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Krishna Pucha April 2, 2021

Neurobiological Changes in Rat Dorsal Root Ganglia due to Osteoarthritis and Exercise Treatment

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Abstract

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Background: Osteoarthritis (OA) is the most common chronic degenerative disease of the joints characterized by the degradation of articular cartilage, subchondral bone sclerosis, synovial inflammation, and the growth of marginal bone spurs. OA often leads to reduced activity and further loss of function in the affected joint. Despite the socioeconomic costs of OA and its associated chronic pain, there are no FDA-approve disease modifying drugs available in the clinic. Exercise is the only disease modifying treatment that has shown evidence to reduce pain in certain patient populations. The objective of this study is to further elucidate the impact of exercise on OA pain progression, specifically in the context of neurobiological changes that occur in the dorsal root ganglia (DRG).

Methods: OA was induced in Lewis Rats in the left knee via a medial meniscal transection (MMT) procedure. Three weeks post-OA induction, sham and MMT animals began a daily exercise regimen for three weeks. Through this six week period, Von Frey measurements were taken at baseline, 2-, 4- and 5-weeks post-surgery to measure pain sensitivity. Six weeks post-surgery, animals were euthanized and DRGs were collected from the thoracic (T9-T12) and lumbar (L1-L4) regions of the spinal column. These tissues were then cryosectioned and stained for the expression of CGRP and TRPV1.

Results: Representative images demonstrated successful isolation and staining of the DRG. No significant differences in the DRGs from the lumbar region were observed between groups, MMT animals demonstrated increased expression of CGRP in DRGs from the thoracic region compared to animals in the MMT with exercise group (MMT Ex). MMT and MMT Ex animals also demonstrated a decreased expression of TRPV1 in DRGs from the thoracic region compared to sham animals. In measures of pain sensitivity, by week 5, MMT Ex animals had higher pain sensitivity compared to non-exercised groups.

Conclusion: Though no changes were detected in DRGs that directly innervate the left knee (lumbar), changes in other regions of the body (thoracic) were observed due to OA induction. Additionally, administering an exercise therapy reversed these changes in MMT animals. These findings show potential evidence of exercise's ability to mitigate neurobiological changes induces by OA and potentially help alleviate the pain associated with the disease.

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Introduction

Osteoarthritis (OA) is the most common chronic degenerative disease of the joints impacting more than a third of adults over the age of 65 in the US (Neogi, 2013; Vos et al., 2015). OA is characterized by the degradation of articular cartilage alongside accompanying phenotypes of subchondral bone sclerosis, synovial inflammation, and the growth of marginal bone spurs, leading to pain and eventual loss of joint function (Loeser et al., 2012). Pain is the driving symptom of this disease, often leading to reduced activity and further loss of function in the affected joint (Clynes et al., 2019). Structural symptoms of the joint and pain worsen as the disease progresses, severely limiting patients' abilities to perform activities of daily living (ADL) and dramatically decreasing quality of life (Clynes et al., 2019).

There are different types of pain driven by distinct processes that occur throughout OA progression. These include acute pain directly caused by tissue damage and local inflammation, as well as chronic pain that can spread to other areas of the body as OA progresses. Initial mechanical insult to the joint can cause intra-articular inflammation and tissue degradation in the joint space that initiates feedback loops of catabolism via enzymes such as matrix metalloproteinases (MMPs). This initial response creates a milieu of pro- and anti-inflammatory mediators that can attract additional innate and adaptive immune cells that release more inflammatory and neurotropic factors. These inflammatory and neurotropic factors drive acute pain by acting on and activating high threshold pain receptors that innervate the subchondral bone (Kidd, 2012; Sellam and Berenbaum, 2010). Additionally, this cocktail of cytokines and chemokines in the inflamed joint decreases the activation thresholds of previously "silent nociceptors" in the subchondral bone that normally do not fire at such high rates, leading to increased pain signaling from the arthritic joint and pain that can outlast inflammation (Dureja et

al., 2017; Martindale et al., 2007; O'Neill and Felson, 2018). This sustained increase in peripheral input causes sensitization in more central levels of the nervous system leading to increased pain responses to noxious stimuli (hyperalgesia) and painful responses to innocuous stimuli (allodynia) (Kidd, 2012; Neogi, 2013).

Another process driving the development of peripheral pain is the angiogenesis that occurs in the subchondral bone due to the local inflammation, further contributing to the inflammation (Gacche and Meshram, 2014). This neovasculature then becomes innervated by new nerve growth. The cellular infrastructure for new nerve growth provided by these new blood vessels increases the concertation of pain receptors in the subchondral bone. The new nerves consist of myelinated A fibers that respond to high intensity mechanical stimuli and are responsible for sharp, acute pain. These signals can originate from structural damage such as bone marrow lesions and exposed bone (Cameron, 2013). Additionally, unmyelinated C fibers, characterized by Substance P and calcitonin gene-related peptide (CGRP) expression, also innervate the neovasculature and respond to low intensity stimuli. Signals sent by these nerve endings are perceived as the slow, burning pain associated with the chronic pain of OA (Cameron, 2013). The increased presence of these fibers in-part marks the transition to a peripherally sensitized state in an arthritic joint, a key step that leads to central sensitization and chronic pain in advanced OA (Mayer et al., 1999; Walsh, 1999; Wojtys et al., 1990). Together these disrupted inflammatory and angiogenic equilibria lead to a broad spectrum of phenotypes in the patient population, contributing to an incomplete understanding of this transition from acute to chronic pain in OA (Bonnet and Walsh, 2005; Eitner et al., 2017; Lingen, 2001; Loeser et al., 2012; Mayer et al., 1999; Schaible et al., 2005; Sellam and Berenbaum, 2010; Vos et al., 2015; Walsh et al., 2010).

In the transition from peripheral to central sensitization and acute to chronic pain, neurobiological changes in the periphery induce similar changes in nervous tissues closer to the central nervous system (CNS). Increased peripheral input in response to local inflammation contribute to the release of factors such as Substance P, brain-derived neurotrophic factor (BDNF) and CGRP in the cell bodies of these nerves located in dorsal root ganglia (DRG). The DRGs consist of the soma of the peripheral afferent fibers and are adjacent and directly connected to dorsal horn neurons in the spinal cord. Neurons in the dorsal horn of the spinal cord then send projections to perception centers in the brain (Miyamoto et al., 2017; Schaible et al., 2005). CGRP, a key focus of this study, has been implicated in the pain and inflammation processes associated with OA progression. Though its function is not fully understood, it has been characterized as a strong vasodilator that plays a protective role in wound healing (Russell et al., 2014). Additionally, CGRP is expressed at multiple levels of the nervous system from the peripheral fibers all the way to the DRG and is implicated in the transmission of nociceptive signals (O'Brien et al., 1989). In patients with hip OA, CGRP expressing nerves were found to be localized in patients with painful OA (Saxler et al., 2007). Additionally, the targeting of this peptide with a CGRP receptor antagonist reduced inflammatory pain in an adjuvant induced arthritis (AIA) rat models (Hirsch et al., 2013). CGRP knock out murine models also demonstrated reduced hypersensitivity to pain (Zhang et al., 2001). Together, these studies demonstrate the close relations between CGRP and pain in OA.

