ATTENUATING TRIGEMINAL NEUROPATHIC PAIN BY REPURPOSING PIOGLITAZONE AND D-CYCLOSERINE IN THE NOVEL TRIGEMINAL INFLAMMATORY COMPRESSION MOUSE MODEL

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ATTENUATING TRIGEMINAL NEUROPATHIC PAIN BY REPURPOSING PIOGLITAZONE AND D-CYCLOSERINE IN THE NOVEL TRIGEMINAL INFLAMMATORY COMPRESSION MOUSE MODEL

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Danielle Nicole Lyons
Lexington, Kentucky, United States of America

Director: Dr. Karin N. Westlund High
Lexington, KY
2014

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Approximately 22% of the United States population suffers from a chronic orofacial pain condition. One such condition is known as trigeminal neuropathic pain frequently reported as continuous aching and burning pain, often accompanied by intermittent electrical shock-like sensations. Dental procedures or trauma are known causes of peripheral trigeminal nerve injury and inflammation. Patients who have this type of facial pain also suffer from emotional distress. For these reasons, trigeminal neuropathic pain needs to be studied in more detail to improve the understanding of the etiology and maintenance of this condition, as well as to develop effective treatment strategies. The first experiment was focused on characterizing the behavioral aspects of the Trigeminal Inflammatory Compression (TIC) mouse model. The findings determined that the TIC injury model induced mechanical and cold hypersensitivity that persist at least 21 weeks. This orofacial, neuropathic pain condition was accompanied by anxiety- and depressive-like behaviors at week 8 post injury. The TIC injury mouse model's chronicity and development of psychosocial impairments demonstrated its usefulness as a facial pain model. The second experiment used the mouse TIC injury model to test the ability of pioglitazone (PIO), a PPARγ agonist used clinically for treatment of diabetes, on alleviating trigeminal pain. A single low dose of PIO had no effect, but a higher dose attenuated facial pain. The third experiment determined that combining ineffective low doses of PIO and D-cycloserine (DCS) produced a potentiated anti-allodynic response of these drugs and attenuated the anxiety associated with the TIC injury. Ex vivo studies revealed that cortical mitochondrial dysfunction occurred after the TIC injury but could be reversed by the combination of DCS/PIO which improves mitochondrial function. Overall, the present studies determined that the novel mouse TIC injury model is a clinically relevant facial neuropathic pain model. The results suggest that PPARγ and brain mitochondria may represent new molecular targets for the treatment of trigeminal neuropathic pain. These studies support the future "repurposing" of
PIO and DCS as well as the combination of the two drugs for this new use in patients with trigeminal neuropathic pain.

KEYWORDS: trigeminal inflammatory compression, mouse model, PPARγ, D-cycloserine, mitochondria
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Dr. Bret N. Smith
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This dissertation is dedicated to my Rock and my Redeemer, my Lord and my Savior, Jesus Christ...

“Let all that I am praise the Lord; may I never forget the good things He does for me.” - Psalm 103:2
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CHAPTER ONE
INTRODUCTION AND BACKGROUND

1.1. Introduction of Continuous Trigeminal Neuropathic Pain

Aristotle believed that pain along with all other sensations e.g. vision/taste were perceived in the heart (Chen 2011; Rey 1993). For a culture in the prime of the dramatic theatre, where masks were used to describe an actor’s emotion, perhaps Aristotle and his fellow philosophers at the time thought that “emotions” were revealed in face, but felt in the heart. However, in the 1500s, autopsies performed by Andreas Vesalius supported the theory that the brain was in fact the site of perception of all sensations including pain. In the 1600s, René Descartes, the “Father of Modern Philosophy,” reasoned that peripheral sensory nerve fibers carried the “pain” signal to the spinal cord. The signal was then thought to be transmitted to the brain along neurons and then to the pineal organ (Descartes’ believed site of pain perception) (Chen 2011; Rey 1993). In 1664, Thomas Willis published the *Cerebri Anatome* which reinforced the brain as the site of pain and the cerebral cortex as the key player in pain perception. Since his discovery, countless scientists have added to the anatomy and theory of pain perception in hopes of answering two major questions: 1) What is pain? and 2) How can pain be stopped?

Although these questions are still being pursued, previous studies have allowed the International Study of Pain (IASP) to form a solid definition:

“Pain is an unpleasant *sensory* and *emotional* experience associated with actual or potential
tissue damage, or described in terms of such damage.” (IASP 2014)

However, this definition barely begins to describe the mechanisms of pain or the many different types of pain that exist. For example, *allodynia* is specific “pain due to stimulus that does not normally provoke pain” while *hyperalgesia* is “increased pain from a stimulus that normally provokes pain” (IASP 2014). The American Academy of Pain Medicine provides a good definition of *chronic pain*:

“Pain that persists due to pain signals continuing to fire in the nervous system for weeks, months, even years (pain resulting from damage to the peripheral nerves or central nervous system.” (AAPM, 2014)

Generally, pain is considered chronic if it lasts longer than 3 months. Surprisingly, 100 million American citizens suffer from some form of chronic pain (AAPM 2014). **Table 1.1** shows that patients with chronic pain are four times more prevalent in the population than patients with diabetes as well as patients suffering from heart disease/stroke (AAPM 2014). These statistics are most likely due to the fact that in every disease “damage” occurs in the infected tissue often times eliciting a painful response. Hence if a person has a chronic disease, then they could also suffer from chronic pain. Due to the numerous chronic diseases, there are multiple classifications of chronic pain including chronic neuropathic pain. According to IASP, *neuropathic pain* is defined as

“Pain caused by a lesion or disease of the somatosensory nervous system.
Note: Neuropathic pain is a clinical description (and not a diagnosis) which requires a demonstrable lesion or a disease that satisfies established neurological diagnostic criteria. The term lesion is commonly used when diagnostic investigations (e.g. imaging, neurophysiology, biopsies and laboratory tests) reveal an abnormality or when there was obvious trauma. The term disease is commonly used when the underlying cause of the lesion is known (e.g. stroke, vasculitis, DIABETES MELLITUS, genetic abnormality). Somatosensory refers to information about the body per se including visceral organs, rather than information about the external world (e.g., vision, hearing, or olfaction). The presence of symptoms or signs (e.g., touch-evoked pain) alone does not justify the use of the term neuropathic. Some disease entities, such as TRIGEMINAL NEURALGIA, are currently defined by their clinical presentation rather than by objective diagnostic testing. Other diagnoses such as postherpetic neuralgia are normally based upon the history. It is common when investigating neuropathic pain that diagnostic testing may yield inconclusive or even inconsistent data. In such instances, clinical judgment is required to reduce the totality of findings in a patient into one putative diagnosis or concise group of diagnoses.” (IASP 2014)

The note in the IASP definition describes neuropathic pain as a “clinical description” because of the difficulties in diagnosing patients with neuropathic pain when no cause of the pain is currently known or observed. Interestingly enough, at least half of the note description was referring to orofacial pain defined as “pain and dysfunction affecting motor and sensory transmission in the trigeminal nerve system” (De Leeuw 2008).
Trigeminal neuropathic pain was first alluded to by Areataues of Cappadocia in the 2nd Century. He not only described migraines, but also described “facial spasms and distortions of the countenance” (Nurmikko & Eldridge 2001). In 1756, Nicolaus André coined the term “tic douloureux,” meaning painful twitch, to describe these facial spasms currently known today as Trigeminal Neuralgia. However, it was not until 1773 when a full account of trigeminal neuralgia was published by John Fothergill. Although trigeminal neuralgia is the most well-known of the trigeminal neuropathic pain conditions, modern day diagnosis has helped better classify the different types of trigeminal neuropathic pain in order to determine proper treatment.

Zakrzewska and colleagues (2013) depicts the most recent classifications of chronic neuropathic facial disorders using the following features to categorize the pain: 1) location, 2) timing, 3) quality severity, 4) aggravating factors, 5) associated factors, 6) examination 7) investigations (scans or sensory testing), and 8) pain management (Zakrzewska 2013). Trigeminal neuralgia, episodic chronic pain, is viewed separately from continuous trigeminal neuropathic pain that exists in the absence of the paroxysmal attacks. For clinicians, continuous trigeminal neuropathic pain can often times be the most difficult to treat because of the amount of peripheral and central factors that contribute to the exact cause of the pain (Blasberg & Greenberg 2008; De Leeuw 2008; Okeson 2005; Zakrzewska 2013). Even though the etiology of every syndrome cannot be defined, understanding the anatomy of the trigeminal system is certainly helpful in determining some problems with trigeminal pain transmission.
1.2. Anatomy of the Trigeminal System

The trigeminal nerve is the fifth cranial containing both a motor and sensory nuclei relaying information to and from the orofacial region. This largest cranial nerve consists of three main peripheral branches: ophthalmic ($V_1$), the maxillary ($V_2$), and the mandibular ($V_3$). Figure 1.1 (De Leeuw 2008) depicts the specific orofacial region innervated by each main branch of the trigeminal nerve. The ophthalmic branch innervates the region comprised of the forehead and the upper 1/3 of the face, including the meninges. The maxillary innervation region is the middle 1/3 of the face below the eye encompassing the sinuses and the mouth region. The mandibular branch innervates the lower parts of mouth, mandible bone with the temporomandibular joint, and ear (De Leeuw 2008).

The relay of neuronal transmission from the periphery for central perception begins with a stimulus activating the peripheral pseudo-unipolar sensory neurons. Noxious input is transduced by the primary afferent nociceptors. Nociceptors are located in the skin, viscera, joints, vasculature, and muscle, although they are typically silent in deep structures. With specificity in function, modality, and density, nociceptors are able to respond to any noxious heat, cold, mechanical, and chemical stimulus. The primary nociceptor subtypes relay their information via primary afferents which include: A-delta afferents, thinly myelinated fibers with a conduction velocity of > 2 m/s and C-fiber afferents, unmyelinated fibers with a slower conduction velocity <2 m/s (Wall & Melzack 1999; Westlund & Willis 2015; Willis & Westlund 1997). These two primary afferent axonal subtypes respond similarly to the nociceptive stimuli with
the exception that the A-delta fibers surpass the C fibers in firing rate and do not respond to chemical stimuli (Voscopoulos & Lema 2010; Wall & Melzack 1999). As compared to A-beta fibers, primary afferents that respond to low threshold mechanical stimuli, the C and A-delta nociceptors have a significantly higher activation threshold in order to respond with an action potential in “normal conditions.” Therefore, slight touch (a low threshold stimulus) across the face elicits firing in the A-beta fibers while a slap across the face (a mix of low and high threshold stimuli) elicits the firing of not only the A-beta fibers, but also the C and A-delta fibers to generate action potentials.

These primary afferent neurons have their cell bodies in the peripheral trigeminal ganglion also known as the Gasserian ganglion, a collection of ganglia from all three branches of the trigeminal system. Together, the peripheral neurons enter the trigeminal dorsal root entry zone and may ascend/descend the brainstem to synapse at different levels of the trigeminal brainstem sensory nuclear complex (TBSNC) depicted in Figure 1 and 2. The nuclei composing the TBSNC are the main sensory nucleus and the spinal trigeminal nucleus (sp5), which is composed of three subnuclei: oralis, interpolaris, and caudalis. Figure1.2 (DaSilva & DosSantos 2012) shows the distribution of the A and C fibers. The A-beta fibers synapse primarily in the main sensory nucleus located in the pons and some in the sp5 oralis. The A-delta and C-fibers synapse onto second order neurons in the sp5 oralis, interporalis and sp5 caudalis, with the majority of the C-fibers synapsing in sp5 caudalis.
The second order neurons in the TBSCN ascend parallel with the medial lemniscus pathway, the sensory fibers arising from spinal levels. They project from the brainstem and synapse onto neurons in the ventroposteromedial (VPM) nucleus of the thalamus, “the sensory relay center,” forming what is known as the trigeminothalamic pathway. From the VPM the third order neurons in the thalamus will synapse onto postcentral gyrus neurons (somatosensory cortex), the actual site of pain perception. **Figure 1.3** (Becerra et al 2006) depicts this pathway using fMRI BOLD imaging from a patient suffering from trigeminal neuropathic pain. When a specific noxious stimulus was applied to the facial region, all areas of the trigeminal system were highlighted implicating involvement of the entire system in chronic facial pain.

However, the neurological system is extremely complicated involving many signaling pathways. The thalamus has received the title “the sensory relay center” because it not only receives input from the spinal trigeminal nucleus and then relays it to the cortex, but also receives input from all levels of the brainstem and the spinal cord and delivers their messages to the higher order areas of the brain. **Figure1.4** (May 2009) illustrates the “pain matrix “thereby highlighting all the areas of the brain influencing pain perception (i.e. primary and secondary somatosensory cortex, insula cortex, prefrontal cortex, cingulate cortex, posterior parietal cortex, amygdala, thalamus, periaqueductal gray, basal ganglia, supplementary motor area, and the nucleus accumbens (not shown in the figure)). This would indicate and support the idea that pain processes are not
only just a response to a peripheral nociceptive input, but are also activation of a central network.

1.3. Biopsychosocial Model of Pain

Wall and Melzack (1999) highlighted this feature of pain when they said pain can best be described in two aspects: “pain sensation” and “pain affect.” This is referring not only to the biological disturbance and emotional distress that a chronic pain patient experiences, but also including the disruptions in psychosocial functioning associated with this condition/syndrome (Engel 1997; Quintner et al 2008; Wall & Melzack 1999).

Perhaps Aristotle’s definition of pain perception occurring in the heart was not exactly wrong. Although physiologically incorrect, Aristotle understood the emotional aspect of pain that the philosophers in the 17th century overlooked. “Pain” does not exist in a vacuum and cannot exclusively be caused by one thing such as the historical biomedical model of pain suggests. This model illustrates a direct cause to the pain that a person perceives. It illustrates pain only as a symptom of an injury that occurred elsewhere in the body (Blasberg & Greenberg 2008; De Leeuw 2008). However, not every painful syndrome appears to have a direct cause. Therefore, pain cannot solely be due to “sensation.” In 1977, George Engel coined the term “biopsychosocial model of illness” in which he challenged the biomedical model of “cause-and-effect” and begin describing illness as the interaction of the biological, psychological, social, and cultural components that are exposed to a person (Engel 1977). All of these factors play
a key role in a person's perception and self-diagnosis of their pain (Quintner et al 2008).

Anxiety related to trigeminal neuropathic pain was first described by an Arab physician, Jujani, in the 11th century (Nurmikko & Eldridge 2001). Today, many patients with chronic trigeminal neuropathic pain also report numerous comorbid physical and psychological conditions as well as psychosocial and economic issues (i.e. medical expenses, loss of work, sleep disturbances, anxiety, depression, and family conflict) (Asmundson & Katz 2009; Burris et al 2010; De Leeuw 2008; Nicholson & Verma 2004; Porto F 2011). Besides the anxiety and depression that are related to pain, some patients may often even experience cognitive impairment due to their pain. (Ji et al 2010). Several studies have supported the amygdala, nucleus accumbens, and pre-frontal cortex, as major players in the observed emotional or cognitive effects (Apkarian 2004; Apkarian et al 2004a; Apkarian et al 2004b; Ji et al 2010) . However, with the pain matrix being so interconnected, often times it is difficult to determine which higher order brain regions are solely responsible for the changes observed in the brain. Therefore, this makes treating the patients with chronic continuous trigeminal pain very difficult.

1.4. Current Treatments

Clinicians have progressed in their ability to provide treatment for patients suffering from chronic orofacial pain. They are beginning to understand the comorbidities of this syndrome and therefore treat not only the “sensation,” but
also the “pain affect.” Pharmacological approaches along with psychological therapy have been implemented.

The first line of pharmacological treatment is purely based on the pain assessment and history of that patient. If local anesthetics fail to relieve pain, many studies show that tricyclic antidepressants such as amitriptyline, doxepin, or nortriptyline improve the pain of patients as well as their anxiety/depression related to their pain. Anticonvulsants, such as gabapentin, are typically not effective in subduing their pain (De Leeuw 2008). Still, pregabalin and tramadol have also been prescribed. However, many patients still struggle to find any medications that attenuate their pain.

To treat the “pain affect,” clinicians and therapist often adapt the biobehavioral model of therapy in order to treat patients suffering with chronic pain. Carlson (2008) states the biobehavioral approach as “integrating the important roles biological factors play in governing human function with the influences of behavioral factors” (Carlson 2008). Cognitive behavioral therapy (CBT) is one type of biobehavioral therapy that focuses on the patient becoming more self-aware or their condition in order to inhibit the external influences potentiating their pain response (Ehde et al 2014). The patient learns a new skill set to better disregard the outside reinforcement of the pain by learning tools for relaxation, routine management, self-care, and the self-awareness of external factors that potentiate their pain (Carlson 2008; Carlson et al 2001; Ehde et al 2014; Ullrich et al 2013).
Although somewhat effective, these therapies are still proving to be unsatisfactory in treating all patients with continuous trigeminal neuropathic pain (Koopman et al 2010). Therefore, a great need exists for new medications for these patients and a greater understanding about this type of neuropathic pain will better help to treat patients suffering with this type of continuous trigeminal neuropathic pain. It is for such reasons that animal models of chronic pain conditions such as trigeminal neuropathic pain need to be studied in more detail to better understand the etiology and maintenance of these conditions as well as to develop effective treatment strategies.

1.5. Trigeminal Inflammatory Compression (TIC) Injury

It is necessary for pre-clinical models to accurately represent the clinical picture of patients with trigeminal neuropathic pain in order to improve the validity of the results obtained from pre-clinical research. The rodent model has been chosen as the main model for inducing orofacial pain. One of the reasons for this is due to the fact that rodents are readily available as well as having a similar trigeminal system anatomy. Figure 1.5A depicts the rodent trigeminal system demonstrating the three main branches that correspond to the three main branches in humans (Leiser & Moxon 2006). Furthermore, it is quite easy to study the histology of the trigeminal brainstem sensory nuclear complex in a rodent because the barrel structure in the thalamus and brainstem corresponds to the whisker barrels on the whisker pad (Erzurumlu et al 2006; Lee et al 2009a; Lee et al 2009b; Li et al 1994; Negredo et al 2009; Zembrzycki et al 2009; ...
Figure 1.5B (Mosconi et al 2010) illustrates the whisker barrels at the trigeminal dorsal horn, thalamus, and cortex in relation to the whisker pad.

The historical model used to study trigeminal pain is the chronic constriction injury of the infraorbital nerve (CCI-ION) (Donegan et al 2013; Kernisant et al 2008; Vos et al 1994). This model induces mechanical allodynia on the whisker pad of rats because chromic gut suture is used to loosely ligate the infraorbital nerve (ION) as shown in Figure 1.6A (Kernisant et al 2008), a nerve diverging off the maxillary branch. Although, this has been the most popular trigeminal neuropathic pain injury model due to the robust induction of chronic hypersensitivity, this model does have its weaknesses. First of all, this injury does not induce hypersensitivity in all animals due to the difficulty of tying the nerve. The discrepancy comes with the tightness of the ligation. For the rats that do develop hypersensitivity, they do not develop allodynia until two to four weeks post injury (Bennett & Xie 1988; Ma et al 2012b; Vos et al 1994). The first two weeks the injured ION is silent, and it is the demyelination and other peripheral and central neuroplastic and glial mediated events that cause the hypersensitivity (Evans et al 2014; Taylor 2001; Uchida et al 2010a; Uchida et al 2010b). Furthermore, this procedure can only be performed in rats. Although, the pCCI-ION model was adapted from the CCI-ION to be a specific surgery to be performed in mice, this model punctures the ION and is difficult to repeat because often times the partial ligation will cause a complete tear of the ION (Xu et al 2008).
Our group, however, developed a new chronic trigeminal neuropathic pain model for mice known as the Trigeminal Inflammatory Compression (TIC) injury. The model is reliable, robust, and reproducible. Instead of ligating the nerve, as in the CCI-ION, a small piece of chromic gut suture is placed in the fissure between the maxillary bone and the infraorbital nerve. Figure 1.6B illustrates the TIC injury with the chromic gut alongside the infraorbital nerve (Ma et al 2012a). Our previous study determined the TIC injury model induces mechanical allodynia in the mice within one week, which persisted through at least 10 weeks in the initial study. Of the mice that receive this surgery, 100% of them develop hypersensitivity. Lastly, we defined this not just as a neuropathic pain model, but it also includes an inflammatory component because microglial activation is observed in the trigeminal dorsal horn making this a very interesting model for future continuing experiments (Ma et al 2012a).

