evaluate sensory functions that are associated with central sensitization (Scheid et al. 2013) mechanical and thermal sensitivity tests described below were performed before (Baseline-Day 0), and at days 3, 5, 7, 14 and 21 post-surgery (n=6 sham; n=6 surgery).

5.3.3.1 Mechanical Threshold

Mechanical threshold was assessed by electronic von Frey (EVF) apparatus (IITC Life Science Inc., #2390 series, Woodland Hills, CA, USA). Briefly, a non-noxious stimulus was applied by the probe onto the ipsilateral and contralateral hind paw using the EVF. A positive response was determined when a reflex withdrawal of the paw occurred. The average of five separate readings from the ipsilateral and contralateral hind paws were taken and recorded for statistical analysis (Horst et al. 2014).

5.3.3.2 Thermal Sensitivity

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 X 22 cm)
maintained at 50°C (±2°C) and withdrawal latency (seconds) was measured and recorded for statistical analysis. The withdrawal latency was defined as the length of time animals took to present heat-avoidance responses such as jumping or licking their hind paws, regardless of the paw side. If no response was present after 30 seconds, the animal was taken off, and a 30-second latency was recorded as the withdrawal latency (Leri et al. 2007).

5.3.4 Spinal Column Preparation for Micro-CT and Histology

Experimental-surgery and sham-intervention animals were euthanized by carbon dioxide and spinal lumbar columns were harvested on day 21 post intervention (n=12).

5.3.4.1 Micro-CT analysis

Spinal samples were fixed for 48 hours in 10% formalin for micro-computerized tomography (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). Isosurface projections were used to identify facet injury sites and verify the loss of cartilage space and new bone formation.

5.3.4.2 Histology

Spinal columns were fixed (10% formalin) for 48 hours, decalcified (Cal-X II, Fisher Scientific, Nepean, ON) for five days and L4-L5 and L5-L6 were cut in the transverse plane by surgical stainless-steel blade. Lumbar facet joints were further
dehydrated and embedded in paraffin-wax for sectioning in the coronal plane. Five micron sections were cut and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Nepean, ON). Hematoxylin and Eosin (H&E) staining was performed for visualization of the L4-L5 and L5-L6 lesion and subsequent pathology.

5.3.5 Brainstem and Muscle Tissue Preparation

The examination of the right brainstem can be justified due to the distribution pattern of the nociceptive information ascending from the spinal cord to supraspinal centers throughout the contralateral spinothalamic tract (Ossipov et al. 2010). After euthanasia and a thorough brain dissection, brainstem tissue (medulla, pons and midbrain) was quickly collected, sectioned in the sagittal plane into two equal halves using the ventral medial fissure as guidance, then, the contralateral half of the brainstem (right side) was snap frozen in liquid nitrogen and stored at -80°C freezer. In addition, after euthanasia, neurosegmentally linked quadriceps (rectus femoris) muscle (L2-L5) (Peyronnard et al. 1986) from both ipsilateral and contralateral sides as well as a non-segmentally linked ipsilateral biceps brachii (C4-C7) (Bácskai et al. 2013) were collected, snap frozen in liquid nitrogen and stored at -80°C. These muscles were chosen due to their similar muscle type II fiber type composition (Fuentes, Cobos, and Segade 1998; Kohn and Myburgh 2007).
5.3.6 Western Blotting (WB)

Western blot analysis was performed on brainstem and muscle homogenates using methods described previously (Sickle et al. 2005). Briefly, frozen tissues (20-30 mg) were placed into glass homogenization tubes with cell lysis buffer (NP40 CLB - FNN0021- Thermo Scientific Fisher – Canada) supplemented with protease inhibitor and serine protease inhibitor phenylmethylsulphonyl fluoride (PMSF) for homogenization. A copper-based assay using the bicinchoninic acid (BCA) protein detection kit (Thermo Scientific, Pierce BCA Protein Assay - Canada) was employed to determine protein concentration from homogenized samples for subsequent sample preparation (Cervone and Dyck 2017). Equal amounts of muscle protein (15 µg) and brainstem protein (20 µg) were loaded per lane onto a sodium dodecyl sulphate-polyacrylamide gel (15%), separated by electrophoresis and transferred for 1 h at 0.2 A to a polyvinylidene difluoride membrane. Membranes were blocked in 5% non-fat skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature followed by overnight incubation at 4°C with primary antibody diluted in 5% bovine serum albumin. After three washes in TBS-T, membranes were incubated at room temperature for 1 h with the secondary antibody (anti-rabbit- 1:2000, A0545-Sigma-Aldrich- Oakville, ON), and detected using enhanced chemiluminescence (PerkinElmer, Waltham, MA). Protein bands were quantified by optical densitometry using a chemiluminescence detection system (Alpha Innotech Fluorchem HD2, Fisher Scientific, Hampton, NH). The density of the bands from muscle and brainstem samples were normalized against their corresponding total protein loaded controls obtained by
Ponceau S membrane staining which was visualized and quantified by optical densitometry using the same detection system (Cervone and Dyck 2017).

The following primary antibodies of N1, extracellular signaling kinase, intracellular kinase sensitive to calcium variation and astrocyte activation were used throughout this study for muscle and brainstem analysis, respectively: Muscle: anti-Substance P antibody- 1:500, (orb215527), anti-CGRP 1:250, (orb182870) and anti- PAR2 antibody – 1:500, (orb385619) - Biorbyt Ltd, San Francisco, CA; anti-phospho-ERK (pERK) 1/2 (Thr202/204) antibody-1:1000, (#9101) and anti-ERK1/2 antibody-1:1000, (#9102)- Cell Signaling Technology, Denver, MA; anti-phospho-CaMKII (Thr286) antibody-1:1000, (#12716) and anti CaMKII (Thr286) antibody- 1:1000, (#4436) - Cell Signaling Technology, Denver, MA; Brainstem: anti-Substance P antibody- 1:500, (orb215527), anti-CGRP 1:250, (orb182870) and anti-glial fibrillary acidic protein (GFAP)- 1:500 (orb 129615)- Biorbyt Ltd, San Francisco, CA; anti-phospho-ERK (pERK) 1/2 (Thr202/204) antibody-1:1000, (#9101) and anti-ERK1/2 antibody-1:1000, (#9102)- Cell Signaling Technology, Denver, MA. Anti-ERK and anti CaMKII antibodies were kindly assigned by Dr. David J. Dyck).

5.3.7 Statistical Analysis

All data are presented as means ± standard error of the means (SEMs). We conducted a two-way repeated measures ANOVA to examine changes in mechanical and thermal thresholds using intervention (experimental lumbar facet injury and sham) and time (0,3,5,7,14 and 21 days post-intervention) as fixed effects. When significance was found, post-hoc Sidak’s or Tukey’s multiple comparisons test were used to inspect
for differences within and between groups across time. For western blotting analysis of both brainstem and muscle samples, after normalization of the data, a ratio between intervention group (experimental lumbar facet injury or sham) relative to the control group with no intervention was employed. This was then reported as relative protein expression and described as fold change on graphs. For brainstem statistical analysis, a Two-way ANOVA was employed to examine changes in neuroplastic markers at the contralateral half of the brainstem using intervention (lumbar facet injury or sham) and time (14 and 21 days post-intervention) as fixed effects. When significance was found multiple comparisons Tukey’s test was used. In addition, a two-way ANOVA was used to inspect for changes in neurogenic inflammatory markers, ERK 1/2 and CaMKII within individual muscle (bilateral rectus femoris and biceps brachii) using intervention (facet injury and sham) and time (7, 14 and 21 days) as fixed effects. When significance was found we conducted post-hoc multiple comparisons Tukey’s test. Statistical analyses were performed in the Prism (V 7.03). Alpha was set at 0.05.