Alongside neuropeptides such as CGRP, membrane receptor proteins are also important in the transmission of pain. Transient receptor potential cation channel subfamily V member 1 (TRPV1) is one of these receptors that is located throughout the nervous system and also plays a key role in the transmission of pain. TRPV1 is non-specific cation channel that responds to wide

variety of noxious stimuli including heat, acidic stimuli and capsaicin, the molecule that produces the spice sensation in foods (Fernihough et al., 2005). It also has been implicated in pain transmission as antagonists of this receptor and knock out/deficiency models result in reduced pain sensitivity in rodent models of inflammatory pain (Walker, 2003; Wang, 2008). Additionally, different inflammatory mediators can sensitize and increase TRPV1 expression in the DRG in various inflammatory and pain models (Amadesi et al., 2006; Fernihough et al., 2005; Ji et al., n.d.; Sugiura et al., 2002). However, other studies found that TRPV1 expression did not to increase at the DRG level in a rat inflammatory arthritis model at both short- and long-term stages (Bär et al., 2004). Additionally, local administration of capsaicin, a ligand for TRPV1, injections at the site of injury causes peripheral desensitization in sensory fibers and has been used clinically to alleviate pain (Wang, 2008). Though there is promising evidence that TRPV1 can be used as a marker for peripheral sensation, more research must be conducted to characterize the impact of peripheral damage and inflammation on TRPV1 expression. Changes in DRGs characterized in-part by these proteins provide the infrastructure to sensitize pain perception in the CNS (central sensitization) and lead to chronic pain and broader complications such as widespread pain (WSP).

Despite the socioeconomic costs of OA and its associated chronic pain, there are no FDA-approve disease modifying drugs available in the clinic. Though many preclinical studies have elucidated key mechanisms of disease progression and tissue regeneration, few have translated into the clinic. Exercise is the only disease modifying treatment that has shown evidence to reduce pain in certain patient populations (Henriksen et al., 2014; Lee et al., 2018). In post-traumatic OA (PTOA) animal models, exercise can lead to structural improvements in the affected joint (Iijima et al., 2016; Li et al., 2016; Zheng et al., 2019). Our lab has

demonstrated the mitigative effects of exercise on OA in slowing joint degradation in a PTOA model (Kaiser, unpublished data). Additionally, exercise can reduce pain in WSP animal models demonstrating its potential to exert its effect on regions not directly related to the cite of injury (Sharma et al., 2010). Exercise is a powerful and multifaceted attenuator of local inflammation as well as inflammation in the DRG making it a potentially powerful tool to mitigate the inflammation that contributes to pain progression in OA (Chang et al., 2015; Chhaya et al., 2019). However, there remain gaps in knowledge on the mechanism of action of exercise's therapeutic and potential pain reliving effects in the context of cellular mechanisms that drive OA pain progression. By being able to better characterize the impact of exercise on chemical and neurobiological components that drive pain progression in OA, we will be able to better elucidate therapeutic targets and bridge gaps in understanding between tissue regeneration, inflammation, and neuroscience

The objective of this study was to test if exercise could potentially facilitate pain resolution and mitigate peripheral and central sensitization. Specifically, I aimed to measure the impact of exercise on cellular changes at the DRG level in a PTOA animal model. If successful, studying cellular impacts of exercise on a PTOA model can allow for the simultaneous targeting of tissue protection, joint inflammation, innervation, and pain stemming from OA, while also demonstrating its potential utility in elucidating therapeutic targets.

The impact of exercise on pain transmission within the peripheral nervous system (PNS) at the level of the DRG has been studied largely in an adjuvant induced arthritis (AIA) models. In contrast, relatively few studies utilize PTOA that models initiate arthritis through surgical trauma of the joint, especially in the context of characterizing neurobiological aspects of OA pain progression (Little and Zaki, 2012). These animal models are more useful in replicating

structural damage that is phenotypic of OA and is relatively understudied when looking at neurobiological changes. Further understanding is needed on the impact of exercise on neurobiological changes in the DRG in the context of sensitization in regions near and nonadjacent to the site of injury in OA animal models. Through central sensitization, patients often develop chronic pain in areas of the body not directly impacted by OA. As exercise not only directly engages the affected joint but rather the body has a whole, this systematic study will be able to provide a unique opportunity to gain preclinical insights on how exercise can exert global therapeutic effects and pave ways to optimize treatments that look to reduce inflammation, reduce systemic pain and, increase functionality of patients effected by OA.

I hypothesize that the implementation of an exercise regimen post-induction of OA in animals will mitigate changes in the DRG that innervate the knee region. I hypothesize exercise treatment in a preclinical model of OA will 1) reduce expression of markers in the DRG associated with central sensitization (CGPR and TRPV1) and 2) lead to reduced pain sensitivity in animals with OA.

Methods and Materials

Animal care and experiments were conducted in accordance with the institutional guidelines of the Atlanta Veteran Affairs Medical Center (VAMC) and were approved by the Atlanta VAMC Institutional Animal Care and Use Committee (IACUC) (Protocol: V002-18).

Animal Training

Two weeks prior to surgery, 24 male Lewis rats (strain code: 004; Charles River) began acclimation to the treadmill environment to become accustomed to the exercise regimen. Animals were placed on the motionless treadmill in darkness for 25 minutes, followed by 5 minutes of walking at 10 m/sec. Each subsequent day increased the exercise duration by 5

minutes until animals were walking for a full 30 minutes. Out of the animals that consistently ran the full length of the exercise routine during training, 12 animals were selected to receive the exercise treatment.

Surgical Methods

OA was induced in the animals by implementing a rat medial meniscal transection (MMT) on the left leg of the animal as previously described (Bendele, 2001). Animals were anesthetized via isoflurane inhalation and SR buprenorphine (ZooPharm,Windsor, CO) was administered subcutaneously as an analgesic. An incision was made along the medial aspect of the animals' left knee exposing the femora-tibial joint. A blunt dissection was made to expose the medial collateral ligament (MCL) which was then cut to expose the meniscus. A full cut of the meniscus was performed at the narrowest point. Soft tissue was then sealed with 4.0 vicryl sutures and the wound closed with surgical staples. A sham procedure dissecting the MCL, but not the meniscus was also performed for animals in the control group. Half of each experimental group (exercise and no exercise) received the MMT treatment (n = 6) and the other half received the sham treatment (n = 6). Animals were monitored during the post-operative period to ensure their recovery and staples were removed 7 days post-surgery.

Von Frey Measurements

Von Frey measurements of allodynia were taken to observe pain perception in the rats. Measurements were at baseline before the MMT surgery and 2-, 4-, and 5-weeks post-surgery (Sup. Fig. 3). The 50% withdrawal threshold method was utilized as previously outlined (Chapman et al., 1985). Rats were acclimated to cages for three days prior to testing. Cages consisted of a clear plexiglass box with a wire mesh floor. Testing began once rats ceased exploration and grooming (~20 mins after placement).Filaments of increasing stiffness were

passed through the wire mesh floor to push the left hind limb of the animal in the cage. A lifting of the paw was considered a positive hit. If an ambiguous response was observed, another test with the same filament was administered after one minute. There were one minute time intervals between tests. Testing started with a moderately stiff filament. Stiffness was increased with a negative hit and decreased with a positive hit. Testing ended with five consecutive filaments following the first positive response, or until the stiffest filament (26 gf) was used.

