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CLINICALLY RELEVANT MODEL OF TEMPOROMANDIBULAR DISORDER

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Neelima Chelliboina

May 2017

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CLINICALLY RELEVANT MODEL OF TEMPOROMANDIBULAR DISORDER

Biology

Missouri State University, May 2017

Master of Science

Neelima Chelliboina

ABSTRACT

Temporomandibular joint disorders (TMD) is a debilitating orofacial pain condition that affects a significant portion of the population and is associated with a decrease in quality of life. Results from the Orofacial Pain Prospective Evaluation and Risk Assessment Study (OPPERA) study provide evidence that risk factors such as neck muscle tension, prolonged jaw opening, and female gender increase the likelihood of developing TMD. Routine visits to an orthodontist or dentist can result in injury to the jaw joint or muscles of mastication. I tested the hypothesis that neck muscle inflammation and female gender increase the risk of developing chronic pain in response to prolonged jaw opening. Young male Sprague-Dawley rats were injected with complete Freund's adjuvant in the trapezius muscle to promote sensitization of trigeminal ganglion neurons. After 8 days, both male and female animals were subjected to near maximal jaw opening for 20 minutes. Mechanical nocifensive thresholds were determined in response to von Frey filaments applied to the skin over the eyebrow, masseter muscle, and joint capsule. Near maximal jaw opening increased nocifensive responses to mechanical stimuli over the masseter area for 14 days but then returned to near baseline levels by day 21. However, muscle inflammation prior to jaw opening resulted in prolonged nociception that was observed up to 28 days post jaw opening. Our findings provide evidence that neck muscle inflammation promotes sensitization of trigeminal neurons such that prolonged jaw opening causes a sustained nocifensive response that was more severe in females.

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

This abstract is approved as to form and content

Paul L. Durham, Ph.D.
Chairperson, Advisory Committee
Missouri State University

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Neelima Chelliboina

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Approved:

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INTRODUCTION

Orofacial Pain

Orofacial pain is defined as pain associated with hard and soft tissues of the head, face, and neck regions. One of the most common causes of orofacial pain is toothache, which is reported to occur in 12.2% of the population Boggero et al. (2016).

Temporomandibular joint pain was reported by 5.3% and face or cheek pain being reported by 1.4%. Many conditions co-exist with temporomandibular joint disorder like fibromyalgia, chronic fatigue syndrome, headache, panic disorder, and irritable bowel syndrome (Aaron et al., 2000, Bevilaqua Grossi et al., 2009, Ohrbach et al., 2011b, Dahan et al., 2015). It is estimated that a third of the population in the nation suffers from chronic pain. Chronic pain usually results in billions of dollars spent annually for health care services, loss of work, decreased productivity, and disability compensation. There is decreased satisfaction with life seen in patients with orofacial pain because this pain negatively affects their physical, emotional, and social functioning (Carlson et al., 1998, De Leeuw et al., 2005, Boggero et al., 2016). The mental health status of these patients will affect the pain experience and conditions like depression and anxiety are often co-morbid in these individuals. There are certain neural markers for fear and anxiety that aggravate chronic pain and add to the disease burden (Zakrzewska, 2013).

The clinician responsibility for treating patients with TMD is threefold. Firstly, they must ask appropriate questions and try to get certain information from the patients. Based on the responses, they should try analyzing and understanding the pathology based on their knowledge of basic and clinical science of orofacial pain. Secondly, a thorough

clinical assessment needs to be performed including physical and laboratory testing. Third, all the findings from the tests must be clearly explained and the treatment options should be clearly presented to the patient, which must be consistent with the standards of care based on the scientific literature. As reported by the National Institutes of Dental and Craniofacial Research (NIDCR), temporomandibular joint disorders are the most prevalent orofacial pain conditions for which patients seek treatment and are typically difficult to treat because of their complex pathophysiology.

Temporomandibular Joint Disorders

Temporomandibular disorders (TMD), a chronic disease that affects between 5-12% of the adult population, is characterized by pain in the temporomandibular joint (TMJ) or jaw joint and the muscles associated with mastication (Poveda Roda et al., 2007, Greenspan et al., 2011a, Ohrbach et al., 2011b, Furquim et al., 2015). TMD affects both men and women with the incidence of pain highest during adolescence (Bonjardim et al., 2009), and individuals often exhibit increased sensitivity to other experimentally induced pains (Maixner et al., 1995, Maixner et al., 1998). Temporomandibular joint disorders (TMJD) are those that affect the joint more than the muscles. They are commonly associated with trauma and are unilateral conditions. The signs of TMJD are seen in about 60–70% of the general population but only one in four people with signs are actually aware of it. Some of the common symptoms associated with these are clicking, tinnitus, headaches, and limited range of motion (Bevilaqua Grossi et al., 2009). There are many diseases and medical problems that are reported more common in women

in the clinical population. Not too surprisingly, the ratio between women and men who seek treatment for the TMJ is 8:1 (Sharma et al., 2011).

Approximately 6 to 12% of the adult United States population suffers from TMD. Among these patients, 80% show signs and symptoms of joint diseases including disc displacement, arthralgia, and osteoarthritis (Park et al., 2015). Patients with TMD usually present symptoms of more neck pain besides pain in the craniomandibular region. Through central command or reflex connectivity between the anatomical areas, disease in one system results in pain or dysfunction of another system (Ries et al., 2014). The pathological pain associated with TMD involves activation of trigeminal ganglion nerves, which provide sensory innervation of the head and face and relay nociceptive signals to the spinal trigeminal nucleus (STN) (Bereiter et al., 2005, Shimizu et al., 2009, Sessle, 2011). During TMD dysfunction, inflammatory molecules including cytokines, calcitonin-gene related peptide (CGRP), and the excitatory amino acid glutamate are released from the nerve terminals in the peripheral tissues to promote neurogenic inflammation (Sessle, 2011). There are three main effects characteristic of neurogenic inflammation which include increased blood flow, increased endothelial permeability, and recruitment and activation of key immune cells. The rise in CGRP and glutamate levels activates peripheral RAMP1 and N-methyl-D-aspartate (NMDA) receptors, respectively, that are located on afferent trigeminal neurons innervating deep tissues of both the joint and muscle tissues (Lennerz et al., 2008, Benemei et al., 2009, Cady et al., 2011, Cornelison et al., 2016). Stimulation of these peripheral receptors promotes a prolonged inflammatory response to facilitate healing and also transmits nociceptive signals to the upper spinal cord to cause pain, which helps to protect the tissues by

inhibiting their use. This type of physiological response to injury or infection leads to the development of peripheral and central sensitization of trigeminal neurons that persists until the tissues are healed (Cheng and Ji, 2008, Ren and Dubner, 2008, Ji et al., 2009, Sessle, 2011). However, in some cases, this sensitized state can persist leading to a pathophysiological state characterized by increased pain (hyperalgesia) and sensitivity to non-noxious stimuli (allodynia) that does not serve a protective or healing function.

Risk Factors for TMD

Multiple risk factors have been identified that promote the development of TMD, which are prevalent chronic orofacial pain conditions often associated with a significant decrease in quality of life. Results from the OPERRA study provide evidence that prolonged jaw opening, neck muscle tenderness, and female gender are commonly reported risk factors associated with the development of TMD (Greenspan et al., 2011a). Paradoxically, routine visits to the dentist for molar extractions or root canals, or to the orthodontist can result in an injury to the TMJ or muscles of mastication if the jaw is held open for prolonged periods of time or is opened to 100% of maximum even for a short period of time (e.g. yawning). Mechanical overloading of the TMJ is a causative factor in the onset of osteoarthritis and related orofacial pain disorders (Nicoll et al., 2010). With respect to TMD, female prevalence is more than twice that of males, yet the reason for this difference is not well understood.

There is emerging evidence in many complex neurological diseases that anxiety greatly influences disease onset, progression, and maintenance of the clinical phenotype (Ohrbach and Dworkin, 1998, Buse et al., 2013). The pathophysiological effects of

unmanaged anxiety can include increased tension in the muscles of the neck and shoulders especially in females (Ohrbach and Dworkin, 1998). Based on immunohistochemical studies, skeletal muscles are predominantly innervated by CGRP-containing neurons that facilitate pain signal transmission to the spinal cord (Tsukagoshi et al., 2006, Dudek et al., 2011, Barry et al., 2015). Thus, chronic muscle overload and tension in the neck and shoulders can lead to persistent fiber contraction, local ischemia, and the release of pro-inflammatory mediators, including CGRP, which can promote sensitization and activation of primary nociceptors (Greenspan et al., 2011b). Excitation of nociceptive neurons, which occurs in response to tonic muscle activity associated with myogenic trigger points, can lead to hypersensitization and lower pain thresholds of second order nociceptive neurons characteristic of central sensitization (Graven-Nielsen and Arendt-Nielsen, 2002). In addition, CGRP levels were reported to be elevated in myogenic trigger points, which are described as ‘a hyperirritable spot in skeletal muscle that is associated with a hypersensitive palpable nodule in a taut band’ (Hong and Simons, 1998, Shah and Gilliams, 2008). Convergence in the upper spinal cord of nerves providing sensory innervation of the neck and shoulder muscles and those emanating from the trigeminal ganglion may help to explain why neck and shoulder pathology is often associated with orofacial pain conditions including TMJD (Sessle, 1999, Bartsch and Goadsby, 2003, Morch et al., 2007). Elevated levels of CGRP in the spinal cord, as can occur in response to prolonged muscle inflammation in the neck (unpublished data from our laboratory), are implicated in the development of central sensitization and a prolonged state of neuronal sensitization (Seybold, 2009, Cornelison et al., 2016). In sum, these findings support a fundamental role of neck muscle tension in promoting the

development of a more persistent sensitized state of trigeminal primary and secondary nociceptive neurons characteristic of TMD. Disability associated with jaw and neck pain interferes greatly with daily activities and can have a negative impact on a patient's lifestyle by diminishing an individual's ability to work and interact in a social environment (Silveira et al., 2015).

Anatomy of Temporomandibular Joint

Temporomandibular joint (TMJ) is a sliding hinge that connects the jawbone to skull (Fig. 1). It is a bilateral synovial articulation between the mandible and temporal bone. The TMJ and its corresponding structures play a major role in guiding mandibular motion and in daily tasks such as chewing, swallowing, and speaking by distributing the mechanical stresses (Murphy et al., 2013). The capsule is a dense fibrous membrane that surrounds the TMJ. The joint capsule is lined by a synovial membrane which contains the synovial fluid and has a lateral ligamentous thickening that is the temporomandibular ligament (Shaffer et al., 2014). The articular surface is a fibrous extension of the capsule, which is a distinctive structure of the temporomandibular joint. The articular disc is a round, biconcave, avascular fibrocartilage structure that is located between the condyle and glenoid fossa (Bag et al., 2014). The articular disc divides the TMJ into two compartments namely upper and lower joint compartments, which are synovial cavities. The lower joint compartment formed between the mandible and articular disc is involved in rotational movement. The upper joint compartment formed between articular disc and temporal bone is involved in translational movement. Anterior to the glenoid fossa and medial to the posterior margin of the zygomatic process is articular eminence.

The movement and stability of the TMJ is provided by the musculature of the head, face, and cervical spine. The muscles of mastication which include masseter, temporalis, medial pterygoid and lateral pterygoid are the primary muscles of the cranial facial area that are involved in the jaw opening and closing, protrusion and retraction, as well as the lateral movements of the mandible (Shaffer et al., 2014). Muscles of mastication and other accessory muscles generally help the opening and closing of the jaw (Bag et al., 2014). One of the most powerful muscles of mastication is the masseter muscle. It has two parts namely, superficial and deep. The superficial originates from the zygomatic process of the maxillary bone and the deep part originates from the zygomatic arch of the temporal bone. Both parts attach to the zygomatic arch of the temporal bone. They play a role in the closing of the mouth or elevating the mandible. The Temporalis is another powerful muscle of the TMJ that arises from the temporal fossa, which is a shallow depression on the lateral aspect of the skull. It inserts into the coronoid process of the mandible. Together with the masseter, the temporalis muscle helps in elevating the mandible and closing of the mouth. The mandibular nerve (V3) branch of the trigeminal nerve innervates these two muscles.

The other muscles, which are innervated by the trigeminal nerve, are the lateral and the medial pterygoid muscles. The lateral pterygoid muscle is a triangular shape muscle that has two heads namely superior and inferior. The superior head originates from the greater wing of the sphenoid and the inferior head from the lateral pterygoid plate of the sphenoid. Both of them attach into the neck of the mandible. Since, it has horizontally oriented muscle fibers, thus it is the major protractor muscle of the mandible. The lateral pterygoid muscle acts bilaterally to cause the protraction of the mandible or

forward movement, and acts unilaterally to produce side-to-side movement of the jaw. Located inferior to the lateral pterygoid muscle is the medial pterygoid, a quadrangular shaped muscle possessing deep and superficial heads. These attach to the ramus of the mandible, near the angle of the mandible and help in elevating the mandible.

Trigeminal System and Nociception

Sensory innervation of the TMJ and associated muscles of mastication is provided by the Trigeminal or fifth cranial nerve that originates from the lateral border of the pons through the sensory and motor roots (Shankland, 2000). There are three branches of the trigeminal nerve namely, Ophthalmic, Maxillary and Mandibular divisions that provide sensory innervation to different areas of the face and neck tissues (Fig. 2). The Ophthalmic branch is purely sensory and innervates the scalp, skin of the eyebrows, eyelids, forehead, nose, tentorium cerebelli, posterior falx cerebri and dura mater (Shankland, 2001a). The disorders primarily associated with this branch are headaches and migraine. The Maxillary nerve is intermediate in size and it gives sensory innervation to all structures in and around the maxillary bone and the midfacial region (Shankland, 2001b). That includes the skin of the midfacial regions, lower eyelid, side of nose and upper lip, the mucous membrane of the nasopharynx, maxillary sinus, soft palate, palatine tonsil, roof of the mouth, maxillary gingivae, and maxillary teeth. Disorders involving this branch include rhinosinusitis and trigeminal neuralgia. The largest branch among these three is the mandibular branch that is a mixed nerve, meaning that there are both sensory and motor roots (Shankland, 2001c). The sensory root innervates the skin of the temporal region and lower third of the face, lower lip, and ear, the mucous membrane of

the anterior two-thirds of the tongue and mouth floor, the muscles of the first brachial arch, and the teeth and gingivae of the mandible . The small motor root supplies efferent innervation to the muscles of the first brachial arch that consist of the muscles of mastication, the tensors veli palatini and tympani, the mylohyoid muscle, and anterior belly of the digastrics muscle. The mandibular nerve relays chemical, mechanical, and thermal information from peripheral head and face tissues to the central nervous system.

