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Towards a Better Understanding of Temporomandibular Disorder

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TOWARDS A BETTER UNDERSTANDING OF

TEMPOROMANDIBULAR DISORDER

A Master's Thesis

Submitted to the Graduate College

Of Missouri State University

In Partial Fulfillment

of the Requirement for the Degree

Master of Science, Biology

By

Jessica R. Cox

May 2019

TOWARDS A BETTER UNDERSTANDING OF TEMPOROMANDIBULAR

DISORDER

Biology

Missouri State University, May 2019

Master of Science

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ABSTRACT

Results from the OPPERA study provided evidence that risk factors such as neck muscle tension, prolonged jaw opening, and female gender increase the likelihood of developing temporomandibular joint disorders (TMJD), which are prevalent, debilitating orofacial pain conditions. Peripheral and central sensitization, which mediate a lowering of the stimulus required for pain signaling, are implicated in the underlying pathology of chronic TMJD. The goal of my study was to investigate cellular changes in the expression of proteins associated with the development of central sensitization. Female Sprague-Dawley rats were injected with complete Freund's adjuvant in the upper trapezius muscles to promote trigeminal sensitization. After 8 days, animals were subjected to near maximal jaw opening for 20 minutes, and spinal cord tissues were collected at several time points until day 28 post jaw opening. Changes in proteins associated with neuronal and glial cell activation were investigated in the medullary dorsal horn using immunohistochemistry. Somewhat surprisingly, consistently increased protein expression was not observed in second-order nociceptive neurons, astrocytes, or microglia in the dorsal horn. Thus, my results are suggestive that this novel model for inducing chronic TMJD pathology is mechanistically different from other reported inflammatory-induced TMJD models. Based on my results, I propose that this model that involves pain signaling in response to prolonged jaw opening in sensitized animals involves dysfunction of descending inhibitory signaling and likely involves changes in the expression of cytokines and miRNAs.

KEYWORDS: temporomandibular joint disorder, trigeminal nerve, peripheral sensitization, central sensitization, risk factors, inflammatory mediators

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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Finally, I would like to thank my parents, Jim and Theresa, for their love, support, guidance and encouragement throughout my entire life. I am thankful for each opportunity you both have guided and encouraged me through. Both of you have been very patient, understanding, and supportive throughout my collegiate years.

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<center>**INTRODUCTION**</center>

Temporomandibular Joint Disorder

Temporomandibular joint disorders (TMD) are common orofacial pain conditions that can affect the temporomandibular joint (TMJ) or jaw joint and muscles of mastication.[46] TMD is the most prevalent type of orofacial pain and occurs in approximately 10% of the population over age of 18.[44, 67] There is a higher prevalence of TMD associated with the female gender with increased prevalence in ages between 20 and 40, which corresponds to the time of childbearing and career development.[47, 67] The common TMD symptoms experienced by millions of American adults include jaw pain/tenderness after or during mastication, jaw clicking, limited jaw movement, tinnitus, and an increased number of headaches or migraine. [5]

Risk Factors Associated with TMD

TMD is often seen as a comorbid condition with other types of chronic pain including headaches and migraine, and neck and shoulder pain as well as psychological complaints such as depression and anxiety.[74] Results from the Orofacial Pain Prospective Evaluation and Risk Assessment (OPPERA) Study provide evidence that prolonged jaw opening, neck muscle tenderness, and female gender are commonly reported risk factors associated with the development of chronic TMD.[26] While genetic factors have been proposed to play a role in the etiology of chronic pain conditions, only a few genes with low correlative values have been associated with development of chronic TMD.[68] One interpretation of these results is that TMD is a complex polygenetic disease that involves not only genes involved in pain signaling but likely genes that regulate the structure and function of key anatomical elements involved in

<center>1</center>

mastication including the jaw joint and associated muscles as well as biopsychosocial behaviors. While it has been shown that there is a low genetic predisposition present in TMD patients, it is now thought that progression to chronic TMD is dependent to a large degree on epigenetic changes that occur in response to one's lifestyle and environment.[22, 70] Some of the most common causes of TMD is direct trauma to the head during sporting activities or motor vehicle accidents and repeated near maximal jaw opening as occurs during yawning or singing.[54, 67] However, a routine visit to the dentist or orthodontist in which a patient is forced to hold open their jaw to near maximum for a prolonged duration should be considered a risk factor of development of TMD. Prolonged jaw opening is common during procedures involving molar extractions, root canals, and orthodontics. Given that on average females are not able to open their mouth as wide as males and their masticatory ligaments and tendons are not as thick, there is an increased risk of injury to the TMJ and associated structures in women. Interestingly, neck muscle tenderness, a commonly reported condition in women, is considered a risk factor for the development of chronic TMD since these muscles can affect the functioning of the masticatory muscles.[41, 75, 76] Neck muscle tension and tenderness that may exhibit multiple taut bands can occur from a multitude of factors including whiplash, improper sleep position, sports injury, overuse, improper positioning of the neck throughout the day or while sleeping, or unmanaged stress. Pain and stiffness in shoulder and neck muscles, especially the upper trapezius, is often cited as a co-morbid condition with TMD patients.

Trigeminal Nerve

Similar to other orofacial pain conditions such as migraine and chronic rhinosinusitis, TMD involves sensitization and activation of the trigeminal nerve.[11] The trigeminal nerve is the fifth cranial nerve and serves to provide sensory innervation of structures throughout the head

and face and provides a pathway for pain transmission from peripheral tissues to the spinal cord.[63-66] The nerve is made up of three branches of which the first two branches the ophthalmic (V1) and maxillary (V2) only are comprised of sensory afferent neurons that respond to thermal, mechanical, and chemical stimuli. While sensitization and activation of V1 and V2 branches are implicated in headache disorders, trigeminal neuralgia, and rhinosinusitis, the underlying pathology associated with TMD primarily involves activation of the third branch, which is referred to as the mandibular (V3) that possesses both sensory and motor neurons.[61] The pain and inflammation associated with TMD involves activation of primary V3 trigeminal ganglion nerves that function to relay nociceptive signals from peripheral tissues such as the TMJ and associated muscles of mastication (masseter) to the upper cervical spinal cord.[1, 6, 13] The nociceptive signal is transferred to second-order neurons in the medullary dorsal horn and then via the ascending pain pathway is transmitted to the thalamus and on to higher brain structures including the cortex for processing and coordinating a protective response to minimize further tissue damage and initiate repair mechanisms.[57, 61]

Trigeminal System

The trigeminal system consists of the trigeminal ganglion, which is part of the peripheral nervous system, and the trigeminocervical complex located in the central nervous system.[4] There are two main cell types that comprise the trigeminal system including neuronal cells that are responsible for conduction of sensory information from the peripheral tissues to the spinal cord and the associated glial cells that function to regulate the excitability state of the neurons.[20] The sensory fibers of the primary trigeminal nerves transmit mechanical, thermal, and chemical information to the cell bodies located in the trigeminal ganglion. The pseudounipolar

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morphology of the neurons within the ganglion can be differentiated into 2 types including large light and small dark neurons. While the large light neurons are myelinated, fast transducing Aδ fibers that provide rapid reflexive response to harmful stimuli, the small dark neurons consist of C fibers that are unmyelinated and thus have slower transduction but are responsible for long-term sensations and mediating prolonged inflammatory responses. Another functional aspect of the ganglion is the supporting glial cells such as the Schwann cells that are associated with Aδ fibers and are responsible for the production of the myelin sheath that promotes faster conduction within the peripheral nervous system. Another prominent glial cell in the ganglion is the satellite glial cell, which surrounds the cell body of both Aδ and C neurons, and as such form a functional unit.[21, 23] Satellite glial cells help to maintain homeostasis by controlling the level of ions and neurotransmitters in the extracellular environment around the neuronal cell body by their selective uptake and thus regulating the excitability state of both types of neurons.[18, 28] Increased communication between neuronal cell bodies and satellite glial cells via gap junctions and paracrine signaling is associated with more chronic pain states as characteristic of most orofacial disorders involving trigeminal nerve activation including TMD. The central nervous system component of the trigeminal system consists of the second order neurons and the two main types of glial cells, which include astrocytes and microglia that are responsible for modulating the excitability level of spinal neurons.[16, 50, 81] Similar to signaling in the trigeminal ganglion, activation of astrocytes and microglia that facilitate neuron-glia communication is implicated in the underlying pathology of chronic pain conditions by promoting and maintaining a heightened state of sensitivity referred to as central sensitization.[14, 37, 56, 80]

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TMJD Pathology

Following an injury to the jaw joint, inflammatory signals are released that activate primary afferent nerves of the trigeminal system, which can lead to the development of a sensitized state within the peripheral tissue. During the sensitized state, neuronal cells can produce an increased pain response (hyperalgesia) to a normal stimulus but will result in a nociceptive response to sub-threshold, non-painful stimuli (allodynia) such as brushing one's teeth or chewing. These pathophysiological phenomena are mediated via cellular changes that lower the amount of stimuli required to activate the nociceptive neurons. Sensitization involves changes in the excitability of neurons by lowering the activation threshold to mechanical, thermal, or chemical stimuli through molecular changes promoted by persistent nociceptive input. This established sensitized state, which is maintained by release of pro-inflammatory cytokines and chemokines from activated glial cells can lead to a persistent (primed) state of hypersensitivity or hyperexcitability of trigeminal neurons.[34, 42]

During states of hypersensitivity, there is a reduced electric threshold of activation required to open ion channels, delayed opening of inhibitory channels, or prolonged the opening of excitatory channels. Prolonged peripheral sensitization can lead to central sensitization that has the potential to become a chronic pathological condition long after the initiating event. Constant input from the sensitized primary afferents leads to nociceptive neuronal hyperexcitability within the medullary horn of the trigeminal nucleus caudalis.[56] A prolonged state of central sensitization is associated with pathological conditions characteristic of TMD. Unfortunately, in some susceptible individuals, peripheral sensitization promotes and sustains central sensitization that drives sensitization of higher-order neurons within the thalamus and development of a chronic pain state that is no longer physiological but rather pathophysiological

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in nature. Thus, while acute inflammation and sensitization of the trigeminal system are necessary to restore homeostasis following injury to the TMJ or associated masticatory structures, prolonged sensitization can lead to changes in pain processing pathways that are no longer associated with healing. During these chronic pain conditions, there is a heightened nocifensive response in the absence of peripheral inflammation.

