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Trigeminal Inflammatory Compression in Rats: A Novel Orofacial Pain Model

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Abstract

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Background: Testing novel therapeutics for trigeminal neuralgia requires reproducible small animal models of chronic orofacial pain. Currently, the models of chronic orofacial pain in rats can cause excessive surgically-related damage that is not necessary to study trigeminal neuralgia therapeutics. The trigeminal inflammatory compression (TIC) model of orofacial pain has the potential to induce chronic orofacial pain with minimal surgically-related damage. This is the first study to investigate the use of the TIC surgery to induce chronic orofacial pain in rats.

Methods: The TIC surgery used in this study involved the placement of a length of chromic gut suture in direct and parallel contact with the infraorbital nerve of experimental rats. Following TIC surgery, increased sensitivity and pain-induced anxiety were characterized. Neuroinflammation of the infraorbital nerve in contact with chromic gut suture was quantified by macrophage and proinflammatory cytokine markers.

Results: Placement of the chromic gut suture in direct and parallel contact with the infraorbital nerve was correlated with reproducible mechanical allodynia in experimental rats. No evidence of pain-induced anxiety caused by the TIC surgery was detected by Light and Dark Box test during the ten week period of the study. Neuroinflammation caused by the TIC surgery was not detected by the presence of macrophages and interleukin-1β at ten weeks post-surgery.

Conclusion: The TIC model in rats is a promising model of chronic orofacial pain. With improved surgical placement of the chromic gut suture and additional behavioral assays of pain, the TIC model will be fully characterized by multiple dimensions of pain and may become a viable model to test novel therapeutics for trigeminal neuralgia.

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Table of Contents

Introduction 1
Pain management and Trigeminal Neuralgia Research 1
Path of the Trigeminal Nerve: Whisker Pad Innervation to Brainstem 5
Animal Models of Chronic Orofacial 7
Neuroinflammation Mechanisms for Neuropathic Pain after Nerve Damage 14
Neuropathic Pain and Pain-Induced Anxiety 18
Behavioral Assays for Pain-Induced Anxiety 21
Trigeminal Inflammatory Compression Model In Rat Study Aims 23
Medical Ethics 24
Materials and Methods 27
Animals 27
Surgery 27
Light and Dark Box Test 28
Von Frey Filaments Test 29
Tissue Preparation 30
Immunohistochemical Analysis 31
Statistical Analysis 32
Results 33
Von Frey Hair Testing 33
Light and Dark Box Test Data 34
Immunohistological Staining of Trigeminal Nerve Tissue 38
Comparison of the Placement of Chromic Gut Suture Ten Weeks Post Surgery With Phenotypic Pain Response 41
Discussion 43
Von Frey Test and Mechanical Allodynia 44
Comparison of Von Frey Data to Alternative Chronic Orofacial Pain Models 44
Light and Dark Box 49
Comparison of Light and Dark Box Testing to Previous Models of Pain 50
Light and Dark Box Test: Need for More Testing 52
The Influences of Chronic Stress and Anxiety on the Pain Perception of Rats 54
Potential Chronic Stress Experienced by Rats in Study 54
Potential Anxiety Experienced by Rats Used in Study 55
Potential Effects of Chronic Stress and Anxiety on Pain Perception 56

Potential Effects of Chronic Stress on Pain Perception via Hypothalamic-Pituitary-Adrenal (HPA) Axis Dysfunction. 57
Potential Effects of Stress on Von Frey Testing 58
Potential Effects of Anxiety on LDB Testing 58
Post-Operative Pain due to TIC Surgery 58
Neuroinflammation in the TIC model in Rats 60
Future Directions 62
Improvements to TIC Surgery 62
Improvements to Von Frey Testing 62
Improvements to LDB Testing 62
Improvements to Understand the Chronic Impact of Chromic Gut Suture on the ION 63
Proposed Alternative Behavioral Assays 63
Summary and Conclusion: 66
Bibliography 68

Tables and Figures

Figure 1. Path of Trigeminal Nerve From Whisker Pad to Foramen Rotundum. 5
Figure 2. Path of Trigeminal Nerve From Whisker Pad to Foramen Rotundum 6
Figure 3. Path of Trigeminal Nerve From Whisker Pad to Brainstem. 7
Table 1. Von Frey Testing for Mechanical Allodynia: Threshold Filament Weight for Positive Behavioral Response (grams). 33
Figure 4. The Percentage of Experimental Animals that Demonstrated Positive Responses to Von Frey Testing From Baseline to POW10 34
Light and Dark Box Test Data 34
Figure 5. Light and Dark Box Test for Anxiety: Time In Light Compartment (Seconds). 36
Figure 6: Light and Dark Box Test for Anxiety: Number of Transitions between the Dark and Light Compartment. 37
Immunohistological Staining of Trigeminal Nerve Tissue 38
Figure 7. IL-1β Staining of Infraorbital Nerve Tissue 39
Figure 8. OX-42 Staining of Infraorbital Nerve Tissue 40
Figure 9. Dissection of the Infraorbital Nerve 42

Introduction

Pain management and Trigeminal Neuralgia Research

Current approaches to chronic pain management in developed countries provide on average unsatisfactory therapeutic value to patients (Borsook & Kalso, 2013; van Hecke, Torrance, & Smith, 2013). Frequently using a "trial and error" approach, physicians will prescribe a variety of pharmaceutical drugs for patients suffering from chronic pain until a drug provides a noticeable decrease in pain, or analgesia, without intolerable side effects (Borsook & Kalso, 2013). Still, even efficacious pharmaceutical drugs cannot provide significant benefit for the majority of chronic patients (Braune, 2004). Even as an often prescribed therapeutic for chronic pain, opioid analgesics have only demonstrated short-term efficacy for pain relief in standardized and epidemiological studies (Ballantyne & Shin, 2008). Another shortcoming of opioid intervention is their loss of analgesic efficacy over time despite dose escalation to overcome pharmacologic tolerance (Ballantyne & Shin, 2008). Moreover, in some patients exposure to opioids can cause increased sensitivity to painful stimuli and adverse effects in the gastrointestinal system outside of the central nervous system (Lee, Silverman, Hansen, Patel, & Manchikanti, 2011; Pappagallo, 2001). The management of chronic pain presents a complex therapeutic burden that increasingly challenges clinicians to provide effective treatments for a diverse patient population (Borsook & Kalso, 2013). Since the current standard approach to pain management is a "trial and error" process, research investigating the neural mechanisms that mediate different chronic pain syndromes will help the development of targeted therapies specific to each patient's condition (Borsook & Kalso, 2013).

Improved interventions must start at the level of treating nociceptive pain and neuropathic pain conditions with different approaches. Nociceptive pain requires a noxious

stimulus to activate sensory receptors called nociceptors that subsequently signal a neuronal pathway to mediate a pain sensation to the central nervous system (CNS) (Brown, 2014). Aδ and C fibers are primary afferent fibers that, along with other fiber types, comprise sensory nerve bundles and mediate these initial pain sensations to the CNS (Basbaum, Bautista, Scherrer, & Julius, 2009). In contrast, neuropathic pain is caused by the injury or dysfunction of the somatosensory system (Brown, 2014). Since neuropathic pain is the result of damage to the somatosensory system and not the direct activation of nociceptors, neuropathic pain will have unique symptomatology compared to nociceptive pain. For example, abnormal sensory perception conditions, such as hyperalgesia and allodynia, are typically associated with neuropathic pain (Bridges, Thompson, & Rice, 2001). Hyperalgesia is the increased pain response to a noxious stimulus, whereas allodynia is the presence of a pain response to a normally non-noxious stimulus (Bridges et al., 2001). Both nociceptive pain and neuropathic pain conditions should require treatment specific toward the source of the pain response. As a prevalent chronic neuropathic pain syndrome, trigeminal neuralgia is the subject of therapeutic research targeted to resolve the specific etiologies of the neuropathic pain syndrome.

Trigeminal neuralgia (TN) is a chronic facial pain condition that is mediated by aberrant activity of the trigeminal nerve (NINDS, 2015). The trigeminal nerve is the fifth cranial nerve that innervates the face and primarily mediates sensory information, including pain and light touch (Vanderah, Gould, & Nolte, 2016). Patients suffering from type 1 TN experience episodic and sudden facial pain (NINDS, 2015). Alternatively, patients suffering from type II TN experience a constant aching and possibly burning facial pain (NINDS, 2015). As a neuropathic pain condition, TN also causes patients to experience facial hyperalgesia and allodynia (Stavropoulou, Argyra, Zis, Vadalouca, & Siafaka, 2014). In addition to pain symptoms, TN

patients also present with anxiety and depression (Macianskyte, Januzis, Kubilius, Adomaitiene, & Sciupokas, 2011). Due range of symptomatology, there is increasing interest into the pathophysiology of TN.

Classically, TN is caused by the compression of the trigeminal nerve in the region as it leaves the pons in the brainstem (Montano et al., 2015; NINDS, 2015). Compression of the trigeminal nerve causes progressive and focal demyelination of nerve fibers (NINDS, 2015). Compression of the trigeminal nerve is most commonly caused by abnormal positioning of vasculature, and less often as a result of tumorigenesis (Montano et al., 2015). Both compression and demyelination of trigeminal nerve fibers have been proposed to explain the resultant increased excitability and irregular neuronal firing associated with TN (Love & Coakham, 2001). Studies have shown that demyelinated axons can cause spontaneous ectopic neuronal firings (Love & Coakham, 2001). Ectopic firings by trigeminal neurons are a likely cause for sudden pain episodes experienced by type 1 TN patients. Furthermore, the close proximity of compressed demyelinated axons allows for ephatic coupling between light touch and pain fibers (Love & Coakham, 2001). Ephatic coupling, or cross talk between neurons, is the communication of electrical signals between the axons of excitable neurons in close contact to each other (Ramon & Moore, 1978). Ephatic coupling between pain and light touch fibers may explain the painful sensations after light facial touch or allodynia experienced by TN patients. Despite on-going research into the pathophysiology of TN, physicians can still help patients with TN manage and alleviate the painful symptoms of the condition.

Although there exists a range of current treatments for TN patients, novel therapeutics are being researched to provide direct and effective treatments with minimal side effects. Initially, TN patients are prescribed pharmaceutical agents to treat pain symptoms non-specific to TN.

However, the non-specific nature of pharmaceutical agents increases the chance of adverse effects and potential drug interactions in patients. The only direct medical interventions are invasive surgical treatments to decrease aberrant trigeminal nerve activity and alleviate TN pain (Montano et al., 2015). Novel advancements in the field of TN treatment continue to focus on treating specific etiologies of TN with minimal invasiveness. Moreover, potential TN treatments must be tested using specific pre-clinical animal models of TN to successfully understand a basal level of the novel treatment's efficacy.

In pre-clinical trials, the efficacy of novel therapeutics for TN must be proven in small animals models. Currently, animals models for TN focus upon inducing neuropathic pain via the chemical and/or physical manipulation of the maxillary branch (V2) or infraorbital nerve (ION) of the trigeminal nerve. In rodents, the ION provides the major innervation for the whisker pad, upper teeth, and dorsal oral cavity. These models of chronic orofacial pain can be evaluated on the basis of duration of pain as indicated by behavioral assays, ability to reproduce classical neuropathic pain symptoms, and risks of manipulating the ION to determine efficacy and safety as a model. In order to properly determine the long-term efficacy of novel therapeutics on chronic orofacial pain, the model used should demonstrate a stable state of induced pain preferably at least 4 weeks, as determined by a review of novel therapeutic research in chronic neuropathic pain in animal models (Deseure, Koek, Adriaensen, & Colpaert, 2003; Deseure, Koek, Colpaert, & Adriaensen, 2002; Hao, Mata, Goins, Glorioso, & Fink, 2003; Meunier et al., 2005). Currently, there are several small animal models of chronic neuropathic orofacial pain that utilize manipulation of the ION. An appropriate model of neuropathic pain should demonstrably elicit hyperalgesia or allodynia or both. Of further interest, a chronic neuropathic orofacial pain model should be characterized by pain-induced anxiety since anxiety experienced

by TN patients as well. In order to properly understand the techniques used in these models to elicit neuropathic pain, the anatomy of the trigeminal nerve must be properly known.

Path of the Trigeminal Nerve: Whisker Pad Innervation to Brainstem

In rodents, sensory fibers that innervate the whisker pad move posteriorly and converge to form the ION prior to entering the infraorbital foramen (Fig. 1). The ION moves posteriorly through the infraorbital foramen to the orbit of the skull. Within the orbit, the ION follows a path posteriorly and medially through the infraorbital groove, which is inferior to the globe of the eye, and into the foramen rotundum (Fig.1). After moving through the foramen rotundum, the ION enters the cranium of the skull. Once in the cranium of the skull, the ION converges with other branches of the trigeminal nerve to form the trigeminal ganglion (Fig. 2), where the trigeminal neuron cell bodies are located but remain outside the CNS. From the trigeminal ganglion, the nerve fibers move posteriorly toward the brain and synapse onto the trigeminal nuclei in the brainstem finally entering the CNS (Fig. 2). The complete dissection of the trigeminal nerve from the brain to the whisker pad is shown in Figure 3 for further reference.

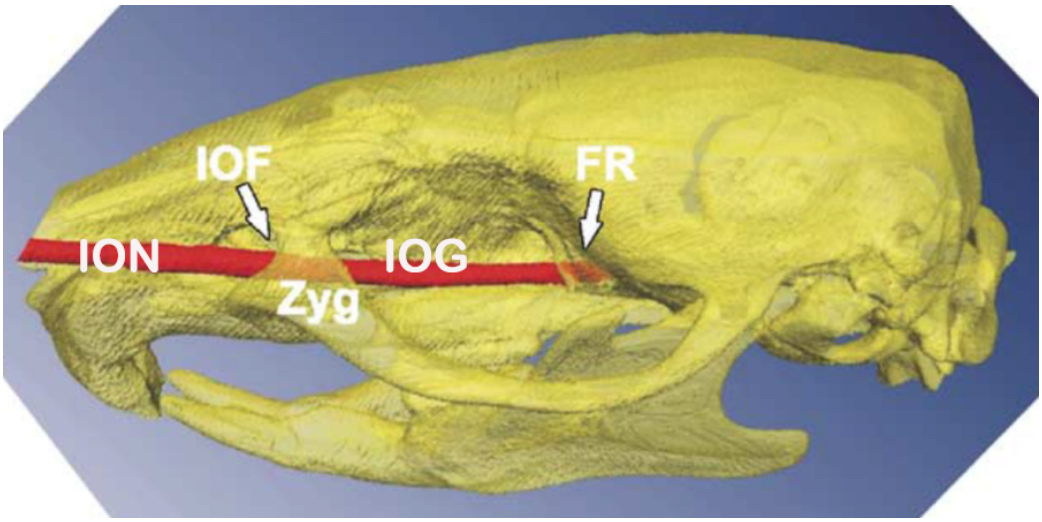


Figure 1. Path of Trigeminal Nerve From Whisker Pad to Foramen Rotundum.
Adapted From J.K. Neubert et al. / Brain Research Protocols 15 (2005) 119–126
Abbreviations Used: ION = Infraorbital Nerve; IOF = Infraorbital Foramen; Zyg = Zygomatic Arch; IOG = Infraorbital Groove; FR = Foramen Rotundum

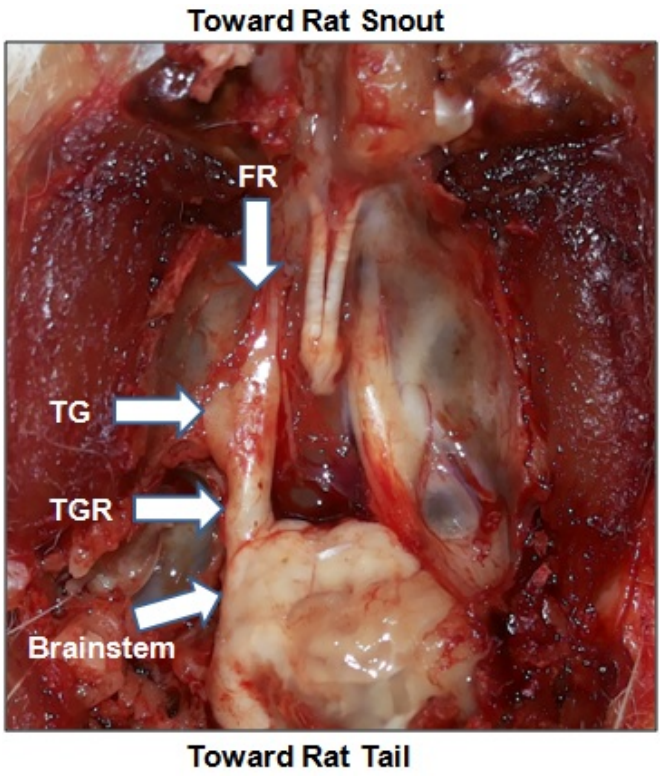


Figure 2. Path of Trigeminal Nerve From Whisker Pad to Foramen Rotundum.
Adapted From Photograph Dissection of the Trigeminal Nerve by Dr. Sukreet Raju, Boulis Lab.
Abbreviations Used: FR = Foramen Rotundum; TG = Trigeminal Ganglion; TGR = Trigeminal Ganglia Nerve Root

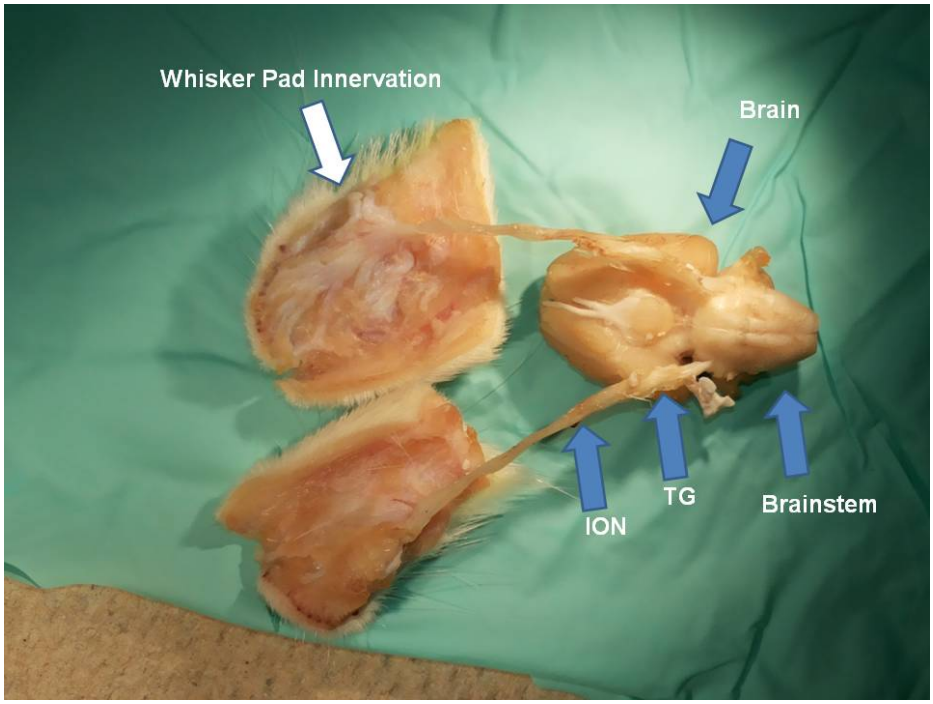


Figure 3. Path of Trigeminal Nerve From Whisker Pad to Brainstem.
Adapted From Photograph Dissection of the Trigeminal Nerve by Dr.Sukreet Raju, Boulis Lab.
Abbreviations Used: ION = Infraorbital Nerve; TG = Trigeminal Ganglion

Animal Models of Chronic Orofacial

Researchers have demonstrated the induction of chronic orofacial pain by injecting lysophosphatidic acid (LPA) or cobra venom into the ION (Ahn et al., 2009; An et al., 2011). Both chemically-induced models demonstrated behavioral indications of chronic pain lasting beyond 60 days. Studies have shown that LPA mediates the initiation of neuropathic pain via the LPA-1 receptor, causing demyelination of neurons (Inoue et al., 2004; Ueda, 2008). Similarly, cobra venom has been shown to cause demyelination of neurons (Zhu, Xu, Rong, Ben, & Gao, 2004) and interrupt nerve conduction via its major components cardiotoxin and phospholipase A_2 (Chang, Chuang, Lee, & Wei, 1972). These chemical agents are distinct from other inflammatory agents, such as Complete Freund's Adjunct and formalin, which interact with primary sensory neurons to elicit an inflammatory response and do not damage the neuron directly through acute exposure. Although these chemically-induced models of orofacial pain elicit behavioral and

biochemical evidence of neuropathic pain, these models do not mimic the vascular compression seen in clinical TN. Thus, the relevant physiological and pain inducing effects of nerve compression are absent in these models. Furthermore, biodistribution studies of LPA and cobra venom after injection into the ION have not been extensively conducted. Biodistribution studies would elucidate other potential tissue effects these chemical could generate. Without such studies, it is possible for these chemical agents to have off-target effects to generate pain in other areas of the rat unrelated to neuropathic pain generated from damage to the ION. This unrelated pain would be indistinguishable from the neuropathic pain of the ION, and any resulting behavioral testing to rat subjects could not conclusively relate pain-related behaviors demonstrated to be a consequence of damage to the ION. Consequently, there are small animal models that have experimented with the use of pure nerve compression of the ION in rats.