Exercise Regimen

Three weeks post-surgery, animals in the exercise group began their daily exercise routine of 30 minutes of running at 10 m/sec, 5 days a week for 3 weeks. Animals in the non-exercise group were not administer any additional treatments. All rats were be euthanized 6 weeks post-surgery via CO_2 asphyxiation (Sup. Fig. 3).

Sample Preparation and Data Collection

Prior to euthanasia, animals underwent trans-cardiac perfusion with ice-cold PBS. Spinal columns were collected from each rat and split on the sagittal plane, exposing the spinal cord and inner wall of the spinal column. The spinal cord columns were then fully submerged in formalin for 72 hours at 4^{o}C. Post-fixation, DRG residing in the T9-T12 and L1-L4 spinal columns from the left half of the spinal column were isolated and collected and pooled into either a lumbar or thoracic group. After washing with PBS 4 times for 10 min, DRG were stored in a 20% sucrose solution at 4^{o}C for cryoprotection for at least 24 hours and then embedded in Tissue-Plus O.C.T. Compound embedding medium (Fisher Healthcare; Waltham, MA) and frozen at -80^{o}C. Five samples from each group were randomly chosen and sectioned into 10 µm thick sections at -20^{o}C onto (slide type name) with a Microm HM 550 cryostat. Slides were then stored at -80^{o}C.

Immunological Staining of DRG

Prior to immunohistochemical (IHC) staining, slides were thawed at room temperature for at least one hour prior. Once thawed, individual slices on the slide were circled with a hydrophobic marker. Slices were then washed 3 times for 5 minutes each in 1X phosphate buffered saline (PBS). Each subsequent washing step followed this procedure. Slides were then submerged in 3% hydrogen peroxide in PBS for 30 minutes, washed with PBS, submerged in 0.1% Triton X-100 in PBS and washed once again.

To block non-specific binding, tissues were submerged in 10% goat serum for in hour followed by an overnight incubation at 4^{o}C with primary antibodies diluted in 1.5% goat serum in PBS. Rabbit anti-rat CGRP polyclonal antibody (1:600; Thermo Fisher; Waltham, MA) and Chicken anti-rat TRPV1 antibody(1:1200; Bio-Rad; Hercules, CA) were used to bind to the proteins of interest, CGRP and TRPV1. Negative control slices were incubated in 1.5% goat serum in PBS with no primary antibodies. Following the overnight incubation, samples were washed and submerged in goat anti-rabbit IgG H&L-Alexa Fluor 488 and goat anti-chicken IgY H&L-Alexa Fluor 594 (1:200; Abcam; Cambridge, UK) in 1.5% goat serum for 1 hour at room temperature. The tissues were then washed and one drop of Fluoroshield mounting medium with DAPI (Abcam; Cambridge, UK) and placed under a coverslip.

Image Analysis

Slides were scanned with a BioTek Lionheart LX Automated Microscope and 3 representative images from each animal was taken for further processing and quantification (Fig. 1a). ImageJ (NIH, Bethesda MD) was used to subtract background fluorescence from each gray-scale image. Lower thresholds were then set at 1000 and 2500 mean grayness value for TRPV1 and CGRP respectively. Images were then converted to binary and duplicated (Fig. 1b). The

original image brightness values were then divided by 255 and multiplied by the binary mask and the lower thresholds was raised to 1 to exclude areas of the image not covered by the mask. Regions of interest (ROI) were then manually isolated to include only cell bodies and mean greyness value of the ROI was measured (Fig 1c).

Statistical Analysis

All figures are presented as calculated mean +/- standard deviation (SD) of each group. Significance was calculated using a one-way analysis of variance (ANOVA) with post-hoc Tukey Honest analysis. Two-way ANOVA with post-hoc Tukey Honest analysis was used to determine interactions between time and pain sensitivity Von Frey measurements. Significance was set at $p < 0.05$ for all analyses. Data were analyzed with GraphPad Prism software version 6.0 (GraphPad Software Inc., La Jolla, CA).

Results

Exercise Compliance

A 100% exercise compliance was maintained throughout the course of the 3-week exercise treatment. All 16 animals selected to receive the exercise treatment completed the 30 minutes of daily walking, 5 days of the week for 3 weeks.

Representative Images of DRG IHC

Representative images from all groups demonstrated expression of CGRP (red coloring) and TRPV1 (green coloring) at varying brightness levels (Sup. Fig. 2). Nuclei were visualized with DAPI (blue coloring) (Figs. 3, 5, 7, 9). Negative control images (Sup. Fig. 1) (images that were incubated with the secondary antibody but not the primary antibody) showed much lower intensity levels, suggesting the absence of secondary antibody cross reactions in the

representative images. Representative images showed circular areas of high expression of both CGRP and TRPV1 (Sup. Fig. 1). Figures visualizing CGRP expression showed small, high intensity blue regions of DNA throughout the tissue and larger, low intensity blue areas in the center of circular regions expressing CGRP. These larger, low intensity blue areas represented the nuclei of the cell bodies in the DRG. The larger circular areas surrounding the smaller blue circles are indicative of the neuronal cell bodies indicating successful isolation of the DRG tissues. Many of the images also depicted low intensity, striated regions. These regions represent white matter tracts that travel though the DRGs.

Quantification of DRG IHC

Expression of CGRP and TRPV1 were quantified across all groups in DRGs from the left lumbar regions and left thoracic region. CGRP expression in DRGs from the left lumbar region (Fig. 9a) did not vary significantly between all four experimental groups. TRPV1 expression in DRGs from the left lumbar region (Fig. 9b) also did not vary significantly between the experimental groups.

CGRP expression within DRGs from the left thoracic region (Fig. 9c) were significantly increased ($p < 0.05$) in the MMT animals compared to the MMT w/ exercise (MMT Ex) group. There were no significant differences between any other experimental groups. TRPV1 expression in DRGs from the left thoracic region (Fig. 9d), was significantly larger expression ($p < 0.01$) in the sham group compared to the MMT and MMT Ex group.

Von Frey Measurements of Pain Sensitivity

50% withdrawal threshold was measured in animals at baseline (pre-surgery) and 2-, 4-, and 5- weeks post-surgery. Administration of exercise treatment in exercise groups began 3-weeks post-surgery. There was a significant interaction between time and 50% withdrawal

threshold with week 5 having an overall lower withdrawal threshold compared to other time points. Within the 5-week time point, animals in the MMT + Exercise experimental group demonstrated a significantly lower withdrawal threshold compared to the non-exercise groups (Sham and MMT), represented by horizontal black lines.