The cell bodies from which all trigeminal nerve fibers originate are located in the trigeminal ganglion (TG). The neurons of this nerve are pseudounipolar, which means that this neuron contains a conducting process that has split into two branches. One branch, the afferent, terminates in the periphery and the other branch, the efferent, projects to the upper spinal cord (Devor, 1999, Durham, 2016). The two main types of sensory neurons are Large Light (LL) and Small Dark (SD). Large light neurons gives rise to A δ fibers, which are myelinated and fast conducting. Small dark neurons give rise to non-myelinated C fibers that transmit signals more slowly and for a longer duration. The TG contain two types of supporting glial cells, namely Schwann cells and satellite glial cells (Hanani, 2005). Schwann cells play an important role in the production of the myelin sheath that surrounds the axons and increase the conduction velocity of the nerve. The Satellite glial cells have a major modulatory role in influencing the excitability state of the neurons in response to peripheral stimuli (Durham and Garrett, 2010, Durham, 2016). Thus, primary trigeminal nerves provide a pathway for transmission of information mediated by peripheral stimuli, including thermal, mechanical, and chemical, to the upper spinal cord region known as the spinal trigeminal nucleus.

Nociception is encrypting and processing of harmful stimuli in the nervous system and therefore protecting itself from potential harm. The nociceptors are specialized peripheral sensory neurons that detect intense stimuli and provide the first line of defense against any potential damage. They are thin myelinated, fast acting (A- δ) fibers or slow acting unmyelinated C fibers that form the majority of the sensory neurons in the peripheral nervous system (Woolf and Ma, 2007). Inflammation of peripheral tissues may be associated with the release of chemical mediators like histamine and tumor necrosis factor from the tissue cells such as mast cells, macrophages, and other immune cells. Nociception caused by a harmful stimuli can trigger nociceptive reflexes at the sight of pain (Sessle, 2008). These reflexes are protective behaviors that can be observed as avoidance behaviors or withdrawal behaviors, and result in the removal of the affected body part from the stimuli. The classic example of this is the hand withdrawal-reflex from a hot stove. Similar behaviors can be observed from other types of noxious stimuli to protect tissues located in the stimulated area.

During inflammation or injury, there is an activation of primary nociceptors and decrease in the activation threshold resulting in peripheral sensitization (Sessle, 2011). This results in an increase in glutamate levels and other excitatory molecules in the spinal trigeminal nucleus. These mediators can cause sustained excitation of second order neurons that promotes increased spontaneous neuronal firing and a lower threshold of activation characteristic of central sensitization. Development of peripheral and central sensitization results in hyperalgesia and allodynia, which are pathophysiological features seen in individuals suffering from chronic pain pathologies like TMD.

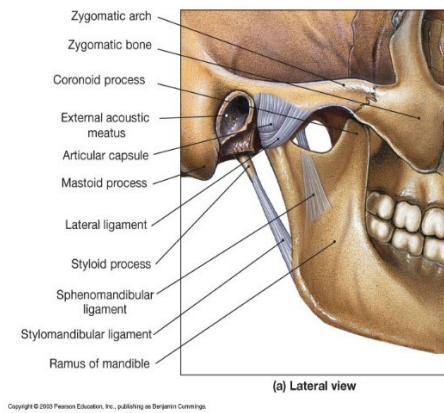
Hypothesis and Goals of Thesis Research

There are a number of animal models that mimic human clinical conditions. Chronic pain conditions are established using an emulsion of heat- killed *Mycobacterium tuberculosis* known as complete Freund's adjuvant (Sato et al., 2005, Takeda et al., 2005). Routine dental visits for molar extractions or root canals can result in the injury to the TMJ or muscles of mastication. Previous studies showed that CFA or prolonged jaw opening increased nocifensive responses to mechanical stimuli over the masseter area for 14 days but then returned to near baseline levels by day 21 (Garrett et al., 2012, Hawkins and Durham, 2016). Based on the findings from the OPERRA study, which was the first large prospective study done on humans to identify the psychological and physiological risk factors that lead to development of TMD, I wanted to test my hypothesis that the risk factors, neck muscle inflammation and female gender, would promote persistent sensitization of trigeminal neurons leading to a more chronic orofacial pain condition in response to prolonged jaw opening.

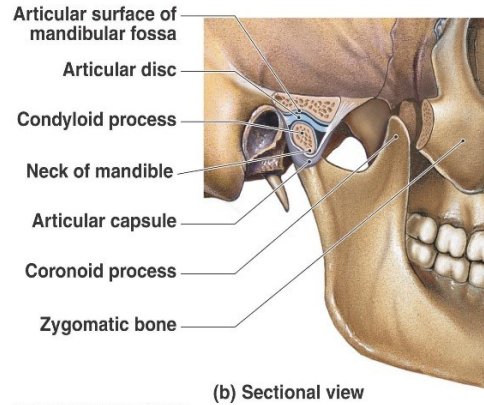
To test my hypothesis, I propose the following goals:

1. To determine the effect of neck muscle inflammation on the nocifensive behavioral response to prolonged jaw opening in three different orofacial regions including the cutaneous tissue above the eyebrow, masseter, and TMJ.
2. To compare the nocifensive withdrawal responses to mechanical stimuli in males and females in three different orofacial regions in sensitized animals.

A



B



C

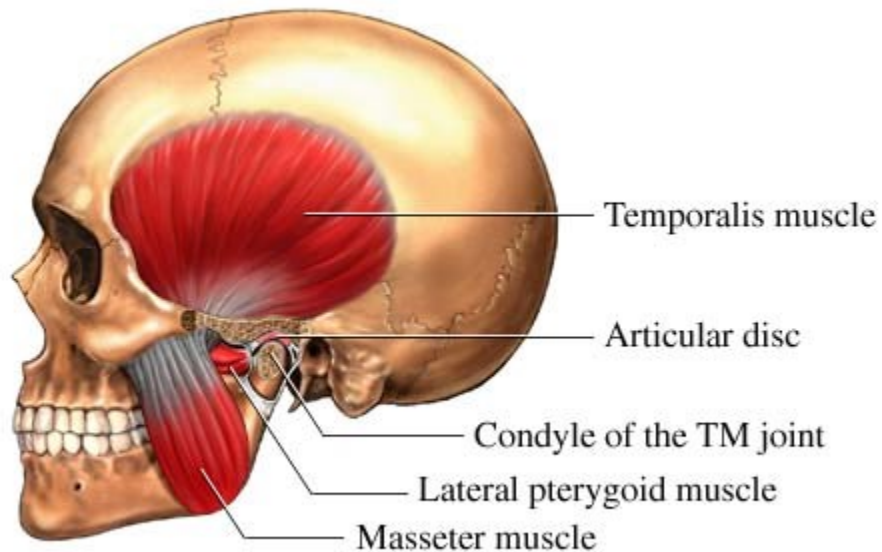


Fig. 1. Anatomy of the Temporomandibular Joint (A) Diagram displaying a lateral view of the joint including supporting ligaments and processes. (B) Cross-sectional view revealing the articular disk and joint cavities. (C) Diagram showing the various muscles associated with the TMJ. <http://www.edu.womenshealtharizona.com>.

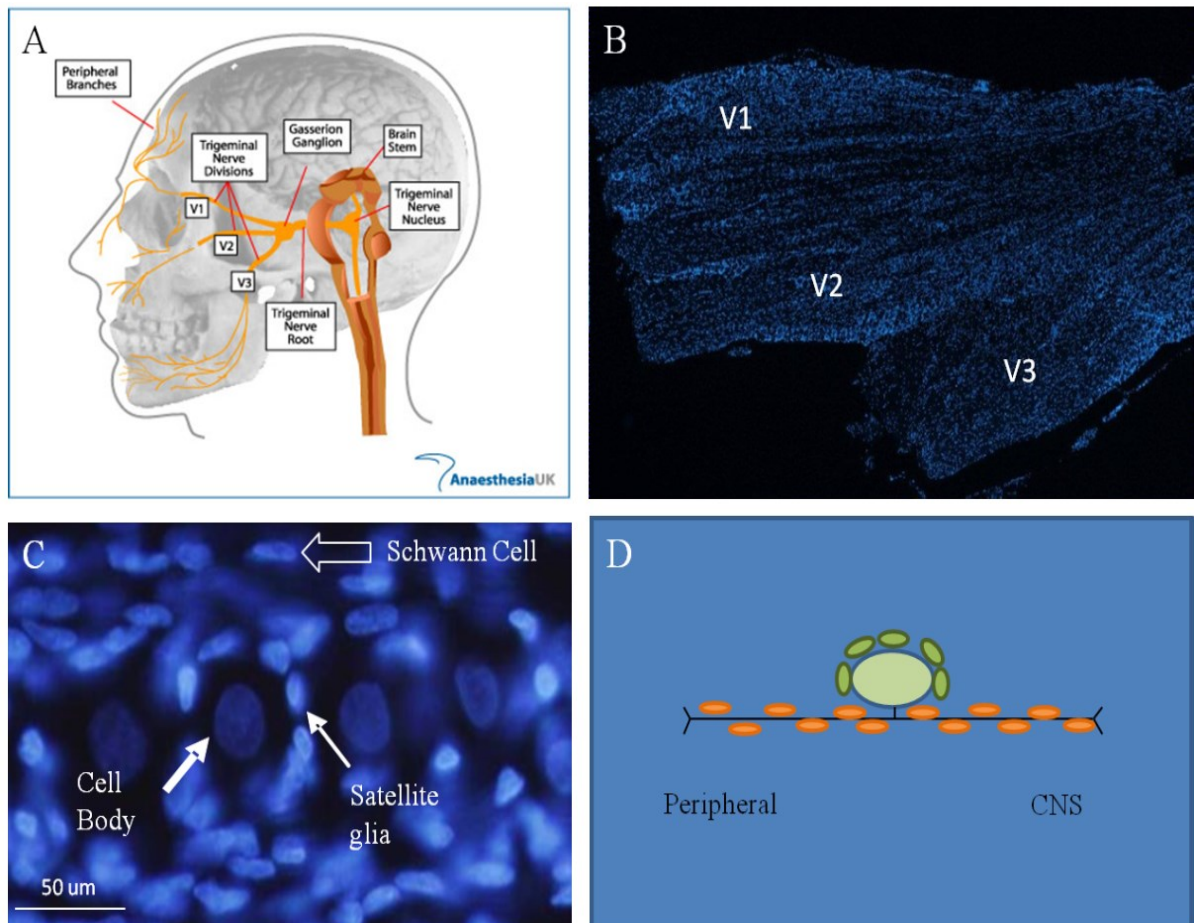


Fig. 2. Anatomy of the Trigeminal Ganglion. (A) Illustration of the location of the trigeminal nerves and trigeminal ganglion. <http://www.frca.co.uk> (B) A cross-section of the trigeminal ganglion stained with the nuclear dye DAPI to identify the three main branches. (C) A 400x image of DAPI-stained tissue showing nucleus of the neuronal cell body, surrounded by satellite glia cells. Also shown are Schwann cells, which are associated with axons. (D) A diagram illustrating a pseudounipolar trigeminal neuron, which provides a pathway to transmit sensory information from the peripheral tissues to the central nervous system (CNS).

MATERIAL AND 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. An effort was made to reduce suffering and number of animals used in the study. Adult male Sprague-Dawley rats (350-500g) and 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 for at least 1 week upon arrival prior to use. Complete Freud's adjuvant (CFA, Sigma Aldrich) was prepared as a 1:1 emulsion in 0.9% saline solution (Fisher-Scientific) immediately prior to use.

Model of TMD

Neck muscle inflammation, which results in a hyperirritable spot in skeletal muscle that is associated with a hypersensitive palpable nodule in a taut band ("trigger point") will be induced by administering complete Freud's adjuvant (CFA) or saline bilaterally into the neck muscle of young adult Sprague Dawley male and female rats. The 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. Once anesthetized, animals were placed ventral surface down, and attached to a nose cone apparatus delivering a steady flow of 3 % isoflurane. The final volume injected was a total 100 μ L CFA (50 μ L bilaterally) and applied as micro-injections containing 10 μ L each (Fig. 3).

In preparation of mechanically induced injury to the TMJ and associated muscles, ligaments, and tendons, animals were anesthetized via inhalation of 5% of Isoflurane and then were placed dorsal side down and attached to a nose cone apparatus delivering a steady flow of 3% isoflurane. A mouth retractor (Fine Scientific Tools, Foster City, CA), was positioned around the bottom and top incisors. The retractor arms were then separated until the desired percentage of opening was reached and held at near maximum incisor opening for 20 minutes (Fig. 4). 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 by utilization of a CaliMax Vernier Caliper (Wiha Tools, Montecillo, MN). It was determined that, on average, the maximum opening capacity without subluxation of the joint was approximately 22 mm in young adult male Sprague-Dawley rats and 20 mm in young adult female Sprague-Dawley rats. To allow for consistency between experiments, this measurement was used as a standard opening measurement throughout the experiment. Once the retractor was placed at the standard opening measurement, the animal was returned to the animal chamber attachment and maintained at 3% isoflurane for the duration of stress application. The breathing patterns and body temperatures were monitored during application and the procedure was aborted

if either varied significantly from normal observations. As a control, some animals did not undergo TMD induction procedures, and were just placed under 3% Isoflurane.

During the period of study, if an animal experienced a subluxation of the TMJ, the animal was euthanized immediately to minimize pain and suffering. Upon successful application of mechanical stress, the retractor was released, removed, and the animal was allowed to recover in its original cage.

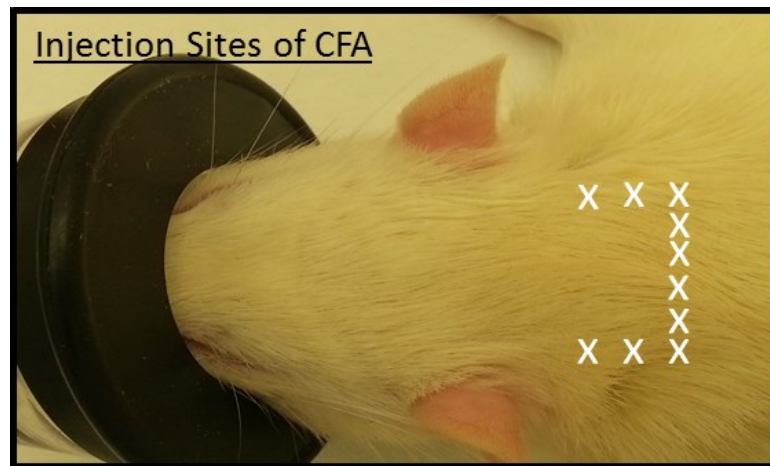
Behavioral Testing

For the nocifensive behavioral testing, animals were allowed to enter the Durham animal holding device (Ugo Basile, Italy) for five minutes on three consecutive days to acclimate the animals to the testing conditions essentially as described previously (Garrett et al., 2012, Hawkins et al., 2015, Hawkins and Durham, 2016). The animals were gently guided into the device and secured using a plastic blockade inserted behind the hind paws. To minimize false responses, animals were conditioned to a mechanical stimulus by gently rubbing the hair follicles and epidermis located over the masseter muscle and joint capsule with a pipette tip. Following acclimation period, mechanical nocifensive thresholds were determined in response to a series of calibrated von Frey filaments applied (North Coast Medical, Inc., Gilroy, CA; 26, 60, 100, 180, grams) in increasing force to the cutaneous area over the V1 area, masseter and joint capsule (Fig. 5). The researcher responsible for directly testing the response to each filament was blinded to the experimental conditions. The filament was placed at the appropriate position and pressure was applied. If there is head withdrawal seen before bending of the filament, the response was considered as positive. The filament of interest for my study was the 100 gram filament since baseline positive responses to this force were

consistently less than one bilaterally, while the 180 gram regularly caused more than three out of five nocifensive head withdrawal responses per side. Each filament was applied five times, and the data reported as the average number of responses obtained from five applications of each specific calibrated filament \pm Standard Error of the Mean (SEM). Baseline thresholds were measured before experimental procedures.