1.6. Scope and Hypothesis of the Dissertation

The study described in this chapter of the dissertation will further characterize the mouse TIC injury model as a suitable and effective model for chronic, trigeminal neuropathic pain studies, and demonstrate its suitability for evaluation of drugs that are efficacious in alleviating the mechanical allodynia in the mice with TIC injury.

Behavioral characteristics of mice with the TIC injury were observed at time points 1, 4, and 8 weeks post injury using cognitive dependent tests ((light-dark preference, open field, and elevated plus maze). Mice with TIC injury were
then given either FDA approved drugs (Pioglitazone and D-cycloserine) in combination and/or separate to observe their inhibitory effects on orofacial neuropathic pain. Finally, assays were performed in isolated mitochondrial preparations from the mice with TIC injury for comparisons to mitochondria from naïve mice (treated vs. untreated) to determine the mitochondrial bioenergetics of each group.

The hypotheses for this study are that:

1) Orofacial neuropathic pain after TIC injury would be accompanied by anxiety- and depression-like behaviors.

2) The activation of peroxisome proliferator-activated receptor gamma (PPARγ) receptor would elicit anti-allodynic effects in the mice with TIC injury. This was tested using the PPARγ agonist, pioglitazone (PIO). The prediction is that: A. pioglitazone would attenuate mechanical allodynia on the whisker pads of the mice with TIC injury, B. PPARγ will be upregulated in the TBSNC, and C. the combination of PIO and D-cycloserine (DCS), an N-methyl-D-aspartate (NMDA) receptor agonist/antagonist, will provide a potentiated analgesic effect.

3) Mitochondrial dysfunction occurring in the mice with TIC is partially responsible for the maintenance of chronic pain. This will be tested using a mitochondrial uncoupler, 2,4-DNP to determine its ability to attenuate the mechanical allodynia on the whisker pads of the mice with TIC injury. Furthermore, this dysfunction
would be corrected with the PIO+DCS combination treatment \textit{ex vivo} utilizing the Seahorse XF\textsuperscript{24} Analyzer.
Table 1.1. Chronic Pain is More Prevalent Than Other Major Diseases.  
*(Table posted by the AAMP).*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of Sufferers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Pain</td>
<td>100 million Americans</td>
<td>Institute of Medicine of The National Academies</td>
</tr>
<tr>
<td>Diabetes</td>
<td>25.8 million Americans (diagnosed and estimated undiagnosed)</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>Coronary Heart Disease</td>
<td>16.3 million Americans</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>(heart attack and chest pain)</td>
<td>Stroke 7.0 million Americans</td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>11.9 million Americans</td>
<td>American Cancer Society</td>
</tr>
</tbody>
</table>
Figure 1.1. Anatomy of Trigeminal Nerve. The trigeminal system is composed of three main nerve branches (V1-opthalamic, V2- maxillary, V3- mandibular) (a). The facial dermatomes will synapse accordingly on the descending spinal nuclei (b). All nerve meet at the trigeminal ganglion and synapse on the Trigeminal Brainstem Sensory Nuclear Complex (TBSNC) composed of main sensory nucleus, and descending spinal trigeminal nuclei (sp5): sp5 oralis, sp5 interpolaris, sp5 caudalis (c). (from de Leeuw, 2008, figure 1-1.)
Figure 1.2. Trigeminal Brainstem Sensory Nuclear Complex (TBSNC) with Specific Nociceptor Fiber Representation. The majority of primary neurons that synapse at trigeminal sp5 caudalis are C-fibers along with a few Aδ delta fibers while the primary neurons that synapse at the main sensory nucleus, sp5 oralis, and sp5 interpolaris are primarily Aβ fibers. (from DaSilva and Dos Santos 2012, figure 1).
Figure 1.3. Functional Magnetic Resonance Imaging (fMRI) of Patient Suffering from Trigeminal Neuropathic Pain. Activation areas (red) are highlighted along the trigeminal pain pathway after a patient suffering with orofacial pain receives a specific stimulus. The image shows that the trigeminal ganglia (TG), trigeminal dorsal horn (spV), thalamus (Th), and somatosensory (SI) cortex are activated after brush, cold, and heat stimuli are applied to the face of patient suffering with orofacial pain. *(from Becerra, Morris et al. 2006, figure 2)*
Figure 1.4. The Matrix of “Pain Sensation” and “Pain Affect.” The pain matrix incorporates multiple brain regions integrating pain sensation and affect. The areas shown are somatosensory cortex 1 and 2 (S1 and S2), posterior parietal cortex (PPC), insula cortex, supplementary motor area (SMA), cingulate cortex (ACC), prefrontal cortex (PFC), amygdala, thalamus, and periaqueductal gray (PAG). (from May, 2009, figure 1).
Figure 1.5. The Trigeminal Sensory System in Rodents. (A) The rodent trigeminal nerve separates into three branches similar to the human trigeminal nerve (from Leiser and Moxon 2006, figure 1). (B) The barrel structure of whisker pads of the rodents correspond to the barrel structures observed in the brainstem, thalamus and cortex (from Mosconi, Woolsey et al. 2010, figure 1).
Figure 1.6. The CCI-ION Injury Model in Rats Compared to the TIC Injury Model in Mice. (A) The CCI-ION model requires a loose ligation of the ION by chromic gut suture that will induce hypersensitivity on the whisker pads of the rats 2-4 weeks after surgery. (B) The TIC injury model places the chromic gut suture alongside the ION to induce hypersensitivity on the whisker pads of the mice within 1 week post-surgery. The arrows point to the ION and the chromic gut suture. (figure A is from Kernisant, Gear et al. 2008, figure 1; figure B is from Ma, Zhang et al. 2012, figure 1).
CHAPTER TWO

INDUCED FACIAL PAIN, ANXIETY- AND DEPRESSION RELATED
BEHAVIORS ASSOCIATED WITH TRIGEMINAL INFLAMMATORY
COMPRESSION (TIC) INJURY

2.1. Introduction

Approximately 22% of the US population suffers from facial and headache pain. Patients with trigeminal neuropathic pain, one type of chronic facial pain, frequently report continuous aching and burning pain sensation that may be accompanied by intermittent electrical shock-like experiences. Patients who have this type of facial pain also suffer from mechanical allodynia and cold hypersensitivity (Baron et al 2010; Zakrzewska 2013). While dental procedures or trauma are known causes of peripheral trigeminal nerve injury and inflammation, in some cases, no clear causes are identified for the origin and maintenance of trigeminal neuropathic pain (Porto F 2011; Renton T 2011).

There are, however, a limited number of models of such pain conditions available for use in laboratory experiments. Historically, one model of neuropathic, facial pain frequently used in rats is known as the chronic constriction injury of the infraorbital nerve (CCI-ION) (Vos et al 1994). This model has been adapted for use in mice and is referred to as the partial CCI-ION (Xu et al 2008). These models involve tying chromic gut suture around the ION, a branch of the maxillary nerve which innervates the whisker pad of rats and mice, causing mechanical hypersensitivity in the whisker pad region. However, the
suture causes deformation of the ION and constricts blood flow thus inducing partial nerve ischemia and death (Bennett & Xie 1988; Kawamura 1997; Kim 1992), features not necessarily observed in patients suffering from trigeminal neuropathic pain. To address these issues, a novel chronic facial neuropathic pain model in mice, named as the Trigeminal Inflammatory Compression (TIC) injury model, was developed in our laboratory to more closely mimic the clinical characteristics of trigeminal neuropathic pain (Ma et al 2012a). As previously reported, the TIC injury model is produced by inserting chromic gut suture between the infraorbital nerve and the maxillary bone. This placement alongside the nerve, rather than constriction of the nerve, has been successful in preventing whole nerve ischemia, demyelination, and death in the small mice (Ma et al 2012a).

Due to its novelty, there is a great need for the TIC injury model to be further evaluated to increase our understanding of the behavioral characteristics of the model. For example, one important aspect of the clinical presentation of trigeminal neuropathic pain not yet evaluated is the common comorbidity of psychological disorders and the emotional distress (Wall & Melzack 1999). In clinical populations, symptoms of anxiety and depression in particular have been consistently observed in patients with chronic trigeminal-mediated pain (Averill et al 1996; Burris et al 2010; Fishbain 1999a; b; M. J. Robinson 2009; McWilliams et al 2003; Nicholson & Verma 2004).

The measurement of constructs such as anxiety and depression in animal models, however, has proven more difficult than making these measurements in
clinical populations. Fortunately, the use of cognitive dependent tests offers a more thorough examination of psychological constructs such as depression and anxiety, and are increasingly used by researchers seeking to understand chronic neuropathic pain conditions (Mao et al 2008; Mogil 2009). Anxiety-like behaviors in animals have been extensively studied, and numerous ones resulted in validate protocols (Belzung & Griebel 2001). Three assays that are particularly well understood in measuring animal behavior associated with psychological constructs are: the light-dark box preference test, the open field exploratory test, and the elevated plus maze task. Furthermore, the activity and rearing behavior in each of these tasks has been previously shown to be affected by the experience of pain (Bouwknecht & Paylor 2002; Crawley 1980; Parent et al 2012; Roeska et al 2008).

The aim of the current study was to further characterize the novel TIC injury model by examining mechanical allodynia and thermal hypersensitivity and by measuring pain-related anxiety- and depressive-like behavior with cognitive dependent tests. My hypothesis was that mice with TIC injury would display greater mechanical allodynia, cold hypersensitivity, and more anxiety- and depressive-like behaviors than that of naïve mice or animals undergoing sham surgical procedures only.
2.2. Materials and Methods

2.2.1. Animals

All experiments were performed with C57Bl/6 male, wild-type mice that weighed between 25 and 35 grams purchased from Harlan Laboratories (Indianapolis, IN). Animals were randomly assigned to receive either experimental (TIC injury model) surgical procedures, sham surgical procedures, or to remain in the naïve cohort. Mice were housed in a well-ventilated mouse housing room (maintained at 27°C) with a reversed 10/14 h dark/light cycle so that testing could be performed in their active period. All mice had access to food and water ad libitum throughout the duration of the experiment. Low soy bean content diet normal chow was provided (Teklab 8626, Harlan, Indiana). All experimental procedures were completed according to the guidelines provided by the National Institute of Health (NIH) regarding the care and use of animals for experimental procedures. Animal protocols were approved by the University of Kentucky’s Institutional Animal Care and Use Committee (IACUC). All animals were housed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and the United States Department of Agriculture (USDA).

2.2.2. Trigeminal Inflammatory Compression (TIC) Injury Surgery

Mice were anesthetized with sodium pentobarbital (70 mg/kg, i.p.). Under the standard sterilized condition, the hair on the top of their head was then
shaved, and ophthalmic cream was applied over their eyes to protect the eye from over-dry. Mice were then fully constrained in a stereotaxic frame. A small 15 mm incision was made along the midline of the head and the orbicularis occuli muscle was gently dissected and retracted away from the bone. Small cotton balls were packed into the orbital cavity to control bleeding, and the infraorbital nerve was located approximately 5 mm deep against the bony fissure. Animals randomly assigned to receive the TIC injury surgery underwent surgical placement of a 2 mm length of chromic gut suture (6-0), inserted between the infraorbital nerve and the maxillary bone infraorbital fissure. Chromic gut suture was inserted specifically in this region to adhere to specific infraorbital nerve bundles in order to prevent the chromic gut suture from being lost in the orbital cavity, but not to pierce the entire infraorbital nerve. Mechanical allodynia was induced in the mouse whisker pad due to the suture physically stimulating the nerve as well as the chromate salt released from the suture. Animals assigned to receive sham surgical procedures did not have the chromic gut suture placement, but only received the skin incision and muscle dissection. Naive animals did not receive any surgery. All mice were aged matched.

2.2.3. Behavioral Tests

All behavioral tests were conducted during the animal’s active cycle (i.e. dark phase of the dark/light cycle) during the hours of 8:00 am to 6:00 pm. During testing, either a red-light or a dim lamp was illuminated to allow light for the experimenters. None of the behavioral tests were conducted on the same day.
2.2.3.1. Assessment of Mechanical Allodynia

Mechanical threshold of the whisker pad was measured before and after surgery with a modified up/down method (Chaplan et al 1994) using a graded series of von Frey fiber filaments (force:0.008 g (size:1.65); 0.02 g (2.36); 0.07 g (2.83); 0.16 g (3.22); 0.4 g (3.61); 1.0 g (4.08); 2.0 g (4.31); 6.0 g (4.74); Stoelting, Wood Dale, IL). One experimenter gently restrained the mouse in their palm (2-5 minutes) with a cotton glove until the mouse was acclimated and calm. A second experimenter applied the von Frey filaments to the mouse’s whisker pad. The 0.16 g (3.22) fiber was applied first. If the mouse responded three or more times out of five trials to the fiber, this was considered to be a positive response and the next lower gram force filament was applied. However, if the mouse responded two or fewer times out of five to the fiber applied, this was recorded as a negative response and then the filament with the next higher gram force was applied. Head withdrawal/front paw sweeping/biting all were considered positive responses. Time between applications of each filament was 2-10 seconds. After one fiber successfully caused positive responses, application of the subsequent fibers continued until four fibers were applied or until the animal responded to the lowest gram force fiber. Data were analyzed with a curve-fitting algorithm that allowed for estimation of the 50% mechanical withdrawal threshold (measured in gram force). The decreased mechanical threshold value is an index of mechanical allodynia. Responses to the von Frey fibers stimulations were recorded on day 7 post surgery (TIC and sham) and continued once a week post-surgery testing both the ipsilateral and contralateral
whisker pads. A cohort of naïve mice were tested intermittently (weeks 2, 4, 8, 10, 11) alongside the sham animals and mice with TIC.

### 2.2.3.2. Assessment of Thermal Hypersensitivity

The protocol used to measure thermal hypersensitivity was adapted from Neubert and colleagues (2005) (Neubert et al 2005). Neubert and colleagues used a metal probe connected to a water bath placed in an operant licking box. The present study utilized a looped copper coil probe (0.065" I.D.; 1/8" O.D.; 0.030" Wall Thickness, Restek Corporation, Bellefonte, PA) with a 5 x 3 x 3 mm tip connected to an insulated rubber tubing and attached directly to an Isotemp bath circulator (39.5 x 24.5 x 39 cm, Isotemp 3016S; Fisher Scientific). The water bath was filled with antifreeze liquid, and digitally set to a specified temperature for testing. The temperature of the copper-wire probe was measured with a Physiotemp temperature monitor (Thermalert Model TH-8; PHYSITEMP INSTRUMENTS INC. Clifton, NJ, USA). The temperatures reported in this study were measured from the tip of the copper-wire probe. The 10-11 °C was chosen to activate cold nociceptors; likewise 45-46.5 °C was chosen to activate heat nociceptors (Neubert et al 2005; Rossi & Neubert 2009). Room temperature (23-24.3 °C) and body skin temperature (32-32.5°C) were chosen to act as controls for this experiment to ensure that the probe was not causing an adverse mechanical response to the animals. The mouse skin was shaved on the ipsilateral V2 (trigeminal nerve second branch) region just behind the whisker pad 24 hours before testing (Cha et al 2012). One experimenter gently restrained
the mouse in their palm with a cotton glove until the mouse was acclimated and calm (5 minutes). The other experimenter applied the looped copper-wire probe to the shaved V2 region (Figure 2.1.A). Head withdrawal latency, the time in seconds from which the stimulus was applied to the time the mouse reacted with head withdrawal/front paw sweeping/biting, was recorded. Three trials for each temperature were conducted with 1 minute intervals between each trial. Only one temperature was recorded per testing day and all thermal testing occurred after post-operative week 8.

2.2.3.3. Acoustic Startle Disturbance

The acoustic startle disturbance test is a well-established measure of anxiety-like behaviors in response to a stressful stimulus (Blaszczyk et al 2010; Blaszczyk et al 2000; Geyer et al 1982). Mice were placed in a vinyl cylinder container (radius: 21.5 cm; depth: 29.9 cm) with room for the animal to move. Using a modified form of an acoustic startle disturbance, one experimenter pressed a © Top Paw Dog Training Clicker (2" Length “blue bone” clicker; Item # 39330 purchased from ©Pet Smart, Lexington, KY) above the animal irregularly for a period of 2 min. The clicker was pressed with force eliciting an average frequency of 430.89 Hz (recorded with a KAYPENTAX COMPUTERIZED SPEECH LAB, Real-Time Pitch; Model #5121, version 3.4.1) ranging from 70-110 dB (Decibel Meter App; Version 1.6; Device Type: iPhone4 iOS Version: 6.1.3). Mice not currently being tested were housed in a separate sound-proof room during the acoustic startle test. Immediately after exposure to the mild
acoustic startle disturbance, mice were either placed in the dark side of the light-dark box or in the central area of the elevated plus maze to begin the test designated for that day. Only one of the operant tests was conducted per day.

2.2.3.4. Two Compartment Light-Dark Box Preference Test

The light-dark box preference task has a long history of use in the measurement of anxiety-like behavior. In this task, anxiety-like behaviors are believed to manifest as a decrease in: 1) total time spent in the light area, 2) number of entries into the light area, and 3) number of rearing/exploratory behavior (Bouwknecht & Paylor 2002; Crawley 1980; Hascoet 1998). The light-dark box consisted of two equally-sized chambers (one illuminated and one darkened; 11 x 19 x 12 cm/each) connected with a 5 x 5 cm doorway in which mice were allowed to freely move between chambers. Immediately after exposure to the mild acoustic startle disturbance (described above), mice were placed in the dark side of the box facing away from the light chamber. Animals remained in the light-dark preference box for a total of 10 minutes. Behaviors measured in this test included: (1) time spent in the light area, (2) number of transitions into the light and dark chambers, defined as at least partial passage between chambers with extension of at least one of the animal’s back leg from one chamber to the next; (3) number of rearing events, a measurement for exploratory behavior, (4) latency of the first transition into the light chamber, and (5) latency of first re-entry (transition) back into the dark chamber. Behaviors were measured at post-operative weeks 1, 4, and 8.
2.2.3.5. Open Field Exploratory Activities

Exploratory behaviors were measured using a Flexfield Animal Activity System (San Diego Instruments, San Diego, CA). This apparatus consists of two Plexiglas chambers (40 x 40 x 36 cm) equipped with Photobeam Activity System (PAS) software coupled to a Compaq 486 computer (Hewlette Packard, Palo Alto, CA). Each chamber contained infrared photobeam sensors with 16 beams on each axis (total of 32 beams) that are arranged 1.25 cm above the chamber floor. Obstruction of these photo beams constitutes movements in the x- and y- plane. The x- and y- plane were divided into 5 zones to define the center and peripheral area. Another set of 16 beams is located 8 cm above the chamber floor to record movements along the z-axis measured i.e. rearing events and rearing duration (Zhang et al 2004). Data were collected in 5-min intervals for a total of 45 minutes to record: (1) number and duration of rearing (2) active time vs. rest time, (3) overall distance travelled, (4) total beam breaks, and (5) time spent in the central verses peripheral areas of the chamber. The sum of time spent in Zone 1-4 equaled the total duration of time spent in the periphery. The duration of time in Zone 5 equaled the duration of time spent in the center. Behaviors were measured at post-operative weeks 1, 4, and 8.
2.2.3.6. Elevated Plus Maze Task

The elevated plus maze task is a widely used test for measuring fear and anxiety-like behavior and has been previously shown to be affected by rodent pain models (Belzung & Griebel 2001; Kontinen et al 1999; Parent et al 2012; Roeska et al 2008; Walf & Frye 2007). The elevated plus maze (Bioseb, Vitrolles, France) consists of four arm cross-shaped device (length: 35 cm, width: 5 cm/each, height from floor: 51 cm); two arms are enclosed on three sides by 15 cm high walls and the other two are not. All arms meet in a central area (5 cm x 5 cm) which allows animals to move freely throughout each zone of the maze. A computer equipped with automated program software (BIOEPM 1.1.14; BIOSEB, France) and linked with a camera head (DFK22AUC03) recorded each animal’s movement throughout the maze. In this test, the open arms represent a potentially threatening environment and thus anxiety-like behavior in response to this threat is believed to manifest as a decrease in the time spent in and the number of entries into the open arms of the maze (Belzung & Griebel 2001). Immediately after the acoustic startle disturbance (described above), mice were placed in the central area of the maze and allowed to explore the maze for a period of 5 minutes. Mouse behaviors were coded and analyzed off line by a blinded observer for: (1) time spent in open arms, (2) number of transitions into open and closed arms, (3) number of head dips into the open arms, defined as the movement of the animals head from the closed arm to the open arm of the maze. Behaviors were measured at post-operative weeks 1, 4, and 8.
2.2.4. Statistical Analysis

The GraphPad Prism 6 statistical program was used for all data analysis (Graph Pad Software, Inc. La Jolla, CA). Results are shown as the mean ± standard error of the mean (S.E.M.). Data were analyzed by a one-way analysis of variance (ANOVA) tests followed by Tukey post hoc test and two-way ANOVA followed by Fishers post hoc test (where was appropriate). A p<0.05 was considered significant for all tests.

