



MSU Graduate Theses

Fall 2015

Calcitonin Gene-Related Peptide Promotes Peripheral and Central Trigeminal Sensitization

Lauren Elise Cornelison

As with any intellectual project, the content and views expressed in this thesis may be considered objectionable by some readers. However, this student-scholar's work has been judged to have academic value by the student's thesis committee members trained in the discipline. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

Follow this and additional works at: <https://bearworks.missouristate.edu/theses>

 Part of the [Biology Commons](#)

Recommended Citation

Cornelison, Lauren Elise, "Calcitonin Gene-Related Peptide Promotes Peripheral and Central Trigeminal Sensitization" (2015). *MSU Graduate Theses*. 971.
<https://bearworks.missouristate.edu/theses/971>

This article or document was made available through BearWorks, the institutional repository of Missouri State University. The work contained in it may be protected by copyright and require permission of the copyright holder for reuse or redistribution.

For more information, please contact BearWorks@library.missouristate.edu.

**CALCITONIN GENE-RELATED PEPTIDE PROMOTES PERIPHERAL AND
CENTRAL TRIGEMINAL SENSITIZATION**

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

Lauren Cornelison

December 2015

Copyright 2015 by Lauren Cornelison

CALCITONIN GENE-RELATED PEPTIDE PROMOTES PERIPHERAL AND CENTRAL TRIGEMINAL SENSITIZATION

Biology

Missouri State University, December 2015

Master of Science

Lauren Cornelison

ABSTRACT

Temporomandibular joint disorder is characterized by peripheral and central sensitization of trigeminal nociceptive neurons. Although CGRP is implicated in the development of central sensitization by stimulating glial activation via its receptor, the mechanism by which CGRP promotes and maintains sensitization of trigeminal nociceptive neurons is not well understood. The goal of my study was to investigate the role of calcitonin gene-related peptide (CGRP) on the initiation and maintenance of a nocifensive withdrawal response to mechanical stimulation following activation of primary trigeminal sensory neurons. For my studies, I used adult male Sprague Dawley rats that were injected with CGRP alone or co-injected with inhibitors and determined changes in nocifensive behavior and inflammatory proteins. Intrathecal injection of CGRP increased nocifensive responses to mechanical stimulation up to 48 hours and this stimulatory effect was blocked by the antagonist peptide CGRP₈₋₃₇ and a protein kinase A inhibitor. Results from my cellular studies provide evidence that elevated levels of CGRP in the spinal cord can promote bidirectional signaling within the trigeminal system, a novel finding that helps to explain how central sensitization can lower the activation threshold of primary nociceptors in TMD patients.

KEYWORDS: TMJ, calcitonin gene-related peptide, trigeminal ganglion, nociception, neuronal sensitization.

This abstract is approved as to form and content

Paul L. Durham, PhD
Chairperson, Advisory Committee
Missouri State University

**CALCITONIN GENE-RELATED PEPTIDE PROMOTES PERIPHERAL AND
CENTRAL TRIGEMINAL SENSITIZATION**

By
Lauren Cornelison

A Masters Thesis
Submitted to the Graduate College
Of Missouri State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science, Biology

December 2015

Approved:

Paul Durham, PhD

Laszlo Kovacs, PhD

Ryan Udan, PhD

Julie Masterson, PhD: Dean, Graduate College

ACKNOWLEDGEMENTS

I would like to thank Dr. Paul Durham for welcoming me into his lab and for his advice and support throughout my education. Additionally, I thank my committee members Dr. Ryan Udan and Laszlo Kovacs for their advice and suggestions during to improve my thesis, as well as the members of my lab for their encouragement and assistance. I would particularly like to thank Jordan Hawkins for teaching me many of the techniques utilized in my experiments.

TABLE OF CONTENTS

Introduction.....	1
Temporomandibular Joint Disorder.....	1
Trigeminal Nerve and Trigeminal Ganglia.....	2
Trigeminal Nociception Pathway	4
Peripheral and Central Sensitization.....	5
Molecules Implicated in Promoting and Maintaining Neuronal Sensitization.....	7
Hypothesis and Goals of Thesis Research	9
Materials and Methods.....	13
Animals and Reagents.....	13
Behavioral Testing.....	14
Immunohistochemistry, Cytokine Array, and Retrograde Dye Studies	16
Results.....	23
Behavioral Testing.....	23
Immunohistochemistry in the Upper Spinal cord	25
Cytokine Arrays	28
P-ERK.....	28
Fast Blue	29
Discussion	49
CGRP Promotes Trigeminal Nociceptor Sensitization.....	49
CGRP-Mediated Sensitization Involves PKA	50
CGRP Induces Astrocyte Activation and Increased Cytokine Expression.....	53
CGRP Promotes Bidirectional Signaling within the Trigeminal System	54
Summary and Future Directions	56
References	59

