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Evaluation of Anti-Migraine Drugs in an in Vivo Rat Model of Chronic Migraine

Shannon Nicole Stiles

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**EVALUATION OF ANTI-MIGRAINE DRUGS IN AN IN VIVO RAT MODEL
OF CHRONIC MIGRAINE**

A Masters Thesis

Presented to

The Graduate College of
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Shannon Nicole Stiles

July 2015

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EVALUATION OF ANTI-MIGRAINE DRUGS IN AN IN VIVO RAT MODEL OF CHRONIC MIGRAINE

Biology

Missouri State University, July 2015

Master of Science

Shannon Nicole Stiles

ABSTRACT

Approximately 2% of the US population is affected by chronic migraine (CM). CM is a recurring, neurological disorder characterized by painful headache, along with autonomic, gastrointestinal, and other somatic symptoms. Prolonged sensitization of the trigeminal system, which is implicated in CM, is comprised of peripheral primary trigeminal ganglion neurons that provide sensory innervation of the head and face and second order neurons in the spinal trigeminal nucleus (STN). The goal of my study was to evaluate anti-migraine drugs for their ability to inhibit ongoing peripheral and central sensitization of trigeminal nociceptors. An in vivo animal model was used to determine if two novel drugs in development and Topiramate, a common drug used to treat frequent migraine, could inhibit nocifensive responses to mechanical stimulation of trigeminal neurons. In addition, the trigeminal ganglion and STN were evaluated on a molecular level to determine if the drugs could inhibit expression of a signaling protein implicated in peripheral and central sensitization. My results provide evidence that the two novel drugs significantly inhibited nocifensive responses and decreased expression of Protein Kinase A (PKA) in the trigeminal ganglion and STN. In contrast, Topiramate did not decrease PKA expression and had no effect on nocifensive responses. Based on my findings, I conclude that these novel drugs may be beneficial in the treatment of CM.

KEYWORDS: kinase, migraine, nociception, sensitization, trigeminal

This abstract is approved as to form and content

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

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Approved:

Paul Durham, Ph.D.

Kyoungtae Kim, Ph.D.

Laszlo Kovacs, Ph.D.

Julie Masterson, Ph.D.: Dean Graduate College

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INTRODUCTION

Chronic Migraine

Worldwide, primary headache disorders are the most common neurological condition for which medical consultation is sought (Stovner et al., 2007). In the United States alone, approximately 12% of the adult population suffers from migraine with and without aura (Stewart et al., 2003, Lipton et al., 2007). The most common symptoms of a migraine attack include a throbbing headache that can last up to 72 hours, increased sensitivity to light (photophobia) and noise (phonophobia), nausea (feeling sick), vomiting (being sick) and lethargy (lack of energy). Some migraineurs experience neurological symptoms that are referred to as migraine aura in which they “see” dark or colored spots, sparkles or ‘stars’, or zigzag lines prior to headache onset. In addition, migraine attacks can be preceded by a feeling of numbness or tingling, weakness, and dizziness or vertigo, disturbances in speech and hearing, and even reported memory changes such as feelings of fear and confusion. Migraine attacks can be further subdivided into two major categories: episodic and chronic (Manack et al., 2011). Although chronic migraine was once thought of as an extension of episodic migraine, recent studies have shown that this is not the case. Acute pain, which is a result of episodic migraines, has been described as transient, self-limiting, and serves a protective biological function. In contrast, chronic pain can cause long-term maladaptive changes in neuroplastic tissues, is not believed to serve a protective biological function, and can become detrimental to one’s overall health by promoting other illnesses (Schulman et al., 2008).

Chronic migraine ranks among the eight most burdensome conditions according to the Global Burden of Disease of the World Health Organization (WHO) (Vos et al., 2012). Due to the debilitating nature of migraine, the WHO has considered a day with chronic migraine comparable to a day with quadriplegia or severe psychosis (Menken et al., 2000). Interestingly, chronic migraine has only recently been classified as its own disorder. The International Headache Society (IHS) included the diagnosis of chronic migraine for the first time in 2004, and appended the definition in 2006 to define chronic migraine as 15 or more “headache days” a month for 3 or more consecutive months, with 8 or more of those headache days fulfilling the IHS criteria for migraine (Headache Classification Subcommittee of the International Headache, 2004, Olesen, 2006). Out of the 12% of the US population that is affected by migraine, 1-2% suffer from chronic migraine (Schramm et al., 2013). This means that approximately 5 million people in the US alone will suffer from chronic migraine in a given year (Schwedt, 2014). Based on data from the American Migraine Prevalence and Prevention Study, the prevalence of chronic migraine was highest among females, in mid-life, and in households with the lowest annual income (Buse et al., 2012, Buse et al., 2013). Thus, finding new and more effective ways to treat this debilitating disorder should be a top priority for the pharmaceutical and medical communities.

Chronic Migraine Pathology

A pathophysiological feature of migraine is prolonged sensitization, a lowering of the activation threshold, of neurons within the central nervous system (CNS) and peripheral nervous system that are responsible for transmission of nociceptive (painful)

stimuli (Bernstein and Burstein, 2012). Peripheral sensitization, which is characterized by an increase in neuronal excitability and a lowering of the threshold for activation, plays an important role in the initiation of a migraine attack. Based on current models, inflammatory mediators are released from trigeminal nerve fibers that provide sensory innervation of the meningeal blood vessels that promote neurogenic inflammation in response to real or perceived ischemic events within the brain (Noseda and Burstein, 2013, Burstein et al., 2015). Neurogenic inflammation is characterized by increased blood flow, increased plasma protein extravasation, and recruitment and activation of immune cells. The release of neuropeptides such as calcitonin gene-related peptide (CGRP) and other pro-inflammatory molecules including nitric oxide and cytokines are responsible for inducing changes in the expression and activity of ion channels and receptors on trigeminal neurons that facilitate their sensitization and activation. With respect to episodic migraine, peripheral sensitization, which is characterized by the throbbing sensation, promotes development of central sensitization via the release of CGRP and other inflammatory mediators within the upper spinal cord. Central sensitization, which is characterized by enhanced excitability of second-order neurons, is responsible for the pain associated with a migraine. More specifically, central sensitization is associated with hyperalgesia (an increased pain response) and also allodynia (a nocifensive response to non-painful stimuli) (Dodick and Silberstein, 2006, Jakubowski et al., 2007). In episodic migraine attacks, central sensitization typically resolves within 4-72 hours if the migraine is not treated with pharmaceuticals. However, in chronic migraine central sensitization can lower the activation threshold of peripheral trigeminal neurons, thus promoting peripheral sensitization of trigeminal neurons and a

maladaptive filtering of a variety of sensory stimuli including strong odors, foods, weather patterns, light, sounds, and even exercise. Interestingly, these types of stimuli are regarded as risk factors or triggers of migraine attacks (Lipton and Bigal, 2005, Friedman and De ver Dye, 2009).

Role of the Trigeminal System in Migraine Pathology

Both peripheral and central sensitization involve the activation of glial cells that are found in close proximity with the cell bodies of primary peripheral and secondary central trigeminal neurons (Ren and Dubner, 1999, Davies et al., 2010). Based on results from our studies as well as other studies, increased neuron-glia interactions within the trigeminal ganglion and medullary horn of the upper spinal cord are thought to contribute to the initiation and maintenance of a sensitized or hyperexcitable state of nociceptive neurons (Xie, 2008, Damodaram et al., 2009, Cady et al., 2010, Villa et al., 2010, Cady et al., 2011, Cady et al., 2013, Cady et al., 2014). Sensitization and activation of the trigeminal nerve, which results in the release of neuropeptides both in peripheral tissues and the CNS that promote inflammatory and nociceptive responses, plays an important role in the underlying pathology of migraine. The trigeminal nerve, also known as the fifth cranial nerve, is the largest cranial nerve in the human body and is responsible for both sensory and motor innervation of the head and face (Shankland, 2000). The trigeminal nerve consists of three distinct branches: the ophthalmic branch, the maxillary branch, and the mandibular branch (Fig. 1). While sensitization and activation of the ophthalmic branch is strongly implicated in migraine pathology, there is less evidence for a direct role of the maxillary and mandibular branches (Bartsch and Goadsby, 2003).

The first division of the trigeminal nerve is the ophthalmic branch (V1). This afferent sensory nerve is responsible for relaying information from the scalp, forehead, upper eyelid, eyebrow, eye, portions of the nasal cavity, the tentorium cerebelli, posterior flax cerebri, and dura mater (Shankland, 2001a). The dura mater is the protective covering of the brain that contains the blood vessels innervated by the V1 branch and thought to be activated during a migraine attack (Bolay et al., 2002). The maxillary branch (V2), which is the second sensory division of the trigeminal nerve, innervates the lower eyelid, midfacial region, nose, maxillary sinus, mucous membrane of the nasopharynx, soft palate, roof of the mouth, maxillary teeth, and palatine tonsil (Shankland, 2001b). The third branch of the trigeminal nerve, the maxillary branch (V3), consists of both afferent sensory neurons and efferent motor neurons. The afferent sensory neurons carry information from the skin of the lower one-third of the face, the ear, lower lip, and temporal region along with the teeth, gingivae of the mandible, and the mucus membrane of the anterior two-thirds of the floor of the mouth and tongue. The efferent motor neurons innervate the anterior belly of the digastric, mylohyoid, tensor tympani, tensor veli palatine, and the muscle of mastication (Shankland, 2001c). Although the V2 and V3 branches are not directly implicated in migraine attacks, activation of these sensory nerves, which occurs in allergic rhinitis and temporomandibular joint disorder, respectively, can promote sensitization of the V1 branch and thus contribute to migraine pathology.

The cell bodies of the pseudounipolar sensory neurons associated with the trigeminal nerve are located within the trigeminal ganglion, which lies in a depression of the temporal bone, known as Meckle's cave, in the floor of the middle cranial fossa

(Shankland, 2000). The sensory trigeminal neurons have peripheral and central processes; the peripheral processes extend to the head and face whereas the central processes synapse with second order neurons in the spinal trigeminal nucleus (STN) (Fig. 1). These nerve fibers can be either A δ fibers, which are myelinated, or unmyelinated C fibers (Lazarov, 2007). Due to their myelination, A δ fibers transmit pain and temperature signals more quickly than C fibers (Giniatullin et al., 2008). In addition to the neurons, there are two types of glial cells that are found in the trigeminal ganglion: Schwann cells and satellite glial cells. Schwann cells are responsible for the myelination of A δ fibers, therefore increasing the speed at which the fibers conduct information, while satellite glial cells surround the individual cell bodies and play an important role in the modulation of the excitability state of the neuron (Thalakoti et al., 2007, Durham and Garrett, 2010).

Afferent fibers from the trigeminal ganglion enter the central nervous system at the level of the pons, where they synapse with neurons in the trigeminal nuclei (Shankland, 2000, 2001c, b, a). The nuclei of the trigeminal nerve extend from the cervical spinal cord to the midbrain and consist of three sensory nuclei and one motor nucleus. Fibers from the trigeminal ganglion project to two of the sensory nuclei, whereas the third sensory nuclei contain fibers that do not pass through the trigeminal ganglion.

The mesencephalic nucleus, which is the most superior nucleus, receives proprioceptive fibers of mastication and mechanoreceptors from the hard palate and temporomandibular joint (Lazarov, 2002). The largest nucleus, which is the principle nucleus, receives projections of mostly myelinated fibers from the trigeminal ganglion. The most inferior nucleus, which is the STN, receives both lightly myelinated and

unmyelinated fibers from V1, V2, and V3 of the trigeminal nerve. Within the STN, there are three subnuclei: the subnucleus oralis, the subnucleus interpolaris, and the subnucleus caudalis, the site implicated in migraine pathology (Nosedá and Burstein, 2013). This region of the medullary horn receives input from the trigeminal nerves that provide sensory innervation of the cerebral and meningeal blood vessels, and thus is a common pathway for migraine pain signaling. Based on work by Burstein and colleagues, V1 primary nociceptive neurons terminate primarily in outer laminae I and II of the medullary horn where they synapse with second order neurons that send projections to the thalamus (Fig. 1). Fiber projections from the thalamus to the cortex complete the ascending pain pathway referred to as the spinothalamic tract that is responsible for the perception of nociceptive stimuli as painful (Burstein et al., 2015).

Proteins Implicated in the Development of Peripheral and Central Sensitization

Changes in the expression and activity level of ion channels and receptors that function to control the level of neuronal excitability is mediated by a number of signaling proteins expressed in neurons and associated glial cells (Sun et al., 2004, Cheng and Ji, 2008, Ji et al., 2009, Cady et al., 2011). Sustained upregulation of key signaling proteins is implicated in the development of a primed state of the nociceptive neurons, which is a pathophysiological state characteristic of chronic pain (Hucho and Levine, 2007). The following proteins are associated with a hyperexcitable state of nociceptive neurons: protein kinase A (PKA), glial fibrillary associated protein (GFAP), and ionized calcium-binding adapter molecule Iba1 (Ji et al., 2013). Increased expression of PKA has been reported in trigeminal ganglia and spinal cord in response to peripheral inflammation of

tissues of the head and face (Levy and Strassman, 2002, Cady et al., 2011). PKA is activated by a number of inflammatory agents that mediate increased intracellular levels of the secondary messenger cAMP (Aley and Levine, 1999, Kim et al., 2006). Following binding of cAMP to PKA, the enzyme can phosphorylate proteins involved in intracellular signaling pathways, including receptors, ion channels, and transcription factors to modulate their activity within the cell. Typically in neurons and glial cells, elevated levels of PKA result in an increase in the synthesis and release of pro-inflammatory molecules such as cytokines and nitric oxide as well as changes in ion channels and receptors to promote a more hyperexcitable state of trigeminal neurons (Hanada and Yoshimura, 2002, Gosselin et al., 2010).

In response to trigeminal activation by thermal, mechanical, or chemical stimuli, trigeminal neurons release inflammatory molecules that can then cause activation of the associated glial cells both in the ganglion and STN (Ren and Dubner, 2008, Ren, 2009, Durham and Garrett, 2010). Furthermore, increased neuron-glia communication via gap junctions and paracrine signaling is thought to play an important role in the initiation and maintenance of peripheral and central sensitization. Within the CNS, activation of astrocytes and microglia is a well-documented cellular phenomenon that directly correlates with the level of neuronal excitability and pain. The intermediate filament protein GFAP is often used as a marker of activated astrocytes while the calcium binding adapter protein Iba1 is utilized as a marker of activated microglia. Changes in GFAP expression coincide with structural changes in astrocytes associated with migration and synthesis and release of cytokines and chemokines (Guo et al., 2007). Elevated levels of Iba1 in microglia are associated with increased proliferation, migration, and an increase

in the production and secretion of pro-inflammatory cytokines and chemokines (Ohsawa et al., 2004, Villa et al., 2010). Thus similar to astrocytes, microglial activation serves a protective role within the CNS to facilitate cellular events to repair damaged tissues and restore tissue homeostasis (Ikeda et al., 2012).