2.3. Results

2.3.1. Animals with TIC Injury Displayed Unilateral Whisker Pads Mechanical Allodynia

All mice (100%) that underwent TIC surgery developed mechanical allodynia on of the ipsilateral but not contralateral whisker pad as determined with von Frey fibers thus confirming the results of our previously published paper for this model (Ma et al 2012a). For all mice with TIC injury, the mean 50% mechanical threshold of the ipsilateral side was 0.03 ± 0.28g an indication of mechanical allodynia; while the mean 50% mechanical threshold of the contralateral side was 3.72 ± 0.12g (n=13) and did not change from baseline (Figure 2.1B). The mechanical threshold of the ipsilateral side of the mice with TIC injury were significantly different from the sham animals (ipsilateral, 3.36 ± 0.07g; contralateral, 3.49 ± 0.05g; n=12) and naïve animals (ipsilateral, 3.55 ± 0.019g; contralateral, 3.50 ± 0.21g, n=5; p<0.0001, two way ANOVA, Fishers
post hoc test) within one week post injury as previously reported (Ma et al 2012a). The chronicity of this model was further shown by decreased mechanical threshold in animals with TIC injury that was present post injury only on the ipsilateral side (until the animals are euthanized at week 21).

The previous study published (Ma et al 2012a) mentioned the presence of a receptive field for each mouse, but was not discussed in detail. It is important to note that all mice with TIC responded to fiber 0.4 g (3.61) regardless of specific receptive field stimulation. In order to find the specific receptive fields of an injured animal, the von Frey fibers <0.16 g (3.22) were applied to the whisker pad sporadically until a withdrawal response occurred. The sham and naïve animals did not respond to fiber 0.4 g (3.61). The location of the receptive field for each animal was always the same every week and rarely changed once established confirming that the location of the chromic gut suture is not only injuring a localized area of axonal fibers along the infraorbital nerve, but also that these fibers correlate with a specific receptive field on the whisker pad.

Three different patterns of sensitivity were identified on the receptive fields of the whisker pads of the injured mice (Figure 2.1C). Mouse #1 is representative of the most typical receptive field observed (50% of the mice with TIC injury had this phenotype). The second most common pattern is represented by Mouse #2 (38% of mice with TIC). However, Mouse #3 (11% of mice with TIC) represents a uniquely different phenotype than the previous two in that its receptive fields are spread sporadically throughout the whisker pad. Interestingly, all mice (100%) subjected to TIC surgery showed a sensitive spot at the 6 o’clock
position on the whisker pad (Figure 2.1C, indicated by the red x with a circle). The different pattern of receptive fields did not change over the course of the animal’s life.

2.3.2. TIC Injury Induced Unilateral Cold Allodynia of the V2 Area

Withdrawal latencies for all thermal stimuli were recorded (n=8; Figure 1D). In the 10-11°C temperature stimuli point, the head withdrawal latency of animals with TIC injury (5.11 ± 0.62s) was significantly different compared to that of sham animals (9.21 ± 0.88s) and naïve animals (9.75 ± 0.71s; p<0.001, one way ANOVA, Tukey post hoc test). The head withdrawal latency of animals with TIC for 45-46.5 °C temperature point was 10.21± 1.20s which was not significant compared to that of sham animals (9.42 ±1.11s) and naïve animals (8.63 ±0.90s; p>0.05). At room temperature stimuli point (23.24.3°C), the head withdrawal latency of animals with TIC injury (15.54 ± 1.52s) were similar to that of sham animals (79 ± 0.83s) and naïve animals (17.17 ± 1.13s; p>0.05). At mouse skin temperature (32-32.5°C) point, the head withdrawal latency of animals with TIC injury was 20.42 ± 2.37s. This was not significantly different from that of the sham (17.25 ± 1.87s) and naïve animals (15.92 ± 1.75s; p>0.05; Figure 2.1D). The results indicated that animals with TIC injury were sensitive to mild cold stimuli, but had normal reactions to heat, room temperature, and skin temperature.
2.3.3. Animals with TIC Injury Displayed Anxiety- and Depressive-Like Behaviors in Two Compartment Light-Dark Preference Testing

Immediately after the 2-min acoustic disturbance the mice were put in the dark side of the light-dark box facing away from the light box and the 10 min experiment began. Total time spent in light and dark sides, light-dark transitions, rearing events and latencies of first cross into light and re-entry into dark were measured over the 10 min. There were no significant differences in time spent in the light or numbers of rearing events in weeks 1 or 4 post injury. There was no significant difference between mice with TIC and sham animals when comparing latency of the first cross into the light, latency to re-enter dark side, or the number of light-dark transitions (Table 2.1). Both mice with TIC and sham animals spent almost 50/50 time in the light or dark side of the box at post-operative week 1 and week 4. Until post injury week 8, mice with TIC injury spent less time in the light, 231.60 ± 25.55s, i.e. they hide in the dark chamber (over 70% of testing time). In contrast, the sham mice spent as long as 330.39 ± 43.72s (over 50%) in the light chamber (TIC vs. sham P<0.05, Two-way ANOVA, Fishers post hoc test, Figure 2.2A). Mice with TIC injury also had significantly less rearing events (9.77 ± 1.89/10min) at week 8 post injury compared to sham animals at week 8 post operation (18.82 ±1.95/10min, Figure 2.2B). When the number of rearing events were analyzed by the first and second five minute intervals of the light-dark box preference, mice with TIC injury still showed significantly fewer numbers of rearing events compared to sham animals (Table 2.1). The results of the light-
dark box preference test indicated that mice with TIC injury develop signs associated with anxiety- and depressive-like behaviors 8 weeks after injury.

2.3.4. Acoustic Disturbance Affected Mice with TIC in the Elevated Plus Maze Task

Immediately after the 2 min acoustic disturbance, both mice with TIC injury and sham groups were subjected to an acoustic disturbance on post-operative weeks 1, 4, and 8. Mice with TIC injury spent a significant time in the open arm (78.90 ± 30.45s) compared to that of sham animals (8.89 ± 3.52s, p<0.01, Two-way ANOVA, Fishers post hoc test) at week 1 post operation (Figure 2.3A). Mice with TIC injury also had a greater number of transitions into the open arms at week 1 (4.00 ± 0.67/5min) and week 4 (2.09 ± 0.69/5min) compared to sham animals (week 1, 1.50 ± 0.42/5min; week 4, 0.25 ± 0.16/5min; p<0.001, Two-way ANOVA, Fishers; Figure 2.3B). The number of head dips into the open arm did not differ between the mice with TIC injury and the sham animals at weeks 1, 4, an 8 post injury (Figure 2.3C). However, in a separate cohort of animals that did not receive the acoustic disturbance stimulation before the elevated plus maze task, a significant difference was not observed in time spent in the open arms and the number of transitions when comparing mice with TIC injury and sham animals (Table 2.2). These results suggest that the mice with TIC injury experience an extinction of fear behavior only after an acoustic disturbance at week 1 and 4 post injury.
2.3.5. Mice with TIC Injury Showed Less Exploratory Activities

Animals with TIC injury also showed a decrease in rearing duration (153.11 ± 18.22s) and number of rearing events (229.89 ± 27.36) at week 8 compared to the sham animals (rearing duration, 224.75 ± 25.09s; rearing events, 337.46 ± 37.67; P<0.05; Figure 2.4A & 4B). Mice with TIC injury had decreased active time (2311.50 ± 63.94s) and increased resting time (388.48 ± 63.94) 8 weeks after injury compared to that of sham animals (active, 2513.30 ± 43.15s; resting, 186.72 ± 43.15s; Figure 2.4C & 4D). Mice with TIC injury also had decreased total distance traveled compared to sham animals (TIC: 1370.20 ± 110.60cm; sham: 1666.00 ± 114.38cm, P<0.05; P<0.01; Two-way ANOVA, Fishers post hoc test; Figure2.4E). A significant difference at week 1 or 4 post injury was not observed (p's>0.5). There was also no difference in total beam breaks or center vs. peripheral time duration at any time point (Figure 2.4F, 4G, & 4H). The open field results parallel the light-dark box data supporting the development of depressive-like behaviors in mice with TIC injury at week 8 post injury.

2.4. Discussion

2.4.1 TIC Injury Model Mimics Clinical Facial Neuropathic Pain

In the present study, we determined that the surgery is 100% efficacious for inducing hypersensitivity in all of the animals receiving the chromic gut suture placement. All mice experienced mechanical allodynia with distinctive receptive
fields (sensitivity spots) that did not change over the course of the animal's life. However, the receptive field pattern variations in the TIC model were due to the relative position of the chromic gut suture, the maxillary bone, and the infraorbital nerve. The development of cold allodynia in the mice with TIC injury, but not heat hypersensitivity was another important feature of this model. Clinic patients with neuropathic pain experience cold hypersensitivity but not heat sensitivity (Baron et al 2010; Zakrzewska 2013). We also initially determined that the chronicity of the TIC injury model lasts at least until 21 weeks. These were the first recorded data indicating a mouse facial pain persisting with this time course without causing ischemia and complete demyelination of the infraorbital nerve (Ma et al 2012a).

Many acute and chronic orofacial models have existed that successful induce hypersensitivity in the mouse. However, many of the acute models do not last longer than a week and fall short of reflecting the full clinical characteristics of chronic facial pain (Bornhof et al 2011; Luccarini et al 2006; Quintans et al 2014). The chronic models of facial pain have also either differed significantly from the clinical picture of patients with trigeminal pain or have had low efficacy in mice (Saito et al 2008; Siqueira-Lima et al 2014; Xu et al 2008; Zhang et al 2012b). The TIC injury model is beneficial because it is not only reliable and reproducible, but it also closely mimics the clinical presentation of this pain condition. Thus, this study provides support improving the validity for using the model.
The TIC model demonstrated several aspects consistent with chronic facial pain in humans. While the mice with TIC had distinct receptive fields to elicit a withdrawal reflex response, human patients also have specific receptive fields triggering pain. Likewise, the pattern of the receptive fields were not always represented the same in all animals, as the pattern of receptive fields from person to person may also vary (Simons & Travell 1981; Siqueira et al 2009; Travell 1981). However, the receptive fields in the mice with TIC did not vary over the course of the mouse’s life providing a reliable sensitivity area for testing evoked hypersensitivity over a chronic period of time. Moreover, as the mice with TIC suffered from only cold hypersensitivity, patients suffering with trigeminal neuropathic pain are not often bothered by heat stimuli, but instead most often complain that light touch, wind, or cold air trigger a shooting pain (De Leeuw 2008; Zakrzewska 2013).

2.4.2. TIC Injury Developed Anxiety- and Depressive -Like Behaviors Similar to Patients Suffering with Chronic Facial Pain

For the light-dark box preference and open field exploratory test, the behavior of the sham mice in weeks 1, 4, and 8 post operation remained relatively consistent, but was significantly altered in the mice with TIC injury at week 8 post injury. In the light-dark box preference test, the mice with TIC injury spent significantly less time in the light side of the box and had a fewer number of rearing events. Similarly for the open field testing, the mice with TIC showed a decrease in number of rearing events, decreased active time with increased
resting time, and a decreased total distance traveled only at post injury week 8. Although, cognitive dependent behavioral testing beyond week 8 is not reported in this paper, these anxiety-and depressive-like behaviors remained consistent throughout the remainder of the animals' lives parallel with the whisker pad sensitization that occurs after the TIC injury.

Previous research has shown that a decreased amount of time spent in the light side of the light-dark box is indicative of anxiety-like behavior while decreased number of rearing events and transitions into the light side are indicative of depressive-like behavior (Costall & Naylor 1997; Cryan & Holmes 2005; Fedorova et al 2003). Open field has been used to measure the exploratory and locomotor activity of animals including number of rearing events, active vs. resting time, and center vs. peripheral time. Decreased number of rearing events, decreased active time, and decreased occupancy center time is considered a reliable index of anxiety-like behavior and a measure of response to anxiolytic agents (Costall & Naylor 1997; Katz & Roth 1979; Ramos et al 1997). Together, the results of the light-dark box and open field testing have supported that the chronicity of the TIC injury producing anxiety- and depressive-like behaviors starting at week 8 post injury. Other chronic pain animal models have also observed similar depressive-like behavior after migraine-induced pain, sciatic nerve injury, etc. (Dellarole et al 2014; Lipton et al 2000; McWilliams et al 2003; McWilliams et al 2004; Robinson et al 1988). Furthermore, Yalcin and colleagues (2011) similarly reported a time course of chronic pain animals revealing the development of depressive-like behaviors also occurring 6-8 weeks
post injury (Yalcin et al 2011). These data compared to other studies reported suggest that it is the chronicity of the TIC injury model that allows the development of the “pain affect” to ensue as another facet observed with similarity to patients with chronic pain.

Wall & Melzack (1999) described chronic pain as having two main features: “pain sensation” and the “pain affect” which has incorporated the emotional distress the patients undergo due to the pain (Wall & Melzack 1999). The “pain affect” has also been described as the sequela of other physical and psychological disorders such as anxiety and depression (Dellarole et al 2014; Maletic & Raison 2009; McWilliams et al 2003; McWilliams et al 2004). These comorbidities have been so prevalent that approximately half of all patients suffering from chronic pain have also suffered from anxiety and depression ((Asmundson & Katz 2009; Asmundson & Taylor 2009; Macianskyte et al 2011; Robinson et al 2009). The relationship between chronic pain, depression, anxiety, and fear is extremely complex with extensive overlap making it difficult to determine a pattern of cause and effect. This complex relationship could be related to the numerous brain structures, such as the somatosensory cortex, prefrontal cortex, nucleus accumbens, insular cortex, anterior cingulate cortex, amygdala, hippocampus, thalamus, and cerebellum involved in pain perception have all also been highlighted as major players in affective states, including depression, anxiety, and fear (Apkarian 2004; Apkarian et al 2004a; Apkarian et al 2004b; Averill et al 1996; Becerra et al 2006; Dellarole et al 2014; Fishbain 1999a; b; Robinson et al 2009). Thus, the TIC injury model seems to be a
representative model of anxiety-related chronic facial pain. This makes it a good model for testing pharmaceutical agents with potential for facial pain relief, and also for testing anxiolytics agents, such selective serotonin reuptake inhibitors (SSRIs), and other antidepressants to attenuate pain as well as comorbid psychological effects.

2.4.3. Decreased Fear Response in Mice with TIC after Acoustic Disturbance

The development of the anxiety- and depressive-like behaviors at week 8 post injury was interesting considering that the elevated plus maze results showed opposite behavior at weeks 1 and 4 post injury. Mice with TIC injury spent more time in the open arm at week 1 post injury and had a significantly greater number of transitions at weeks 1 and 4 post injury compared with that of sham mice. One could argue that these results represented anxiety-like behavior in our sham animals (Table 2.2); likewise, the sham animals were not significantly different from the naïve animals in time spent in open arm and number of transitions. Therefore, the data strongly support the idea that the TIC injury was the cause of the behaviors observed. Interestingly, our results in the elevated plus maze suggest that after acoustic disturbance, the mice with TIC injury were showing evidence for decreased anxiety at week 1 and possibly week 4 post injury (Bailey & Crawley 2009; Davis et al 1997). Mice with TIC that were not exposed to the acoustic disturbance, however, did not show a significant difference in time spent in the open arm or number of transitions compared with that of sham animals also not exposed. These results raise some interesting
questions: 1) Why was there decreased anxiety-like behaviors at week 1 post injury and increased anxiety-like behaviors at week 8 post injury in the mice with TIC only after an acoustic disturbance? 2) Were the behaviors in the elevated plus maze task not anxiety-like behaviors at all? I offer possible reasons for these results in the discussion below.

One explanation is that the mice with TIC were exposed to two additional stressful stimuli in an already sensitized state. The first stressful stimulus was the mild acoustic startle stimulation. An acoustic startle disturbance has been shown to create a “stress-induced analgesia” by inducing endogenous opioids release, thereby, creating the possibility that the animal is in a less painful condition (Frew & Drummond 2008; Lewis et al 1980; Nencini et al 1984; Terman et al 1984; Vitale et al 2005; Watkins & Mayer 1982). The acoustic stimulus has also been shown to activate the HPA axis which would not only cause an increase in activity, but also would similarly decrease neuropathic pain (Buijs et al 1993; Buijs & Van Eden 2000; Gonzales et al 2008; Kosten & Ambrosio 2002). Since the separate cohort of animals who did not receive the acoustic disturbance did not have the same behavioral results, the acoustic disturbance might have induced a temporary “stressed-induced analgesia” as a potential reason for the increased time spent in open arms and increased number of transitions.

Although, the acoustic stimulation did not appear to have much effect on the mice behaviors in the light-dark box preference test, it was possibly an important stressor in the elevated plus maze test, the second stressful stimulus. This test was more stressful than the light-dark box and open field, and the
former could have triggered anxiety-like activities that the latter two did not (Cruz et al 1994; Rodgers 1997; Rodgers & Dalvi 1997; Salas et al 2003; Walf & Frye 2007). Thus, this would contribute to the results observed in the elevated plus maze at weeks 1 and 4 after injury in the mice with TIC. The increased number of transitions into the open arm possibly suggests hyperactivity, and the increased time spent in open arm could suggest a temporary decrease in pain sensation.

Another possible explanation for these data was that there was a decrease in fear/defensive behavior due to poor decision making in the injured animals after the acoustic startle stimulation in the elevated plus maze task. This could be due to lack of fear associated with increased release of endogenous opioids or due to major sites in the brain which might be undergoing plasticity at different time points following the injury. Neugebauer and colleagues (2004) described the amygdala as being a multi-functional integration site (Neugebauer et al 2004). The pre-frontal cortex, amygdala, and ventral hippocampus are just a few areas of the brain crucial in fear and stress responsive behaviors (Ji et al 2010; Neugebauer et al 2004; Quirk et al 2000; Rea et al 2013). Rodents that have received excitotoxic lesions localized to the pre-frontal cortex, amygdala, and ventral hippocampus have also shown a significant more time spent in open arms and increased number transition compared with that of sham animals (Quinn et al 2002; Shah & Treit 2003; Ventura-Silva et al 2013). Negative emotions such as anxiety and depression facilitated pain, but the negative emotions associate with fear and stress promoted the inhibitory pathways of the amygdala which then inhibited pain. This interpretation allows for a possible
coexistence of depression, fear, and pain. Therefore, if certain pharmaceutical agents were devised or used that not only treated depression, but also pain, then perhaps our understanding of these mechanisms and treatment would be improved.