LIST OF TABLES

Table 1. Summary of Antibodies and Conditions Used for Immunohistochemistry.....	20
Table 2. Summary of Changes in PKA Expression in the Upper Spinal Cord.....	30
Table 3. Summary of Changes in GFAP Expression in the Upper Spinal Cord	31
Table 4. Summary of Changes in Iba1 Expression in the Upper Spinal Cord.....	32

LIST OF FIGURES

Figure 1. Schematic depiction of a pseudounipolar neuron that are found in a trigeminal ganglion.....	11
Figure 2. Identification of the neuronal and glial cell nuclei in adult rat trigeminal ganglion.....	12
Figure 3. Devices used for behavioral testing.....	21
Figure 4. Schematic of experimental timeline for behavioral testing.....	22
Figure 5. No difference in nocifensive withdrawals between naïve and vehicle controls.....	33
Figure 6. Increased nocifensive withdrawals after CGRP injection.....	34
Figure 7. Decrease in number of nocifensive withdrawals in animals that received inhibitor co-injections in a time-dependent manner.	35
Figure 8. No detectable staining pattern in observed in tissues that were incubated with secondary antibodies only.....	36
Figure 9. Increased expression of PKA in upper spinal cord in response to CGRP injection two hours prior.....	37
Figure 10. Increased expression of PKA in upper spinal cord in response to CGRP injection 48 hours prior.....	38
Figure 11. Increased expression of PKA in upper spinal cord in response to CGRP injection 72 hours prior.....	39
Figure 12. Increased expression of GFAP in upper spinal cord compared to naïve control in response to CGRP injection two hours prior.	40
Figure 13. Increased expression of GFAP in upper spinal cord in response to CGRP injection 48 hours prior.....	41
Figure 14. Increased expression of GFAP in upper spinal cord compared in response to CGRP injection 72 hours prior.	42
Figure 15. Increased expression of Iba1 in upper spinal cord compared to naïve control in response to CGRP injection two hours prior	43

Figure 16. No change in expression of Iba1 in upper spinal cord in response to CGRP injection 48 hours prior	44
Figure 17. No change in expression of Iba1 in upper spinal cord in response to CGRP injection 72 hours prior	45
Figure 18. Intrathecal injection of CGRP caused prolonged elevation of cytokine expression in the upper spinal cord tissue.....	46
Figure 19. Elevated levels of CGRP stimulate increased expression of p-ERK in trigeminal ganglion neurons.	47
Figure 20. Diffuse fluorescence in the trigeminal ganglion following injection of Fast Blue dye intrathecally.....	48

INTRODUCTION

Temporomandibular Joint Disorder

The temporomandibular or jaw joint is comprised of bilateral hinges on the side of the face, and allows the motions necessary for speech, eating, and yawning. The hinges themselves are an articulation between the glenoid fossa of the temporal bone of the skull and the condylar processes of the mandible. Surrounding the joint is a fibrous membrane called the capsule. The fiber extends between the two articular surfaces, forming the temporomandibular disc, and posterior to the hinge splits vertically; this region is called the retrodiscal tissue. Two synovial sacs lubricate the joint, above and below the disc. Many muscles and ligaments attach to the mandible, and the retrodiscal tissue is heavily innervated. Given the complexity of this structure coupled with the frequency of jaw use, disorders involving this joint are quite common.

Temporomandibular disorder (TMD) is a condition involving pain in muscles of mastication and the temporomandibular joints, often accompanied by limited jaw movement (Dworkin et al., 1990; Poveda Roda et al., 2007; Manfredini et al., 2011). Temporomandibular joint disorders (TMJD) specifically affect the joint rather than the muscles. TMJD is more often unilateral and more frequently associated with trauma than the more general TMD, which may be associated with stress or entirely idiopathic. Nomenclature of these disorders is not universal, and some professionals may simply split TMD into categories of myogenous (muscle-related) or arthrogenous (joint-related). Millions of American adults experience symptoms associated with TMD, such as jaw pain, restriction of movement, swelling, headache, and tinnitus (Bevilaqua Grossi et al.