In response to peripheral activation of trigeminal nerves the neuropeptide calcitonin gene-related peptide (CGRP) and other inflammatory mediators facilitate excitation of second-order neurons and glial cells involved in central sensitization and persistent pain.[12, 62] Elevated CGRP levels in the spinal cord are known to promote the development of central sensitization due to changes in ion channels, receptors, and inflammatory genes in second-order neurons and glial cells.[38] CGRP is involved in the initiation and maintenance of central sensitization by activation of CGRP receptors present on secondary neurons and glial cells within the spinal cord that couple to activation of protein kinase A (PKA). Following activation of CGRP receptors, there is an increase in the secondary messenger cAMP, which then causes activation of the intracellular signaling protein PKA. Persistent elevated active PKA levels leads to a sensitized state of second-order neurons as well as activation of astrocytes and microglia that establish a sustained primed state in which normally non-stimulating levels of neurotransmitters can now cause activation.[71]

The central dogma of acute pain signaling is that peripheral sensitization promotes cellular changes that lead to central sensitization of second-order nociceptive neurons involved in pain transmission to the thalamus.[4, 17, 61] However, in most chronic migraine and TMD patients, the current thought is that central sensitization is maintained without any evidence of peripheral tissue damage or trauma. While peripheral sensitization results from interactions of nociceptors

with inflammatory substances released when a tissue is inflamed or damaged[61], central sensitization can be maintained in the absence of trigeminal nociceptor activation. In support of this notion, findings from our studies have provided evidence of bidirectional signaling within the trigeminal system.[12] In this model, CGRP was injected intracisternally (upper spinal cord) to mimic higher CGRP levels reported in cerebral spinal fluid of chronic migraine patients. Somewhat surprisingly, elevated CGRP levels resulted in cellular changes associated with central sensitization but also mediated increased neuron-glia communication with the ganglion associated with the development and maintenance of peripheral sensitization of nociceptive neurons. The evidence of bidirectional signaling within the trigeminal system may explain how trigeminal peripheral nociceptive neurons become sensitized during chronic orofacial pain conditions many days/weeks after resolution of inflammation. Thus, peripheral nociceptor sensitization can occur even in the absence of any physical trauma or signs of inflammation.

As previously stated, there are several inflammatory molecules involved in the process of initiating and maintaining neuronal sensitization. CGRP, which is expressed in both peripheral and central neurons, is a known prominent inflammatory mediator that functions as a potent vasodilator and potentiates protein extravasation, stimulates the release of cytokines from satellite glial cells, performs autocrine signaling to stimulate synthesis of itself in the trigeminal ganglion neurons, and reduces the threshold for depolarization of nociceptive neurons and glia.[3] Chronic elevation of CGRP peripherally would lead to persistent pain and destruction of the TMJ capsule, which is common in TMD. Within the spinal cord, central sensitization involves increased interactions between neurons and spinal glia cells, specifically astrocytes and microglia. Astrocytes are the most abundant cells within the brain and regulate the neuronal environment by maintaining the blood-brain barrier and modulating ion, fluid, and

neurotransmitter concentrations. Astrocytes can promote and sustain sensitization of central neurons through the release of cytokines, chemokines, and other pro-inflammatory mediators while microglia perform functions similar to peripheral macrophages within the brain and spinal cord.[49] When activated these specialized immune-like cells release inflammatory cytokines and chemokines that can promote and sustain a persistent sensitized or primed state of neurons characteristic of chronic pain conditions. These pro-inflammatory molecules often cause activation of the nuclear transcription factor NF-κB, which binds to the promoter of cytokines and chemokines to enhance their expression and hence, sustain elevated levels.[43] Astrocytes and microglia work together to control the excitability state of secondary nociceptive neurons by maintaining a balance within the extracellular environment around the neurons. Towards this end, these cells are known to express transport proteins for the uptake of potassium ions and glutamate, which are molecules that promote activation of neuronal cells involved in pain transmission.[82] In addition, elevated levels of glial fibrillary associated protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) are associated with activated astrocytes and microglia.[7, 55]

Hypothesis and Goals of Research

Animal models of acute and chronic orofacial pain have been developed that mimic human clinical pain conditions to study the cellular and molecular mechanisms involved in mediating inflammation and pain signaling involving the trigeminal system. Many of these models rely on the injection of inflammatory agents into the joint capsule or masticatory muscles.[8, 9, 23, 24, 40] Thus, these models are mimicking a traumatic peripheral event that elicits an inflammatory reaction to promote tissue injury and minimize jaw activity. Interestingly, almost

all animal studies involving TMD pathology have been performed only in male animals, yet, TMD is more prevalent in females. More recently in our laboratory, we developed a chronic model of TMD pathology based on risk factors known to promote chronic TMD in humans, which included neck muscle tension/inflammation, prolonged jaw opening, and female gender (Figure 1). The model involves neck muscle inflammation prior to prolonged jaw opening. The neck muscle inflammation promotes a sensitized state of trigeminal nociceptors such that prolonged jaw opening results in a sustained increase in trigeminal nociception to mechanical stimulation of the masseter muscle (Figure 2). The goal of my thesis project was to investigate the changes in key signaling proteins in neuronal and glial cells involved in initiating and maintaining the prolonged state of sensitization observed in this novel model of chronic TMD pathology. Based on previous studies in our laboratory, I hypothesized that there would be an increase in the pro-inflammatory proteins within the upper spinal cord to promote central sensitization, and hence lower the activation threshold required for peripheral activation.

To test my hypothesis, I propose the following goals that involve utilizing immunohistochemistry to investigate changes in protein expression in spinal cord tissues in a novel model of chronic TMJD:

1. Determine changes in the levels of CGRP since it is implicated in the establishment of central sensitization.

2. Study expression of PKA, the downstream intracellular pathway activated by CGRP in mediating central sensitization in neurons and glial cells.

3. Determine if astrocytes and microglia are activated in this chronic model.

4. Determine changes in the levels of the transcription factor NF-KB since increased expression of this protein is associated with stimulated release of cytokines and chemokines.
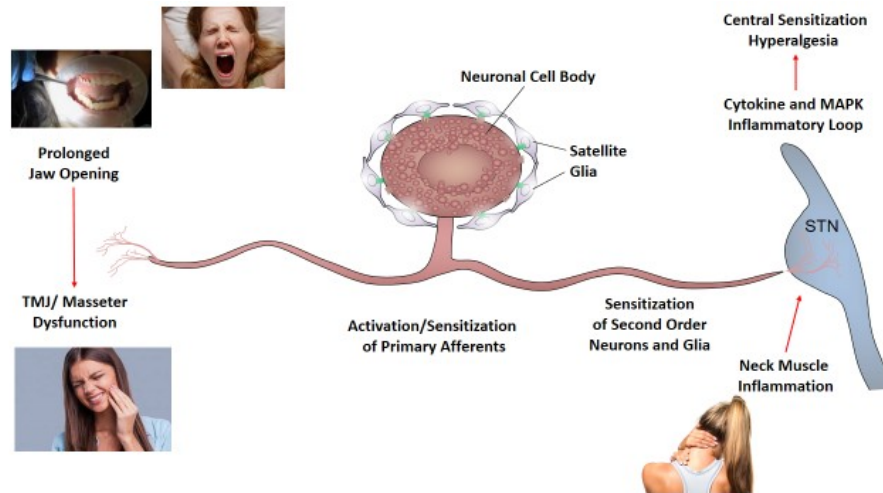
Figure 1 Schematic Representation of a Chronic TMJD Model. See text for more details. Neck muscle inflammation leads to sensitization among the ascending pain signaling pathway causing the brain to detect the painful sensation. A second risk factor (prolonged jaw opening) is introduced leading to more sensitized inflammation which activates the second order neurons and glia cells causing pain signaling along the primary afferent neurons which leads to greater ascending pain modulation and increased sensitization.
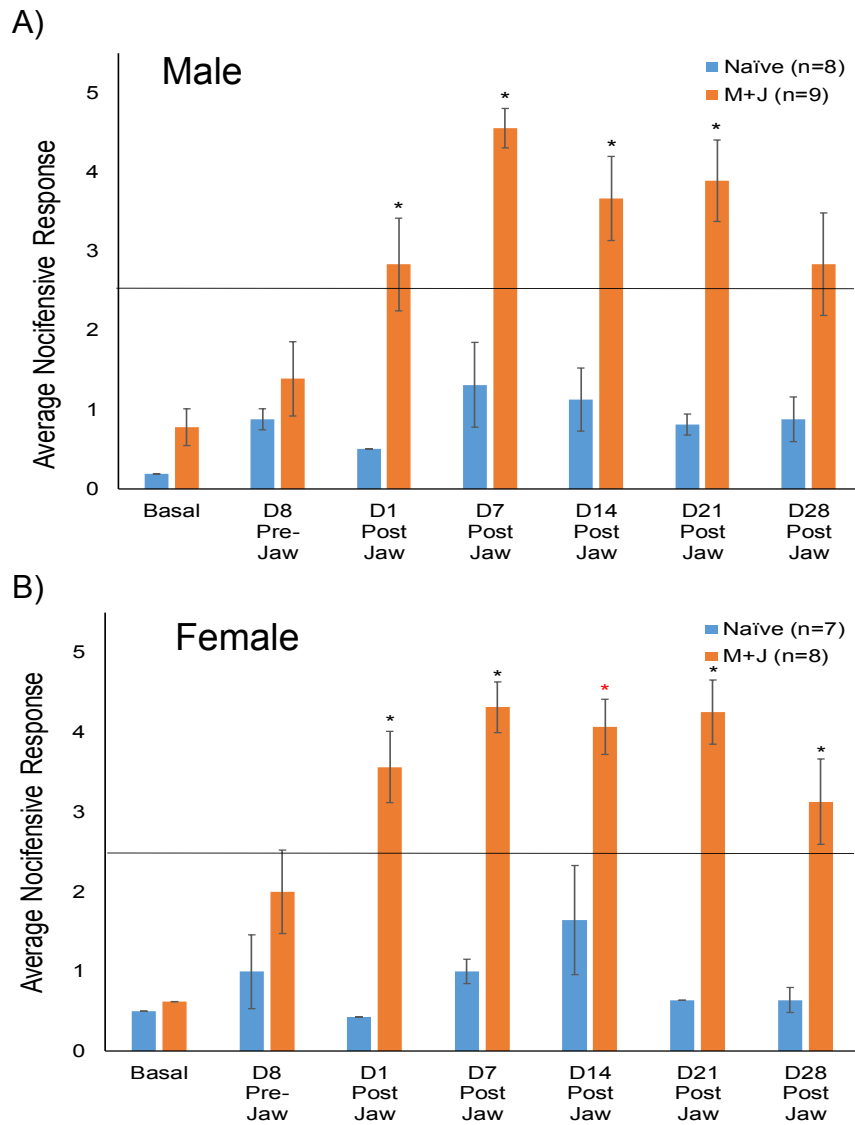
Figure 2 Neck Muscle Pathology and Prolonged Jaw Opening Mediated Sustained Sensitized Masseter Nociceptive State. The average number of head withdrawals in response to mechical stimultion over the masseter muscle. See text for more details. (A) Male nociceptive state. (B) Female nociceptive state.