Measurements were also taken 2 hours, 1 day, 3 days, 5 days, 7 days, 14 days, 21 days and 28 days post-injury. On day 28, the animals are tested and then euthanized. Statistical analysis was performed on data with $n = 8$ or greater for each experimental condition. Outliers were determined by SPSS Statistics 21 software (IBM, North Castle, NY). To determine normality of behavioral data sets, a Shapiro-Wilk test was utilized, while a Levene's test was used to determine equal or unequal variance. It was determined that data sets were in violation of these assumptions and therefore non-parametric statistical analysis was used to determine significant changes. To determine if the observed effects were statistically significant, a Kruskal-Wallis ANOVA was performed followed by a Mann-Whitney U post hoc test with a Bonferroni correction ($\alpha_{\text{altered}} = 0.05/6$, $P < 0.008$) for pairwise comparisons at each time point. Additionally, a Friedman ANOVA was performed within each group, followed by a Wilcoxon Signed Ranks test with a Bonferroni correction ($\alpha_{\text{altered}} = 0.05/9$, $P < 0.006$) to evaluate comparisons made in a single group from baseline readings. All statistical tests was conducted utilizing SPSS Statistics.

A)



B)



Fig 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.

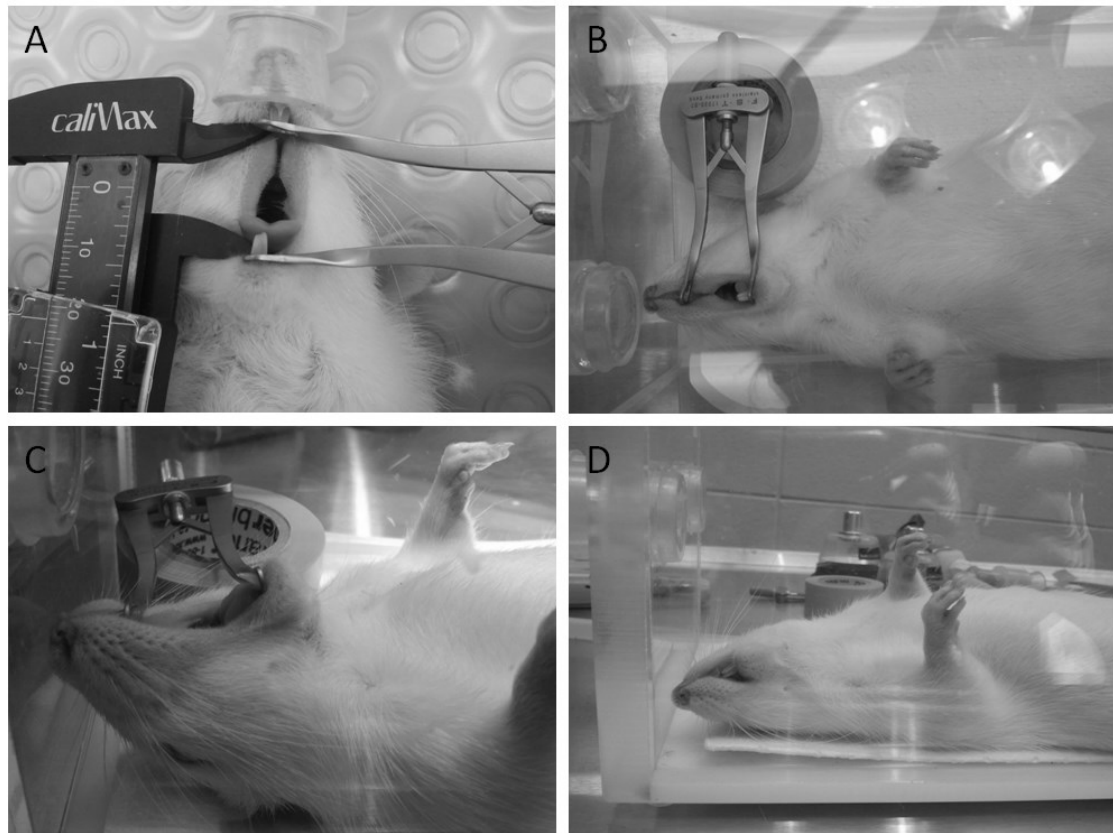


Fig. 4. Mechanically-Induced TMJ Injury Model. (A) Picture of lower jaw being retracted to near maximum jaw opening. (B) Animals resting in chamber after retraction distance was set. (C) Close-up image illustrating the position of retractor during procedure. (D) Picture of isoflurane control condition.

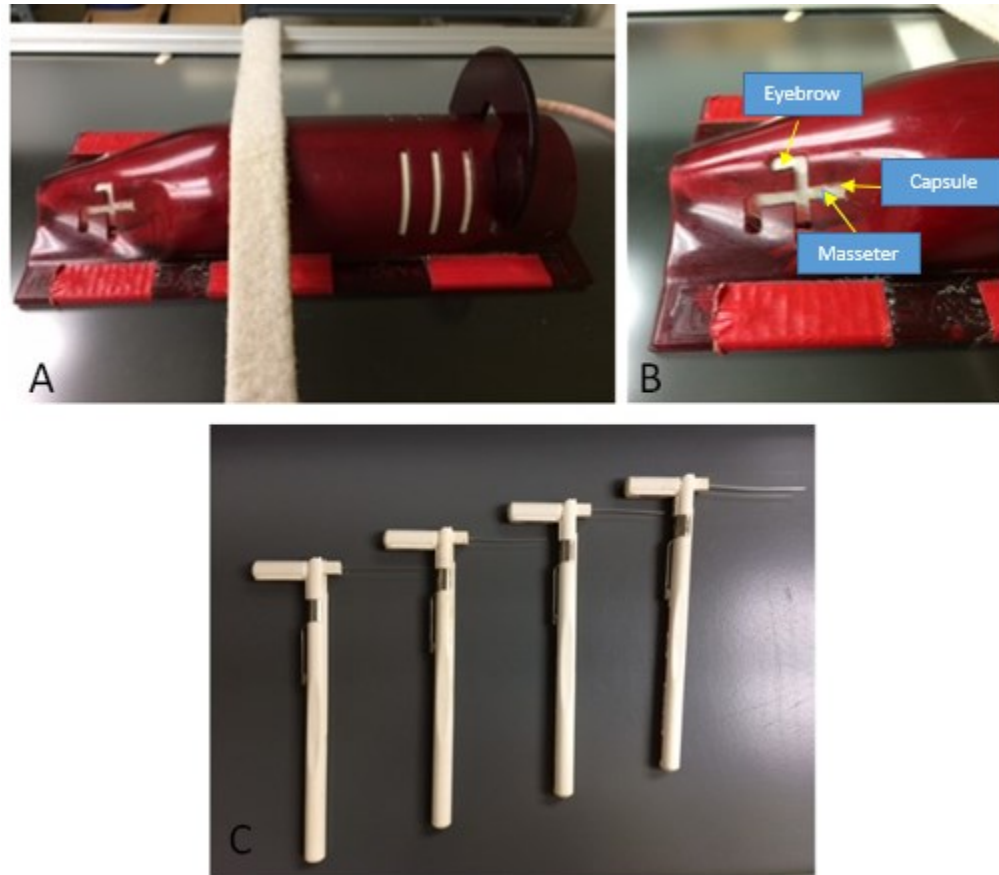


Fig. 5. Behavioral Testing Model. (A) Adult Sprague-Dawley rat secured in Durham holding device. (B) An enlarged image showing the cutout area used to access regions of the head and face with arrows indicating the eyebrow, masseter, and capsule where pressure was applied to determine mechanical sensitivity. (C) Series of von Frey filaments used in my study. From left to right: 26 grams, 60 grams, 100 grams, and 180 grams.

RESULTS

Nocifensive Response to Mechanical Stimulation

Baseline nocifensive thresholds were established for a series of force stimuli prior to either muscle injections or prolonged jaw opening. The data are reported as the number of reactions to the 60 gram force applied to the eyebrow of the V1 region and 100 gram force applied to the cutaneous area over the masseter and joint capsule of the V3 dermatome of trigeminal nerve. Responses to these filaments were reported because animals rarely respond to this force at baseline readings, whereas the subsequent higher force filaments always elicited a strong response. Once the average number of withdrawal responses for both right and left sides (5 each) was established, each animal was given a score based on the averages of the 10 total measurements. If the average was between 0-1.9 head withdrawal responses, these animals were given a score of one. If the number was between 2-2.9, they were given a score of two, if it was between 3-3.9, they were assigned a score of three, and if the average value was between 4-4.9, they were given a score of four. Animals that had an average value of 5 were given a score of five. Some animals went into an antinociception state and hence were not even responsive to the 180 g filaments. These animals were given the highest score of six. The score averages were calculated for each experimental group of animals. Animals were considered to have normal or mild sensitivity if they were in the 0-1.9 range, moderately sensitive if between 2-3.9 and severely sensitive if the average was greater than 4.

Masseter Region

The average for the number of nocifensive head withdrawal responses to mechanical stimulation of the masseter in naïve male animals did not differ significantly between groups (Fig. 6A). All values were in the normal or mild range (0-1.9) of sensitivity. In contrast, the group of male animals that received CFA neck muscle injections (Fig. 7A) interestingly did not exhibit any significant differences in nocifensive behaviors in areas over the masseter muscles at the earlier time points but were elevated at days 5 and 7 days post muscle injections ($p = 0.000$). The highest level of sensitivity was seen on day 5 with the value reaching the severe range (4-6). However, by 14 days post injections, mechanical nocifensive sensitivity over the masseter muscles decreased and returned to near baseline mild levels by day 21. As seen in Fig. 8A, prolonged jaw opening caused a change to moderate sensitivity at multiple time points but none of the values reached significance from basal levels. There was a significant increase in trigeminal sensitivity in animals with neck muscle tension and prolonged jaw opening at multiple time points, with sustained moderate to severe sensitivity starting at day 1 and continuing into day 21 (Fig. 9A). A summary of the results in nocifensive response to mechanical stimulation of the masseter muscle in male animals is shown in Fig. 10E. Similar to the males, no significant temporal change was observed in the sensitivity of the masseter in naïve (Fig. 6B). The female animals injected with CFA in the trapezius was significant on day 7 and exhibited moderate sensitivity at some time points (Fig. 7B). A similar trend was seen in female animals subjected to prolonged jaw opening (Fig. 8B). In contrast, the combination of trapezius inflammation and prolonged jaw opening resulted in a significant change in the level of sensitivity starting on day 1 and continuing

in the severe range even at day 28, which was the longest time point recorded in my study (Fig. 9B). A summary of the female results are presented in Fig. 10B. My findings provide evidence that neck muscle tension/tenderness should be considered a risk factor for developing chronic pain in the masseter muscle following prolonged jaw opening in both male and female animals. Furthermore, my results are suggestive that females may experience a more severe and longer-lasting state of pain and thus female gender should be considered a risk factor for chronic masseter sensitivity associated with TMD.

TMJ Capsule

The average for the number of nocifensive head withdrawal responses to mechanical stimulation of the TMJ capsule in naïve male animals did not differ significantly between groups (Fig. 11A). All the values were either in the mild or moderate sensitivity. The group of animals that were treated with CFA injections in the trapezius muscle showed no significant difference to mechanical sensitivity at various different time points (Fig. 12A). As seen in Fig. 13A, prolonged jaw opening caused a change to moderate sensitivity at multiple time points but none of the values reached significance from basal levels. However, there was a significant increase in trigeminal sensitivity in animals with neck muscle tension and prolonged jaw opening seen only at day 3, day 14 and day 21 time points, with sustained moderate to severe sensitivity starting at day 1 and continuing into day 28 (Fig. 14A). A summary of the results in nocifensive response to mechanical stimulation of the TMJ capsule in male animals is shown in Fig. 15A. Similar to the males, no significant temporal change was observed in the sensitivity of the TMJ capsule in naïve (Fig. 11B) or female animals injected with

CFA in the trapezius (Fig. 12B) even though CFA animals exhibited moderate sensitivity at some time points. A similar trend was seen in female animals subjected to prolonged jaw opening where most of them showed mild or low moderate sensitivity (Fig. 13B). In contrast, the combination of trapezius inflammation and prolonged jaw opening resulted in a significant change sensitivity on day 7 and day 28. The sensitivity started on day 3 and continuing in the moderate range even at day 28, though the levels were not as consistently elevated at all time points (Fig. 14B). A summary of the female results are presented in Fig. 15B. My findings provide evidence that neck muscle tension/tenderness should be considered a risk factor for developing chronic pain in the TMJ capsule following prolonged jaw opening in both male and female animals.

V1 Region

The average for the number of nocifensive head withdrawal responses to mechanical stimulation of the V1 dermatome above the eyebrow in naïve male animals did not differ significantly between groups (Fig. 16A). All the values were in the mild sensitivity. The group of animals that were treated with CFA injections in the trapezius muscle showed no significant difference to mechanical sensitivity at various different time points (Fig. 17A). As seen in Fig. 18A, prolonged jaw opening caused no change in sensitivity at any of the time points and only reached moderate levels on day 3. Somewhat surprisingly, there was no significant increase in trigeminal sensitivity in animals with neck muscle tension and prolonged jaw opening at any the time points (Fig. 19A). A summary of the results in nocifensive response to mechanical stimulation of the V1 region in male animals is shown in Fig. 20A. Similar to the males, no significant

temporal change was observed in V1 sensitivity in naïve (Fig. 16B) or female animals injected with CFA in the trapezius (Fig. 17B). A similar trend was seen in female animals and no changes are seen in animals subjected to prolonged jaw opening (Fig. 18B). The combination of trapezius inflammation and prolonged jaw opening also failed to show any significant changes in the level of sensitivity (Fig. 19B). A summary of the female results are presented in Fig. 20B. My findings provide evidence that while neck muscle tension/tenderness and prolonged jaw opening resulted in a sustained state of nociception in the V3 dermatome regions, the masseter and TMJ capsule, the same risk factors did not mediate a change in trigeminal sensitivity in the V1 nerves.