5.4 Results

5.4.1 Spine Lumbar Facet Histology and Micro-CT

Animals exposed to experimental facet injury or sham maintained their body mass at every time point assessed after experimental intervention and no difference in mass between groups were detected (data not shown). Mechanical compression injury to the most lateral aspect of the lumbar facet joint segments L4-L5 and L5-L6 created structural changes in bone, cartilage and subchondral bone (Fig.10).
**Figure 10**: Illustrating radiological (micro-CT) and histological (H&E staining) images of the lumbar facet articular joint injury (L4-L5; L5-L6) 21 days postoperatively. Injury sites are outlined in a square, contralateral facets are contained in a circle. **A and B** are micro-CT images at two levels in one animal exposed to facet injury. **C** shows the injured L4-L5 facet joint with H&E staining from the box outlined in **E** and **D** shows the contralateral L4-L5 facet joint with H&E staining from the circle outlined in **E**. **G** illustrates the injured L5-L6 facet joint with H&E staining from the box outlined in **F** and **H** illustrates the contralateral L5-L6 facet joint with H&E staining from the circle outlined in **F**. Images (C, D, G and H) at 100X magnification. Images (E and F) at 10X magnification. Scale bars (C;D;G;H) = 125 microns. Scale bars (E;F) = 60 microns

At 21 days post facet compression, isosurface projections of the micro-CT images and 2D slices showed deformation of the compressed facet, bone fragments and enlargement of the facet by periarticular bone formation (Fig. 10-A,B). In addition, there was irregular multifocal loss of the subchondral bone plate continuity interspersed with areas of sclerosis and periarticular new bone formation at L4-L5 and L5-L6 (Fig.10-A,B). Histology of the left (compressed) side confirmed that hyaline cartilage was replaced with a mixture of new bone, fibrocartilage, and fibrous connective tissue, resembling a fracture callus. There was fibrosis of the joint capsule, as well as irregular bone resorption and deposition at the base of the lumbar facet (Fig.10-C, E-G). The contralateral facet remained unaffected (Fig.10-D, E, F and H).
5.4.2 Mechanical Threshold

The animals exposed to lumbar facet injury had increased tactile sensitivity at the ipsilateral hind paw at all time points post-surgery \([F (5, 25) = 23.64, p<.001]\) (Fig.11-A). In addition, an effect of surgical intervention \([F (1, 5) = 36.17, p=.001]\) and an interaction effect between time and surgery \([F (5, 25) = 7.45, p<.001]\) were found. The decrease of mechanical threshold varied between 45% and 65% three days \((p= .018)\) until 21 days \((p<0.001)\) post-facet injury compared to baseline . In contrast, mechanical threshold of sham animals decreased 25% at 7 days post surgery and was similar to baseline at all other time points\((p=.041)\). Facet-injury animals were more mechanically sensitive than sham controls at day 5 \((p=.017)\), 14 and 21 \((p <.001, \text{respectively})\) post surgery, with no difference between these groups at baseline\((MD = 2.12; \text{95\% CI [-14.49 to 18.73]; p>0.999}).
Figure 11: Comparison between the sham-operation and experimental L4-L6 facet injury groups on the bilateral hindpaw mechanical threshold and thermal threshold. (A) illustrates the effect of the facet injury on the reduction of the mechanical threshold and (B) in the contralateral hindpaw. (C) illustrates the comparison between ipsilateral and contralateral hindpaw on the mechanical threshold of animals exposed to experimental lumbar facet joint. (D) illustrates the effect of the facet injury on the reduction of the thermal threshold. Data is represented as mean ± SEM. Significance was set at α = 0.05. # denotes significance compared to baseline pre-intervention (day 0) within each group. Between groups significance within the same time-point is denoted by * (p<.05), ** (p<0.01) and *** (p<0.001).
It is also noteworthy that rats exposed to lumbar spine facet injury had decreased threshold at their contralateral hind paw \[F (5, 25) = 4.03, p=0.008\] (Fig.11-B). Three days post facet injury, increased mechanical sensitivity about 46\% (p<.001) was shown compared to baseline. Similar decrease of mechanical threshold between 30\% to 40\% was present at 5 \(p =.005\), 7 \(p=0.019\) 14 \(p =.049\) and 21 \(p =.002\) days post facet injury compared to baseline. There was no difference between the mechanical thresholds of the contralateral limbs of facet injury and sham animals \[F (1, 5) = 5.92, p=.059\].

When ipsilateral and contralateral hind paw were contrasted in rats submitted to facet injury (Fig.11-C), no difference between ipsilateral and contralateral 3 days (MD = 5.49; 95\% CI [-8.16 to 19.15]; p>.999), 5 days (MD = -10.63; 95\% CI [-24.28 to 3.02]; p=.345) and 7 days (MD = -12.62; 95\% CI [-26.28 to 1.03]; p=.101) post facet injury was observed (Fig.11-C). However, significant difference between ipsilateral and contralateral existed at 14 days (MD = -15.98; 95\% CI [-29.63 to -2.32]; p=.009) and 21 days (MD = -15.48; 95\% CI [-29.14 to -1.82]; p=.001) post facet injury.

5.4.3 Thermal Threshold

Lumbar spine facet injury led to thermal heat hyperalgesia denoted by the increase of thermal tactile sensitivity at time points assessed \[F (5, 25) = 4.91, p=.002\] (Fig.11-D). An increase of thermal sensitivity between 40-60\% (p<.05) was present at days 3, 5, 7, 14 and 21 days post facet injury, suggesting the presence of thermal hyperalgesia in animals exposed to spine facet injury. In addition, difference of thermal threshold between spine facet injury and sham animals was present \[F (5, 25) = 3.72, p=.008\]
p=.011], reaching significance at days 14 (p=.001) and 21 (p=.002) post intervention (Fig. 11-D). Importantly, there were no differences in the baseline thermal threshold between spine facet injury and sham groups (p>.999).

5.4.4 Concentration of Proteins in the Brainstem

Western Blot analysis revealed that spine facet injury did not alter the concentration of SP and CGRP in brainstem tissue at the protein level ([F (1, 17) = .22, p=.644], [F (1, 17) = .61, p=.443] (Fig. 12-A,B). In contrast, phosphorylated ERK 1/2 in the contralateral half of the brainstem revealed a marked increase (93%) after facet injury compared to sham controls [F (1, 17) = 5.40, p=.032] while total ERK was not affected by facet injury [F (1, 17) = 2.56, p=.127] (Fig. 12-C,D). In a similar manner, GFAP was increased 35% in facet injury rats compared to sham [F (1, 17) = 5.12, p=.036] (Fig. 12-E).
Figure 12: Representative densitometric images of western blots and the relative expression of indicated proteins (bar graphs) at the contralateral brainstem (opposite to injury side) at 14 and 21 days post intervention (sham or experimental L4-L6 facet injury). (A) SP in the brainstem. (B) CGRP in the brainstem. (C) p-ERK 1/2 in the brainstem. (D) t-ERK 1/2 in the brainstem. (E) GFAP in the brainstem. Data is represented as mean ± SEM. “a” denotes a main effect of the intervention between groups (two-way ANOVA). All data was internally normalized to Ponceau staining. Fold
change was determined by the ratio between interventions (sham or experimental) relative to naïve control (with no intervention). Significance was set at alpha of 0.05.

5.4.5 Concentration of SP, CGRP and PAR2 within Neurosegmentally and non-Neurosegmentally Linked Myotome

Western blot outcomes revealed an increase of 50% and 30% in the relative protein concentration of SP and PAR2 (respectively) between experimental lumbar facet injury and sham groups within the ipsilateral quadriceps muscle [F (1, 26) = 9.37, p=.005], [F (1, 26) = 5.57, p=.026] (Fig. 13 A,C). In contrast, no effect of facet injury was denoted in the relative expression of CGRP in the ipsilateral quadriceps muscle [F (1, 26) =.002, p=.960] (Fig. 13 B).
Figure 13: Representative densitometric images of western blots showing relative protein concentration of neurogenic inflammation proteins (bar graphs) in response to lumbar facet injury in neurosegmentally linked ipsilateral or contralateral quadriceps muscle and non-segmentally linked biceps brachii muscle. (A, B and C) western blot densitometry SP, CGRP and PAR2 (respectively) and their corresponding Ponceau control staining as well as their relative concentrations (bar graphs) in the indicated muscle at 7, 14 and 21 days post sham surgery or experimental lumbar facet injury (Exp). Two-way ANOVA denotes a main effect of the experimental lumbar facet injury compared to sham-operation “*”. † denotes significant difference between experimental lumbar facet injury and sham within the same time point. Data is represented as mean ±
SEM. All data was internally normalized to Ponceau staining. Fold change was determined by the ratio between interventions (sham or experimental) relative to naïve control (with no intervention). Significance was set at $\alpha = 0.05$.

In addition, at the neurosegmentally-linked contralateral quadriceps, an increase (30-35%) in the concentration of SP [$F(2, 24) = 4.500, p=.021$], CGRP [$F(1, 24) = 6.548, p=.017$] and PAR2 [$F(1, 24) = 5.681, p=.025$] between experimental facet injury and sham groups was observed (Fig. 13 A-C). An increase of SP about 90% ($p=.041$) at day 7 and 65% ($p=.046$) at day 14 in the lumbar facet injury group compared to the sham group was denoted (Fig. A). No difference between groups was present at day 21 ($p =.999$) (Fig. A). In complete contrast, the relative expression of SP, CGRP and PAR2 in remote non-segmentally-linked biceps brachii were unaffected by facet injury ([$F(1, 26) = 3.34, p=.089$], [$F(1, 26) =.340, p=.564$], [$F(1, 26) = 2.46, p=.128$]) respectively (Fig. 13 A-C).

5.4.6 Concentration of ERK 1/2 and CaMKII within Neurosegmentally and non-Neurosegmentally Linked Myotome

Western blot outcomes in the ipsilateral quadriceps revealed a significant difference in the relative concentration of p-ERK [$F(2, 26) = 7.34, p=.003$] and p-CaMKII [$F(2, 26) = .93, p=.032$] (Fig. 14 A and C). 21 days after facet injury, p-ERK 1/2 increased 61% ($p=.03$) compared to 21 days in the sham group (Fig. 14 A). In addition, an increase within sham group (50-70%) between sham 7 days to sham 14 ($p=.007$) and to sham 21 days ($p<.001$) was also observed (Fig. 14 A). In a similar manner, a significant increase
(64-77%) in the relative expression of phosphorylated-CaMKII between facet injury 7
days to sham 7 days (p<.001), to sham 14 (p=.002) and facet injury 14 days (p=.003) as
well as to sham 21 days (p<.001) in the ipsilateral quadriceps muscle was observed (Fig
14 C).