Treatment of Migraine

The treatment of migraine is generally divided into acute and preventative (Silberstein, 2006, Cady et al., 2012a). While acute treatments are used intermittently to reverse a migraine attack that has already begun, preventative therapeutics are typically taken on a daily basis to quiet the nervous system so as to minimize or block the development of future migraines. Despite a wide array of treatments being available, migraine continues to be a leading cause of disability because of being misdiagnosed, underdiagnosed, and inadequately treated (Lipton et al., 2011). The primary goals of migraine pharmacology are to reduce the frequency and severity of attacks and thus significantly decrease the amount of physical, social, and psychological disability. Many of the drugs currently used to treat episodic or chronic migraine were originally developed to reduce the frequency, duration, and severity of seizures associated with epilepsy (Mathew, 2001). One such drug is Topiramate, which was initially investigated for its ability to block epileptic seizures and only later was approved as a prophylactic treatment of frequent migraine. Results from open-label and small controlled studies provided evidence that Topiramate, a structurally distinct antiepileptic drug, could be efficacious in migraine prevention (Diener et al., 2004, Diener et al., 2007). More recently, data from 2 multicenter, randomized, double-blind, placebo-controlled trials

established the efficacy of Topiramate to significantly reduce the frequency and severity of migraine attacks (Silberstein, 2005). In addition, Topiramate exhibited high responder rates, good tolerability, and a lack of major contraindications. Topiramate is arguably the best studied and most efficacious migraine preventive for patients with frequent episodic migraine. Although the exact cellular mechanisms of action are not known, Topiramate is thought to function by reducing neuronal hyperexcitability and release of inflammatory molecules through several possible mechanisms: a state-dependent blocking of sodium channels, augmentation of GABA activity, antagonism of AMPA/kainate glutamate receptors, or inhibition of carbonic anhydrase. However, even though Topiramate is generally well-tolerated in most patients, there have been reports of severe adverse events and relatively high drop-out rates in both clinical trials and clinical practice.

Another class of drugs that are used in the treatment of migraine is the opiates even though they are not recommended as first-line therapy because of the high risk of developing medication overuse headache (Saper and Da Silva, 2013). Their use is appropriate in patients that cannot tolerate, do not respond to, or have contraindications to standard migraine abortive drugs since they exert a strong analgesic response (Loder et al., 2013). Opioids are most commonly utilized in patients that are seen in the emergency department of hospitals since often headache specialists will not be on staff. In general, guidelines from the American Academy of Neurology and American Headache Society advocate for the limited use of opioids by clinicians in the treatment of migraine because they are not as effective as other migraine approved drugs and treat the symptoms rather than the migraine process. For example, results from a large double-blind placebo-controlled study compared sumatriptan/naproxen, butalbital/ acetaminophen/ caffeine/

codeine, and placebo in a population of severely impacted migraine subjects who in the past had used a butalbital-containing product (opioid) (Derosier et al., 2012). Compared to the butalbital combination and placebo groups, the subjects who took sumatriptan/naproxen reported a greater incidence of being pain-free or achieving pain relief. Despite our advances in the treatment of migraine, there still remains a clear need for safer and more effective drugs for managing acute and chronic migraine.

Goals of My Study

The primary goal of my study was to determine the effect of two novel anti-migraine drugs, designated as Drug A and Drug B, and compare their mechanism of action to Topiramate and the opioid Buprenorphine in an animal model that mimics trigeminal system pathology associated with frequent and chronic migraine. I wanted to test the hypothesis that these novel drugs would be able to inhibit ongoing peripheral and central sensitization of trigeminal nociceptors, and hence pain transmission. To accomplish my goal, I utilized behavioral and cellular techniques to investigate the effects of each of these drugs in a well-established model of chronic trigeminal nerve activation. Briefly, the pro-inflammatory agent, complete Freund's adjuvant (CFA), was injected in the TMJ capsule to cause sustained sensitization of trigeminal neurons and the ability of each drug to inhibit ongoing sensitization investigated.

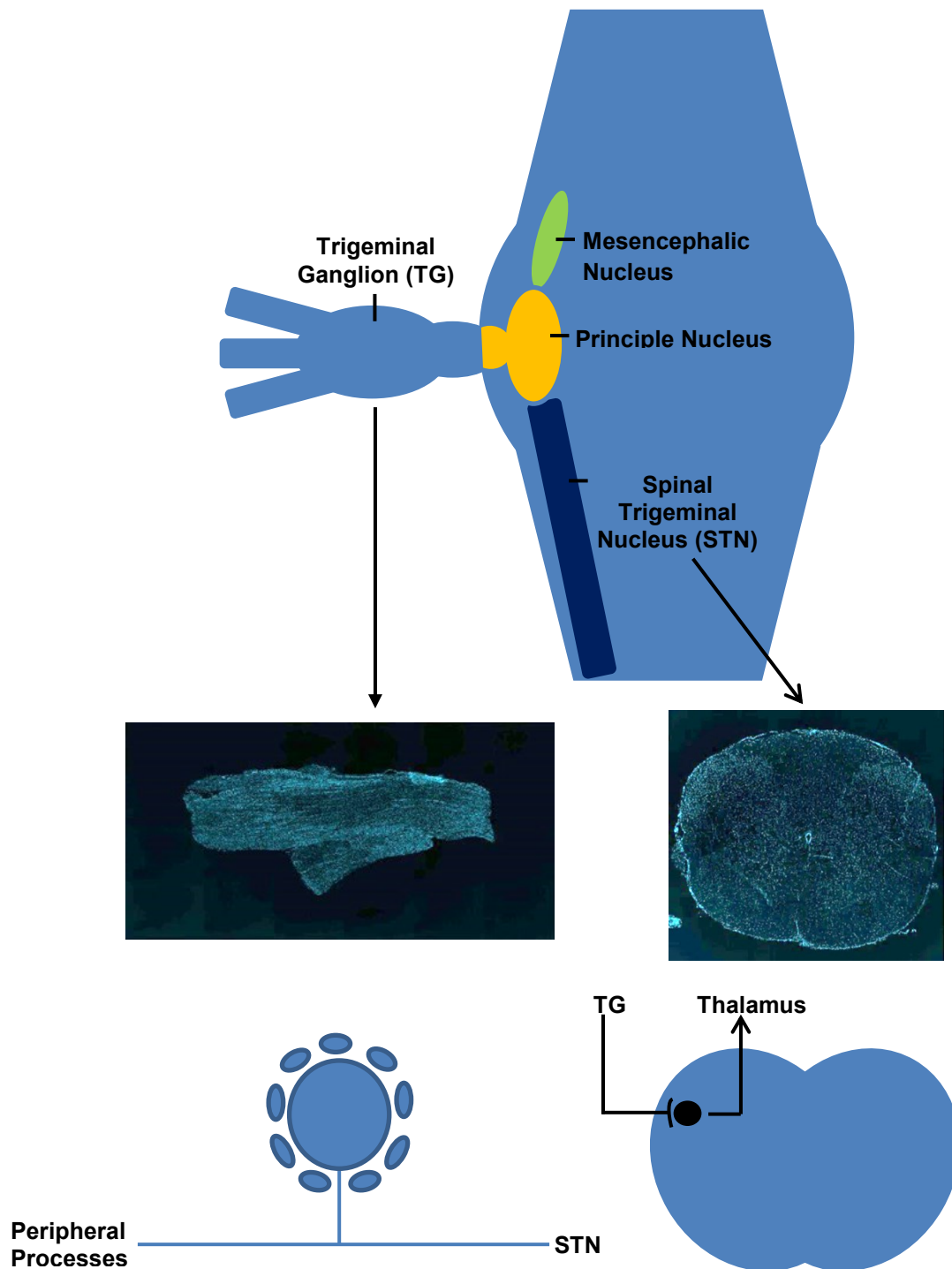


Figure 1. Schematic representation of trigeminal nociceptive pathway from peripheral tissue through the ganglion and then to the second order neurons and finally the thalamus. See text for details.

METHODS

Animals and Drugs

All of the animal procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Missouri State University (Protocol number 14-021.0-A approved on 03/13/2014). Every effort was made to minimize animal suffering and to use the minimum number of animals required for this study. Adult male Sprague-Dawley rats (350-500 g; Charles River Laboratories Inc., Wilmington, MA) were individually housed in clean plastic cages on a 12-hour light/dark cycle with unrestricted access to food and water. Upon arrival in our facility, all animals were housed for up to 7 days before being released for my studies.

The novel drugs, designated simply as Drug A and Drug B, used in my study were kindly provided by the pharmaceutical company Upsher-Smith Laboratories (Maple Grove, MN). Drug A was diluted to a concentration of 10 mg/mL in dimethyl sulfoxide (DMSO) while Drug B was diluted to a concentration of 30 mg/mL in 20% TPGE (vitamin E). Topiramate, which was purchased by Upsher-Smith and provided to our laboratory, was prepared at a 50 mg/mL concentration in DMSO. Buprenorphine hydrochloride (0.3 mg/mL) was obtained from Patterson Veterinary (Devens, MA). All drugs were made up as stock solutions in appropriate vehicle solvent and stored at -20 °C for future use. Complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO, USA) was prepared just prior to administration as a 1:1 CFA/0.9% sodium chloride (saline) emulsion.

Behavioral Testing in Response to Prolonged Trigeminal Activation

The measurement of changes in nocifensive response to mechanical stimulation in the orofacial region of the rat was performed as described in recent publications from the Durham Lab (Garrett et al., 2012, Cady et al., 2014, Hawkins et al., 2015). For three consecutive days prior to testing, the rats were acclimated to the Durham Holder (Ugo Basile, Milan, Italy) (Fig. 2). Each rat was guided into the Durham Holder and kept there for 5 minutes to familiarize the rat with the device. While in the device, a pipette tip was used to stimulate the area over each TMJ region (30 sec on each side for the duration of the 5 min) to acclimate the rat to having the TMJ region touched with a filament. If a rat was consistently uncooperative during this acclimation period, the animal was removed from the study.

Prior to injection, basal mechanical withdrawal responses were measured in the Durham Holder using different force von Frey filaments (60, 100, 180 grams). The filaments were applied five times on the left and right side of the animal to stimulate the trigeminal neurons that innervate the TMJ region. Both the researcher who recorded the response and the researcher who physically stimulated the animal were blinded to the experimental conditions being tested. A positive response was indicated by head withdrawal prior to the bending of the filament. If the animal had less than three withdrawal responses to the 60 g filament, the 100 g and then 180 g filaments were used until the animal had three or more withdrawal responses to a given filament (Fig. 3).

After baseline threshold nocifensive responses were obtained, the animals were anesthetized by inhalation of 5% isoflurane. As shown by previous studies in our laboratory, bilateral injection of CFA promotes a sustained (chronic) activation of the

trigeminal system (Garrett and Durham, 2009, Cady and Durham, 2010, Cady et al., 2014). Based on prior results using this model, anaesthetized animals were given bilateral injections of either 50 μ L CFA (1:1 CFA/saline) or saline as a vehicle control in the TMJ capsule. Animals received Drug A (10 mg/kg; intraperitoneal or IP) or Drug B (30 mg/kg; per os or PO gavage) on days 3 and 4 post CFA injection, or Topiramate (50 mg/kg, IP) daily up through day 4 post CFA injection, or equivalent volume of vehicle (Fig. 4). DMSO was used as the vehicle since it was used as the drug solvent. Dosage and mode of administration for Topiramate was based on a previously published study (Wieczorkiewicz-Plaza et al., 2004) on the effect of Topiramate on mechanical allodynia in rats. Other male Sprague-Dawley rats were left untreated to serve as naïve controls. The rationale for administration of Drug A and Drug B on days 3 and 4 post CFA injection is based on the knowledge that nocifensive behaviors peak around day 5 post-injection. Topiramate was administered daily since it is approved as a prophylactic drug for frequent migraine. The opioid drug, Buprenorphine hydrochloride (0.05 mg/kg), was injected subcutaneously in the hindquarter two hours prior to mechanical testing. In a previous study, our laboratory used Buprenorphine as a control drug since it is known to block peripheral pain transmission (Garrett et al., 2012). Using the same method that was used for the baseline responses, the number of nocifensive responses were recorded 2 hours and 3, 5, 10, and 14 days post CFA injection for each experimental condition. The data were reported as the average number of responses obtained from a total of 10 applications of each specific calibrated filament (5 applications on each side of the face).

Cellular Evaluation

Using the same injection method as stated earlier, 50 μ L of CFA or saline alone (control) was injected into both TMJ capsules to cause prolonged activation of trigeminal sensory neurons. Some animals received Drug A (10 mg/kg, IP) or Drug B (30 mg/kg, PO) on days 3 and 4 post CFA injection, or Topiramate (50 mg/kg, IP) daily up through day 4 post CFA injection, or equivalent volume of vehicle to determine if the drugs are capable of blocking the sustained stimulatory effect of CFA on trigeminal neurons that promote prolonged peripheral and central sensitization (Fig. 5).

Five days post CFA injection, animals were sacrificed by CO₂ asphyxiation and decapitation. Both trigeminal ganglion as well as the upper spinal cord tissue (4-5 mm posterior to the obex) containing the STN were removed and immediately placed in 4% paraformaldehyde at 4°C overnight. Following paraformaldehyde fixation, tissues were sucrose fixed in 12.5% sucrose at 4°C for one hour, then placed in a 25% sucrose solution at 4°C overnight. Tissues were removed from the sucrose solution the following day and stored at -20°C for further immunohistochemical analysis.

Prior to cryosectioning, the ganglia were mounted in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA) such that the ventral surface was in contact with the upper surface of a Superfrost Plus slide (Fisher Scientific, Pittsburgh, PA), quickly frozen, and stored at -20°C. Fourteen micron longitudinal sections of the entire trigeminal ganglion tissue were serially prepared using a cryostat (Microm HM 525, Thermo Scientific, Waltham, MA) set at -24°C. A total of 20 slides were prepared to ensure that sections from the middle of the ganglion, which contain cell bodies from all three branches (V1-V3), were obtained. The slides that contained sections

from the middle of the ganglion were chosen for immunohistochemistry. For the spinal cord samples, tissues were positioned with the caudal side in contact with the slide, covered with OCT, quickly frozen, and stored at -20°C. Spinal cord sections (14 µm) containing the STN were sectioned transversely at a distance of 4-5 mm posterior to the obex using a cryostat set at -24°C. As with the trigeminal ganglion, multiple slides were prepared to ensure that the sections used for immunohistochemistry were from the correct area of the tissue (STN; 71 sections \approx 1 mm). In addition, each slide contained at least one section for each experimental condition: naïve, vehicle, CFA, and CFA + Drug A, CFA + Drug B, or CFA + Topiramate.

Slides containing sections of ganglia or spinal cord, which had been stored at -20°C, were hydrated with 1x Phosphate Buffered Saline Solution (PBS; Sigma-Aldrich) for 5 minutes and then incubated in a solution of 0.1% Triton X-100 and 5% donkey serum (Jackson Immuno Research Laboratories, West Grove, PA) for 20 minutes. Next, slides were rinsed 5 times with PBS and then incubated with primary antibodies diluted in 5% donkey serum for 3 h at room temperature and then with secondary antibodies diluted in PBS for 1 h at room temperature. A complete list of the antibodies used in my study is shown in Table 1. As a control, some slides were incubated with only secondary antibodies. None of the secondary antibodies used in my study exhibited staining levels above those observed when primary antibodies were omitted. Sections were mounted using Vectashield medium (H-1200) containing 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to co-stain cell nuclei. Images (20x) were collected using a Zeiss Axiocam mRm camera mounted on a Zeiss Imager Z2 fluorescent

microscope equipped with an ApoTome. Image acquisition was performed using Zeiss Zen Blue 2011 (Thornwood, NY).