Current rat models of pure nerve compression of the ION induce a pain-state mimicking TN. However, the available rat models do not elicit an extended chronic pain state and put the rats at risk for nervous system damage beyond the ION, even though this damage may not interfere with pain sensory perception. The use of a small round plastic filament to compress the trigeminal nerve root has been studied (Luo et al., 2012). However, the insertion of this plastic filament involves advancing the filament through the foramen rotundum until the filament is within approximately 0.3 cm of the pons (Luo et al., 2012). Starting from the trigeminal ganglion in the brainstem, the ION nerve must pass through the foramen rotundum before innervating the whisker pad and other facial areas (Fig. 2). Procedurally, the depth that the filament would be positioned to avoid the pons was based upon an average distance from the entrance of the foramen rotundum (Luo et al., 2012). Thus, this procedure poses the risk for unnecessary harm for the pons of each rat due to slight anatomical differences between rats and increased

probability for surgical error given the extremely precise nature of the blind filament placement. Additionally, the filament compression model demonstrated behavioral indications of pain only lasting up to 28 days, a short period to evaluate the effects of novel therapeutics (Luo et al., 2012). Alternatively, the nerve compression effects of a 4% agar solution injection into the dorsal surface of the trigeminal nerve root have been studied (Jeon et al., 2012). In terms of longevity of pain duration, the agar model demonstrates behavioral indicators of pain up to 40 days (Jeon et al., 2012). Still, like the filament model, the agar solution model also poses the risk of central nervous system damage. The agar solution was injected using an implanted cannula (Jeon et al., 2012). However, the implantation of the cannula damaged portions of the brain including the cortex (Jeon et al., 2012). Since these pure nerve compression models discussed involved the potential damage to portions of the brain or midbrain that may be critical to pain detection, these models have the potential to cause greater damage to the rats than necessary for conducting research for TN. As with the chemical models, any additional damage could produce a source of pain not related to damage of the ION and inhibit pain signals via the ION. In order to cause direct compression of the ION nerve for localized damage, researchers in the field have predominantly utilized the chronic constriction injury of the ION (CCI-ION) model to study TN.

The CCI-ION model induces localized neuropathic pain via the ligation of the ION by tying two separate chromic gut sutures around the nerve spaced approximately 3 to 5 mm apart. The CCI-ION model was originally an adapted model of sciatic neuropathic pain, which involved the chromic gut suture ligation of the sciatic nerve (Bennett & Xie, 1988). Typically, chromic gut suture is used for the nerve ligations of the CCI-ION model. Chromic gut suture was initially shown in chronic constriction of the sciatic nerve to induce increasing sensitivity to painful stimuli as the diameter of the suture increased (Maves, Pechman, Gebhart, & Meller,

1993). Comparatively, chromic gut suture ligation produces a greater damage to the nerve in question compared to ligation by plain gut or silk suture (Kajander, Pollock, & Berg, 1996; Maves et al., 1993; Robinson & Meert, 2005). The increased efficacy of the chromic gut suture ligation to produce neuropathic pain is attributed to the release of chromium salts/ions and pyrogallol (Maves et al., 1993; Shapiro & Risbud, 2014). Chromium gut salts have been associated with the activation of Schwann cells, macrophages, and microglia (Shapiro & Risbud, 2014). Additionally, there is speculation that a possible reduction in pH caused by chromium salts near the ligated nerve may activate capsaicin-sensitive afferents in the nerve (Maves et al., 1993). Unpublished data have suggested that infusions of pyrogallol to the sciatic nerve cause thermal hyperalgesia (Maves et al., 1993). No studies have been performed to evaluate how the chromic gut suture specifically affects the structure and function of the ION nerve after ligation. Still, the induction of mechanical hyperalgesia and allodynia by chromic gut suture ligation of the ION has been relied upon by numerous studies of TN in small rodents. Yet, the multiple variations of the CCI-ION model still cause unnecessary harm to animal subjects used in this research.

Despite demonstrating reproducible and long-term chronic orofacial pain, the basic CCI-ION model causes great disruption to the anatomical structures and tissues surrounding the ION in rat. The CCI-ION model, using chromic suture ligation, was originally an adaptation of the CCI model for the sciatic nerve (Vos, Strassman, & Maciewicz, 1994). The initial CCI-ION model demonstrated the induction of mechanical allodynia in the whisker pad region up to a remarkable 80 days (Vos et al., 1994). This specific CCI-ION model will be referred to as the Vos CCI-ION model. The original procedure for the CCI-ION model ligated the ION as it passed from the foramen rotundum anteriorly under the globe of the eye and through the infraorbital

foramen (Vos et al., 1994). The CCI-ION procedure involves breaking and removal of the zygomatic bone, maxillary, frontal, and lacrimal bones of the face to expose the ION (Gregg, 1973; Vos et al., 1994). After these facial bones have been dissected away, the eye and other contents of the orbital cavity must be temporarily moved to gain full access to the ION for ligation. Finally, ligation of the nerve in the Vos CCI-ION model requires that the ION be retracted and stretched to an extent in order for the sutures to be placed properly (Vos et al., 1994). The damage to the facial bones of the rat can cause pain in the rats, extending any period of post-surgical pain. Potential damage caused by over manipulation of the orbital cavity could cause long-term harm to the sight of the rats. After the CCI-ION surgery, rats experienced chromorrhea, or red tears, hypersalivation, and scratch marks on the whisker pad region (Vos et al., 1994). Furthermore, the CCI-ION model requires excessive manipulation of the ION simply due to the surgical procedure alone and could potentially be unrelated to the principle methods the model proposes to generate neuropathic pain.

Retraction of the nerve may result in neuronal injury beyond the intended injury due to ligation. Retraction of the ION nerve in this region can cause harm to the anterior ethmoid nerve, which passes perpendicular to the ION as the ION passes through the inferior orbital fissure toward the infraorbital fissure (Kernisant, Gear, Jasmin, Vit, & Ohara, 2008). Damage to the anterior ethmoid nerve may cause sensory deficits to the nose and anterior internal nasal passages, affecting routine behaviors by the rat subjects (Rybka & McCulloch, 2006). Excessive retraction of the ION can cause unintentional axonotmesis, a class 2 nerve injury, and Wallerian degradation (Belzberg, 2006). Axonotmesis is a nerve injury that disrupts the continuity of neuronal axons and axoplasmic flow of the neuron without disrupting the endoneurium of the nerve (Belzberg, 2006). If axonotmesis occurs in the ION to a considerable degree that break

axons in the nerve, Wallerian degradation, or the distal degeneration of the axon after transection, may occur, resulting in a loss of sensory function by the ION (Sircar, 2008). If excessive retraction causes a sensory deficit in the ION innervated whisker pad, any resulting behavioral assays assessing pain sensitivity in the region will be conducted to no avail. To reduce the degree of nerve retraction as well as the movement of orbital contents, a curved syringe needle has been used to aid in the placement of suture around the ION during ligation (Kernisant et al., 2008). However, the use of curved syringe needle requires that the curvature of the needle be close to 90 degrees and variations of the syringe needle curvature have been stated to increase changes of injury to the surrounding anatomy of the ION (Kernisant et al., 2008). This potential for variation with instrumentation poses increased surgical complexity to the CCI-ION surgery. Variations of the original Vos CCI-ION surgery that avoid many of the specifically stated potential sources of damage to rat subjects have been used in neuropathic pain research.

Alternative CCI-ION models to the Vos CCI-ION model still face challenges to inducing a chronic neuropathic pain state in rat subjects without collateral damage to surrounding structures. Rather than approach access to the ION proximal to the infraorbital fissure, ligation of the ION distal to the infraorbital foramen has been performed using a midline incision of the snout (Henry, Freking, Johnson, & Levinson, 2007). Since this more distal ligation of the ION is also distal to the eye, there is no movement of contents in the orbit during the procedure. However, an incision into the midline of the snout near the whisker pad may disrupt sensory function in the area, limiting the efficacy of behavioral assays that measure sensitivity of the region to determine pain related to ligation of the ION. Additionally, distal ION ligation compresses a lesser number of ION fibers since the nerve will branch to innervate the whisker pad and nose after exiting the infraorbital foramen. Thus, distal ION ligation may affect fewer

ION branches, which are insufficient to induce a state of neuropathic pain. In order to limit damage to region innervated by the ION, an intra-oral approach to access the distal region of the ION for ligation has been used (Imamura, Kawamoto, & Nakanishi, 1997) . However, the use of an incision inside the mouth to approach the ION inferiorly risks directly altering the feeding habits of the rat subjects. Given the current studies inducing neuropathic pain via manipulation of the ION in rats, an ideal method for inducing pain in rat subjects must attempt to cause the least disruption to the ION and surrounding structures as possible. The trigeminal inflammatory compression (TIC) model developed in mice has demonstrated long-term efficacy to induce neuropathic pain with minimal surgical invasiveness (F. Ma, Zhang, Lyons, & Westlund, 2012).

Adapting the TIC model for rat subjects would allow for the investigation ION neuropathic pain without excessive harm. The TIC model involves the insertion of small length of chromic gut suture in parallel to the ION between the nerve and bone of the infraorbital groove, causing mild compression of the ION (F. Ma, Zhang, Lyons, et al., 2012). The TIC model was proposed in reference to previous research which found that chromic gut suture placed along the sciatic nerve caused allodynia and hyperalgesia in rats (Maves et al., 1993). Accordingly, the TIC model in mice induced mechanical allodynia up to 10 weeks (F. Ma, Zhang, Lyons, et al., 2012). Though the procedure to expose the ION for the TIC model is similar to the Vos CCI-ION model, the TIC model procedure does not include the breaking of facial bones and requires minimal movement of orbital contents and the ION itself. Since there is no ligation of the ION in the TIC model, there is minimal retraction of the ION to place the chromic gut suture. Thus, the limited access needed for the placement of the suture does not require excessive disruption of the surrounding structures to the ION. Moreover, the TIC model was developed in mice due to the potential for excessive damage with the Vos CCI-ION model

since mice will have a small operating space and large degree of vasculature in the facial region (F. Ma, Zhang, Lyons, et al., 2012). These risks will additionally be minimized further in a larger rat model with a larger operating space. By lessening the surgical invasiveness of a pain-inducing surgery, the TIC model also reduces the risk for additional damage to the rat's ability to perform daily functions, namely whisker sensory perception, sight, and chewing ability. Finally, I propose that the TIC model adapted for a rat will provide a reliable model of orofacial neuropathic pain without excessive damage to the rats tested.

Neuroinflammation Mechanisms for Neuropathic Pain after Nerve Damage

Peripheral nerve injury repair is resolved by the actions of immune and inflammatory cells. Predominately, peripheral nerve injury involves the disruption of neuronal axons and denervation of surrounding Schwann cells (Scholz & Woolf, 2007). Once peripheral nerve injury has occurred, resident macrophages immediately migrate and become active to operate at the site of nerve injury, helping to remove tissue and myelin debris (Stoll, Jander, & Myers, 2002). As an initial and short-term inflammatory response, neutrophil granulocytes are attracted to the site of nerve injury and release chemical mediators such as cytokines to recruit more macrophages to the region (Perkins & Tracey, 2000). Further recruitment of macrophages from the peripheral blood supply is mediated by the resident active macrophages and denervated Schwann cells that release matrix metalloproteases to act on endoneurial blood vessels to increase their permeability (Scholz & Woolf, 2007). Additionally, injured axons release factors such as substance P and calcitonin gene-related peptide that cause swelling and increased blood flow to the region, aiding the infiltration of blood-borne T lymphocytes, mast cells, and macrophages (Scholz & Woolf, 2007). While macrophages clear cellular debris from the site of injury, Schwann cells aid both the anterograde Wallerian degeneration of injured axons as well as the growth of new axons

(Scholz & Woolf, 2007). As part of the immune and inflammatory response, Schwann cells, active macrophages, and mast cells release prostaglandins and proinflammatory cytokines such as interleukin 1β (IL-1β) that contribute to sensory disturbances after injury (Scholz & Woolf, 2007). The accumulation of immune and inflammatory cells at the site of injury sets the foundation for the pain sensitization of primary afferent nociceptors.

Neuropathic pain via peripheral nerve injury is primarily mediated by immune and inflammatory responses at the site of injury. Both immune and inflammatory responses have been implicated in the induction of allodynia and hyperalgesia. Pain sensitization of primary afferent nociceptors is also part of the nerve repair process and is initiated by pro-inflammatory cytokines and other chemical mediators released by cells recruited after injury. In addition to acting as an initial responder to nerve injury, neutrophil granulocytes are noted to participate in the presence of thermal hyperalgesia via the 15-lipoxgenase pathway product 8R,15S-diHETE and the action of prostaglandins (Perkins & Tracey, 2000). Macrophages release prostaglandins E_2 and I_2 that have been implicated in presence of thermal hyperalgesia as well (Nathan, 1987; Syriatowicz, Hu, Walker, & Tracey, 1999). Specifically, evidence of macrophage infiltration in the trigeminal nerve via OX-42 staining has been an indicator of immune and inflammatory response in models of orofacial pain (Kernisant et al., 2008; F. Ma, Zhang, Lyons, et al., 2012). Schwann cells also participate in pain sensitization after peripheral nerve injury. Though primarily necessary for the re-growth of injured axons, nerve growth factor (NGF) released by Schwann cells has been associated with the sensitization of nociceptors and thermal hyperalgesia (Malin et al., 2006). Moreover, Schwann cells are known to release other chemicals such as tumor-necrosis factor (TNF), prostaglandin E_2, and interleukin 6 that mediate pain sensitization (Moalem & Tracey, 2006). Like Schwann cells, mast cells secrete algesic compounds,

substances that elicit pain, during peripheral nerve injury repair. Mast cells release histamine, which has been implicated in pain sensitization and shown to recruit macrophages and neutrophils (Moalem & Tracey, 2006). At the site of injury, responding immune and inflammatory cells release other chemical mediators involved in pain sensitization such as bradykinin, adenosine triphosphate, prokineticin 2, and interleukins, including interleukin 1β (IL-1β) (Lattanzi et al., 2015; Scholz & Woolf, 2007; Thacker, Clark, Marchand, & McMahon, 2007).

Research has shown that IL-1β plays a significant role in mediating pain sensitization that causes neuropathic pain in the form of hyperalgesia and allodynia after peripheral nerve injury. During times of stress and nerve injury, IL-1β is up-regulated and produced by macrophages and monocytes after acted upon by endogenous molecules (Dinarello, 2011; Nadeau et al., 2011; Thacker et al., 2007). The primary transcript of IL-1β must be cleaved either by intracellular cysteine protease capase-1or extracellular serine protease produced by neutrophils (Dinarello, 2011). The pain sensitization actions of IL-1β are dependent upon the activation of the interleukin 1 type 1 receptor (IL-1R1) (Nadeau et al., 2011; Thacker et al., 2007). Activation of IL-1R1 has been shown to aid in the translocation of nuclear factor-κB into the nucleus to increase gene expression of inflammatory molecules and the production of algesic mediators such as bradykinin, prostaglandins, NGF, and pro-inflammatory cytokines (Sommer & Kress, 2004; Thacker et al., 2007). Neutrophil infiltration at the site of neuronal injury has also been shown to partly dependent upon the action of IL-1R1 (Nadeau et al., 2011). In addition to acting at the neuronal soma, IL-1β acts at the level of the neuronal axons after injury to promote hyperalgesia and mechanical allodynia (Zelenka, Schafers, & Sommer, 2005). The release of algesic mediators via an IL-1β pathway may play a role in the changing the neuronal excitability

of primary afferent nocicieptors (Thacker et al., 2007). Additionally, the IL-1β via IL-1R1 has been shown to increase current through NMDA and AMPA receptors, promoting neuron excitability (T. Liu, Jiang, Fujita, Luo, & Kumamoto, 2013). Specifically, the ability of IL-1β to modulate neuron excitability has been studied in trigeminal nerve injury (Martin, Anton, Gornbein, Shanahan, & Merrill, 1993; Takeda, Takahashi, & Matsumoto, 2008; Takeda et al., 2007).

Hyperalgesia and allodynia following trigeminal nerve injury is mediated by the action of IL-1β to increase neuronal excitability. Following trigeminal nerve injury, peripheral inflammation mechanisms, via the presence of substance P, result in the activation of satellite glial cells encircling trigeminal ganglia (TRG) neurons (Martin et al., 1993; Takeda, Takahashi, et al., 2008; Takeda et al., 2007). Trigeminal injury is also associated with the up-regulation of IL-1RI in small and medium sized TRG neurons innervating the face (Takeda et al., 2007). The secretion of IL-1β by activated satellite glial cells has been shown to increase the magnitude of depolarization of the trigeminal nerve and the spontaneous firing rate of TRG neurons (Takeda, Takahashi, et al., 2008; Takeda et al., 2007). Evidence that chronic IL-1β exposure potentiates sodium channels in TRG neurons may explain this increase in magnitude of depolarization (L. Liu, Yang, Liedtke, & Simon, 2006). Moreover, IL-1β has been shown to increase the excitability of Aδ and C fibers in an inflamed trigeminal nerve via decreasing total potassium currents by inhibition of voltage gated potassium channels (Takeda, Kitagawa, Takahashi, & Matsumoto, 2008). As stated previously, the primary afferent Aδ and C fibers initially mediate pain sensations from the peripheral nervous system to the central nervous system (Basbaum et al., 2009). This effect of IL-1β to increase neuronal excitability is enhanced by the up-regulation of IL-1R1. Studies have also shown that IL-1β may act directly or indirectly upon peripheral

group 1 mGluR receptors, a metabotropic glutamate receptor, and AMPA/Kainate glutamate receptors to mediate mechanical allodynia in the orofacial region of rats (Ahn et al., 2004; Ahn et al., 2005). The glutamate receptors activated by IL-1β are located in TRG neurons in close proximity to the rat whisker pad (Ahn et al., 2004; Ahn et al., 2005).

Neuropathic Pain and Pain-Induced Anxiety

Beyond the physical discomfort, such as mechanical allodynia, caused by damage to the somatosensory system, chronic neuropathic pain induces a linked anxiety state. In a primary care patient screening study, greater than 50% of patients with chronic pain demonstrated one or more anxiety disorders (Kroenke et al., 2013). Termed an affective dimension of pain, the unpleasant emotional feelings associated with pain pertain to distress or fear that patients suffering from pain may have when thinking about the short- and long-term implications (Price, 2000). Patients suffering from chronic pain feel the affective dimension of pain increase in presence over time (Price, 2000). Chronic pain causes affected individuals to feel an intense fear of pain recurrence or situations in which they may feel increased pain, resulting in anxiety-related behaviors (do Nascimento & Leite-Panissi, 2014). Moreover, generalized anxiety disorder prevalence was shown to be about 40% of patients with chronic pain and 14% of patients without chronic pain (Manchikanti et al., 2002). Though the relationship between chronic pain and anxiety has been observed clinically, research is still being conducted to identify the underlying biological mechanisms that mediate this connection.