Discussion

This study aimed to determine the impact of exercise in alleviating neurobiological change in the DRG due to the progression of OA. DRGs were successfully isolated and sectioned, and I was able to clearly distinguish neuronal cell bodies as bright circles with dark centers. These dark centers were dimly stained blue in sections with DAPI indicating the nuclei of these cell bodies. Previous studies that have sectioned DRGs have also observed these morphologies support descriptions (Haberberger et al., 2019). In DAPI stained sections, bright smaller nuclei were present through the tissue indicating nuclei of accessory cells in the DRGs. Non-neuronal cell types in the DRGs mostly include satellite cells that envelope the DRGs, endothelial cells, fibroblasts and various immune cells (Haberberger et al., 2019). Additionally, I confirm the presence of TRPV1 and CGRP in DRGs from both lumbar and thoracic regions of the rat spinal columns.

There were no significant differences in CGRP or TRPV1 expression in the DRGs isolated from the left lumbar region of the animals across all groups, contrary to my hypothesis. Though previous studies have shown significant increases in CGRP and TRPV1 expression OA animal models (Bullock et al., 2014; Fernihough et al., 2005; Miyamoto et al., 2017), there are conflicting reports and other studies have reported opposite findings as well. One study cited no changes in TRPV1 expression in the DRGs from the left lumbar region in an OA animal model and another cited decreased expression of CGRP RNA in the DRGs from the left lumbar region

in an OA animal model (Bar, 2004; Im, 2010). A key difference between our study and previously published studies, is that most previous studies utilized an monoiodoacetate (MIA) chemically induced inflammatory arthritis model, a common AIA model. Many differences exist between AIA and PTOA models, such as the MMT utilized in this study. MIA is a toxic chemical the causes chondrocyte death when injected into the joint space and produces rapid, widespread cell death and apoptosis. This results in phenotypes that that mimic the end stage OA structural damage and inflammation, but do not replicate the slow progression of OA and the accompanying pain development that is characteristic of the disease (Bapat et al., 2018; Pitcher et al., 2016). Though certain aspects of OA induction between these two OA models are similar, differences in sensory and sensitization pathways differ and should be taken into considered (Brederson et al., 2018). Though we see the development of distinct characteristics of early-stage OA at 3-weeks, more phenotypes characteristic of moderate clinical OA are seen at 6 and severe morphological phenotypes are seen at 12 weeks (Doan et al., 2021). Joint degradation, pain sensitization and inflammation develop differently in PTOA models compared to AIA models that induce late stage, severe phenotypes more rapidly (Ferland et al., 2011; Pitcher et al., 2016; TenBroek et al., 2016). Dramatic neurobiological changes might not always be apparent as quickly as they are in AIA models. Thus, it is possible that the time point chosen in this study was insufficient to determine the full extent of neurobiological changes in the DRG resultant of OA. A 12-week study would potentially allow us to capture the extent of these OA induced neurobiological changes. One study did look to measure CGRP in a PTOA model and saw significant increases in CGRP expression, but at the level of the spinal cord and not in DRGs at 4 weeks post-surgery (Ferland et al., 2011). Ferland et al. also processed the tissue allowing for the collection of whole protein content as opposed to staining sections of the DRGs, potentially

counting for the conflicting results seen between ours and this study. Future studies with longer timepoints for severe OA to develop may be necessary to fully characterize the cellular impacts of PTOA in the DRG for these types of animal models.

Though significant changes were not seen in DRGs from the left lumber region of the animals, we observed a significant increase in CGRP expression in the DRGs from the thoracic region of MMT animals. This CGRP expression was significantly lower in MMT animals that received the exercise treatment. This data suggests that though we do not see direct sensitization in the neuronal regions impacted by OA in our animal model, more global mechanisms might potentially be at play. It is possible that the MMT model can initiate processes that relate to central sensitization and the spreading of pain. Central sensitization also can contribute to widespread pain (WSP) and chronic widespread pain (CWP), defined as pain occurring centrally in the spine or in other regions of the body other than the affected joints (Don L Goldenberg, 2020; Guérard et al., 2020). Previous studies have demonstrated these phenomena of central sensitization and WSP pain in rodent models, demonstrating that MIA knee-joint injection can lead to increased expression of Fos, a cell activity marker (Havelin et al., 2016), increased MMP activity and scar formation, known as astrogliosis, in the spinal cord (Burston et al., 2013). Another study found MIA injection into the temporomandibular joint led to tactile hypersensitivity in the forepaw and hind paw (Sannajust et al., 2019). Uniquely in this study, I found evidence indicating that our PTOA model could lead to increased neurobiological markers of central sensitization as indicated by increased CGRP expression in the DRGs from the left thoracic region of MMT animals. In humans, central sensitization is associated with increased serologic biomarkers of OA (Arendt-Nielsen et al., 2014)). Additionally, increased pain sensitization, measured via pressure pain thresholds and temporal summations, correlates

significantly with WSP in patients with knee OA, demonstrating the global impact of OA (Carlesso et al., 2017; Guérard et al., 2020). A widespread impact of OA demonstrates the need for therapies that can exert an effect not just on the local region directly affected by OA, but also the body as a whole.

TRPV1 expression in the DRGs from the left thoracic region was decreased in both MMT groups compared to sham animals. These data are potentially contradictory to the general consensus of a role of TRPV1 in facilitating sensitization of pain due to peripheral damage and inflammation (Amadesi et al., 2006; Fernihough et al., 2005; Ji et al., n.d.; Sugiura et al., 2002). Peripheral delivery of capsaicin can desensitize TRPV1 receptors, but there is no evidence that this can lead to decreased expression these receptors (Wang, 2008). More complex processes other than central sensitization, therefore, may be at play in a complex condition such as OA. More research will be required to fully understand why a decrease in TRPV1 expression is being observed due to PTOA induction in regions non-adjacent to the affected joint.

In addition to demonstrating potential markers of central sensitization, we demonstrated that exercise mitigated the OA induced increase in CGRP expression in the left thoracic DRGs. Exercise in previous studies can mitigate local pain stemming from tissue damage and inflammation (Henriksen et al., 2014; Lee et al., 2018). Exercise can also relieve osteoarthritic and inflammatory pain in patients with central sensitization by increasing endogenous analgesia through activating inhibitory projections descending from the periaqueductal gray (PAG) (Ellingson et al., 2016; Lluch Girbés et al., 2013). Previous studies have shown similar results of exercise mitigating WSP in rodent models (Sharma et al., 2010). In our studies, we were able to demonstrate that exercise can act on regions not directly affected by OA and produce mitigative affects by reducing neurobiological markers of central sensitization.

In order to connect neurobiological observations of central sensitization to pain sensitization, we measured secondary allodynia in animals with Von Frey assays. After 5 weeks of OA induction, all groups showed increased pain sensitivity in the hind paw. Additionally, animals that received the exercise treatment demonstrated significantly greater sensitivity to innocuous stimuli. However, these results may be attributed more to exercise induced increased reflexivity in these animals as opposed to increased pain perception in these animals (Cobos et al., 2012). To better quantify pain sensitivity, other proxies measuring heat sensitivity, such as the Paw withdrawal test, or behavioral measures, such as the facial grimace scale, will have to be explored and tested in our PTOA model (Turner et al., 2019).