Statistical Analysis

For the behavioral studies, the data are reported as the average number of withdrawal responses \pm SEM to the von Frey filament force at each time point. The number of animals used for each experimental condition are provided in Tables 2 and 3. Statistical analysis was performed using a Factorial with Independent t-test with Bonferroni correction between groups in SPSS 20 software. Differences are considered to be significant at $p < 0.05$.

The relative staining intensity measurements in trigeminal ganglia and spinal cord tissues were based on previously published protocols from the Durham Lab (Cady et al., 2010, Cady et al., 2011, Cady et al., 2014, Hawkins et al., 2015). Zen software (Carl Zeiss) was utilized to evenly balance the background of each image prior to analysis. For trigeminal ganglia, four randomly chosen 20x images containing a similar number of neuronal and glial cells in the V1/V2 region, as identified by the nuclear dye DAPI, were opened in ImageJ software (NIH) and analyzed for each experimental condition that were repeated in at least four independent experiments. This resulted in a minimum of 16 fluorescent images being analyzed for the intensity measurements. The fluorescent staining intensity in spinal cord tissue was determined by measuring the mean gray intensity from ten regions, in laminae I-III, of staining in the medullary horn and subtracting the background intensity from acellular regions of the medullary horn in a minimum of four independent experiments. This resulted in a minimum of 40 fluorescent

images being analyzed for the intensity measurements in the spinal cord tissues for each experimental condition. As with the trigeminal ganglia, ImageJ software (NIH) was used for this analysis.

The fold-change in staining intensity is defined as the mean change in relative intensity in the experimental condition when compared to the mean of the unstimulated control (naïve) tissue whose average value was made equal to one. Changes in immunostaining intensity were evaluated using a One-Way ANOVA with Tukey post-hoc tests between groups. Results were reported as mean \pm SEM and were considered significant when $p < 0.05$. All statistical tests were performed using SPSS (Version 20, IBM, Chicago, IL).

Table 1. Summary of Antibodies Used for Immunohistochemistry.

Antibody	Host	Supplier	Dilution	Incubation
PKA	Rabbit	Abcam	1:500	3 h
Iba-1	Goat	Abcam	1:400	3 h
GFAP	Goat	Abcam	1:5000	3 h
NeuN	Mouse	Millipore	1:10,000	3 h
Alexa 488	Rabbit	Invitrogen	1:200	1 h
Alexa 568	Goat	Invitrogen	1:200	1 h
Alexa 647	Mouse	Invitrogen	1:200	1 h

Table 2. Summary of Treatments and Number of Animals Used for Behavioral Studies.

Group Number	Treatment	Number Used
1	Naïve Control	10
2	CFA TMJ + Saline IP	10
3	CFA TMJ + Saline PO	10
4	CFA TMJ + Saline SC	10
5	CFA TMJ+ Drug A IP	10
6	CFA TMJ + Drug B PO	10
7	CFA TMJ + Topiramate IP	10
8	CFA TMJ + Buprenorphine SC	10

Table 3. Summary of Treatments and Animals Used for Immunohistological Studies.

Group Number	Treatment	Number Used
1	Naïve Control	4
2	Saline TMJ+ Saline IP	4
3	Saline TMJ+ Saline PO	4
4	Saline TMJ+ PRX201145 IP	4
5	Saline TMJ+ PRX201260 PO	4
6	Saline TMJ + Topiramate IP	4
7	CFA TMJ + Saline IP	4
8	CFA TMJ + Saline PO	4
9	CFA TMJ+ PRX201145 IP	4
10	CFA TMJ + PRX201260 PO	4
11	CFA TMJ + Topiramate IP	4



Figure 2. A rat being acclimated to the Durham Holder prior to testing nocifensive response to mechanical stimulation of the cutaneous region near the TMJ. Slits were cut into the commercially available device to allow for mechanical stimulation of orofacial region.

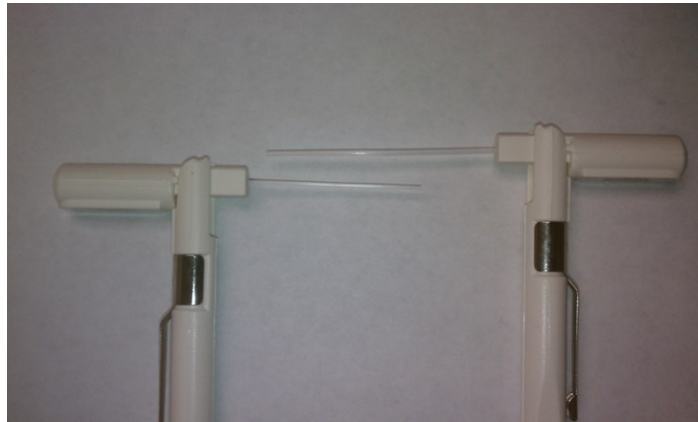
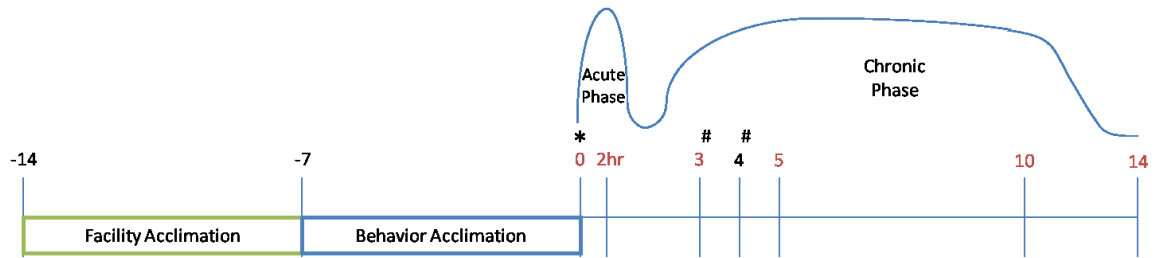
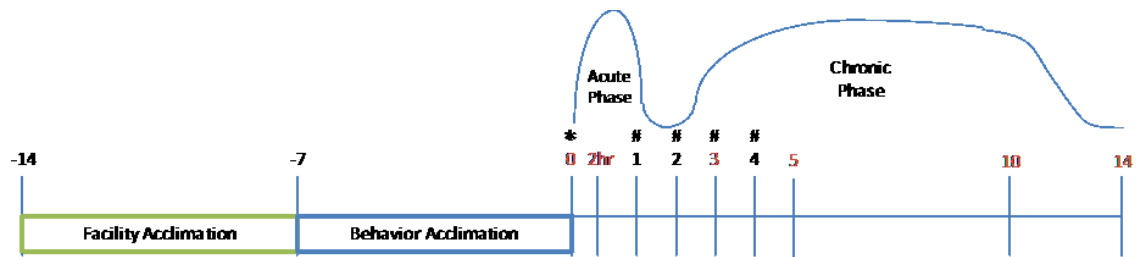


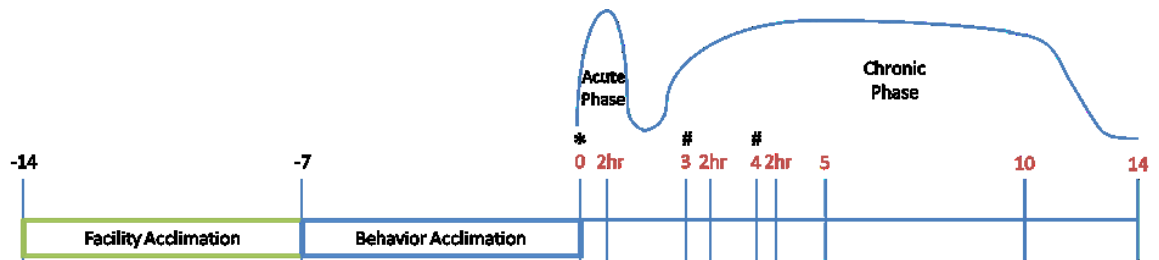
Figure 3. Picture of two von Frey filaments used in my study. The 60 g von Frey filament is shown on the left while a 100 g von Frey filament is shown on the right. Notice that the filament on the right is thicker than the one on the left, so it produces a greater force when applied to the animal.



* = CFA or Saline Injection
 # = Vehicle, Drug A, or Drug B Treatment
 RED = von Frey Testing



* = CFA or Saline Injection
 # = Vehicle or Topiramate Treatment
 RED = von Frey Testing



* = CFA or Saline Injection
 # = Vehicle or Buprenorphine Treatment
 RED = von Frey Testing

Figure 4. Relative behavioral timelines in days for treatment with Drug A and Drug B (A), Topiramate (B), and Buprenorphine (C) are shown. The CFA pain response curve is shown above the timeline.

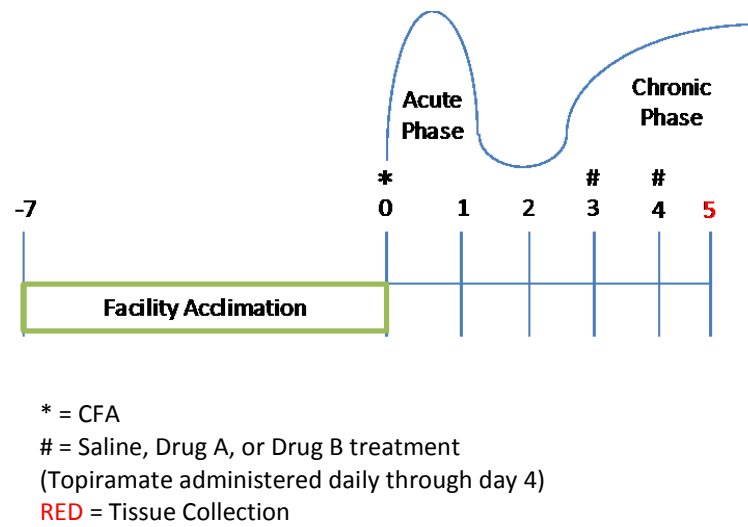


Figure 5. A relative timeline in days for CFA injection, treatments, and tissue collection. The CFA pain response curve is shown above the timeline.

RESULTS

Behavioral Testing in Response to Prolonged Trigeminal Activation

Behavioral testing was performed to determine whether the administration of Drug A, Drug B, or Topiramate could reduce the number of nocifensive withdrawal responses to mechanical stimulation of trigeminal sensory neurons in a chronic inflammatory model. Each von Frey filament (60, 100, 180 g) was applied 5 times to the cutaneous area in the TMJ region on the left and right side of each animal and the number of withdrawals was recorded. A positive, nocifensive response was defined as occurring if the animal withdrew from a given filament 3 or more times out of 5 tests. As seen in Figure 6, no difference was observed in the baseline values for any of the experimental conditions with all being below 1.0 on average. Since rats injected with CFA exhibited a large and consistent increase in the number of nocifensive responses to the 100 g filament at the 2 hour time point while the saline and vehicle control did not, the 100 g filament was used for testing at all other later time points.

Animals injected with 50 μ L of saline followed by treatment with the vehicle withdrew from the 100 g filament an average of 0.4 times at baseline, 0.8 times 2 hours post saline injection, 0.5 times on day 3, 0.8 times on day 5 following injection of vehicle, 0.3 times on day 7, 0.6 times on day 10, and 0.8 times on day 14. In comparison, animals injected with 50 μ L saline followed by Drug A (10 mg/kg) on days 3 and 4 withdrew from the 100 g filament an average of 0.4 times at baseline, 0.3 times 2 hours after saline injection, 0.4 times on day 3, 1.1 times on day 5 following injection of Drug A, 0.8 times on day 7, 0.8 times on day 10, and 0.9 times on day 14. However, animals

injected with 50 μ L of CFA withdrew from the 100 g filament an average of 0.5 times at baseline, 4.3 times 2 hours after CFA injection, 4.3 times on day 3, 4.5 times on day 5 following injection of vehicle, 4.9 times on day 7, 4.8 times on day 10, and 3.9 times on day 14. Finally, animals injected with 50 μ L CFA followed by Drug A (10 mg/kg) withdrew from the 100 g filament an average of 0.8 times at baseline, 4.1 times 2 hours after CFA injection, 4.9 times on day 3, 1.8 times on day 5 following injection of drug A, 3.3 times on day 7, 3.2 times on day 10, and 3.8 times on day 14. On day 5, Drug A administration caused a significant decrease in the number of nocifensive withdrawals when compared to CFA values. However, I observed no difference between CFA alone and CFA followed by treatment with Drug A on subsequent days.

The average number of withdrawal responses for both the left and right side to the 100 g filament for administration of Drug B on days 3 and 4 post CFA injection can be seen in Figure 7. Animals injected with 50 μ L of saline and vehicle withdrew from the 100 g filament an average of 0.1 times at baseline, 0.5 times 2 hours after saline injection, 0.4 times on day 3, 0.4 times on day 5 following gavage of vehicle (vitamin E), 0.7 times on day 7, 0.4 times on day 10, and 0.4 times on day 14. Animals injected with 50 μ L saline followed by Drug B (30 mg/kg) withdrew from the 100 g filament an average of 0.6 times at baseline, 0.9 times 2 hours after saline injection, 0.6 times on day 3, 0.9 times on day 5 following gavage of Drug B, 1.0 times on day 7, 0.7 times on day 10, and 1.1 times on day 14. Animals injected with 50 μ L of CFA withdrew from the 100 g filament an average of 0.5 times at baseline, 4.5 times 2 hours after CFA injection, 4.6 times on day 3, 4.5 times on day 5 following gavage of vehicle (vitamin E), 4.1 times on day 7, 4.3 times on day 10, and 4.1 times on day 14. Finally, animals injected with 50 μ L of CFA

followed by Drug B (30 mg/kg) withdrew from the 100 g filament an average of 0.5 times at baseline, 4.4 times 2 hours after CFA injection, 4.5 times on day 3, 1.3 times on day 5 following gavage of drug B, 1.6 times on day 7, 2.4 times on day 10, 3.7 times on day 14. On days 5, 7, and 10, Drug B administration caused a significant decrease in the number of nocifensive withdrawals when compared to CFA values. However, no difference between CFA and Drug B was observed on day 14.

The average number of withdrawal responses for both the left and right side to the 100 g filament following daily administration of Topiramate can be seen in Figure 8. Animals injected with 50 μ L of saline and vehicle withdrew from the 100 g filament an average of 0.4 times at baseline, 0.8 times 2 hours after saline injection, 0.5 times on day 3, 0.8 times on day 5 following injection of vehicle, 0.3 times on day 7, 0.6 times on day 10, and 0.8 times on day 14. Animals injected with 50 μ L saline followed by Topiramate (50 mg/kg) withdrew from the 100 g filament an average of 1.3 times at baseline, 0.7 times 2 hours after saline injection, 0.8 times on day 3, 1.2 times on day 7 following injection of Topiramate, 1.9 times on day 10, and 1.4 times on day 14. Animals injected with 50 μ L CFA withdrew from the 100 g filament an average of 0.5 times at baseline, 4.3 times 2 hours after CFA injection, 4.3 times on day 3, 4.5 times on day 5 following injection of vehicle, 4.9 times on day 7, 4.8 times on day 10, and 3.9 times on day 14. Finally, animals injected with 50 μ L CFA followed by Topiramate (50 mg/kg) withdrew from the 100 g filament an average of 0.5 times at baseline, 4.6 times 2 hours after CFA injection, 4.8 times on day 3, 4.5 times on day 5 following Topiramate injection, 4.7 times on day 7, 4.9 times on day 10, and 4.7 times on day 14. In contrast to Drug A and

Drug B results, Topiramate did not inhibit CFA mediated nocifensive responses on any of the days post administration.