Currently, the relationship between chronic pain and a pain-induced anxiety state has been extensively been studied in rodent models. Studies have shown that induction of chronic inflammatory or neuropathic pain in small rodents has also induced anxiety-related behaviors (do Nascimento & Leite-Panissi, 2014; Narita, Kaneko, et al., 2006; Parent et al., 2012; Zhang et al.,

2014). Analgesic drugs, such as tramadol, morphine, and gabapentin, have been shown to reduce anxiety behaviors in rats with induced neuropathic pain while not altering the anxiety state of healthy rats (Caspani, Reitz, Ceci, Kremer, & Treede, 2014; Roeska, Doods, Arndt, Treede, & Ceci, 2008; Wallace, Segerdahl, Blackbeard, Pheby, & Rice, 2008). Thus, the anxiety experienced by rats with neuropathic pain may be induced by their pain state. The amygdala and the anterior cingulate cortex (ACC) are brain structures that play roles in mediating pain-induced anxiety.

Neuropathic pain has been shown to alter the functionality of the amygdala to partially mediate pain-induced anxiety. The amygdala has been notably shown to be involved in the generation of fear and anxiety-related behaviors (Davis, Rainnie, & Cassell, 1994). In addition to receiving various polymodal sensory inputs from the thalamus and cortical regions (Shi & Davis, 1999), the amygdala receives direct nociceptive input via the spinal cord and brainstem (Gauriau & Bernard, 2002) and indirect nociceptive input via the spinothalamic and spinohypothalimic pain pathways (Neugebauer, Li, Bird, & Han, 2004). This nociceptive-related information input may alter the output of the amygdala to generate anxiety behavior. In mice, chronic inflammatory pain or neuropathic pain is reportedly related to the reduction of δ-opioid receptor G-protein activation in the amygdala (Narita, Kaneko, et al., 2006). Since δ-opioid receptor antagonists and the knock down of the δ-opioid receptor resulted in increased anxiety behaviors in mice, activation of the δ-opioid receptor has been implicated to have an anxiolytic effect (Filliol et al., 2000; Narita, Kaneko, et al., 2006). Thus, nociceptive input to the amygdala may cause anxiety behavior mediated by the down regulation of δ-opioid receptors. Within the amygdala, the output central nucleus of the amygdala (CeA) is a regulator of motor and emotional behavior following a stress-related input into the amygdala from cortex,

hypothalamus, and thalamus (LeDoux, Iwata, Cicchetti, & Reis, 1988; Wu, Kim, & Zhuo, 2008). The CeA integrates different modalities of sensory information with nociceptive information to elicit an emotional response (Neugebauer et al., 2004). Thus, the CeA may have a role in the development of pain-induced anxiety behavior. In relation to pain-induced anxiety specifically, peripheral nerve injury reduces GABAergic inhibition of the CeA, resulting in hyperexcitabilty (as distinguished by increased burst firing pattern) that correlates to increased anxiety behavior in rats (Jiang et al., 2014).The CeA has additionally been implicated in pain-induced anxiety mediated by corticosterone in rats. Peripheral neuropathic pain has been noted to cause increased levels of corticotrophin releasing factor (CRF) and glucorticoid receptors (GR) in the CeA of rats (Ulrich-Lai et al., 2006). It is important to note that this increase in CRF is independent of the HPA axis (Ulrich-Lai et al., 2006).Furthermore, administration of corticosterone into the CeA increases anxiety-related behavior (Greenwood-Van Meerveld et al., 2001).

Research suggests that the anterior cingulate cortex is involving in both the processing of pain and pain affect (Devinsky, Morrell, & Vogt, 1995; Kuroda, Yorimae, Yamada, Furuta, & Kim, 1995; Neugebauer, Galhardo, Maione, & Mackey, 2009; Yen et al., 2005). Thus, the ACC may be involved in the mediation of pain-induced anxiety. It has been proposed that information regarding the “unpleasant” nature of pain is transmitted via the medial/intralaminar thalamus to the ACC (Treede, Kenshalo, Gracely, & Jones, 1999). Like the amygdala, the ACC has been implicated in mediating anxiety behavior (Kim et al., 2011). Pain-induced anxiety may be mediated by various mechanisms involving the anterior cingulate cortex. Building upon premise of δ-opioid receptor mediated pain-induced anxiety, evidence suggests that neuropathic pain in mice may cause the down-regulation of δ-opioid receptors in the cingulate cortex leading to astrogliogenesis from progenitor cells in the cingulate cortex (Narita, Kuzumaki, et al., 2006).

Furthermore, the increased presence of astrocytes in the cingulate cortex has been related to increased anxiety behavior in mice (Narita, Kuzumaki, et al., 2006). Once a pain-induced anxiety state has been established via the ACC, changes in synaptic plasticity in the ACC may promote the long-term maintenance and strengthening of the pain-induced anxiety state. Both pre-synaptic and post-synaptic long term potentiation (LTP) of excitatory transmission at the thalamacortical synapse of the ACC has been noted to be established in rats with chronic pain-induced anxiety (Koga et al., 2015).

Behavioral Assays for Pain-Induced Anxiety

Though chronic pain-induced anxiety is not a unitary emotional state, researchers have studied anxiety by observing specific anxiety-related activities. Animal anxiety is a defensive and adaptive response to the seemingly imminent threats from the environment (Rodgers, 1997). Pathological anxiety in animals will occur if the initial adaptive response to pain is unsuccessful (Steimer, 2011). Thus, after an animal's response given a specific set of potential threats in the environment has been studied, the degree of the animal's response can indicate a certain level of anxiety. The fundamental neural connections that mediate anxiety related behaviors have been evolutionarily conserved in vertebrates and mammals since such behaviors are important for survival (LeDoux, 1995; Steimer, 2011). Consequently, the anxiety-related behaviors are similar across mammalian species and vary according to the specific nature of the threatening stimulus in the environment (Rodgers, 1997). The similarity of anxiety-related behaviors and their underlying neural mechanisms has prompted the use of has prompted the use anxiety-related behaviors in animals to be studied as a surrogate for human anxiety behaviors. To study human anxiety and human anxiety disorders in animals, researchers conduct animal anxiety-related

behavioral assays that rely on the analysis of anxiety-related defensive behaviors (Rodgers, 1997).

During anxiety-related behavioral assays, animals are challenged with anxiogenic environments in the laboratory setting and the resulting behavior of the animal is quantified (Steimer, 2011). Thus, animal changes in anxiety-related behavior can be studied as result of pharmacological or physiological manipulation. Furthermore, the validity of anxiety-related behavioral assays has been established by their ability to detect changes in adaptive behavior changes due to clinical anxiolytic drugs, namely benzodiazepines (Rodgers, 1997). Recently, anxiety-related behavior assays have been used to detected adaptive behavioral changes due to chronic pain.

The light and dark box (LDB) anxiety-related behavior assay has been used to indicate the presence of behavioral changes induced by neuropathic pain. The anxiety-related behaviors demonstrated in the LDB test have been used to assess chronic pain induced anxiety (Parent et al., 2012). Chronic orofacial pain, chronic visceral pain, and neuropathic pain in rodent models have been shown to increased anxiety related behaviors in the LDB test (do Nascimento & Leite-Panissi, 2014; Matsuzawa-Yanagida et al., 2008; Zhang et al., 2014). The LDB test is a simplistic assay for rodents that is based upon the innate nature of a rodent to explore and fear a novel and unfamiliar environment (Bourin & Hascoet, 2003; Crawley & Goodwin, 1980). During the LDB test, a rodent is placed into a box with two compartments: a light compartment with transparent walls and a dark compartment with opaque walls (Crawley & Goodwin, 1980). Once the LDB test has begun, the rodent is free to move between the compartments. In a given period of time during the LDB test, the amount of time spent in the light compartment (TL) and number of transitions between compartments (NT) are two primary endpoints to evaluate

anxiety-related behavior. Increased time spent in the illuminated chamber without change in number of transitions is indicative of decreased anxiety (Costall, Jones, Kelly, Naylor, & Tomkins, 1989). Increased number of transitions without a change in time spent in either compartment has also been found to be indicative of decreased anxiety (Crawley & Goodwin, 1980). Specifically, rats with orofacial inflammatory pain spent decreased time spent in the light compartment and decreased in number of transitions, which are both associated with increases in pain-induced anxiety (do Nascimento & Leite-Panissi, 2014). Furthermore, studies evaluating the mechanisms of how chronic pain-induced anxiety have successfully relied upon the LDB test to indicate level of anxiety (Narita, Kaneko, et al., 2006; Narita, Kuzumaki, et al., 2006).

Trigeminal Inflammatory Compression Model In Rat Study Aims

This study aims to evaluate the efficacy of the trigeminal inflammatory compression model in a rat subject to elicit chronic orofacial pain. I hypothesize that the placement of chromic gut suture alongside the infraorbital branch of the trigeminal nerve will cause chronic orofacial pain as indicated by the presence of nerve inflammation at the site of injury, facial mechanical allodynia, and pain-induced anxiety.

Efficacy of the TIC model in rats to elicit an observable chronic neuropathic pain state will be determined as a reflection of the Von Frey test for detection of mechanical allodynia and the LDB test for pain-induced anxiety. The Von Frey test is a behavioral assay that has been used to assess mechanical allodynia in previous models of orofacial pain (Ahn et al., 2009; An et al., 2011; Christensen, Gautron, Guilbaud, & Kayser, 2001; Jeon et al., 2012; Kernisant et al., 2008; Krzyzanowska et al., 2011; Luo et al., 2012; Vos et al., 1994; Xu, Aita, & Chavkin, 2008). The Von Frey test assesses facial pain sensitivity by analyzing a rat subject's aversive response when filaments of varying diameter designed to bend at the application of specific corresponding

amounts of force are applied to the rat subject's whisker pad (Vos et al., 1994). Von Frey filaments are applied in order of increasing required force until an aversive response is observed. The force of the filament that elicits an aversive response is termed the pain threshold. A reduced pain threshold to a non-noxious stimulus is indicative of mechanical allodynia. Related to behavior, the LDB test has been used to detect changes in the anxiety states of rat subjects based upon the presence of pain (do Nascimento & Leite-Panissi, 2014; Matsuzawa-Yanagida et al., 2008; Zhang et al., 2014). The LDB test assesses the anxiety state of the rat subject by analyzing the spontaneous nature of the rat subject to explore or avoid unfamiliar environments (Bourin & Hascoet, 2003). In the LDB test, increased exploratory behavior is indicative of a lower anxiety state (Bourin & Hascoet, 2003).

Finally, efficacy of the TIC model in rats to elicit inflammatory nerve damage to the ION at the site of injury will be determined by the presence of Interleukin -1β, a proinflammatory cytokine, and OX-42, a macrophage marker, in ION tissue in contact with chromic gut suture. Immunohistochemical techniques will be used to identify the presence of these markers of inflammation and immune response.

Medical Ethics

Dedicated effort has been made to minimize and justify necessary pain induced by the TIC model in rats in this study. The TIC model in rats is an attempt to create a small animal model of orofacial pain with limited excessive damage compared to other orofacial pain models. Limiting the necessary pain and intrusiveness of a chronic orofacial pain TIC model is of utmost ethical importance. As it is a privilege to work with animals for research purposes, researchers using animals have an ethical obligation to humanely care for the needs and welfare of animals used (Tannenbaum, 1999). Any medical research using animals must consider the relevancy that

potential practical and theoretical results of research will have to human health (OLAW, 2015). Thus, pain must only be induced in animals when pain is necessary and unavoidable for research applications (Code, 2013). With much regret, there are no alternative non-animal models of chronic orofacial pain that will accurately determine the efficacy of potential analgesic treatments in the complex biochemical and physiological environment of mammals, namely humans. Thus, the use of rodents in the research of chronic orofacial pain presented here is scientifically justified and information attained from this research is valuable. Justification for the creation of a novel chronic orofacial pain is that a novel rodent model will be able to effectively assess the potential treatments for severe orofacial pain in humans while reducing excessive harm to rodents as seen in previous models of orofacial pain. It is the goal of this study to only induce chronic orofacial pain that has been deemed necessary in duration and severity to create a rodent model of pain capable of accurately testing novel analgesic therapeutics to deem worthiness of human use. The TIC model in rats presented here is an effort to minimize rodent pain needed for orofacial pain research. Accurate characterization and recording of pain elicited in rat subjects used in this study will be done using proven behavioral assays for mechanical allodynia and anxiety. Unfortunately, no analgesics can be administered to provide relief of pain in this study since the study is being conducted to determine the effectiveness of the TIC surgery to elicit orofacial pain in rodents. Administration of analgesics during the study, outside of the acute post-operative phase, would confound the results of the study and make the results of this study in-comparable to previous research in the field. All efforts have been taken to ensure potential causes of environmental pain be minimized, namely excessive handling. The ten week timeline for pain-inducing experimentation was deemed necessary for the observation of chronic neuropathic pain. Each animal was monitored daily by both a member of this research team and

by the veterinary staff of the Division of Animal Resources at Emory University. The experimental use of the animals was done in compliance with guidelines for animal care and experimental use set by the National Institutes of Health. All animal use protocols were reviewed and accepted by the Institutional Animal Care and Use Committee at Emory University.

Materials and Methods

Animals

18 male Sprague Dawley rats (Charles River Laboratory), weighing 350-480g at the beginning of the study, housed in ventilated (10-15 changes of fresh air per hour) and temperature controlled (72°F ± 1°F) single cage housing with a reversed light and dark cycle (lights on from 4:00PM to 4:00AM). Humidity in each housing room was controlled to 40%-50%. Each rat had access to unlimited food and water. There was a 14 day minimal acclimation period before rats were handled for experimentation and behavioral testing.

Rats were randomly assigned to be in either a control group or a surgical group. In the surgical group (n=9), rats received TIC surgery on the left trigeminal infraorbital nerve (IoN). In the control group (n=9), rat received a sham surgery on the left side where the ION was exposed but unaltered. Behavioral testing was done at 9:00AM. Von Frey testing was conducted once per week one week prior to surgery and at weeks one, two, three, four, six, eight, and ten post-surgery. LDB testing was conducted one day prior to Von Frey testing weekly.

Surgery

Prior to surgery, each rat was anesthetized using isofluorane (maximum 5% concentration mixed with oxygen gas). The snout of each rat was placed into a nose chamber on the stereotactic frame that allowed for the flow of isofluorane during surgery. After each rat was sufficiently anesthetized, each rat was firmly immobilized in the prone position using a stereotactic frame (David Kopf Instruments; Tujunga, CA) and ear bars. The hair between the eyes of each rat was shaved properly. Using an 11-surgical blade, an incision was made from the Bregma anteriorly too approximately 3mm anterior to the medial zygomatic arch, identified via palpation. Using a combination of Metzenbaum scissors and a cotton-tipped wooden applicator, the skin and

subcutaneous tissue were bluntly dissected from the outer skull. Further dissection was conducted laterally to expose the superior orbital rim, medial zygomatic arch, and the globe of the eye. The globe of the eye was gently retracted to expose the distal intraorbital position of the infraorbital nerve for the nerve passes through the infraorbital foramen. Following visualization of the infraorbital nerve, a single 4mm length of 3-0 chromic gut suture was placed between the nerve and the maxillary bone. In the sham-surgery, the infraorbital nerve was only exposed and no chromic gut suture was placed. Closure of the subcutaneous tissue was done using 5-0 Vicryl™ (Ethicon, Inc; Somerville, NJ) suture, and closure of the skin was done using 4-0 Ethilon™ (Ethicon, Inc; Somerville, NJ) suture. A subcutaneous injection of buprenorphine was administered at 0.05mg/kg dosage during the surgery. Half of the buprenorphine dosage was given prior to incision, while the other half of the dosage was given following closure of the skin.

Light and Dark Box Test

The apparatus used for this study was made of a rectangular Plexiglas box (11.5in by 11.5in by 19in) with two compartments of equal area divided by a black rectangular piece of Plexiglas (11.5in by 0.25in by 11.5in) with a hole at the floor level large enough for a rat to move through with ease. One compartment had black walls and a black floor and was referred to as the dark compartment. The other compartment was completely transparent to light and referred to as the light compartment. The top of the dark compartment was covered with a laboratory drape that blocked light transmission into the box. The top of the light compartment was left open and received room light (approximately 50 lux). During LDB testing, each rat was placed into the light compartment, and the subsequent behavior of the rat was monitored for five minutes from above the apparatus and recorded by a Logitech® HD Pro Webcam C920. The time each animal

spent in either compartment was recorded along with number of total transitions between compartments, instances of facial grooming, and amount of fecal matter and urine. An animal was considered to be in a compartment if all four paws of the animal were in a given compartment. Each video for LDB testing was scored by a research team member. After LDB testing, each animal was returned to the housing facility, and each compartment of the apparatus was cleaned using 70% ethanol solution. LDB testing was conducted once per week one week prior to surgery and at one, two, three, four, six, eight, and ten weeks post-surgery. Each rat was habituated to the LDB test for 2 weeks prior to baseline testing.

Von Frey Filaments Test

Touch Test® Sensory Evaluators were used to conduct the Von Frey test. During Von Frey testing, each rat was held in a horizontal position and immobilized from the neck to tail by a single researcher as done previously (Luiz et al., 2010; F. Ma, Zhang, Lyons, et al., 2012; Seino, Seo, Maeda, & Someya, 2009). Care was taken to ensure that each rat being tested was not exhibiting signs of stress such as the accumulation of porphyrin around the eyes and squealing sounds while held by a researcher. After a rat being tested demonstrated a calm demeanor, a single Von Frey filament was pressed against the left whisker pad until the filament's max applicable force was achieved causing the filament to bend slightly. Behavior following the application of the Von Frey filament was recorded as a positive aversive reaction or a negative reaction. For each filament, three positive aversive reactions out of five applications were considered in total positive response for that particular filament. Filaments of 2g, 4g, 8g, and 10g were tested on each rat. The filament weight of 10g was chosen as the maximum weight used since any larger weight filament tested was found to move the head of the rat. Consequently, a larger filament weight than 10g would have evoked a response due to nociceptive pain rather

than mechanical allodynia. If no positive response was demonstrated after the use of all the filament weights previously mentioned, the rat recorded to be non-responsive to Von Frey testing. The Von Frey Filament test was conducted on each whisker pad of the rat being tested for all types of filament. Each rat was habituated to the Von Frey Filament test and holding by a researcher for 2 weeks prior to baseline testing.

Tissue Preparation

After Week 10 of the study, each rat was anesthetized using isofluorane, sacrificed using an injection of 1 mL of EUTHASOL® (pentobarbital sodium and phenytoin sodium) solution, and perfused trancardially using first 4°C saline solution followed by a 4°C solution of 4% paraformaldehyde in deionized water. The head of each rat was excised and preserved in 4% paraformaldehyde in deionized water until dissection. For experimental rat subjects, the left infraorbital nerve in contact with chromic gut suture was dissected out and the right infraorbital nerve was dissected out in the same corresponding location. For control rat subjects, the left infraorbital nerve was dissected out in a corresponding manner to that of the experimental rat subjects. Each nerve sample was placed in fixative 4°C solution of 4% paraformaldehyde in deionized water for 24h. Nerve samples were then placed in 4°C solution of 30% sucrose in deionized water for a minimum of 7 days. Samples will then be embedded into Optimal Tissue Cutting compound and frozen at -80°C. Nerve samples were cryosectioned using a Leica CM 1950 Cryostat at 12 μm and mounted onto Superfrost® Plus glass slides (VMR). Nerve sample slides were preserved at -80° C until use for immunohistochemical staining.