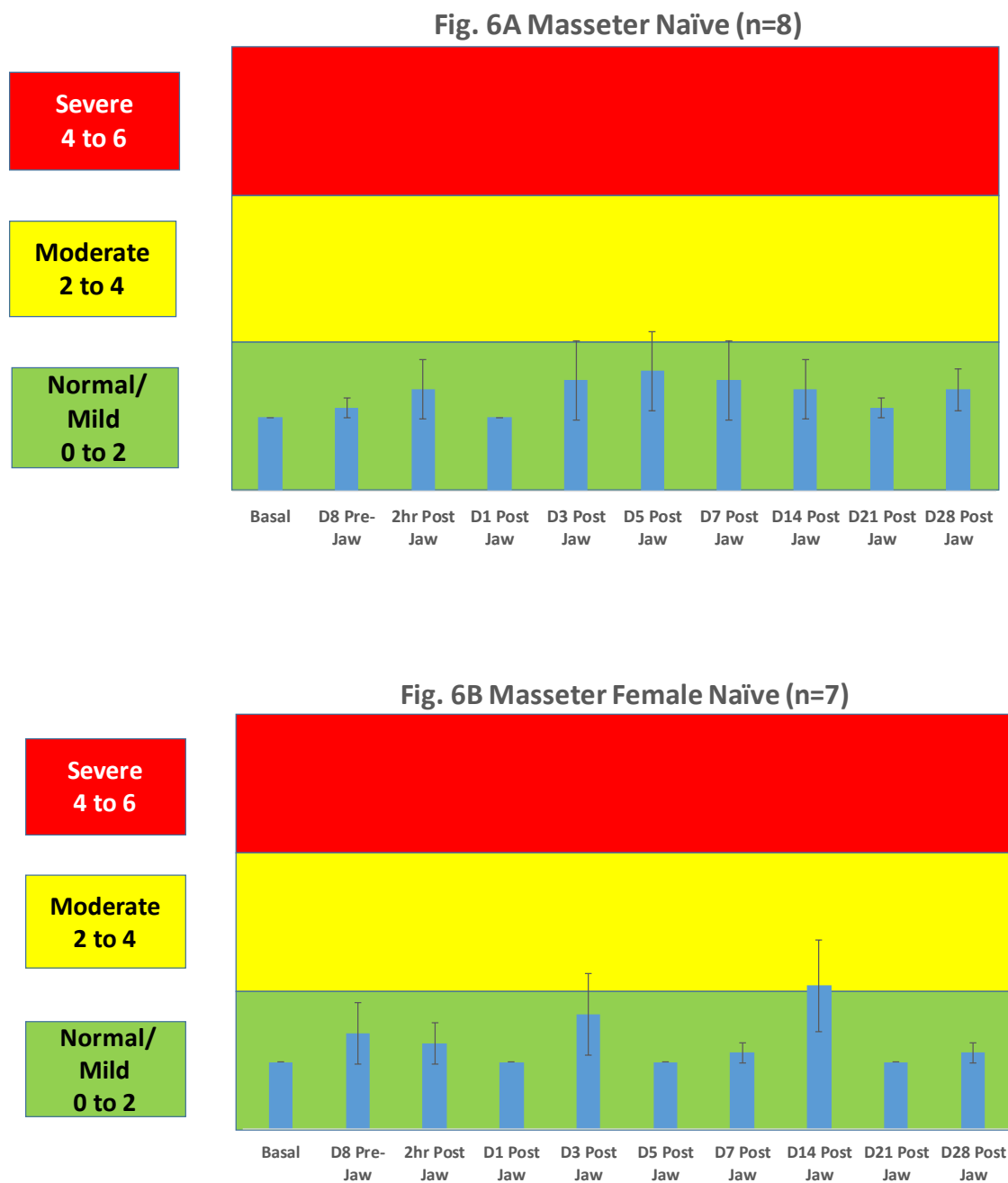


Fig 6. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the masseter muscle in male and female naïve Sprague-Dawley rats.

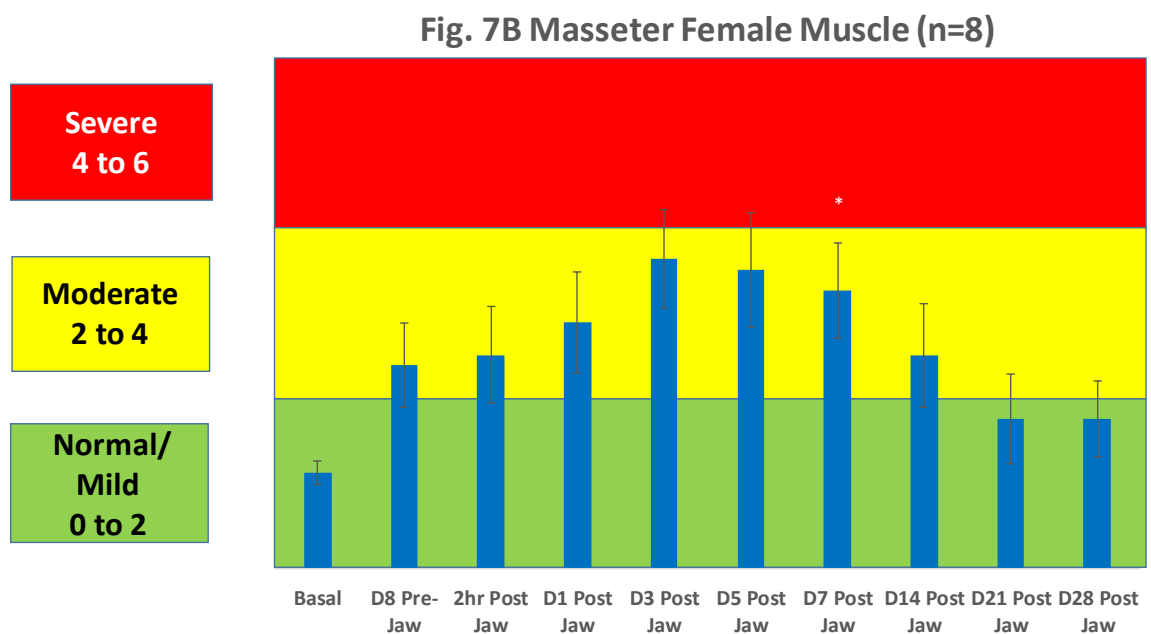
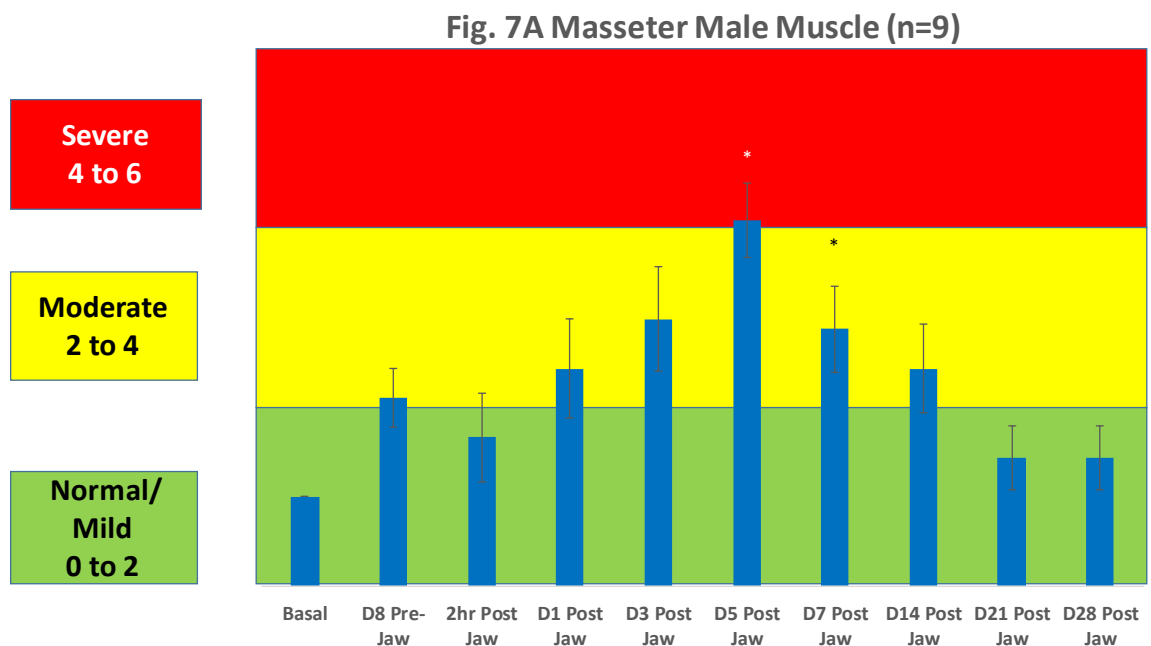


Fig 7. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the masseter muscle in male and female Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

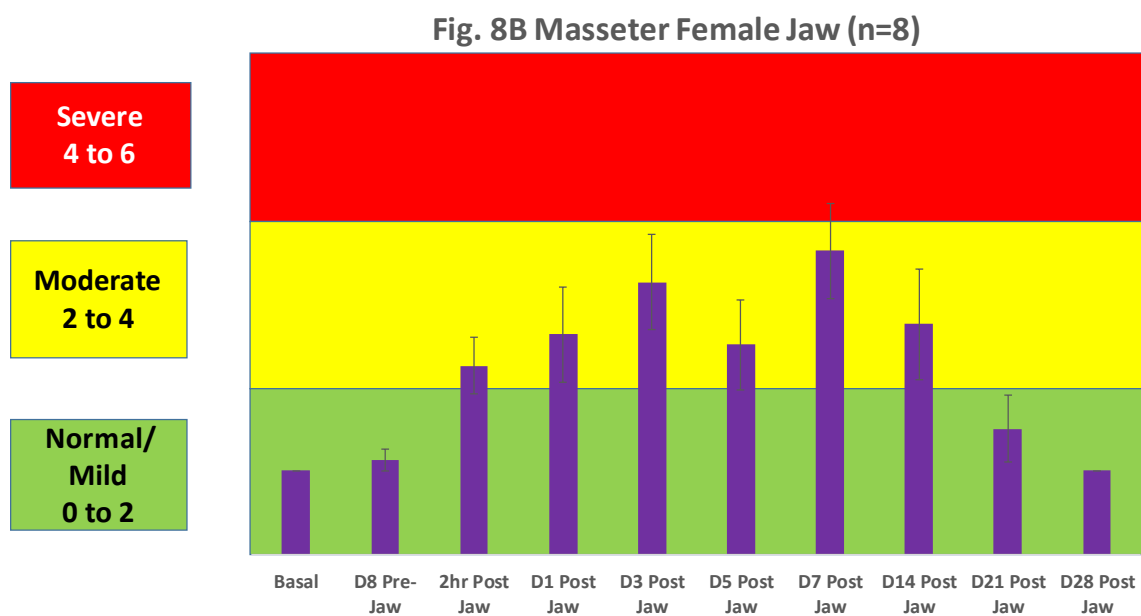
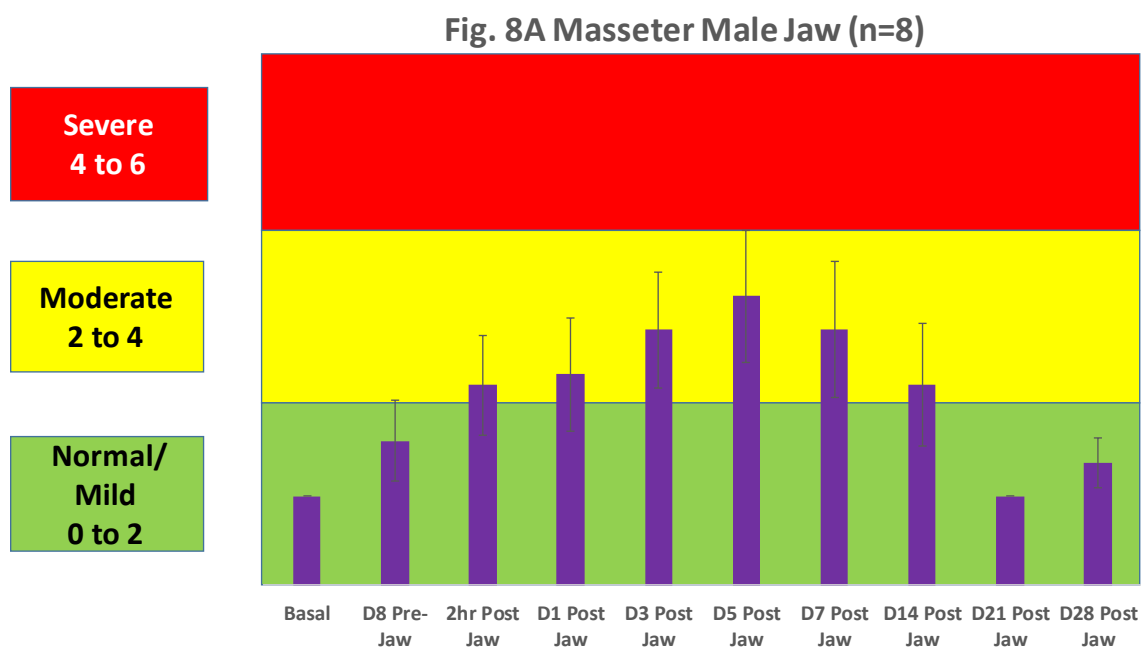


Fig 8. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the masseter muscle in male and female jaw Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