2009; Mujakperuo et al., 2010; Ohrbach et al., 2011). Men and women are both impacted, with the highest incidence of pain during adolescence (Manfredini et al., 2011). TMD symptoms are often comorbid with other disorders that are indicative of a systemically sensitized state, such as fibromyalgia (Light et al., Aaron et al., 2000; Dahan et al., 2015; Furquim et al., 2015).

Trigeminal Nerve and Trigeminal Ganglion

The trigeminal nerve is the most complex of the cranial nerves (Shankland, 2000). It originates in a sensory root as well as a motor root at the border of the pons, and is further divided into three major sections: the ophthalmic, maxillary, and mandibular. The ophthalmic branch, also referred to as V1, provides only sensory input and is responsible for innervating the scalp, forehead, upper eyelid, eyebrow, eye, portions of the nasal cavity, the tentorium cerebelli, posterior flax cerebri and dura mater (Shankland, 2001a). Headache disorders can be primarily attributed to this portion of the trigeminal nerve. The second division is also purely afferent in function and intermediate in size. It innervates the maxillary bone and the midfacial region, including the skin of the midfacial regions, lower eyelid, side of nose and upper lip, the mucous membrane of the nasopharynx, maxillary sinus, soft palate, palatine tonsil, roof of the mouth, maxillary gingivae, and maxillary teeth (Shankland, 2001b). It is responsible for transmission of pain arising in the sinus cavities. The third and largest division of the nerve is the mandibular, which performs both afferent and efferent functions. It is composed of two separate roots, a large sensory root and a smaller motor root. The sensory root innervates the skin of the temporal region and lower third of the face, lower lip, and ear, the mucous

membrane of the anterior two-thirds of the tongue and mouth floor, the muscles of the first brachial arch, and the teeth and gingivae of the mandible (Shankland, 2001c). The small motor root supplies efferent innervation to the muscles of the first brachial arch which consist of the muscles of mastication, the tensors veli palatini and tympani, the mylohyoid muscle, and anterior belly of the digastrics muscle. This portion of the nerve is activated during TMD (Sessle, 2011).

The cell bodies of the sensory fibers making up the trigeminal nerve are located in the trigeminal ganglion (TG). These neurons fall into two basic morphological types known as small dark (SD) and large light (LL) neurons. Large light neurons give rise to myelinated A δ fibers, which transmit signal more quickly. Unmyelinated, slower transmitting C-fibers extend from small dark neurons. Both of these neuron types are pseudounipolar, with two diverging axonal branches (Fig. 1). The peripheral process extends to the cranial or facial area the neuron innervates, while a central process ascends to the spinal trigeminal nucleus and synapses with second order neurons (Devor, 1999; Rathee et al., 2002). The trigeminal ganglion is also populated by two types of supportive glial cells. Schwann cells provide the myelination to neuronal fibers, contributing to increased signal speed, while satellite glia modulate the environment surrounding individual neuronal cell bodies and modulate their excitability state. As seen in Figure 2, a neuron and its surrounding satellite glia comprise a single functional unit, which develops during the first weeks after birth and functions to maintain homeostasis (Hanani, 2005; Durham and Garrett, 2010a). Importantly, increased neuron-glia communication via paracrine signaling and gap junctions are associated with diseases of the head and face involving trigeminal nerve activation (Durham and Garrett, 2010b).

Trigeminal Nociception Pathway

Sensory fibers exit the trigeminal ganglion to synapse with second order neurons in the trigeminal nuclei (Bereiter et al., 2000; Takeshita et al., 2001). The most inferior of the three sensory nuclei is the spinal trigeminal nucleus (STN), and receives information from both neuronal types in all three branches of the trigeminal nerve. This nucleus can be subdivided into the subnuclei, from rostral to caudal, oralis, interpolaris, and caudalis (Davies et al.; 2010). The trigeminal nucleus caudalis (TNC) is the most inferior and processes nociceptive signal from the jaws, teeth, and face (Wilcox et al., 2015). From the TNC, second order neurons relay nociceptive signal to the thalamus, where third order neurons transmit the information to the somatosensory cortex (Shankland, 2000; Bernstein and Burstein, 2012).