2.5. Conclusion

In summary, due to the efficacy and persistence that is translationally relevant to the chronicity of the clinical injury, the TIC injury model is a good chronic trigeminal pain model that can be used to determine different signaling cascades initiated during the course of facial pain. This will help not only to identify molecular targets, but also to define differences between facial pains versus somatic pain relayed by the spinal cord after nerve injury. Since the TIC injury model has been developed in mice, gene therapies can be used in order to target particular cytokines, proteins, and receptors in the hopes of revealing underlying mechanisms and how specific gene knock-outs influence trigeminal pain. Furthermore, since this model displays anxiety- and depressive-like behaviors by 8 weeks, it is a useful model to study certain pharmaceutical agents that perhaps can help attenuate not only depression/anxiety, but also fear, anxiety, and depression related to pain.
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<th>Light/dark Transitions (#)</th>
<th>Latency to re-enter dark side (s)</th>
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**Table 2.2. Elevated Plus Maze Data.**

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<th>Transitions into Open Arms (#)</th>
<th>Head Dips into Open Arms (#)</th>
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**Figure 2.1. TIC Injury Induced Mechanical and Cold Allodynia.** The mice with TIC were tested on the whisker pad for mechanical allodynia and thermal hypersensitivity in the V2 area behind the whisker pad (A). The ipsilateral whisker pad of the mice with TIC had a significant decrease in mechanical threshold than that of their contralateral side and the sham and naïve mice.
starting within the first week post injury lasting until euthanasia, 21 weeks post injury (B). The patterns variations of the receptive fields are shown in (C) with 100% of the mice receiving the TIC surgery developing allodynia. Mice with TIC are only hypersensitive to cold temperatures (D). n= 7-10; Two way ANOVA, Fishers post hoc test ****p<0.0001; One way ANOVA, Tukey post hoc ***p<0.001.
Figure 2.2. Mice with TIC Had Anxiety- and Depressive-Like Behavior in the Light-dark Box. Mice with TIC spent significantly less time in the light side of the light-dark box at post injury week 8 compared with that of the sham animals (A). Mice with TIC showed a decrease number of rearing events at post injury week 8 as compared to the sham mice (B). Naïve animals behavioral baselines are indicated by the dotted line; n= 8-21, Two way ANOVA Fishers post hoc test **p<0.01,*p<0.05.
Figure 2.3. Acoustic Disturbance Affected Mice with TIC in the Elevated Plus Maze. Mice with TIC spent significantly more time in the open arms post injury week 1 compared with that of the sham animals (A). Mice with TIC showed a significantly more number of transitions into the open arms at post injury week
1 and 4 as compared to the sham mice (B). Head dips into the open arms were not significantly from mice with TIC compared to that of sham mice (C). Naïve animals behavioral baselines are indicated by the dotted line; n= 8-11, Two way ANOVA Fishers post hoc test ***p<.001, **p<0.01,*p<0.05.
Figure 2.4. Mice with TIC Injury Showed Decreased Exploratory Activity in Open Field. Mice with TIC had a significantly fewer rearing events and rearing compared to sham mice post injury week 8 (A&B). Mice with TIC had significantly less active time and increased resting time post-op week 8 compared with that of sham mice (C&D). Mice with TIC traveled less overall distance post-op week 8 compared with that of sham mice (E). No significant
difference was detected in beam breaks (F), central (G) or peripheral (H) time duration. N=8-19, Two-way ANOVA, Fishers post hoc test, **p<0.01, *p<0.05.
CHAPTER THREE
RAPID EFFECTS OF PPAR-AGONISTS ON ATTENUATION OF TRIGEMINAL PAIN

3.1. Introduction

Trigeminal neuropathic pain is an orofacial pain condition characterized by continuous aching and burning sensation caused by trigeminal nerve damage (Zakrzewska 2013). Dental procedures or trauma can cause trigeminal peripheral nerve injury and inflammation, but in some cases, the cause is unknown. Clinicians most often resolve treating this continuous trigeminal neuropathic pain with inadequate anticonvulsants and antidepressant (Asmundson & Katz 2009; De Leeuw 2008; Robinson et al 2009; Roditi et al 2009). Therefore, there is a great need for discovery of new drug targets.

Peroxisome proliferator-activated receptor (PPAR) is a nuclear receptor with three isoforms: alpha, beta/delta and gamma. PPAR is widely expressed in adipose, liver, cardiac, endometrial stromal cells, immune cells, neurons, and glia of the peripheral and central nervous system (Cimini et al 2005a; Cimini et al 2005b; Cristiano et al 2005; Cullingford et al 2002; Gray et al 2012; Li et al 2010; McKinnon et al 2012; Park et al 2007; Sarruf et al 2009). After ligand-activation, the PPAR transcription factors of the nuclear hormone receptor superfamily plays a major regulatory role in energy homeostasis and metabolic function (Michalik & Wahli 2006). These receptors form a heterodimer with retinoid X receptor (RXR) controlling gene expression of PPAR response elements (PPRE) on DNA.
(Berger & Moller 2002; Moore et al 2001; Sanz et al 2012; Szanto et al 2004). In particular, the activation of the peroxisome proliferator-activated receptor isoform gamma (PPARγ) has shown to have multiple downstream effects.

PPARγ is activated by endogenous lipids or by thiazolidinediones, such as rosiglitazone and pioglitazone (PIO), which is FDA approved for the treatment of type 2 diabetes. These agonists have been shown to regulate fatty acid metabolism (Nagashima et al 2005; Szanto et al 2004; Willson et al 2001). However, more recent studies suggest PPARγ activation plays a role in another major pathway that suppresses neuroinflammatory mediators, such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), thereby decreasing microglial activation and certain cytokines such as TNF-α (tumor necrosis factor alpha) and IL-6 (Interleukin 6) (Berger & Moller 2002; Combs et al 2000; Maeda & Kishioka 2009; Sadeghian et al 2012). As well as showing a reduction in paw edema after capsaicin injection, PPARγ activation also reduces mechanical allodynia and thermal hyperalgesia in the sciatic nerve injury animal model (Fehrenbacher et al 2009; Ghosh et al 2007; Maeda & Kishioka 2009; Morgenweck et al 2010; Morgenweck et al 2013; Park et al 2007). Furthermore, PPARγ has been shown to be upregulated in Schwann cells after nerve injury (Cao et al 2012; Zhang et al 2010). Other studies have found that the PPARγ receptor is upregulated specifically within two weeks after an optic nerve injury in a mouse and then decreases down to normal levels for later time points (Zhu et al 2013).
Although PPARγ activation has been highly implicated in decreasing specific types of neuropathic and inflammatory pain, the effects of PPARγ activation on trigeminal pain has never been studied. Although, Moreno et al. indicated PPAR to be in the trigeminal nucleus (Moreno et al 2004), but no one has explored the function of this receptor in the Trigeminal Brainstem Sensory Nuclear Complex (TBSNC) which is composed of four nuclei name rostral to caudal: main sensory nucleus (principal nucleus 5), spinal trigeminal oralis, spinal trigeminal interpolaris, and spinal trigeminal caudalis (De Leeuw 2008; Sessle 2000). The TBSNC consists of second order neurons that relay tactile and painful stimulation to the thalamus which then transmit the signals to the sensory cortex (layer IV) corresponding to the relevant head/neck/facial region making up the trigeminothalamic pathway (DaSilva & DosSantos 2012; De Leeuw 2008; Sessle 2000). Therefore, the TBSNC is vital to trigeminal nociception transmission.

The aim of this current study is to determine the role of PPARγ in trigeminal neuropathic pain utilizing our novel Trigeminal Inflammatory Compression (TIC) injury model in mice (Ma et al 2012a). Our hypotheses are that 1) PPARγ is more immunoreactive in the mice with TIC injury and 2) PIO attenuates trigeminal TIC injury pain dependent on PPARγ activation. We evaluated: 1) PPARγ immunoreactivity in the TBSNC with/without TIC injury, 2) systemic administration of PIO, PPARγ agonist, in the mice with TIC injury to assess the attenuation of trigeminal pain, and 3) systemic administration of PPARγ
antagonists to better determine whether PIO acts through PPARγ dependent pathways in the attenuation of pain.

3.2. Materials and Methods

3.2.1. Animals

See chapter 2; section 2.2.1.

3.2.2. Trigeminal Inflammatory Compression (TIC) Surgery

See chapter 2; section 2.2.2.

3.2.3. Assessment of Mechanical Allodynia on the Mouse Whisker Pad

See chapter 2; section 2.2.3.1.

3.2.4. Immunohistological Study

Mice with TIC, 3 weeks post injury and aged matched naïve mice were anesthetized with isoflurane and decapitated. This early time point was based on previous literature that indicated PPARγ changes occur at earlier time points (Cao et al 2012; Zhu et al 2013). The brainstem was dissected and then placed in 4% paraformaldehyde in 0.1 M phosphate buffer solution (PB, pH 7.4) for 42 hours. This was followed by a 24 hour soak in 30% sucrose in PB. The brainstems were then embedded in OCT Compound (Tissue-Tek, Sakura, Torrance, CA) and sectioned with a cryostat at the thickness of 40 microns and
sequentially placed in 24-well plates filled with Ethylene Glycol based anti-freeze solution, stored at -20°C for immunohistological study. To insure the integrity of the stain and control for variability, all tissues were simultaneously processed for staining on the same day. On the day of immunostaining, the tissues were washed with 0.1M PBS (pH 7.4) and pretreatment with 3% hydrogen peroxide in 0.1M PBS (pH 7.4) for 15 min to destroy endogenous peroxidase activity in erythrocytes. Tissues were then blocked using 0.5% Triton X-100 in PBS (15min) to permeabilize cell membranes and reduce cell surface tension to increase the antibody penetration. The 5% normal goat serum in the PBS (40min) blocked nonspecific antigen-antibody combinations. Sections were incubated overnight at 4°C with rabbit polyclonal anti-PPAR-gamma IgG (1st antibody) (1:6000 dilutions; H-100 Santa Cruz Biotechnology, Dallas, TX). The succeeding day, the sections were incubated at room temperature with a secondary bioatinylated goat anti-rabbit IgG (1:200; company, place) for 40 minutes. The sections were then incubated with avidin-biotin complex (ABC) reagent for 40 minutes. Finally the antibody-antigen interaction was visualized via a peroxidase-catalyzed reaction. After mounting the sections to gel-coated Super Plus glass slides, they were allowed to air dry for at least 4 hours before they were dehydrated through graded ethanol and xylene. Then the slides were cover-slipped using Permount mounting medium (Fishers Scientific, Waltham, MA). The slides were imaged using Nikon E1000 microscope (Nikon Instruments, Inc., Melville, NY) equipped with a Nikon DXM1200F digital camera and the Act-1 Program.
3.2.5. Image Analysis

The immunostaining intensity of the trigeminal brainstem sensory nuclear complex (main sensory, oralis, interpolaris, and caudalis) was analyzed (n=3; 9-12 tissues/animal) using ImageJ (1.46, NIH). Each subnuclei of the trigeminal brainstem sensory nuclear complex was identified as a region of interest and analyzed for mean fluorescent intensities. To identify PPARγ immunoreactivity differences, each subnuclei of the complex was analyzed separately. The mean fluorescent intensities of the trigeminal dorsal horn were measured in mice with TIC injury and then compared to that in naïve mice.

3.2.6. Drug Preparation and Administration

PPARγ agonist, pioglitazone, was dissolved in normal saline followed by 30 seconds of vortex. Then the solution was sonicated for 20 minutes before use. Benzafibrate, PPARα agonist, was dissolved in 1% carboxymethylcellulose and was then vortexed for 30 seconds. Fenofibrate, PPARα agonist; GW0742, PPARβ/δ agonist and GW9662, PPARγ antagonist were dissolved in 10% dimethyl sulfoxide (DMSO) and vortexed for 30 seconds before administration. All drugs were fresh prepared on the day just before administration.

Drugs were administered after mechanical allodynia was confirmed at least 8 weeks post operation to observe the efficiency of each drug in a chronic neuropathic pain condition. All doses were chosen based on drug safety, and drug efficacy in previous studies (Fehrenbacher et al 2009; Feinstein et al 2005;

Given systemically with the following doses: pioglitazone at doses 100 mg/kg and 300 mg/kg (<10ml/kg volume) was injected intraperitoneal (<10 ml/kg volume) and 600 mg/kg was given oral gavage (at the volume of <10 ml/kg). Lower doses of pioglitazone (≤100 mg/kg) have been reported in previous studies, but when no effect was observed at 100 mg/kg, doses were increased accordingly. For a mouse, the reported LD50 of PIO given systemically ranges from 181 mg/kg-1200 mg/kg, therefore the 600 mg/kg dose was administered orally (United States Pharmacopeial Convention, 2013]).

PPARα agonists, benzofobrate (100 mg/kg p.o.; LD50 500 mg/kg) and fenofibrate (200 mg/kg i.p.; LD50 1200 mg/kg), and PPARβ/δ agonist, GW0742 (1 mg/kg and/or 6 mg/kg i.p.) were given systemically to serve as PPAR activation controls. The LD50 of GW0742 has not been reported.

In a separate cohort of mice, the PPARγ antagonist, GW9662 (30 mg/kg i.p), was injected 30 minutes before the PPARγ agonist, pioglitazone (300 mg/kg i.p.) was given. This dose of PIO was chosen because it had the maximum effect on the elevation of mechanical threshold in TIC injury animals. GW9662 was employed to block PIO from binding to PPARγ to see if the effect of PIO is specific via activation of PPARγ (Feinstein et al 2005; Lea et al 2004; Maeda & Kishioka 2009).

During each drug testing, 50% mechanical threshold was measured at time points of 0, 0.5, 1, 2, 3, 4 hours post inject on the ipsilateral whisker pad
only. The 600 mg/kg oral dosing of PIO was measured out until 6 hours because an attenuated effect was observed starting at the 4th hour post injection time point. To increase animal “n” number in the treatment groups for these experiments, mice were tested with these drugs using the Latin square type crossover method with at least 1 week interval between each drug testing and allow sufficient time for the effect of a previous treatment to wear off. Vehicle for the PIO study was normal saline. For all other drugs, 10% DMSO was administered to the mice with TIC injury to serve as a vehicle. One experimenter was blinded to the drugs given to the animals for each experiment.

3.2.7. Statistical Analysis

The data were expressed as means ± S.E.M. The GraphPad Prism 6 statistical program was used for data analysis (Graph Pad Software, Inc., La Jolla, CA). All behavioral data including drug studies were analyzed using a Two-Way ANOVA with a Fishers post hoc test; Histological studies were analyzed by a Two-Way ANOVA with a Tukey post hoc test; (where is appropriate) p≤0.05 is considered significant.
3.3. Results

3.3.1 Mice with TIC Injury Demonstrated Unilateral Whisker Pad Mechanical Allodynia

The 50% baseline mechanical threshold was initially the same on both side of the whisker pad of mice with/without TIC (3.51 ± 0.18 g for the left; 3.74 ± 0.45g for the right). The mice with TIC injury experienced unilateral mechanical allodynia on the ipsilateral whisker pad within one week post operation lasting until the euthanasia day (week 14 post-injury). The mean 50% mechanical threshold of the ipsilateral whisker pad for the mice with TIC injury was 0.24 ± 0.92 g making it statistically significant compared to that on the contralateral whisker pad (3.51 ± 0.18 g; n=8; p<0.0001, two-way ANOVA, Fishers post hoc test, Figure 3.1). In contrast, the sham operation control mice did not show any changes in the mechanical threshold after the surgery. Compared to sham operation control mice, the mean mechanical thresholds of the ipsilateral whisker pad of the mice with TIC injury was statistically significant (0.24 ± 0.92g vs. 3.51 ±0.36g; n=8; p<0.0001, two-way ANOVA, Fishers post hoc test).
3.3.2. The Effect of PPAR Agonists on Mechanical Allodynia of Mice with TIC Injury

3.3.2.1. PPARγ Agonist, Pioglitazone, Attenuated Mechanical Allodynia in Mice with TIC Injury

At 8 weeks post operation, pioglitazone (300 mg/kg i.p.) effectively attenuated mechanical allodynia of the ipsilateral whisker pad, the 50% mechanical threshold from 0.24 ± 0.092 g before drug treatment increased to 1.61 ± 0.54 g at hour 1 peaking at hour 2 (2.72 ± 0.74 g) lasting for 3 hours (2.33 ± 0.98 g; Figure 3.2A). This was statistically significant from those of the mice with TIC injury treated with saline injection (2.72 ± 0.74 g vs 0.10 ± 0.04 g; two-way ANOVA, Fishers post hoc test, ****p<0.0001; n=3-7). The p.o. administration of pioglitazone (600 mg/kg p.o.) also attenuated mechanical allodynia in the mice with TIC injury (5hr: 0.87 ± 0.32 g; 6hr: 0.92 ± 0.45 g; two-way ANOVA, Fishers post hoc test, *p<0.05; n=3-7) compared that to the saline treated mice with TIC injury (0.03 ± 0.00 g; two-way ANOVA, Fishers post hoc test, *p<0.05; n=3-7) which is significantly different at 5 and 6 hours post injection. However, this dose was not as effective as the 300 mg/kg i.p. of pioglitazone. The 100 mg/kg i.p. dose of pioglitazone did not have any effect on the mice with TIC injury compared to that of the saline treated mice (0.56 ± 1.40 g vs 0.10 ± 0.04 g). The 300 mg/kg dose elicited hypothermic side effects that were most likely an indication that the dose was too high. However, the 100 mg/kg and 600 mg/kg doses did not elicit any observable overt side effects. These results
demonstrated that PPARγ receptor is a key player in alleviating whisker pad mechanical allodynia in the mice with TIC injury.

3.3.2.2. PPARβ/δ Agonist Moderately Attenuated Mechanical Allodynia in the Mice with TIC Injury

Allodynia in the Mice with TIC Injury The administration of GW0742, PPARβ/δ agonist (6 mg/kg i.p.), partially attenuated mechanical allodynia in mice with TIC injury compared to that of the mice with TIC injury treated with vehicle. The effect reached peak at hour 2 post injection (1.59 ± 0.55 g vs. 0.06 ± 0.02 g; two-way ANOVA, Fishers post hoc test; ****p<0.0001, n= 4-6; Figure 3.2B). The GW0742 administration of (1 mg/kg i.p.) provided no attenuation of mechanical allodynia (0.51 ± 1.31 g). These results showed that the activation of PPARβ/δ isoform play some role in the control of mechanical alldodynia induced by the TIC injury.

3.3.2.3. PPARα Agonist Had No Effect on the Mice with TIC Injury

Two PPARα agonists were used in this experiment. The first one is benzafibrate which is a pan-PPAR agonist with the highest affinity for the alpha subunit. Benzafibrate at 100 mg/kg i.p. injection, had no effect on mechanical allodynia in mice with TIC injury (treated: 0.55 ± 1.34 g vs. vehicle: 0.71 ± 1.52 g; two-way ANOVA, Fishers post hoc test; p>0.05, n= 3-6; Figure 3.2C). The second and more specific PPARα agonist, fenofibrate, was administered at 200 mg/kg i.p., no attenuation effect on mechanical alldodynia effect (0.56 ± 1.28 g) was observed with this drug at this particular dose. These results demonstrated
that activation of PPARα at these doses do not contribute to the attenuation of the mechanical allodynia in the mice with TIC injury.

3.3.2.4. PPARγ Antagonist, GW9662, Blocked Analgesic Effects of Pioglitazone

GW9662, a potent antagonist of PPARγ, at the dose of 30 mg/kg (i.p.) successfully blocked the actions of pioglitazone (300 mg/kg i.p.) in alleviating mechanical allodynia in the mice with TIC (GW9662+PIO: 0.53 ± 1.29 g vs. PIO only: 1.55 ± 1.41 g; two-way ANOVA, Fishers post hoc test, N=4-7; ****p<0.0001, Figure 3.2D). These results provide evidence that PIO is acting through a PPARγ dependent mechanism to attenuate mechanical allodynia. Due to the fact that mechanical allodynia in the mouse whisker pad was attenuated post 3 hours after PIO injection, support is given to the theory that PPARγ is working through nongenomic mechanisms to attenuate trigeminal pain.