METHODS

Animals and Reagents

Animal protocols were approved by the Institutional Animal Care and Use Committee at Missouri State University (IACUC ID: 17.001.0) and conducted in compliance with all guidelines established by the National Institutes of Health Animal Welfare Act. Adult female Sprague-Dawley rats (250-300g) were purchased from Charles River Laboratories Inc. (Wilmington, MA) or purchased from Missouri State University (internal breeding colonies). Animals were housed in clean, plastic cages (VWR, West Chester, PA) in an animal holding room maintained at ambient temperature (22-24 C) with access to food and water ad libitum. The holding room was on a 12-hour light/dark cycle starting at 7 A.M. These animals were allowed to acclimate in this environment at least one week before use. Complete Freund's adjuvant (CFA, Sigma Aldrich, St. Louis, MO) was prepared as a 1:1 emulsion in 0.9% saline solution (Fisher-Scientific, Pittsburgh, PA) immediately prior to use.

Model of Chronic TMJD

CFA was administered bilaterally into the upper trapezius muscles of young adult Sprague-Dawley female rats to induce low-grade neck muscle inflammation (Figure 3). Prior to the injection of CFA, animals were anesthetized with 5% isoflurane (Webster Veterinary, Devens, MA) using oxygen as the carrier gas and a Vetequip isoflurane mixing apparatus connected to the animal chamber. Upon completion of anesthetization, the rats were placed ventral surface down and a nose cone apparatus was used to deliver a constant flow of 3% isoflurane. A total of 100 µl CFA was delivered via five 10 µl intramuscular injections in both

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the right and left upper trapezius muscles using a 26 ½ gauge needle (Becton Dickinson, Franklin Lakes, NJ) and a 50 µl Hamilton syringe (Hamilton Company, Reno, NV).

Following the CFA injections, eight days later the animals were subjected to prolonged jaw opening as described previously[30, 31] (Figure 4) to induce injury to the TMJ and associated muscles, ligaments, and tendons anesthetized animals (5% isoflurance vs 3%). Under anesthesia via inhalation of 5% isoflurane, animals were placed dorsal side down and attached to a nose cone apparatus delivering a steady flow of 3% isoflurane. A commercially available mouth retractor (Fine Scientific Tools, Foster City, CA), was positioned around the top and bottom incisors. The retractor arms were separated until near maximal jaw opening was achieved and then maintained for 20 minutes in this position. Using a CaliMax Vernier Caliper (Wiha Tools, Montecillo, MN) the opening measurements were taken from the gingival line on the lingual surface of the upper incisors to the gingival line on the lingual surface of the lower incisor. On average, the maximum opening capacity without subluxation of the joint was approximately 20 mm for adult female Sprague-Dawley rats. Upon placement of the retractor, the animals were returned to the animal chamber and were maintained in an anesthetized state with 3% isoflurane for the duration of the jaw opening procedure. The breathing patterns and body temperatures were monitored throughout the application, and the procedure was aborted if either physiological parameter varied significantly from normal levels. Following mechanical stressing, the retractor was released, removed, and the animal was allowed to recover in its cage prior to further experimentation.

Immunohistochemistry

The methods utilized for immunohistochemistry were similar to prior studies from our

lab.[12, 40] The brain stem and upper spinal cord (6 mm posterior to the obex) were removed at 2 hours, 1 day, 14 days, and 28 days post prolonged jaw opening (Figure 5). The tissues were immediately placed in 4% paraformaldehyde to preserve them for immunohistochemistry.

To investigate cellular changes in protein expression in neuronal and glial cells, immunohistochemical staining of tissues was performed. The tissues were incubated in 4% paraformaldehyde at 4°C overnight then were transferred into 15% sucrose for 1 hour at 4°C or until they sank to the bottom of the centrifuge tube. Following the 15% sucrose incubation tissues were moved to 30% sucrose and incubated at 4°C overnight. The tissues were removed from the sucrose and stored at -20°C until sectioning. Prior to sectioning, tissues were embedded in Optimal Cutting Temperature compound (OCT; Sakura Finetek, Torrance, CA). Transverse sections 14 μm thick were taken between 4 and 5 mm caudal to the obex (about 75 sections from top of tissue) using a cryostat (Richard Allan Scientific Co, San Diego, CA) set to -24°C. Tissue sections were placed on Superfrost Plus microscope slides (Fisher Scientific, Pittsburg, PA) with the caudal side facing down and stored at -20°C.

The slides containing sectioned tissues from naïve and each experimental condition were rehydrated by incubation in 1X Phosphate Buffered Saline (PBS; 10 mM phosphate, 138 mM NaCl, 27 mM KCl) for 5 minutes then blocked and permeabilized in a solution of 0.1% Triton X-100 in 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 20 minutes at room temperature. During this incubation, primary antibodies of interest were prepared according to the manufacturer's recommended dilutions (Table 1) in 5% donkey serum. Following the donkey + triton incubation, slides were thoroughly rinsed with PBS, and the primary antibodies were incubated with tissues for either 3 hours at room temperature or overnight at 4°C in a humidified chamber. After the primary antibody incubation, the slides were

rinsed with PBS and 1% Tween 20. The tissues were then incubated with Alexa Fluor-conjugated secondary antibodies prepared in PBS at a 1:200 dilution for 1 hour at room temperature to visualize immunoreactive proteins. Vectashield medium (H-1200) containing 4', 6- diamidino-2-phenylindole was used to mount the tissue sections and visualize cell nuclei using fluorescent microscopy.

A Zeiss Axiocam mRm camera (Carl Zeiss, Thornwood, NY) mounted on a Zeiss Imager Z1 fluorescent microscope equipped with an ApoTome was used to collect 100X images of the medullary horn of the spinal cord tissue (Figure 6). Once the images were acquired, they were analyzed using Image J software as previously described.[12, 40] Fluorescent intensity was measured in ten rectangular regions of approximately equal area in laminas I-III in the medullary horn. Five random acellular regions outside of the region of interest were measured and the average of these values was subtracted from the medullary horn values to account for background signal. SPSS Statistics 21 software (IBM, North Castle, NY) was used to determine outliers. It was determined using a parametric independent sample T-test that most of the data was normally distributed, so a Shapiro-Wilk test was used followed by a Leavean's test to determine equal or unequal variances. For the data that was not normally distributed a nonparametric test was used to determine statistically significance followed by a Mann-Whitney U post hoc test. All statistical tests was conducted utilizing SPSS Statistics.

Table 1 Summary of Antibodies and Conditions Used for Immunohistochemistry

Protein	Dilution	Incubation time	Incubation Temperature	Company	Catalog number
CGRP	1:1000	3 Hours	20-22°C	Sigma-Aldrich, Inc.	C 8189
GFAP	1:5000	3 Hours	20-22°C	Abcam, Inc.	Ab53554
Iba-1	1:1000	3 Hours	20-22°C	Wako Chemicals USA, Inc.	019 - 19741
NeuN	1:1000	3 Hours	20-22°C	EMD Millipore, Corp.	MAB377
P-ERK	1:500	OVN	4°C	Bioworld Technology, Inc.	BS5016
PKA	1:500	3 Hours	20-22°C	Abcam, Inc.	Ab76238
cFos	1:500	OVN	20-22°C	Abcam, Inc.	Ab190289
CNX43	1:500	OVN	20-22°C	Abcam, Inc.	Ab11370
NF-KB	1:500	OVN	20-22°C	AbCam, Inc.	Ab16502
RAMP1	1:500	OVN	20-22°C	Bioss	Bs-1567R
Alexa-Fluor 488	1:200	1 Hour	20-22°C	JacksonImmuno Research, Inc.	125719
Alexa-Fluor 647	1:200	1 Hour	20-22°C	JacksonImmuno Research, Inc.	122180
DAPI	N/A	N/A	N/A	Vector Laboratories	H-1200

A)
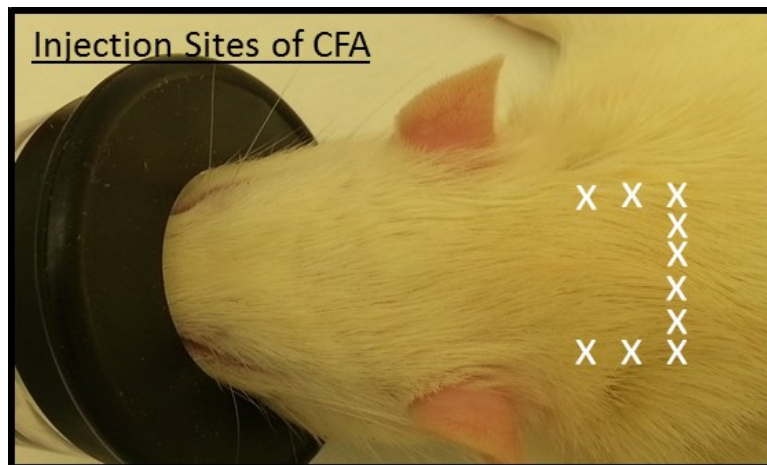
B)