Figure 14: Representative densitometric images of western blots showing relative
protein concentration of intracellular signalling kinases (bar graphs) in response to
lumbar facet injury in neurosegmentally linked ipsilateral or contralateral quadriceps
muscle and non-segmentally linked biceps brachii muscle. (A, B, C and D) western blot
densitometry of p-ERK, ERK, p-CaMKII and CaMKII (respectively) and their corresponded Ponceau control staining as well as their relative concentrations (bar graphs) in the indicated muscle at 7, 14 and 21 days post sham surgery or experimental lumbar facet injury (Exp). Two-way ANOVA denotes a main effect of the experimental lumbar facet injury compared to sham-operation “*” and a main effect of time “a”. † denotes significant difference between experimental lumbar facet injury and sham within the same time point. †† denotes significant difference between 7 days post experimental lumbar facet injury and 7, 14 and 21 days post sham surgery. # denotes significant difference between time points within sham or experimental lumbar facet injury. Data is represented as mean ± SEM. All data was internally normalized to Ponceau staining. Fold change was determined by the ratio between interventions (sham or experimental) relative to naïve control (with no intervention). Significance was set at α = 0.05.

At the contralateral quadriceps muscle, no effect of facet injury was noticed in the relative concentration of p-ERK 1/2 [F (1, 24) = .027, p=.869] (Fig. 14 A). However, an effect of time was present [F (2, 24) = 4.797, p=.017] inferring that a possible acute effect of either facet injury or sham intervention is likely influencing the main effect of time of p-ERK 1/2 since p-ERK 1/2 at 7 days demonstrated the highest levels of ERK activation (Fig. 14 A). In contrast, analysis of the p-CaMKII in the contralateral quadriceps muscle revealed a marked increase (38%) after lumbar facet injury compared to sham [F (1, 24) = 5.412, p=.028] (Fig. 14 C). Importantly, facet injury did not alter the relative concentration of p-ERK 1/2 [F (1, 26) = 0.9731, p=.333] nor p-
CaMKII \( F(1, 26) = 0.1727, p=0.681 \) at the non-segmentally linked biceps brachii muscle (Fig. 14 A and C).

Western Blot analysis of the total ERK and total CaMKII revealed no effect of facet injury within the ipsilateral quadriceps \( F(1, 26) = 0.002, p=0.957 \), \( F(1, 26) = 0.441, p=0.512 \), the contralateral quadriceps \( F(1, 24) = 2.492, p=0.127 \), \( F(1, 24) = 2.242, p=0.147 \) nor the biceps brachii \( F(1, 26) = 0.03, p=0.844 \), \( F(1, 26) = 0.862, p=0.361 \) (Fig. 14 B and D).

### 5.5 Discussion

The results of this study support the hypothesis that spine facet injury and the ensuing chronic OA in (L4-L6) induced CS and increased the relative expression of neurogenic (SP, CGRP and PAR2) mediators in both the ipsilateral and contralateral bilateral rectus femoris muscles. This was not observed in the sham intervention that included acute soft tissue injury. Since increased neuropeptide concentrations were not found in the biceps brachii muscle, this strongly supports a neurosegmental (rectus femoris) effect of NI rather than in a random or diffuse pattern. These findings provide evidence that NI was arising from a segmental spine disorder addressing that a segmental lumbar spine pathology may mediate neurogenic mechanism in muscles within the lumbar segment myotome.
5.5.1 The Effects of Facet Injury on Neurogenic Inflammation

The present study is the first to report that axial lumbar spine facet injury and the associated CS elicited NI at the ipsilateral and contralateral muscles residing within the common neuromeric field through NI mechanism. In the present study, we detected an increase in the relative expression of SP and PAR2 after lumbar facet injury within the bilateral neurosegmentally-linked myotomes. By contrast, the CGRP concentration in the ipsilateral quadriceps presented no changes and was similar between the experimental facet injury and sham groups, contrasting to the contralateral quadriceps, that showed CGRP was elevated in the contralateral quadriceps post-experimental facet injury compared to sham.

Observation of increase NI has been previously reported in both somatic and visceral tissues post osteoarthritis-induced and inflammation (Duarte et al. 2018; Rees et al. 1996; Decaris et al. 1999; Ustinova, Gutkin, and Pezzone 2006; Pan et al. 2010). The increase of NI at bilateral symmetrical and/or heterologous joints after induction of monoarthritis led to the development of joint inflammation, metabolic changes at the articular cartilage and reduction of the nociceptive mechanical threshold (Rees et al. 1996; Donaldson et al. 1995; Decaris et al. 1999; Duarte et al. 2018) Similarly, after colonic inflammation, increase of NI, mast cell infiltration and decrease of the mechanical threshold were observed at the neurosegmentally-linked bladder tissue (Ustinova et al. 2006; Pan et al. 2010; Fitzgerald et al. 2013).

SP and CGRP have been shown to be important players in the mechanism leading to NI (Donaldson 2009). The manifestation of NI after the induction of
experimental monoarthritis has been related to a cascade of events. The first step involves the increase of SP and CGRP production in the bilateral dorsal root ganglia, followed by dorsal horn sensitization in the spinal cord and finally subsequent bilateral antidromic release of SP and CGRP via dorsal root reflex within knee joint afferents innervated by the same neural segment(s) (Ahmed et al. 1995; Staton et al. 2007; Donaldson 2009). The antidromic impulse (dorsal root reflex) and the subsequent release of neuropeptides has been demonstrated to be an efferent response of the central neuronal hyperactivity (CS) (Carlton 2014; Schaible and Grubb 1993; Nagy et al. 2009). The antidromic impulse manifests preferentially in neurosegmentally connected tissues and has previously been shown in both somatic and visceral tissues (Eccles et al. 1961; Decaris et al. 1999; Lin et al. 2000; Pan et al. 2010).

In muscles, there is less evidence for dorsal root reflex mediating NI (Eccles et al. 1961; Sluka 2002). Eccles (Eccles et al. 1961) showed that type 1 afferents mediate dorsal root reflex impulses while Sluka showed that capsaicin-sensitive afferents mediate muscle joint NI through the dorsal root reflex (Sluka 2002). Reinert et al. (1998) showed the ability of muscle inflammation to increase the density of SP and CGRP in muscle afferent terminals which was explained by increase expression of these neuropeptides at the dorsal root ganglia triggered by NGF and pro-inflammatory cytokines (Reinert et al. 1998). The release of neuropeptides from nociceptive nerve endings has been suggested as a central player in a cascade leading to neurogenic inflammation and muscle pain (Sluka 2002). Recent evidence showed that rats exposed to L5 nerve root ligation, an experimental model used to mimic clinical neuropathic pain
disorder, had higher protein expression of SP and CGRP at neurosegmentally-linked quadriceps muscle (Ota et al. 2014). Although the mechanism leading to such alteration was not clearly explained it is possible that dorsal root reflex mediating increase of SP and CGRP within neurosegmentally-linked quadriceps muscle post neuropathic pain may be supporting their findings.

PAR 2 belongs to class of G-protein coupled receptors present mainly in nociceptive afferent nerves, immune cells including T-cells, macrophages, neutrophils and mast cells (Huesa et al. 2016; Lindner et al. 2000; Russell and McDougall 2009). PAR2 is activated by mastocyte degranulation and its inflammatory role involves an autocrine and paracrine action with the additional release of SP, CGRP, TNF-alpha and bradykinin from mast cells or afferent terminal endings contributing to the local pro-inflammatory profile (Steinhoff et al. 2000; Vergnolle et al. 2003, 2001). Thus, the observation of enhanced PAR2 expression within the bilateral quadriceps muscles add support to the neurogenic inflammatory profile in the neurosegmentally linked rectus femoris muscles.

The lack of changes of CGRP in the ipsilateral rectus femoris is difficult to explain since NI was supported by increases in SP and PAR2. It has been shown that CGRP is quickly degraded while SP has a slow degradation rate in skeletal muscle (Russell et al. 1996; Steinhoff et al. 2000). In addition, since Steinhoff et al. (2000) evidenced that PAR2 has a seven-fold higher influence in the release of SP from peripheral afferents than CGRP, either a fast degradation rate or the lower release of
CGRP could explain our observation of increased SP without a mirrored increase in CGRP in the ipsilateral muscle (Steinhoff et al. 2000).