3.3.3. PPARγ Was More Immunoreactive in the TBSNC of Mice with TIC injury

The entire trigeminal brainstem sensory nuclear complex (TBSNC) (composed of the main sensory trigeminal nucleus, and spinal trigeminal oralis, spinal trigeminal interpolaris, and spinal trigeminal caudalis was sectioned and stained for PPARγ immunoreactivity. The spinal trigeminal dorsal horn (sp5) sections were imaged and analyzed for PPARγ immunoreactivity (mean intensities) in mice three weeks after TIC injury and along with naïve controls. Although the PPARγ positive neurons were expressed in the TBSNC of both
mice with TIC and naïve mice, the PPARγ-like immunoreactivity greatly increased in the injured animals. **Figure 3.3** depicts the mean intensities of the immunoreactivity of PPARγ throughout the trigeminal brainstem complex in the mice with TIC injury compared to that of the naïve mice. There was a significantly higher optical intensity for PPARγ immunoreactivity in the TBSNC of the mice with TIC injury. The main sensory nuclei (85.65 ± 19.37), spinal trigeminal oralis (65.43 ± 12.68), and spinal trigeminal caudalis (93.59 ± 27.62) on the ipsilateral side of TIC injury was compared to that of the naïve mice (main sensory nucleus: 67.21 ± 14.01; sp5 oralis: 46.42 ± 18.46; sp5 caudalis: 71.76 ± 17.31; p<0.05; two-way ANOVA, Fishers post hoc test, n=3 with 9-12 sections/animal). The immunostaining intensity in the spinal trigeminal interpolaris of the mice with TIC injury (75.61 ± 17.48) did not show significant difference from those of the naïve mice (65.64 ± 15.78). However, PPARγ immunoreactivity in the spinal trigeminal caudalis of the mice with TIC injury was greater than that of the rest subnuclei of the trigeminal brainstem complex and significantly different from the spinal trigeminal oralis (p<0.01) and the spinal trigeminal interpolaris (p<0.05; two-way ANOVA, Fishers post hoc, n=3 with 9-12 sections/animal). Furthermore, there was a bilateral increase in PPARγ immunoreactivity only in the spinal oralis (TIC contralateral: 67.22 ± 10.89).

**Figure 3.4** shows tissue slices of the entire TBSNC in a naïve mouse compared to that of a mouse with TIC injury. Sample tissues representing each level of the TBSNC from a mouse with TIC injury are depicted in figure panels B-D (x20).
Figure 3.5 depicts the ipsilateral sp5 interpolaris in the mice with TIC injury compared to that in naïve mice. The white arrows indicate the projection of whisker barrels of sp5 that have been shown to correlate to the receptive fields on whisker pads of the mice (Zembrzycki et al 2013). As indicated at 20x image, this is primarily where positive PPARγ neurons were located indicated by the black arrows. There also appears to be glial nuclei stained for PPARγ consistent with previous findings (Cao et al 2012; Maeda & Kishioka 2009; Sadeghian et al 2012). Although PPARγ immunoreactivity also appears in the TBSNC of naïve mice, the mean staining intensity was much less compared to that of the mice with TIC injury.

3.4. Discussion

This study determined that the nuclear receptor, peroxisome proliferator receptor-gamma isoform (PPARγ), plays a significant role in trigeminal pain transmission. Histology revealed that 3 weeks after the Trigeminal Inflammatory Compression (TIC) injury, compared to the other subnuclei of the trigeminal brainstem complex, a most intense PPARγ immunoreactivity appeared in the spinal trigeminal caudalis where the primary pain fibers actually synapse onto. Systemic administration of a PPARγ agonist, pioglitazone (PIO), attenuated the mechanical allodynia in the mice with TIC injury at doses of 300 mg/kg i.p. and 600 mg/kg p.o. However, 100 mg/kg of PIO (i.p.) had no effect on the mice with TIC injury. Furthermore, these studies revealed that administering a PPARγ antagonist, GW9662 (30 mg/kg i.p.) prior to the optimal dose of PIO (300 mg/kg
i.p.) blocked the analgesic effect of PIO indicating that PIO is acting through a PPARγ mechanism. Additionally, the PPARα agonists, benzafibrate and fenofibrate, had no effect on the allodynic mice. However, the PPARβ/δ agonist, GW0742 had a minimal attenuation of allodynia effect to the mice with TIC injury. Taken together, these results confirm PPAR’s role in trigeminal pain transmission.

Based on these findings, PPARγ and PPARβ/δ function to inhibit pain transmission once activated. However, PPARα does not appear to be a key player in trigeminal neuropathic pain. Some controversial studies show that PPARα activation have an inhibitory effect on nociception after nerve injury or inflammation (Benani et al 2004; LoVerme et al 2006; Maeda & Kishioka 2009; Oliveira et al 2007). No study has ever reported PPARα activation eliciting an analgesic effect in trigeminal neuropathic pain. However, in this study, the doses of the two PPARα agonists could have been increased to possibly provide an analgesic effect. Future studies should be conducted to observe if higher doses comparable to pioglitazone will attenuate the mechanical allodynia observed in the mice with TIC injury. On the other hand, PPARβ/δ agonists have been reported to attenuate inflammatory pain (Gill et al 2013; Hall et al 2008), but little about this receptor isoform has been reported. The biological role of PPARβ/δ has remained elusive due, in part, to its broad tissue expression and the lack of good chemical tools with which to study its physiological function. Future studies could be conducted to observe PPARβ/δ immunoreactivity in along the trigeminal dorsal horn.
Unlike PPARβ/δ, PPARγ has been identified more thoroughly. Upon PPARγ activation, it will inhibit not only inflammation, but also neuropathic pain after an injury (Fehrenbacher et al 2009; Ghosh et al 2007; Maeda & Kishioka 2009; Morgenweck et al 2010; Morgenweck et al 2013; Park et al 2007). Furthermore, little is known about PPARγ in the trigeminothalamic pathway. Moreno and colleagues (Moreno et al 2004) confirmed PPARγ was present in the dorsal horn of the brainstem and spinal cord, and Maedo and colleagues (2005) (Maeda et al 2005) confirmed Moreno’s findings by using immunohistochemistry to identify PPARγ in the sciatic nerve, dorsal root ganglia, and dorsal horn supporting PPAR’s representation along the nociceptive pathway.

This study was not only the first to identify PPARγ immunoreactivity throughout the trigeminal brainstem sensory nuclear complex with/without trigeminal nerve injury, but this study also was the first study to demonstrate that PPARγ activation attenuates trigeminal hypersensitivity in the TIC injury model. This study revealed that PPARγ immunoreactivity increases three weeks after the Trigeminal Inflammatory Compression (TIC) injury consistent with other findings in which PPARγ becomes upregulated within weeks after a nerve injury (Cao et al 2012; Zhu et al 2013). Additionally, the results of this study support previous studies demonstrating PPARγ’s presence in neuronal and glial cells (Maeda & Kishioka 2009). Although this study identified PPARγ immunoreactivity in putative neurons, future double-labeling experiments in the TBSNC with PPARγ, neuronal, and glial markers (neuN, IB-4, OX-42, etc.) would serve as
more conclusive evidence of PPARγ’s presence in neurons and/or glial cells, supporting its role in neurogenic inflammation.

Furthermore, PPARγ activation has been shown to transrepress NF-κB thereby downregulating pro-inflammatory cytokines such as IL-6 and TNF-α (Berger & Moller 2002; Combs et al 2000; Maeda & Kishioka 2009; Sadeghian et al 2012; Scholz & Woolf 2007). Previous studies have even reported that PPARγ deficient mice are more vulnerable to inflammatory diseases (Adachi et al 2006; Cuzzocrea et al 2004; Straus & Glass 2007). In our previous study (Ma et al 2012a), we demonstrated microglial activation at the trigeminal dorsal horn of mice with TIC injury. Therefore, one possible reason for the increase in PPARγ immunoreactivity at the TBSNC observed in the mice with TIC injury could be the inflammatory response occurring in the trigeminal dorsal horn. The upregulation of PPARγ at the trigeminal nucleus would be a way to combat the neural immune system and downregulate the pro-inflammatory cytokines once PPARγ is activated.

Interestingly, PPARγ immunoreactivity was most intense in the spinal trigeminal caudalis of the mice with TIC injury while barely detectable in naïve mice. Since the spinal trigeminal caudalis has been identified as the primary nucleus for nociceptive signaling (De Leeuw 2008; Sessle 2000), the upregulation might partially explain why the PPARγ receptor agonist, PIO, has analgesic effects on mechanical allodynia in mice with TIC injury. With the upregulation of PPARγ, a stronger anti-inflammatory cell signaling cascade could be initiated once PIO binds to its receptor.
However, there is much debate about the actions of PIO. Some scholars believe that PIO activates PPARγ to induce transcription while others believe there are PPARγ transcription independent mechanisms that occur to decrease allodynia (Fehrenbacher et al. 2009; Feinstein et al. 2005; Lea et al. 2004; Maeda & Kishioka 2009; Thal et al. 2011). Still, others suspect and have possibly demonstrated that PIO can act on other intracellular receptors such as acting on a mitochondrial membrane protein known as mitoNEET (Yonutas & Sullivan 2013). However, in present study, when PPARγ antagonist, GW9662, blocked PIO’s analgesic actions, it was then concluded that PIO attenuates trigeminal nociception by acting through PPARγ. More studies need to be conducted to elucidate the action of PIO non PPARγ dependent pathways.

3.5. Conclusion

Overall this novel study determined that PPARγ activation by PIO plays the most potent role in attenuating mechanical allodynia in the mice with TIC injury. This experiment was the first to map PPARγ in the trigeminal brainstem sensory nuclear complex as well as show an increase in PPARγ immunoreactivity after three weeks post injury. Taken together, these studies provide a new target, PPARγ, for attenuation of trigeminal pain which raises the possibility for repurposing the FDA approved diabetic therapeutic drug, PIO, for the treatment of patients suffering from orofacial pain.
Figure 3.1. Mice with TIC Injury Developed Unilateral Mechanical Allodynia on the Ipsilateral Whisker Pad. The 50% mechanical threshold on whisker pads of the mice with TIC injury and the sham mice were measured bilaterally for detecting mechanical allodynia. The 50% mechanical threshold on the ipsilateral whisker pad of mice with TIC injury was dramatically decreased within one week of injury lasting until euthanasia, week 14. The mechanical threshold on contralateral whisker pad of the mice with TIC injury was unaffected by the surgery. The mechanical threshold on the whisker pads of the sham operation mice did not change (n=9/group; ****p<0.0001, two-way ANOVA, Fishers post hoc test).
Figure 3.2. Pioglitazone (PIO) Attenuate Mechanical Allodynia in the Mice with TIC Injury. Hypersensitivity was blocked by specific PPARγ antagonism with (A) PIO rapidly elevating the 50% mechanical threshold in the mice with TIC at higher doses (300 mg/kg and 600 mg/kg), but was ineffective at 100 mg/kg (n=3-7). (B) GW0742, PPARβ agonist, attenuated mechanical allodynia in the mice with TIC at a dose of 6 mg/kg, but it still not as effective as PIO (n=4-6). (C) PPARα agonists, benzafibrate and fenofibrate, were not effective in alleviating mechanical allodynia in the mice with TIC (n=3-6). (D) PPARγ antagonist, GW9662, blocked the effects of PIO at a dose of 30 mg/kg (n=4-7). *p<0.05, ****p<0.0001; two-way ANOVA, Fishers post hoc test.
Figure 3.3. PPARγ Immunoreactivity Increased in TBSNC of Mice with TIC Injury. The Trigeminal Brainstem Sensory Nuclear Complex (TBSNC) was stained for anti-PPARγ antibody in the mice with TIC injury and naïve mice. The images show the individual subnuclei of the TBSNC (main sensory/principle five, spinal oralis, spinal interpolaris, spinal caudalis) and PPARγ immunoreactivity in each nucleus. The mice with TIC injury had an increased PPARγ immunoreactivity in the main sensory, spinal oralis and spinal caudalis on the ipsilateral side compared to those in the ipsilateral side of the naïve mice (n=3 with 9-12 sections/animal ;*P<0.05, two-way ANOVA, Fishers post hoc). The spinal trigeminal nucleus oralis expressed bilateral immunoreactivity that was significant greater than tissue from naïve mice (*P<0.05, two-way ANOVA,
Fishers post hoc). PPARγ immunoreactivity on the ipsilateral side of the mice with TIC injury also was significantly higher in the spinal caudalis compared to that of spinal trigeminal oralis and interpolaris (#p<.05, ##p<0.01, two-way ANOVA, Fishers post hoc).
Figure 3.4. PPARγ Immunoreactivity Localized Throughout the Trigeminal Brainstem Sensory Nuclear Complex in Mice with TIC Injury. **(A)** The image depicts the TBSNC rostral to caudal in naïve mice compared to mice with TIC. The subnuclei are indicated as follows **(B)** principal 5 (Pr5), **(C)** spinal subnucleus oralis (Or5), **(D)** spinal subnucleus interpolaris (Inter5), and **(E)** spinal subnucleus caudalis (Caud5).
Figure 3.5. PPARγ Immunoreactivity at the Spinal Trigeminal Nucleus Interpolaris in Sham Mice vs. Mice with TIC Injury. The upper panel depicts the ipsilateral side of the naïve mice at 10x (A) and 20x (B). The lower panel depicts the ipsilateral side of the mice with TIC at 10x (C) and 20x (D). The black arrows indicate the PPARγ positive cells and the white arrows indicate the projection of whisker barrels that correspond to the receptive fields on the mouse whisker pad where most PPARγ positive cells were found. As shown by the pictures, the staining intensity of PPARγ immunoreactivity was more intense in the tissue from the TIC injured mice than that of the tissue from the naïve mice.
CHAPTER FOUR
LOW-DOSE COMBINATION OF PIOGLITAZONE AND D-CYCLOSERINE
ATTENUATED OROFACIAL PAIN BY IMPROVING MITOCHONDRIAL DYSFUNCTION

4.1. Introduction

Chronic trigeminal neuropathic pain is an orofacial pain condition characterized by chronic aching and burning sensation sometimes overlaid with sharp, electric-like shooting pain caused by trigeminal nerve damage. This injury could be due to the compression of the trigeminal nerve by an arteriole pulsation or could also be caused by a peripheral nerve injury initiated by dental trauma or unknown cause (Burchiel 1993; Cruccu et al 1990; De Leeuw 2008; Devor et al 2002a; Devor et al 2002b; Jannetta 1967a; b; Love & Coakham 2001). This type of injury and pain is very difficult to treat so new therapeutics are needed that target key players in the ensuing cell stress that follows.

The chemiosmotic theory states that during the oxidation of mitochondrial complexes, “free energy” in the form of protons (H⁺ ions) are released into the intermembrane space creating the proton motive force (Sheinin et al 2001). In healthy mitochondria, the protons will flow back through complex V (ATP synthase) inducing ATP synthesis. However, a disruption to any specific complex along the mitochondrial electron transport chain (mETC) will lead to one of the following energy-dissipating pathways (EDP): 1) decreased electron transport along the mETC, 2) increased proton concentration in the intermembrane space,
3) lowered ATP production, or 4) increased reactive oxygen species (Brand & Nicholls 2011; Starkov 2008; Starkov et al 2004; Wallace & Starkov 2000). Cell stress can often be triggered by mitochondrial dysfunction, referring to any energy dissipating pathway (EDP) decreasing mitochondrial bioenergetics (Brand & Nicholls 2011; Starkov 2008).

Studies have indicated mitochondrial dysfunction is a major player not only in cell stress, but also in the etiology of inflammatory and chronic neuropathic pain that occurs after peripheral nerve injuries (Bouillot et al 2002; Ferrari & Levine 2010; Joseph & Levine 2006; Kim et al 2004; Shin et al 2003; Sui et al 2013). Joseph and Levine (2006) showed that after administering inhibitors to the specific complexes of the mitochondrial electron transport chain (mETC), mechanical allodynia was attenuated in animals with sciatic nerve injury (Joseph & Levine 2006).

Other studies have shown that after spinal cord or brain injury, mitochondrial dysfunction and oxidative stress also occur within 24 hours of the injury (Sullivan et al 2007; Sullivan et al 2004a; Sullivan et al 2004b). However, administration of a mild mitochondrial uncoupler, such as 2, 4-Dinitrophenol (2,4-DNP), has been shown to protect the cortex from mitochondrial dysfunction and reactive oxygen species (ROS) production in mice after spinal cord injury (Brand & Esteves 2005; Mahmud et al 1996; Pandya et al 2007; Patel et al 2009). Mild uncouplers, such as 2,4-DNP, decrease the proton concentration gradient in the intermembrane space by creating protonophores in the mitochondrial membrane to allow H⁺ ions to freely flow across the mitochondrial membrane into the matrix.
without crossing through the ATP synthase. In naïve animals, this lowers ATP production and alters the mitochondrial oxygen consumption. However, during cellular stress, mild mitochondrial uncouplers have been shown to decrease the production of mitochondrial ROS and increase bioenergetics (Brand & Esteves 2005; Jin et al 2004; Rolo & Palmeira 2006; Sullivan et al 2004b).

Although studies have shown that ROS are produced in the trigeminal nucleus after peripheral nerve injury and that ROS play a major role in pain transmission (Alp et al 2010; Viggiano et al 2004; Viggiano et al 2005; Viggiano et al 2010), whether peripheral trigeminal nerve injury can induce mitochondrial dysfunction have never been studied to date.

Furthermore, FDA approved drug, (R)-(+) 4-Amino-3- isoxazolidinone (D-cycloserine) (DCS), known under the same Seromycin® (DCS capsules, USP, 250 mg) is a broad spectrum antibiotic used alternatively for tuberculosis. It is a derivative of the naturally occurring amino acid serine and acts as a partial agonist at the strychnine insensitive glycine recognition site of the NMDA receptor complex (Furukawa & Gouaux 2003; Hood et al 1989; Monahan et al 1989; Sheinin et al 2001). Binding of DCS to the NMDA complex enhances glutamate activation and increases calcium influx, thus enhancing excitatory neurotransmission (Heresco-Levy & Javitt 1998; Tomek et al 2013). However, DCS when administered in higher doses has been shown to act as an NMDA antagonist to reduce hypersensitivity in sciatic nerve injury in rats (Millecamps et al 2007). Clinical trials showed that DCS is effective in the extinction of acquired fear when used as an adjuvant to exposure therapy for anxiety disorders (e.g.
post-traumatic stress disorder, phobias, obsessive-compulsive disorder) (Davis et al 2006; Heaton et al 2010; Norberg et al 2008). Only one clinical case study to date has ever observed the anti-allodynic effects of DCS for alleviation of chronic facial pain (Antal & Paulus 2011). Although DCS seems to be a prospective new therapy for the treatment of orofacial pain, it has never before been thoroughly tested in animals or a mechanism defined.

Pioglitazone (PIO), FDA approved, is a prescription drug (Actos) of the class thiazolidinedione (TZD) with hypoglycemic (anti-hyperglycemic, anti-diabetic) action to treat type 2 diabetes. Pioglitazone is a selective agonist of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ). However, studies have shown the PIO reduces hypersensitivity in the sciatic nerve injury animal model (Fehrenbacher et al 2009; Ghosh et al 2007; Maeda & Kishioka 2009; Morgenweck et al 2010; Morgenweck et al 2013; Park et al 2007). Many have theorized that PIO is acting through PPARγ to decrease microglial activation and oxidative stress (Collino et al 2006; Combs et al 2000; Sadeghian et al 2012; Thal et al 2011). However, more recent data has shown that PIO is also acting through a PPARγ independent mechanism on the mitochondria by directly activating mitoNEET to decrease mitochondrial oxidative stress (Geldenhuys et al 2014; Wiley et al 2007a; Wiley et al 2007b; Yonutas & Sullivan 2013). Recent studies in our laboratory have shown that PPARγ agonist, pioglitazone (PIO), attenuates mechanical allodynia in the whisker pads of mice after the Trigeminal Inflammatory Compression (TIC) injury primarily by a PPARγ dependent pathway (see chapter 3). However, our data also suggest that since
the analgesic effect occurs within 2 hours, a PPARγ nongenomic mechanism is a possibility. However, PIO’s actions in isolated mitochondria after a peripheral trigeminal nerve injury have never been studied.

In this study, a trigeminal inflammatory compression (TIC) mouse model was employed to investigate the effect of DCS or PIO, and DCS/PIO combination on the relief of neuropathic nociception and anxiety associate with trigeminal neuropathic pain. The effect of DCS/PIO combination on isolated brain mitochondria after a peripheral trigeminal nerve injury was also explored.
4.2. Materials and Methods

4.2.1. Animals

See Chapter 2; section 2.2.1.