Certain limitations of this project should be taken into consideration. The number of steps and the time duration needed to process the tissues from was not ideal and potentially lead to loss of protein expression in the samples. Tissue disection procedures to isolate DRGs were also not always precise leading to damage in certain samples, inclusion of white matter tracts and large variations in the size of the samples. Additionally, though the pooling of samples from each of the spinal regions was necessary for logistical reasons, being able to account for each individual DRG position would have potentially provided useful insights. The use of representative images from IHC, as opposed to quantifying protein expression from the entire tissue also increases the probability that certain samples were misrepresented. Finally, the relatively low power of this study could have prevented certain statistical differences between groups.

Future steps for this project will involve tuning standardizing DRG isolation procedures to limit large variations between samokes ensure correct data interpretation. Though many papers studying the impact of knee OA on DRGs isolate DRGs from the L1-L6 spinal columns, 49% of the neuronal population that innervate the knee reside solely in the L4 DRG (Fernihough

et al., 2005). Continuations of this project will focus on this single DRG as opposed to pooling those from the entire region. Additionally, more quantitative techniques that collect whole protein content will be utilized to measure protein expression in order to reduce inter- and intrasample variability. Additionally, inflammatory markers in the DRG such as the presence of CD86+ macrophages will be observed in order to better connect pain sensitization, local and global inflammation, and neurobiological changes in our PTOA model.

The biological mechanisms that reside in the DRGs can initiate the transition between peripheral pain and chronic pain. DRGs are integral in connecting the peripheral and central nervous system and act as the gateways to central sensitization. Changes in this region due to changes in peripheral input and protein expression can act as an amplifier for all pain signals that pass through it into the dorsal horn neurons in the spinal cord, even when peripheral inflammation subsides and damage resolves (Aranda-Villalobos et al., 2013; Im et al., 2010). These cellular changes can impact pain perception in the brain which in turn can further exacerbate hyperalgesia and allodynia, increase receptive fields of the effected DRGs and also decrease descending inhibitory input from the brain (Eitner et al., 2017; Thakur et al., 2014). Together, these processes lead to central sensitization, a fundamental change in how the body perceives pain and open the doors to the development of chronic pain. I was able to demonstrate the potential ability of exercise to mitigate these impacts in the context of centralized neurobiological changes. Being able to target these mechanisms at the level of the DRG, such as through exercise therapy for OA, can pave the way for therapeutics that act upon biochemical sensitization processes in the DRG. With a better understanding of how exercise modulates the changes we observed, we will be better equipped to manage the local and global symptoms associated with OA.

Figures

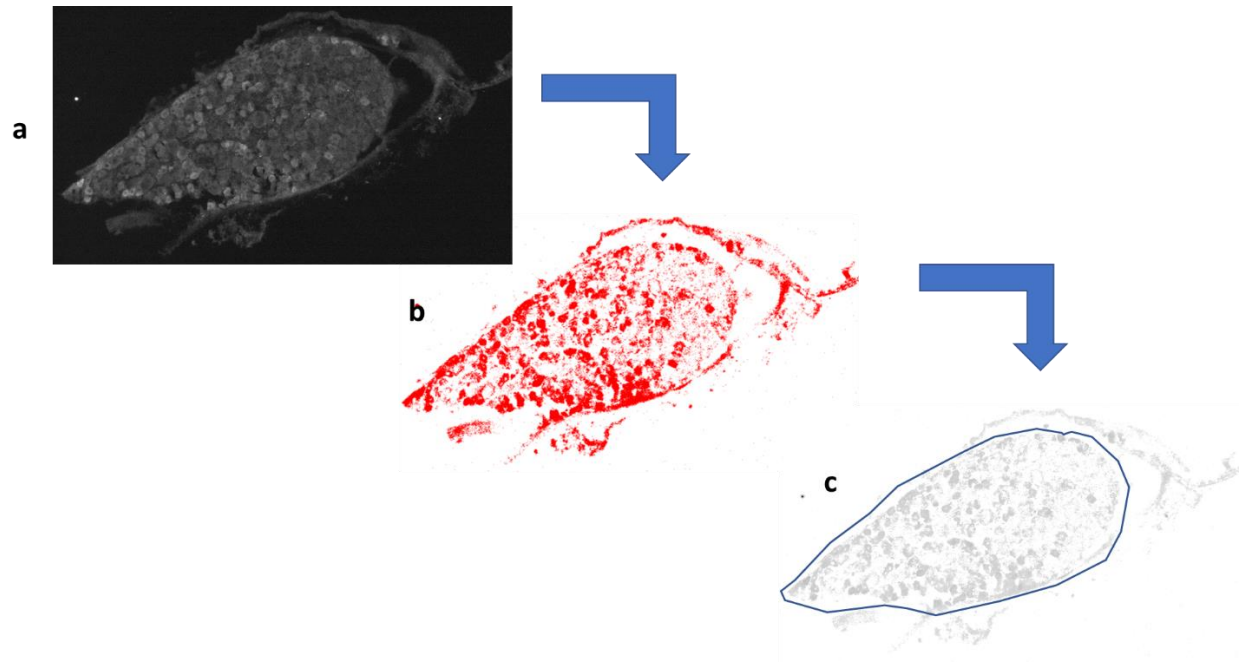


Figure 1: Image processing (a) Raw gray scale images were exported from the fluorescence microscope. (b) Background subtraction and thresholding were applied as previously described in the methods section to create an image mask (c) The mask is applied to the original image and an ROI is manually applied to isolate cell bodies of the DRG