The findings from this study show novel evidence of the modulation of extracellular signal-regulated kinases (ERK) and Ca\textsuperscript{2+}-CaM-dependent protein kinase (CaMKII) in skeletal muscle post spine facet OA-like pathology and associated CS. Phosphorylated CaMKII was increased at 7 days after the induction of spine OA-like pathology in the ipsilateral rectus femoris, and contralaterally within the rectus femoris at 7 and 14 days post-surgery, versus sham animals. p-ERK was increased at 21 days within the ipsilateral rectus femoris compared to sham animals, however, no changes of p-ERK 1/2 nor p-CaMKII were observed in the biceps brachii muscle.

It is not clear what caused the increased expression of these. Since both ERK and CaMKII are sensitive to intracellular calcium mobilization, inflammation and muscle fiber twitch (Eilers et al. 2014; Widegren et al. 2001), this observation raises the possibility that the segmental effect of NI, as well as the possible presence of other mechanisms involving pro-inflammatory cytokines and chemokines, may be mediating their modulation. Further investigation into this topic is necessary to elucidate these mechanisms more fully.

5.5.2 Lumbar Spine Facet Injury Leads to Early OA-like Pathology and Supraspinal Sensitization

In the present study, a 21-day-old L4-L6 facet injury resulted in histological and tomographic abnormalities in the subchondral bone, periarticular bone and cartilage.
are irreversible and likely to be progressive resulting in osteoarthritic facets and spondylosis.

Rats showed a bilateral secondary mechanical hind paw alldynia and thermal hyperalgesia with early time point onset (3 days) lasting 21 days after facet injury but longer studies are required. These results are in line with recent findings in the literature regarding post-traumatic facet joint injury (Crosby et al. 2014; Henry et al. 2012) that support the hypothesis that continued local inflammation triggers neuroplastic changes in the spinal cord that correspond to both secondary mechanical alldynia and thermal hyperalgesia (Weisshaar and Winkelstein 2014; Henry et al. 2012). In addition, we demonstrated increased expression of the anti-glial fibrillary acid protein (GFAP) as well as increased of p-ERK1/2 in the brain stem at 14 and 21 days post facet injury. Henry et al., (2012) in a similar study inducing facet joint injury (L4-L5) showed the presence of CS due to increased expression of SP and activated ERK in the dorsal horn of the spinal cord (Henry et al. 2012). Activation of both GFAP and p-ERK in the spinal cord have previously supported the presence of CS and the behavioral changes peripherally such as secondary mechanical alldynia and thermal hyperalgesia after inflammatory and spine OA (Sun et al. 2006; Cruz and Cruz 2007; Henry et al. 2012). The presence of behavioral changes related to CS supports the presence of spinal segmental sensitization. The manifestation of supraspinal changes suggests a possible transition from acute to chronic (supraspinal) sensitization. Further long-term studies are needed to understand the relationship between axial lumbar spine injury and long-term supraspinal neuroplasticity.
5.5.3 Lumbar Spine Degeneration Induces Neurosegmental Response

Despite the presence of CS characterized by neuroplastic increase of ERK and GFAP in brainstem, secondary mechanical allodynia and thermal hyperalgesia, post lumbar facet OA-like pathology, the presence of SP, CGRP and PAR2 only in muscles under the segmental facet injury myotome and not in myotome level above the injury (biceps brachii) supported the study hypothesis that neurogenic inflammation is related to the primary lumbar spine OA-like pathology. It has been described that CS once present provokes neuroplastic changes within the entire CNS (Park et al. 2006; Gwilym et al. 2009; Latremoliere and Woolf 2009). Carlton demonstrated experimentally that CS post spinal cord injury at the thoracic level produced mechanical allodynia and thermal hyperalgesia above, at and below the contusion level (Carlton et al. 2009). In addition, astrocyte activation was shown in multiple levels of the spinal cord (Carlton et al. 2009).

Due to the importance of the segmental sensitization on the manifestation of the dorsal root reflex (Rees et al. 1996; Sluka, Willis, and Westlund 1995; A. P. Malykhina 2007; Duarte et al. 2019), finding an increase of neurogenic inflammation in biceps brachii would not be a surprise if segmental sensitization in upper spinal cord levels (cervical segments) was manifested. However, the increase of neurogenic inflammation markers only within neurosegmentally linked myotomes 21 days post experimentally-induced lumbar OA and the presence of lumbar segment sensitization, supported by the secondary hindpaw mechanical allodynia and thermal hyperalgesia, suggests that these responses were mediated via neurosegmental pathways. These observations were not noted within non-segmentally linked biceps brachii. It may be interesting to investigate if
the spread of sensitization to upper spinal cord levels, supported for instance by the manifestation of mechanical and thermal allodynia and hyperalgesia, respectively, in the forepaw and the manifestation of neurogenic inflammation within the biceps brachii may be evidenced in a longer-term study.

5.5.4 Novelty and Possible Implication of these Findings on Myofascial Pain Syndrome

In contrast to previous studies addressing that experimentally induced local muscle injury as the primary origin of increase muscular biochemical milieu in MPS patients (Shah et al. 2005), the present study did not provoke any direct muscle injury to the quadriceps but still found increase of NI in this muscle that is neurosegmentally linked to the injury site. These changes were evident in rats exposed to lumbar spine facet injury with evidence of CS (Table 4).

Thus, this study raises a novel proposal that pathologic change in spinal facets may lead to NI in neurosegmentally linked muscles. This raises the question whether a chronic primary spine pathology might drive NI and its consequences, including pain and inflammation in neurosegmentally linked myotomes and possible joints.
Table 4: Summary table of temporal changes of behavioral (mechanical and thermal) and expression of markers related to central sensitization (brainstem) and related to neurogenic inflammation and intracellular inflammation/muscle twitch (muscle).

<table>
<thead>
<tr>
<th></th>
<th>Behavioural Tests of CS</th>
<th>Brainstem markers of CS</th>
<th>Ipsilateral quadriceps:</th>
<th>Contralateral quadriceps:</th>
<th>Remote-located biceps brachii</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3 days post spine intervention</strong></td>
<td>Decreased of mechanical an thermal threshold in the bilateral hindpaw compared baseline</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td><strong>5 days post spine intervention</strong></td>
<td>Decreased of mechanical an thermal threshold in the bilateral hindpaw compared baseline</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
<td>No data</td>
</tr>
<tr>
<td><strong>7 days post spine intervention</strong></td>
<td>bilateral hindpaw mechanical allodynia and thermal hyperalgesia in compared to sham</td>
<td>No data</td>
<td>Increase of SP, PAR2 and p-CaMKII compared to sham</td>
<td>increase of SP, PAR2, CGRP and p-CaMKII compared to sham</td>
<td>No changes</td>
</tr>
<tr>
<td><strong>14 days post spine intervention</strong></td>
<td>bilateral hindpaw mechanical allodynia and thermal hyperalgesia in compared to sham</td>
<td>Increase of p-ERK and GFAP compared to sham</td>
<td>Increase of SP and PAR2 compared to sham</td>
<td>Increase of SP, PAR2, CGRP and p-CaMKII compared to sham</td>
<td>No changes</td>
</tr>
<tr>
<td><strong>21 days post intervention</strong></td>
<td>bilateral hindpaw mechanical allodynia and thermal hyperalgesia in compared to sham</td>
<td>Increase of p-ERK and GFAP compared to sham</td>
<td>Increase of SP PAR2 and p-ERK compared to sham</td>
<td>Increase of PAR2, CGRP compared to sham</td>
<td>No changes</td>
</tr>
</tbody>
</table>
5.6 Limitations

Despite the innovative character of the present study approaching the effects of the lumbar facet joint injury upon brain stem tissue, the investigation of brainstem nucleus in the medulla oblongata, pons and midbrain were not addressed. The current study did not discriminate between brain regions, rather pooled them together. It is possible therefore, that the expression of the target proteins assessed through this study may display a different expression pattern within each anatomical nucleus, which further immunohistochemical investigation could reveal. In addition, we did not investigate the effects of blocking CS through intrathecal glutamate antagonist or SP receptor antagonists, for instance, on the release of the neurogenic inflammation within neurologically connected myotomes. Indeed, we did not perform a surgical dorsal rhizotomy to block the communication between the central and peripheral nervous system. Thus, we cannot rule out a systemic effect mediating the changes observed in the present study, although our data, together with that of others strongly support the role of the dorsal root reflex modulating the expression of neuropeptides in segmentally linked connected tissues. Both quadriceps (rectus femoris) and biceps brachii although mainly composed of fast-twitch fiber type present a significant regional variation in the distribution of muscle fiber types in rats (Fuentes et al. 1998; Kohn and Myburgh 2007). Thus, we cannot exclude that variation in muscle fiber composition influenced the observed results despite our efforts to systematically collected samples from both muscles in similar areas. In addition, pre-clinical approaches that have been used for MTrP diagnoses such as palpation, ultrasound or EMG recording were not explored.
Since the lack of a gold-standard method of diagnosis, this study relied on the biochemical signature of the MTrP that have been previously shown in MPS patients (Shah et al. 2005). Lastly, we found a high effect of the sample size in certain biomarkers assessed through this study, which emphasizes the needed to enlarge the N for future studies in this field.