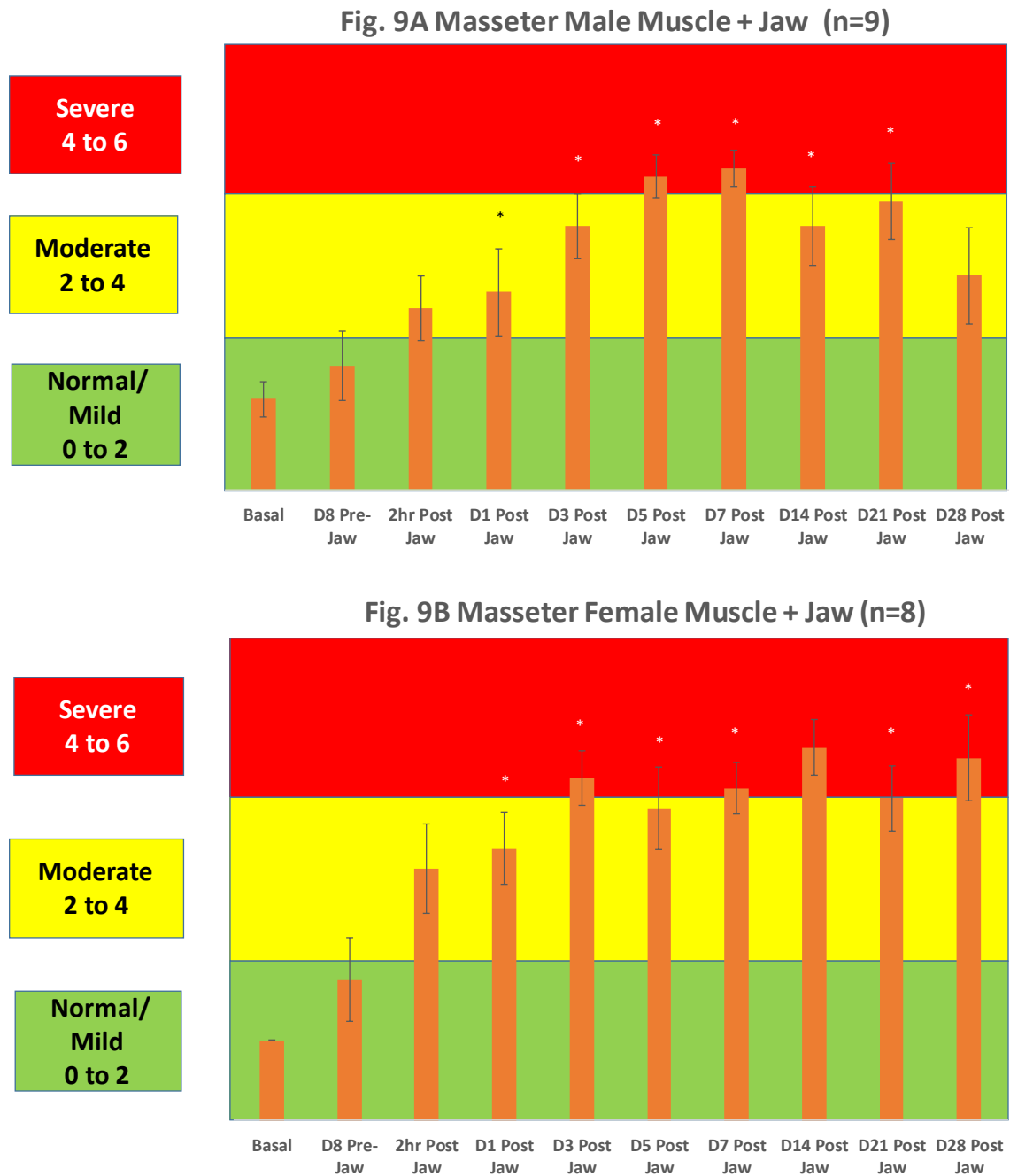


Fig 9. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the masseter muscle in male and female muscle + jaw Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

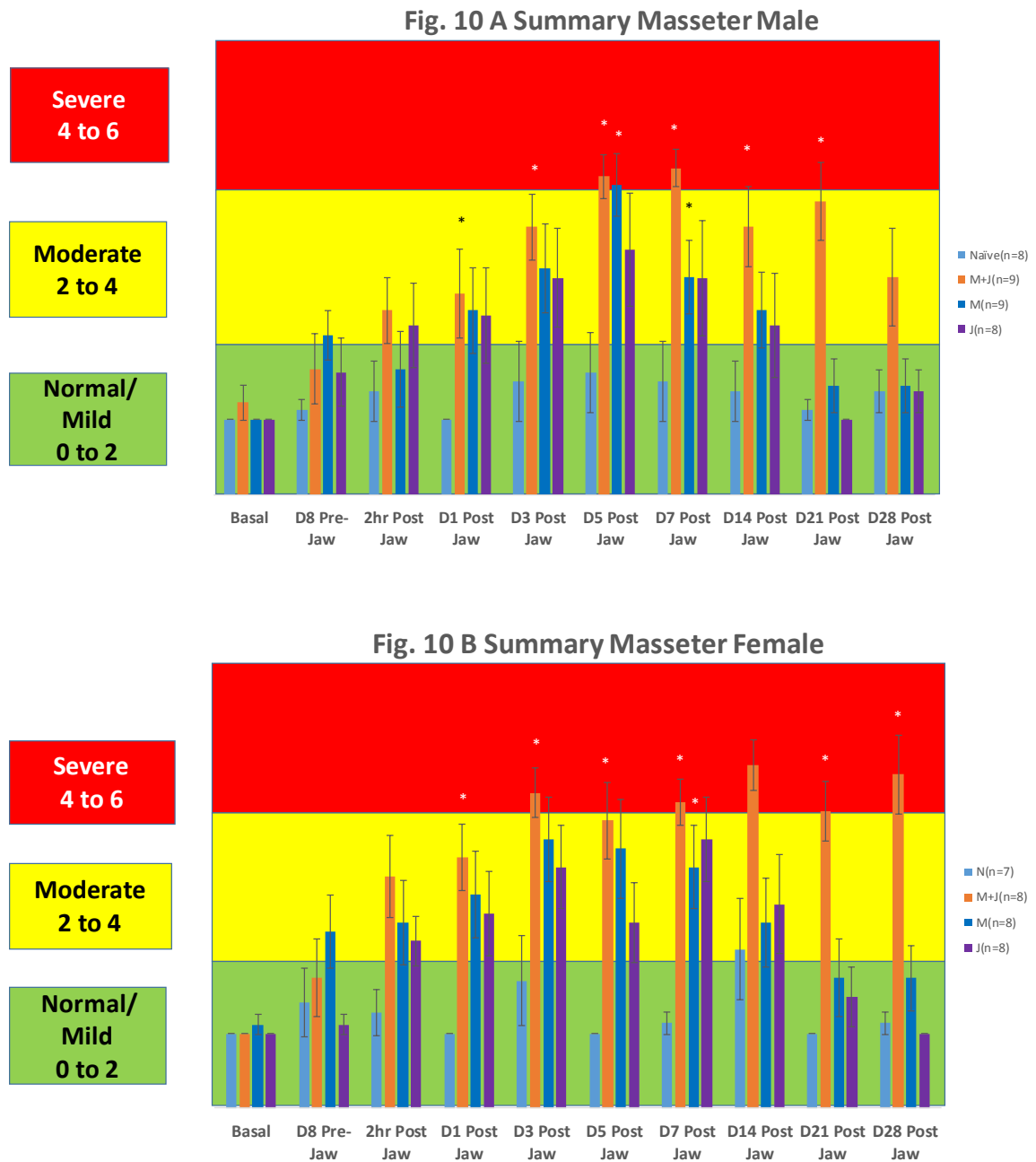


Fig 10. Summary of temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the masseter muscle in male and female Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

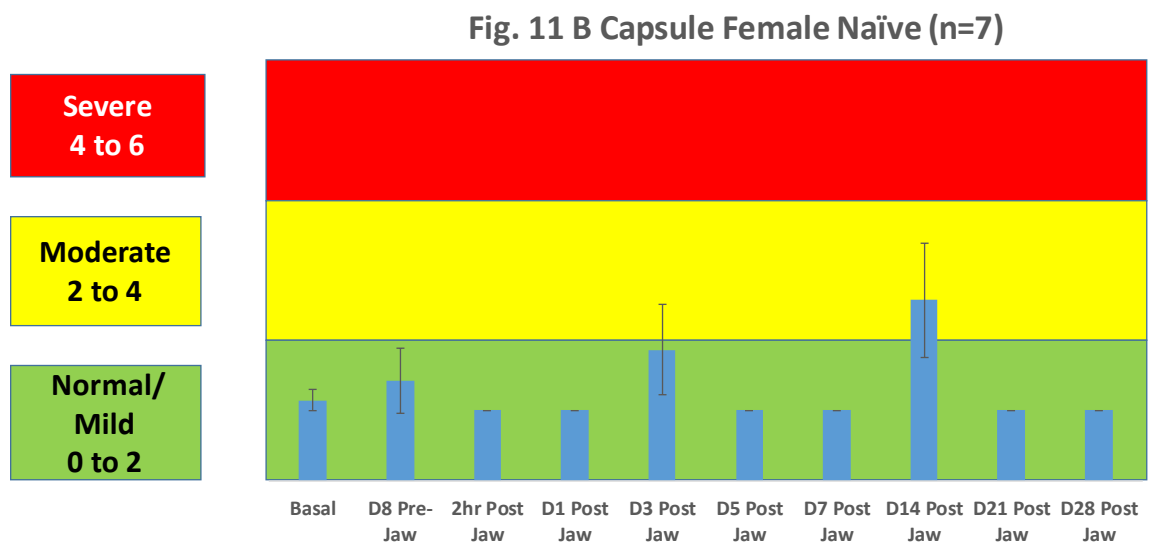
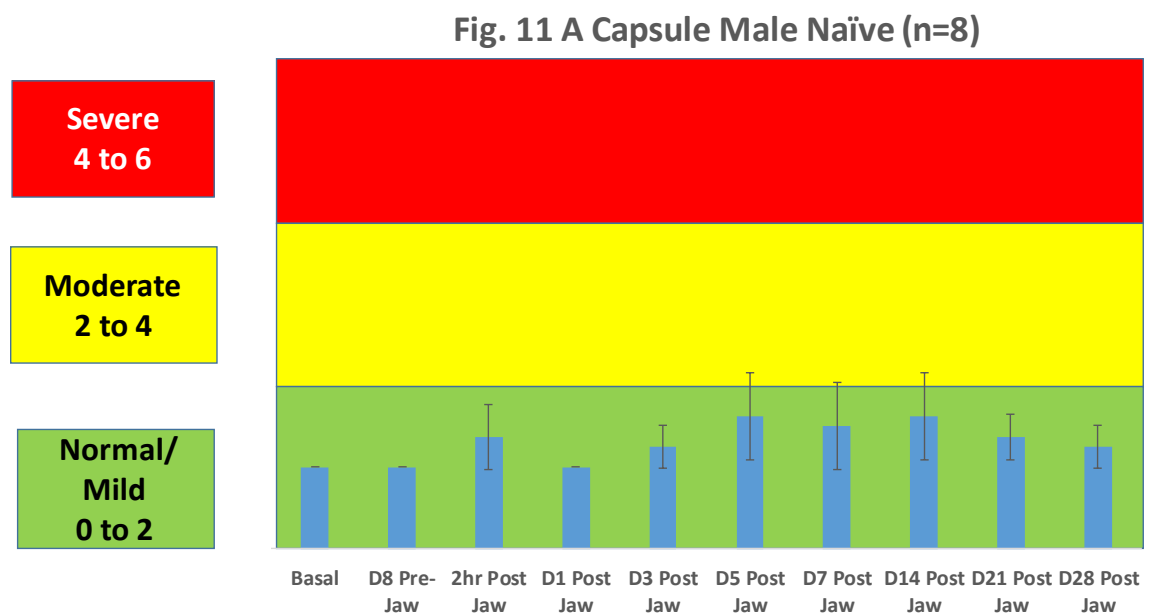


Fig 11. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the TMJ capsule in male and female naïve Sprague-Dawley rats.

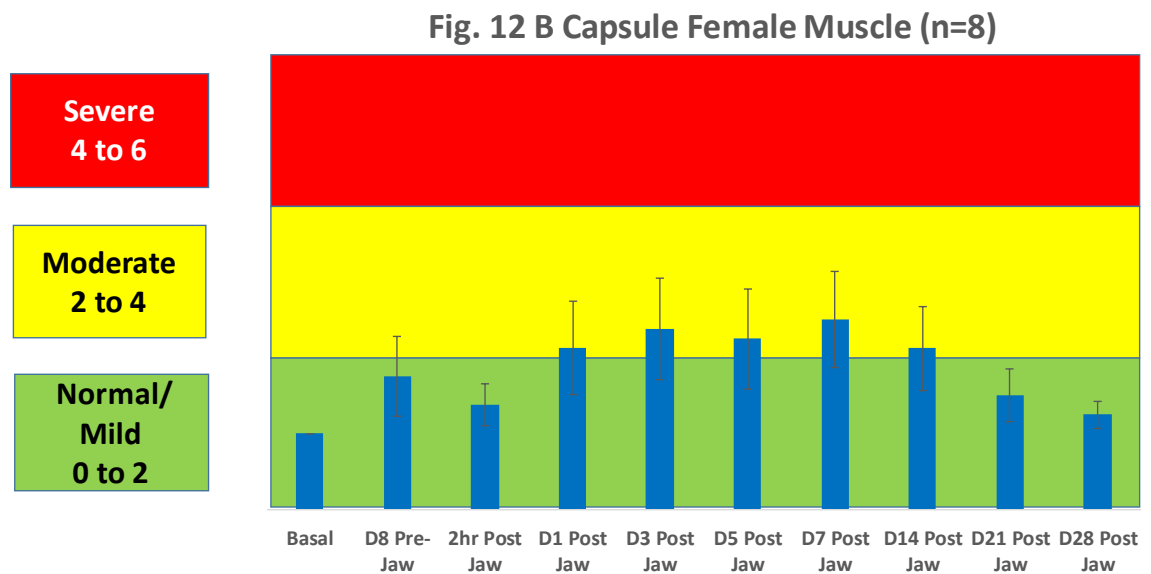
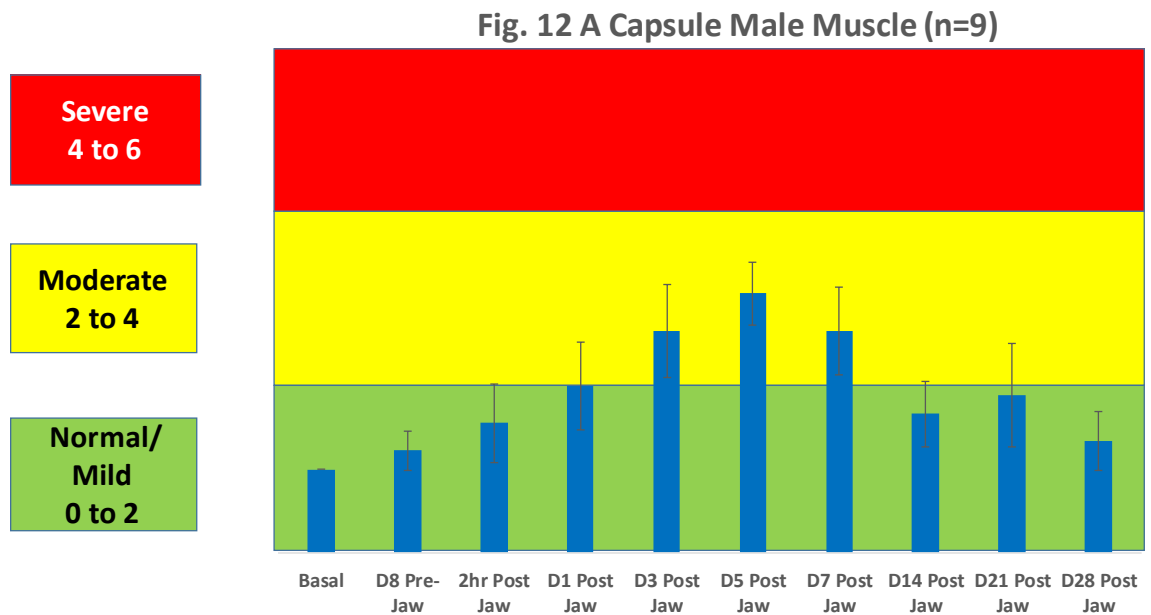


Fig 12. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the TMJ capsule in male and female Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