4.2.2. Trigeminal Inflammatory Compression (TIC) Injury

See Chapter 2; section 2.2.2.

4.2.3. Behavioral Assays

4.2.3.1. Detecting Mechanical Allodynia with von Frey Fiber Test

See chapter 2; section 2.2.3.1.

The assessment of mechanical allodynia after the drug administration was only conducted on the ipsilateral whisker pad 8 weeks after injury. This time point after injury was chosen since anxiety-and depression-like behavior develops 6-8 weeks after injury, as discussed in chapter 2 (Yalcin et al 2011). For one-time injections, mechanical threshold was measured at 0, 0.5, 1, 2, 3, and 4 hours drug injection, with hours 5, 6, 7, and 8 post injection evaluated if drug effect persisted. For daily injections, the mechanical thresholds of the mice whisker pads were measured once daily at the same testing time each day. This time point was determined by the peak effect time point of the one-time injection, and usually was between 2.5 - 3 hours after injection. One experimenter was blinded to the drugs given at all times.
4.2.3.2. **Light-Dark Box Preference Testing**

This test is used to observe anxiety-like behavior in the mice with TIC injury (saline vs. drug treated). We have already determined that mice with TIC injury developed an anxiety-and depressive-like behavior at the 8th week post injury indicated by a prolonged duration of the occupancy in the light side of the box (see chapter 2, section 2.2.3.4).

4.2.4. **Drug Preparation and Administration**

4.2.4.1. **Drug Preparation**

D-cycloserine (DCS), NMDA agonist/antagonist, was easily soluble in normal saline (0.9% NaCl, 10 mg/ml). Pioglitazone (PIO), PPARγ agonist, was dissolved in normal saline and vortexed for 30 sec before 20 minutes in the sonicator. 2, 4-Dinitrophenol (2,4-DNP) was dissolved in 10% DMSO solution followed by a 30 second vortex.

4.2.4.2. **Drug Administration**

For subcutaneous (s.c.) injections drug was administered at a volume of ≤ 5 ml/kg/site using a sterile syringe with a 25G needle. For intraperitoneal (i.p.) injection with 25G needle syringe, at a volume of ≤ 10 ml/kg (Turner et al 2011). One experimenter was blinded to the drug treatments given for each experiment described.
4.2.4.2.1. Experiment 1: Treatment of Mechanical Allodynia with NMDA Receptor Agonist/Antagonist, DCS

The mice with TIC were given subcutaneous (s.c.) injections of the following DCS doses in mg/kg (40, 60, 80, 100, 160, and 320 in 150 µl/mouse). These doses were chosen based on previous published studies (Davis et al 2006; Kushner et al 2007; Lanthorn 1994; Millecamps et al 2007). All drugs were administered at least 8 weeks after TIC injury since anxiety-like behaviors were expected to develop 6-8 weeks post the TIC injury (see chapter 2). The goal was to observe the anti-anxiety effects of the drugs. Mechanical allodynia was assessed every hour starting at 0 hour time point, for 4 hours. The vehicle control mice with TIC injury received a subcutaneous injection of normal saline (150µl/mouse).

At least 8 weeks after the TIC injury, mice started receiving daily subcutaneous injections of a low dose of DCS (40 mg/kg, 60 mg/kg, and 80 mg/kg). In parallel to the mice with TIC injury, sham mice were also given the same injections to observe the DCS effects as control. Mechanical threshold was measured daily 2.5-3 hours after injection. On the 7th day, the mice receiving the 80 mg/kg dose went through the light/dark box preference testing. The control mice with TIC injury and sham mice received a daily subcutaneous injection of normal saline (150µl).
4.2.4.2.2. *Experiment 2: One-time Injection of Combination of DCS/PIO*

In a separate cohort of animals, at least 8 weeks after TIC injury, mice received one of the three injections 1) PIO, 100 mg/kg i.p., 2) DCS, 80 mg/kg s.c., or 3) Combination injection: PIO, 100 mg/kg i.p. injection quickly followed by DCS, 80 mg/kg s.c. injection. As mentioned in chapter 3, the reported LD50 of PIO given systemically in a mouse ranges from 181 mg/kg-1200 mg/kg (United States Pharmacopeial Convention, 2013). Although, no carcinogenic effects are observed in chronic feedings of 100 mg/kg in a mouse, we did not want to risk the integrity of the experiment. Therefore, only chronic doses of DCS were given with a bolus of PIO (100 mg/kg) on the 7th day.

Control mice with TIC received an i.p. injection of normal saline (200 µl/mouse). Mechanical allodynia was evaluated at 0, 0.5, 1, 2, 3, 4, 5, and 6 hour post injection time points.

4.2.4.2.3. *Experiment 3: 7-day Treatment of 80 mg/kg Dose of DCS Followed by a Bolus of 100 mg/kg Dose of PIO on the 7th Day*

At least 8 weeks after the TIC injury, mice started receiving daily subcutaneous injections of 80 mg/kg dose of DCS. On the 7th day, in addition to the 80 mg/kg DCS dose, the mice were given a one-time bolus of PIO, 100 mg/kg (i. p.). In parallel to the mice with TIC injury, sham mice were also given the same injections. The vehicle control mice with TIC injury and sham mice received a daily subcutaneous injection of normal saline (150 µl). Mechanical
threshold was measured daily at 2.5-3 hours after injection. On the 7th day, the mice with TIC injury (saline vs. drug group) were placed in the light/dark box for preference testing.

4.2.4.2.4. Experiment 4: Single Dose of Mitochondrial Uncoupler 2,4-DNP

At least 8 weeks after TIC injury, the BALB/C mice were given either 5 mg/kg i.p. dose of 2,4, DNP or an i.p. injection of 10% DMSO (250 µl) as vehicle control. Mechanical allodynia was assessed at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 hour time point after injection.

4.2.5. Mitochondrial Isolation Assays

Isolated Mitochondrial assays were taken from previously described methods (Pandya et al 2013; Pandya et al 2007; Sauerbeck et al 2011a; Sauerbeck et al 2012; Sauerbeck et al 2011b).

4.2.5.1. Isolated Mitochondria Preparation

Twenty-eight weeks post TIC injury; mice were euthanized with CO2 and rapidly decapitated. Naïve age matched mice were also euthanized as controls. The brainstems and brain were quickly dissected and placed on an ice cold dissecting plate where the entire cortex was divided from the rest of the brain. Then the entire cortex and whole brainstem were utilized for this experiment in
order to obtain enough tissue and mitochondria to perform the Seahorse assay. The cortex and brainstem were homogenized in separate vials each containing 2 ml of 4°C mitochondrial isolation buffer (MIB) containing (215 mM mannitol, 75mM sucrose, 0.1% BSA, 20mM HEPES, 1mM EGTA, pH adjusted to 7.2 using KOH). The EGTA (ethylene glycol tetraacetic acid), a calcium chelator, was added to improve the mitochondria isolation (Pandya et al 2013; Patel et al 2009). Tissue homogenates were centrifuged twice at 1300 x G for 3 minutes at 4°C. The resultant supernatant was then removed and centrifuged at 13,000 x G for 10 minutes at 4°C. The mitochondrial/synaptosomal pellets were burst in a nitrogen bomb chamber (1200 psi for 10 minutes at 4°C). After the nitrogen burst, the mitochondrial pellets were placed atop of a discontinuous Ficoll gradient (7.5% - 10%) and centrifuged at 100,000 X G for 30 min at 4°C. The mitochondrial pellet was then resuspended in EGTA-free MIB at 10,000 x G for 10 minutes at 4°C. The final pellet was resuspended at a concentration of 10 mg/ml in EGTA-free MIB and was stored on ice until further use. A BCA protein assay kit determined the protein concentrations in a Biotek Synergy HT plate reader by measuring absorbance at the optical wave length of 560nm (Winooski, Vermont).

4.2.5.2. Bioscience Seahorse XF²4 Flux Analyzer Assay

The Seahorse XF²4 Flux Analyzer (Seahorse Bioscience, Massachusetts, United States) was used to measure the mitochondrial bioenergetics in isolated mitochondria preparation as previously described (Pandya et al 2013; Sauerbeck
et al 2011b). The day before the experiment, the stock mitochondrial substrates and inhibitors were prepared (500 mM pyruvate, 250 mM malate, 30 mM ADP, 1 mg/ml oligomycin-A, 1 mM FCCP, 1 mM rotenone, and 1 M succinate with the pH adjusted to 7.2). The XF Calibrant solution (1ml) was added to each well of a 24-well calibration sensor cartridge. This sensor cartridge was then positioned on the 24-well calibration plate and placed in a 37°C incubator overnight, the calibration sensor cartridge ports A to D were loaded with the appropriate mitochondrial substrates/inhibitors at 10x concentrations at the following day. The mitochondrial respiration buffer (MRB) consisted of 215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl, 2.5 mM KHPO₄ adjusted to a pH of 7.2. The volume of MRB in the mitochondrial plate was based upon the original 500µl MRB volume.

The brain mitochondrial samples (10 µg) of both mice with TIC and naïve mice were analyzed together on a single plate. After being resuspended in MRB, 50 µl of the mitochondrial samples were added in each experimental well with control wells (totaling 4 wells) only obtaining 50 µl of MRB. The XF24 plate was centrifuged at room temperature for 4 minutes at 3,000 rpm. For half of the TIC and naïve mitochondrial samples (totaling 10 wells), 475 µl of MRB at 37°C was added to each well. For the other half of the TIC and naïve mitochondrial samples (totaling 10 wells) 475 µl of MRB (37°C) containing 50 nM of DCS and 50 nM of PIO were added to each making a total volume in each well equal to 525 µl. The plates were then placed in the Seahorse XF²4 flux analyzer for mitochondrial bioenergetics analysis following the calibration.
Sauerback and colleagues (Sauerbeck et al 2011b) provide a detailed explanation of the cyclic protocol in which the appropriate substrates/inhibitors are added to the wells and the measurement of the oxygen consumption rate is recorded for each well. The substrates/inhibitors listed above were added from port A to port D. Pandya and colleagues (2014) describes the State III response after 5mM pyruvate, 2.5 mM malate, and 1 mM ADP were measured (Port A). State III is a good indicator of how healthy the mitochondria providing a complex I driven ADP phosphorylation rate and ATP synthesis. State IV response is in the presence of all the State III substrates/inhibitors including the addition of 1 μM oligomycin A (Port B). With the oligomycin A addition, State IV is inhibiting the complex V (ATP synthase) action. The Seahorse analyzer will collect data and report as percent Oxygen Consumption Ratio (OCR) at each State. Therefore, State III/State IV is known as the Respiratory Control Ratio (RCR) which is used to determine how well coupled electron transport is to the production of ATP. A RCR that is greater than 5 is reported for healthy mitochondria.

4.2.6. Statistical Analysis
GraphPad Prism 6 software package (GraphPad Software, Inc. La Jolla, CA) was used for graphing and statistical analysis of data from all behavioral tests, drug administrations, and mitochondrial assays. Data is shown as mean ± standard error of the mean (S.E.M.). Data was analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test, or one-way ANOVA followed by a
Fishers post hoc test, or by standard, two-tailed, unpaired t-test (where is appropriate). A p≤0.05 was considered a significant for all tests.

4.3. Results

4.3.1. Unilateral Facial Mechanical Allodynia in Mice with TIC Injury

Baseline 50% mechanical thresholds were taken before surgery and were similar for mice with TIC injury and sham (TIC: 3.84 ± 0.35 g vs. sham: 3.47 ± 0.00 g). However, one week post TIC injury the 50% mechanical threshold on the ipsilateral whisker pad of the mice decreased to 0.37 ± 0.39 g and this was significantly different from that on the contralateral side whisker pad (3.45 ± 0.04 g vs. 0.37±0.39 g; p<0.0001, two-way ANOVA, Bonferroni post hoc test; Figure 4.1). The unilateral lowered mechanical threshold started within the first week, reached the maximum mechanical allodynia (threshold at the 0 g) at week 1 and lasted until the animals were euthanized. The sham mice did not display any differences in the mechanical threshold of the whisker pad before and post-surgery. The 50% mechanical threshold of the ipsilateral whisker pad of sham mice was 3.41 ± 0.02 g and contralateral whisker pad was 3.43 ± 0.05 g. Comparing the mechanical threshold of the ipsilateral side whisker pad between the mice with/without TIC injury, there is also a significant difference (p<0.0001, two-way ANOVA, Bonferroni post hoc test; n=9).
4.3.2. *D*-cycloserine Attenuated Mechanical Allodynia But Did Not Relieve Anxiety Behaviors Associated with Hypersensitivity

Single doses of DCS (40 mg/kg, 60 mg/kg, 80 mg/kg, 100 mg/kg, 160 mg/kg, and 320 mg/kg) were given in ascending sequence with a one week interval between each treatment, given to the mice with TIC injury to determine a DCS dose response curve (*Figure 4.2A*). Only the higher DCS doses, 160 mg/kg and 320 mg/kg, were effective in alleviating mechanical allodynia on the whisker pad of the mice with TIC injury. At 3 - 4 hour post injection, the mechanical threshold of the 160 mg/kg dose treatment group was elevated (hr 3: 0.51± 0.13 g; hr 4: 0.43 ± 0.12 g) and was statistically significant different from that of saline treatment group. (hr 3: 0.00 ± 0.00 g; hr 4:0.01 ± 0.00 g; p<0.0001, two-way ANOVA, Bonferroni post hoc test). The mechanical threshold of the 320 mg/kg dose group was elevated at 3 hour post injection (0.17 ± 0.11 g) and was statistically significant different from that of the saline control mice group (0.00 ± 0.00; p<0.0001, two-way ANOVA, Bonferroni post hoc test; n=4-8).

As shown in *Figure 4.2B*, the daily dose of 80 mg/kg attenuated the mechanical allodynia in ipsilateral whisker pad of the mice with TIC injury on the 6th day of injection (1.10 ± 0.62 g; n=4-7). Interestingly, the 40 mg/kg and 60 mg/kg dose of DCS had no effect on the mice with TIC injury, but the sham mice that received the 40 mg/kg dose of DCS became hypersensitive to the von Frey fiber stimuli on the whisker pad bilaterally (0.37 ± 0.36 g; indicated by the orange line in *Figure 4.2B*). The decreased 50% mechanical threshold was significantly different from that of the shams that received the 60 mg/kg dose (3.76 ± 0.23 g)
and the 80 mg/kg dose treatment (3.43 ± 0.05 g; p<0.001; two-way ANOVA, Bonferroni post hoc test; n=4 - 7).

Previous studies have reported DCS is involved in alleviating anxiety-related disorders (Antal & Paulus 2011; Davis et al 2006; Norberg et al 2008). The light-dark box preference test was used to determine if the 7-day treatment of DCS would attenuate the anxiety-like behaviors that developed in the mice with TIC injury. Figure 4.2C depicts the time of light side occupancy of the mice with TIC injury injected with saline (235.30 ± 55.80 sec) vs. the mice with TIC + DCS (271.30 ± 65.16 sec; p=0.69; unpaired t test; n=5). Although there is no statistical significant difference in the DCS treated mice compared to the saline treated, the DCS treated mice did spend substantially more time in the light side. This indicated that DCS had minimal effect at the dose tested and would be more effective in relieving the anxiety-like behaviors in a higher doses range.

4.3.3. Attenuation of Mechanical Allodynia and Anxiety by Combination of Ineffective Low Doses of D-cycloserine and Pioglitazone

A single dose of 100 mg/kg of PIO (i.p.) was determined to be ineffective, but the 300 mg/kg dose of PIO was effective in alleviating mechanical allodynia in mice with TIC injury (see chapter 3). There are no negative interactions reported between PIO and DCS in the literature. There is also no mention of the effect of the two drug combination on orofacial neuropathic pain as was explored with our TIC injury mouse model. Figure 4.3A depicts the one-time drug dose combination of 100 mg/kg (i.p.) of PIO and 80 mg/kg (s.c.) of DCS compared to
these same doses given alone in the mice with TIC injury. The mechanical allodynia was attenuated in the mice with TIC injury treated with the drug combination of PIO + DCS (the 50% mechanical threshold was 0.94 ± 0.34 g) and was significantly different compared to that in the saline treated animals (0.00 ± 0.00 g; at hour 3 post injection p<0.001, two-way ANOVA, Bonferroni post hoc; n=5-9; Figure 4.3B). The effect peak at 2 hour and lasted for 4 hours post injection. The effect of the drug combination was also significantly different from that of a single dose of 100 mg/kg PIO (0.03 ±0.01 g) or a single dose of 80 mg/kg DCS (0.06 ± 0.02 g). These one-time doses given alone had no effect on mechanical allodynia in the mouse with TIC injury.

Following the same experimental method as above for 7-day dosing with 80 mg/kg (s.c.) of DCS, the study was repeated with the addition of a 100 mg/kg (i.p.) bolus of PIO given on the 7th day. The drug combination on the 7th day had a greater attenuating effect on mechanical allodynia compared to the effect in saline treated mice. Two hours following the injection, the mice were placed in the light-dark box for the preference test. The mice treated with the drug combination (DCS + PIO) spent a significantly increased amount of time in the light side compared to saline treated mice (drug: 221.0 ± 52.33 sec vs saline: 75.28 ± 36.65 sec; p<0.05, unpaired t-test; Figure 4.3C). Taken together, these data demonstrated that combination of PIO and DCS at low dose has a potentiating effect. Additionally, this drug combination provided a reversal of anxiety-like behaviors associated with the TIC injury, suggesting it could be very
beneficial in clinic patients who suffer from chronic orofacial pain and anxiety-associated with pain.

4.3.4 2.4-DNP Attenuated Mechanical Allodynia in Mice with TIC Injury

The mitochondrial uncoupler, 2,4-DNP, has been proven to be neuroprotective after traumatic brain injury (Pandya, 2007). At week 8 post injury, 5 mg/kg of 2,4-DNP, single dose injection (i.p.) effectively attenuated mechanical allodynia on the whisker pad of the mice with TIC injury (Figure 4.4). The effect started from 2 hour post injection (1.03 ± 0.15 g) and lasted for 5 hour (3hr: 1.64 ± 0.21g; 4hr: 1.17 ± 0.09g; 5hr: 0.83 ± 0.07g). This difference was statistically significant compared to mice with TIC injury given vehicle (10% DMSO) (0.02 ± 0.00g; *p<0.05, ****p<0.0001; two-way ANOVA, Bonferroni post hoc test). The effectiveness of this drug in increasing mechanical threshold suggests that mitochondrial dysfunction could be a key factor in maintaining the chronic mechanical allodynia in the mice after TIC injury. Thereby, drugs that uncouple the altered mitochondrial function could be beneficial for relieving chronic pain.

4.3.5. Cortex Mitochondria Had a Decreased Respiratory Control Ratio in Mice with TIC Indicating Cortical Mitochondrial Dysfunction

The mitochondrial isolation assay and Seahorse XF®24 analyzer was used to determine the mitochondrial oxygen consumption of isolated cortical mitochondria in the mice with TIC injury. The State III OCR of the mitochondria from mice with TIC injury (382.40 ± 10.47) significantly increased compared to the State III OCR of the mitochondria from naïve animals (253.9 ± 11.99;
p<0.001, two-way ANOVA; **Figure 4.5A**). The oxygen consumption rate (OCR) of State IV increases in the mitochondria from mice with TIC injury compared to the mitochondria from the naïve controls (TIC: 86.78 ± 1.93 vs. 47.14 ± 8.54; p<.05, two-way ANOVA; **Figure 4.5B**). This data suggest that the cortical mitochondria from mice with TIC injury had increased mitochondrial bioenergetics. However, the Respiratory Control Ratio (RCR), defined as RCR = State III/State IV, significantly decreased in the mitochondria from mice with TIC injury compared to the RCR in the mitochondria of naïve controls (TIC: 4.41 ± 0.07 vs. naïve: 6.64 ± 1.01; p<0.05, two-way ANOVA; **Figure 4.5C**). These results indicate mitochondrial dysfunction occurs in the mice with TIC injury since the RCR is less than 5.