5.7 Conclusion

The findings of the present study establish a relationship between primary spine pathology and neurogenically mediated inflammatory mechanisms acting within neurosegmentally linked muscle tissue. This has an important implication because it broadens our understanding of the underlying mechanisms responsible for the clinical manifestation of chronic musculoskeletal pain. These findings collectively shed light on the Neurogenic Hypothesis of the MPS and MTrPs pathophysiology. The Neurogenic Hypothesis suggests that central sensitization caused by any previous pathology leads to a significant antidromically-driven increase of neuropeptide expression within skeletal muscle tissues that are neurosegmentally innervated (Srbely et al. 2008, 2010). This theory stands in contrast to the current prevailing hypothesis that the primary pathology for chronic myofascial pain exists within the muscle itself. In practical terms, this work suggests that therapeutic considerations in the management of chronic myofascial pain should consider targeting the reduction of central sensitization and peripheral NI subsequent to persistent noxious/ inflammatory activity from facet or spine degenerative disorders.
Chapter 6: Integrated Discussion

6.1 Overall Summary of Findings

In the present thesis, we investigated the expression of neurogenic inflammatory neuropeptides within neurosegmentally-linked musculoskeletal tissue (cartilage and skeletal muscle) following experimentally induced lumbar spine facet OA, as well as spontaneous OA models using an ageing rat population. We report increased expression of neurogenic inflammatory peptides within heterologous neurosegmentally-linked ipsilateral knee cartilage tissues subsequent to the experimental induction of lumbar spine OA. We observed similar increases in neurogenic inflammatory peptides within myotomes that were neurosegmentally linked to primary spine OA pathology, both experimentally induced as well as spontaneously present within an aging rat population. Our collective findings offer support for our overall hypothesis that spine OA leads to neurogenic inflammatory responses within neurosegmentally linked heterologous tissues (both cartilage and muscle tissue). These mechanisms may have important implications in the pathophysiology of OA and MPS, respectively.

6.2 Study 1

Study 1 demonstrated increased expression of SP in chondrocytes in the tibiofemoral cartilage compared to sham and control rat. The greatest increase in SP was observed within chondrocytes in the superficial zone of the articular cartilage. This pattern was not observed in the sham intervention or control rats, which presented a more uniform distribution of chondrocytes expressing SP throughout all layers. Furthermore, we observed early OA changes in the ipsilateral tibiofemoral articular
cartilage. These findings provide novel evidence to suggest that the regional differences in chondrocyte expression of SP may be an important determinant in the early stages of the pathophysiology of cartilage degeneration.

Observations from our first study also demonstrated that SP expression in the superficial cartilage zone parallels early signs of OA in the most superficial cartilage zone, supporting the findings of Panula (Panula et al. 1998). The idea that changes in the superficial zone of the cartilage may represent initial stages of OA is further supported by Panula et al. (1998) who demonstrated that in experimentally induced OA, de-arrangement of the collagen network within the superficial zone of the cartilage precedes macroscopic OA evidence (Panula et al. 1998).

The mechanisms for these observations are unclear, given that cartilage is aneural (Grässel 2014). The pattern of SP distribution observed in our study closely parallels the pattern of nerve infiltration in osteoarthritic cartilage previously reported in humans (Walsh et al. 2010). It was previously reported by Walsh et al. (2010) that expression of nerve growth factor (NGF), as well as other vascular growth factors, were present in higher concentrations in the superficial zone of human arthritic cartilage, compared to non-arthritic controls; increased NGF was not observed in the middle or deep zones (Walsh et al. 2010). Importantly, NGF was co-localized with small, unmyelinated nerve fibers and CGRP (Walsh et al. 2010). Since the nerve fibers observed by Walsh et al. (2010) were unmyelinated, and the fact that C-fibers propagate antidromic release of SP during NI (Rees et al. 1996; McDougall 2006), we postulate that the observed increase in SP in our study could similarly be mediated via neurogenesis of C-fibers
within the superficial zone. The study by Walsh et al. (2010) provides rationale for the mechanism by which aneural cartilage may develop the capacity to express SP, highlighting a possible central mechanism in the pathophysiology of cartilage degeneration and OA (Rees et al. 1996; Lin, Zou, and Willis 2000; Lucy F. Donaldson 2009; Walsh et al. 2010).

While upregulation of SP in superficial zones may reflect early stages of cartilage degeneration and repair, the presence of SP in cartilage in deeper zones may have beneficial effects to cartilage health. Meta-analyses have concluded that low-impact exercise is important to maintaining healthy joint cartilage and is beneficial to patients suffering from OA (Musumeci et al. 2015; Dubin 2016). Karahan et al. (2002) has also reported that routine exercise leads to increased expression of SP in chondrocytes within the middle and deep zones of tibiofemoral cartilage in dogs (Karahan et al. 2002), where it may play an important role in mechanotransduction and the maintenance of articular cartilage (Millward-Sadler et al. 2003; Opolka et al. 2012). Notably, the pattern of SP expression exhibited by the exercise group in the Karahan et al. (2002) study is analogous to the sham and control groups in our study (Karahan et al. 2002), both of which did not exhibit articular degeneration. These collective observations may offer support to the idea that, while enhanced expression of SP in the middle and deep zones could be essential to the maintenance of cartilage health, increased concentrations of SP in the superficial zones of articular cartilage may represent an early degenerative stage in the pathophysiology of cartilage degeneration.

Multiple joint OA is prevalent, affecting approximately 40-80% of patients (Spector, 148
Hart, and Doyle 1994; Keenan et al. 2006). Given that symmetric spread of OA to contralateral joints is the most common pattern of spread (Spector, Hart, and Doyle 1994; Keenan et al. 2006) future research should investigate whether similar changes in SP expression are observed within the contralateral heterologous cartilage tissues.

Future directions should also advance our findings by investigating the longer-term implications of these regional changes in SP expression within the cartilage tissues. Our study demonstrated early OA changes, and future studies should elucidate subsequent biochemical and structural changes in cartilage that may be detectable through biochemical assay and/or imaging.

Given that NI is mediated by persistent nociceptive stimulation, future work should also investigate whether therapeutic interventions targeting pain and inflammation can meaningfully impact the expression of SP by mitigating the neurogenic response within neurosegmentally linked cartilage tissues. Previous research has shown that modulating the DRR can be achieved by dorsal rhizotomy and administration of spinal glutamate-receptor antagonists (Sluka et al. 1994; Sluka et al. 1994). Future studies should similarly investigate whether neurogenic mechanisms observed in our study can be blocked either by dorsal rhizotomy or by spinal administration of glutamate receptors antagonists. This line of research may have important implications to targeted therapeutic intervention strategies aiming to manage the progression of chronic, polyarticular OA.
6.3 Study 2

Emerging evidence suggests that neurogenic mechanisms may play an essential role in the pathophysiology of chronic MPS (Srbely et al. 2010). Previous research has shown that muscles of patients presenting with chronic MPS express a unique biochemical milieu dominated by the presence of SP (Shah et al. 2005, 2008). SP is a neuropeptide released peripherally by small unmyelinated fibers via DRR initiated by sensitization of the dorsal horn (Sluka et al. 1995; Rees et al. 1996; Willis 1999). In contrast to the Integrated Hypothesis of MPS proposed originally by Simons (Gerwin et al. 2004), the Neurogenic Hypothesis suggests that MPS is an expression of neurogenic inflammatory responses within muscle tissues that are neurosegmentally linked to a primary pathology residing within the common neuromeric field of the affected muscle (Srbely et al. 2010).

Both spine OA and MPS show strong correlations with age (Ita et al. 2017; Jarraya et al. 2018; Zhuang, Tan, and Huang 2014; Weiner 2007), and often coexist clinically (Weiner 2007; Malanga and Cruz Colon 2010). Weiner offered an explanation for the coexistence by proposing that the persistent inflammation from the spine OA may impair muscular function within myotomes supplied by the common spinal segments (Weiner 2007). We hypothesize that spine OA may be a common primary pathology contributing to the high prevalence of MPS in the elderly population by initiating and/or facilitating neurogenic inflammatory mechanisms within neurosegmentally linked myotomes (Neurogenic Hypothesis) (Srbely et al. 2010).

The primary objective of Study 2 was to investigate the association between the
clinical presence of naturally occurring lumbar spine OA and expression of neuropeptides associated with NI (SP and PAR2) within neurosegmentally linked myotomes, using a model of naturally occurring spine OA in a geriatric rat population. Consistent with our hypothesis, geriatric rats presenting with advanced OA changes in the lumbar spine (L3-L5) expressed significantly increased concentrations of SP within the neurosegmentally linked quadriceps muscle (L2-L5 innervation), when compared with young healthy animals free of OA.

Our findings align with previous research demonstrating robust neurosegmentally arranged expressions of NI and primary pathologies of both somatic and visceral origin. In particular, given the high association between MPS with degenerative axial spine diseases (Weiner 2007), our findings add empirical support to the Neurogenic Hypothesis which proposes that primary spine OA pathology may evoke NI within neurosegmentally linked myotomes, leading to subsequent inflammatory cascades that mediate chronic MPS (Srbely et al. 2010, 2013).