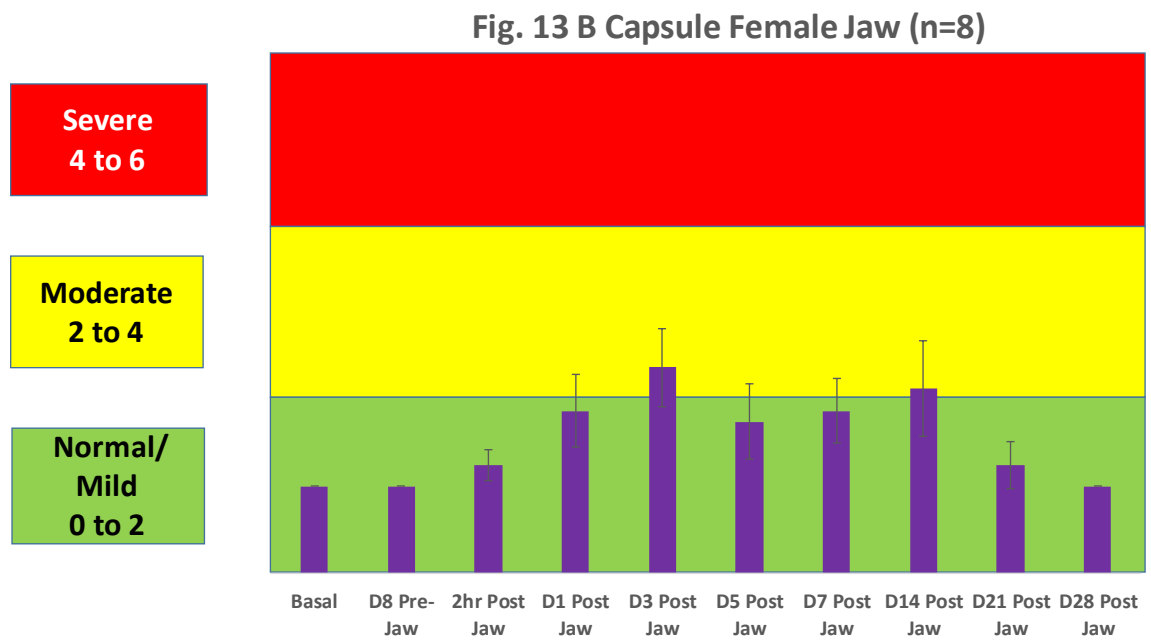
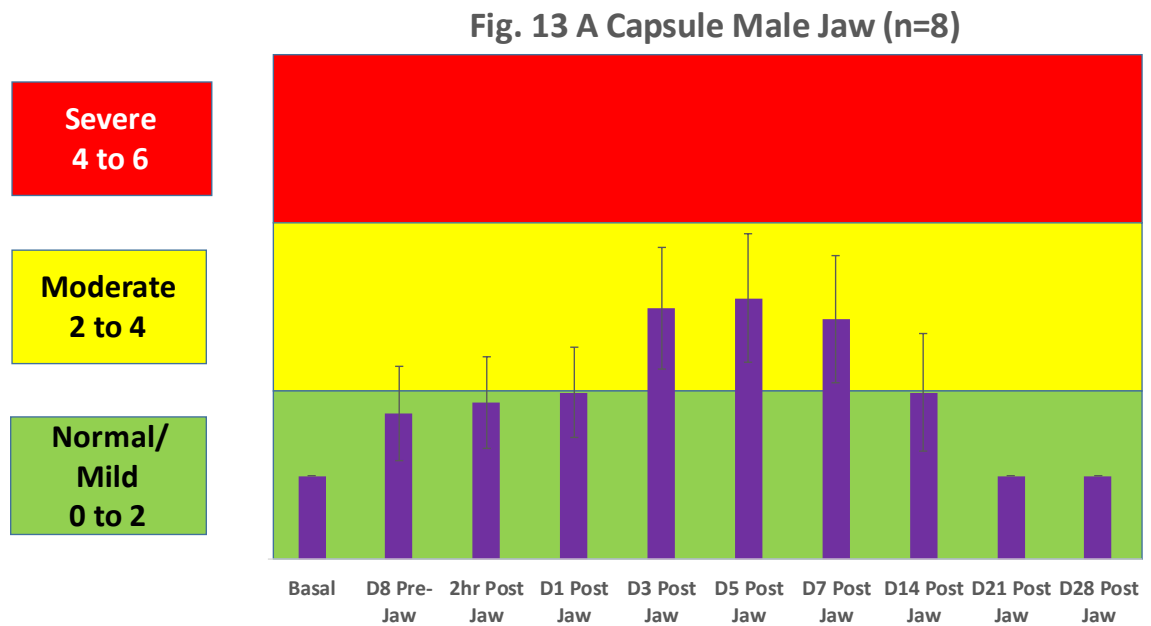


Fig 13. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the TMJ capsule in male and female jaw Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

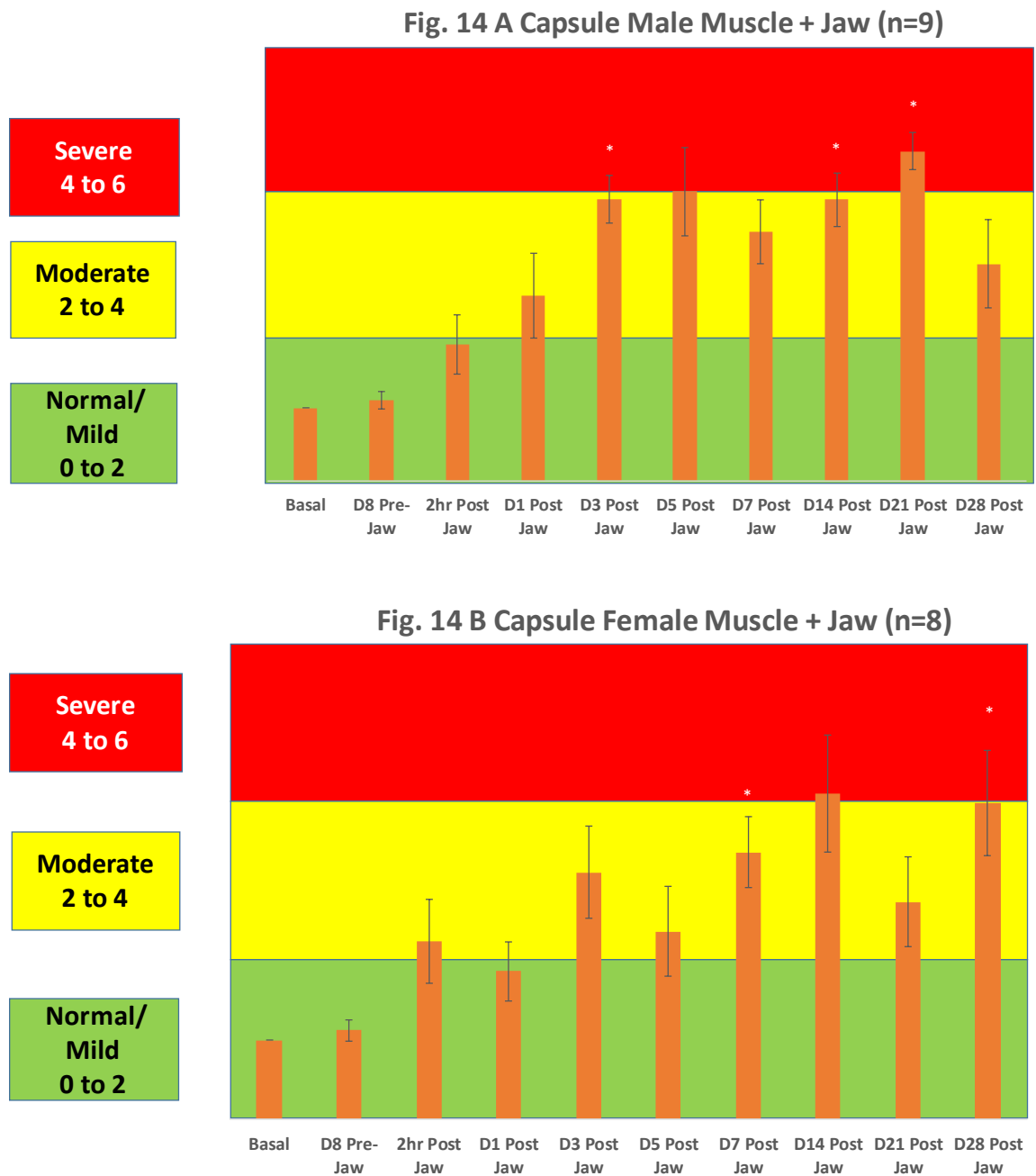


Fig 14. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the TMJ capsule in male and female muscle + jaw Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

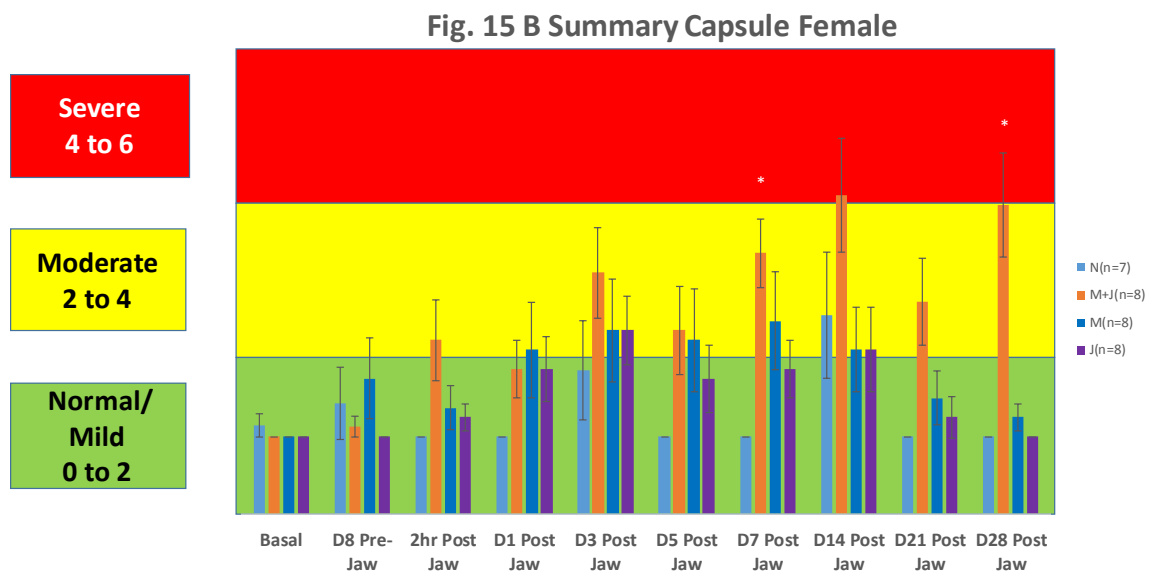
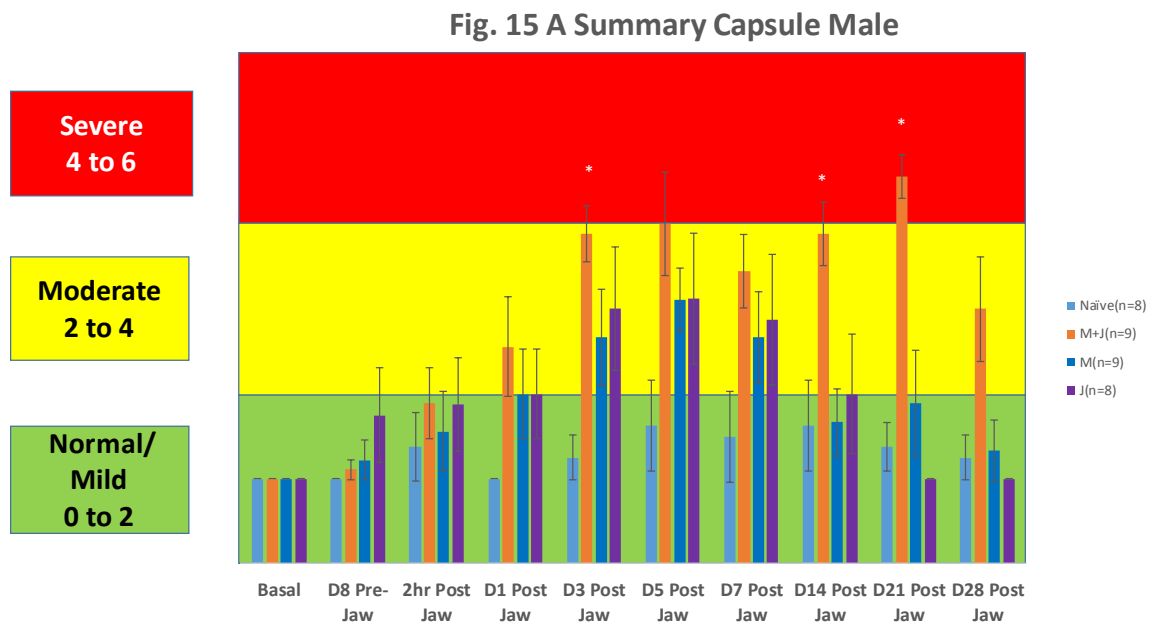


Fig 15. Summary of temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the TMJ capsule in male and female Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

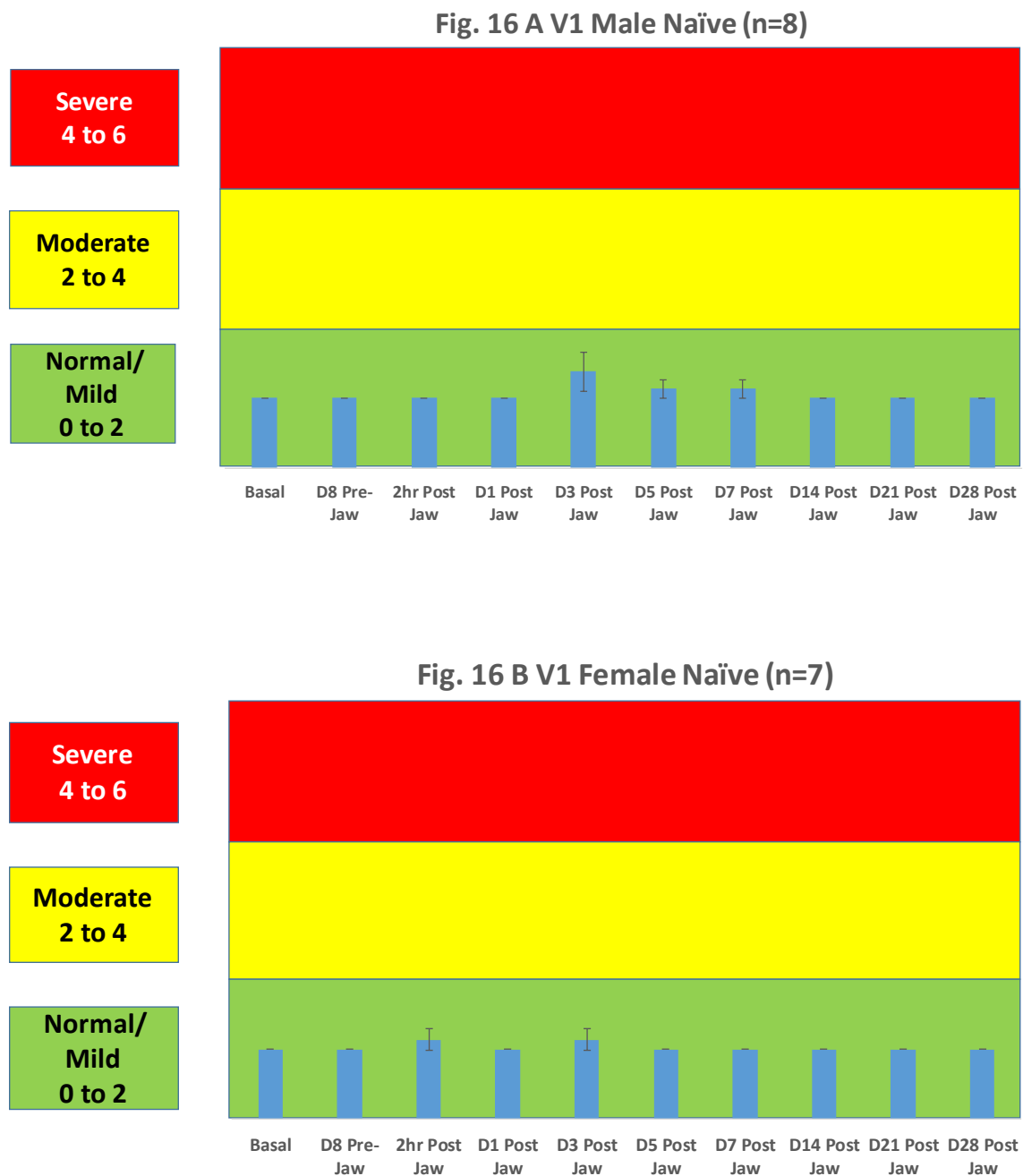


Fig 16. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the eyebrow region that is innervated by V1 trigeminal neurons in male and female naive Sprague-Dawley rats.