The DCS/PIO drug combination (added ex vivo to the MRB) did not significantly change State III or State IV OCR. However, the drug combination did significantly increase the RCR of the cortical mitochondria of mice with TIC (cortex: 6.00 ± 0.71) compared to untreated mitochondria of mice with TIC (cortex: 4.407 ± 0.07, p<0.5, unpaired t-test; **Figure 4.5C**). The drug combination significantly decreased the RCR of the cortical mitochondria of naïve mice (4.08 ± 0.21) compared to that of untreated cortical mitochondria from naïve controls (6.64 ± 1.008; p<0.5, two-way ANOVA; **Figure 4.5C**).
4.3.6. Drug Combination Treatment Increased Respiratory Control Ratio in Brainstem Mitochondria of Mice with TIC Injury

Isolated brainstem mitochondria of the mice with TIC injury were analyzed along with the mitochondria from age matched naïve mice. The OCR of State III and State IV of the mitochondria from mice with TIC injury (state III: 502.10 ± 48.26; state IV: 70.44 ± 9.43) was not significant compared to the mitochondria of naïves (state III: 513.90 ± 27.81; state IV: 85.87 ± 9.17; Figure 4.6A & 6B). The RCR of the mitochondria of the mice with TIC injury (7.43 ±0.34) compared to the mitochondria from naïve mice was not significantly different (6.24 ± 0.35; Figure 4.6C). However, with the two drug combination, the only significant change observed was that the mitochondria drug treated mice with TIC injury had an increased RCR compared to the mitochondria from the untreated mice with TIC injury (treated: 9.00 ± 0.59 vs. untreated: 7.43 ± 0.34, p<0.05, two-way ANOVA, Figure 4.6C).

4.4. Discussion

In this study, it was determined that higher doses of single injections of DCS (160 mg/kg and 320 mg/kg) were efficacious in alleviating mechanical allodynia on the whisker pad of mice with TIC injury. However, low doses of DCS such as 40 mg/kg induced hypersensitivity in the sham operation control animals. Chronic treatment with the 80 mg/kg dose of DCS for 7 days partially attenuated mechanical alldynia on the whisker pads of the mice with TIC injury on the 6th day. This chronic treatment dose, however, did not improve the anxiety-like
behavior associated with TIC injury as shown by the light-dark box preference test. The combination of the low dose of DCS (80 mg/kg) and PIO (100 mg/kg) was shown to significantly increased the 50% mechanical threshold levels of the mice with TIC injury compared to that in saline treated mice with TIC injury as well as that in the ineffective single dose of 100 mg/kg PIO or 80 mg/kg DCS. Furthermore, the 7-day dose regiment of 80 mg/kg dose of DCS was repeated with the exception of the additional bolus of 100 mg/kg dose of PIO given on the 7th day. This was to mimic the DCS (single drug only) 7-day regiment followed by the light-dark box preference test. Single dose of drug combination treatment improved not only mechanical allodynia, but also the anxiety-like behavior associated with the TIC injury.

In this study, the mild mitochondrial uncoupler, 2,4-DNP, significantly elevated the 50% mechanical threshold on the whisker pads of the mice with TIC injury. Although this provides evidence that mitochondrial dysfunction occurs in the mice with TIC injury, ex vivo studies were conducted in order: 1) to confirm the mitochondrial dysfunction after the TIC injury and 2) to determine if the drug combination (DCS\PIO) is acting specifically on the mitochondria to attenuate mechanical allodynia. The mitochondrial isolation assays determined that State III and State IV OCR increased in cortical mitochondrial, but did not change in brainstem mitochondria. However, the RCR did in fact decrease in the isolated cortical mitochondria. Furthermore, when treated with the drug combination, the RCR of the cortex mitochondria in the naïve mice decreased while those of the
mice with TIC injury increased not only in the cortex mitochondria but also in the brainstem mitochondria.

Although clinical trials have been conducted with DCS for alleviation of chronic back pain, anxiety/stress disorders, and fear related to pain (Davis et al 2006; Heaton et al 2010; Norberg et al 2008), only one study has been published that observes its attenuation of chronic orofacial pain (Antal & Paulus 2011). DCS is known to have an affinity for a specific glycine binding site on the NMDA receptor. Previous studies that have shown that at lower doses, DCS will act as a partial agonist on the NMDA receptor producing hypersensitivity, but higher doses, DCS can act as a partial antagonist of NMDA (Kushner et al 2007; Lanthorn 1994). Similarly, the present study found that DCS acts as an agonist at low doses (40 mg/kg) inducing hypersensitivity in naïve animals and as an antagonist at high doses (160, 320 mg/kg) in the mice with TIC injury for alleviation of chronic pain. As explanation for the dual effect, Mony and colleagues speculated that many ligands in high doses desensitizes the NMDA receptor (Mony et al 2009).

Although previous studies have determined that PIO, a PPARγ receptor agonist, attenuates neuropathic pain and inflammatory nociception due to peripheral nerve injury (Fehrenbacher et al 2009; Ghosh et al 2007; Maeda & Kishioka 2009; Morgenweck et al 2010; Morgenweck et al 2013; Park et al 2007), the present study was the first to use the drug combination of DCS and PIO to treat orofacial neuropathic pain and anxiety associated with TIC injury in a mouse model. It was determined that using ineffective low doses of both DCS and PIO,
when combined together, attenuated trigeminal neuropathic pain in the mice with TIC injury. This potentiated effect also improved the anxiety-like behavior in the mice with TIC injury whereas the DCS only treatment did not. This indicated that the drug combination is having a potentiated effect most likely in higher order brain regions to attenuate not just pain, but anxiety-related to pain (Dellarole et al 2014; Lipton et al 2000; McWilliams et al 2003; McWilliams et al 2004; Robinson et al 1988). Furthermore, the DCS/PIO combination produced no overt side effects observable in the mice with TIC injury. Therefore this drug combination could be a very beneficial treatment for patients who are suffering from depression, anxiety, or other psychological conditions due to their chronic pain status.

Although DCS and PIO could be acting through the NMDA receptor and PPARγ separately, another explanation for this potentiated effect is that these drugs are acting through alternative receptors altogether. In fact, some studies have supported a role for PIO acting through PPARγ independent pathways directly on mitoNEET, a mitochondrial protein. mitoNEET is vital for mitochondrial respiration and for increasing mitochondrial bioenergetics (Geldenhuys et al 2014; Wiley et al 2007a; Wiley et al 2007b; Yonutas & Sullivan 2013). Furthermore, Korde and Maragos (2012) successfully identified a NMDA-like receptor directly on the mitochondrial membrane (Korde & Maragos 2012). This evokes the questions: Is the DCS/PIO combination alleviating trigeminal pain by correcting mitochondrial dysfunction through these mechanisms?
Although mitochondrial dysfunction has been shown to be responsible for maintaining chronic pain after neuronal injury (Bouillot et al 2002; Ferrari & Levine 2010; Joseph & Levine 2006; Kim et al 2004; Shin et al 2003; Sui et al 2013), this is the first study to demonstrate that mitochondrial dysfunction occurs in a chronic trigeminal neuropathic pain model. First, this study determined that a mild mitochondrial uncoupler, 2,4-DNP, attenuated mechanical allodynia on the whisker pad of the mice with TIC injury. This was supportive reasoning that mitochondrial dysfunction was occurring in the mice with TIC. However, it did not answer the question of whether mitochondrial function could be improved with the DCS and PIO drug combination.

To further investigate the role of mitochondria in neuropathic pain after TIC injury, isolated mitochondrial assays were performed at 28 weeks post injury. This study observed the mitochondrial respiration rates of States III and IV in particular. These data showed that the cortical mitochondrial of the mice with TIC injury have increased State III respiration as well as an increased State IV respiration, thereby significantly decreasing the RCR compared to naïve mice. However, there were no significant changes in the brainstem mitochondria compared to naive mice. This data could be interpreted in many ways.

First, this data could support an adaptive mechanism that is occurring in the cortical mitochondrial of the injured animals. Since the mitochondria was analyzed 28 weeks post injury, it is sufficient to say that in a chronic state the increased State III respiration is needed to provide sufficient ATP production for brain function. However, while State III indicates complex I driven ADP
phosphorylation and the general mitochondrial oxidation, State IV is a sufficient indicator of electron leak (Brand & Nicholls 2011; Chance & Williams 1955a; b). Since State III respiration also increased, this could be supportive of increased electron leak that then leads to increased ROS production. However, further studies need to be conducted to confirm mitochondrial ROS in the mice with TIC injury. Since the respiratory control ratio (RCR) (State III/State IV) was less than 5 in the cortical mitochondrial of the mice with TIC injury, this would support the idea that the complexes of electron transport chain are not very well coupled to net production of ATP, indicating mitochondrial dysfunction (Brand 1990; Brand et al 1978; Brand & Nicholls 2011).

Furthermore, the drug combination (DCS/PIO) increased RCR in the cortical and brainstem mitochondria, but decreased RCR in the cortical mitochondria of the naïve mice. The DCS/PIO combination was able to improve RCR suggesting that the DCS/PIO combination improves mitochondrial dysfunction after injury, but is not beneficial in naïve mice. An underlying detailed mechanism for the DCS and PIO combination effect is unknown and needs to be investigated in future studies. Future studies could confirm if this drug combination is acting through protein-dependent (NMDA-like receptor in mitochondria for DCS and mitoNEET for PIO) or protein-independent mechanisms to improve mitochondrial bioenergetics. Future studies looking at specific complex activity of the mETC, in particular the redox state of complex I could be beneficial in uncovering the mechanism. (Starkov 2006).
Additionally, mitochondrial dysfunction was only detected in the cortical mitochondria, but not in the brainstem. This could be due to the fact that there is a large area of cortex dedicated to the trigeminal somatotopic map, and thus, a higher number of mitochondria in the entire cortex affected compared to the brainstem. The trigeminal dorsal horn, on the other hand, is a small portion of the brainstem. Nevertheless, the drug combination was acting to increase RCR at both levels to ADP phosphorylation. This paralleled improvement of both aspects of pain demonstrated with the behavioral tests.

Lastly, unlike 2,4-DNP which was removed from the market due to increased fatality rates, DCS and PIO, given at their low ineffective dose combination, did not induce any observed side effects in the mice. The DCS\PIO combination could be a great value in the clinic with patients suffering from continuous trigeminal neuropathic pain.

4.5. Conclusion

This study demonstrated that the combination of DCS/PIO attenuated not only orofacial neuropathic pain, but also the anxiety behaviors associated with the TIC injury through receptor independent mechanisms. These mechanisms were supported by the DCS/PIO combination improving the cortical mitochondrial dysfunction present in the mice with TIC injury.
Figure 4.1. TIC Injury Induced Unilateral Whisker Pad Mechanical Allodynia.

The 50% mechanical threshold (in gram force) was measured bilaterally on the whisker pads of the mice with TIC injury and the sham mice. The mechanical threshold was decreased on the ipsilateral whisker pad of mice with TIC injury within one week post injury. The mechanical threshold of contralateral whisker pad was unaffected by surgery. The mechanical threshold of the ipsilateral and contralateral whisker pads of the sham mice also did not change. TIC n=9; TIC (ipsi) vs. TIC (con.) or naïve ****p<0.0001, two-way ANOVA, Bonferroni post hoc test.
Figure 4.2. DCS Attenuated Mechanical Allodynia, but Does Not Reverse Anxiety-Like Behavior in the Mice with TIC Injury. (A) Dose response curve for DCS showed that higher doses of DCS (160 mg/kg and 320 mg/kg) are
effective at alleviating the mechanical allodynia on the whisker pad of the mice with TIC injury. (n=6) (B) The mice were given a 7-day treatment of lower doses of DCS (s.c.). Only the 80 mg/kg dose elevated the mechanical threshold in the mice with TIC injury. The 40 mg/kg dose of DCS lowered the mechanical threshold on the whisker pad of the sham mice while the 60 mg/kg dose had no effect on sham mice or mice with TIC injury (n=8). (C) On day 7, two hours post-injection, the mice with TIC injury treated with 80 mg/kg dose of DCS or went through the light-dark box preference test. There is no difference in the time of the light side occupancy between drug treated and vehicle treated group. *p<0.05, ***p<0.01; ****p<0.0001; two-way ANOVA, Bonferroni post hoc test. Figure C: Unpaired t-test, n.s.; n=5.
Figure 4.3. The Combination of DCS and PIO Attenuated Mechanical Allodynia and Anxiety-Like Behavior in the Mice with TIC Injury. (A) One-time dose of DCS (80 mg/kg) + PIO (100 mg/kg) provided a potentiated effect in elevating mechanical threshold of the whisker pad of the mice with TIC injury which was significantly different than that of the single dose of either DCS (80 mg/kg) only or PIO (100 mg/kg) only (n=6-8). The mechanical threshold of the drug combination treatment group was also significantly different from the vehicle treated group. (B) After a 7-day dose of DCS (80 mg/kg), a bolus of PIO (100 mg/kg) was given on the 7th day. The mechanical threshold was dramatically increased in the mice with TIC injury. This was significantly different from that of the vehicle treated mice with TIC injury. (C) Two hours following injection of the PIO bolus on day 7, the drug and the vehicle treated mice with TIC injury went through in the light-dark box preference test (n=8/group). The drug treated group showed a significant increase in the time spent in the light chamber compared to that of the vehicle treated group (*p<0.05, ***p<0.001; ****p<0.0001; two-way ANOVA, Bonferroni post hoc test. Figure C: Unpaired t-test, (*p<0.05.; n=5).
Figure 4.4. A Mild Mitochondrial Uncoupler Attenuated the Mechanical Allodynia in the Mice with TIC Injury. 2,4-DNP (5 mg/kg) significantly increased the 50% mechanical threshold of the mice with TIC injury compared to that of the vehicle treated mice. This effect started at one hour, peaked at 3 hour and lasted for 5 hours post injection. (n=8; (*p<0.05, ****p<0.0001; two-way ANOVA, Bonferroni post hoc test.).
Figure 4.5. Isolated Cortical Mitochondria from the Mice with TIC Injury Showed an Increased State III and State IV OCR, but Decreased RCR which is Reversed With the Drug Combination of DCS + PIO. Cortical mitochondria isolated from mice with TIC injury had an increased oxygen consumption rate (OCR) for State III (A) and increased OCR in State IV respiration (B). This is
significantly different compared to those of the cortical mitochondria from naïve mice. The respiratory control ratio (RCR = StateIII/StateIV)) decreased in the cortical mitochondria of the mice with TIC injury (C). However, the drug combination of DCS and PIO decreased the OCR in State IV, therefore increased the RCR (n=8/group; *p<0.05, **p<0.01; two-way ANOVA, Bonferroni post hoc test; #p<0.05, unpaired t-test).
Figure 4.6. The Drug Combination (DCS +PIO) Increased the Brainstem Mitochondrial RCR in the Mice With TIC Injury. There are no significant differences in the OCR of State III (A) or State IV (B) for the isolated brainstem mitochondria from the mice with TIC injury compared to that of the naïve mice. The respiratory control ratio (RCR) did not significant change in the isolated
brainstem mitochondria from the mice with TIC injury compared to that of naïve mice (C). However, due to the drug combination treatment (50 nM DCS+ 50nM PIO, ex vivo) increased the OCR of the brainstem mitochondria of mice with TIC injury at the state III as showed in (A). The RCR of the brainstem mitochondria from the mice with TIC injury did increase (n=4/group; *p<0.05; two-way ANOVA, Bonferroni post hoc test).
CHAPTER FIVE
OVERALL DISCUSSION AND CONCLUSIONS

The aims of the study were to develop a new orofacial neuropathic pain model specifically for mice that provides a suitable model for study of orofacial pain. With this model, the studies sought to identify certain molecular targets that play a major role in the etiology and maintenance of trigeminal neuropathic pain.

5.1. Trigeminal Inflammatory Compression (TIC) Injury Is a Clinically Relevant Orofacial Neuropathic Pain Model

This series of studies expands our understanding of the novel facial mouse pain model known as Trigeminal Inflammatory Compression (TIC) injury. This chronic neuropathic pain model was described in our previous study (Ma et al 2012a), but the cold, anxiety- and depressive-like behavioral characteristics of this injury were not yet completely characterized. The TIC model demonstrated several aspects consistent with chronic facial pain in humans. The first study of this thesis determined that the surgery is 100% efficacious for producing facial hypersensitivity in all of the animals receiving the chromic gut suture. The placement of the suture provides mechanical allodynia with distinctive receptive fields (on the affected whisker pad). Likewise, the pattern of receptive fields were not always the same in all animals (depending on the nerve fascicle contacted by the chromic gut suture), as the pattern of receptive fields from person to person may also vary (Simons & Travell 1981; Travell 1981). The development of cold
allodynia on the whisker pad of the mice with TIC injury, but not heat hypersensitivity is consistent with the clinical profile. Clinic patients suffering with trigeminal neuropathic pain are not often bothered by heat stimuli, but instead most often complain that light touch, and cold stimuli (De Leeuw 2008; Zakrzewska 2013).

Furthermore, the studies determined that the chronicity of the TIC injury model persists at least through 21 weeks. These were the first recorded data indicating a mouse facial pain persisting through this long time course without causing ischemia and complete demyelination of the infraorbital nerve (Ma et al 2012a). Accompanying the hypersensitization were the anxiety- and depressive-like behaviors. These were supported by the performance of the mice in operant tests (light-dark box, open field, elevated plus maze). The anxiety-depressive-like behavior was worsened by the acoustic disturbance.

This again fits into the definition of chronic pain described Wall & Melzack (1999) with the two main features of “pain sensation” and “pain affect” which are incorporated into the emotional distress suffered by patients with pain (Wall & Melzack 1999). The “pain affect” has also been described as the sequelae of other physical and psychological disorders such as anxiety and depression (Dellarole et al 2014; Maletic & Raison 2009; McWilliams et al 2004). These comorbidities have been so prevalent that approximately half of all patients suffering from chronic pain reportedly also suffered from anxiety and depression (Asmundson & Taylor 2009; Carleton et al 2009; Macianskyte et al 2011; Robinson et al 2009). The relationship between chronic pain, depression,
anxiety, and fear is extremely complex with extensive overlap making it difficult to determine a pattern of cause and effect. This complex relationship is related to the numerous brain structures, such as the somatosensory cortex, prefrontal cortex, nucleus accumbens, insular cortex, anterior cingulate cortex, amygdala, hippocampus, thalamus, and cerebellum that have all been highlighted as major players involved not only in pain perception but also in affective states, including depression, anxiety, and fear (Apkarian 2004; Apkarian et al 2004a; Apkarian et al 2004b; Becerra et al 2006; Dellarole et al 2014; Heim et al 2004; Robinson et al 2009). Thus, the TIC injury model is indeed a representative model of anxiety-related chronic facial neuropathic pain.

5.2. **PPARγ Is an Important Molecular Target for the Treatment of Trigeminal Neuropathic Pain**

Our experiment determined that the nuclear receptor, peroxisome proliferator receptor-gamma isoform (PPARγ), plays a significant role in trigeminal pain transmission. Immunohistological study revealed that 3 weeks after the Trigeminal Inflammatory Compression (TIC) injury, a most intense PPARγ immunoreactivity appeared in the spinal trigeminal caudalis where the primary nociceptive fibers synapse. Systemic administration of a PPARγ agonist, pioglitazone (PIO), attenuated the mechanical allodynia in the mice with TIC injury at doses of 300 mg/kg i.p. and 600 mg/kg p.o. However, 100 mg/kg of PIO (i.p.) had no effect. Furthermore, these studies revealed that administering a PPARγ antagonist, GW9662 (30 mg/kg i.p.) prior to the optimal dose of PIO (300
mg/kg i.p.) blocked the analgesic effect of PIO indicating that PIO is acting through a PPARγ activation mechanism. Additionally, the PPARα agonists, benzafibrate and fenofibrate, had no effect on the allodynic mice. However, the PPARβ/δ agonist, GW0742 had a minimum alleviation of allodynia effect to the mice with TIC, but it is not as effective as the PPARγ agonists. Taken together, these results confirm PPAR’s role in trigeminal pain transmission, particularly signaling through the γ isoform.