Figure 3 Induction of Prolonged Neck Muscle Inflammation. (A) This figure depicts the ten different injection sites of CFA in the Trapezius muscle. (B) Image of the setup for anesthetizing the animals.
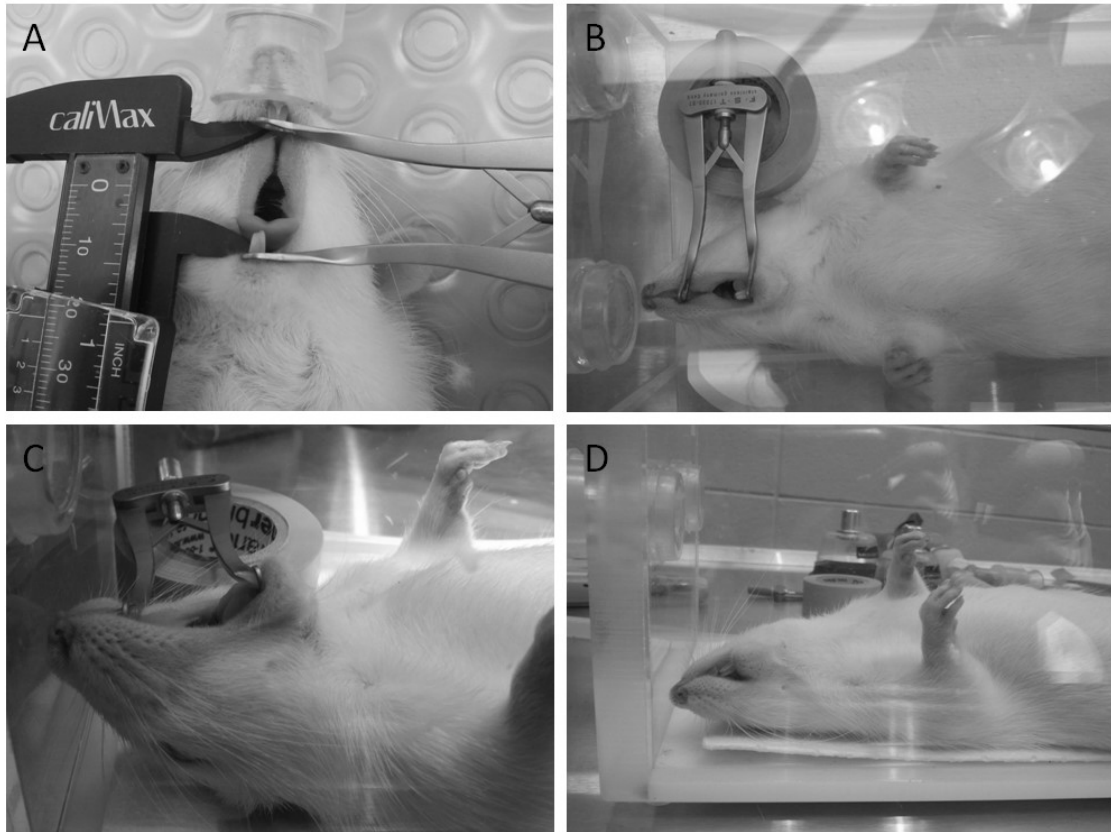
Figure 4 Mechanically-Induced Chronic TMJD Model. (A) Picture of lower jaw being retracted to near maximum jaw opening. (B) Animals resting in chamber after retraction distance was set. (C) Close-up image illustrating the position of retractor during procedure. (D) Picture of isoflurane control condition.
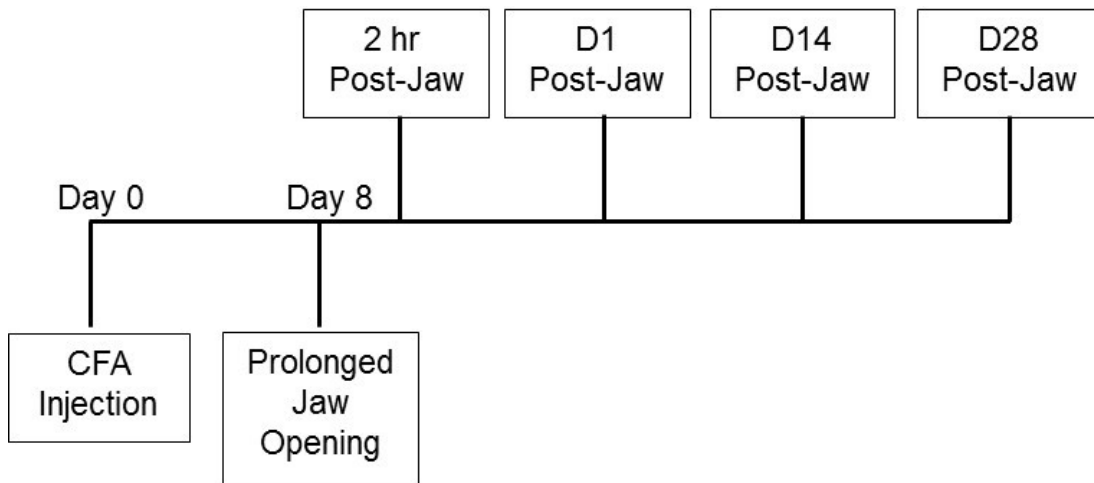
Figure 5 Experimental Timeline for Immunohistochemistry Studies.
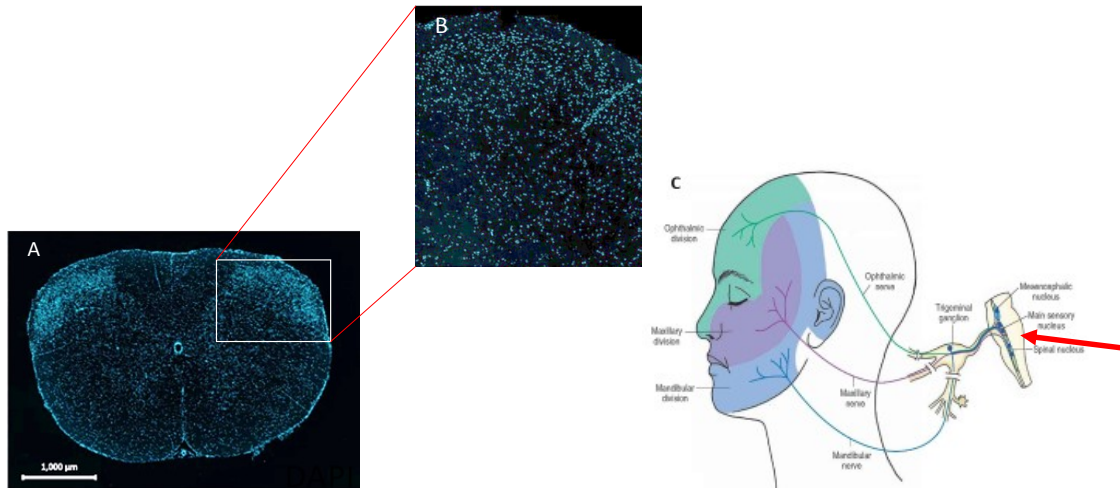
Figure 6 Identification of the Neuronal and Glial Cell Nuclei in the Rat Upper Spinal Cord. **(A)** Entire upper spinal cord section from an untreated animal stained with DAPI to identify all nuclei at 40X magnification. **(B)** Magnification (100X) of neuron containing the medullary horn stained with DAPI. **(C)** Trigeminal nerve pathway showing sensory innervation pattern in the head and face and projection through ganglion to upper spinal cord region known as spinal trigeminal nucleus (http://www.orofacialpain.org.uk/education/trigeminal-nerve/). Arrow indicates region of spinal cord shown in panels A and B.

RESULTS

CGRP

Temporal changes in the expression of the pro-inflammatory neuropeptide CGRP in neuronal cells located in the spinal trigeminal nucleus were investigated using immunohistochemistry. In spinal cord tissues from naïve animals, CGRP staining was detected predominantly in the outer lamina of the medullary dorsal horn at the 2 hour and 14 day time points (Figures 7 and 8). The relative intensity of CGRP staining was not elevated over naïve levels 2 hours or 14 days post jaw opening in sensitized animals (M+J; where M refers to neck muscle injections and J refers to prolonged jaw opening). A summary of the fold-change ± SEM of the relative intensity of CGRP immunostaining compared to naïve levels, whose mean was set equal to one, is shown in Table 2 for all conditions. At 2 hours post prolonged jaw opening, there were no significant changes in the localization pattern or intensity of staining in the M+J animals when compared to naïve levels (fold 1.06, $p = 0.202$). The same was true of day 14 animals with no significant changes detected in the staining intensity or pattern for CGRP in the M+J animals (fold 0.98, $p = 0.748$). Similarly, no differences were observed in CGRP levels in M+J animals (fold 0.89, $p = 0.447$) and naïve animals at day 28. However, a small yet significant increase in the intensity of CGRP immunostaining in M+J animals was found one day post jaw opening when compared to naïve levels (fold 1.30, $p = 0.007$).

PKA

Levels of PKA in the medullary dorsal horn were evaluated to determine if increased intracellular signaling downstream of CGRP receptor activation could be mediating sensitization of trigeminal nociceptive neurons. In naïve animals, low levels of PKA immunostaining were

detected within the medullary horn of the upper spinal cord in a pattern similar to that of CGRP (Figure 9 and 10). In contrast to the CGRP results, no significant differences in PKA staining pattern or intensity was observed between the M+J treated animals and naïve animals at any time point. A summary of the average fold-change ± SEM of the relative intensity of PKA immunostaining in M+J animals compared to naïve levels (mean set equal to one) is shown in Table 3 for all conditions. At 2 hours post prolonged jaw opening, there were no significant changes in the intensity in the M+J animals (fold 1.02, p = 0.844). The same was true of day 14 animals with no significant changes in the intensity in the M+J animals (fold 1.20, p = 0.086). Similarly, no significant changes were observed in the relative staining intensity between the M+J animals (fold 0.88, p = 0.401) and the naïve animals for day 1 or day 28 (fold 1.10, p = 0.237) post prolonged jaw opening.