Immunohistochemical Analysis

Nerve sections were brought to room temperature for 10 minutes and subsequently hydrated in 0.1M phosphate buffered saline (PBS, pH 7.4) for 10 minutes. After hydration, nerve sections exposed to 3% solution of hydrogen peroxide in 0.1M PBS solution with 1% Triton (PBST) at room temperature for 10 minutes. Nerve sections were then washed three times with PBST at room temperature for 10 minutes per wash. Following PBST washes, the nerve sections were blocked with 5% normal goat serum in PBST for 30 minutes at room temperature. Subsequently, the nerve sections were incubated overnight with rabbit anti-Interleukin 1β (1:500, Abcam ab1211) or anti-OX42 (1:500, Abcam ab9722) antibodies for immunolocalization of biomarkers inflammation and macrophage infiltration, respectively. After, sections were washed three times with PBST at room temperature at 10 minutes per wash. The nerve sections were incubated with secondary goat anti-rabbit antibodies (Polyclonal DyLight 680 Conjugate, Thermo Scientific Pierce) for 2 hours. After secondary antibody incubation, the sections were then washed three times with PBST at room temperature at 10 minutes per wash. The nerve sections were incubated with VECTASTAIN® ABC Reagent for 1 ½ hours to set up an immunoperoxidase detection system. After application of the ABC Reagent, nerve sections were washed three times with PBS at room temperature at 10 minutes per wash. The nerve sections were then incubated with Vector® SG Substrate reagent, a substrate for peroxidase enzyme, for 20 minutes at room temperature. Subsequently, the nerve sections were washed three times with PBS at room temperature at 10 minutes per wash. The sections were rinsed in 95% ethanol and then 100% ethanol. Finally, the nerve sections were soaked in Histo-Clear solution for 5 minutes, coverslipped with micro cover glass (VMR), and visualized using a Nikon Eclipse E400 Microscope with a Nikon DS-Fi1 camera.

Statistical Analysis

All quantitative measurements were shown as mean ± SEM. The statistical test used was for analysis of variance (2 Way ANOVA) with use of GraphPad PRISM software, Version 4 for Windows (GraphPad Software, Inc.; San Diego, CA). Only probability values after statistical analysis of less than 0.05 were significant.

Results

Von Frey Hair Testing

At baseline, all control and experimental animals were unresponsive to any filament weight tested by control or experimental animals. One out of nine experimental animals did not survive after the TIC surgery due to post-surgical complications. At post-operative week (POW) 1, six out of eight experimental animals had a positive response to a filament weight tested compared to zero out of nine control animals (Figure 4). All control animals did not demonstrate a positive response to any filament weight throughout the course of the study. At POW2, five out of eight experimental animals had a positive response to a filament weight tested with one experimental animal becoming unresponsive. At POW3 through POW6, five out of eight experimental animals consistently had a positive response to a filament weight tested (Figure 4). At POW 8, four out of eight experimental animals had a positive response to a filament weight tested with one experimental animal becoming unresponsive. At POW 10, two out of eight experimental animals had a positive response to a filament weight tested with two experimental animals becoming unresponsive (Figure 4). The threshold filament weight for each animal is provided in Table 1.

	Animal 10	Animal 11	Animal 12	Animal 13	Animal 14	Animal 15	Animal 16	Animal 17	Animal 18
Baseline	NR	NR	NR	NR	NR	NR	NR	NR	NR
POW1	2g	2g	4g	4g	8g	NR	8g	N/A	NR
POW2	2g	2g	2g	2g	8g	NR	NR	N/A	NR
POW3	4g	2g	2g	4g	2g	NR	NR	N/A	NR
POW4	4g	2g	2g	4g	8g	NR	NR	N/A	NR
POW6	2g	2g	2g	4g	4g	NR	NR	N/A	NR
POW8	2g	2g	2g	2g	NR	NR	NR	N/A	NR
POW10	NR	2g	2g	NR	NR	NR	NR	N/A	NR

Table 1. Von Frey Testing for Mechanical Allodynia: Threshold Filament Weight for Positive Behavioral Response (grams).

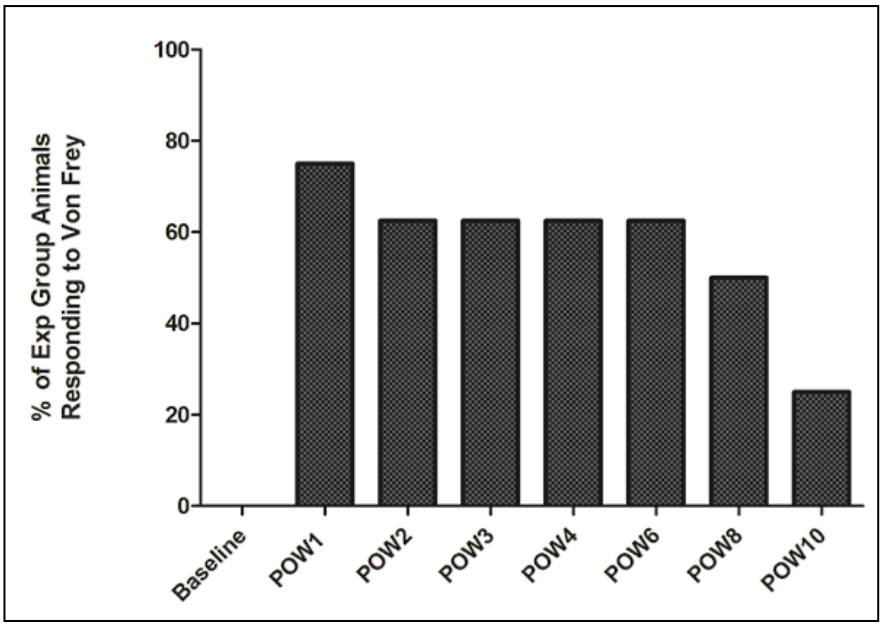


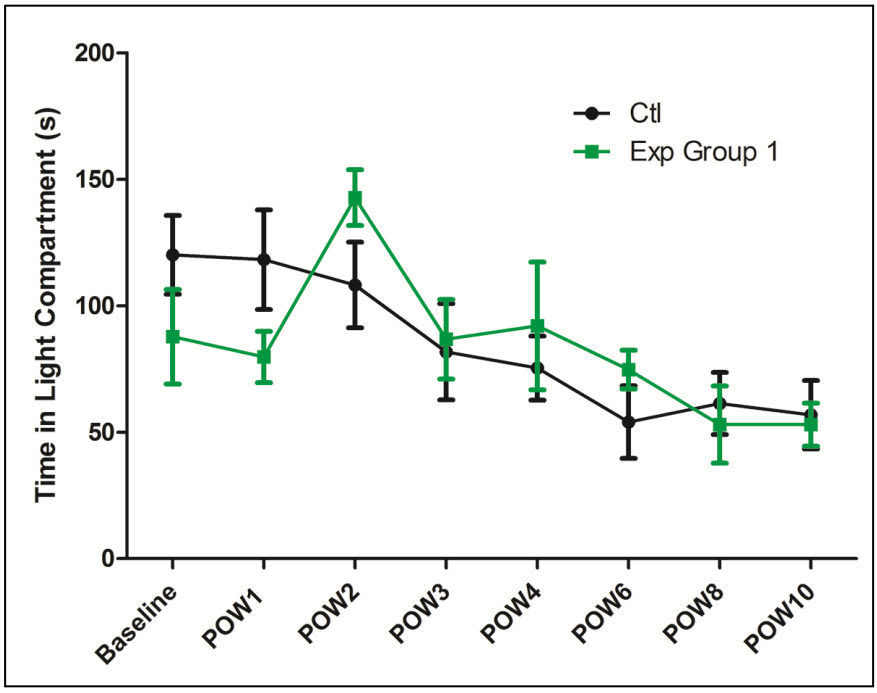
Figure 4. The Percentage of Experimental Animals that Demonstrated Positive Responses to Von Frey Testing From Baseline to POW10

Light and Dark Box Test Data

During the entire course of the study, there were no significant differences ($p > 0.05$) of time in the light compartment (TL) between experimental and control animals. For the baseline week and POW1, experimental animals spent less time on average in the light compartment (-32.35s Baseline; -38.47s POW1) compared to the control animals (Figure 5A). In POW 2, experimental animals spent on average more TL (34.52s POW2) than the control animals. From POW3 through POW6, the experimental groups continued to spend on average more TL compared to control animals, though the differences were not significant (Figure 5A). From POW 8 through POW 10, both experimental animals and control animals spent on average a similar TL. However, from POW8 through POW 10, experimental animals with positive Von Frey responses did spend on average decreased TL compared to control animals (-35.83s POW8; -32.89s POW10) (Figure 5B). Although not statistically significant, both experimental and control animals demonstrated a negative trend in time spent in the light compartment of the box.

During the entire course of the study, there were no significant differences ($p > 0.05$) of number of transitions (NT) between experimental and control animals. At baseline, both experimental and control animals demonstrated on average a similar NT. From POW1 to POW2, experimental animals increased on average the NT compared to control animals (+2.25 POW1; +4.21 POW2)(Figure 6A). From POW2 to POW3, both experimental animals and control animals demonstrated on average a decrease in NT (+4.21 POW2; +1.85 POW3). From POW 3 through POW 10, both experimental animals and control animals demonstrated on average a relatively constant NT (Figure 6A). During POW 8 through POW 10, experimental animals with positive Von Frey responses on average had fewer NT than control animals (-2.56 POW8; -4.33 POW10) (Figure 6B).

A)



B)

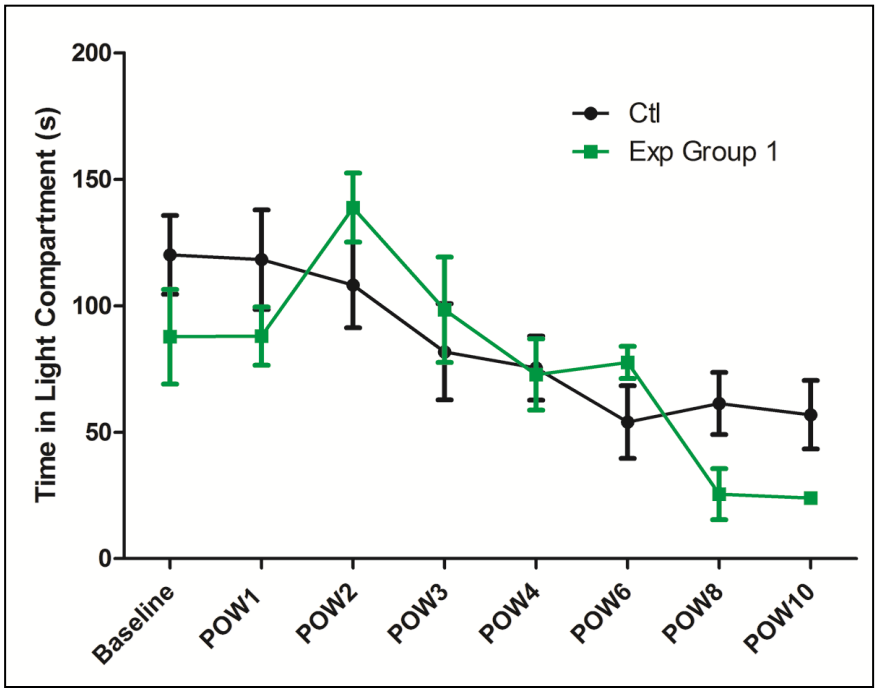
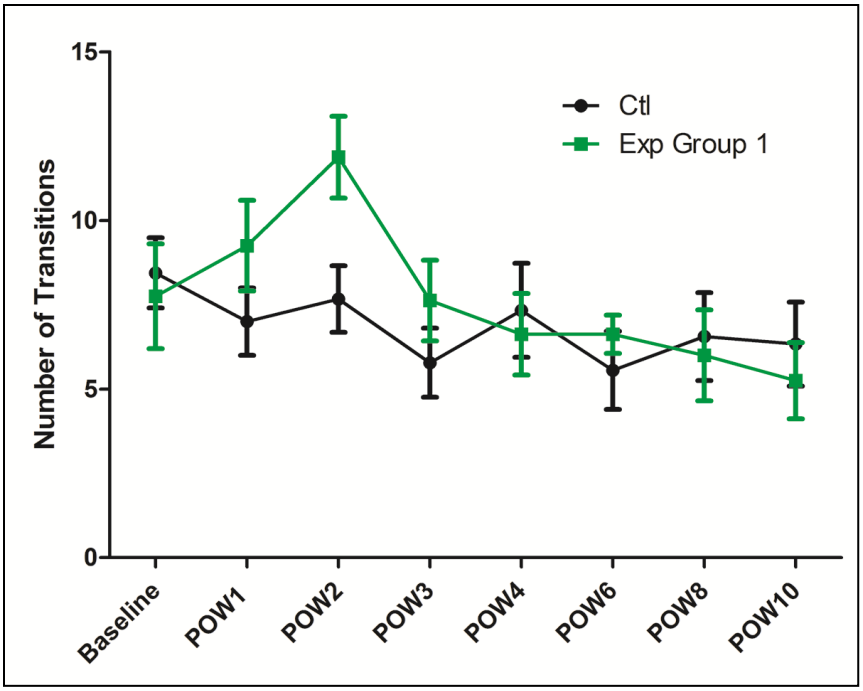


Figure 5. Light and Dark Box Test for Anxiety: Time In Light Compartment (Seconds). Data on the line graph shows means ± S.E.M. (A)Time Spent in Light Compartment (Seconds) By Control and All Experimental Animals and (B)Time Spent in Light Compartment (Seconds) By Control and Only Experimental Animals With Positive Von Frey Responses.

A)



B)

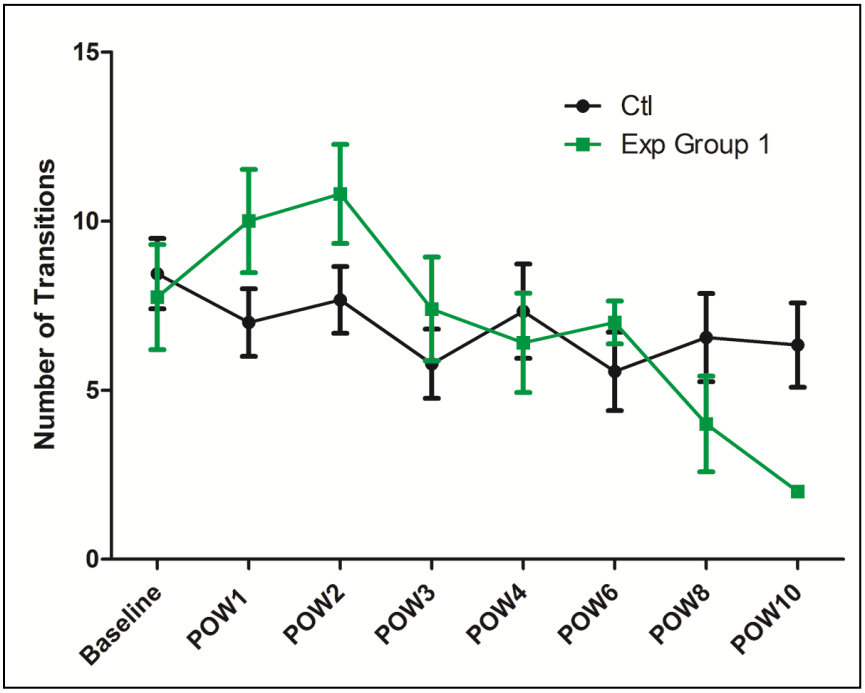


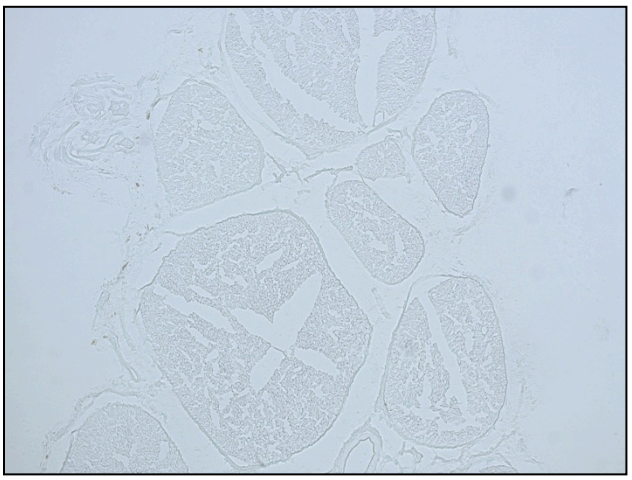
Figure 6: Light and Dark Box Test for Anxiety: Number of Transitions between the Dark and Light Compartment. The data in the line graph is means ± S.E.M. (A) Time Spent in Light Compartment (Seconds) By Control and All Experimental Animals and (B) Time Spent in Light Compartment (Seconds) By Control and Only Experimental Animals With Positive Von Frey Responses.

Immunohistological Staining of Trigeminal Nerve Tissue

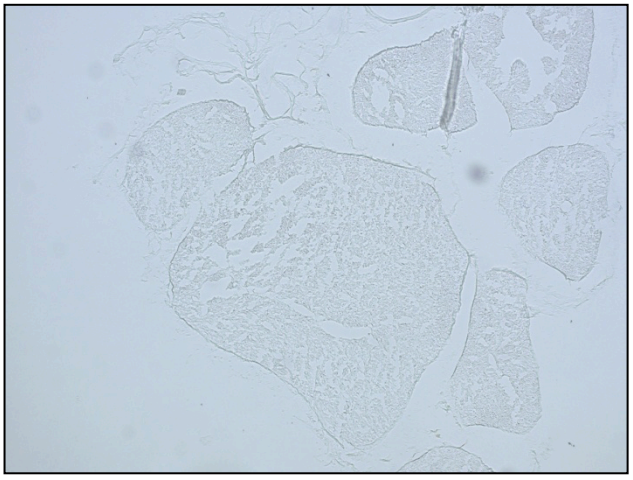
Of the tissue analyzed at POW10, most of the animal tissue studied demonstrated minor staining for IL-1β. There was no difference in degree of staining for IL-1β present between the ION of the control animals, the surgically manipulated ION of the experimental animals, and the contralateral ION of the experimental animals (Figures 7A, 7B, and 7C). Moreover, there was no difference in staining for IL-1β within the stated groups dissected for tissue analysis.

Similarly at POW10, the majority of animal tissue studied demonstrated minor staining for OX-42. There was animal tissue that did stain positively for OX-42, but this positive staining was seen in only a few animals spread randomly amongst the various tissue groups studied. For immunohistochemical staining of OX-42, there was no overall difference in degree of staining present between the ION of the control animals, the surgically manipulated ION of the experimental animals, and the contralateral ION of the experimental animals (Figure 8A, 8B, and 8C). Moreover, there was no difference in staining for OX-42 within the stated groups dissected for tissue analysis.