Similar to the cellular organization of the TG, the STN is comprised of both neuronal and glial cells (Milligan and Watkins, 2009; Woolf, 2011). The glial cells located within the central nervous system include astrocytes, microglia, and oligodendrocytes. Astrocytes modulate the extracellular environment by regulating the concentrations of ions and neurotransmitters, control energy supply to neurons, and regulate synaptic formation and transmission (Watkins, et al. 2007). Microglia are resident phagocytic cells of the brain and spinal cord and play a key role in the development of chronic inflammatory and pain states. They express major histocompatibility complex class II receptors and function to monitor the environment for potential damage caused by increased expression of cytokines and chemokines. Once activated, microglia may migrate, proliferate, release inflammatory mediators, and alter receptor expression (Raivich, 2005; Ji et al., 2013; Lee, 2013). Both astrocytes and

microglia may be induced by neurotransmitters or other inflammatory molecules into an active state that results in the release of pro-inflammatory mediators (Miller et al., 2009; Ikeda et al., 2012).

Peripheral and Central Sensitization

Sensitization is a characteristic of neurons that have undergone molecular changes to reduce the amount of mechanical, chemical, or thermal stimuli required to cause their activation. In current pain models, peripheral sensitization of primary nociceptive neurons is thought to promote and sustain central sensitization of second order neurons involved in pain transmission to the thalamus (Sessle, 2011; Bernstein and Burstein, 2012). Within the trigeminal ganglion, satellite glia may become activated following neuronal dissemination of pain signals in response to chemical, thermal, or mechanical stimuli within peripheral tissues. Once activated, satellite glial cells produce molecules such as cytokines and chemokines, which have the potential to alleviate or exacerbate inflammation in the peripheral nervous system (Gosselin et al., 2010; Vause and Durham, 2010; Villa et al., 2010). Pro-inflammatory signaling from glia in combination with consistent nociceptive signals from the peripheral nervous system may chronically alter the excitability of second order neurons, leading to long-term hypersensitivity to noxious stimuli (Durham and Garrett, 2010b). This hypersensitivity might manifest as ion channels opening at lower voltages, excitatory ion channels staying open longer, or inhibitory ion channels taking longer to become active. Development of this sensitized state (central sensitization) leads to a decrease in the activation threshold and is a key

pathological feature of many trigeminal nerve mediated diseases including migraine, TMD, and allergic rhinitis (Woolf, 2011).

Peripheral sensitization results from the interaction of nociceptors with inflammatory substances released when tissue is damaged or inflamed (Sessle, 2011). This sensitization state may reduce the threshold of depolarization required for generation of action potentials within nociceptive neurons. Such electrical activity that facilitates depolarization subsequently promotes release of peptides and neurotransmitters that function to protect the site from further injury through development of hyperalgesia, defined as increased sensitivity to painful stimuli, and allodynia, which is the experience of pain arising from a non-painful stimulus, such as a light touch. In addition release of these pro-inflammatory molecules promotes healing by increasing blood flow, vascular permeability, and recruitment of white blood cells. While transient peripheral sensitization of nociceptive neurons that lasts for 24-48 hours is considered a normal physiological response, prolonged sensitization (>1 week) can lead to pathology. The different molecules comprising this inflammatory mixture may be promising targets for pain management strategies when peripheral sensitization becomes a chronic state.

Central sensitization refers to an increase in the excitability state of neurons in the dorsal horn of the spinal cord following high levels of activity in the nocifensive afferents (Ikeda et al., 2012; Furquim et al., 2015). This results in normally subthreshold activity becoming adequate to generate action potentials in the dorsal horn, further promoting hyperalgesia (increased pain sensitivity). Once central sensitization has been triggered by nociceptor activity, non-nociceptive afferent nerves may produce activation of the dorsal horn, thus causing allodynia (painful response to non-painful stimuli) that functions to

extend the receptive field in peripheral tissues. In acute models of pain, peripheral sensitization has been shown to promote cellular changes that mediate central sensitization (Dodick and Silberstein, 2006; Taylor and Corder, 2014). However, in chronic pain states, central sensitization persists in the absence of evidence of peripheral tissue damage. A major focus of my thesis research was to determine if central sensitization can mediate cellular changes to promote sensitization of peripheral nociceptive neurons.