To test the effect of an opioid in my model, I measured the average number of withdrawal responses for both the left and right side to the 100 g filament following administration of Buprenorphine on days 3 and 4. As seen in Figure 9, animals injected with 50 μ L of saline withdrew from the 100 g filament an average of 0.4 times at baseline, 0.8 times 2 hours after saline injection, 0.5 times on day 3, 0.5 times on day 4, 0.8 times on day 5, 0.3 times on day 7, 0.6 times on day 10, and 0.8 times on day 14. Animals injected with 50 μ L of CFA withdrew from the 100 g filament an average of 0.5 times at baseline, 4.3 times 2 hours after CFA injection, 4.3 times on day 3, 4.3 times on day 4, 4.5 times on day 5, 4.9 times on day 7, 4.8 times on day 10, and 3.9 times on day 14. Animals injected with 50 μ L of CFA followed by Buprenorphine (0.05 mg/kg) withdrew from the 100 g filament an average of 0.5 times at baseline, 5.0 times 2 hours after CFA injection, 0.0 times on day 3 and day 4 (Buprenorphine was administered 30 minutes prior to testing), 5.0 times on day 5, 3.5 times on day 7, 3.8 times on day 10, and 4.0 times on day 14. On days 3 and 4, which correspond to the delivery of Buprenorphine, there was a significant decrease in the number of nocifensive withdrawals when compared to CFA values on those days. However, Buprenorphine caused only a transient inhibition since no difference was observed between CFA and drug treatment on days 5, 7, 10, or 14.

Effect of Drugs on PKA Levels in the Trigeminal Ganglia

Trigeminal nerves were dissected, fixed, and stained for changes in PKA levels in the V1/V2 region of the trigeminal ganglion using immunohistochemistry. In naïve

animals, PKA immunostaining was detected at low levels in neurons in the trigeminal ganglion. Additionally, animals that received saline injections followed by injection of either DMSO or Drug A exhibited low levels of PKA as well (Fig. 10). However, in animals that received CFA injections followed by injection of DMSO, significantly elevated levels of PKA (2.46 ± 0.22 , $p < 0.01$) were detected in the trigeminal ganglion compared to levels in naive rats (1.00 ± 0.08). In animals that received CFA injections followed by treatment with Drug A, PKA was detected at decreased levels (1.21 ± 0.19 , $p < 0.01$) in the trigeminal ganglion compared to levels in animals that had received CFA + DMSO injections (2.46 ± 0.22).

Animals that received saline injections followed by gavages of Vitamin E or Drug B exhibited low levels of PKA in the V1/V2 region of the trigeminal ganglion (Fig. 11). Conversely, the animals injected with CFA followed by a Vitamin E gavage exhibited high levels of PKA expression (2.38 ± 0.17 , $p < 0.01$) in neurons when compared to animals that received the saline + Vitamin E treatment (1.00 ± 0.14). Animals that were treated with Drug B following CFA injections had greatly decreased levels of PKA (1.10 ± 0.12 , $p < 0.01$) in the trigeminal ganglion compared to levels in animals that had received CFA + Vitamin E treatment (2.38 ± 0.17 , $p < 0.01$).

For this condition, the naïve animals exhibited low levels of PKA in the V1/V2 region of the trigeminal ganglion. In addition, PKA immunostaining was detected at low levels in neurons and satellite glia cells in the trigeminal ganglion of animals that received saline injections followed by either DMSO or Topiramate injections (Fig. 12). Animals injected with CFA followed by a DMSO injection exhibited high levels of PKA expression (2.88 ± 0.33 , $p < 0.01$) when compared to naïve animals

(1.10 ± 0.10). Interestingly, the animals that were injected with CFA then treated with Topiramate expressed higher levels of PKA (2.74 ± 0.24 , $p < 0.01$) in the trigeminal ganglion.

Effect of Drugs on PKA Levels in the Spinal Trigeminal Nucleus

The upper spinal cord tissue containing the STN was dissected out and changes in PKA levels were determined using immunohistochemistry. In animals injected with saline followed by DMSO or Drug A injections, low levels of PKA were detected in the spinal trigeminal nucleus (Fig. 13). In contrast, the level of PKA staining was significantly higher in animals injected with CFA followed by DMSO (1.96 ± 0.09 , $p < 0.01$) compared to levels in animals that had received saline + DMSO injections (1.00 ± 0.07). Animals that received CFA injections followed by treatment with Drug A had lower levels of PKA expression (1.33 ± 0.11 , $p < 0.01$) in the STN compared levels in animals that had received CFA + DMSO injections (1.96 ± 0.09 , $p < 0.01$).

Animals that received saline injections followed by gavages of Vitamin E or Drug B exhibited low levels of PKA in the spinal trigeminal nucleus (Fig. 14). Expression of PKA was significantly increased (2.31 ± 0.26 , $p < 0.01$) in the STN of animals that received CFA injections followed by Vitamin E gavage when compared to PKA levels in animals that received the saline + Vitamin E treatment (1.00 ± 0.10). Animals that were treated with Drug B following CFA injections had greatly decreased levels of PKA (1.16 ± 0.11 , $p < 0.01$) in the outer laminae of the STN compared to levels in animals that had received CFA + Vitamin E treatment (2.31 ± 0.26).

The naïve animals exhibited low levels of PKA in the STN. Similarly, PKA immunostaining was detected at low levels in the outer laminae of animals that received saline injections followed by either DMSO or Topiramate injections (Fig. 15). Animals injected with CFA followed by a DMSO injection exhibited significantly higher levels of PKA expression (1.79 ± 0.13 , $p < 0.01$) when compared to naïve animals (1.00 ± 0.07). Interestingly, the animals that were injected with CFA then treated with Topiramate still had higher levels of PKA expression (1.84 ± 0.14 , $p < 0.01$) in the STN.

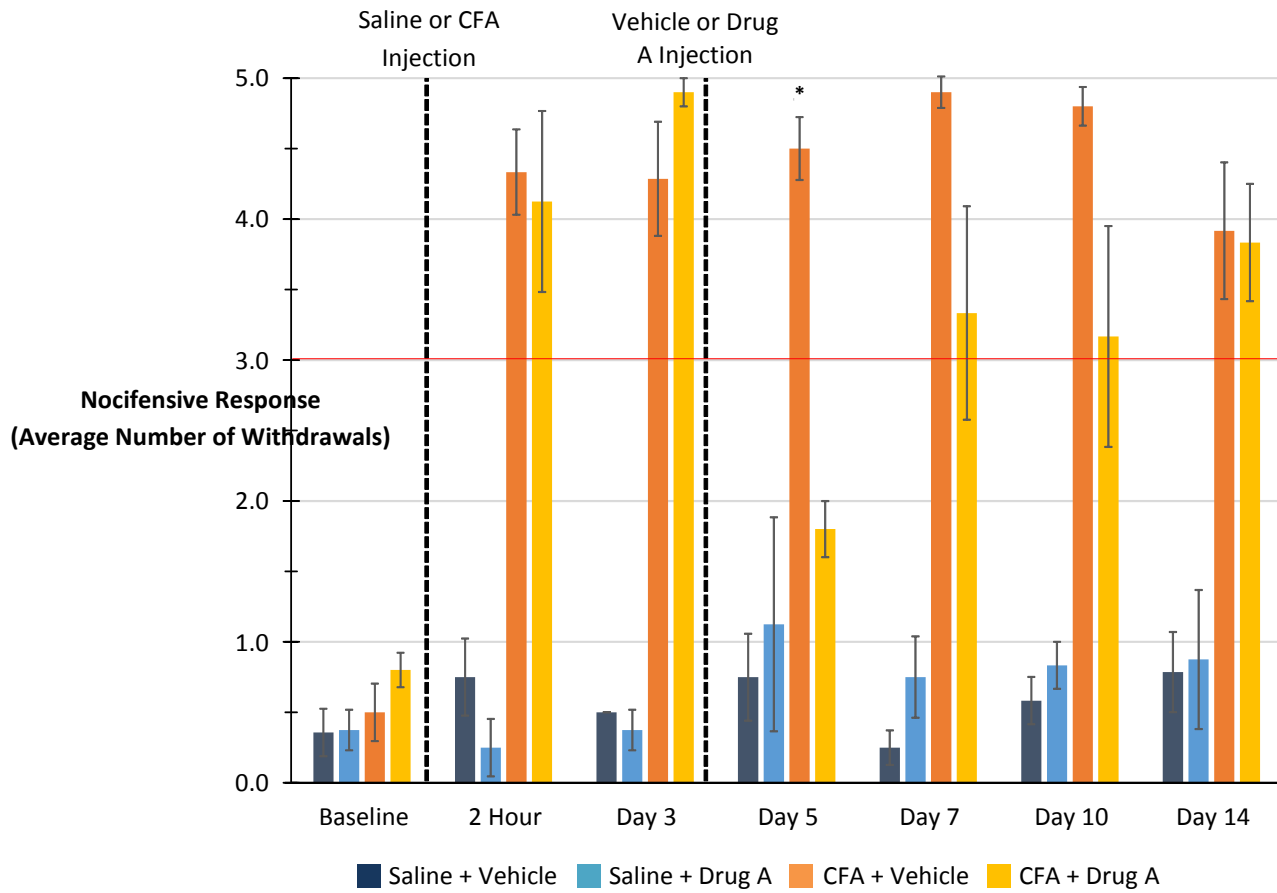


Figure 6. Drug A transiently inhibits nocifensive response to mechanical stimulation of the trigeminal nerve. The cut-off for a positive nocifensive response was set at 3 withdrawals from 5 applications. Values are reported as the average number of withdrawal responses to mechanical stimulation (out of 5 applications to each side; 100 g von Frey filament). * = $p < 0.01$ when compared to CFA value.

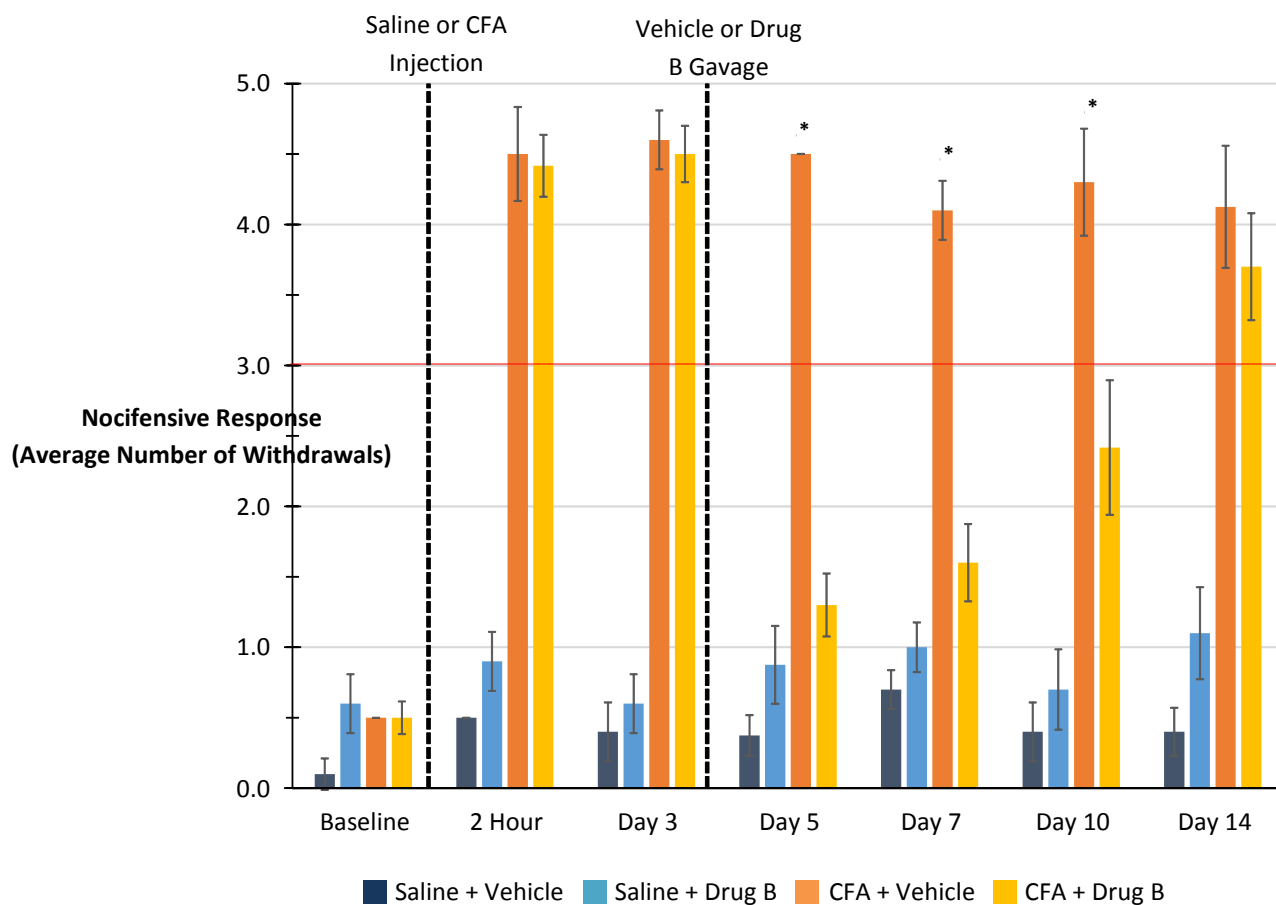


Figure 7. Drug B mediated sustained inhibition of CFA-mediated nocifensive response to mechanical stimulation of the trigeminal nerve. Red line at 3.0 responses was set as the cut-off for a positive nocifensive response. Values are reported as the average number of withdrawal responses to mechanical stimulation (out of 5 applications to each side; 100 g von Frey filament). * = $p < 0.01$ when compared to CFA value.