Experimental evidence validates the presence of neurogenic mechanisms subsequent to experimentally induced OA (Decaris et al. 1999), neuropathic pain (Ota et al. 2014) and visceral-inflammation (Ustinova, Gutkin, and Pezzone 2006). Similarly, previous research using animal models has demonstrated that experimentally inducing pathologies within a tissue leads to increased expression of neuropeptides linked to NI within distinct and unaffected tissues. Using animal models, Levine (1985) showed vasodilation, plasma extravasation, and hyperalgesia within contralateral homologous joints after unilateral injections of saline within the hindpaw (Levine et al. 1985). Kidd
and Bileviciute reported similar findings, adding that neuropeptide infiltration in the synovial membrane was associated with changes in vascular tissue and leukocyte infiltration, creating a favorable inflammatory microenvironment (Bileviciute et al. 1993; Kidd et al. 1995).

Similar mechanisms have also been observed in visceral tissues. Experimentally induced colitis was shown to enhance the release of substance P in the neurosegmentally linked bladder, leading to heightened mechanical sensitivity of bladder afferents and chronic cystitis (Ustinova et al. 2006). The authors attributed this to the sensitization of the primary bladder afferents subsequent to the induction of chronic colitis. Pan also confirmed these findings and demonstrated that nociceptive information from the spinal cord could be transmitted to a bladder via DRR mechanism mediated by DRG unmyelinated neurons (Pan et al. 2010).

Our results also align with the findings presented by Shah et al. who similarly reported increased expression of SP and CGRP within the local biochemical milieu of affected muscles in MPS patients (Shah et al. 2005, 2008). These observations add further evidence to support the Neurogenic Hypothesis, suggesting a potentially foundational role for NI in mediating the pathophysiology of MPS. Epidemiological studies have also confirmed that gender is a risk factor for OA. Given that we employed male rats, future research should advance our findings in a female population. Additionally, given that SP and CGRP have been shown to enhance pro-inflammatory activity by direct action on immune cells, future research should advance our findings by further characterizing the pro-inflammatory profile within affected muscle tissues. In
particular, next steps should explore the correlation between the inflammatory profile reported by Shah (Shah et al. 2005, 2008) and NI-induced inflammatory cascades, providing insight into the underlying role of NI in mediating the signature inflammatory response within muscles affected by chronic MPS.

6.4 Study 3

Although the findings of Study 2 demonstrate a strong association between spine OA and expression of neuropeptides within neurosegmentally linked heterologous muscle tissue, these observations do not provide insight into causal relationship between spine OA and neuropeptide expression. In order to address the question of causality, our third study adopted a controlled experimental design wherein we experimentally induced lumbar facet spine OA using the same surgical facet compression technique employed in Study 1, and assessed the expression of neurogenically mediated proinflammatory peptides (SP, CGRP and PAR2) within neurosegmentally linked bilateral heterologous muscle tissue. In addition to assessing the expression of proinflammatory peptides, we aimed to also assess the protein concentration of activated intracellular kinases (ERK1/2 and CaMKII). Previous studies have demonstrated that these kinases are intimately involved in muscle function and modulation of their expression within muscle may provide insight into our understanding of the pathogenesis of the characteristic local contractures often seen within muscles of patients afflicted with MPS.
6.4.1 SP and PAR2 Findings and Importance

Our study demonstrated robust increases in SP within the neurosegmentally linked rectus femoris muscle ipsilaterally, and contralaterally, in animals exposed to facet compression injury at L4-L5 and L5-L6 spinal levels. These findings support our hypothesis that experimentally induced spine pathology evokes neurogenic inflammatory responses within neurosegmentally linked heterologous muscle tissue (rectus femoris). No changes in expression were observed within the non-segmentally linked muscle (biceps brachii), enabling us to conclude that these mechanisms are mediated via neurosegmental pathways.

Similar patterns of enhanced ipsilateral expression were also observed with PAR2, a G-protein coupled receptor found on immune cells (macrophages, neutrophils, T-cell) involved in downstream inflammatory cascades. Increased expression of PAR2 suggests the presence of a robust inflammatory response within the neurosegmentally linked rectus femoris muscle, which was not present in non-segmentally linked biceps brachii. Once again, these findings support our hypothesis that robust neurosegmental mechanisms mediate NI responses within heterologous muscle tissue, and may highlight an important pathway in the clinical manifestation of chronic inflammatory muscle pain. Interestingly, increased expression of both SP and PAR2 was also observed in the contralateral rectus femoris muscle. This observation supports the fact that nociceptive pathways decussate at the spinal level (Paxinos and Mai 2012; Basbaum et al. 2009), providing the neural pathway for antidromic release of SP within contralateral myotomes.
These observations have important implications to our understanding of the pathophysiology of MPS. MPS is the most common form of chronic musculoskeletal pain and, despite its prevalence, the underlying physiologic mechanisms are still poorly understood. Recent research has shown that the biochemical milieu of affected muscles in MPS patients is characterized by enhanced expression of a variety of proinflammatory mediators including SP, CGRP, TNF-α, IL-1β, IL-6 (Shah et al. 2005, 2008). SP, in particular, is a potent vasodilator and proinflammatory mediator that has been implicated as a key player in the pathophysiology of chronic musculoskeletal pain (Shah et al. 2005, 2008; Littlejohn and Guymer 2018) and myofascial pain syndrome (Pedersen-Bjergaard et al. 1989; De Stefano et al. 2000). It has direct influence on cAMP (Öhlen et al. 1987; Suvas 2017), which has been identified as a key factor in the induction of muscle hyperalgesia and muscle inflammation (Sluka 2002). In an experimental model of chronic regional pain syndrome (CRPS) Wei et al. reported that SP binding to its NK1 receptor increased the expression of pro-inflammatory TNF-α, IL-1B and IL-6 in cutaneous tissue (Wei et al. 2009). Using this model, it was observed that administration of NGF antagonists minimized TNF-α expression and hindlimb hyperalgesia, while minimal effects were noted on edema and other cytokine expression. These observations suggest that, while NGF and TNF-α may contribute to abnormal nociception, SP may be strongly implicated in edema formation and in mediating the pro-inflammatory pathway (Wei et al. 2009; Ota et al. 2014).

The potential role of PAR2 in mediating the pathophysiology of MPS is less clear. Although the role of PAR2 in mediating inflammation has been well-established in the
literature, its precise role in mediating intramuscular inflammation is yet unresolved. Future research should aim to elucidate the mechanisms by which PAR2 could influence the upregulation of well known intramuscular inflammatory mediators (TNFa, bradykinin, IL-1B and IL-6) previously reported within the biochemical milieu of muscles from MPS patients.

6.4.2 CGRP Findings and Importance

CGRP is also released alongside SP from small unmyelinated peripheral terminals during neurogenically mediated inflammation. In contrast to SP, we observed significant increases in CGRP only within the contralateral rectus femoris muscle; similar increases in intramuscular expression of CGRP were not observed ipsilaterally. Although unexpected, one possible explanation for this might be the fact that CGRP is rapidly degraded within skeletal muscle while SP has a slower degradation rate (J. S. Russell et al. 1996; Steinhoff et al. 2000). In addition, Steinhoff et al. (2000) evidenced that PAR2 has a seven-fold higher influence in the release of SP from peripheral afferents than CGRP (Steinhoff et al. 2000). Furthermore, in contrast to SP, CGRP alone does not lead to muscle hyperalgesia; only when it is applied in combination with SP has it been shown to evoke significant pain and inflammation in humans (Pedersen-Bjergaard et al. 1991). Future research should elaborate on this unique observation by studying the fate of CGRP within muscle tissue, and its precise role in the pathophysiology of chronic inflammatory muscle disease.