Furthermore, the combination of low doses of DCS (80 mg/kg) and PIO (100 mg/kg) were shown to significantly elevate the 50% mechanical threshold of the mice with TIC injury. The single dose of DCS only at 80 mg/kg or PIO at 100 mg/kg was shown to be ineffective. In combination, they produced a potentiated effect. Treatment with the single dose combination improved not only mechanical allodynia, but also the anxiety-like behavior associated with the TIC injury.

Therefore, the question is: What defines a molecule as a potential target for the treatment of inflammation? Several features should be associated with the molecular signature of an inflammatory target. First, the cellular or sub-cellular expression of the molecule of interest could be changed upon inflammation. Second, the activation of this molecule could cause signs of decreased inflammation. Third, the functions of the molecule of interest could be regulated by other inflammatory mediators. Finally, the inhibition or activation of this molecule should downregulate pro-inflammatory mediators that cause inflammation. After reviewing the data, this study demonstrated that PPAR-
gamma meets all the criteria, thereby supporting this receptor as a potential therapeutic target to treat trigeminal neuro-inflammatory pain.

This study is not only the first study to identify PPARγ immunoreactivity throughout the trigeminal brainstem sensory nuclear complex with/without trigeminal nerve injury, but this study also is the first to demonstrate that PPARγ activation attenuates trigeminal neuropathic/inflammatory pain. In conclusion, PPARγ is suggested as a potential therapeutic target in the trigeminal pain neuraxis. And from this study also arises the possibility of repurposing the FDA approved diabetic therapeutic drug, PIO, for the treatment of patients suffering from orofacial pain.

5.3. Mitochondrial Dysfunction is One Underlying Mechanism of Trigeminal Neuropathic Pain

In this study, the mild mitochondrial uncoupler, 2,4-DNP, significantly elevated the 50% mechanical threshold on the whisker pads of the mice with TIC injury. This provides evidence that mitochondrial dysfunction occurs in the mice with TIC injury. *Ex vivo* studies were conducted in order to determine: 1) the cause of the mitochondrial dysfunction, and 2) whether the drug combination (DCS/PIO) is acting on the mitochondria to attenuate mechanical allodynia. The mitochondrial isolation assays determined that the OCR of State III and State IV increased in cortical mitochondria, but did not change in brainstem mitochondria. However, the RCR did in fact decrease in the isolated cortical mitochondria. Furthermore, when treated with the DCS/PIO combination *ex vivo*, on the
isolated brain mitochondria preparation, the RCR of the cortex mitochondria in
the naïve mice decreased while those of the mice with TIC injury increased not
only in the cortex mitochondria but also in the brainstem mitochondria. Although
mitochondrial dysfunction has been shown to be responsible for maintaining
chronic pain after neuronal injury (Bouillot et al 2002; Ferrari & Levine 2010;
Joseph et al 2004; Joseph & Levine 2006; Kim et al 2004; Park et al 2006;
Schwartz et al 2009; Shin et al 2003; Sui et al 2013), this is the first study to
demonstrate that mitochondrial dysfunction occurs in chronic trigeminal
neuropathic pain. This study determined that a mild mitochondrial uncoupler, 2,4-
DNP, attenuated mechanical allodynia on the whisker pad of the mice with TIC
injury. The experiment suggests that the DCS/PIO combination could be acting in
the brain after TIC injury to improve the brain mitochondrial respiration capacity
and leads to highly efficient functional performance of the brain, thereby,
controlling pain related behavior and pain related anxiety.

5.4. Limitations and Future Studies

Several limitations exist for this study. First, all of our animals were male
C57Bl/6 mice. C57Bl/6 mice were chosen for their moderate levels of anxiety-
like behavior (Belzung 2001; Belzung & Griebel 2001; Kulesskaya et al 2014;
Kulesskaya & Voikar 2014). In order to collect more consistent results, females
or other strains were not tested in this study. However, future studies should be
conducted to observe the behaviors of different species. Since facial neuropathic
pain in humans is diagnosed more often in females, behavioral studies

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performed in chapter two need to be repeated in female mice with TIC injury (Koopman et al 2009; Macfarlane et al 2002a; b; Macfarlane et al 2001; Rauhala et al 2000).

Secondly, the operant behavioral tests were only conducted weeks 1, 4, and 8 post injury. This was done to prevent over-testing of the animals since many previous studies suggest only testing occasionally (Walf & Frye 2007). However, while anxiety- and depressive-like behaviors could have developed at time points earlier than week 8 post injury that our experimental design would not have detected, Yalcin (2007) reported that anxiety-like behavior starts to develop at 6 weeks post injury. Therefore, a new experimental design could utilize different time points such as 2, 6, 10 post injury to observe earlier and later time points than 8 weeks post injury to help determine which week precisely the anxiety-and depressive-like behavior starts to occur.

Thirdly, although depressive-like behavior was observed in mice with TIC injury, we did not conduct any traditional operant depressive tests such as forced swimming test, tail suspension test, and sucrose consumption test (Wang et al 2012; Zhang et al 2012a). Because we wanted to measure anxiety-like behavior along with depressive-like behavior, assays such as the forced swimming and tail suspension tests were not used because they subject the animals to an extremely life threatening, stressful environment (Borsini & Meli 1988; Sakakibara et al 2005). However, the sucrose consumption test would be an ideal operant test to use to measure depression in the mice with TIC injury as well as observe their reward-seeking behavior ((Wang et al 2012; Wyvell &
Berridge 2000). Since the mice with TIC revealed poor decision making ability in the elevated plus maze test after exposure to the acoustic startle stimulus, the sucrose test would also serve the purpose of determining the anhedonic behavior of mice with TIC injury. Taken together, future studies combined with the data collected here could provide better characterization of the cognitive impairment of the mice with the TIC injury.

Another limitation of these studies is that all of the drug tests using PPAR agonists/antagonists were one time dose administrations. In order to insure efficacy in reducing mechanical allodynia in the mice with TIC injury, higher doses were administered to the animals than preferable. The only overt side effect that was observed was hypothermia when the animals were given the 300 mg/kg dose of PIO. Since toxic doses of PIO are report to be >181 mg/kg, chronic feeding of mice at such high doses was not safe to perform. Therefore, it would be beneficial in future studies to observe chronic treatment of PIO in the mice with TIC injury at lower doses that have been previously published (Lee et al 2006; Morgenweck et al 2013; Takamura et al 1999). Morgenweck and colleagues (2013) pretreated their mice with PIO (10 mg/kg i.p. or 30 mg/kg p.o. twice daily) before the spared nerve injury and observed partial inhibition of mechanical allodynia (Morgenweck et al 2013). An additional experiment would be to modify the treatments in this study for chronic feeding. Instead of pretreating the mice with TIC with chronic low doses of PIO, treatment of PIO would start at least 8 weeks post injury. This would be a beneficial way to test PIO’s effects on mechanical allodynia in the mice with TIC after chronic
hypersensitivity and anxiety-like behaviors have already been established (see chapter 2). Thereby, this study would answer the question as to whether PIO could be translatable to the clinic to attenuate pain in patients suffering with continuous trigeminal neuropathic pain.

Furthermore, previous studies have speculated that by providing chronic treatment, PIO will act through PPARγ receptor signaling as a transcription factor thereby downregulating pro-inflammatory cytokines rather than acting through PPARγ receptor activated non-genomic pathways to reduce neurogenic inflammation (Fehrenbacher et al 2009; Feinstein et al 2005; Gardner et al 2005). Since PPARγ's transcription actions takes longer than PPARγ's rapid nongenomic actions, this leads to strong speculation that low doses will be more effective over a period of time rather acting rapidly as it did with the one-time dose given in this study.

The histology done in this study was helpful not only to show localization of PPARγ immunoreactivity in the TBSNC, but also to demonstrate that PPARγ is upregulated three weeks after injury in the TBSNC. Future studies could observe the alteration of immunoreactivity of PPARγ at different time points after TIC injury such as 6, 9, 12 weeks post. This could be performed simultaneously with a double-labeling of cytokines (IL-6, TNF-α) as well as glial markers (IB-4, OX-42). This would provide insight into the relationship that is possibly occurring between the neural immune system and the upregulation of PPARγ.

For the DCS/PIO combined treatment study, one limitation is that only one drug dose combination was used (80 mg/kg DCS + 100 mg/kg PIO). Future
studies should be conducted at different dose combinations to determine if lower doses of both drugs would elicit a similar or more effective anti-allodynic effect. In this study, the mice with TIC were only given 7 days of DCS treatment and on the 7th day received a bolus of PIO. It would be more interesting to determine if simultaneous chronic feeding of both DCS and PIO resulted in a greater increase in mechanical threshold. As stated above, a future study would be to determine an effective chronic treatment dose of PIO. Once determined, the low dose of PIO and DCS could be given chronically together. Based on the data from the study in which the one-time low dose combination of DCS/PIO attenuated mechanical allodynia, speculation would lead to the theory that chronic treatment of even lower doses will elicit an effect over time. However, questions would arise of the mechanism: 1) Are the drugs acting through their receptors (DCS→NMDAR and PIO→PPARγ), 2) Are they acting through receptor independent pathways (mitochondrial actions), or more likely 3) Are they acting through both receptor dependent and independent pathways?

This current study observed the receptor independent actions of the drug combinations and its effects on improving mitochondrial function after the TIC injury. Another limitation of this study is that only State III, State IV, and the RCR were conducted for mitochondrial dysfunction in the Seahorse assay. Alternatively, future studies could determine specifically if complex I is dysfunctional by measuring the NADH/NAD+ ratio before and after nerve injury. Since complex I is important for State III and State IV electron transport respiration, this would be a beneficial experiment to run in order to determine if
the mitochondrial dysfunction that is observed is due to alteration of the complex I redox state (Pandya et al 2013; Sauerbeck et al 2011a). Furthermore, the activity of the remaining complexes in the mETC could be observed in future studies using the Oxytherm method to determine if one or more complex is responsible for the mitochondrial dysfunction that occurs after TIC injury in the mitochondrial cortex (Mustafa et al 2010; Sullivan et al 2004b).

Since there are no changes observed in the calcium dynamics of the isolated mitochondria (cortex and brainstem), measuring total oxidative stress could also provide explanation for the mitochondrial dysfunction observed. By measuring reactive oxygen/nitrogen species using spectrophotometric assays along with the H₂O₂ production (Sullivan et al 2004b), we could not only determine if oxidative stress is occurring in the mitochondria, but what particular oxidative stress species is responsible. Taken together with the experiments listed above, a mechanism for mitochondrial dysfunction could be deduced.

Furthermore, future studies could determine more specifically how the DCS/PIO drug combination is acting on the mitochondria to reverse dysfunction. It could be from any of the mechanisms listed above and should be studied in more detailed. Future studies could analyze the cortical mitochondria from the mice with TIC treated with chronic feeding of the DCS/PIO combination to observe if there is greater reversal of mitochondrial dysfunction.
5.5. *Significance and Innovation*

Overall, these studies advanced the field of orofacial pain by providing:

1) Detail characterization of a novel model useful as a tool in identifying molecular targets that play a major role in the etiology and maintenance of trigeminal neuropathic pain.

2) First report that identifies upregulated PPARγ in the TBSNC after injury.

3) First report that PIO attenuates trigeminal neuropathic pain through activation of PPARγ receptor signaling.

4) First report that the DCS/PIO combination attenuates trigeminal pain as well as anxiety related to pain.

5) First demonstration that mitochondrial dysfunction occurs in the cortex of the mice after TIC injury and that this dysfunction can be improved by the DCS/PIO combination.

The characterization of the TIC injury model is an advancement in the field of trigeminal pain research because orofacial pain mouse models previously described do not have characteristics as closely matching those of patients with chronic facial neuropathic pain (Bornhof et al 2011; Luccarini et al 2006). Since this model has stronger translational features than the other models display, the TIC injury model can be used to study pharmaceutical agents that perhaps can help relieve facial pain and anxiety-related to pain. Finally, since this model is induced in mice, the model could be used for genetic studies of trigeminal
neuropathic pain. Developing specific gene knockout mice will be helpful in determining other molecular targets that are important in the etiology and maintenance of trigeminal neuropathic pain.

These studies will be useful when treating orofacial pain patients in the future. By determining that PPARγ is a molecular target and that PPARγ activation will attenuate trigeminal pain, the potential exists to repurpose the FDA approved diabetic therapeutic drug, PIO. It is true that caveats exist when discussing the possibility of using PIO as a chronic therapeutic option. Currently, PIO has been withdrawn from the market in Germany and France due to increased health factors including bladder cancer, edema, and cardiac issues (Kostapanos et al 2013). However, it is important to mention that clinical and animal studies performed in non-diabetic subjects that were given chronic treatments of PIO did not suffer from the same side effects (i.e. no change in LDL levels and no overt signs of edema and cardiac risk) (Aithal et al 2008; Fullert et al 2002; Szapary et al 2006). This leads to strong speculation that PIO may not, in fact, be as toxic in patients who suffer from a metabolic disorder.

Furthermore, although the doses that were used in this study were high, when comparing the mouse doses to human doses, they were in normal range prescribed for patients. Clinical dose for PIO ranges from 15 mg/kg to 45 mg/kg / day. Shaw and colleagues determined a relatable equation factoring the body weight and surface area of an average mouse and human that better estimates that the 300 mg/kg PIO dose in mice is equivalent to about 25 mg/kg PIO for a human(Reagan-Shaw et al 2008). Therefore, this highest dose given to the mice
in this study, falls within normal dosage range for humans. Whether this dose will be efficacious in treatment of the pain that is suffered by patients with chronic orofacial pain will need to be determined. Furthermore, although DCS is being tested for the treatment of sciatic pain and the anxiety-related to pain in humans (Davis et al 2006; Millecamps et al 2007; Norberg et al 2008), this data support the repurposing of DCS for the treatment of trigeminal neuropathic pain, specifically in combination with PIO.

Furthermore, new drug therapies/deliveries can be challenged. For example, a clinical trial could be conducted with the combination of both DCS/PIO could determine an effective dose combination that would reduce the side effects of PIO while potentiating the inhibitory effect on neuropathic pain and the anxiety-related to pain. Many patients will often receive high doses of medication, or sometimes two pills, an analgesic and an anti-depressant drug (De Leeuw 2008; Zakrzewska 2013). However, this study determines that combination of the two, as a double edged sword, act as both an analgesic and anti-depressant (see chapter 4).

The two drugs together could propel the chronic pain field forward for many reasons. One being it challenges the way the clinicians, basic scientists, and industry drug developers think about old drugs. If there is a way to save money by utilizing available drugs, that not only attenuate pain, but together have a potentiated effect, then the money invested in drug development could be better spent and discovery shifted into drug repurposing. This will save time and money in the long run while treating patients more quickly. Furthermore, the present
study supports dual therapy treatment. Recent studies have observed the synergistic effects of PIO with pravastatin to decrease inflammation (Wei et al 2007) as well as the synergistic effects of DCS and valproic acid for the extinction of fear (Kuriyama et al 2011).

Finally, this was the first study to determine that mitochondrial dysfunction is a key play in trigeminal neuropathic pain, highlighting the mitochondria also is a drug target for treating facial pain. This study also determined ex vivo that the combination of DCS/PIO is acting independently of their known receptors to improve mitochondrial dysfunction. Studies have linked mitochondrial dysfunction to neuronal injury and neuropathy (Bouillot et al 2002; Ferrari & Levine 2010; Joseph et al 2004; Joseph & Levine 2006; Kim et al 2004; Pandya et al 2007; Park et al 2006; Schwartz et al 2009; Shin et al 2003; Sui et al 2013; Sullivan et al 2007), and others to depression (Gardner & Boles 2008; Kato 2011; Kato & Kato 2000; Rezin et al 2009). This was the first study to determine that a drug combination targeting mitochondria can in fact attenuate both of these aspects of pain in vivo. Perhaps these studies will not only encourage a more in depth study of mitochondria and its link to trigeminal neuropathic pain, but also that the drug combination itself can be utilized in so many different diseases related to mitochondrial dysfunction. This suggests other drugs that target the improvement of mitochondrial dysfunction could be utilized. Future studies need to be conducted to confirm the specific pathway(s) that DCS/PIO are utilizing to elicit these effects.
5.6. Conclusion

This study was successful in characterizing a novel orofacial neuropathic pain mouse model, TIC, and determining the usefulness of the similarities it has with the clinical syndrome of continuous trigeminal neuropathic pain. This study supports the repurposing of PIO and DCS as useful pharmaceuticals in treating trigeminal neuropathic pain, particularly in a low dose combination by acting to improve mitochondrial dysfunction. Overall, this study accentuates the need for new pharmaceutical treatments for patients suffering with trigeminal pain and provides two novel molecular targets (PPARγ and mitochondria) as options to attenuate continuous trigeminal neuropathic pain.
### Appendix 1. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAALAC</td>
<td>Association for Assessment and Accreditation of Laboratory Animal Care International</td>
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<tr>
<td>AAPM</td>
<td>the American Academy of Pain Medicine</td>
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<tr>
<td>ACC</td>
<td>Cingulate cortex</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATPase</td>
<td>Adenylypyrophosphatase</td>
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<tr>
<td>BOLD</td>
<td>Blood-oxygen-level dependent</td>
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<td>CCI-ION</td>
<td>Chronic constriction injury of the infraortibal nerve</td>
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<td>CBT</td>
<td>Cognitive behavioral therapy</td>
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<td>2,4-DNP</td>
<td>2,4-Dinitrophenol</td>
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<tr>
<td>DCS</td>
<td>D-cycloserine</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EDP</td>
<td>an energy-dissipating pathway</td>
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<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FCCP</td>
<td>Trifluorocarbonylcyanide Phenylhydrazone</td>
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<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>IASP</td>
<td>the International Association for the Study of Pain</td>
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<tr>
<td>ION</td>
<td>The infraortibal nerve</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>IL-6</td>
<td>Interleukin 6</td>
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<td>mETC</td>
<td>the mitochondrial electron transport chain</td>
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<td>MIB</td>
<td>Mitochondrial isolation buffer</td>
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<td>MRB</td>
<td>Mitochondrial running buffer</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>NMDA</td>
<td>N-Methyl-D-aspartate</td>
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<td>NIH</td>
<td>National Institute of Health</td>
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<tr>
<td>OCR</td>
<td>Oxygen Consumption Ratio</td>
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<td>PAG</td>
<td>Periaqueductal gray</td>
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<tr>
<td>pCCI-ION</td>
<td>Partial chronic constriction injury of the infraortibal nerve</td>
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<td>PFC</td>
<td>Prefrontal cortex</td>
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<td>PIO</td>
<td>Pioglitazone</td>
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<td>P.O.</td>
<td>per os</td>
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<td>PMF</td>
<td>Proton motive force</td>
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<td>PPAR-α</td>
<td>Peroxisome proliferator-activated receptor alpha</td>
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<td>PPAR-β/δ</td>
<td>Peroxisome proliferator-activated receptor beta/delta</td>
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<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
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<tr>
<td>PPRE</td>
<td>Peroxisome proliferator-activated receptor response elements</td>
</tr>
<tr>
<td>PPC</td>
<td>posterior parietal cortex</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RCR</td>
<td>the Respiratory Control Ratio</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>S1/S2</td>
<td>Somatosensory cortex 1 and 2</td>
</tr>
<tr>
<td>SMA</td>
<td>Insula cortex, supplementary motor area</td>
</tr>
<tr>
<td>S.C.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>TBSNC</td>
<td>Trigeminal brainstem sensory nuclear complex</td>
</tr>
<tr>
<td>TIC</td>
<td>Trigeminal Inflammatory Compression</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>USDA</td>
<td>the United States Department of Agriculture</td>
</tr>
<tr>
<td>VPM</td>
<td>Ventroposteromedial nucleus of the thalamus</td>
</tr>
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PUBLICATIONS

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Journal Papers Submitted
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Journal Papers In Progress
Lyons, DN., Zhang, L., Ma, Fei, Westlund, KN. Working Title: Rapid Effects of PPAR-agonists on Alleviation of Orofacial Pain.

Lyons, D, Kniffin, TK, Zhang L, Ma, F, Danaher, R., Miller, C., Carlson, C., Westlund KN. Working Title: Low-dose Combination of Pioglitazone and D-cycloserine Attenuated Orofacial Pain by Acting as a Mild Mitochondrial Uncoupler.

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