NF-κB

Levels of transcription factor, NF-κB, were evaluated to determine if cytokine activation could be involved in promoting the sensitized state of trigeminal nociceptive neurons, which was examined using immunohistochemistry. In the upper spinal cord of the naïve animals, low levels of NF-κB immunostaining were detected within the medullary horn (Figure 11 and 12). The sensitized animals, which were the M+J treated animals, showed no significant differences from the naïve animals. A summary of the fold-change ± SEM of the relative intensity of NF-κB immunostaining compared to the naïve levels is shown in Table 4 for all conditions. At 2 hours post prolonged jaw opening, there were no significant changes in the intensity of the staining in the M+J animals when compared to the naïve animals (fold 0.81, p = 0.261). The same was true of day 14 animals with no significant changes in the intensity or pattern for NF-κB in the treated

animals compared to the naïve animals (fold 0.98, *p* = 0.874). The same was true for M+J

animals (fold 0.86, *p* = 0.265) and naïve animals at day 28 post prolonged jaw opening. The

staining patterns of NF-κB for day 1 was not determined in this study.

GFAP

Examination of astrocyte activity in the upper spinal cord was completed through

analysis of GFAP immunostaining levels. In naïve animals, low levels of GFAP immunostaining

were detected within the medullary horn of the upper spinal cord (Figure 13 and 14). Summaries

of the average fold-change ± SEM of the relative intensity of GFAP immunostaining compared

to naïve levels (mean set equal to one) for all conditions are shown in Table 5. The M+J treated

animals showed no significant differences from the naïve animals. At 2 hours post-prolonged-

jaw opening, there were no significant changes in the intensity in the M+J animals (fold 0.78, *p*

= 0.343). The same was true of day 14 animals with no significant changes in the intensity in

the treated animals (fold 1.40, *p* = 0.073). There were no significant changes in the intensity

between the M+J animals (fold 0.98, *p* = 0.879) and the naïve animals for day 1 shown in Table

2 (staining not pictured). The same was true for M+J animals (fold 0.83, *p* = 0.303) and naïve

animals at day 28 post prolonged jaw opening.

Iba1

Activation of microglia within the upper spinal cord was assessed through

immunostaining of the microglial marker Iba1. In naïve animals, low levels of PKA

immunostaining were detected within the medullary horn of the upper spinal cord (Figure 15 and

16). Summaries of the average fold-change ± SEM of the relative intensity of Iba1

immunostaining compared to naïve levels (mean set equal to one) for all conditions are shown in Table 6. The M+J treated animals showed no significant differences from the naïve animals. At 2 hours post-prolonged-jaw opening, there were no significant changes in the intensity in the M+J animals (fold 0.95, $p = 0.717$). The same was true of day 14 animals with no significant changes in the intensity in the treated animals (fold 1.01, $p = 0.462$). There were no significant changes in the intensity between the M+J animals (fold 1.40, $p = 0.418$) and the naïve animals for day 1 shown in Table 2 (staining not pictured). The same was true for M+J animals (fold 0.75, $p = 0.448$) and naïve animals at day 28 post prolonged jaw opening.

Table 2 Summary of Fold Changes in CGRP Expression in the Medullary Horn of the Upper Spinal Cord. All data are reported as the average fold change when compared to naïve levels, which was made equal to 1 ± the standard error of the mean.

Protein	Time point	Naïve	M+J
CGRP	2hr	1.00 ± 0.02	1.06 ± 0.05
CGRP	Day 1	1.00 ± 0.05	1.30 ± 0.04 *
CGRP	Day 14	1.00 ± 0.03	0.98 ± 0.05
CGRP	Day 28	1.00 ± 0.04	0.89 ± 0.07

* = significantly different from the naïve
Significant is defined by $p < 0.05$
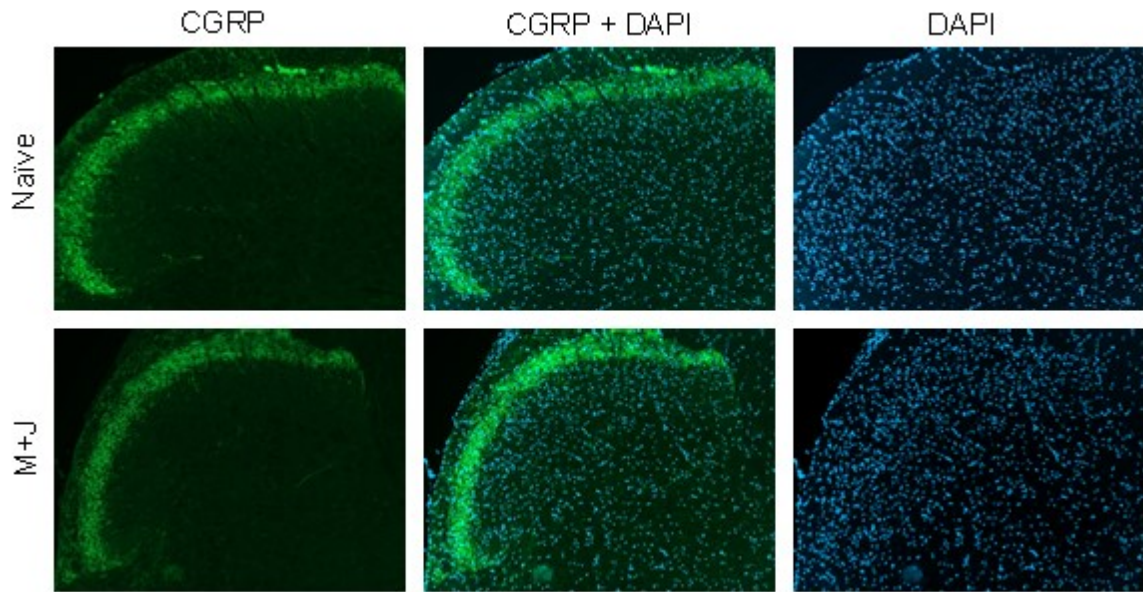
Figure 7 No Significant Changes in CGRP Levels in the Upper Spinal Cord 2 hours after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of CGRP in naïve (top) and M+J (bottom) (M+J; where M refers to neck muscle injections and J refers to prolonged jaw opening) animals. Sections were co-stained with the nuclear dye DAPI (right panels) and merged imaged of CGRP and DAPI staining is shown in the middle panels.

Figure 8 No Significant Changes in CGRP Levels in the Upper Spinal Cord 14 days after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of CGRP in naïve (top) and M+J (bottom) animals. Sections were co-stained with the nuclear dye DAPI (right panels) and merged imaged of CGRP and DAPI staining is shown in the middle panels.

Table 3 Summary of Fold Changes in PKA Expression in the Medullary Horn of the Upper Spinal Cord. All data are reported as the average fold change when compared to naïve levels, which was made equal to $1 \pm$ the standard error of the mean.

| Protein	Time point	Naïve	M+J
PKA	2hr	1.00 ± 0.04	1.02 ± 0.09
PKA	Day 1	1.00 ± 0.04	0.88 ± 0.16
PKA	Day 14	1.00 ± 0.09	1.20 ± 0.10
PKA	Day 28	1.00 ± 0.09	1.10 ± 0.03

Significant is defined by $p < 0.05$

Figure 9 No Significant Changes in PKA Levels in the Upper Spinal Cord 2 hours after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of PKA in naïve (top) and M+J (bottom) animals. Sections were co-stained with the nuclear dye DAPI (right panels) and merged imaged of PKA and DAPI staining is shown in the middle panels.
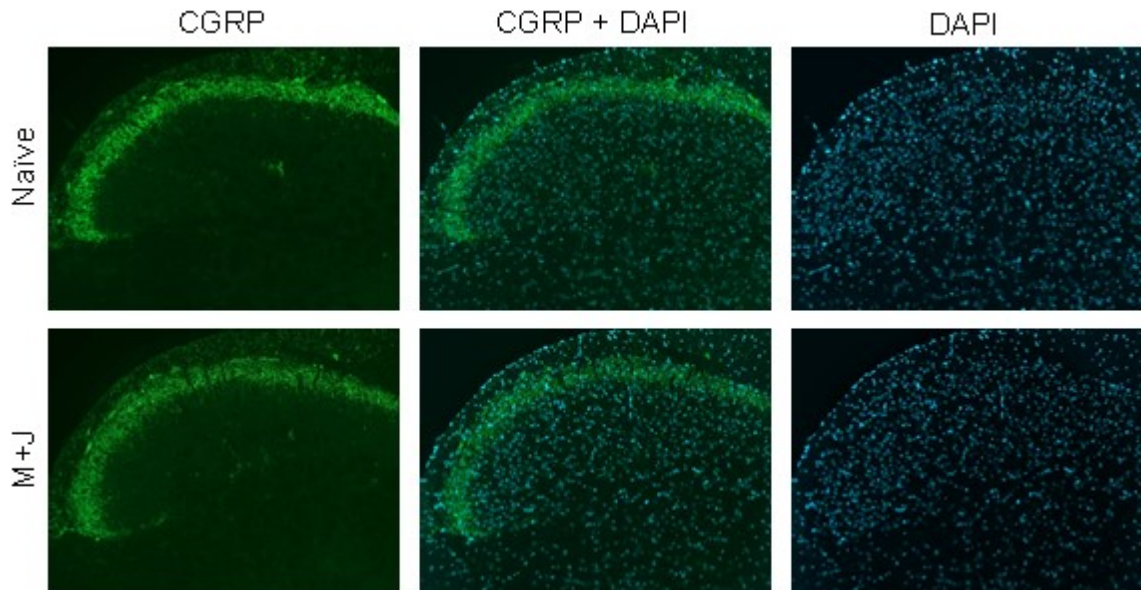
Figure 10 No Significant Changes in PKA Levels in the Upper Spinal Cord 14 days after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of PKA in naïve (top) and M+J (bottom) animals. Sections were co-stained with the nuclear dye DAPI (right panels) and merged imaged of PKA and DAPI staining is shown in the middle panels.

Table 4 Summary of Fold Changes in NF-KB Expression in the Medullary Horn of the Upper Spinal Cord. All data are reported as the average fold change when compared to naïve levels, which was made equal to 1 ± the standard error of the mean.