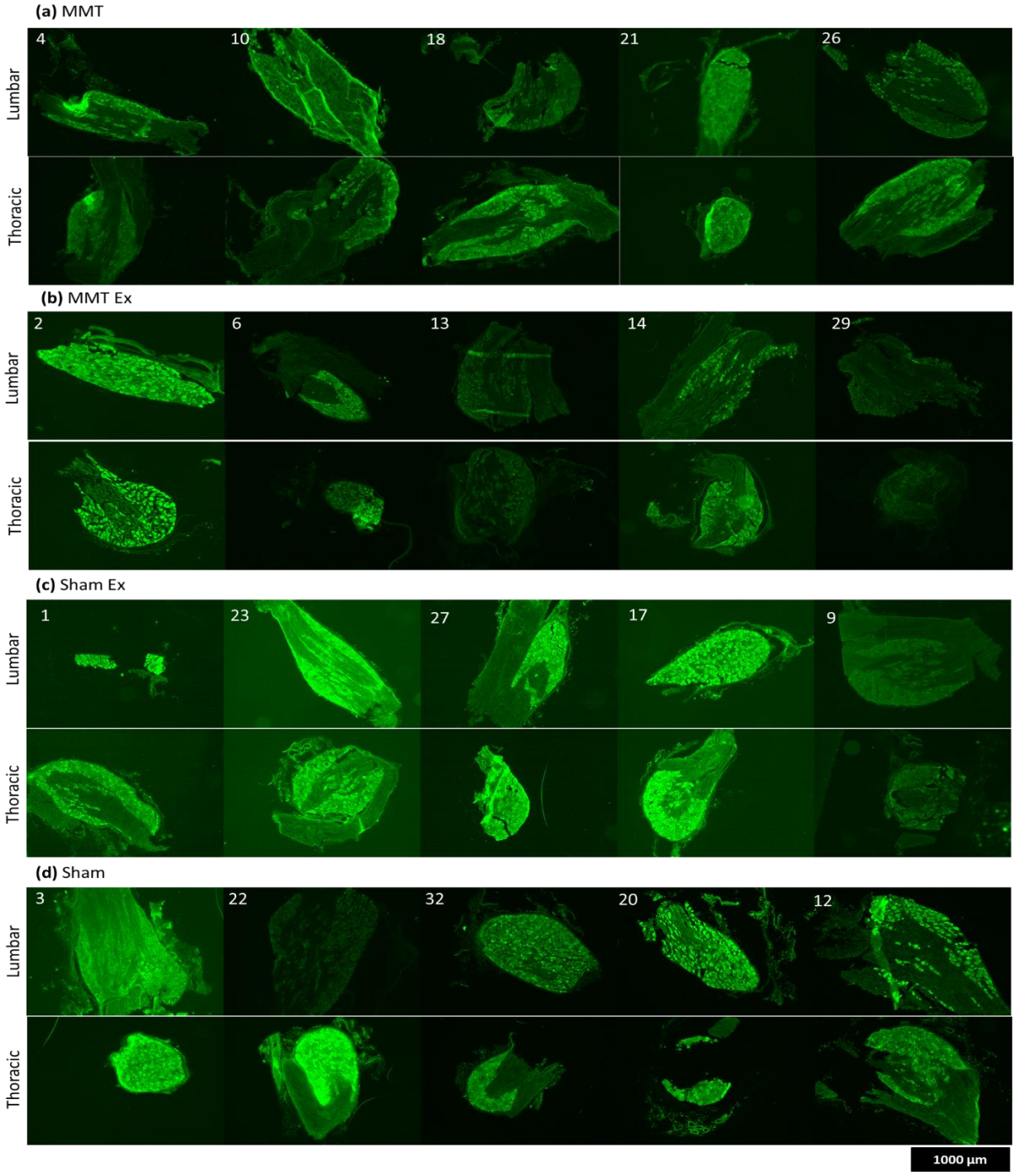


Figure 2: Representative images of TRPV1 expression in DRGs A single representative image was selected from each group of 3 images that were used to quantify a single data point. Numbers in the top left corner of each image indicate the animal number from which the DRG was collected. Circular staining patterns of green coloring (TRPV1) indicate the presence of successful DRG isolation in (a) MMT (b) MMT animals that received exercise treatment (MMT

Ex) (c) sham animals that received exercise treatment (sham ex) and (d) sham animals. Dimmer green regions represent featured of the DRG tissue other than the cell bodies such as white matter tracts and connective tissue. Black scale bar (1000 μm) in the bottom right is representative of all images.

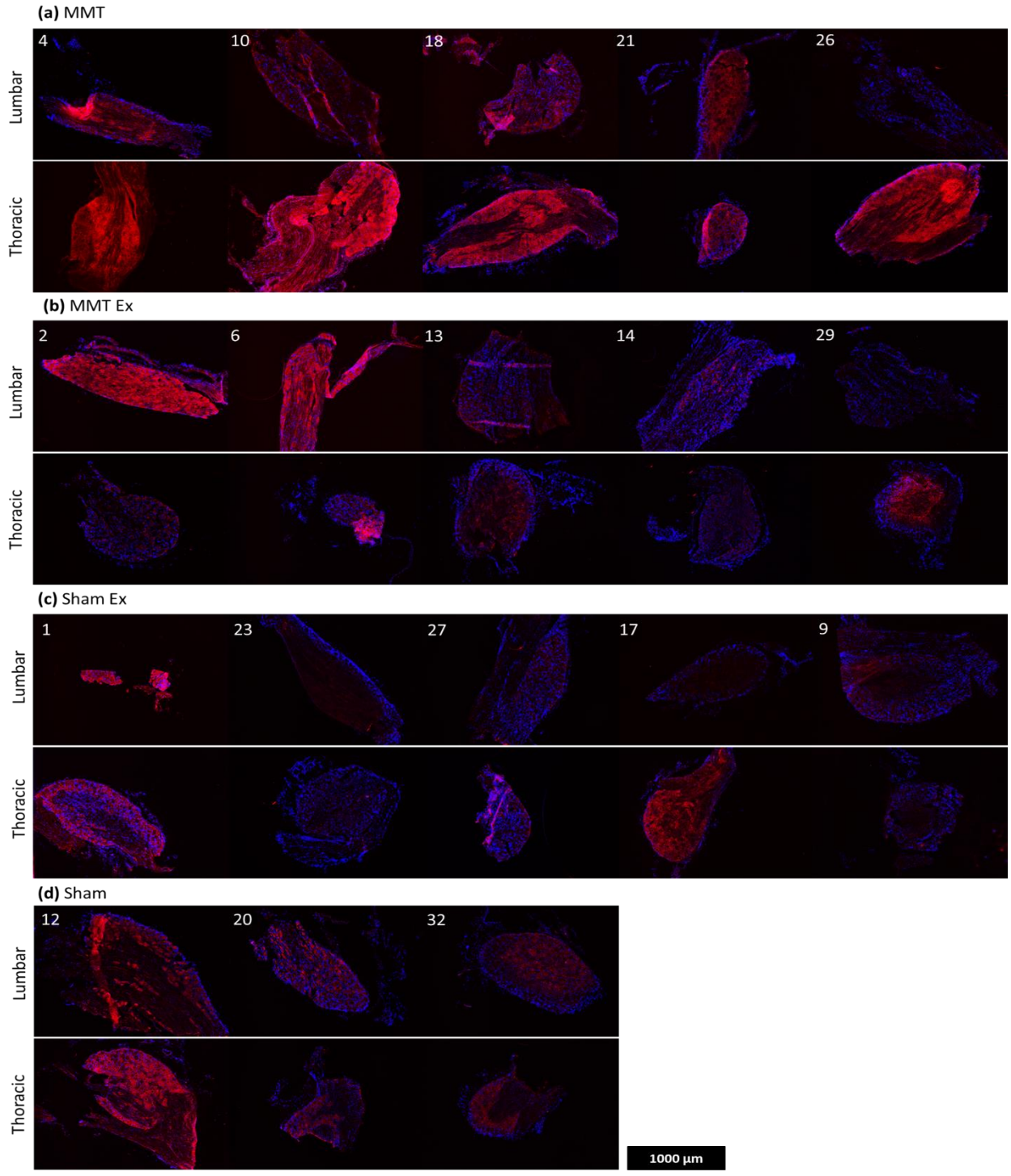


Figure 3: Representative images of CGRP expression with DAPI in DRGs A single representative image was selected from each group of 3 images that were used to quantify a single data point. Numbers in the top left corner of each image indicate the animal number from which the DRG was collected. Circular staining patterns of red coloring (CGRP) indicate the presence of successful DRG isolation in (a) MMT (b) MMT animals that received exercise treatment (MMT Ex) (c) sham animals that received exercise treatment (sham ex) and (d) sham

animals. Bright blue points visualize the nuclei of accessory cells including satellite cells along the outer edges of the tissue, fibroblasts, and various immune cells in all experimental groups (a-d). Dimmer red regions represent featured of the DRG tissue other than the cell bodies such as white matter tracts and connective tissue. Black scale bar (1000 µm) in the bottom right is representative of all images.