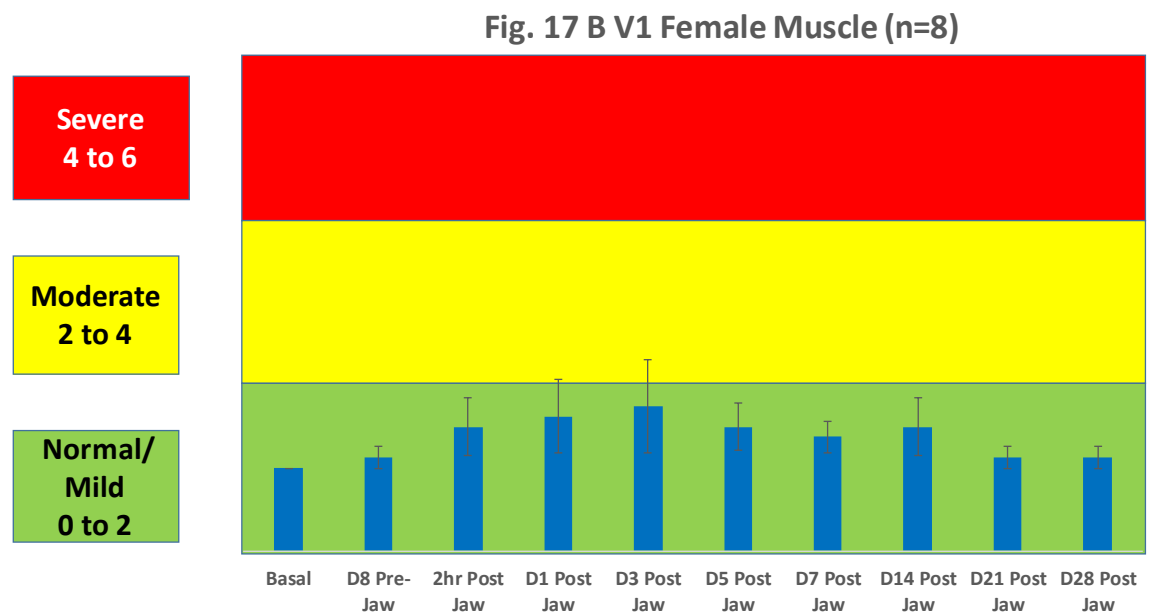
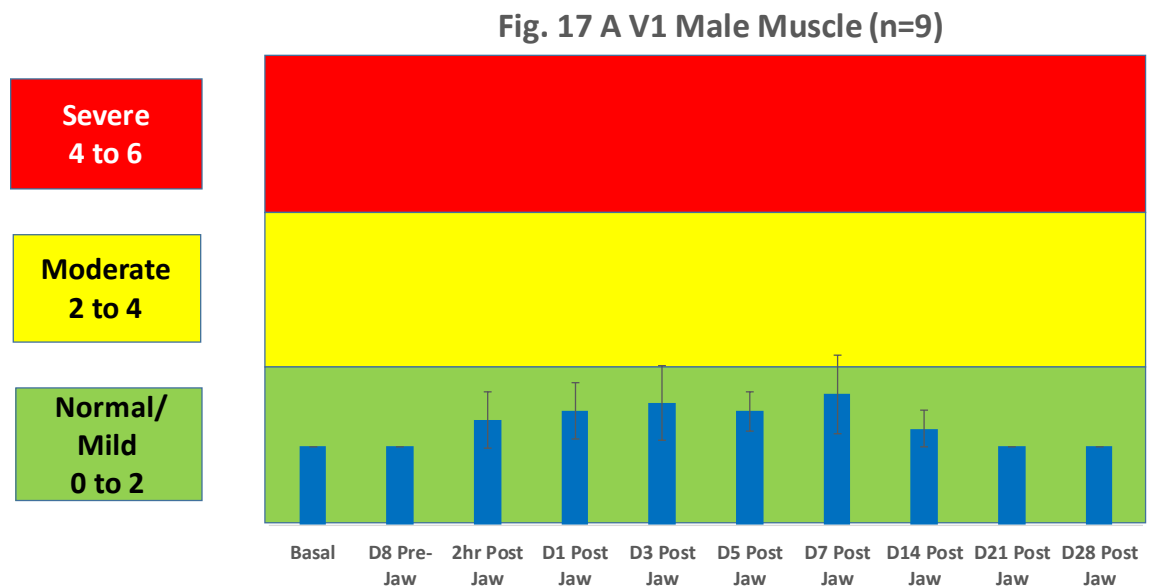


Fig 17. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the eyebrow region that is innervated by V1 trigeminal neurons in male and female muscle Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

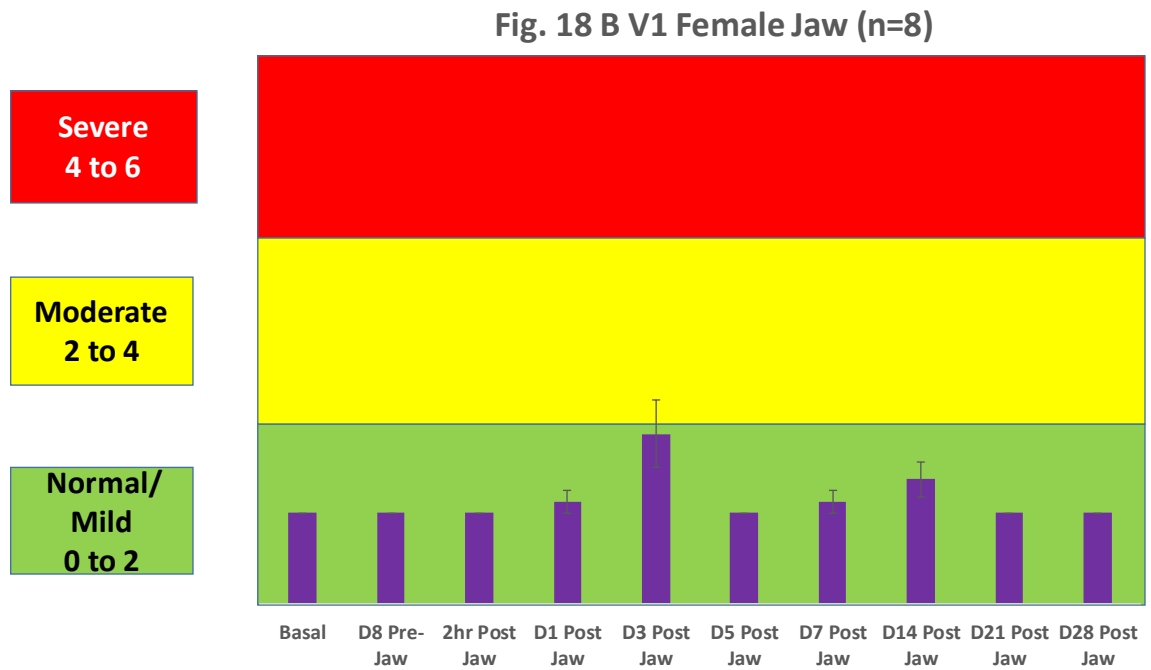
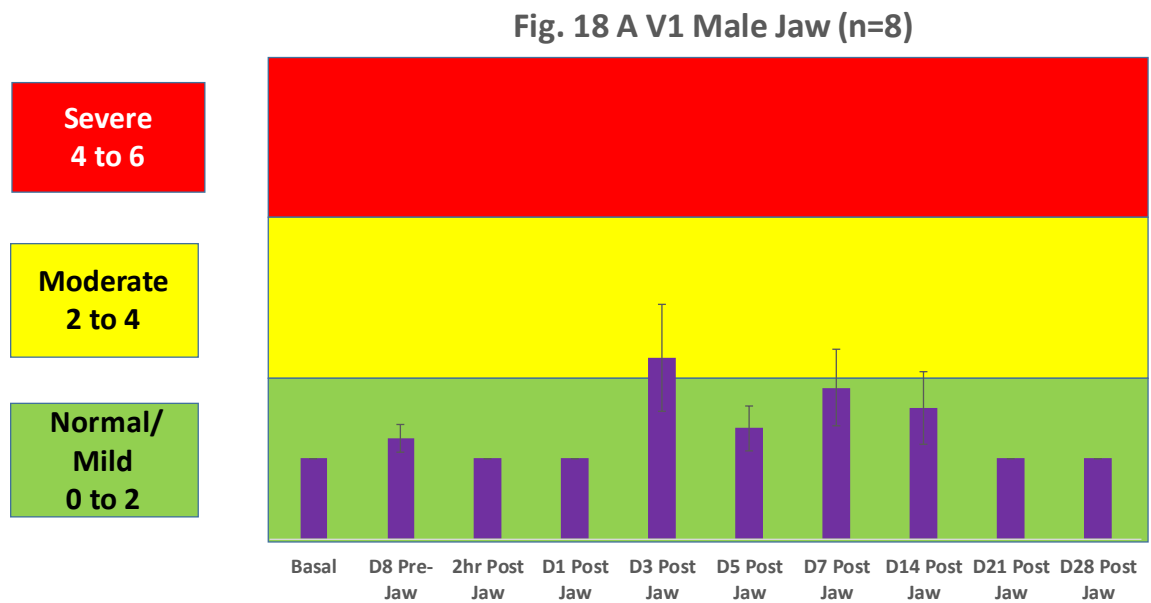


Fig 18. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the eyebrow region that is innervated by V1 trigeminal neurons in male and female jaw Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

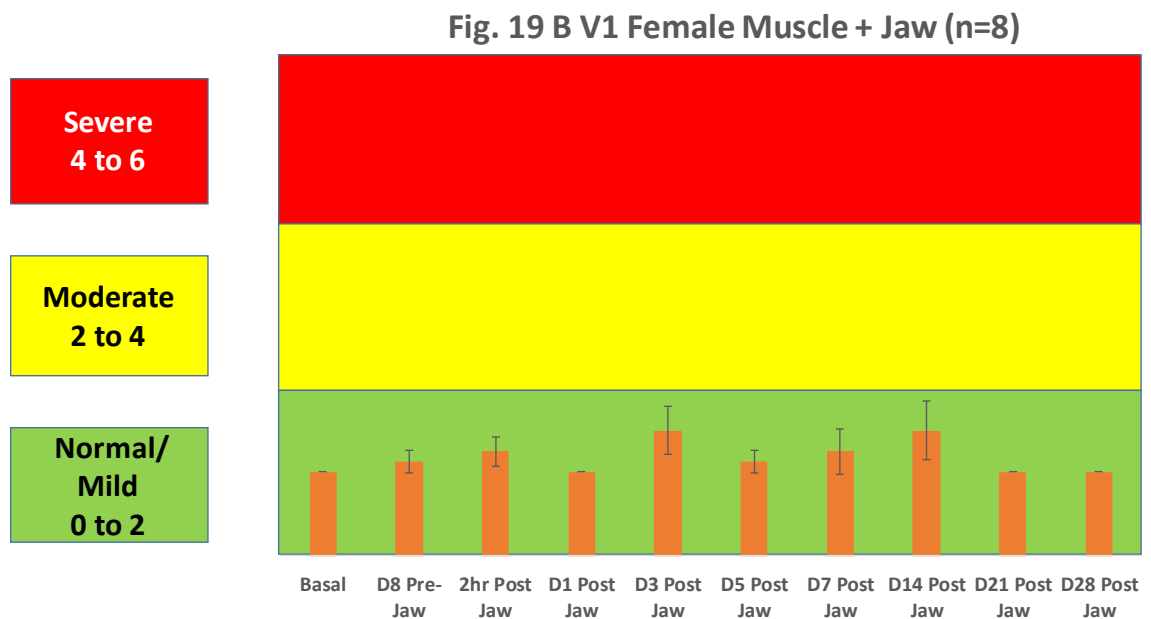
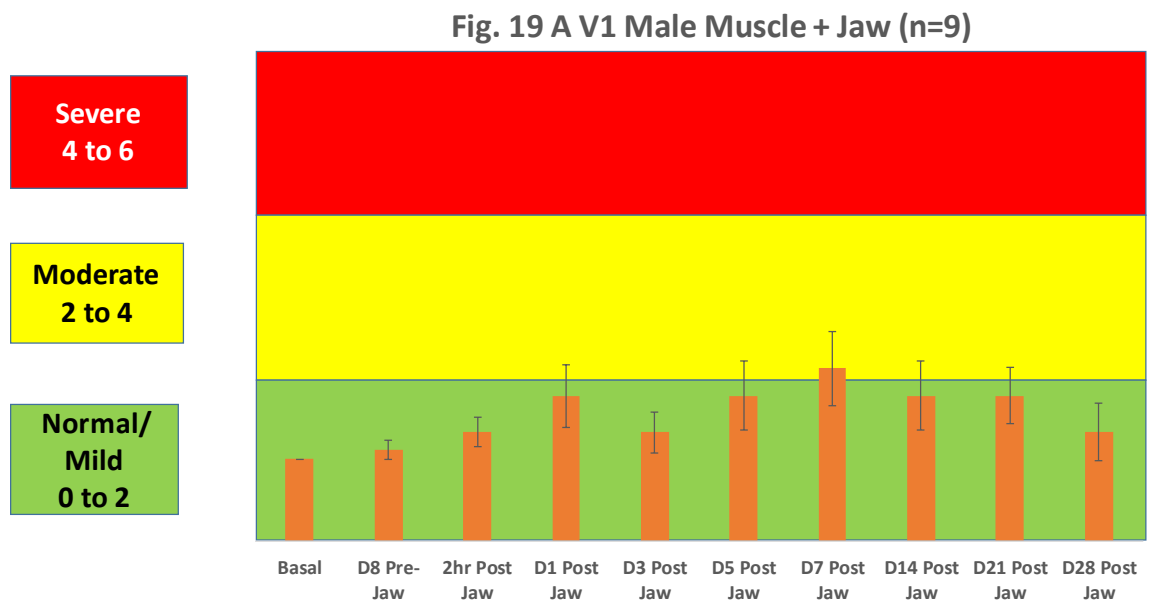