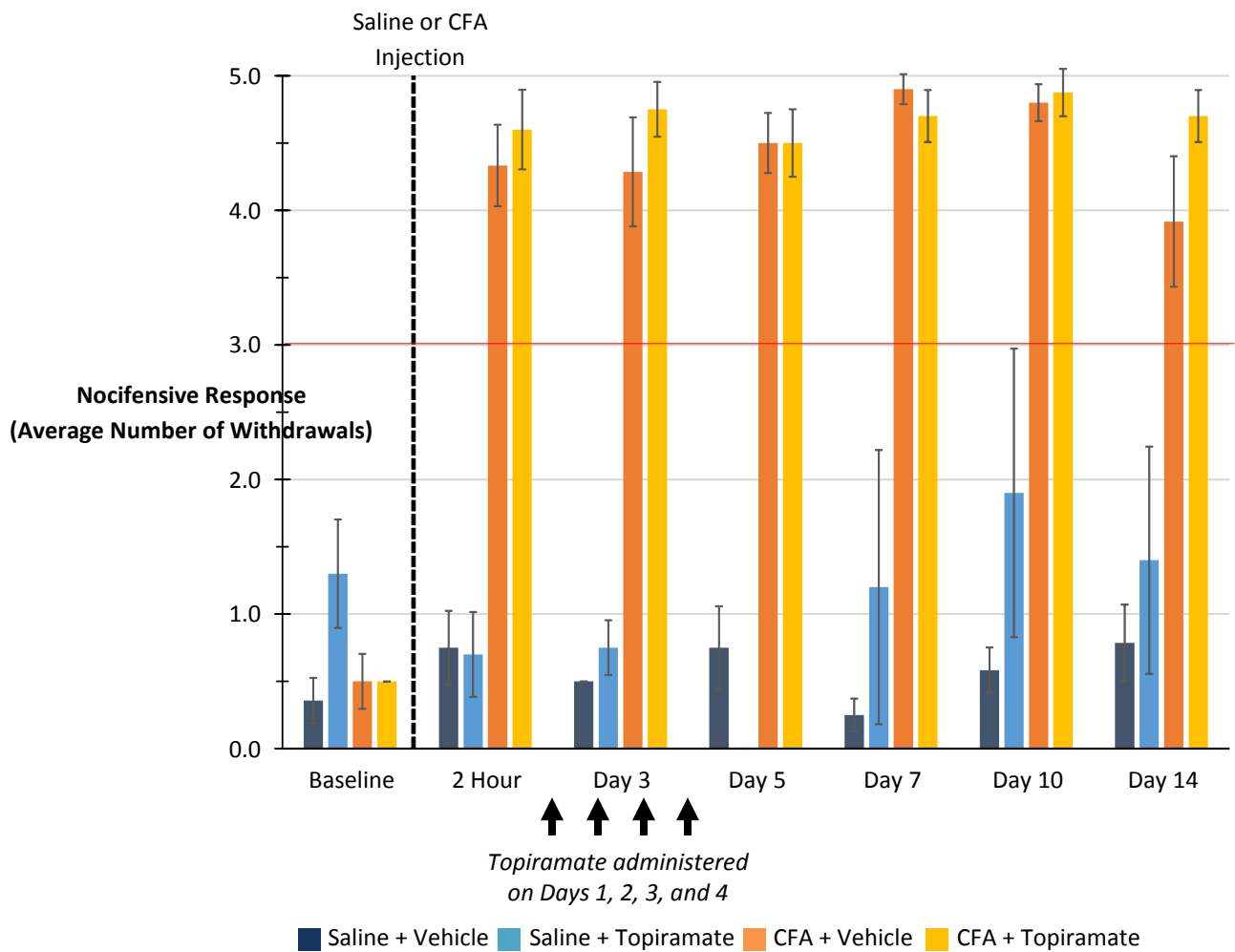


Figure 8. Topiramate does not inhibit nocifensive response to mechanical stimulation of the trigeminal nerve. Red line at 3.0 responses was set as the cut-off for a positive nocifensive response. Values are reported as the average number of withdrawal responses to mechanical stimulation (out of 5 applications to each side; 100 g von Frey filament).

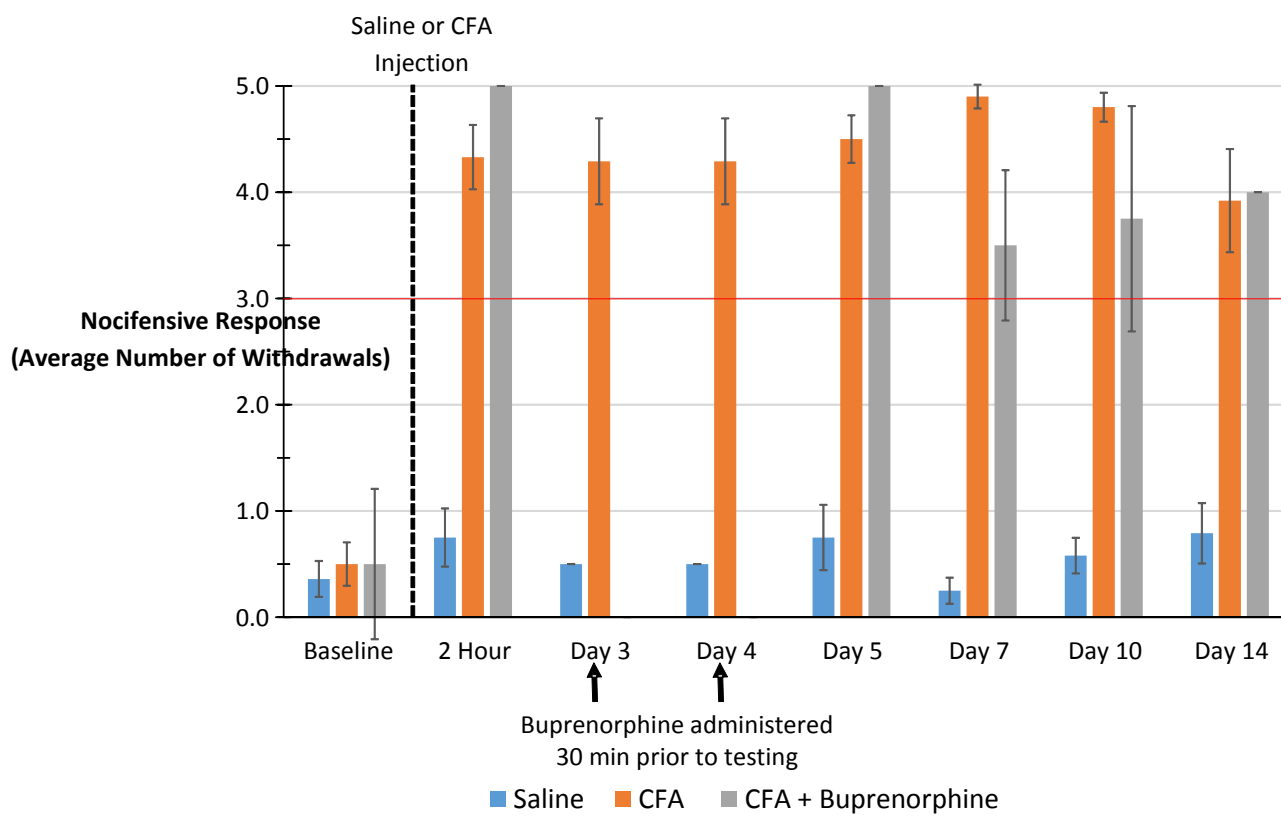
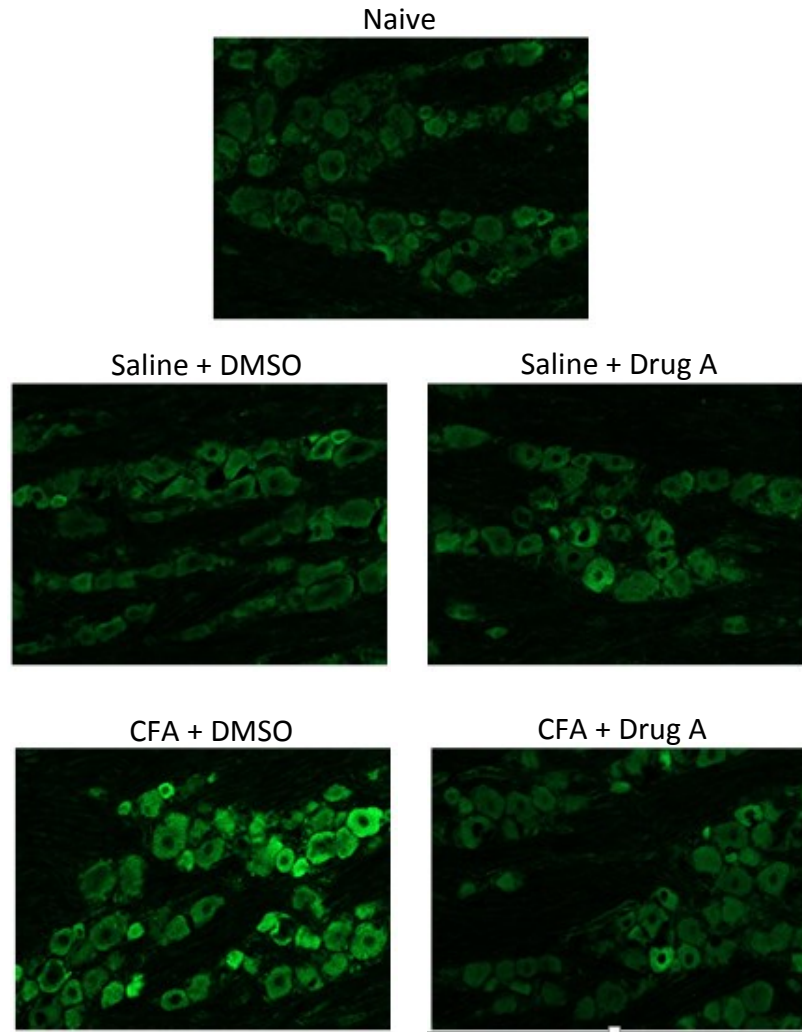
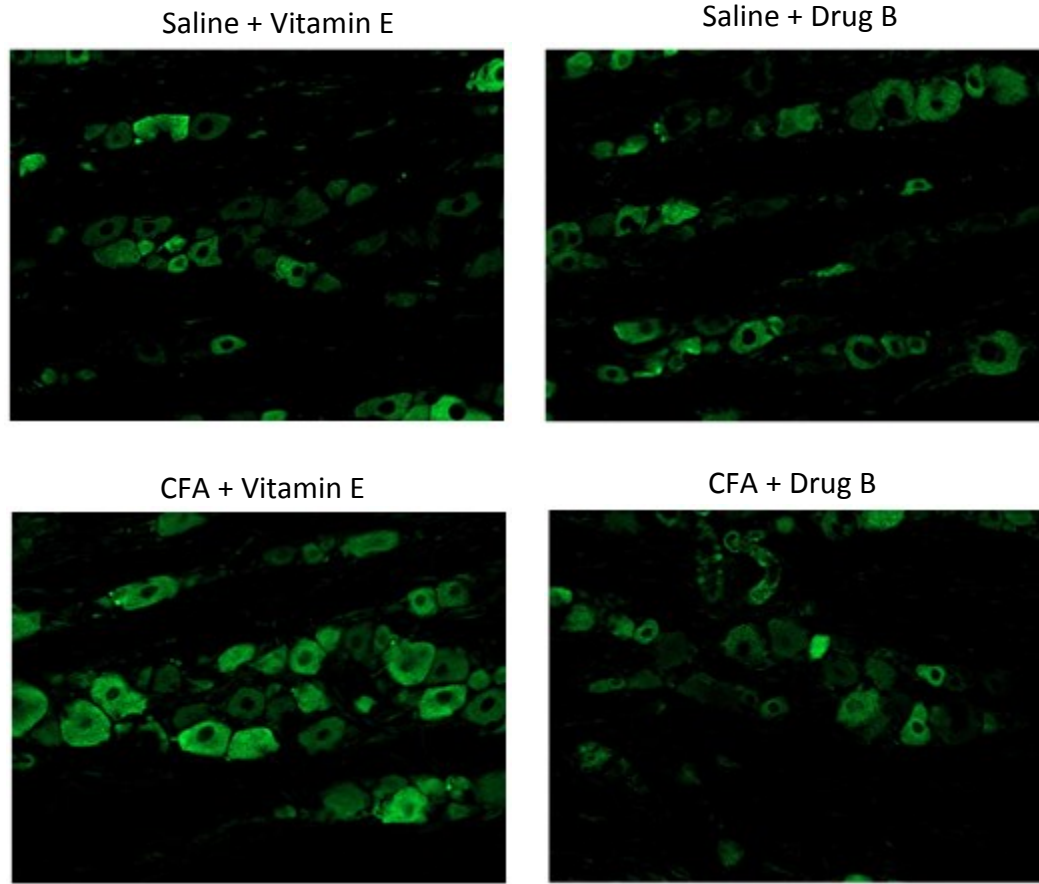


Figure 9. Buprenorphine treatment completely inhibits CFA-mediated nocifensive response to mechanical stimulation of the trigeminal nerve. Red line at 3.0 responses was set as the cut-off for a positive nocifensive response. Values are reported as the average number of withdrawal responses to mechanical stimulation (out of 5 applications to each side; 100 g von Frey filament).



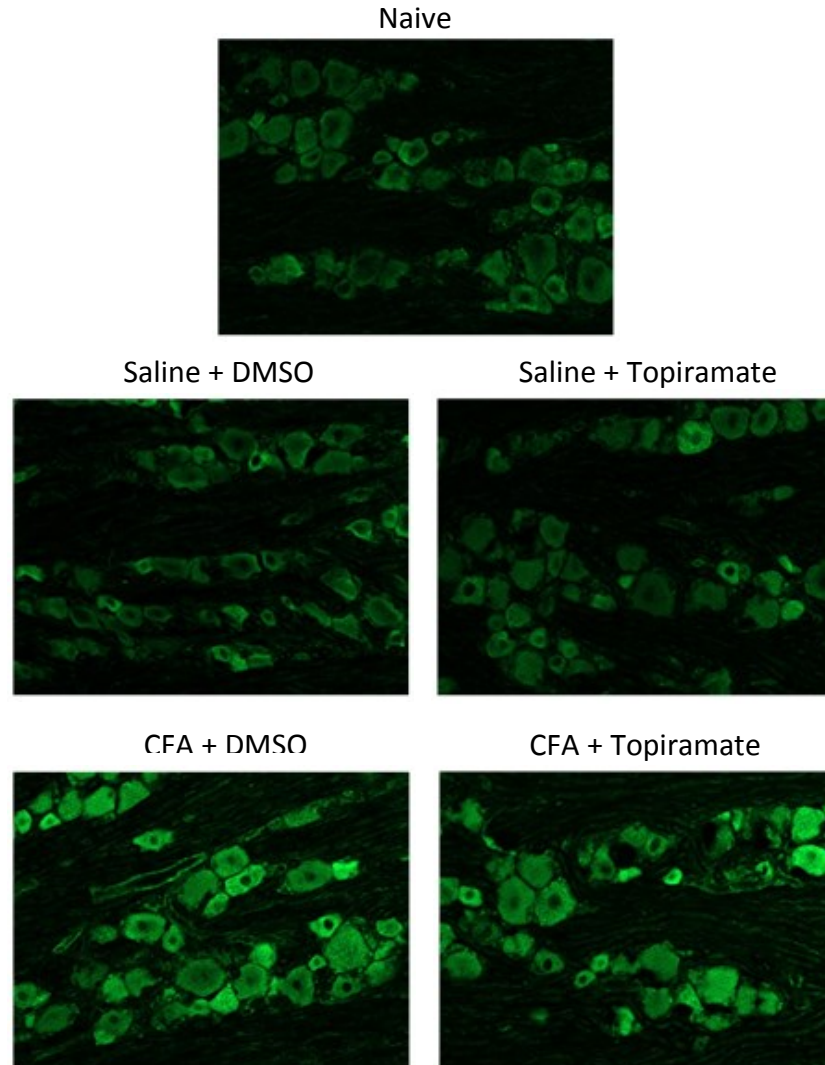
Condition	Relative Intensity of PKA Staining (Mean +/- SEM)
Naïve	1.00 ± 0.08
Saline + DMSO	1.09 ± 0.13
Saline + Drug A	1.13 ± 0.11
CFA + DMSO	2.46 ± 0.22 *
CFA + Drug A	1.21 ± 0.19 #
	* = $p < 0.01$ when compared to Naïve
	# = $p < 0.01$ when compared to CFA

Figure 10. Drug A inhibits CFA-mediated stimulation of PKA expression in trigeminal ganglion neurons. Drug A was administered on day 3 and 4 post CFA injection and trigeminal ganglia removed on day 5 for immunohistochemistry. A representative image is shown for each experimental condition.