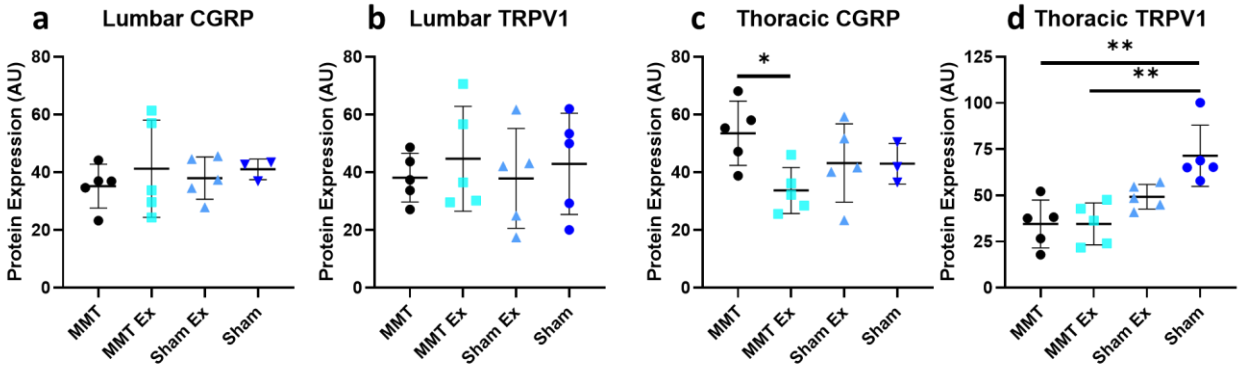


Figure 4: Quantitative analysis of TRPV1 and CGRP expression Three representative images were selected from each animal and protein expression from these images was averaged to create a single data point. Five animals from each group were randomly selected to be included in this analysis. No significant differences were observed between experimental groups in (a) left lumbar DRG CGRP expression and (b) left lumbar DRG TRPV1 expression. In the (c) left thoracic DRG CGRP expression, a significant difference was observed between MMT animals and MMT animals that received the exercise treatment (MMT Ex). In (d) left thoracic DRG TRPV1 expression, a significant difference was observed between Sham animals and both MMT experimental groups (MMT and MMT Ex). Data shown as mean +/- SD. n = 5 for all experimental groups except for sham CGRP expression in DRGs from left lumbar and thoracic regions (where n = 3) *$p < 0.05$; **$p \leq 0.01$.