A)



B)



C)

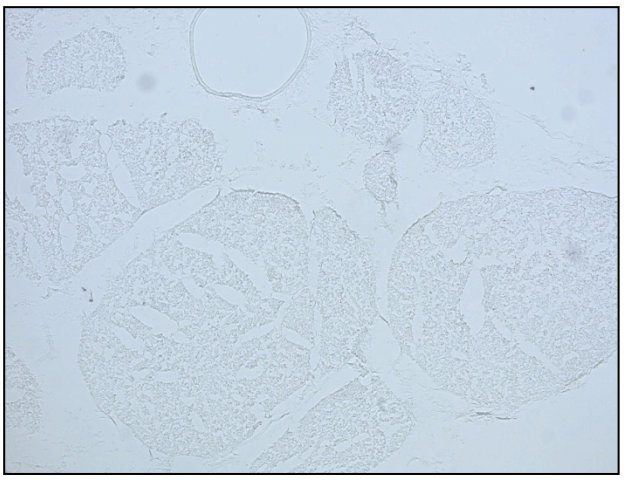
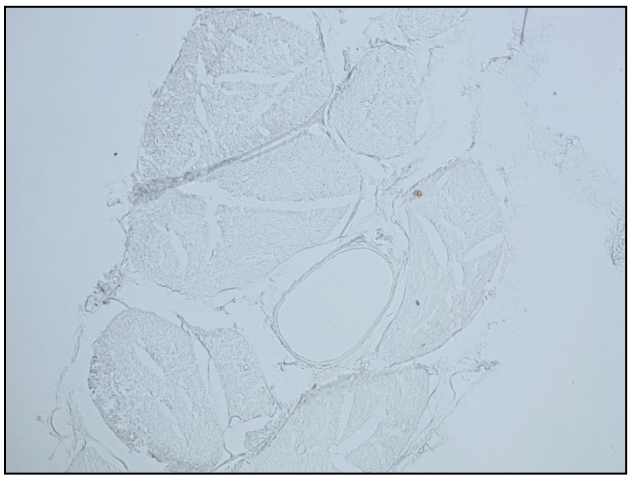
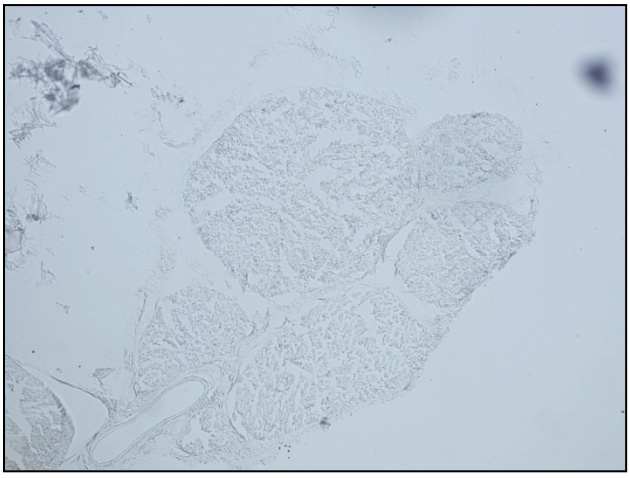


Figure 7. IL-1β Staining of Infraorbital Nerve Tissue
A) IL-1β Staining of Infraorbital Nerve Tissue From Control Animal
B) IL-1β Staining of Surgically Manipulated Infraorbital Nerve Tissue From Experimental Animal
C) IL-1β Staining of Contralateral Infraorbital Nerve Tissue From Experimental Animal

A)



B)



C)

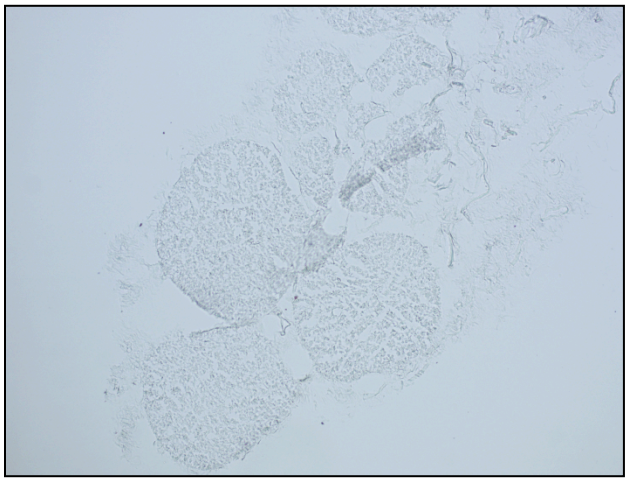
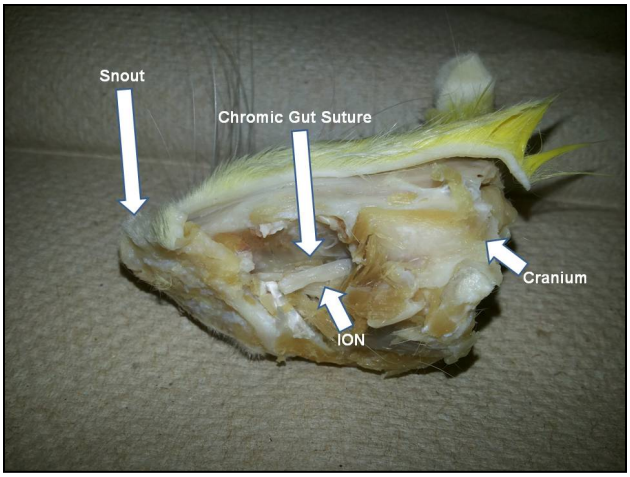


Figure 8. OX-42 Staining of Infraorbital Nerve Tissue
A) OX-42 Staining of Infraorbital Nerve Tissue From Control Animal
B) OX-42 Staining of Surgically Manipulated Infraorbital Nerve Tissue From Experimental Animal
C) OX-42 Staining of Contralateral Infraorbital Nerve Tissue From Experimental Animal

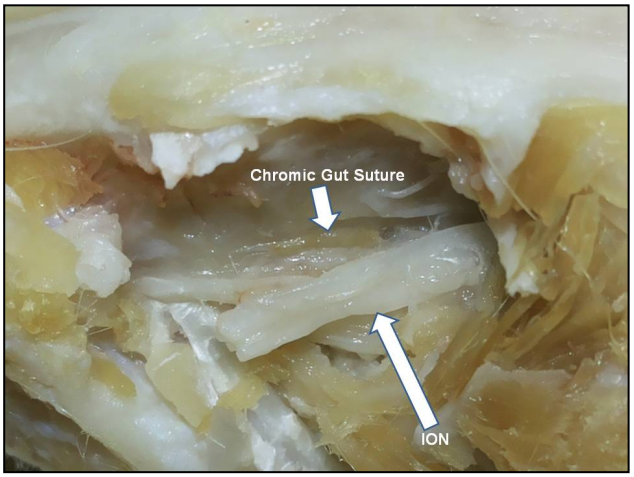
Comparison of the Placement of Chromic Gut Suture Ten Weeks Post Surgery With Phenotypic Pain Response

Upon post-mortem dissection of the experimental animals, chromic gut suture was in full parallel contact with the trigeminal nerve and adjacent to the infraorbital groove for four out of eight animals (experimental animals 10, 11, 12, and 13)(Figure 9C). All four animals in which the chromic gut suture remained in contact with the trigeminal nerve demonstrated positive Von Frey filament responses consistently through POW8. For the remaining four experimental animals, the chromic gut suture appeared embedded upon the maxillary bone and was in minimal contact with the trigeminal nerve (Figure 9B). Only one animal in which the chromic gut suture was embedded upon infraorbital groove demonstrated positive Von Frey filament response consistently through POW6.

A)



B)



C)

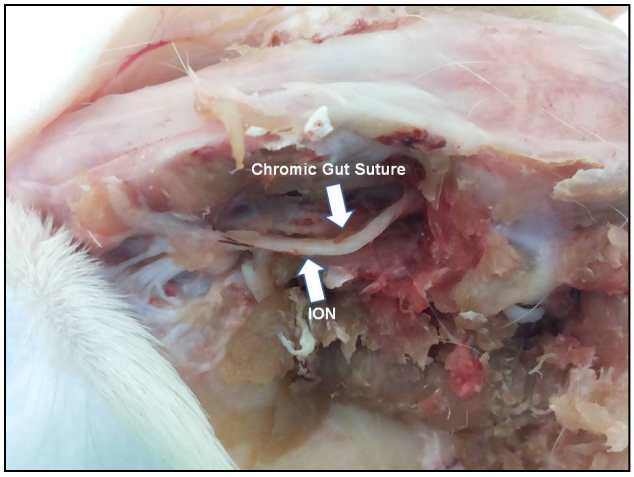


Figure 9. Dissection of the Infraorbital Nerve
A) Lateral View of Rat Skull with ION and Chromic Gut Exposed
B) Magnified Lateral View of Rat Skull with Chromic Gut Embedded in Infraorbital Groove
C) Magnified Lateral View of Rat Skull with Chromic Gut Embedded In Contact With ION
Pictures taken by Dr. Sukreet Raju

Discussion

The trigeminal inflammatory compression (TIC) model for neuropathic orofacial pain was initially validated in mice (F. Ma, Zhang, Lyons, et al., 2012). After studying the insertion of chromic suture between the infraorbital nerve and maxillary bone in mice, the mice developed mechanical allodynia up to POW10 with immunohistochemical evidence of neuronal injury in the trigeminal ganglion neurons (F. Ma, Zhang, Lyons, et al., 2012). Currently, the TIC model has yet to be assessed as a potential orofacial pain model using other small animals, such as rats. As a model for chronic orofacial pain, the TIC model has the potential to elicit continual and localized neuropathic pain originating from damage to the ION without excessive ION retraction and disruption to structures surrounding the ION compared to the CCI-ION model. However, the small operating space near the ION of the mice increases the risk of accidental injury to the mice during the TIC surgery. Comparatively, a TIC model in rats would be safer for the rats used due to an increased operating space near the ION compared to mice. This larger operating space in rats will likely reduce accidental injury during the TIC surgery. The study conducted here investigated the effectiveness of the trigeminal inflammatory compression approach in a rat model.

We hypothesized that following TIC surgery the experimental group animals would develop chronic neuropathic pain sequela when compared to the control animals. The degree of mechanical allodynia due to the TIC surgery done on these animals was characterized via Von Frey testing. The degree of neuropathic pain-induced anxiety due to the TIC surgery was characterized by the LDB test. Potential trigeminal nerve inflammation caused by chromic gut suture in parallel contact to the nerve was determined via staining of OX-42, a microglia marker, and Interleukin 1-Beta, an inflammation marker.

Von Frey Test and Mechanical Allodynia

This study provides evidence that the TIC surgery may induce chronic mechanical allodynia in rats. Five out of eight experimental animals demonstrated increased sensitivity to non-noxious Von Frey stimulation on the ipsilateral side of the face by exhibiting aversive responses to lower filament weights at POW6 as compared to baseline responses (Figure 4). Inclusive in these five experimental animals, two experimental animals demonstrated mechanical allodynia the entire length of the study through POW10 (Table 1). The chromic gut suture was observed to be in contact with the ION for each of these two experimental animals at POW10. None of the experimental animals exhibited mechanical allodynia on the contralateral whisker pad at any point during the course of the study. Thus, the absence of contralateral response suggests that the TIC surgery did not induce a bilateral pain state. Additionally, all control animals did not show mechanical allodynia on the ipsilateral side of the face at any point during the course of the study. The consistent absence of mechanical allodynia for control animals during the course of the study demonstrated that repeated exposure to Von Frey testing in conjunction with the sham surgery did not affect the irritability or sensitivity of the control animals. Since only experimental animals demonstrated mechanical allodynia on the ipsilateral whisker pad, these results suggest that localized facial mechanical allodynia was induced in the experimental animals due to the TIC surgery. Moreover, with continual placement of the chromic gut suture in contact with the ION, the TIC surgery may have produce chronic facial mechanical allodynia.

Comparison of Von Frey Data to Alternative Chronic Orofacial Pain Models

Due to the variety of methods used to quantify mechanical allodynia in orofacial pain animal models, there is difficulty in directly comparing the extent of induced mechanical

allodynia between this study and others. Von Frey testing and air puff pressure testing have been primarily used to study mechanical allodynia. Air puff pressure testing involves the application of various air pressure intensities to the rat's whisker pad to evoke withdrawal behavior (Ahn et al., 2009; Jeon et al., 2012). Differences in application and type of force tested on rat's whisker pad between Von Frey testing and air puff pressure testing make comparisons between the assays difficult to quantify. Only the presence of mechanical allodynia can be accurately compared between the two tests. With regard to Von Frey testing, several studies (Christensen et al., 2001; Kernisant et al., 2008; Vos et al., 1994) evaluating mechanical allodynia using Von Frey testing have utilized the rank-order system developed by Vos et al. (1994). In the rank order system, each filament is applied to the rat's whisker pad and the resulting response is quantified to a response score by attributing one point for each of the following recognized behaviors: 1) Detection, rat turns to and investigates the stimulus applied 2) Withdrawal Reaction, rat turns away from stimulus applied 3) Escape/Attack, rat physically moves away from stimulus applied or attacks stimulus applied 4) Asymmetric Face Grooming, rat strokes whisker pad three times immediately after stimulus has been applied (Vos et al., 1994). Each response score was attributed to a response category: score of 0 is no response, score of 1 is non-aversive response, score of 2 is mild aversive response, score of 3 is strong aversive response, and score of 4 is prolonged aversive behavior (Vos et al., 1994). The response score to a given filament weight is then observed over time. Other studies using Von Frey testing have utilized the Up-Down Method (An et al., 2011; F. Ma, Zhang, Lyons, et al., 2012). In the Up-Down Method, if after a filament was applied the rat demonstrated aversive behavior (as defined by the researcher), a weaker filament is then applied and tested. Alternatively, if after a filament is applied the rat demonstrates no response, a stronger filament is applied and tested. After a series of four positive

responses have occurred after the initial filament was applied, a 50% mechanical withdrawal threshold calculation is made using the Dixon formula (Dixon, 1980). Some researchers have created methods of Von Frey filament testing analysis that seem to be specific to their studies of orofacial pain (Krzyzanowska et al., 2011; Xu et al., 2008). This study uses Von Frey testing to observe a minimal pain threshold, in which successive filaments are applied to the rat whisker pad in order of increasing weight until a positive aversive response has been observed. This method has also been implemented successfully in model of orofacial pain by Luo et al. (2012). Though there are many methods to quantify orofacial mechanical allodynia in rats, comparisons related to the time course and spatial localization of mechanical allodynia induced by various models can be made.

In this study, the TIC model in rats in this study demonstrated localized ipsilateral mechanical allodynia. In comparison, other models of orofacial pain demonstrated both ipsilateral and contralateral mechanical allodynia, also known as mirror-image allodynia. A majority of rodent models of orofacial pain demonstrate contralateral mechanical allodynia that is to a lesser degree than the ipsilateral pain, including the original TIC model for mice (Ahn et al., 2009; An et al., 2011; Christensen et al., 2001; Jeon et al., 2012; Krzyzanowska et al., 2011; F. Ma, Zhang, Lyons, et al., 2012; Vos et al., 1994). The presence of contralateral orofacial pain in these models does not seem to depend upon whether the pain is induced by chemical or physical manipulation of the ION. Research has shown that mirror-image allodynia may be mediated by the intensity of an immune response to nerve inflammation (Chacur et al., 2001). In this study, the TIC model in rats may not have induced enough of an immune response to the site of ION injury to elicit contralateral allodynia, and thus, did not elicit mirror-image allodynia.

Consistent ipsilateral allodynia in rats induced by the TIC surgery appeared to originate at the 7 days post-operative, similar to other rat models of orofacial pain (Figure 4). The time point at which consistent ipsilateral allodynia is observed may be indicative of when the chemical or physical manipulation of the ION elicits allodynia and also when the post-operative pain is unlikely to contribute to allodynia. This time point shall be referred to as the origination of induced allodynia. An early origination of induced allodynia may be evidence of a short post-operative pain and recovery from period from surgery. The origination of induced allodynia is between 7 and 12 days for rat orofacial pain models using chemical irritants on the ION (Ahn et al., 2009; An et al., 2011) and between 3 and 7 days for rat models using physical compression of the ION (Jeon et al., 2012; Luo et al., 2012). Comparatively, the origination of induced allodynia is 12 days for the rat CCI-ION model (Vos et al., 1994) and 3 days for the mouse TIC model (F. Ma, Zhang, Lyons, et al., 2012). In comparison to other rat models of orofacial pain, the TIC model in rats presented here has an average origination of induced allodynia and thus does have a comparatively average period of post-operative pain and recovery period. However, mechanical allodynia in this study was only first recorded at 7 days post-operative whereas earlier recordings of mechanically allodynia may have revealed earlier evidence of induced allodynia.

The duration of induced mechanical allodynia in responsive rats via the TIC surgery in this study demonstrated similar longevity compared to other rat models of orofacial pain. First, as seen in other rat models of orofacial pain, no effect was seen in control animals in this study. In comparison to models strictly based on trigeminal nerve root compression without chemical agents, the orofacial pain rat model using trigeminal compression via a plastic filament showed statistically significant mechanical allodynia in rats up to four weeks post surgery (Luo et al.,

2012). Additionally, the orofacial pain rat model using trigeminal compression via agar solution showed statistically significant mechanical allodynia in rats up to 40 days post surgery (Jeon et al., 2012). Furthermore, in comparison to models strictly based upon chemical irritation of the infraorbital nerve, the orofacial pain model utilizing the injection of cobra venom into the ION showed statistically significant mechanical allodynia in rats up to 60 days post surgery (An et al., 2011). Moreover, the orofacial pain model utilizing injection of LPA into the trigeminal ganglion showed statistically significant mechanical allodynia in rats up to 100 days post surgery via injection of LPA into the trigeminal ganglion (Ahn et al., 2009). As a model that utilizes the compression of the infraorbital nerve and chromic gut suture as a chemical irritant, the TIC rat model studied here demonstrated mechanical allodynia in 50% of experimental animals up to 56 days and 25% of experimental animals up to 70 days (Figure 4). Using similar approach of physical and chemical disruption to the infraorbital nerve, the original CCI-ION model demonstrated in rats that chronic constriction of the infraorbital nerve using chromic gut suture induced mechanical allodynia up to 80 days (Vos et al., 1994). Interestingly, the TIC model evaluated here did not elicit chronic orofacial pain in rats as effectively as the original TIC model in mice. As stated before, the TIC model in mice demonstrated the presence of significant mechanical allodynia in all animals used for experimentation for more than 10 weeks (F. Ma, Zhang, Lyons, et al., 2012).

Differences between the TIC model in rats of this study and the original TIC model in mice may be attributed to improper adjustments when adapting the TIC procedure from mice to rats. In mice, the TIC model utilized the placement of a 2 mm length of 6-0 chromic gut suture adjacent to the ION (F. Ma, Zhang, Lyons, et al., 2012). To adapt to the size of a rat, the TIC model in this study doubled the length and increased the diameter of the suture used, resulting in

the placement of a 4 mm length of a 3-0 chromic gut suture. It is possible that the size increase of chromic gut suture used in this study was not enough of a size increase to effectively cause inflammation and damage to the larger rat ION to cause sustained neuropathic pain. After necropsy of the experimental animal and Von Frey data analysis was conducted, the majority of experimental animals that demonstrated positive responses consistently during the course of the study had retained placement of the chromic gut suture alongside the ION. Conversely, the majority of experimental animals that did not demonstrate positive responses consistently during the course of the study had not retained placement of the chromic gut suture alongside the ION. Thus, the ability of the chromic gut suture to retain placement alongside the ION is believed to be crucial to the production of neuropathic pain. If chromic gut suture was not large enough to fit securely between the maxillary bone and the ION, the chromic gut suture may have been more vulnerable to movement and displacement. Further increase in both the length and diameter of the chromic gut suture used for the TIC model in rats may help the chromic gut suture to retain its position and induce chronic mechanical allodynia.

Light and Dark Box

This study provided limited evidence to support that the TIC surgery induced chronic anxiety in rats. Since there was no statistical difference for TL and NT between the experimental and control animals, there is no statistically suggestive evidence that the TIC surgery induced chronic anxiety in rats at any time point during the course of the study. Comparison between control animals and experimental animals that responded positively to Von Frey testing did not reveal significant differences in LDB testing behavior since the number of positively responding animals decreased in size during the course of the study. However, the trends in this study may have achieved statistical significance if a larger number of animals were used and demonstrated

positive responses to Von Frey testing. Discussion of the data collected from LDB testing will be based upon average trends observed as a guide for future experiments. On average, the decreased TL and NT of all experimental animals compared to control animals at POW1 may be indicative of decreased exploratory behavior and increased anxiety of experimental animals at POW1 (Figure 5A and Figure 6A). However POW2, all experimental animals had on average increased TL and increased NT compared to control animals (Figure 5A and Figure 6A). Thus, the TIC surgery may have elicited temporary pain-induced anxiety at POW1 that was relieved at POW2 (See Section on Post-Operative Pain). There was no difference in TL or NT between all experimental and control animals between POW3 and POW6. Thus, the TIC surgery most likely did not cause any pain-induced anxiety during this time frame. At POW 10, experimental animals 11 and 12 with positive Von Frey responses did spend on average decreased TL and NT compared to control animals, indicative of decreased exploratory behavior and increased anxiety (Figure 5B and Figure 6B). Thus, at POW10 in those particular animals, the TIC surgery may have elicited pain-induced anxiety due to the presence of the suture as additionally supported by the presence of orofacial mechanical allodynia in these animals at this time point.

There was an overall negative trend in exploratory/anxiety behavior, indicated by decreased TL and NT, of both animal cohorts during the study. This may be explained by either an unknown environmental factor or that animals used in the study became habituated over time to LDB testing developed a preference for the dark compartment.