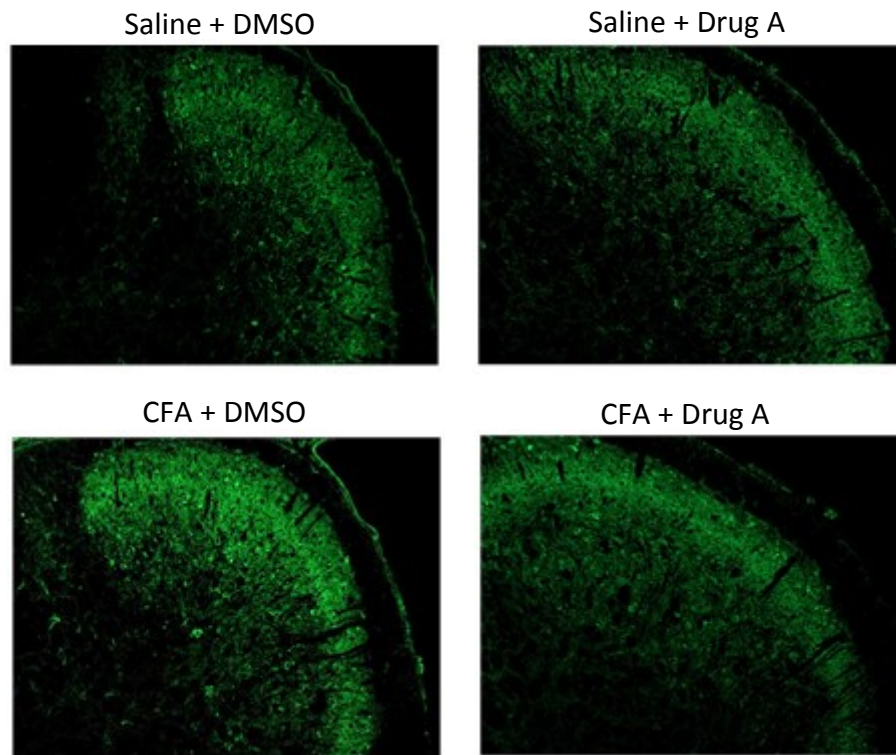
Condition	Relative Intensity of PKA Staining (Mean +/- SEM)
Saline + Vitamin E	1.00 ± 0.14
Saline + Drug B	0.98 ± 0.09
CFA + Vitamin E	2.38 ± 0.17 *
CFA + Drug B	1.10 ± 0.12 #
	* = $p < 0.01$ when compared to Saline + Vitamin E
	# = $p < 0.01$ when compared to CFA + Vitamin E

Figure 11. Drug B inhibits CFA-mediated stimulation of PKA expression in trigeminal neurons. Drug B was administered on day 3 and 4 post CFA injection and trigeminal ganglia removed on day 5 for immunohistochemistry. A representative image is shown for each experimental condition.



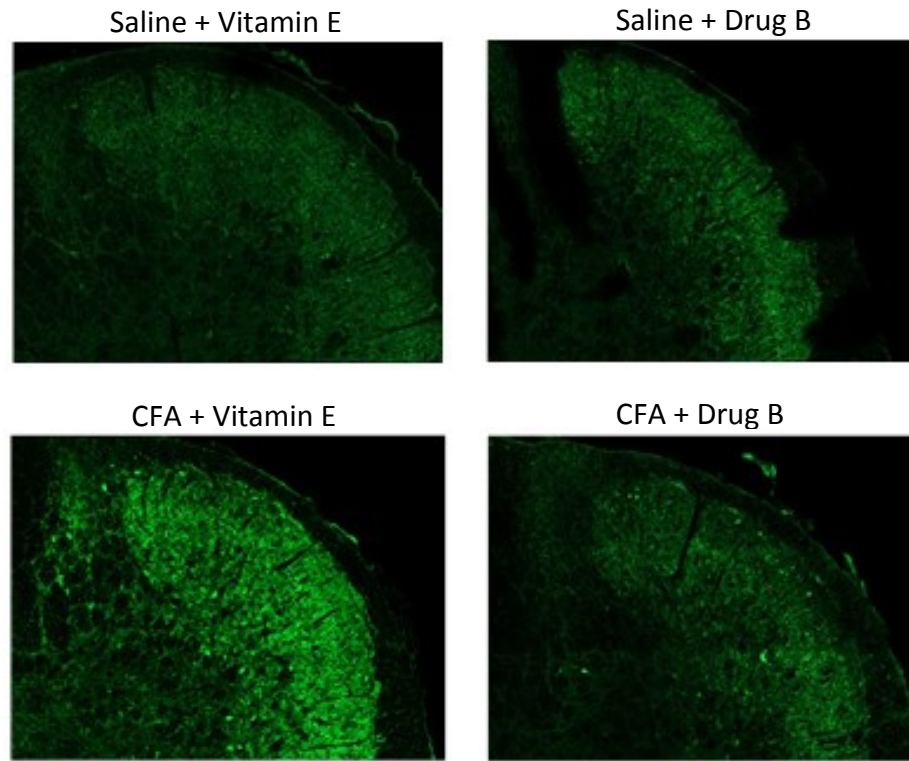
Condition	Relative Intensity of PKA Staining (Mean +/- SEM)
Naïve	1.10 ± 0.10
Saline + DMSO	1.16 ± 0.11
Saline + Topiramate	1.18 ± 0.13
CFA + DMSO	2.88 ± 0.33 *
CFA + Topiramate	2.74 ± 0.24
	* = $p < 0.01$ when compared to Naïve

Figure 12. Topiramate does not inhibit CFA-mediated stimulation of PKA expression in trigeminal neurons. Topiramate was administered on day 3 and 4 post CFA injection and trigeminal ganglia removed on day 5 for immunohistochemistry. A representative image is shown for each experimental condition.



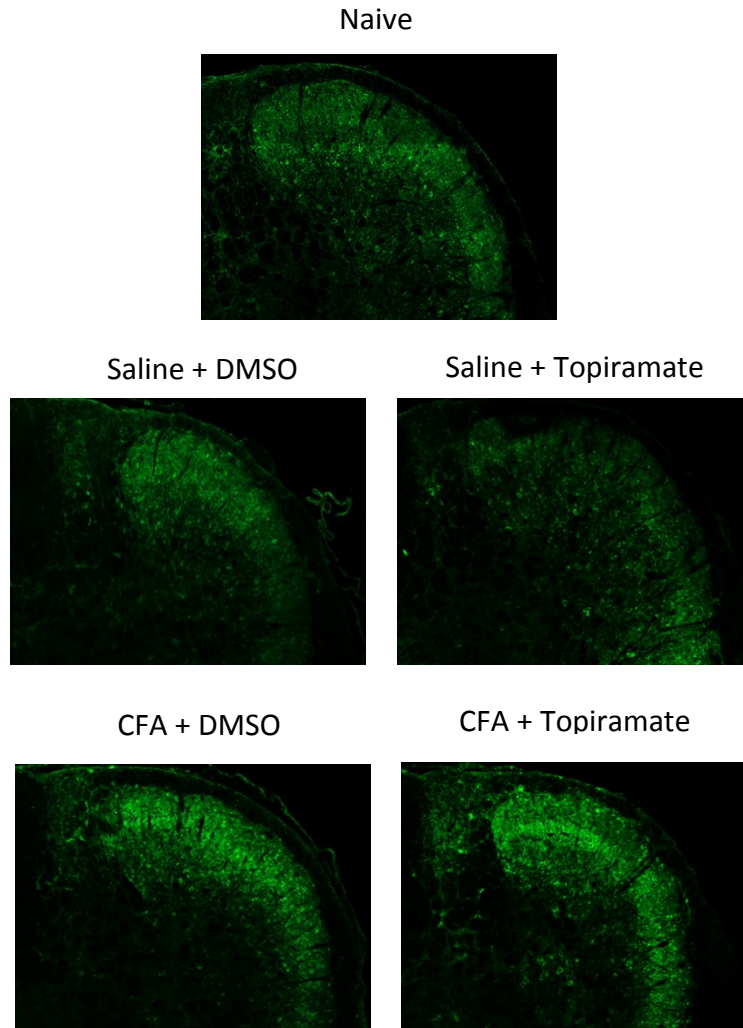
Condition	Relative Intensity of PKA Staining (Mean +/- SEM)
Saline + DMSO	1.00 ± 0.07
Saline + Tonabersat	1.11 ± 0.15
CFA + DMSO	1.96 ± 0.09 *
CFA + Drug A	1.33 ± 0.11 #
	* = $p < 0.01$ when compared to Saline + DMSO
	# = $p < 0.01$ when compared to CFA

Figure 13. Drug A inhibits CFA-mediated stimulation of PKA expression in spinal trigeminal nucleus. Drug A was administered on day 3 and 4 post CFA injection and upper spinal cord tissue containing the spinal trigeminal nucleus removed on day 5 for immunohistochemistry. A representative image is shown for each experimental condition.



Condition	Relative Intensity of PKA Staining (Mean +/- SEM)
Saline + Vitamin E	1.00 ± 0.10
Saline + Drug B	1.14 ± 0.17
CFA + Vitamin E	2.31 ± 0.26 *
CFA + Drug B	1.16 ± 0.11 #
	* = $p < 0.01$ when compared to Saline + Vitamin E
	# = $p < 0.01$ when compared to CFA

Figure 14. Drug B inhibits CFA-mediated stimulation of PKA expression in the spinal trigeminal nucleus. Drug B was administered on day 3 and 4 post CFA injection and upper spinal cord tissue removed on day 5 for immunohistochemistry. A representative image is shown for each experimental condition.



Condition	Relative Intensity of PKA Staining (Mean +/- SEM)
Naïve	1.00 ± 0.07
Saline + DMSO	1.03 ± 0.09
Saline + Topiramate	0.94 ± 0.11
CFA + DMSO	1.79 ± 0.13 *
CFA + Topiramate	1.84 ± 0.14
	* = $p < 0.01$ when compared to Naïve

Figure 15. Topiramate does not inhibit CFA-mediated stimulation of PKA expression in the spinal trigeminal nucleus. Topiramate was administered on day 3 and 4 post CFA injection and upper spinal cord tissue removed on day 5 for immunohistochemistry. A representative image is shown for each experimental condition.

DISCUSSION

Effect of Anti-Migraine Drugs on Nociception

A main goal of my study was to determine if the novel anti-migraine drugs designated as Drug A and Drug B could inhibit ongoing peripheral and central sensitization and nociception in an animal model of chronic migraine. A characteristic feature of frequent and chronic migraine is prolonged sensitization of primary and secondary sensory trigeminal neurons that provide innervation to most of the head and face and facilitate transmission of painful stimuli from the peripheral tissues to the CNS. I chose to use the inflammatory chemical mediator CFA, which is a heat-killed bacterium, to promote sustained peripheral tissue inflammation and nociception of trigeminal neurons. The physiological and cellular effects of CFA injection into the TMJ capsule or masseter muscles of mastication have been well characterized by our laboratory as well as other research laboratories (Ambalavanar et al., 2006, Suzuki et al., 2007, Garrett and Durham, 2009, Cady and Durham, 2010, Cady et al., 2010, Garrett et al., 2012, Cady et al., 2014, Romero-Reyes et al., 2015) . Following CFA injection into the capsule or muscle, there is an acute phase of nociception to mechanical stimulation that resolves by 48 hours post injection. However, a more chronic phase of nociception returns on day 4 reaching a peak on day 5 and then is sustained for up to 14 days. The prolonged nature of the inflammatory response to CFA is responsible for the development of a more persistent state of peripheral and central sensitization of the trigeminal neurons associated with increased nociception. Thus, CFA-mediated changes

in the trigeminal nociceptive neurons mimic some aspects of frequent and chronic migraine and provides a useful model for studying novel anti-migraine drugs.

I found that treatment with Drug A and B on days 3 and 4 post CFA injection could significantly inhibit the average number of nocifensive responses to mechanical stimulation of the trigeminal neurons on day 5, which is the time of peak sensitization. In my model, CFA was injected into both TMJ capsules and nocifensive withdrawal responses to mechanical stimulation monitored 2 hours post injection and then on days 3, 5, 7, 10, and 14 post injection. CFA caused a rapid increase in nocifensive behavioral response within 2 hours following injection that was sustained throughout the 14 day duration of my experiment. The 2 hour time point was used to determine whether the CFA injections were performed correctly since the acute nocifensive response is known to be followed by a more prolonged inflammatory and nociceptive response in the trigeminal system. While Drug A was able to repress CFA-mediated nocifensive response only on day 5, pretreatment with Drug B, which is a structural isomer of Drug A, significantly inhibited the number of withdrawals on days 7 and 10. These data support the notion that the structural change to Drug B extended the therapeutic window of this drug beyond that of the parent compound and thus may be more beneficial in the treatment of frequent and chronic migraine. I can only speculate on why Drug B has a greater duration of efficacy. It is possible that the modification of the parent Drug A compound increases the half-life of Drug B in the animal or it produces a more stable secondary metabolite that prolongs its ability to inhibit trigeminal nociception. However, it should be noted that neither of the drugs completely abort the nocifensive response since even in animals receiving Drug B exhibited an increase in the number of

nocifensive withdrawals on day 14 post CFA injection. A likely explanation for the prolonged CFA response on day 14 is that there is still ongoing inflammation in the TMJ capsule that is sustaining both peripheral and central sensitization. Importantly, I did not observe any negative side effects such as changes in grooming or feeding behaviors in animals receiving a single dose of either drug. Results from my study are suggestive that Drug A and Drug B would need to be administered on a regular basis to achieve full inhibition of nociception caused by trigeminal sensory neuron activation.