6.4.3 SP, CGRP and PAR2 Have Direct Impact at NMJ

The neuromuscular junction (NMJ) is the site of communication between motor
nerve axons and muscle fibers (Gerwin, Dommerholt, and Shah 2004; Hughes, Kusner, and Kaminski 2006) where signals are transmitted from the motor neuron to the skeletal muscle primarily via release of acetylcholine (ACh) (Hughes, Kusner, and Kaminski 2006). Emerging evidence has shown, however, that other synaptically active molecules can be released from afferent nerve terminal and muscle fibers, leading to a neuromodulatory effect at the NMJ (Malomouzh 2012). In addition to their potential role in mediating muscle inflammation, research suggests that SP and CGRP, in particular, may have the capacity to directly modulate activity at the neuromuscular junction (NMJ). SP and CGRP are released antidromically by both Aδ and C-fibers, as well as by α-motor fibers (Akasu et al. 1983; Wali 1985; Sakaguchi et al. 1991; Gaydukov, Bogacheva, and Balezina 2016; Malomouzh 2012). Increased presence of SP and CGRP at the NMJ has been shown to facilitate twitch responses via spontaneous release of acetylcholine (ACh) from the presynaptic membrane of the NMJ (Wali 1985; Gaydukov, Bogacheva, and Balezina 2016; Malomouzh 2012). In addition, Matteoli reported that high doses of SP can result in accumulation of ACh within the synaptic cleft by blocking the nicotinic ACh receptor, leading to the characteristic increase in muscle activity commonly recorded from affected muscles of patients presenting with MPS (Matteoli, Haimann, and De Camilli 1990; Gerwin, Dommerholt, and Shah 2004). Adding to the excess release of ACh, previous research has shown that high doses of SP cannot be fully degraded by muscle peptidases, further accentuating the sensitizing effect at the NMJ (Russell et al. 1996). In contrast to the mechanism of SP, CGRP was shown to enhance pre-synaptic release of ACh by mobilizing calcium (Gaydukov et al. 2016).
Another family member of protease activated receptors, PAR1, has been observed at the NMJ (Lanuza et al. 2007). PAR1 has been identified at the post synaptic terminal of the NMJ (Lanuza et al. 2007). In neonatal and adult rat muscles, the role of PAR1 has been linked to the formation and maintenance of the neuromuscular contacts supporting the expression of ACh receptors at the post synaptic region (Faraut et al. 2004; Lanuza et al. 2007) but there is still limited research on the role of PAR1/2 at the NMJ. PAR2 is highly prevalent within Aδ and C-fibers and actively participates in the maintenance of NI via degranulation of mastocytes, which further promotes additional release of SP and CGRP. In this way, PAR2 may also modulate NMJ function indirectly by mediating the expression of SP and CGRP but further research is needed to elucidate how these pro-inflammatory mediators and neuropeptides may directly impact NMJ function to explain the formation of discrete extrafusal fiber contracture knots characteristic of chronic myofascial pain.

6.4.4 Role of ERK 1/2 and CaMKII in Muscle

Study 3 also aimed to investigate whether experimentally induced lumbar spine OA-like pathology enhances protein concentration of activated form of intracellular kinases (ERK 1/2 and CaMKII) involved in muscle function and muscle plasticity within neurosegmentally linked muscle. Since phosphorylation of ERK 1/2 is involved in intracellular cascades leading to inflammation and CaMKII is sensitive to fluctuations of intracellular calcium levels following muscle contraction their increase may provide a rationale for the pathophysiological mechanisms of inflammation and muscle
tenderness in patients with chronic skeletal muscle disorder.

Similar to SP and PAR2, we observed significant increases in the phosphorylated form of both ERK 1/2 and CaMKII concentration bilaterally within the neurosegmentally linked rectus femoris muscle.

While the precise mechanism by which they were increased is still unclear, it appears that NI mechanisms may have played a key role in their enhanced activated expression, in light of the fact that they were not upregulated within the non-segmental biceps brachii. Given that both kinases are sensitive to intracellular calcium mobilization, inflammation and muscle fiber twitch (Widegren et al. 2001; Eilers et al. 2014), a number of factors within skeletal muscle could have been responsible for eliciting the activation of the ERK 1/2 cascade and CaMKII, including the presence of inflammation, cytokines, mechanical stress (contraction) and changes in intracellular calcium levels (Widegren et al. 2001; Yu et al. 2001; Supinski and Callahan 2018).

Upregulation of ERK has important implications to the expression of intramuscular inflammation. The phosphorylated (activated) form of ERK 1/2 (p-ERK 1/2) is involved in the downstream activation of nuclear transcription factors associated with inflammation such as CREB and NFk-B (Wojtaszewski et al. 1999; Parkington et al. 2004; Wang, Chabot, and Quirion 2011). Inflammatory stress and muscle damage is typically associated with neutrophil and macrophage recruitment which promotes the induction of pro-inflammatory cytokines interleukin-1 beta (IL-1b), IL-6, and tumor necrosis factor-alpha (TNF-a) (Szelenyi and Urso 2012).
ERK can also act synergistically with SP to promote inflammation. SP has been shown to regulate the activation of inflammatory signaling pathways via ERK 1/2 activation in macrophages, neutrophils, smooth muscle cells and lymphatics muscles. This cascade of intracellular events mediated by SP and ERK 1/2 is dependent on intracellular increase in calcium (Tanabe et al. 1996). Previous studies have shown that application of SP agonists to intestinal smooth and lymphatic muscle lead to muscle contraction and inflammatory responses; however, when ERK 1/2 antagonists are applied, both contraction and inflammation were significantly diminished (Suvas 2017; Chakraborty et al. 2011). SP-NK1 coupling causes the formation of inositol triphosphate, which leads to an increase in the level of intracellular calcium and formation of diacylglycerol, subsequently activating upstream protein kinase C and later phosphorylating ERK1/2 (Suvas 2017; Chakraborty et al. 2011). Since the expression of NK1 has been previously confirmed within skeletal muscle (Pinto et al. 2004), our findings raise the possibility that similar signaling cascades involving SP and p-ERK 1/2 may occur within skeletal muscle, leading to inflammation and discrete extrafusal fiber contraction commonly observed in patients with chronic MPS. Future research is needed to advance our understanding of these pathways and inform potentially novel therapeutic directions in the management of chronic MPS.

Study 3 also demonstrated increases in the activation of calcium/calmodulin-dependent kinases (CaMKII) bilaterally within neurosegmentally linked muscle tissue, but not within non-segmentally linked myotomes (biceps brachii). CaMKII are important transducers of intracellular calcium signals (Chin 2005; Kawasaki 2004; Cervone and
Dyck 2017). Following exercise or muscle contraction, the phosphorylated form of CaMKII significantly increases within skeletal muscle (Eilers et al. 2014; Eilers et al. 2014), the degree to which being related to the duration, amplitude and frequency of intracellular Ca\(^{2+}\) (Chin 2005). After prolonged skeletal muscle activation, autophosphorylation of CaMKII has been reported, suggesting that CaMKII may be a stimulation-frequency decoder in skeletal muscle (Chin 2005). CaMKII has mostly been studied in muscle activity-related exercise adaptations as increasing muscular oxidative capacity has been shown to be associated with CaMKII activation (Rose and Hargreaves 2003; Chin 2005). In contrast, experimental model of dystrophic muscle disease revealed decrease of CaMKII in most muscle fibers presenting signs of dystrophy, however increase CaMKII expression was shown in regenerating muscle fibers (Gao and McNally 2015; Chin 2005).

We have shown that increase of CaMKII phosphorylation followed a similar pattern of expression within neurosegmentally linked muscle as such NI peptides. Thereby, it is feasible to speculate that a possible connection between NI and CaMKII may exist and future studies should aim to elucidate the direct relationship between NI and phosphorylation of CaMKII, as well as whether they may have any impact on the functional aspects of muscle as such increasing force production and contraction. This line of research may provide additional evidence to support the Neurogenic Hypothesis and provide a rationale for the pathophysiological mechanisms of inflammation and muscle tenderness in patients with chronic skeletal muscle disorder.
6.4.5 ERK 1/2 and GFAP at Brainstem

The aim of this analysis was to investigate the expression of ERK 1/2 and GFAP (a marker of astrocyte activation) within the contralateral brainstem following experimentally induced lumbar spine OA-like pathology. This analysis enabled us to investigate whether the experimentally induced spine OA-like pathology evoked supraspinal changes in sensitization. This is an important area of research in the field of chronic musculoskeletal pain given that sensitization of supraspinal centers typically leads to chronic widespread pain syndromes, such as fibromyalgia (Gracely et al. 2002). Advancing our understanding of these mechanisms is an important line of research in this field as it may provide important insight into potential therapeutic pathways. We observed significantly increased protein expression of p-ERK 1/2 and GFAP within the contralateral (right) brainstem region following left sided lumbar spine OA-like pathology. No effects were seen in sham surgery and controls and suggest that persistent nociception from the spine OA-like pathology evoked neuroplastic changes supraspinally within brainstem regions.

Astrocytes are the most common type of glial cell and highly prevalent within the CNS (Sun et al. 2006). Astrocyte activation is intimately involved in the onset and maintenance of CS and associated secondary hyperalgesia and allodynia behaviours (Hald 2009; Sun et al. 2006). Our findings of significantly increased of GFAP concentration support the upregulation of astrocyte activity, which correlates to the behavioral outcomes we observed in these animals, including thermal hyperalgesia and mechanical allodynia.
Similarly, upregulation of p-ERK 1/2 concentration within the CNS is a sequela to intense and persistent nociceptive stimulation, commonly associated with chronic pain disorders (Cruz and Cruz 2007; Gao and Ji 2009). Once activated, phosphorylated ERK 1/2 can be translocated into the nucleus to trigger transcriptional factors, such as cAMP-response element binding protein (CREB) necessary for the transcription of many neuronal genes and long-term synaptic plasticity (Cruz and Cruz 2007). Gao reported that activation of neuronal ERK 1/2 is the most sensitive marker of CS after noxious stimuli and tissue injury (Gao and Ji 2009). Our experimental model demonstrated significantly increased p-ERK 1/2 concentration within the contralateral brainstem, again supporting the hypothesis that experimentally induced spine-OA like pathology can evoke neuroplastic changes within supraspinal centers of the CNS.