Comparison of Light and Dark Box Testing to Previous Models of Pain

LDB testing of experimental and control animals in this study did not reveal pain-induced anxiety in contrast to other pain models in rats. Chronic neuropathic pain models via chronic constriction injury of the sciatic nerve demonstrated a significant decreased TL in comparison to

control animals at least week 4 post-surgery and no weeks prior (Narita, Kaneko, et al., 2006; Narita, Kuzumaki, et al., 2006). Furthermore, control animals in chronic sciatic neuropathic pain models consistently demonstrated no significant changes in TL over the course of 4 weeks post-surgery (Narita, Kaneko, et al., 2006; Narita, Kuzumaki, et al., 2006). Thus, chronic sciatic neuropathic pain most likely elicits pain-induced anxiety at week 4 post-surgery. A chronic model of visceral pain via colonic injection of zymosan have demonstrated consistent and significant decreased TL in comparison to sham-injection animals from day 1 post-injection to at least 4 weeks post-injection (Zhang et al., 2014). Similar to chronic sciatic neuropathic pain models, sham-injection animals in this model of chronic visceral pain consistently demonstrated no significant changes in TL over the course of 4 weeks post-surgery (Zhang et al., 2014). Thus, this model of chronic visceral pain most likely elicits pain-induced anxiety as well at week 4 post-surgery. No conclusive evidence in literature has suggested that anxiety due to chronic inflammatory pain via intraplanar injection of CFA has been demonstrated via LDB testing (Narita, Kaneko, et al., 2006; Parent et al., 2012). Still, a rat model of inflammatory orofacial via intraarticular injection of CFA into the tempomandibular joint (TMJ) has demonstrated both a significant decrease in TL and NT compared to sham-injection animals until least 10 days post-injection (do Nascimento & Leite-Panissi, 2014). However, both CFA-injected animals and sham-injected animals in this model of inflammatory orofacial pain on average demonstrated increasing TL from day 3 to day 10 post-injection compared to baseline (do Nascimento & Leite-Panissi, 2014). Similarly, both CFA-injected animals and sham-injected animals in this model of inflammatory orofacial pain demonstrated increases in NT from day 1 to day 10 post-injection (Nascimento 2014). Comparatively, the TIC model in rats used in this study did demonstrated a

negative trend in both TL and NT for experimental and control animals over the course of the study and did not conclusively elicit pain-induced anxiety over the course of the study.

Light and Dark Box Test: Need for More Testing

In this study, the LDB was the behavioral assay we used to characterize pain induced anxiety. However, there are other behavioral assays, such as the elevated plus maze and the open field assay, used to characterize pain-induced anxiety as caused by inflammatory or neuropathic pain. Both the elevated plus maze and open field tests are based upon the behavioral tendency of a rat to move toward enclosed spaces rather than more exposed spaces (Prut & Belzung, 2003; Walf & Frye, 2007). Although it is not uncommon to successfully demonstrate pain-induced anxiety via a single behavioral assay (Caspani et al., 2014; Roeska et al., 2008; Wallace et al., 2008), several studies have demonstrated that using multiple behavioral assays including the LDB test can characterize anxiety-related behaviors in rats without discrepancies between assays (do Nascimento & Leite-Panissi, 2014; Grivas, Markou, & Pitsikas, 2013; Narita, Kaneko, et al., 2006). Thus, in place of using only the LDB in isolation, the use of the LDB in conjunction with other behavioral assays may provide greater evidence of an induced-anxiety state in rats. Still, not all studies using multiple behavioral assays have found consistency between assays in characterizing anxiety-related behaviors. This may be explained if each behavioral assay only detected changes in a specific anxiety-related behavior.

There is evidence to suggest that each behavioral assay may assess different and partially overlapping aspects of anxiety-related behavior or emotionality with respect to chronic pain (Parent et al., 2012; Ramos, 2008). Each behavioral assay for anxiety will reveal a specific emotional reaction elicited by a certain set of stimuli, and the emotional reaction will be representative of a particular emotional state (Rodgers 1997). Thus, each behavioral assay will

only reveal changes in a specific anxiety-related behavior. The specific type of anxiety-related behavior altered by a given pharmaceutical or surgical change to an experimental animal's physiology will be different depending on the biochemical change induced in the animal. Thus, the anxiety induced in animals by experimental means will likely be reflected in one of a limited number of behavioral assays that observes a specific type of anxiety-related behavior.

Beyond selection of the correct behavioral assay for anxiety, other influences need to be considered that will affect the effectiveness of the assay used. The performance of an animal in a behavioral assay for anxiety may be altered by influences unrelated to the biochemical means by which anxiety was induced. Some animals may have a greater innate tendency behave anxiously. The tendency for anxious behavior by laboratory animals may be dependent on variable environmental factors, such as variable events affecting the rats immediately prior to testing such as sleep or familiarity to the handling experimenter, that are not taken into context when performing behavioral assays (Izidio, Lopes, Spricigo, & Ramos, 2005). Interestingly, it has been proposed that the emotional state of the tested animal fluctuates with time and that performing different anxiety-related behavioral assays at different time points may measure the innate tendency of an animal for anxious behavior at a particular emotional state at that time (Ramos, 2008). Additionally, the order of multiple behavioral assays if performed in succession may influence the results of each assay performed (Acevedo, Nizhnikov, Molina, & Pautassi, 2014).

In consideration of these recent theories regarding rodent anxiety-related behavior, any pain-induced anxiety caused by the TIC surgery in the experimental animals may have only influenced a certain aspect of the animal's anxiety behavior. Consequently, the LDB assay may not have been able to adequately assess this specifically altered aspect of the experimental animal's anxiety behavior. Future studies seeking to understand how the TIC surgery may affect

the anxiety behavior of an animal would include an analysis of which behavioral assay was able to quantify the specific aspect of anxiety behavior altered by this intervention. If this behavior assay is elucidated, then that particular behavioral assay could be used consistently to quantify changes in anxiety-related behavior caused by the TIC surgery. A multitude of behavioral assays that would properly characterize multiple aspects of anxiety behaviors would then not be necessary.

The Influences of Chronic Stress and Anxiety on the Pain Perception of Rats

Potential Chronic Stress Experienced by Rats in Study

Experimental and sham animals used in this study experienced chronic stressors due daily living conditions, surgery, and routine experimental handling for behavioral assays. Physiological stress is defined the response of an organisms given an internal or external challenge to an organism's homeostasis (Kagias, Nehammer, & Pocock, 2012). Living environmental conditions and routine handling may have increased stress in all rats during the study. Prior to surgery, all rats were subject to daily stressors such as environmental noise, housing conditions, and routine handling. Environmental noise in the animal facility setting has been shown in rats to cause a physiological changes and a stress response (Jain & Baldwin, 2003; Wilson & Baldwin, 1998, 1999). In the rat cage, lack of sufficient environmental enrichment that the rat can interact with may cause increased stress in rats as indicated by corticosterone levels (Belz, Kennell, Czambel, Rubin, & Rhodes, 2003). Routine handling, such as required lifting of the rat and movement of the cage necessary for cage cleaning, have also been shown to increase corticosterone levels and blood pressure as related to a stress response (Balcombe, Barnard, & Sandusky, 2004). After surgery, experimental and sham animals may have experienced a period of post-operative stress. Experimental animals most likely

experienced chronic stress due to chronic pain, inflammation, and immune activation induced by the TIC surgery. Moreover, orofacial pain may have influenced eating and drinking habits of the rats.

Similar to routine handling, handling of the rats during Von Frey and LDB testing was likely an additional source of stress to experimental and sham animals. The regular application of Von Frey filaments and physical manipulation of rats during Von Frey testing was likely to have contributed to the stress of the rats, influencing their response to the testing. During Von Frey testing, each animal was handled with the hands of a researcher into a submissive position intended to limit the animal's movement to only neck movement. The Von Frey filaments were applied and the rat's response was observed. Due to the constraining nature of the testing for the rats, many rats would make exaggerated movements to free themselves and thus sometimes would need to be repositioned between administrations of successive filament weights, contributing additional stress to the rat. Additional physical manipulation beyond routine handling was needed to place the rats inside the LDB testing box.

Potential Anxiety Experienced by Rats Used in Study

Rats used in this study most likely experienced anxiety caused by handling. As defined previously, animal anxiety is a defensive and adaptive response to the seemingly imminent threats from the environment (Rodgers, 1997). During the study, handling and movement of the rats may have increased anxiety in the rats. Once a rat is lifted against its own will to an unfamiliar location, the rat may experience anxiety related to the unknown presence of threats in the new environment. Once a rat is transferred to a new location, the rat will move to explore its new environment. Whether the new environment is the arms of a researcher or a testing environment, the rat's movements are restricted to limit exploratory behavior in the laboratory

setting. Unwanted exploratory behavior by rats may include escaping the confined area that a rat has been placed in while in a laboratory setting. Limiting exploratory behavior is typically achieved by physical manipulation of the rat by a researcher, which may be uncomfortable to the animal. During unwanted exploratory behavior, any movement by the rat is subject to the imminent threat of physical manipulation by the researcher. Thus, any physical manipulation that distressingly impedes autonomous movement by the rat will likely increase the anxiety of the rat. Physical manipulation limiting unwanted exploratory behavior was necessary in particular during Von Frey and LDB testing to ensure consistent body positioning.

Potential Effects of Chronic Stress and Anxiety on Pain Perception

Studies in rats have shown that stress and anxiety can alter pain perception antagonistically. Moreover, there seems to be a dichotomy between the effects of acute stress and chronic stress in relation to pain perception. Acute stress has been shown to suppress pain through different pain inhibitory mechanisms, a phenomenon known as stress-induced analgesia. Acute periods of minor stress have been shown to activate endogenous opioid system to relieve pain while acute periods of severe stress have been shown to activate non-opioid systems, such as GABAergic, glutamatergic, and cannabinergic systems, to relieve pain (Butler & Finn, 2009; Hohmann et al., 2005; Marek, Mogil, Sternberg, Panocka, & Liebeskind, 1992; Parikh et al., 2011; Terman, Shavit, Lewis, Cannon, & Liebeskind, 1984). However, chronic stress in rats has been shown to increase pain perception in the form of hyperalgesia, mediated by decreased sensitivity to endogenous opioids (da Silva Torres et al., 2003; Gamaro et al., 1998; Quintero et al., 2000). Specifically, chronic stress has been shown to induce orofacial hyperalgesia (Gameiro et al., 2005). Similarly, prolonged states of anxiety have been shown to cause hyperalgesia,

mediated by a cholecystokininergic system in the frontal cortex (Andre et al., 2005; Colloca & Benedetti, 2007).

Potential Effects of Chronic Stress on Pain Perception via Hypothalamic-Pituitary-Adrenal (HPA) Axis Dysfunction.

The accumulation chronic stressors on rats may alter the degree of pain experienced by the rats via HPA dysfunction. Each environmental stressor may contribute to the activation of the HPA axis in rats, which results in the production of corticosterone to mediate the stress response (Blackburn-Munro & Blackburn-Munro, 2001). Glucocorticoids such as corticosterone are known to suppress immune response and inflammatory responses associated with stress (Chrousos, 1995; Glaser & Kiecolt-Glaser, 2005). Excessive stimulation of the HPA axis by chronic stressors has been shown to disrupt negative feedback mechanisms that regulate activity of the axis, leading to a hyperactive HPA axis and elevated basal glucocorticoid concentrations (Blackburn-Munro & Blackburn-Munro, 2001). This abnormal state of the HPA axis will be referred to as HPA dysfunction. Rats experiencing HPA dysfunction due to chronic stressors may have an inability to mount an additional corticosterone response to future stresses (Blackburn-Munro & Blackburn-Munro, 2001). Additionally, rat HPA dysfunction leading to an increased HPA response may increase the rat's resistance to inflammation pathology and compromise normal function of the immune system (Blackburn-Munro & Blackburn-Munro, 2001; Chrousos, 1995). Since the development of neuropathic pain after nerve injury is dependent upon inflammatory and immune reactions, neuropathic pain may not cause intense sensations of pain if there is HPA dysfunction, as determined by increased corticosterone levels in the blood. Thus, if the rats used in this study experienced HPA dysfunction, any neuropathic pain sensations felt by the rats would be diminished in comparison to rats with normal HPA function.

Potential Effects of Stress on Von Frey Testing

Chronic stressors experienced by the animals in this study may have influenced the results of the Von Frey testing. Since there ways that chronic stressors in this study that could have influenced the pain perceptions of the rats used, the degree to which chronic stressors influenced the pain perception and pain-induced anxiety of rats during the study can only be speculated. However, the effect of HPA dysfunction on pain perception may explain trends seen in the Von testing. By POW10, three experimental animals that had demonstrated mechanical allodynia at all of the previous time points no longer demonstrated mechanical allodynia. Therefore it is plausible that orofacial neuropathic pain experienced by these experimental rats may have been attenuated after POW8 due to HPA dysfunction, resulting in the loss of observable mechanical allodynia.

Though chronic stressors may have influenced the pain perception of all rats used in this study, the consistent procedural nature of Von Frey filament testing for all animals supports the conclusion that a portion of the experimental animals did indeed demonstrate mechanical allodynia as a result of the TIC surgery during the study.

Potential Effects of Anxiety on LDB Testing

Anxiety experienced by the animals in this study may have influenced the results of the LDB testing. As stated previously, increased anxiety may have been experienced by rats due to routine and experimental handling. Increased anxiety due to handling may have contributed to the decreasing time in the light box spent by both experimental and control animals during the course of the study. Alternatively, these LDB testing trends may also be explained by habituation of the rats to the testing environment.

Post-Operative Pain due to TIC Surgery

Evidence from LDB testing and Von Frey testing suggest that post-operative pain after the TIC surgery and not the sham surgery was present until approximately POW1. During the study, one experimental animal was observed to have temporary mechanical allodynia at only POW1 and no later time points. In comparison, five out of eight experimental animals demonstrated a degree of mechanical allodynia up to POW8. Chronic mechanical allodynia seen in experimental animals was most likely due to the presence of chromic gut suture while temporary mechanical allodynia at POW1 was most likely due to post-operative pain specifically due to the TIC surgery. Post-operative pain in experimental animals with chronic mechanical allodynia may have been indistinguishable from pain induced by the presence of chromic gut suture. As previously noted, control animals did not experience induced mechanical allodynia at any point during the study. Thus, there was not post-operative pain as indicated by temporary mechanical allodynia present in the control animals. Evidence of post-operative pain can also be seen in LDB testing. Although the majority of experimental animals from POW2 to POW6 demonstrated increased mechanical allodynia compared to control animals, there was no apparent difference in anxiety between the two animal cohorts during the same time range. Thus, orofacial pain (as indicated by mechanical allodynia) exhibited by experimental animals did not induce an anxiety state during the study as detected by LDB testing. However, in comparison to control animals, the behavior of experimental animals in POW1 was suggestive of pain-induced anxiety related to the TIC surgery while the behavior of experimental animals in week two was not suggestive of pain-induced anxiety. Control animals did not demonstrate a similar behavioral pattern during POW1 and POW2. Thus, the TIC surgery is likely to have caused post-operative pain that resulted in the presence of pain-induced anxiety at POW1.

Neuroinflammation in the TIC model in Rats

This study provided no evidence to support the presence of pro-inflammatory cytokine IL-1β and macrophage infiltration of the dissected infraorbital nerve (dION) at the site of interaction with chromic gut suture at POW10. The presence or absence of an immune and inflammatory response at earlier time points due to the placement of the chromic gut suture cannot be extrapolated from this study. Since there was no difference between the ipsilateral dION of experimental animals, the contralateral dION of experimental animals, and the ipsilateral dION of control animals at POW10, the following conclusions can be made: (A) the sham surgery did not influence the inflammatory and immune response at the dION, (B) the TIC surgery did not influence the inflammatory and immune response at the contralateral dION, and (C) the placement of chromic gut suture alongside the ION did not induce an immune and inflammatory response at POW10. Consequently, any differences in behavioral responses between control and experimental animals in the study at POW10 were unlikely related to differences in immune and inflammatory response at the region of the dissected dION. Mechanical allodynia and pain-induced anxiety demonstrated in experimental animals 11 and 12 were most likely not due to an inflammatory and immune response at the dION.

In contrast to the TIC model in mice, the TIC model in rats presented here did not elicit an immune or inflammatory response at the ION due to the placement of chromic gut suture. In the TIC model in mice, there was evidence of macrophage infiltration in the ION adjacent to chromic gut suture at 10 weeks post-surgery (F. Ma, Zhang, Lyons, et al., 2012). The lack of macrophage infiltration noted in this study may be due to placement of an inappropriately sized chromic gut suture not large enough to induce chronic macrophage infiltration. Additionally, as demonstrated by half of the experimental animals, the inability of the chromic gut suture to stay

in proper placement during this study may also have contributed to the lack of macrophage infiltration. Beyond the TIC model in mice, little research has attempted to directly characterize the presence of immune or inflammatory response at the site of nerve injury due to chromic gut suture at any time point. Following CCI-ION surgery, increased levels of ErbB3, which is involved in axon-Schwann cell communication for re-myelination after injury, were seen at the site of ION injury (F. Ma, Zhang, & Westlund, 2012). However, there is evidence to suggest that following peripheral nerve injury in rats the levels of pro-inflammatory cytokines, such as IL-1β and IL-6, reach peak concentrations at the site of injury at 7 days post-injury before gradual decline (Okamoto, Martin, Schmelzer, Mitsui, & Low, 2001). Thus, there is no consensus in the literature concerning chronic immune and inflammatory response at the site of nerve injury in rats. Although there little research has been conducted to study biochemical changes at the site of nerve injury, most research concerning trigeminal nerve injury models of orofacial pain have consistently characterized immune and inflammatory response in the trigeminal ganglion and the medullary brainstem, where the spinal trigeminal nucleus is located (Aita, Byers, Chavkin, & Xu, 2010; Jeon et al., 2012; Kernisant et al., 2008; F. Ma, Zhang, Lyons, et al., 2012; Takeda, Takahashi, et al., 2008). The spinal trigeminal nucleus contains the first synapse for relay of the pain signal from the trigeminal nerve.

Future Directions

Improvements to TIC Surgery

The primary issue of the TIC surgery noted in this study was the inability the chromic gut suture to stay in place alongside the ION. In future studies of the TIC model in rats, a larger diameter and length of chromic gut suture should be utilized to ensure the placement of the chromic gut suture. Moreover, separate research may be conducted to observe the placement of the chromic gut suture at multiple time points post-surgery.

Improvements to Von Frey Testing

The primary issue of Von Frey testing noted in this study was the stressful nature of the testing to rats. In future studies, rather than researchers holding the rats for Von Frey testing, researchers should place the rats in a plastic box or a metal mesh cage during testing to limit escaping movements and limit the need for excessive handling (Christensen et al., 2001; Luo et al., 2012; Vos et al., 1994). Additionally, more Von Frey filaments, both heavier and lighter weights, should be used so that minor changes in the presence of mechanical allodynia can be studied.

Improvements to LDB Testing

The primary issues of LDB testing were that rats may have become habituated to the dark compartment over the course of the study and that LDB testing may not have properly assess the anxiety-behavior induced by the TIC surgery. Conducting the LDB testing at a reduced frequency or only when mechanical allodynia is persistent may help to deter habituation. Studies suggest that if rats experience a period of acute stress prior to LDB testing may in enhanced anxiety behavior (Bourin & Hascoet, 2003). Thus, future studies should see if stimulation of the ipsilateral whisker pad of rats prior to LDB testing could trigger a more robust anxiety response.

In addition to LDB testing, future studies should utilized multiple behavioral assays for anxiety such as the elevated plus maze and open field test discussed previously.

Improvements to Understand the Chronic Impact of Chromic Gut Suture on the ION

In order to properly characterize an immune and inflammatory response at site of injury in the TIC model, tissue analysis for immune and inflammatory markers should take place at several time points earlier than the final time point during the study. Previous studies concerning evaluating nerve injury inflammation after peripheral nerve injury typically measure inflammatory markers at 3, 7, and 14 days post-injury (W. Ma & Eisenach, 2003; Nadeau et al., 2011; Okamoto et al., 2001). Several biomarkers related to immune and inflammatory response should be used including, IL-6. Chronic constriction injury to the ION has been shown to elevate IL-6 concentrations at the site of ION injury at day 3 post-surgery (Anderson & Rao, 2001). Moreover, proven neuronal markers of nerve injury, such as activating transcription factor 3 (ATF3), should be considered for use (Tsujino et al., 2000). Additionally in future studies, structural changes in the ION as a result of the chromic gut suture should be observed as well at several time points. Structural changes to the ION primarily observed following ION injury in models of orofacial pain are edema in axonal bundles, demyelination of axon bundles, and vascular permeability related to inflammation (Ahn et al., 2009; An et al., 2011; Jeon et al., 2012; F. Ma, Zhang, Lyons, et al., 2012; F. Ma, Zhang, & Westlund, 2012).