Protein	Time point	Naïve	M+J
NF-KB	2hr	1.00 ± 0.12	0.81 ± 0.12
NF-KB	Day 1	ND	ND
NF-KB	Day 14	1.00 ± 0.09	0.98 ± 0.08
NF-KB	Day 28	1.00 ± 0.06	0.86 ± 0.11

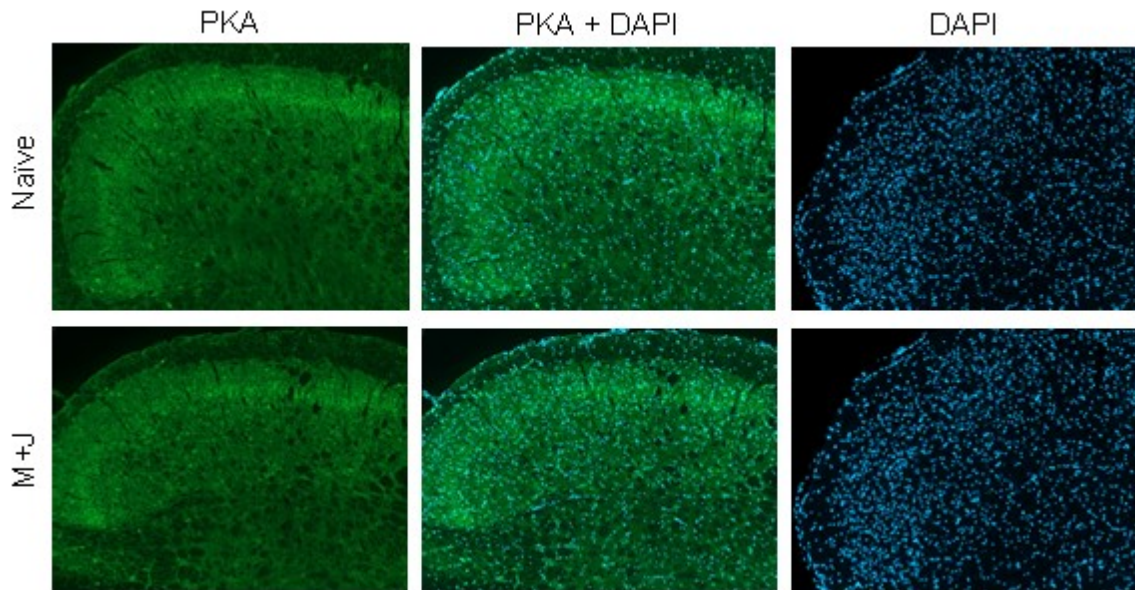Significant is defined by $p < 0.05$
ND = not determined
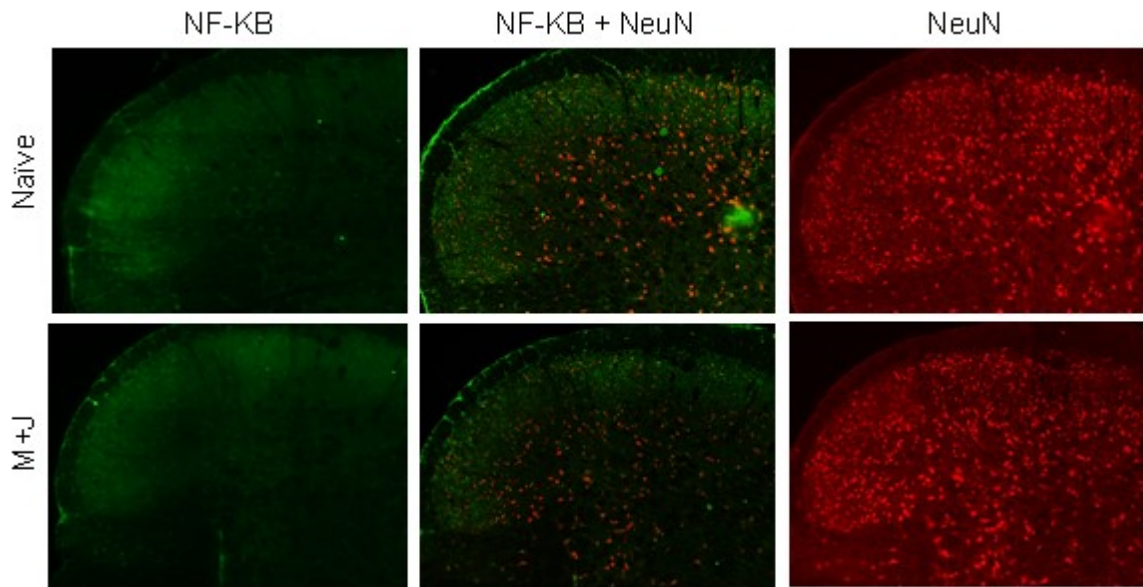
Figure 11 No Significant Changes in NF-KB Levels in the Upper Spinal Cord 2 hours after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of NF-KB in naïve (top) and M+J (bottom) animals. Sections were co-stained for neuronal nuclear expression of NeuN (right panels) and merged imaged of NF-KB and NeuN staining is shown in the middle panels.
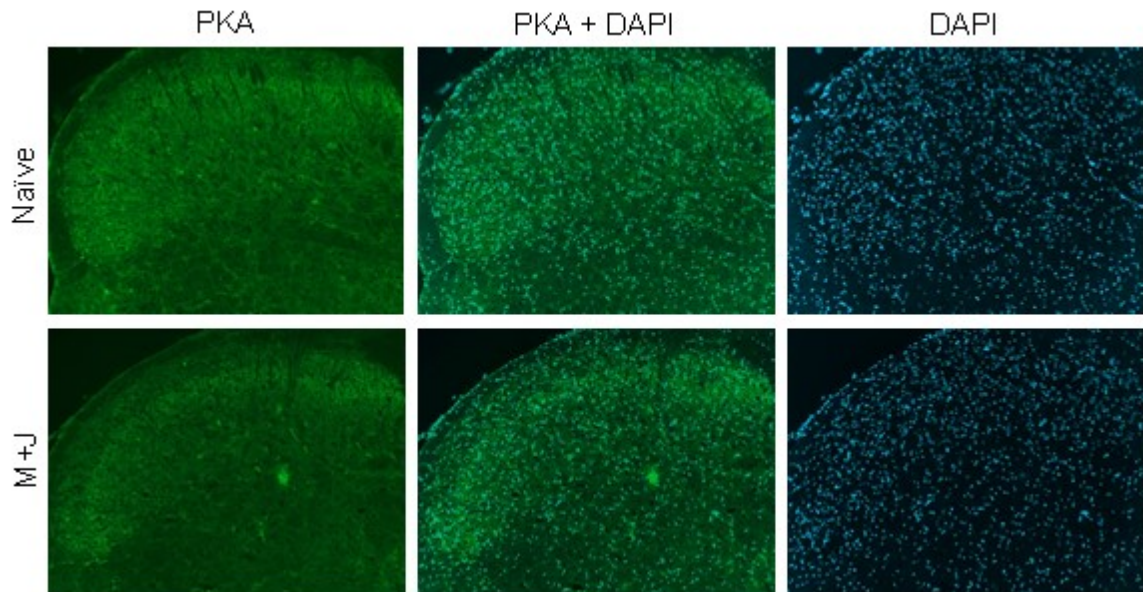
Figure 12 No Significant Changes in NF-KB Levels in the Upper Spinal Cord 14 days after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of NF-KB in naïve (top) and M+J (bottom) animals. Sections were co-stained for neuronal nuclear expression of NeuN (right panels) and merged imaged of NF-KB and NeuN staining is shown in the middle panels.

Table 5 Summary of Fold Changes in GFAP Expression in the Medullary Horn of the Upper Spinal Cord. All data are reported as the average fold change when compared to naïve levels, which was made equal to 1 ± the standard error of the mean.

| Protein	Time point	Naïve	M+J
GFAP	2hr	1.00 ± 0.17	0.78 ± 0.16
GFAP	Day 1	1.00 ± 0.18	0.98 ± 0.05
GFAP	Day 14	1.00 ± 0.09	1.40 ± 0.17
GFAP	Day 28	1.00 ± 0.16	0.83 ± 0.09