Both astrocytes and ERK1/2 activation have been previously reported to increase within the spinal cord as well as brainstem tissues following peripheral inflammatory disorders (Raghavendra et al. 2004; Cruz and Cruz 2007; Kumabe et al. 2017). In a pre-clinical study of neuropathic pain in rats, spinal cord activation of both GFAP and ERK 1/2 lasted for 21 days post nerve ligation, consistent with the observation of sensorial mechanical hyperalgesia throughout the period of the study (Cruz and Cruz 2007). Coincidently, we also observed increased concentration of GFAP and p-ERK 1/2 within the brainstem after a similar period of time post surgery. Kumabe reported similar findings of increase GFAP and ERK 1/2 post masticatory masseter muscle-induced inflammation in rats (Kumabe et al. 2017). Such changes persisted for around 1 week after local inflammation had subsided suggesting that a possible transition between
acute to chronic pain may involve these neuromodulators (Kumabe et al. 2017).

These observations align with previous imaging studies in chronic pain populations. Previous functional neuroimaging studies in humans have reported activation of several supraspinal regions including the contralateral brainstem in patients with chronic pain and OA (Gwilym et al. 2009; Pujol et al. 2017). Our observations contribute biomarker evidence to support these imaging studies demonstrating supraspinal neuroplastic changes in chronic pain populations, and future research should advance this thread by further elucidating the biomarker spectrum.

Several studies have shown the role of SP as an upstream mediator of ERK activation in non-skeletal muscle tissues as lymphatic muscles and immune cells. However, the relationship between SP and ERK has not been the subject of investigation in skeletal muscle tissue. Future studies may want to investigate this intracellular downstream cascade possibly initiated by SP-NK1 activating ERK signal pathway, culminating in the increase of pro-inflammatory cytokines post nuclear transcription. In addition, other pro-cytokines often present in the biochemical milieu of patients with MPS may also be investigated which may also inform the relationship between cytokines and ERK activation.

We have shown that increase of CaMKII phosphorylation was greater at 7 and 14 days following experimental spine injury. Since the modification of this kinase was only present in segmentally-connected myotomes, it is feasible to speculate that a possible connection between NI and CaMKII may be present. Future studies in vitro should aim
to investigate the possible relationship between NI and phosphorylation of CaMKII as well as whether they together may have any impact in functional aspects of muscle as such increasing force production and contraction. This may add more support into the Neurogenic Hypothesis that provides a rationale into the pathophysiological mechanisms of inflammation and muscle tenderness in patients with chronic skeletal muscle disorder.

6.5 Future Directions

The findings of these studies introduce several novel directions for research in this field.

6.5.1 Correlation to Findings of Shah et al. (2005;2008)

Recent studies demonstrate that patients presenting with MPS exhibit unique biochemical milieu within affected muscles, characterized by elevated SP, CGRP and pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Shah et al. 2005, 2008).

The Neurogenic Hypothesis suggests that chronic MPS can be evoked via neurogenic inflammatory responses to a primary pathology within the common neuromeric field (Srbely et al. 2010). Our study demonstrates significantly increased protein expression of proinflammatory neuropeptides and kinases (SP, PAR2, CGRP and p-ERK1/2) within neurogenically linked heterologous myofascial tissues. Given the ability for p-ERK to upregulate the transcription of proinflammatory cytokines, our findings introduce yet another potential factor contributing to the inflammatory profile of chronic MPS patients. Future studies should aim to further resolve the characteristic
inflammatory profile, with a specific aim to correlating these findings in animal models to those previously reported by Shah, in chronic MPS patients.

6.5.2 Preemptive Mitigation of NI

Our findings provide compelling evidence that NI mechanisms may be important in upregulating proinflammatory neuropeptides and kinases within neurosegmentally linked heterologous muscle tissue. In order to further advance this line of inquiry, next steps should aim to abolish NI by mitigating the DRR through blockage of afferent nociceptive input from the primary spine pathology. This could be achieved in animal models using dorsal rhizotomy prior to facet compression surgery, for example. A subsequent finding of decreased expression of proinflammatory neuropeptides and kinases using this model would implicate the role of neurogenic mechanisms in our findings. Similarly, inhibition of CS via application of glutamate receptor antagonists, NK1 antagonists and/or capsaicin may also be employed to pre-empt subsequent DRR and neurogenic inflammatory responses in both cartilage and muscle tissue.

6.5.3 Muscle Structure and Function

A clinical diagnostic feature of MPS is the presence of MTrP, characterized as local contractures within taut bands of muscle. Despite its prevalence, the underlying physiologic mechanisms responsible for these phenomena are still unresolved.

Limited existing research points to the possibility that SP and CGRP (Wali 1985; Takami et al. 1985) may induce excitatory effects by acting directly on the NMJ, resulting in the excess presynaptic release of ACh and local muscle contracture
characteristic of chronic MPS. This presents a novel pathway by which local muscle contractures may form within affected muscles. Future work should advance this line of questioning to elucidate the mechanism(s) responsible, both local and centrally mediated, that contribute to the formation of these localized contractures. Along similar lines, further testing using in situ preparations should also investigate whether persistent exposure of the NMJ to SP and CGRP, as with chronic persistent nociceptive input from spinal OA, may affect muscle function, including contraction force, fatigue, and motor unit recruitment patterns, which may address an important gap in our understanding of neuromuscular maladaptation commonly observed with MPS.

Our study also demonstrates increased protein expression of p-CaMKII within muscle tissue (rectus femoris) that is neurosegmentally linked to the primary spine-OA pathology. Previous research has shown that overexpression of p-CaMKII results in an enhanced efficiency of muscle contraction and relaxation through increased expression of SERCA pump, which is responsible for the removal of Ca\(^{2+}\) from the cytosol of muscle cells (Eilers et al. 2014). Furthermore, repeated muscle contractions are thought to facilitate the activation of p-CaMKII (Eilers et al. 2014). Our findings of increased concentration of p-CaMKII may offer yet another future direction for research to explain the persistent, self-sustaining contractures observed with chronic MPS.

Finally, the effect of CS on muscle structure has yet to be investigated. Previous studies suggested that stiffness due to increase muscle connective tissue may be subsequent to local inflammation in chronic musculoskeletal pain (Langevin and Sherman 2006; Ota et al. 2014). Muscle fibrosis may contribute to muscle stiffness and
muscle tightens commonly present in the clinical assessment of MTrP (Lacomba et al. 2010; Littlejohn and Guymer 2018; Gerwin 2008). Investigating the presence of increase connective tissue in neurosegmentally-linked muscles with presence of NI may be an interesting next step to further elucidate the pathophysiology of MPS and MTrP. Inspecting for the presence of extracellular matrix proteins in the muscle could be addressed through histochemical techniques as Sirius Red and HE. A pro-fibrotic transforming growth factor beta (TGF-β) could also be used to inspect for its presence and quantify its levels in muscle through IHC and WB or ELISA.

6.5.4 Biomarker of CS

CS has been linked to a number of conditions associated with chronic pain including OA, fibromyalgia and MPS (Arendt-Nielsen et al. 2010; Arendt-Nielsen and Graven-Nielsen 2003; Hocking 2010; Srbely et al. 2010; Pujol et al. 2017; Gracely et al. 2002).

A key limitation in both experimental and clinical domains is the absence of a quantifiable outcome measure for CS and this contributes to the diagnostic uncertainty of chronic pain conditions. Our findings suggest that several emerging biomarkers may be useful in the diagnosis and management of CS and chronic pain. Future studies may also strive to correlate biochemical assays with neuroimaging analysis in experimental spine OA-like pathology to add further support to changes observed in neuroimaging studies.
6.6 Conclusion

This thesis was the first to explore the relationship between spinal OA, both experimentally induced as well as naturally occurring, and neurogenic inflammatory mechanisms within neurosegmentally linked heterologous musculoskeletal tissues, including both muscle and cartilage. We contribute novel data demonstrating a causal association between the experimental induction of spinal OA and robust neurogenic inflammatory responses within heterologous tissues, including both cartilage and muscle. These findings advance the field of chronic MPS research by providing additional empirical evidence to support the Neurogenic Hypothesis which states that MPS is the physiologic expression and clinical manifestation of neurogenic inflammation within skeletal muscle that is neurogenically linked to a distinct, heterologous primary pathology (somatic or visceral) residing within the common neuromeric field of the primary pathology.

Similarly, our findings of increased SP expression within the surface layers of articular cartilage contribute novel insight into potential impact of neurogenic inflammatory mechanisms in the pathophysiology of cartilage degradation, with possible implications to the pathophysiology of OA. These collective findings inform future research aiming to elucidate the pathophysiologic mechanisms of chronic pain and degenerative joint disease. Given the aging demographic in society and the growing burden of chronic musculoskeletal disease, this line of research is of urgent priority to reducing the burden of chronic musculoskeletal disease in society.
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