Proposed Alternative Behavioral Assays

There are several alternative methods by which hyperalgesia, allodynia, and pain-induced anxiety may be characterized as related to orofacial pain. Mechanical and thermal hyperalgesia have been characterized in models of orofacial pain using the pin-prick test and application of heat stimuli via infrared laser, respectively (Ahn et al., 2009; Jeon et al., 2012; Luiz et al., 2010;

Luo et al., 2012). In addition to mechanical allodynia, cold allodynia has been characterized specifically in the TIC model of mice using the application of a cold acetone solution and recording the resulting response (F. Ma, Zhang, Lyons, et al., 2012). Recently, the studying ultrasonic vocalizations, an indicator of chronic pain, in addition to mechanical allodynia may provide a more definitive characterization of chronic pain states (Kurejova et al., 2010). As stated previously, anxiety related behavioral assays such as the elevated plus maze assay and the open field assay can be used in conjunction with the LDB assay to properly characterize pain-induced anxiety through observation of different anxiety related behaviors. Each of these tests to detect hyperalgesia, allodynia, and pain-induced anxiety has been used with great frequency to characterize orofacial pain. Still, new behavioral assays are being developed that utilized operant behavior to better understand the behavior of rats in a pain state.

The Orofacial Pain Assessment Device (OPAD) studies the operant behavior of a rat with orofacial pain in a reward/conflict paradigm. Operant behavior is behavior that is changed voluntarily altered according to the known short-term consequences of that behavior (Murphy, Mills, Caudle, & Neubert, 2014). As an example, a rat will tap a pedal with increased frequency if tapping the pedal results in the rat getting more food. In the operant behavior studied using the OPAD device is the reward/conflict behavior of whether a rat that has fasted will feed on a rewarding food if the feeding requires touching both whisker pads to heat stimuli of controllable temperature or mechanical stimuli (Neubert et al., 2005). Consequently, orofacial pain will deter rats from feeding through the OPAD. The OPAD device measures pain sensitivity objectively compared to the Von Frey testing, in which positive responses may vary between studies. Additionally, the OPAD device limits excessive investigator handling associated with Von Frey

testing. Similar to the OPAD, the place escape/avoidance paradigm behavioral assay (PEAP) utilizes operant behavior to characterize increased pain sensitivity.

Comparable to the LDB test, the PEAP studies how increased pain sensitivity will modify operant anxiety-related behavior (Fuchs & McNabb, 2012). In the PEAP, each rat is placed in a box with a light and dark compartment (Fuchs & McNabb, 2012). However, when the rat is in the dark compartment, a suprathreshold Von Frey filament stimulus (a stimulus that generates a force greater than that needed to elicit a pain response) is applied to an area proposed have increased pain sensitivity (Fuchs & McNabb, 2012). Alternatively, when the rat is in the light compartment, a suprathreshold stimulus is applied to the same area on the opposite side of the body (Fuchs & McNabb, 2012). The PEAP observes how a noxious stimulus modifies the anxiety-related innate behavior of a rat to explore or behave aversively towards a novel environment as stated previously. In the PEAP, an increased time spent in the light compartment is indicative of a strong aversion to a noxious stimulus despite an innate aversion toward highly illuminated environments (Fuchs & McNabb, 2012).

Summary and Conclusion:

The advancement of novel therapeutics for TN requires small animal models of orofacial pain to demonstrate pre-clinical efficacy of the therapeutic. Although current small animal models of orofacial pain demonstrate the efficacy to elicit chronic pain, each model poses avoidable harm to the animals used for orofacial pain research. This study investigated the efficacy of the TIC model in rats with the intent to characterize a novel model of chronic orofacial pain in rodents that inflicted only necessary harm and pain. Previously studied in mice, the TIC model was proven to elicit chronic orofacial pain with the placement of a length of chromic gut suture alongside the infraorbital nerve, a sensory branch of the trigeminal nerve that mediates facial pain sensations (F. Ma, Zhang, Lyons, et al., 2012). Placement of chromic gut suture in contact with the infraorbital nerve was proposed to injure the infraorbital nerve via an inflammatory reaction and elicit neuropathic orofacial pain. This study evaluated the effectiveness of the TIC model to cause chronic and localized orofacial pain in rats with minimization of avoidable disturbance to structures surrounding the infraorbital nerve. The TIC surgery in rats induced mechanical allodynia in 62.5% of rats for six weeks post-surgery. The TIC surgery in rats did not elicit significant pain-induced anxiety during the course of the study. However, data trends from LDB testing suggest that the TIC surgery in rats may elicit pain-induced anxiety at ten weeks post-surgery. There was no evidence that the TIC surgery in rats caused an immune and inflammatory response at the site of injury to the infraorbital nerve at ten weeks post-surgery. The results of this study suggest that the TIC model in rat is a promising small animal model of orofacial pain, but only after adjustments are made to improve the TIC surgery in rats and behavioral assays used to characterize pain. An effective TIC model of

orofacial pain rats will allow future researchers to test novel drug or gene-based therapeutics to treat patients that are refractory to current treatments of TN.

Bibliography

Acevedo, M. B., Nizhnikov, M. E., Molina, J. C., & Pautassi, R. M. (2014). Relationship between ethanol-induced activity and anxiolysis in the open field, elevated plus maze, light-dark box, and ethanol intake in adolescent rats. *Behav Brain Res, 265*, 203-215. doi: 10.1016/j.bbr.2014.02.032

Ahn, D. K., Jung, C. Y., Lee, H. J., Choi, H. S., Ju, J. S., & Bae, Y. C. (2004). Peripheral glutamate receptors participate in interleukin-1beta-induced mechanical allodynia in the orofacial area of rats. *Neurosci Lett, 357*(3), 203-206. doi: 10.1016/j.neulet.2003.12.097

Ahn, D. K., Kim, K. H., Jung, C. Y., Choi, H. S., Lim, E. J., Youn, D. H., & Bae, Y. C. (2005). Role of peripheral group I and II metabotropic glutamate receptors in IL-1beta-induced mechanical allodynia in the orofacial area of conscious rats. *Pain, 118*(1-2), 53-60. doi: 10.1016/j.pain.2005.07.017

Ahn, D. K., Lee, S. Y., Han, S. R., Ju, J. S., Yang, G. Y., Lee, M. K., . . . Bae, Y. C. (2009). Intratrigeminal ganglionic injection of LPA causes neuropathic pain-like behavior and demyelination in rats. *Pain, 146*(1-2), 114-120. doi: 10.1016/j.pain.2009.07.012

Aita, M., Byers, M. R., Chavkin, C., & Xu, M. (2010). Trigeminal injury causes kappa opioid-dependent allodynic, glial and immune cell responses in mice. *Mol Pain, 6*, 8. doi: 10.1186/1744-8069-6-8

An, J. X., He, Y., Qian, X. Y., Wu, J. P., Xie, Y. K., Guo, Q. L., . . . Cope, D. K. (2011). A new animal model of trigeminal neuralgia produced by administration of cobra venom to the infraorbital nerve in the rat. *Anesth Analg, 113*(3), 652-656. doi: 10.1213/ANE.0b013e3182245add

Anderson, L. C., & Rao, R. D. (2001). Interleukin-6 and nerve growth factor levels in peripheral nerve and brainstem after trigeminal nerve injury in the rat. *Arch Oral Biol, 46*(7), 633-640.

Andre, J., Zeau, B., Pohl, M., Cesselin, F., Benoliel, J. J., & Becker, C. (2005). Involvement of cholecystokininergic systems in anxiety-induced hyperalgesia in male rats: behavioral and biochemical studies. *J Neurosci, 25*(35), 7896-7904. doi: 10.1523/JNEUROSCI.0743-05.2005

Balcombe, J. P., Barnard, N. D., & Sandusky, C. (2004). Laboratory routines cause animal stress. *Contemp Top Lab Anim Sci, 43*(6), 42-51.

Ballantyne, J. C., & Shin, N. S. (2008). Efficacy of opioids for chronic pain: a review of the evidence. *Clin J Pain, 24*(6), 469-478. doi: 10.1097/AJP.0b013e31816b2f26

Basbaum, A. I., Bautista, D. M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell, 139*(2), 267-284. doi: 10.1016/j.cell.2009.09.028

Belz, E. E., Kennell, J. S., Czambel, R. K., Rubin, R. T., & Rhodes, M. E. (2003). Environmental enrichment lowers stress-responsive hormones in singly housed male and female rats. *Pharmacol Biochem Behav, 76*(3-4), 481-486.

Belzberg, A. J. (2006). Peripheral Nerve Injury. In R. T. Johnson, J. V. Griffin & J. C. McArthur (Eds.), *Current Therapy in Neurologic Disease* (Vol. 1, pp. 241-254). Philadelphia, PA: Mosby Elsevier.

Bennett, G. J., & Xie, Y. K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain, 33*(1), 87-107.

Blackburn-Munro, G., & Blackburn-Munro, R. E. (2001). Chronic pain, chronic stress and depression: coincidence or consequence? *J Neuroendocrinol, 13*(12), 1009-1023.

Borsook, D., & Kalso, E. (2013). Transforming pain medicine: adapting to science and society. *Eur J Pain, 17*(8), 1109-1125. doi: 10.1002/j.1532-2149.2013.00297.x

Bourin, M., & Hascoet, M. (2003). The mouse light/dark box test. *Eur J Pharmacol, 463*(1-3), 55-65.

Braune, S. (2004). [Evidence-based pharmacotherapy of neuropathic pain syndromes]. *MMW Fortschr Med, 146*(50), 49-51.

Bridges, D., Thompson, S. W., & Rice, A. S. (2001). Mechanisms of neuropathic pain. *Br J Anaesth, 87*(1), 12-26.

Brown, J. A. (2014). The neurosurgical treatment of neuropathic facial pain. *Otolaryngol Clin North Am, 47*(2), 343-349. doi: 10.1016/j.otc.2013.10.003

Butler, R. K., & Finn, D. P. (2009). Stress-induced analgesia. *Prog Neurobiol, 88*(3), 184-202. doi: 10.1016/j.pneurobio.2009.04.003

Caspani, O., Reitz, M. C., Ceci, A., Kremer, A., & Treede, R. D. (2014). Tramadol reduces anxiety-related and depression-associated behaviors presumably induced by pain in the chronic constriction injury model of neuropathic pain in rats. *Pharmacol Biochem Behav, 124*, 290-296. doi: 10.1016/j.pbb.2014.06.018

Chacur, M., Milligan, E. D., Gazda, L. S., Armstrong, C., Wang, H., Tracey, K. J., . . . Watkins, L. R. (2001). A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats. *Pain, 94*(3), 231-244.

Chang, C. C., Chuang, S. T., Lee, C. Y., & Wei, J. W. (1972). Role of cardiotoxin and phospholipase A in the blockade of nerve conduction and depolarization of skeletal muscle induced by cobra venom. *Br J Pharmacol, 44*(4), 752-764.

Christensen, D., Gautron, M., Guilbaud, G., & Kayser, V. (2001). Effect of gabapentin and lamotrigine on mechanical allodynia-like behaviour in a rat model of trigeminal neuropathic pain. *Pain, 93*(2), 147-153.

Chrousos, G. P. (1995). The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N Engl J Med, 332*(20), 1351-1362. doi: 10.1056/NEJM199505183322008

Animal Welfare Act of 1990 § 2143 (2013).

Colloca, L., & Benedetti, F. (2007). Nocebo hyperalgesia: how anxiety is turned into pain. *Curr Opin Anaesthesiol, 20*(5), 435-439. doi: 10.1097/ACO.0b013e3282b972fb

Costall, B., Jones, B. J., Kelly, M. E., Naylor, R. J., & Tomkins, D. M. (1989). Exploration of mice in a black and white test box: validation as a model of anxiety. *Pharmacol Biochem Behav, 32*(3), 777-785.

Crawley, J., & Goodwin, F. K. (1980). Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacol Biochem Behav, 13*(2), 167-170.

da Silva Torres, I. L., Cucco, S. N., Bassani, M., Duarte, M. S., Silveira, P. P., Vasconcellos, A. P., . . . Ferreira, M. B. (2003). Long-lasting delayed hyperalgesia after chronic restraint stress in rats-effect of morphine administration. *Neurosci Res, 45*(3), 277-283.

Davis, M., Rainnie, D., & Cassell, M. (1994). Neurotransmission in the rat amygdala related to fear and anxiety. *Trends Neurosci, 17*(5), 208-214.

Deseure, K., Koek, W., Adriaensen, H., & Colpaert, F. C. (2003). Continuous administration of the 5-hydroxytryptamine1A agonist (3-Chloro-4-fluoro-phenyl)-[4-fluoro-4-[[(5-methyl-pyridin-2-ylmethyl) -amino]-methyl]piperidin-1-yl]-methadone (F 13640) attenuates allodynia-like behavior in a rat model of trigeminal neuropathic pain. *J Pharmacol Exp Ther, 306*(2), 505-514. doi: 10.1124/jpet.103.050286

Deseure, K., Koek, W., Colpaert, F. C., & Adriaensen, H. (2002). The 5-HT(1A) receptor agonist F 13640 attenuates mechanical allodynia in a rat model of trigeminal neuropathic pain. *Eur J Pharmacol, 456*(1-3), 51-57.

Devinsky, O., Morrell, M. J., & Vogt, B. A. (1995). Contributions of anterior cingulate cortex to behaviour. *Brain, 118 (Pt 1)*, 279-306.

Dinarello, C. A. (2011). A clinical perspective of IL-1beta as the gatekeeper of inflammation. *Eur J Immunol, 41*(5), 1203-1217. doi: 10.1002/eji.201141550

Dixon, W. J. (1980). Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol, 20*, 441-462. doi: 10.1146/annurev.pa.20.040180.002301

do Nascimento, G. C., & Leite-Panissi, C. R. (2014). Time-dependent analysis of nociception and anxiety-like behavior in rats submitted to persistent inflammation of the temporomandibular joint. *Physiol Behav, 125*, 1-7. doi: 10.1016/j.physbeh.2013.11.009

Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H. W., Simonin, F., . . . Kieffer, B. L. (2000). Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet, 25*(2), 195-200. doi: 10.1038/76061

Fuchs, P. N., & McNabb, C. T. (2012). The place escape/avoidance paradigm: a novel method to assess nociceptive processing. *J Integr Neurosci, 11*(1), 61-72. doi: 10.1142/S0219635212500045

Gamaro, G. D., Xavier, M. H., Denardin, J. D., Pilger, J. A., Ely, D. R., Ferreira, M. B., & Dalmaz, C. (1998). The effects of acute and repeated restraint stress on the nociceptive response in rats. *Physiol Behav, 63*(4), 693-697.

Gameiro, G. H., Andrade Ada, S., de Castro, M., Pereira, L. F., Tambeli, C. H., & Veiga, M. C. (2005). The effects of restraint stress on nociceptive responses induced by formalin

injected in rat's TMJ. *Pharmacol Biochem Behav, 82*(2), 338-344. doi: 10.1016/j.pbb.2005.09.003

Gauriau, C., & Bernard, J. F. (2002). Pain pathways and parabrachial circuits in the rat. *Exp Physiol, 87*(2), 251-258.

Glaser, R., & Kiecolt-Glaser, J. K. (2005). Stress-induced immune dysfunction: implications for health. *Nat Rev Immunol, 5*(3), 243-251. doi: 10.1038/nri1571

Greenwood-Van Meerveld, B., Gibson, M., Gunter, W., Shepard, J., Foreman, R., & Myers, D. (2001). Stereotaxic delivery of corticosterone to the amygdala modulates colonic sensitivity in rats. *Brain Res, 893*(1-2), 135-142.

Gregg, J. M. (1973). A surgical approach to the ophthalmic-maxillary nerve trunks in the rat. *J Dent Res, 52*(2), 392.

Grivas, V., Markou, A., & Pitsikas, N. (2013). The metabotropic glutamate 2/3 receptor agonist LY379268 induces anxiety-like behavior at the highest dose tested in two rat models of anxiety. *Eur J Pharmacol, 715*(1-3), 105-110. doi: 10.1016/j.ejphar.2013.05.048

Hao, S., Mata, M., Goins, W., Glorioso, J. C., & Fink, D. J. (2003). Transgene-mediated enkephalin release enhances the effect of morphine and evades tolerance to produce a sustained antiallodynic effect in neuropathic pain. *Pain, 102*(1-2), 135-142.

Henry, M. A., Freking, A. R., Johnson, L. R., & Levinson, S. R. (2007). Sodium channel Nav1.6 accumulates at the site of infraorbital nerve injury. *BMC Neurosci, 8*, 56. doi: 10.1186/1471-2202-8-56

Hohmann, A. G., Suplita, R. L., Bolton, N. M., Neely, M. H., Fegley, D., Mangieri, R., . . . Piomelli, D. (2005). An endocannabinoid mechanism for stress-induced analgesia. *Nature, 435*(7045), 1108-1112. doi: 10.1038/nature03658

Imamura, Y., Kawamoto, H., & Nakanishi, O. (1997). Characterization of heat-hyperalgesia in an experimental trigeminal neuropathy in rats. *Exp Brain Res, 116*(1), 97-103.

Inoue, M., Rashid, M. H., Fujita, R., Contos, J. J., Chun, J., & Ueda, H. (2004). Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. *Nat Med, 10*(7), 712-718. doi: 10.1038/nm1060

Izidio, G. S., Lopes, D. M., Spricigo, L., Jr., & Ramos, A. (2005). Common variations in the pretest environment influence genotypic comparisons in models of anxiety. *Genes Brain Behav, 4*(7), 412-419. doi: 10.1111/j.1601-183X.2005.00121.x

Jain, M., & Baldwin, A. L. (2003). Are laboratory animals stressed by their housing environment and are investigators aware that this stress can affect physiological data? *Med Hypotheses, 60*(2), 284-289.

Jeon, H. J., Han, S. R., Park, M. K., Yang, K. Y., Bae, Y. C., & Ahn, D. K. (2012). A novel trigeminal neuropathic pain model: compression of the trigeminal nerve root produces prolonged nociception in rats. *Prog Neuropsychopharmacol Biol Psychiatry, 38*(2), 149-158. doi: 10.1016/j.pnpbp.2012.03.002

Jiang, H., Fang, D., Kong, L. Y., Jin, Z. R., Cai, J., Kang, X. J., . . . Xing, G. G. (2014). Sensitization of neurons in the central nucleus of the amygdala via the decreased GABAergic inhibition contributes to the development of neuropathic pain-related anxiety-like behaviors in rats. *Mol Brain, 7*(1), 72. doi: 10.1186/s13041-014-0072-z

Kagias, K., Nehammer, C., & Pocock, R. (2012). Neuronal responses to physiological stress. *Front Genet, 3*, 222. doi: 10.3389/fgene.2012.00222

Kajander, K. C., Pollock, C. H., & Berg, H. (1996). Evaluation of hindpaw position in rats during chronic constriction injury (CCI) produced with different suture materials. *Somatosens Mot Res, 13*(2), 95-101.

Kernisant, M., Gear, R. W., Jasmin, L., Vit, J. P., & Ohara, P. T. (2008). Chronic constriction injury of the infraorbital nerve in the rat using modified syringe needle. *J Neurosci Methods, 172*(1), 43-47. doi: 10.1016/j.jneumeth.2008.04.013

Kim, S. S., Wang, H., Li, X. Y., Chen, T., Mercaldo, V., Descalzi, G., . . . Zhuo, M. (2011). Neurabin in the anterior cingulate cortex regulates anxiety-like behavior in adult mice. *Mol Brain, 4*, 6. doi: 10.1186/1756-6606-4-6

Koga, K., Descalzi, G., Chen, T., Ko, H. G., Lu, J., Li, S., . . . Zhuo, M. (2015). Coexistence of two forms of LTP in ACC provides a synaptic mechanism for the interactions between anxiety and chronic pain. *Neuron, 85*(2), 377-389. doi: 10.1016/j.neuron.2014.12.021

Kroenke, K., Outcalt, S., Krebs, E., Bair, M. J., Wu, J., Chumbler, N., & Yu, Z. (2013). Association between anxiety, health-related quality of life and functional impairment in primary care patients with chronic pain. *Gen Hosp Psychiatry, 35*(4), 359-365. doi: 10.1016/j.genhosppsych.2013.03.020

Krzyzanowska, A., Pittolo, S., Cabrerizo, M., Sanchez-Lopez, J., Krishnasamy, S., Venero, C., & Avendano, C. (2011). Assessing nociceptive sensitivity in mouse models of inflammatory and neuropathic trigeminal pain. *J Neurosci Methods, 201*(1), 46-54. doi: 10.1016/j.jneumeth.2011.07.006

Kurejova, M., Nattenmuller, U., Hildebrandt, U., Selvaraj, D., Stosser, S., & Kuner, R. (2010). An improved behavioural assay demonstrates that ultrasound vocalizations constitute a

reliable indicator of chronic cancer pain and neuropathic pain. *Mol Pain, 6*, 18. doi: 10.1186/1744-8069-6-18

Kuroda, R., Yorimae, A., Yamada, Y., Furuta, Y., & Kim, A. (1995). Frontal cingulotomy reconsidered from a WGA-HRP and c-Fos study in cat. *Acta Neurochir Suppl, 64*, 69-73.