Significant is defined by $p < 0.05$
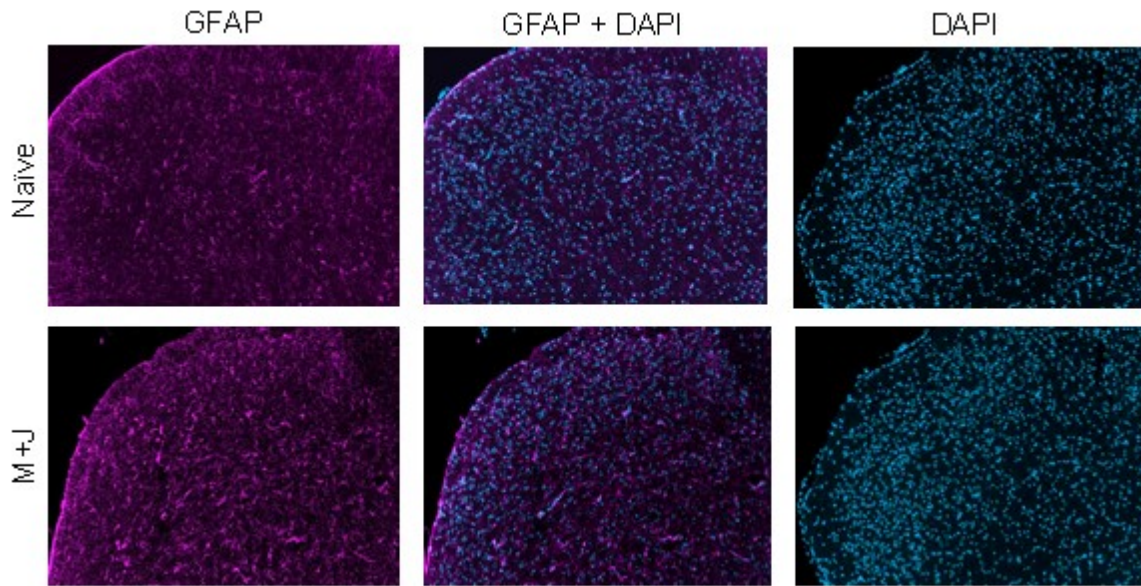
Figure 13 No Significant Changes in GFAP Levels in the Upper Spinal Cord 2 hours after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of GFAP in naïve (top) and M+J (bottom) animals. Sections were co-stained with the nuclear dye DAPI (right panels) and merged imaged of GFAP and DAPI staining is shown in the middle panels.
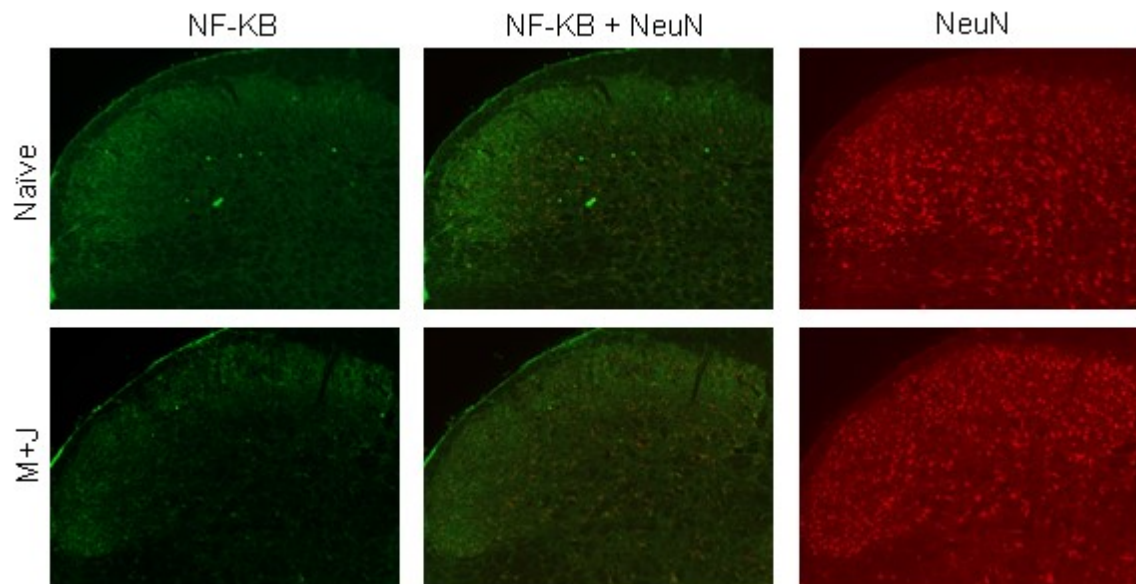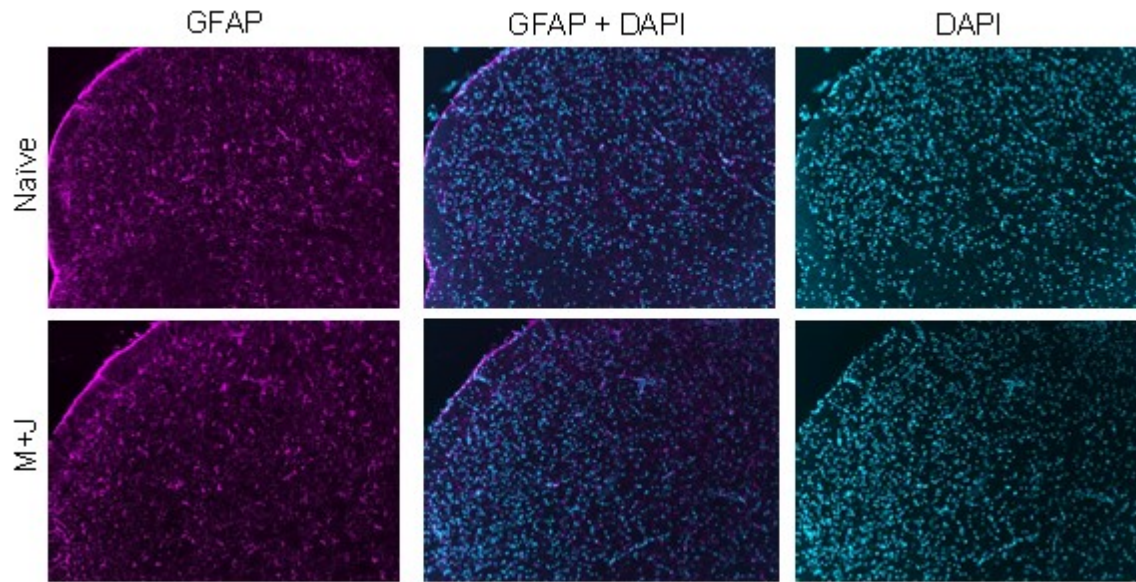
Figure 14 No Significant Changes in GFAP Levels in the Upper Spinal Cord 14 days after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of GFAP in naïve (top) and M+J (bottom) animals. Sections were co-stained with the nuclear dye DAPI (right panels) and merged imaged of GFAP and DAPI staining is shown in the middle panels.

Table 6 Summary of Fold Changes in Iba1 Expression in the Medullary Horn of the Upper Spinal Cord. All data are reported as the average fold change when compared to naïve levels, which was made equal to $1 \pm$ the standard error of the mean.

| Protein	Time point	Naïve	M+J
Iba1	2hr	1.00 ± 0.17	0.95 ± 0.16
Iba1	Day 1	1.00 ± 0.20	1.40 ± 0.33
Iba1	Day 14	1.00 ± 0.37	1.01 ± 0.47
Iba1	Day 28	1.00 ± 0.33	0.75 ± 0.06

Significant is defined by $p < 0.05$
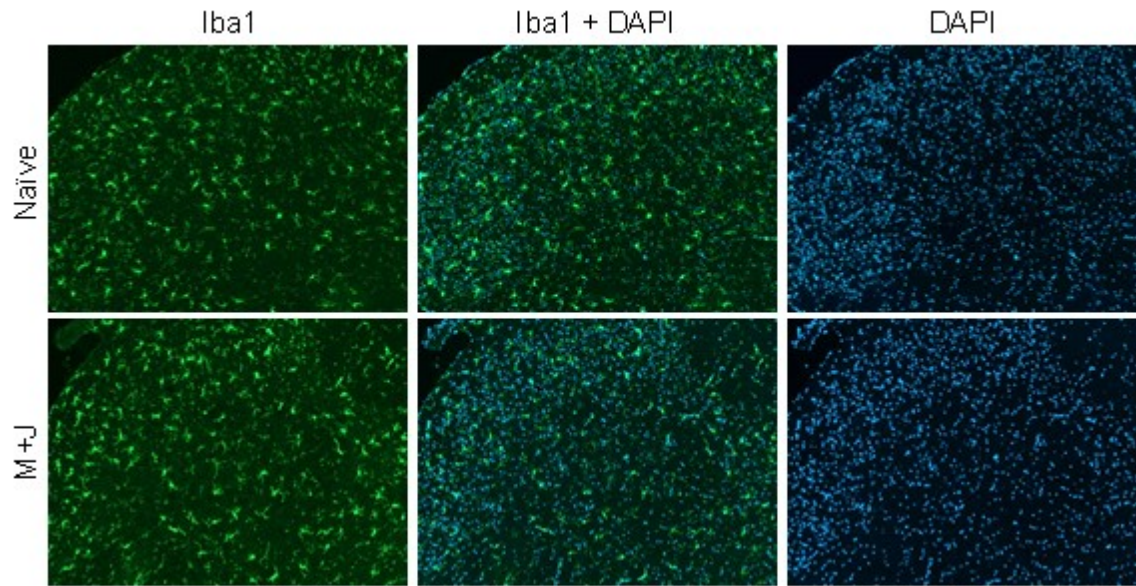
Figure 15 No Significant Changes in Iba1 Levels in the Upper Spinal Cord 2 hours after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of Iba1 in naïve (top) and M+J (bottom) animals. Sections were co-stained with the nuclear dye DAPI (right panels) and merged imaged of Iba1 and DAPI staining is shown in the middle panels.

Figure 16 No Significant Changes in Iba1 Levels in the Upper Spinal Cord 14 days after Undergoing Prolonged Jaw Opening after CFA Capsule Injection. Images (100X) from the representative region of medullary horn stained for expression of Iba1 in naïve (top) and M+J (bottom) animals. Sections were co-stained with the nuclear dye DAPI (right panels) and merged imaged of Iba1 and DAPI staining is shown in the middle panels.
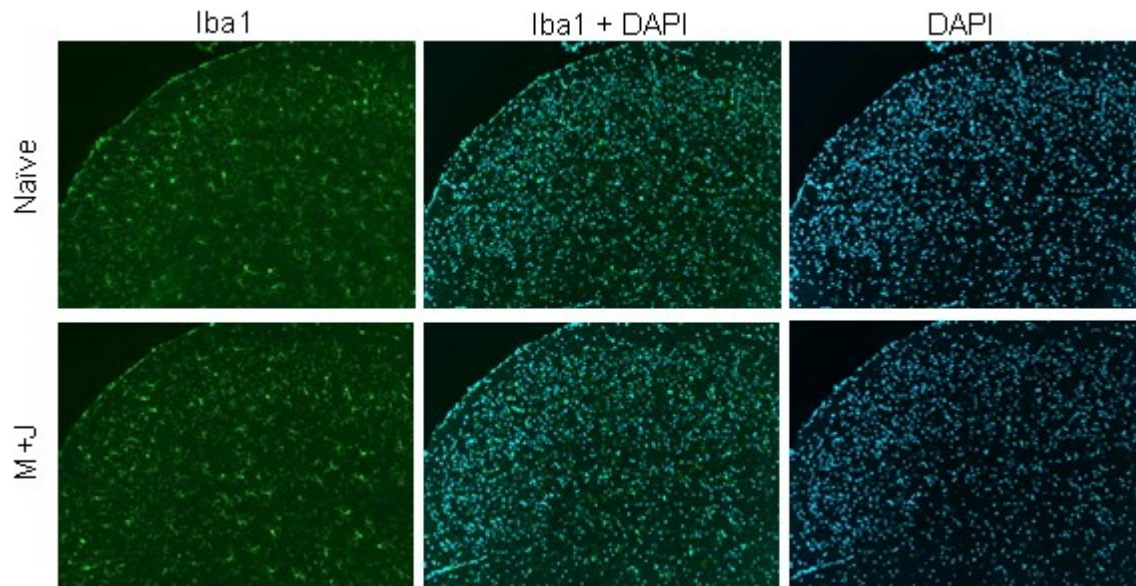
DISCUSSION