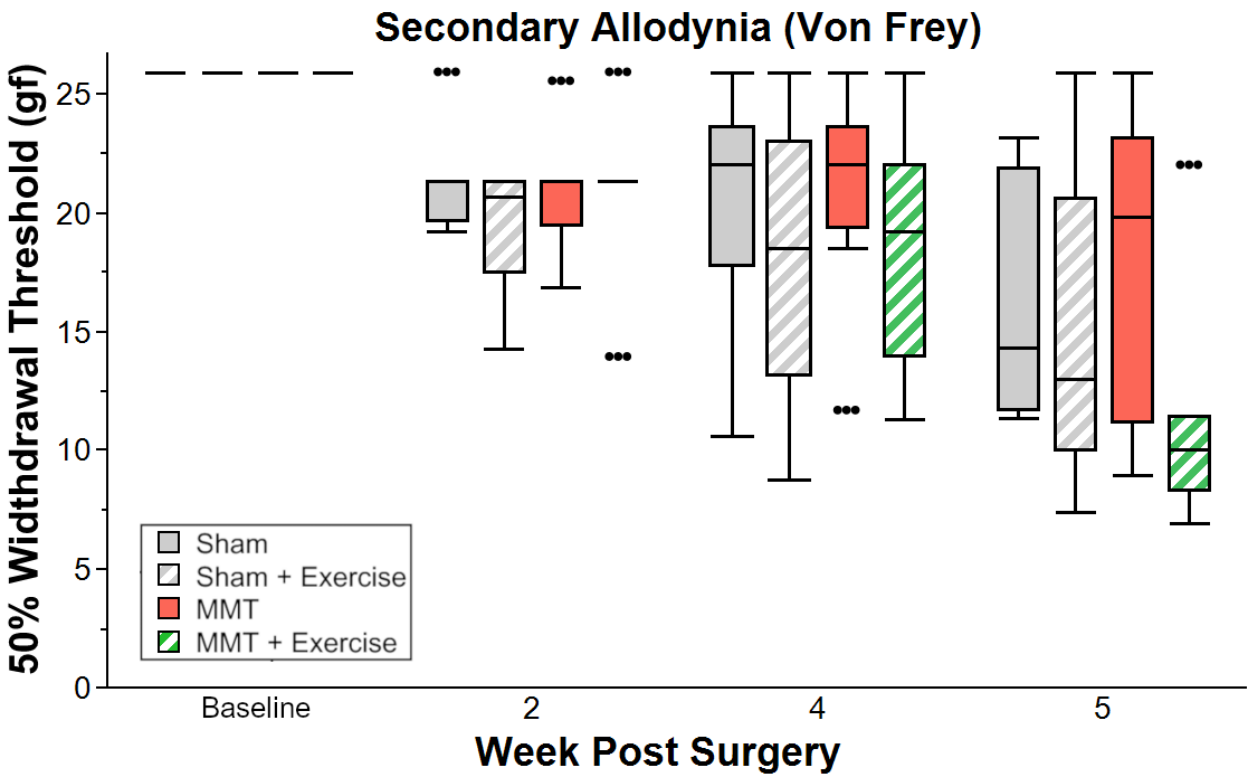
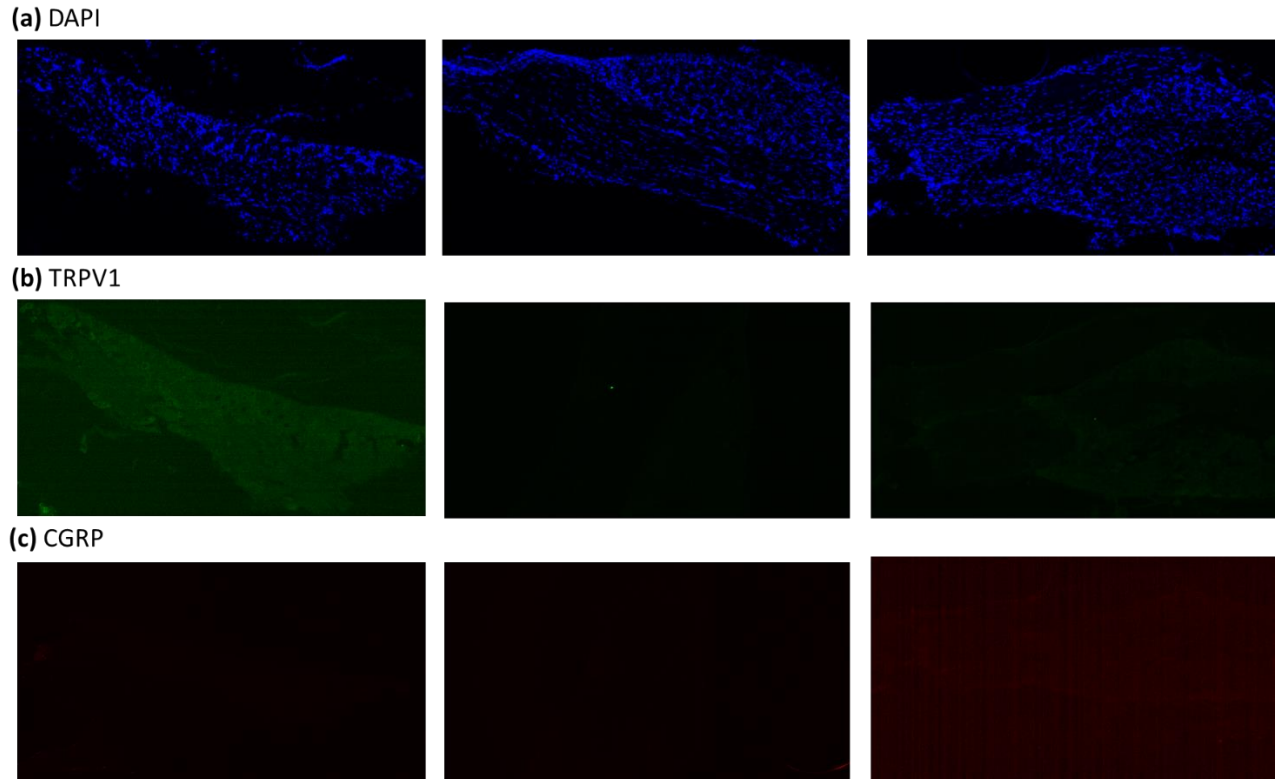
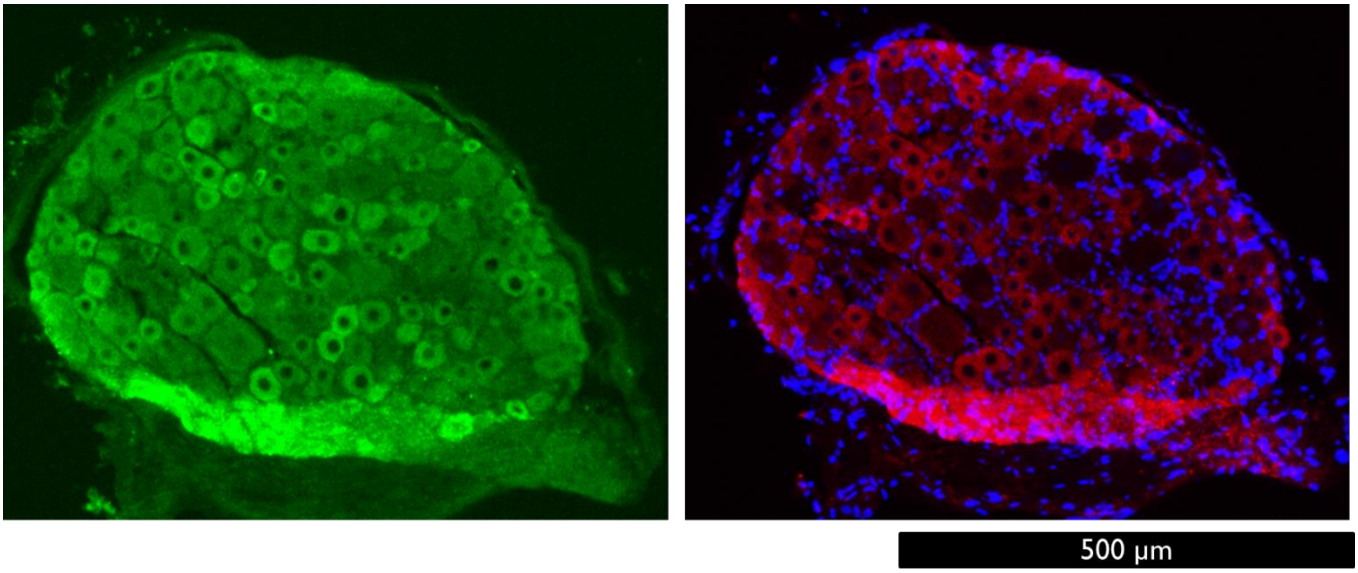


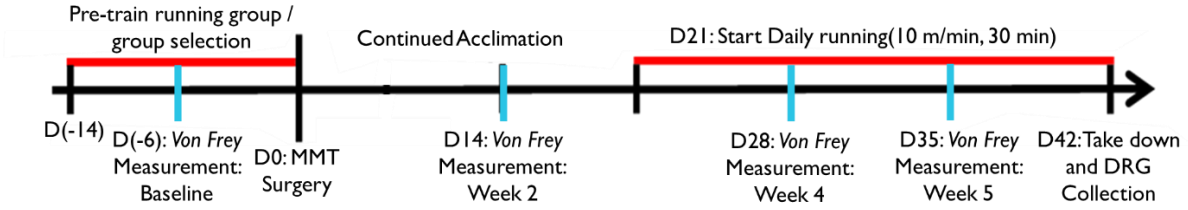
Figure 5: Von Frey measurements of Secondary Allodynia 50% withdrawal threshold was measured in animals at baseline (pre-surgery) and 2-, 4-, and 5- weeks post-surgery. Administration of exercise treatment in exercise groups began 3-weeks post-surgery. Asterisk on the X axis represent significant differences between time points. Horizontal bars represent significant within timepoint group differences. Data represented as box and whisker plots. n = 5 for all experimental groups. *$p < 0.05$.



Supplemental Figure 1: Negative Control Images Negative control sections were created by not exposing sections to primary antibody. The presence of blue coloration in the (a) DAPI images demonstrates the presence of isolated tissues. The significant reduction in brightness of green in (b) TRPV1 images and red in (c) CGRP images indicates the absence of secondary antibody cross reactions. The absence of circular patterns seen in Fig. 2 and Fig. 3 indicate that the dim green fluoresce in the (b) TRPV1 images is not bona fide and most likely due to low levels of autofluorescence of the tissues.



Supplemental Figure 2: DRG Morphology Circular staining patterns of green coloring (TRPV1) and red coloring (CGRP) indicate the presence of successful DRG isolation. Bright blue points visualize the nuclei of accessory cells including satellite cells along the outer edges of the tissue, fibroblasts, and various immune cells in all experimental groups.



Supplemental Figure 3: Experimental Timeline

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