Lattanzi, R., Maftei, D., Marconi, V., Florenzano, F., Franchi, S., Borsani, E., . . . Negri, L. (2015). Prokineticin 2 upregulation in the peripheral nervous system has a major role in triggering and maintaining neuropathic pain in the chronic constriction injury model. *Biomed Res Int, 2015*, 301292. doi: 10.1155/2015/301292

LeDoux, J. E. (1995). Emotion: clues from the brain. *Annu Rev Psychol, 46*, 209-235. doi: 10.1146/annurev.ps.46.020195.001233

LeDoux, J. E., Iwata, J., Cicchetti, P., & Reis, D. J. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *J Neurosci, 8*(7), 2517-2529.

Lee, M., Silverman, S. M., Hansen, H., Patel, V. B., & Manchikanti, L. (2011). A comprehensive review of opioid-induced hyperalgesia. *Pain Physician, 14*(2), 145-161.

Liu, L., Yang, T. M., Liedtke, W., & Simon, S. A. (2006). Chronic IL-1beta signaling potentiates voltage-dependent sodium currents in trigeminal nociceptive neurons. *J Neurophysiol, 95*(3), 1478-1490. doi: 10.1152/jn.00509.2005

Liu, T., Jiang, C. Y., Fujita, T., Luo, S. W., & Kumamoto, E. (2013). Enhancement by interleukin-1beta of AMPA and NMDA receptor-mediated currents in adult rat spinal superficial dorsal horn neurons. *Mol Pain, 9*, 16. doi: 10.1186/1744-8069-9-16

Love, S., & Coakham, H. B. (2001). Trigeminal neuralgia: pathology and pathogenesis. *Brain, 124*(Pt 12), 2347-2360.

Luiz, A. P., Schroeder, S. D., Chichorro, J. G., Calixto, J. B., Zampronio, A. R., & Rae, G. A. (2010). Kinin B(1) and B(2) receptors contribute to orofacial heat hyperalgesia induced by infraorbital nerve constriction injury in mice and rats. *Neuropeptides, 44*(2), 87-92. doi: 10.1016/j.npep.2009.10.005

Luo, D. S., Zhang, T., Zuo, C. X., Zuo, Z. F., Li, H., Wu, S. X., . . . Li, Y. Q. (2012). An animal model for trigeminal neuralgia by compression of the trigeminal nerve root. *Pain Physician, 15*(2), 187-196.

Ma, F., Zhang, L., Lyons, D., & Westlund, K. N. (2012). Orofacial neuropathic pain mouse model induced by Trigeminal Inflammatory Compression (TIC) of the infraorbital nerve. *Mol Brain, 5*, 44. doi: 10.1186/1756-6606-5-44

Ma, F., Zhang, L., & Westlund, K. N. (2012). Trigeminal nerve injury ErbB3/ErbB2 promotes mechanical hypersensitivity. *Anesthesiology, 117*(2), 381-388. doi: 10.1097/ALN.0b013e3182604b2b

Ma, W., & Eisenach, J. C. (2003). Cyclooxygenase 2 in infiltrating inflammatory cells in injured nerve is universally up-regulated following various types of peripheral nerve injury. *Neuroscience, 121*(3), 691-704.

Macianskyte, D., Januzis, G., Kubilius, R., Adomaitiene, V., & Sciupokas, A. (2011). Associations between chronic pain and depressive symptoms in patients with trigeminal neuralgia. *Medicina (Kaunas), 47*(7), 386-392.

Malin, S. A., Molliver, D. C., Koerber, H. R., Cornuet, P., Frye, R., Albers, K. M., & Davis, B. M. (2006). Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. *J Neurosci, 26*(33), 8588-8599. doi: 10.1523/JNEUROSCI.1726-06.2006

Manchikanti, L., Fellows, B., Pampati, V., Beyer, C., Damron, K., & Barnhill, R. C. (2002). Comparison of psychological status of chronic pain patients and the general population. *Pain Physician, 5*(1), 40-48.

Marek, P., Mogil, J. S., Sternberg, W. F., Panocka, I., & Liebeskind, J. C. (1992). N-methyl-D-aspartic acid (NMDA) receptor antagonist MK-801 blocks non-opioid stress-induced analgesia. II. Comparison across three swim-stress paradigms in selectively bred mice. *Brain Res, 578*(1-2), 197-203.

Martin, F. C., Anton, P. A., Gornbein, J. A., Shanahan, F., & Merrill, J. E. (1993). Production of interleukin-1 by microglia in response to substance P: role for a non-classical NK-1 receptor. *J Neuroimmunol, 42*(1), 53-60.

Matsuzawa-Yanagida, K., Narita, M., Nakajima, M., Kuzumaki, N., Niikura, K., Nozaki, H., . . . Suzuki, T. (2008). Usefulness of antidepressants for improving the neuropathic pain-like state and pain-induced anxiety through actions at different brain sites. *Neuropsychopharmacology, 33*(8), 1952-1965. doi: 10.1038/sj.npp.1301590

Maves, T. J., Pechman, P. S., Gebhart, G. F., & Meller, S. T. (1993). Possible chemical contribution from chromic gut sutures produces disorders of pain sensation like those seen in man. *Pain, 54*(1), 57-69.

Meunier, A., Latremoliere, A., Mauborgne, A., Bourgoin, S., Kayser, V., Cesselin, F., . . . Pohl, M. (2005). Attenuation of pain-related behavior in a rat model of trigeminal neuropathic pain by viral-driven enkephalin overproduction in trigeminal ganglion neurons. *Mol Ther, 11*(4), 608-616. doi: 10.1016/j.ymthe.2004.12.011

Moalem, G., & Tracey, D. J. (2006). Immune and inflammatory mechanisms in neuropathic pain. *Brain Res Rev, 51*(2), 240-264. doi: 10.1016/j.brainresrev.2005.11.004

Montano, N., Conforti, G., Di Bonaventura, R., Meglio, M., Fernandez, E., & Papacci, F. (2015). Advances in diagnosis and treatment of trigeminal neuralgia. *Ther Clin Risk Manag, 11*, 289-299. doi: 10.2147/TCRM.S37592

Murphy, N. P., Mills, R. H., Caudle, R. M., & Neubert, J. (2014). Operant Assays for Assessing Pain in Preclinical Rodent Models: Highlights from an Orofacial Assay. In B. Taylor & D. P. Finn (Eds.), *Behavioral neurobiology of chronic pain* (pp. pages cm). New York: Springer.

Nadeau, S., Filali, M., Zhang, J., Kerr, B. J., Rivest, S., Soulet, D., . . . Lacroix, S. (2011). Functional recovery after peripheral nerve injury is dependent on the pro-inflammatory cytokines IL-1beta and TNF: implications for neuropathic pain. *J Neurosci, 31*(35), 12533-12542. doi: 10.1523/JNEUROSCI.2840-11.2011

Narita, M., Kaneko, C., Miyoshi, K., Nagumo, Y., Kuzumaki, N., Nakajima, M., . . . Suzuki, T. (2006). Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. *Neuropsychopharmacology, 31*(4), 739-750. doi: 10.1038/sj.npp.1300858

Narita, M., Kuzumaki, N., Narita, M., Kaneko, C., Hareyama, N., Miyatake, M., . . . Suzuki, T. (2006). Chronic pain-induced emotional dysfunction is associated with astrogliosis due to cortical delta-opioid receptor dysfunction. *J Neurochem, 97*(5), 1369-1378. doi: 10.1111/j.1471-4159.2006.03824.x

Nathan, C. F. (1987). Secretory products of macrophages. *J Clin Invest, 79*(2), 319-326. doi: 10.1172/JCI112815

Neubert, J. K., Widmer, C. G., Malphurs, W., Rossi, H. L., Vierck, C. J., Jr., & Caudle, R. M. (2005). Use of a novel thermal operant behavioral assay for characterization of orofacial pain sensitivity. *Pain, 116*(3), 386-395. doi: 10.1016/j.pain.2005.05.011

Neugebauer, V., Galhardo, V., Maione, S., & Mackey, S. C. (2009). Forebrain pain mechanisms. *Brain Res Rev, 60*(1), 226-242. doi: 10.1016/j.brainresrev.2008.12.014

Neugebauer, V., Li, W., Bird, G. C., & Han, J. S. (2004). The amygdala and persistent pain. *Neuroscientist, 10*(3), 221-234. doi: 10.1177/1073858403261077

NINDS. (2015, Februrary 23, 2015). Trigeminal Neuralgia Fact Sheet. Retrieved March 14, 2015, from http://www.ninds.nih.gov/disorders/trigeminal_neuralgia/detail_trigeminal_neuralgia.htm

Okamoto, K., Martin, D. P., Schmelzer, J. D., Mitsui, Y., & Low, P. A. (2001). Pro- and anti-inflammatory cytokine gene expression in rat sciatic nerve chronic constriction injury model of neuropathic pain. *Exp Neurol, 169*(2), 386-391. doi: 10.1006/exnr.2001.7677

Public Health Service Policy on Humane Care and Use of Laboratory Animals (2015).

Pappagallo, M. (2001). Incidence, prevalence, and management of opioid bowel dysfunction. *Am J Surg, 182*(5A Suppl), 11S-18S.

Parent, A. J., Beaudet, N., Beaudry, H., Bergeron, J., Berube, P., Drolet, G., . . . Gendron, L. (2012). Increased anxiety-like behaviors in rats experiencing chronic inflammatory pain. *Behav Brain Res, 229*(1), 160-167. doi: 10.1016/j.bbr.2012.01.001

Parikh, D., Hamid, A., Friedman, T. C., Nguyen, K., Tseng, A., Marquez, P., & Lutfy, K. (2011). Stress-induced analgesia and endogenous opioid peptides: the importance of stress duration. *Eur J Pharmacol, 650*(2-3), 563-567. doi: 10.1016/j.ejphar.2010.10.050

Perkins, N. M., & Tracey, D. J. (2000). Hyperalgesia due to nerve injury: role of neutrophils. *Neuroscience, 101*(3), 745-757.

Price, D. D. (2000). Psychological and neural mechanisms of the affective dimension of pain. *Science, 288*(5472), 1769-1772.

Prut, L., & Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol, 463*(1-3), 3-33.

Quintero, L., Moreno, M., Avila, C., Arcaya, J., Maixner, W., & Suarez-Roca, H. (2000). Long-lasting delayed hyperalgesia after subchronic swim stress. *Pharmacol Biochem Behav, 67*(3), 449-458.

Ramon, F., & Moore, J. W. (1978). Ephaptic transmission in squid giant axons. *Am J Physiol, 234*(5), C162-169.

Ramos, A. (2008). Animal models of anxiety: do I need multiple tests? *Trends Pharmacol Sci, 29*(10), 493-498. doi: 10.1016/j.tips.2008.07.005

Robinson, I., & Meert, T. F. (2005). Stability of neuropathic pain symptoms in partial sciatic nerve ligation in rats is affected by suture material. *Neurosci Lett, 373*(2), 125-129. doi: 10.1016/j.neulet.2004.09.078

Rodgers, R. J. (1997). Animal models of 'anxiety': where next? *Behav Pharmacol, 8*(6-7), 477-496; discussion 497-504.

Roeska, K., Doods, H., Arndt, K., Treede, R. D., & Ceci, A. (2008). Anxiety-like behaviour in rats with mononeuropathy is reduced by the analgesic drugs morphine and gabapentin. *Pain, 139*(2), 349-357. doi: 10.1016/j.pain.2008.05.003

Rybka, E. J., & McCulloch, P. F. (2006). The anterior ethmoidal nerve is necessary for the initiation of the nasopharyngeal response in the rat. *Brain Res, 1075*(1), 122-132. doi: 10.1016/j.brainres.2005.12.112

Scholz, J., & Woolf, C. J. (2007). The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci, 10*(11), 1361-1368. doi: 10.1038/nn1992

Seino, H., Seo, K., Maeda, T., & Someya, G. (2009). Behavioural and histological observations of sensory impairment caused by tight ligation of the trigeminal nerve in mice. *J Neurosci Methods, 181*(1), 67-72. doi: 10.1016/j.jneumeth.2009.04.020

Shapiro, I. M., & Risbud, M. V. (2014). *The intervertebral disc : molecular and structural studies of the disc in health and disease*. Wien: Springer.

Shi, C., & Davis, M. (1999). Pain pathways involved in fear conditioning measured with fear-potentiated startle: lesion studies. *J Neurosci, 19*(1), 420-430.

Sircar, S. (2008). Nerve Degeneration *Principles of Medical Physiology*: Thieme New York.

Sommer, C., & Kress, M. (2004). Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neurosci Lett, 361*(1-3), 184-187. doi: 10.1016/j.neulet.2003.12.007

Stavropoulou, E., Argyra, E., Zis, P., Vadalouca, A., & Siafaka, I. (2014). The Effect of Intravenous Lidocaine on Trigeminal Neuralgia: A Randomized Double Blind Placebo Controlled Trial. *ISRN Pain, 2014*, 5. doi: 10.1155/2014/853826

Steimer, T. (2011). Animal models of anxiety disorders in rats and mice: some conceptual issues. *Dialogues Clin Neurosci, 13*(4), 495-506.

Stoll, G., Jander, S., & Myers, R. R. (2002). Degeneration and regeneration of the peripheral nervous system: from Augustus Waller's observations to neuroinflammation. *J Peripher Nerv Syst, 7*(1), 13-27.

Syriatowicz, J. P., Hu, D., Walker, J. S., & Tracey, D. J. (1999). Hyperalgesia due to nerve injury: role of prostaglandins. *Neuroscience, 94*(2), 587-594.

Takeda, M., Kitagawa, J., Takahashi, M., & Matsumoto, S. (2008). Activation of interleukin-1beta receptor suppresses the voltage-gated potassium currents in the small-diameter

trigeminal ganglion neurons following peripheral inflammation. *Pain, 139*(3), 594-602. doi: 10.1016/j.pain.2008.06.015

Takeda, M., Takahashi, M., & Matsumoto, S. (2008). Contribution of activated interleukin receptors in trigeminal ganglion neurons to hyperalgesia via satellite glial interleukin-1beta paracrine mechanism. *Brain Behav Immun, 22*(7), 1016-1023. doi: 10.1016/j.bbi.2008.03.004

Takeda, M., Tanimoto, T., Kadoi, J., Nasu, M., Takahashi, M., Kitagawa, J., & Matsumoto, S. (2007). Enhanced excitability of nociceptive trigeminal ganglion neurons by satellite glial cytokine following peripheral inflammation. *Pain, 129*(1-2), 155-166. doi: 10.1016/j.pain.2006.10.007

Tannenbaum, J. (1999). Ethics and Pain Research in Animals. *ILAR Journal, 40*(3), 97-110. doi: 10.1093/ilar.40.3.97

Terman, G. W., Shavit, Y., Lewis, J. W., Cannon, J. T., & Liebeskind, J. C. (1984). Intrinsic mechanisms of pain inhibition: activation by stress. *Science, 226*(4680), 1270-1277.

Thacker, M. A., Clark, A. K., Marchand, F., & McMahon, S. B. (2007). Pathophysiology of peripheral neuropathic pain: immune cells and molecules. *Anesth Analg, 105*(3), 838-847. doi: 10.1213/01.ane.0000275190.42912.37

Treede, R. D., Kenshalo, D. R., Gracely, R. H., & Jones, A. K. (1999). The cortical representation of pain. *Pain, 79*(2-3), 105-111.

Tsujino, H., Kondo, E., Fukuoka, T., Dai, Y., Tokunaga, A., Miki, K., . . . Noguchi, K. (2000). Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. *Mol Cell Neurosci, 15*(2), 170-182. doi: 10.1006/mcne.1999.0814

Ueda, H. (2008). Peripheral mechanisms of neuropathic pain - involvement of lysophosphatidic acid receptor-mediated demyelination. *Mol Pain, 4*, 11. doi: 10.1186/1744-8069-4-11

Ulrich-Lai, Y. M., Xie, W., Meij, J. T., Dolgas, C. M., Yu, L., & Herman, J. P. (2006). Limbic and HPA axis function in an animal model of chronic neuropathic pain. *Physiol Behav, 88*(1-2), 67-76. doi: 10.1016/j.physbeh.2006.03.012

van Hecke, O., Torrance, N., & Smith, B. H. (2013). Chronic pain epidemiology and its clinical relevance. *Br J Anaesth, 111*(1), 13-18. doi: 10.1093/bja/aet123

Vanderah, T. W., Gould, D. J., & Nolte, J. (2016). *Nolte's The human brain : an introduction to its functional anatomy* (Seventh edition. ed.). Philadelphia, PA: Elsevier.

Vos, B. P., Strassman, A. M., & Maciewicz, R. J. (1994). Behavioral evidence of trigeminal neuropathic pain following chronic constriction injury to the rat's infraorbital nerve. *J Neurosci, 14*(5 Pt 1), 2708-2723.

Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc, 2*(2), 322-328. doi: 10.1038/nprot.2007.44

Wallace, V. C., Segerdahl, A. R., Blackbeard, J., Pheby, T., & Rice, A. S. (2008). Anxiety-like behaviour is attenuated by gabapentin, morphine and diazepam in a rodent model of HIV anti-retroviral-associated neuropathic pain. *Neurosci Lett, 448*(1), 153-156. doi: 10.1016/j.neulet.2008.10.005

Wilson, L. M., & Baldwin, A. L. (1998). Effects of environmental stress on the architecture and permeability of the rat mesenteric microvasculature. *Microcirculation, 5*(4), 299-308.

Wilson, L. M., & Baldwin, A. L. (1999). Environmental stress causes mast cell degranulation, endothelial and epithelial changes, and edema in the rat intestinal mucosa. *Microcirculation, 6*(3), 189-198.

Wu, L. J., Kim, S. S., & Zhuo, M. (2008). Molecular targets of anxiety: from membrane to nucleus. *Neurochem Res, 33*(10), 1925-1932. doi: 10.1007/s11064-008-9679-8

Xu, M., Aita, M., & Chavkin, C. (2008). Partial infraorbital nerve ligation as a model of trigeminal nerve injury in the mouse: behavioral, neural, and glial reactions. *J Pain, 9*(11), 1036-1048. doi: 10.1016/j.jpain.2008.06.006

Yen, C. P., Kung, S. S., Su, Y. F., Lin, W. C., Howng, S. L., & Kwan, A. L. (2005). Stereotactic bilateral anterior cingulotomy for intractable pain. *J Clin Neurosci, 12*(8), 886-890. doi: 10.1016/j.jocn.2004.11.018

Zelenka, M., Schafers, M., & Sommer, C. (2005). Intraneural injection of interleukin-1beta and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain. *Pain, 116*(3), 257-263. doi: 10.1016/j.pain.2005.04.018

Zhang, M. M., Liu, S. B., Chen, T., Koga, K., Zhang, T., Li, Y. Q., & Zhuo, M. (2014). Effects of NB001 and gabapentin on irritable bowel syndrome-induced behavioral anxiety and spontaneous pain. *Mol Brain, 7*, 47. doi: 10.1186/1756-6606-7-47

Zhu, B., Xu, W. D., Rong, P. J., Ben, H., & Gao, X. Y. (2004). A C-fiber reflex inhibition induced by electroacupuncture with different intensities applied at homotopic and heterotopic acupoints in rats selectively destructive effects on myelinated and unmyelinated afferent fibers. *Brain Res, 1011*(2), 228-237. doi: 10.1016/j.brainres.2004.03.034