A major goal of my study was to compare the therapeutic benefit of Drug A and Drug B to currently used medications for the treatment of frequent migraine attacks. Towards this end, I chose to study the effect of the drug Topiramate, which is approved by the Federal Drug Administration, in my model of sustained trigeminal sensitization and activation. However in contrast to Drug A and Drug B, Topiramate administered on a daily bases via intraperitoneal injection did not inhibit the nocifensive response to mechanical stimulation at any of the time periods. The choice of daily administration is based on the recommended clinical dosing regimen for Topiramate in which it is taken daily to reduce the frequency, duration, and severity of frequent episodic migraine attacks (Brandes et al., 2004, Diener et al., 2007, Cady et al., 2012b). Given that Topiramate is proposed to function by reducing neuronal hyperexcitability and release of inflammatory molecules (Silberstein, 2006, Bolcskei et al., 2009), my findings are somewhat surprising. Although Topiramate is approved for the treatment of frequent migraine, a significant number of patients report no benefit from taking this drug and many discontinue to use it for managing their migraine attacks because of the sometimes severe negative side effects. My findings are in contrast to findings from a previous study that

Topiramate administered on a daily basis reduced mechanical sensitivity and shortened the period of allodynia (nociception in response to a non-painful stimulus) in rats (Wieczorkiewicz-Plaza et al., 2004). In their study, Topiramate (50 mg/kg, i.p.) administered daily diminished mechanical sensitivity and shortened the period of allodynia and reached statistical significance on the 4th day. However, it should be noted that their model differs in several important ways from my model. For example, their model did not attempt to mimic the pathological features of migraine since they were using a neuropathic pain model caused by nerve injury to the sciatic nerve (not the trigeminal nerve). Additionally, they were studying changes in dorsal root ganglion nociceptive neurons by investigating withdrawal responses to mechanical stimulation of the hind paw. Furthermore, whereas I used Topiramate as a prophylactic drug and administered it daily on days 1-4 (before the “chronic” period), the previous study administered Topiramate daily for the duration of their experiment (administered daily – 1 hr prior to each test).

In addition to Topiramate, I also wanted to study the effect of an opioid in my model since opioids are known to inhibit nociception caused by inflammation in peripheral tissues (Watkins et al., 2005, Kreek et al., 2012, Doebling et al., 2013, Saper and Da Silva, 2013). As reported in a published study from the Durham laboratory (Garrett et al., 2012), the opioid drug buprenorphine hydrochloride injected subcutaneously in the hindquarter at a final concentration of 0.05 mg/kg one hour prior to mechanical testing was shown to significantly inhibit the number of nocifensive withdrawals. Opioid analgesics such as buprenorphine are known to suppress pain by blocking pain transmission via sensory neurons from peripheral tissues to the central

nervous system and activating neurons in the descending pain inhibition pathway. In addition, drugs that target opioid receptors are commonly used by emergency room doctors as an anti-migraine therapy. I found that administration of buprenorphine 30 minutes prior to behavioral testing was able to completely abolish the CFA-mediated nocifensive response on days 3 and 4. However the inhibitory effect of buprenorphine was transient since 24 hours after administration the number of nocifensive responses was equivalent to the CFA injected animals and remained that way throughout the remainder of the study (day 14). Thus, buprenorphine is only beneficial in relieving nociception temporarily. This finding is not too surprising given that opioids are not known to decrease peripheral inflammation or ongoing peripheral sensitization of nociceptors but rather transiently inhibit central pain processing mechanisms (Baron, 2009, Okada-Ogawa et al., 2009). Hence, opioids should only be used as a last resort treatment option since there is no resolution of the underlying inflammatory process and thus, the associated nociceptive response will return once the drug effects have diminished.

Regulation of PKA in Trigeminal Ganglion and Upper Spinal Cord Tissue

To investigate cellular events that are likely involved in the development of peripheral and central sensitization, I chose to focus on changes in the expression of the signaling protein PKA in trigeminal ganglia and upper spinal cord tissue containing the STN. Elevated levels of PKA are implicated in the initiation and maintenance of peripheral and central sensitization (Aley and Levine, 1999, Hu et al., 2003, Hucho and Levine, 2007, Seybold, 2009). Similar to previous studies, I found that CFA injection into the TMJ capsule, which is innervated primarily by V3 neurons, caused a significant

increase in level of PKA expression in trigeminal ganglion neurons throughout the ganglion (V1, V2, and V3) when compared to naïve untreated and vehicle only treated animals. Our laboratory has previously described this type of cross-excitation within the trigeminal ganglion that likely facilitates sensitization of other neurons that likely functions to expand the receptive field following tissue injury (Thalakoti et al., 2007, Durham and Garrett, 2010). Treatment with Drug A or Drug B on days 3 and 4 post CFA injection into the TMJ capsule greatly inhibited PKA expression in neurons on day 5, the time of peak nocifensive response to CFA. In contrast, treatment with the dilution vehicle did not cause repression of CFA-mediated increases in PKA staining intensity or number of positive cells. In agreement with my behavioral results, administration of Topiramate did not inhibit elevated levels of PKA in response to CFA. The modulatory effects of PKA are thought to involve activation of pathways and transcription factors that regulate the expression and activity level of ion channels and receptors in nociceptive neurons and increase expression of pro-inflammatory molecules in both neurons and glial cells (Seybold, 2009). My findings provide evidence that the inhibitory effect of Drug A and Drug B are likely mediated, at least in part, to repression of PKA levels in trigeminal primary nociceptive neurons. Furthermore, these results may help to explain why I observed that Drug A and Drug B could inhibit nocifensive withdrawal responses to mechanical stimulation but not Topiramate.

I also found that CFA injection into the TMJ capsule caused a large increase in the level of PKA expression in the spinal trigeminal nucleus when compared to levels in naïve unstimulated animals and animals receiving saline (control for CFA injection) or DMSO (control for drugs). Within the spinal cord, activation of the PKA pathway has

been shown to lead to central sensitization of second order nociceptive neurons and can promote bidirectional sensitization of primary nociceptors (Kohno et al., 2008, Cady et al., 2011, Hawkins et al., 2015). With respect to migraine and TMJ pathology, the PKA pathway is thought to play a central role since this signal transduction pathways is activated by the neuropeptide CGRP, which is released from primary trigeminal sensory neurons in response to peripheral tissue injury. The cellular effects of CGRP are likely to be mediated by both neurons and glial cells since the CGRP receptor is expressed on neuronal cell bodies, astrocytes, and microglia (Durham, 2008, Durham and Vause, 2010). Following binding of CGRP to its receptor, there is a large increase in the secondary messenger cAMP that causes activation of PKA, which can promote cellular changes via phosphorylation of proteins that function to regulate the excitability state of neurons and glial and the synthesis and release of pro-inflammatory molecules that sustain central sensitization. In my study, Drug A and Drug B were found to repress the stimulatory effect of CFA on PKA expression in the spinal trigeminal nucleus when compared to DMSO treated animals. In contrast, treatment with Topiramate did not inhibit the CFA-induced change in PKA. These findings are in agreement with both my behavioral data and results observed in the trigeminal ganglion and support the notion that Drug A and Drug B can transiently inhibit nociception by repressing CFA stimulation of the PKA pathway and promotion of central sensitization.

Summary and Future Directions

Based on my findings, I propose that both Drug A and Drug B would be beneficial as a therapeutic agent for frequent episodic and chronic migraine by inhibiting

sustained peripheral and central sensitization of trigeminal nerves. The mechanism of action of Drug A and Drug B is unique compared to Topiramate, a drug approved as a therapy for frequent episodic migraine. The cellular data (inhibition of PKA in ganglia and spinal cord) supports the notion that Drug A and Drug B inhibition of nocifensive signaling involves suppressing the sustained development of peripheral and central sensitization, which are key physiological events associated with frequent episodic and chronic migraine. While both Drug A and Drug B were shown to be effective in my model of prolonged trigeminal nerve sensitization, the longer repressive effect of Drug B supports its further characterization as a promising anti-migraine therapy for frequent and chronic migraine. Based on my behavioral data that demonstrated transient inhibition of mechanical sensitivity, I propose that Drug A and Drug B would need to be administered on a daily basis to provide a therapeutic benefit to patients experiencing either frequent episodic or chronic migraine. Within the migraine community, preventing the progression of episodic to chronic migraine needs to be considered a top priority in the management of migraine patients since patients without effective acute therapy are at greater risk of developing CM (Cady et al., 2004). Given that Topiramate is the only drug with FDA approval for the treatment of frequent episodic migraine and BOTOX (onabotulinumtoxin type A) is the only drug approved for the treatment of chronic migraine, there remains a great need for the development of safer and more effective anti-migraine medications.

There are many unanswered questions with respect to the mechanism of action of Drug A and Drug B that would be of interest to investigate. Initially, it would be important to determine the effect of different doses and time of administration on efficacy

and safety. Could these drugs be used as preventative (prophylaxis) therapy in addition to be utilized as abortive drugs? I found that these drugs could inhibit PKA expression but it is likely that other signaling pathways known to promote peripheral and central sensitization such as the mitogen-activated protein kinase or MAP kinase pathway would be a cellular target. Given the hydrophobic nature of the drugs, it is likely that their inhibitory effects would extend beyond the trigeminal system so it would be important to determine if they block nociception in tissues innervated by the dorsal root ganglion (e.g., knee, ankle, wrist, shoulder, and hip joints). Also, it is possible that these drugs may influence nociception at higher levels within the brain such as the thalamus. If higher doses of the drugs were tested, it would be essential to investigate changes in cognitive ability to clearly differentiate the mechanism of action of these drugs when compared to Topiramate, which is known to cause cognitive impairment. Our laboratory has been developing more clinically relevant animal models that more closely mimic key pathophysiological features of episodic and chronic migraine (risk factors and triggers), so it would be of interest to know how Drug A and Drug B perform in these novel models.

REFERENCES

- Aley KO, Levine JD (1999) Role of protein kinase A in the maintenance of inflammatory pain. *J Neurosci* 19:2181-2186.
- Ambalavanar R, Moutanni A, Dessem D (2006) Inflammation of craniofacial muscle induces widespread mechanical allodynia. *Neurosci Lett* 399:249-254.
- Baron R (2009) Neuropathic pain: a clinical perspective. *Handbook of experimental pharmacology* 3-30.
- Bartsch T, Goadsby PJ (2003) The trigeminocervical complex and migraine: current concepts and synthesis. *Current pain and headache reports* 7:371-376.
- Bernstein C, Burstein R (2012) Sensitization of the trigeminovascular pathway: perspective and implications to migraine pathophysiology. *Journal of clinical neurology* 8:89-99.
- Bolay H, Reuter U, Dunn AK, Huang Z, Boas DA, Moskowitz MA (2002) Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. *Nature medicine* 8:136-142.
- Bolcskei H, Farkas B, Kocsis P, Tarnawa I (2009) Recent advancements in anti-migraine drug research: focus on attempts to decrease neuronal hyperexcitability. *Recent Pat CNS Drug Discov* 4:14-36.
- Brandes JL, Saper JR, Diamond M, Couch JR, Lewis DW, Schmitt J, Neto W, Schwabe S, Jacobs D (2004) Topiramate for migraine prevention: a randomized controlled trial. *JAMA* 291:965-973.
- Burstein R, Nosedá R, Borsook D (2015) Migraine: multiple processes, complex pathophysiology. *J Neurosci* 35:6619-6629.
- Buse DC, Loder EW, Gorman JA, Stewart WF, Reed ML, Fanning KM, Serrano D, Lipton RB (2013) Sex differences in the prevalence, symptoms, and associated features of migraine, probable migraine and other severe headache: results of the American Migraine Prevalence and Prevention (AMPP) Study. *Headache* 53:1278-1299.
- Buse DC, Manack AN, Fanning KM, Serrano D, Reed ML, Turkel CC, Lipton RB (2012) Chronic migraine prevalence, disability, and sociodemographic factors: results from the American Migraine Prevalence and Prevention Study. *Headache* 52:1456-1470.

- Cady RJ, Denson JE, Durham PL (2013) Inclusion of cocoa as a dietary supplement represses expression of inflammatory proteins in spinal trigeminal nucleus in response to chronic trigeminal nerve stimulation. *Mol Nutr Food Res* 57:996-1006.
- Cady RJ, Denson JE, Sullivan LQ, Durham PL (2014) Dual orexin receptor antagonist 12 inhibits expression of proteins in neurons and glia implicated in peripheral and central sensitization. *Neuroscience* 269:79-92.
- Cady RJ, Durham PL (2010) Cocoa-enriched diets enhance expression of phosphatases and decrease expression of inflammatory molecules in trigeminal ganglion neurons. *Brain Res* 1323:18-32.
- Cady RJ, Glenn JR, Smith KM, Durham PL (2011) Calcitonin gene-related peptide promotes cellular changes in trigeminal neurons and glia implicated in peripheral and central sensitization. *Mol Pain* 7:94.
- Cady RJ, Hirst JJ, Durham PL (2010) Dietary grape seed polyphenols repress neuron and glia activation in trigeminal ganglion and trigeminal nucleus caudalis. *Mol Pain* 6:91.
- Cady RJ, Shade CL, Cady RK (2012a) Advances in drug development for acute migraine. *Drugs* 72:2187-2205.
- Cady RK, Schreiber CP, Farmer KU (2004) Understanding the patient with migraine: the evolution from episodic headache to chronic neurologic disease. A proposed classification of patients with headache. *Headache* 44:426-435.
- Cady RK, Voirin J, Farmer K, Browning R, Beach ME, Tarrasch J (2012b) Two center, randomized pilot study of migraine prophylaxis comparing paradigms using pre-emptive frovatriptan or daily topiramate: research and clinical implications. *Headache* 52:749-764.
- Cheng JK, Ji RR (2008) Intracellular signaling in primary sensory neurons and persistent pain. *Neurochemical research* 33:1970-1978.
- Damodaram S, Thalakoti S, Freeman SE, Garrett FG, Durham PL (2009) Tonabersat inhibits trigeminal ganglion neuronal-satellite glial cell signaling. *Headache* 49:5-20.
- Davies AJ, Kim YH, Oh SB (2010) Painful Neuron-Microglia Interactions in the Trigeminal Sensory System. *The Open Pain Journal* 14-28.
- Derosier F, Sheftell F, Silberstein S, Cady R, Ruoff G, Krishen A, Peykamian M (2012) Sumatriptan-naproxen and butalbital: a double-blind, placebo-controlled crossover study. *Headache* 52:530-543.

- Diener HC, Bussone G, Van Oene JC, Lahaye M, Schwalen S, Goadsby PJ (2007) Topiramate reduces headache days in chronic migraine: a randomized, double-blind, placebo-controlled study. *Cephalalgia* 27:814-823.
- Diener HC, Tfelt-Hansen P, Dahlof C, Lainez MJ, Sandrini G, Wang SJ, Neto W, Vijapurkar U, Doyle A, Jacobs D (2004) Topiramate in migraine prophylaxis--results from a placebo-controlled trial with propranolol as an active control. *J Neurol* 251:943-950.
- Dodick D, Silberstein S (2006) Central sensitization theory of migraine: clinical implications. *Headache* 46 Suppl 4:S182-191.
- Doehring A, Oertel BG, Sittl R, Lotsch J (2013) Chronic opioid use is associated with increased DNA methylation correlating with increased clinical pain. *Pain* 154:15-23.
- Durham PL (2008) Inhibition of calcitonin gene-related peptide function: a promising strategy for treating migraine. *Headache* 48:1269-1275.
- Durham PL, Garrett FG (2010) Emerging importance of neuron-satellite glia interactions within trigeminal ganglia in craniofacial pain. *Open Pain J* 3:3-13.
- Durham PL, Vause CV (2010) Calcitonin gene-related peptide (CGRP) receptor antagonists in the treatment of migraine. *CNS drugs* 24:539-548.
- Friedman DI, De ver Dye T (2009) Migraine and the environment. *Headache* 49:941-952.
- Garrett FG, Durham PL (2009) Differential expression of connexins in trigeminal ganglion neurons and satellite glial cells in response to chronic or acute joint inflammation. *Neuron Glia Biol* 1-12.
- Garrett FG, Hawkins JL, Overmyer AE, Hayden JB, Durham PL (2012) Validation of a novel rat-holding device for studying heat- and mechanical-evoked trigeminal nocifensive behavioral responses. *J Orofac Pain* 26:337-344.
- Giniatullin R, Nistri A, Fabbretti E (2008) Molecular mechanisms of sensitization of pain-transducing P2X3 receptors by the migraine mediators CGRP and NGF. *Molecular neurobiology* 37:83-90.
- Gosselin RD, Suter MR, Ji RR, Decosterd I (2010) Glial cells and chronic pain. *Neuroscientist* 16:519-531.
- Guo W, Wang H, Watanabe M, Shimizu K, Zou S, LaGraize SC, Wei F, Dubner R, Ren K (2007) Glial-cytokine-neuronal interactions underlying the mechanisms of persistent pain. *J Neurosci* 27:6006-6018.