The goal of my thesis research was to investigate the cellular mechanisms associated with sustained mechanical sensitivity in the masseter muscle in a novel model of TMJD pathology. To mediate persistent pain signaling of trigeminal neurons, adult female rats that had ongoing neck muscle inflammation were subjected to near maximal jaw opening. I hypothesized that the underlying pathology would involve the development of central sensitization and a subsequent lowering of the activation threshold of trigeminal neurons by increasing expression of pro-inflammatory proteins and neuron-glia interactions. However, a somewhat surprising finding of my study was the lack of evidence to support my hypothesis, which however supports the notion that our chronic TMJD model is not mechanistically similar to other established inflammation-induced TMJD models. Previous studies from our laboratory utilized injection of the inflammatory mediator, complete Freund's adjuvant, into both TMJ capsules to cause prolonged joint inflammation and enhanced trigeminal nociception for up to 14 days in non-sensitized animals.[8, 9, 23, 24, 40] The CFA-induced TMJD model is likely relevant to human situations when there is direct injury to the TMJ that would occur from a blow to the face during a sporting event or a car accident. In response to tissue injury of the joint and disc, neurogenic inflammation would result in the release of potent vasodilators and pro-inflammatory molecules such as CGRP and nitric oxide and cause release of cytokines and chemokines from the synoviocytes and recruitment of immune cells to facilitate tissue repair.[10, 35, 36, 59, 69, 73] In the model of TMJD used in my study, neck muscle inflammation is initiated one week prior to prolonged jaw opening to simulate known reported risk factors for the development of chronic TMJD in humans.[53, 67] Our laboratory developed this chronic model to investigate the cellular and molecular changes that lead to a prolonged state of trigeminal nociceptor sensitization. Based on my results,

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development of chronic TMJD in sensitized animals exposed to prolonged jaw opening, which can occur while yawning, singing, or having a routine dental procedure, does not involve upregulation of proteins associated with maintenance of central sensitization or activation of astrocytes or microglia. Hence, this is a unique TMJD model that promotes trigeminal sensitization via a different mechanism that does not follow the typical inflammatory peripheral to central sensitization neuron-glia signaling pathway reported in acute TMJD models.[61] Thus, my findings may help to explain why patients with severe neck stiffness and evidence of taut bends in the trapezius muscles do not respond as well to commonly used anti-inflammatory medications such as nonsteroidal anti-inflammatory drugs or NSAIDs.[27]

A key feature of our chronic TMJD model is trapezius neck muscle inflammation, which is a common complaint of orofacial pain patients prior to and during major pain episodes. Anatomically, there is convergence of sensory stimuli from the primary trigeminal neurons and neck muscle neurons at the level of the upper spinal cord such that both sets of these neurons can cause activation of the ascending nociceptive second order neurons.[2, 51, 60, 78] Interestingly, the signaling pathways involved in muscle pain in general and neck muscle pain in particular have not been well studied. Although not a focus of my study, my results are suggestive that neck muscle inflammation promotes a sensitized state of trigeminal nociceptors that does not involve cellular and molecular changes implicated in central sensitization and facilitation of ascending pain signaling from second order trigeminal neurons to the thalamus and higher brain structures. Furthermore, my results provide evidence that astrocytes and microglial cells are not activated in response to neck muscle inflammation or following prolonged jaw opening in our chronic TMJD model. This finding is quite surprising given the large number of studies that implicate activation of astrocytes and microglia in the initiation and maintenance of a persistent or primed

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state of nociceptive neurons.[16, 48, 57, 77, 79, 80] Alternatively, neck muscle inflammation likely

mediates downregulation or dysregulation of the inhibitory descending pain modulation

pathway, which would result in an enhanced or hyperexcitable state of the trigeminal system

(Figure 17). With respect to pain modulation and establishing threshold levels, nociceptive

neuron sensitivity could be enhanced because of changes in the ascending signaling pathway via

increased ion channel and receptor activity and increased neuron-glia interactions, which could

be thought of as a gain-of-function.[11, 19, 32, 52, 56] However, a loss of function would also be

expected to play a major role in modulating the activation threshold of trigeminal neurons to

peripheral stimuli, which can occur if there is a reduction or loss of signaling in the descending

inhibitory pathway. Hence, dysregulation of the descending pathway would generate a

hypervigalent nervous system that would be more responsive to inflammatory stimuli. I can only

speculate that neck muscle inflammation mediates increased sensitization as a protective

mechanism since most of the major senses are located in the head and face. Therefore, if one

does not have full range of motion of one's head because of neck inflammation, the nervous

system responds by increasing the sensitivity of incoming peripheral stimuli to facilitate a

hypervigalent state characteristic of TMD and migraine patients. The descending inhibitory

pathway plays an important physiological role by quieting the nervous system and controlling

the amount of sensory input that reaches the higher brain centers. Thus, minimizing the

allostatic load on the system. This inhibitory pathway is activated turning states of deep sleep,

meditation involving deep breathing, and in response to our natural endorphins and even

morphine to inhibit peripheral sensory stimuli from reaching higher brain areas.[11] This pathway

is also responsible in part to the "runner's high" that allows elite athletes to block pain signaling

and is elicited during extreme cases of stress when the fight and flight response is initiated.

Recent studies in our laboratory have provided evidence that non-invasive vagus nerve stimulation (nVNS) activates this pathway and is able to inhibit trigeminal nociceptor sensitivity in a model of chronic migraine.[29] The mechanism is likely to involve activation of the periaquaductal grey and rostral ventral medulla pathway and release of serotonin to cause activation of inhibitory interneurons within the spinal cord. Upon activation of serotonin receptors, these interneuronal cells release the inhibitory neurotransmitter GABA (gamma-aminobutyric acid), which upon binding to GABA receptors on the second order neurons, mediates an increase in intacellular chloride levels to cause hypopolarization and effectively prevent activation of ascending nociceptive neurons. Whether the inclusion of grape seed extract, which we have shown inhibits the development of a sensitized trigeminal system and pain signaling in response to prolonged jaw opening in our chronic TMJD model, functions similarly to nVNS to enhance facilitation of the descending inhibitory pathway will be a focus of future studies.

In summary, I did not observe an increase in the expression of proteins implicated in the development of central sensitization in neurons or glial cells in the medullary dorsal horn at any time point following prolonged jaw opening in sensitized animals. Specifically, CGRP and PKA were not consistently elevated even though these proteins are reported to promote the development of central sensitization in other pain models.[62, 71] Similarly, I investigated changes in the transcription factor NF-KB since it has been reported to stimulate the synthesis of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 and contribute to central sensitization.[15, 25, 33, 39, 45, 58, 72] However, I did not see an increase in the level of nuclear localization of NF-KB in neurons or glial cells. Additionally, I did not detect changes in the levels of GFAP or Iba1, which are biomarkers of activated astrocytes and microglia,

respectively. Although not reported in my thesis, I also looked at the expression of several other pro-inflammatory proteins including the transcription factor c-Fos, the gap junction protein connexin 43, and the activated forms of the mitogen-activated protein kinases P-ERK (phospho-ERK) and P-p38 (phospho-p38), but did not observe increased levels in our chronic TMJD model.

In conclusion, results from my study did not support my hypothesis that the prolonged sensitization observed in our chronic TMJD models would be mediated by pro-inflammatory proteins implicated in central sensitization and glial cell activation. Rather, my findings support the notion that the underlying pathology in our TMJD model likely involves dysregulation of the descending inhibitory pathway. In future studies it will be of interest to investigate in our model if there are corresponding changes in cytokine mRNA and protein levels, which can be studied using microarray analysis, qPCR, and specific ELISA. Other studies will focus on understanding the cellular and molecular mechanisms by which dietary inclusion of a grape seed extract enriched in polyphenols and nVNS function to inhibit trigeminal mechanical sensitivity in our chronic TMJD model.
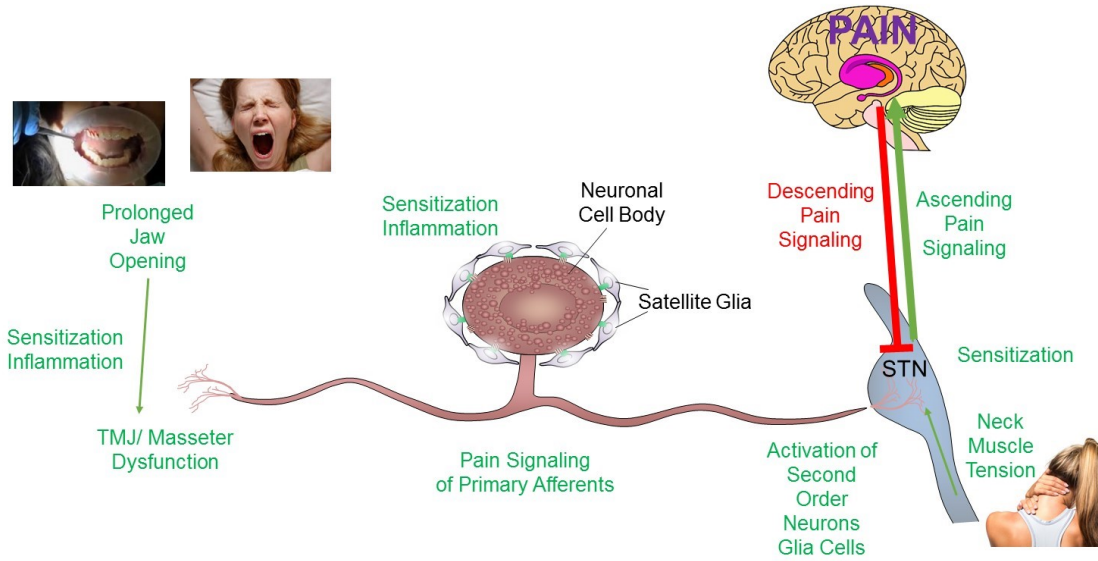
Figure 17 Dysregulation of Descending Modulation Likely Mediating Trigeminal Sensitization in Chronic TMJD Model. See text for detailed description.

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