Fig 19. Temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the eyebrow region that is innervated by V1 trigeminal neurons in male and female muscle + jaw Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

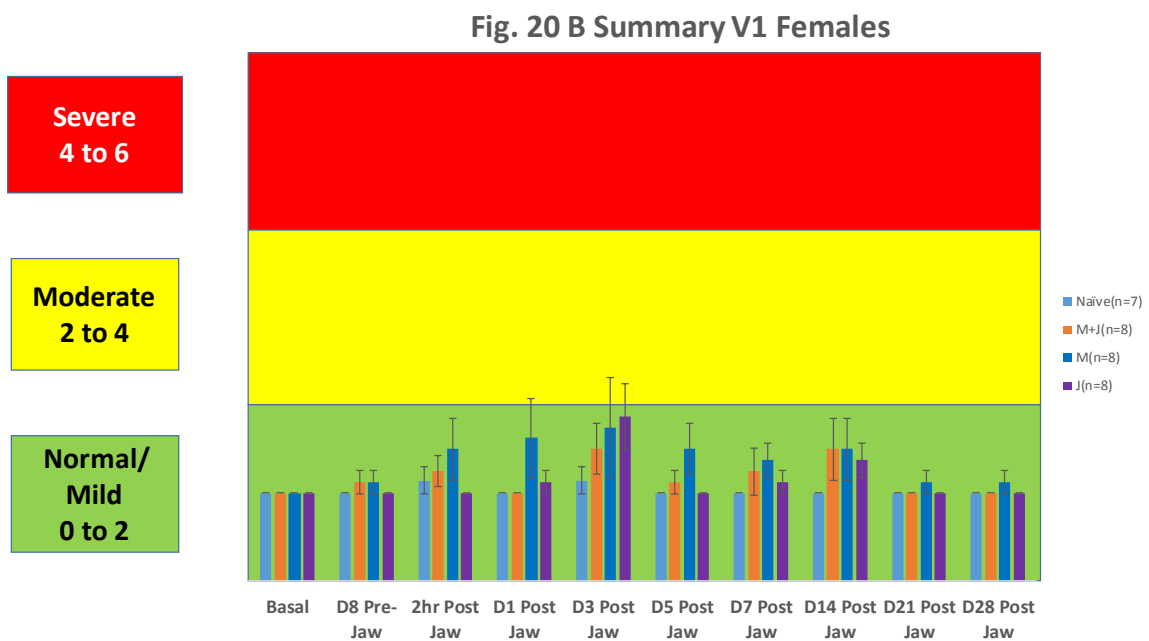


Fig 20. Summary of temporal change in nocifensive head withdrawal responses to mechanical pressure applied to the cutaneous tissue over the eyebrow region that is innervated by V1 trigeminal neurons in male and female Sprague-Dawley rats. Significant difference from the naïve animals is indicated by an asterisk.

DISCUSSION

A major novel finding from my study was that muscle inflammation prior to prolonged jaw opening was sufficient to promote development of a state of chronic nociception that was more severe and of greater duration in female animals. My results are in agreement with data from the NIH funded OPPERA study in which jaw injury, neck/shoulder muscle tension and tenderness, and female gender were strongly associated with development of chronic TMD in humans (Ohrbach et al., 2011a). Unfortunately, prolonged jaw opening, which can occur during yawning, or normal orthodontic and dental procedures, can lead to persistent inflammation and pain in the TMJ itself or in the surrounding muscles. The foundation for my study was based on a published procedure developed by the Durham lab that involved the use of a retractor to hold open the jaw of young adult male Sprague-Dawley rats to near maximal without causing subluxation of the joint (Hawkins and Durham, 2016). In that study, prolonged jaw opening for 20 minutes resulted in a sustained increase in the nocifensive response to mechanical stimulation of trigeminal neurons that resolved by around 14 days. In my study, I wanted to test the hypothesis that neck muscle tension caused by low-grade inflammation in the trapezius would act as a risk factor to increase the likelihood of a more severe and longer-lasting state of nociception. In addition, I thought that female animals may be more susceptible to both types of risk factors given the higher prevalence of chronic TMD reported by female individuals in the United States (Bonjardim et al., 2009). My findings could have important implications for dentists, orthodontists, and other health care professionals that provide dental care to patients. Based on my results, I would recommend that patients be evaluated for ongoing neck and shoulder tenderness/pain

prior to performing a dental procedure, especially if it will require near maximal jaw opening for a prolonged period of time such as during molar extractions. Being a dentist myself, I would recommend that if a patient is experiencing neck muscle stiffness/discomfort that the amount of time the mouth is held open to near maximum should be limited with sufficient rest periods to minimize harm to the TMJ and associated tissues. Furthermore, female patients should be carefully evaluated for ongoing neck muscle pathology and if severe enough, it may be prudent to not perform the procedure given the increased risk for developing chronic TMD pain. To my knowledge, my findings provide the first evidence to support the notion that neck muscle inflammation and female gender increase the risk of chronic TMD following prolonged jaw opening.

In agreement with my findings, neck muscle tension or tenderness to palpation is often reported as a risk factor for TMD, migraine, and other orofacial pain conditions that involve sensitization of trigeminal nociceptive neurons (Ohrbach et al., 2011b). Interestingly, skeletal muscles have been referred to as a silent organ since often times one does not even know there is ongoing tension and inflammation until someone touches the specific region and hits a taut band of muscle fibers. Results from my study provide evidence that low-grade sustained inflammation of the trapezius is sufficient to promote sensitization of trigeminal neurons. This level of inflammation did not appear to negatively affect the animal's ability to eat, drink, or move about the cage, but was associated with a lowering of the activation threshold of primary trigeminal nociceptors. A likely explanation for this sensitizing effect is that centrally-projecting nerve fibers from the dorsal root ganglion neurons innervating the trapezius localize to the same region in the upper spinal cord as the efferent fibers from all branches of the trigeminal

ganglion (Sessle, 1999). Thus, there is a convergence of sensory nerve fibers projecting from the trapezius and ganglion such that cross communication is possible to modulate the effect on second order nociceptive neurons and promote central sensitization. This type of interaction would facilitate a coordinated response to potentially harmful noxious stimuli. One of the molecules that likely plays a key role in promoting sensitization of the second order neurons is the neuropeptide calcitonin gene-related peptide (CGRP) (Seybold, 2009), which is released in the spinal cord upon activation of the dorsal root ganglion neuron providing innervation to the trapezius. Recent findings from our lab have demonstrated that elevated levels of CGRP can cause central and peripheral sensitization of trigeminal neurons via a protein kinase A dependent mechanism (Cornelison et al., 2016). Thus, bidirectional signaling with the upper spinal cord is likely responsible for the increased sensitivity of trigeminal neurons that provide innervation to the muscles, ligaments, and tendons involved in jaw movements. Based on my findings, I propose that unresolved neck muscle tension/inflammation would lead to a sensitized state of both spinal and ganglion trigeminal nociceptors, and hence an increased risk for development of a chronic pain state in response to prolonged jaw opening.

Females are more likely to seek medical treatment for orofacial pain conditions and report greater pain scores. In my study, I found that sensitized male and female animals were susceptible to prolonged nociception in the TMJ capsule and masseter following jaw opening but female animals exhibited a more consistently high level of nociception for a longer duration. With respect to TMD, females are more than 2 times more likely to report symptoms when compared to age matched males. While hormonal

differences related to the menstrual cycle may account for why the female rats were more sensitized (Nekora-Azak, 2004, Cairns, 2010), I did not observe changes in the level of sensitization in the naïve female animals during any time in my study. As an alternative explanation, the greater degree of nociception observed in the females following prolonged jaw opening may be due to anatomical differences in the structures of the jaw and associated tissues involved in mastication. In my model, near maximal jaw opening for 20 minutes may cause a more prolonged and severe form of injury to the ligaments and tendons and possibly even the masseter muscle given the smaller size of these structures. It will be of interest in future studies to investigate the relative level of inflammation in these tissues using the method involving Evan's Blue. The dye is injected in the tail vein and allowed to circulate for 10 minutes. Wherever there is ongoing peripheral inflammation characterized by protein plasma extravasation (leakage), this large dye will localize and then can be detected via visual observation and quantitated by measuring its absorbance with a spectrophotometer.

Another goal of my study was to compare the sensitivity level in tissues innervated by different branches of the trigeminal ganglion in response to prolonged jaw opening in sensitized animals. While both the TMJ and masseter muscle are innervated by the V3 or mandibular branch of the trigeminal ganglion nerves, the cutaneous tissue over the eyebrow is innervated by sensory fibers emanating from the V1 branch. Prior to my study, I had predicted that the nocifensive response would be similar in all the tissues given that results from prior studies had demonstrated cross-excitation of all the branches following activation in one branch (Thalakoti et al., 2007, Damodaram et al., 2009). However, the level of nociception was greatest over the masseter muscle with

comparable levels over the TMJ capsule, which supports the notion that neck muscle tension may be more closely associated with TMD pathology since the activity of the head and neck muscles needs to be coordinated during mastication. The rationale for studying changes in the sensitivity level in V1 neurons is based on reports that TMD pathology is often co-morbid with migraine (Ballegaard et al., 2008, Bevilaqua Grossi et al., 2009, Dahan et al., 2015). While prolonged jaw opening did not elicit a sustained nocifensive response from V1 neurons, it is likely that these nerves are still in a sensitized state characterized by a lower threshold of activation to other stimuli. In support of this notion, I conducted a pilot study using an extract of California Bay leaves, which is known to cause excitation of V1/V2 trigeminal neurons (Nassini et al., 2012), as a triggering factor in day 28 animals that were in a chronic pain state. In these animals, exposure to this pungent odor was sufficient to cause a robust nocifensive response that was greater in intensity when compared to even the neck muscle sensitized, jaw injury animals. Thus, results from my pilot study provided evidence that ongoing TMD pathology should be considered a risk factor for inducing V1 trigeminal activation, which is a key physiological feature of migraine attacks.

While I focused on understanding the changes in nociception in my model, there are many unanswered questions that would be of interest to pursue in future studies. In particular, immunohistochemistry could be used to determine cellular changes in the expression of key proteins in the trigeminal ganglion and spinal trigeminal nucleus that are implicated in peripheral and central sensitization, respectively. Proteins of interest that are likely to be upregulated in neuronal cells include CGRP, protein kinase A, and the MAP kinases – ERK, JNK, and p38 (Ji, 2004, Seybold, 2009). In addition, I would

expect that sustained activation of glial cells in the trigeminal ganglion and spinal cord will temporally correlate with increased nociception (Ji et al., 2013). Another area that would be of interest to explore would be the effects on biopsychosocial behaviors in my model of chronic TMD. Given the severity and duration of the nociceptive response, it is likely that female animals will exhibit decreases in cognitive function (novel object recognition), anxiety levels (elevated maze), and social interactions. Although not included in my thesis project, I helped to develop these assays in our laboratory and believe results from these studies would provide important translational information to clinicians. TMD is commonly characterized by comorbidities; including headache/migraine, back pain, widespread pain and fibromyalgia, and psychosocial challenges including depression, anxiety, and multiple nonspecific physical symptoms (Aaron et al., 2000, Dahan et al., 2015). Often TMD is characterized by impairment in daily activities, excess reliance on health care, and dependence on narcotic analgesics (Hersh et al., 2008). Thus, based on the study of Dworkin and colleagues (Suvinen et al., 2005), patients with TMJD should be evaluated according to both the physical disorder and the psychosocial illness impact factors. Only through using an integrated and multidimensional approach concerning physical and psychosocial factors in TMJ pain and dysfunction will successful management of TMD be achievable by reducing the disease burden. Furthermore, another area that would be important to investigate is the potential of a non-pharmacological therapeutic to decrease nociception as well as the disease burden mediated by prolonged trigeminal nociception.

Given the prevalence, significant morbidity, and major social and economic ramifications of TMD, there is a critical need for improved therapeutic and preventative

measures for TMD. Although much work has been done to identify the key molecules whose levels are elevated during TMJ and masseter inflammation and pain, few novel therapeutic strategies to treat TMD have resulted from these studies. Furthermore, chronic inflammatory pain associated with TMD can persist for months or even years after the primary tissue damage has healed, and current treatments with steroids and COX inhibitors have only produced limited relief of this pain in a portion of patients and are frequently associated with significant adverse effects (Cairns, 2010). A potential reason for the lack of specific therapies may be due to the limitations of the previous animal models of TMD pathology, which relied on injection of inflammatory agents in the masseter or TMJ capsule. Interestingly, none of the prior animal models results in a sustained or chronic state of nociception. In contrast, my model, which more closely mimics the pathophysiological events associated with human TMD, can be utilized in future studies to identify novel cellular targets for inhibiting the development of peripheral and central sensitization. I am particularly interested in knowing the effect of treatment with monoclonal antibodies against CGRP on nociception and behavioral responses in my model since they are in development for chronic migraine. However, there still remains a great need for the identification of novel modalities to more effectively manage the pathological events associated with TMD. One approach towards this goal is to utilize natural products to modulate CGRP levels in the spinal cord and ganglion. The use of nutraceuticals is increasing in the general population and health care providers are often questioned by patients about the use of more “natural” treatments. The use of natural products offers a novel method for preventing and treating chronic inflammatory diseases (Vuorelaa et al., 2004, Newman and Cragg, 2007). In support of

this notion, we have provided evidence that inclusion of GSE in the diet of rats was sufficient to cause upregulation of the anti-inflammatory protein MKP-1 and suppress CGRP expression in the STN, and repress the stimulatory effects of CFA injection into the TMJ on expression of active p38, OX-42, and GFAP (Cady et al., 2010). Results from our study provide evidence that dietary inclusion of GSE would be beneficial as a natural therapeutic option for TMJD by suppressing development of peripheral and central sensitization. Another therapeutic approach may involve the injection of small amounts of botulinum toxin type A (BOTOX) into the trapezius to determine if reducing the tension will prevent the development of a sensitized state of trigeminal neurons. In conclusion, I believe that my model will be useful for further understanding at the cellular level the transition from an episodic to a chronic pain state and provide a clinically relevant model for identification of novel therapies for managing TMD.

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