- Hanada T, Yoshimura A (2002) Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev* 13:413-421.
- Hawkins JL, Denson JE, Miley DR, Durham PL (2015) Nicotine stimulates expression of proteins implicated in peripheral and central sensitization. *Neuroscience* 290:115-125.
- Headache Classification Subcommittee of the International Headache S (2004) The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* 24 Suppl 1:9-160.
- Hu HJ, Glauner KS, Gereau RWt (2003) ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. I. Modulation of A-type K⁺ currents. *J Neurophysiol* 90:1671-1679.
- Hucho T, Levine JD (2007) Signaling pathways in sensitization: toward a nociceptor cell biology. *Neuron* 55:365-376.
- Ikeda H, Kiritoshi T, Murase K (2012) Contribution of microglia and astrocytes to the central sensitization, inflammatory and neuropathic pain in the juvenile rat. *Mol Pain* 8:43.
- Jakubowski M, Levy D, Kainz V, Zhang XC, Kosaras B, Burstein R (2007) Sensitization of central trigeminovascular neurons: blockade by intravenous naproxen infusion. *Neuroscience* 148:573-583.
- Ji RR, Berta T, Nedergaard M (2013) Glia and pain: is chronic pain a gliopathy? *Pain* 154 Suppl 1:S10-28.
- Ji RR, Gereau RWt, Malcangio M, Strichartz GR (2009) MAP kinase and pain. *Brain research reviews* 60:135-148.
- Kim C, Vigil D, Anand G, Taylor SS (2006) Structure and dynamics of PKA signaling proteins. *Eur J Cell Biol* 85:651-654.
- Kohno T, Wang H, Amaya F, Brenner GJ, Cheng JK, Ji RR, Woolf CJ (2008) Bradykinin enhances AMPA and NMDA receptor activity in spinal cord dorsal horn neurons by activating multiple kinases to produce pain hypersensitivity. *J Neurosci* 28:4533-4540.
- Kreek MJ, Levran O, Reed B, Schlussman SD, Zhou Y, Butelman ER (2012) Opiate addiction and cocaine addiction: underlying molecular neurobiology and genetics. *J Clin Invest* 122:3387-3393.

- Lazarov NE (2002) Comparative analysis of the chemical neuroanatomy of the mammalian trigeminal ganglion and mesencephalic trigeminal nucleus. *Progress in neurobiology* 66:19-59.
- Lazarov NE (2007) Neurobiology of orofacial proprioception. *Brain research reviews* 56:362-383.
- Levy D, Strassman AM (2002) Distinct sensitizing effects of the cAMP-PKA second messenger cascade on rat dural mechanonociceptors. *J Physiol* 538:483-493.
- Lipton RB, Bigal ME (2005) Migraine: epidemiology, impact, and risk factors for progression. *Headache* 45 Suppl 1:S3-S13.
- Lipton RB, Bigal ME, Diamond M, Freitag F, Reed ML, Stewart WF (2007) Migraine prevalence, disease burden, and the need for preventive therapy. *Neurology* 68:343-349.
- Lipton RB, Bigal ME, Diamond M, Freitag F, Reed ML, Stewart WF (2011) Migraine prevalence, disease burden, and the need for preventive therapy. *Neurology* 77:1905.
- Loder E, Weizenbaum E, Frishberg B, Silberstein S, American Headache Society Choosing Wisely Task F (2013) Choosing wisely in headache medicine: the American Headache Society's list of five things physicians and patients should question. *Headache* 53:1651-1659.
- Manack AN, Buse DC, Lipton RB (2011) Chronic migraine: epidemiology and disease burden. *Current pain and headache reports* 15:70-78.
- Mathew NT (2001) Antiepileptic drugs in migraine prevention. *Headache* 41 Suppl 1:S18-24.
- Menken M, Munsat TL, Toole JF (2000) The global burden of disease study: implications for neurology. *Archives of neurology* 57:418-420.
- Nosedá R, Burstein R (2013) Migraine pathophysiology: anatomy of the trigeminovascular pathway and associated neurological symptoms, CSD, sensitization and modulation of pain. *Pain* 154 Suppl 1.
- Ohsawa K, Imai Y, Sasaki Y, Kohsaka S (2004) Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity. *Journal of neurochemistry* 88:844-856.
- Okada-Ogawa A, Porreca F, Meng ID (2009) Sustained morphine-induced sensitization and loss of diffuse noxious inhibitory controls in dura-sensitive medullary dorsal horn neurons. *J Neurosci* 29:15828-15835.

- Olesen J (2006) International Classification of Headache Disorders, Second Edition (ICHD-2): current status and future revisions. *Cephalalgia* 26:1409-1410.
- Ren K (2009) Neuron, Glia and Reciprocal Relationships in Pain Processing. *Open Pain J* 2:7-31.
- Ren K, Dubner R (1999) Central nervous system plasticity and persistent pain. *J Orofac Pain* 13:155-163; discussion 164-171.
- Ren K, Dubner R (2008) Neuron-glia crosstalk gets serious: role in pain hypersensitivity. *Curr Opin Anaesthesiol* 21:570-579.
- Romero-Reyes M, Pardi V, Akerman S (2015) A potent and selective calcitonin gene-related peptide (CGRP) receptor antagonist, MK-8825, inhibits responses to nociceptive trigeminal activation: Role of CGRP in orofacial pain. *Experimental neurology* 271:95-103.
- Saper JR, Da Silva AN (2013) Medication overuse headache: history, features, prevention and management strategies. *CNS drugs* 27:867-877.
- Schramm SH, Obermann M, Katsarava Z, Diener HC, Moebus S, Yoon MS (2013) Epidemiological profiles of patients with chronic migraine and chronic tension-type headache. *The journal of headache and pain* 14:40.
- Schulman EA, Lake AE, 3rd, Goadsby PJ, Peterlin BL, Siegel SE, Markley HG, Lipton RB (2008) Defining refractory migraine and refractory chronic migraine: proposed criteria from the Refractory Headache Special Interest Section of the American Headache Society. *Headache* 48:778-782.
- Schwedt TJ (2014) Chronic migraine. *BMJ* 348:g1416.
- Seybold VS (2009) The role of peptides in central sensitization. *Handb Exp Pharmacol* 451-491.
- Shankland WE (2001a) The trigeminal nerve. Part II: the ophthalmic division. *Cranio* 19:8-12.
- Shankland WE, 2nd (2000) The trigeminal nerve. Part I: An over-view. *Cranio* 18:238-248.
- Shankland WE, 2nd (2001b) The trigeminal nerve. Part III: The maxillary division. *Cranio* 19:78-83.
- Shankland WE, 2nd (2001c) The trigeminal nerve. Part IV: the mandibular division. *Cranio* 19:153-161.
- Silberstein SD (2005) Topiramate in migraine prevention. *Headache* 45 Suppl 1:S57-65.

- Silberstein SD (2006) Preventive treatment of migraine. *Trends Pharmacol Sci* 27:410-415.
- Stewart WF, Lipton RB, Kolodner K (2003) Migraine disability assessment (MIDAS) score: relation to headache frequency, pain intensity, and headache symptoms. *Headache* 43:258-265.
- Stovner L, Hagen K, Jensen R, Katsarava Z, Lipton R, Scher A, Steiner T, Zwart JA (2007) The global burden of headache: a documentation of headache prevalence and disability worldwide. *Cephalalgia* 27:193-210.
- Sun RQ, Tu YJ, Lawand NB, Yan JY, Lin Q, Willis WD (2004) Calcitonin gene-related peptide receptor activation produces PKA- and PKC-dependent mechanical hyperalgesia and central sensitization. *J Neurophysiol* 92:2859-2866.
- Suzuki I, Harada T, Asano M, Tsuboi Y, Kondo M, Gionhaku N, Kitagawa J, Kusama T, Iwata K (2007) Phosphorylation of ERK in trigeminal spinal nucleus neurons following passive jaw movement in rats with chronic temporomandibular joint inflammation. *J Orofac Pain* 21:225-231.
- Thalakoti S, Patil VV, Damodaram S, Vause CV, Langford LE, Freeman SE, Durham PL (2007) Neuron-glia signaling in trigeminal ganglion: implications for migraine pathology. *Headache* 47:1008-1023.
- Villa G, Ceruti S, Zanardelli M, Magni G, Jasmin L, Ohara PT, Abbracchio MP (2010) Temporomandibular joint inflammation activates glial and immune cells in both the trigeminal ganglia and in the spinal trigeminal nucleus. *Mol Pain* 6:89.
- Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V, Abraham J, Ackerman I, Aggarwal R, Ahn SY, Ali MK, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Bahalim AN, Barker-Collo S, Barrero LH, Bartels DH, Basanez MG, Baxter A, Bell ML, Benjamin EJ, Bennett D, Bernabe E, Bhalla K, Bhandari B, Bikbov B, Bin Abdulhak A, Birbeck G, Black JA, Blencowe H, Blore JD, Blyth F, Bolliger I, Bonaventure A, Boufous S, Bourne R, Boussinesq M, Braithwaite T, Brayne C, Bridgett L, Brooker S, Brooks P, Brugha TS, Bryan-Hancock C, Bucello C, Buchbinder R, Buckle G, Budke CM, Burch M, Burney P, Burstein R, Calabria B, Campbell B, Canter CE, Carabin H, Carapetis J, Carmona L, Cella C, Charlson F, Chen H, Cheng AT, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahiya M, Dahodwala N, Damsere-Derry J, Danaei G, Davis A, De Leo D, Degenhardt L, Dellavalle R, Delossantos A, Denenberg J, Derrett S, Des Jarlais DC, Dharmaratne SD, Dherani M, Diaz-Torne C, Dolk H, Dorsey ER, Driscoll T, Duber H, Ebel B, Edmond K, Elbaz A, Ali SE, Erskine H, Erwin PJ, Espindola P, Ewoigbokhan SE, Farzadfar F, Feigin V, Felson DT, Ferrari A, Ferri

CP, Fevre EM, Finucane MM, Flaxman S, Flood L, Foreman K, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabbe BJ, Gabriel SE, Gakidou E, Ganatra HA, Garcia B, Gaspari F, Gillum RF, Gmel G, Gosselin R, Grainger R, Groeger J, Guillemin F, Gunnell D, Gupta R, Haagsma J, Hagan H, Halasa YA, Hall W, Haring D, Haro JM, Harrison JE, Havmoeller R, Hay RJ, Higashi H, Hill C, Hoen B, Hoffman H, Hotez PJ, Hoy D, Huang JJ, Ibeanusi SE, Jacobsen KH, James SL, Jarvis D, Jasrasaria R, Jayaraman S, Johns N, Jonas JB, Karthikeyan G, Kassebaum N, Kawakami N, Keren A, Khoo JP, King CH, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lalloo R, Laslett LL, Lathlean T, Leasher JL, Lee YY, Leigh J, Lim SS, Limb E, Lin JK, Lipnick M, Lipshultz SE, Liu W, Loane M, Ohno SL, Lyons R, Ma J, Mabweijano J, MacIntyre MF, Malekzadeh R, Mallinger L, Manivannan S, Marcenes W, March L, Margolis DJ, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGill N, McGrath J, Medina-Mora ME, Meltzer M, Mensah GA, Merriman TR, Meyer AC, Miglioli V, Miller M, Miller TR, Mitchell PB, Mocumbi AO, Moffitt TE, Mokdad AA, Monasta L, Montico M, Moradi-Lakeh M, Moran A, Morawska L, Mori R, Murdoch ME, Mwaniki MK, Naidoo K, Nair MN, Naldi L, Narayan KM, Nelson PK, Nelson RG, Nevitt MC, Newton CR, Nolte S, Norman P, Norman R, O'Donnell M, O'Hanlon S, Olives C, Omer SB, Ortblad K, Osborne R, Ozgediz D, Page A, Pahari B, Pandian JD, Rivero AP, Patten SB, Pearce N, Padilla RP, Perez-Ruiz F, Perico N, Pesudovs K, Phillips D, Phillips MR, Pierce K, Pion S, Polanczyk GV, Polinder S, Pope CA, 3rd, Popova S, Porrini E, Pourmalek F, Prince M, Pullan RL, Ramaiah KD, Ranganathan D, Razavi H, Regan M, Rehm JT, Rein DB, Remuzzi G, Richardson K, Rivara FP, Roberts T, Robinson C, De Leon FR, Ronfani L, Room R, Rosenfeld LC, Rushton L, Sacco RL, Saha S, Sampson U, Sanchez-Riera L, Sanman E, Schwebel DC, Scott JG, Segui-Gomez M, Shahraz S, Shepard DS, Shin H, Shrivastava R, Singh D, Singh GM, Singh JA, Singleton J, Sleet DA, Sliwa K, Smith E, Smith JL, Stapelberg NJ, Steer A, Steiner T, Stolk WA, Stovner LJ, Sudfeld C, Syed S, Tamburlini G, Tavakkoli M, Taylor HR, Taylor JA, Taylor WJ, Thomas B, Thomson WM, Thurston GD, Tleyjeh IM, Tonelli M, Towbin JA, Truelsen T, Tsilimbaris MK, Ubeda C, Undurraga EA, van der Werf MJ, van Os J, Vavilala MS, Venketasubramanian N, Wang M, Wang W, Watt K, Weatherall DJ, Weinstock MA, Weintraub R, Weisskopf MG, Weissman MM, White RA, Whiteford H, Wiersma ST, Wilkinson JD, Williams HC, Williams SR, Witt E, Wolfe F, Woolf AD, Wulf S, Yeh PH, Zaidi AK, Zheng ZJ, Zonies D, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA (2012) Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380:2163-2196.

Watkins LR, Hutchinson MR, Johnston IN, Maier SF (2005) Glia: novel counter-regulators of opioid analgesia. *Trends Neurosci* 28:661-669.

Wieczorkiewicz-Plaza A, Plaza P, Maciejewski R, Czuczwar M, Przesmycki K (2004)
Effect of topiramate on mechanical allodynia in neuropathic pain model in rats.
Pol J Pharmacol 56:275-278.

Xie YF (2008) Glial involvement in trigeminal central sensitization. Acta Pharmacol Sin
29:641-645.