Molecules Implicated in Promoting and Maintaining Neuronal Sensitization

Hypersensitivity in the central nervous system is dependent on both inter and intra-cellular signaling. Within cells, protein signaling may impact transcription, mRNA translation, and sensitivity of ion channels, production of neurotransmitter, and other functions that directly control sensitivity. One protein that is directly implicated in the initiation and maintenance of a prolonged sensitized state in the spinal cord is calcitonin gene-related peptide (CGRP), a 37 amino-acid neuropeptide formed through alternative splicing of the calcitonin gene (Rosenfeld et al., 1983; Benemei et al., 2009; Seybold, 2009). It is found in both central and peripheral neurons and was initially shown to function as a powerful vasodilator (Brain et al., 1985). More recently, increased levels of CGRP have been reported in serum and saliva of migraine patients (Bellamy et al., 2006; Cady et al., 2009) and in the capsule of TMJD patients (Holmlund et al., 1991; Spears et al., 2005). CGRP is now known to play a central role in promoting inflammation and nociception via its ability to promote increased blood flow, protein plasma extravasation, recruitment of immune cells, and decrease of the threshold for depolarization in

nociceptive neurons and associated glial cells (Durham and Vause, 2010). There are two isoforms of the peptide in both rats and humans, α and β (Fischer and Born, 1985). The α form is the predominant form found in trigeminal nerves, and will be referred to hereafter simply as CGRP. Its receptor complex is comprised of three separate proteins: a G-protein-coupled receptor (GPCR), calcitonin receptor-like receptor (CLR) and an accessory protein, receptor activity-modifying protein 1 (RAMP1) that confers ligand-binding specificity (Benemei et al., 2007; Durham, 2008). Following binding of CGRP to its receptor, the membrane bound enzyme adenylate cyclase is activated and causes an increase in the intracellular levels of the second messenger cAMP.

Protein Kinase A (PKA) is an extremely versatile kinase whose activity is dependent on the presence of cyclic adenosine monophosphate (cAMP) in the cell (Kim et al., 2006). Cyclic AMP binds to PKA's regulatory subunit, altering its affinity and causing it to dissociate, thus exposing PKA's catalytic subunit. The catalytic subunit may then phosphorylate a variety of target proteins, including CREB, a transcription factor implicated in neuroplasticity. In addition, elevated PKA levels in the cytosol are correlated with sensitization and activation of nociceptive neurons and glial cells via modulation of receptor expression and ion channel activity (Sun et al., 2004). CGRP is also known to cause activation of mitogen activated protein (MAP) kinases in trigeminal neurons, which are associated with increased inflammation and nociception and are used as biomarkers of neuronal and glial cell activation (Cady et al., 2011). Similar to PKA, MAP kinases are activated through a G-protein coupled receptor (GPCR) mediated mechanism, triggering a kinase cascade that can also impact transcription factors

including CREB, leading to increased synthesis of pro-inflammatory genes that contain CRE cis regulatory elements (e.g., CGRP, cytokines, chemokines) (Ji et al., 2009).

The expression of cytokines, a broad category of small signaling proteins is known to be regulated by CGRP, PKA, and MAP kinases (Miller et al., 2009; Vause and Durham, 2010, 2012; Ji et al., 2013). This large family includes chemokines, interleukins, and tumor necrosis factor, which function to modulate inflammatory responses to changes in chemical stimuli caused by infection or tissue injury. Cytokines may be both pro and anti-inflammatory. Pro-inflammatory cytokines include IL-1 β , IL-6, and TNF- α , all of which have been implicated in mechanisms of pathological pain (Kress, 2010; Song et al., 2014). Changes in cytokine expression and signaling by various cell types represent one mechanism by which normal inflammation due to an insult might transition to a maladaptive pain state. Within the central nervous system, astrocytes and microglia are known to synthesize and release cytokines to control the environment around neurons while in the trigeminal ganglion, satellite glial cells perform that function (Takeda et al., 2007; Damodaram et al., 2009; Gosselin et al., 2010; Ji et al., 2013).

Hypothesis and Goals of Thesis Research

Within the pain community, peripheral sensitization is traditionally considered to be the cause of central sensitization. However, it is possible that within some pathological conditions, central mechanisms may lower the activation threshold of primary nociceptive neurons within the trigeminal ganglion. For example, elevated levels of CGRP in the cerebral spinal fluid have been reported during and in between migraine

episodes. I hypothesize that elevated levels of CGRP with the cerebral spinal fluid of the upper spinal cord will promote sensitization of both central and peripheral nociceptive neurons, and thus provide novel evidence of bidirectional signaling within the trigeminal system.

Goals:

1. Determine if increased levels of CGRP in the upper spinal cord can lead to peripheral sensitization of primary trigeminal neurons to mechanical stimulation.
2. Investigate the mechanism by which CGRP promotes peripheral sensitization using inhibitors of the CGRP receptor and downstream signaling proteins.
3. Directly demonstrate bidirectional signaling from central processes of trigeminal neurons to ganglion cell bodies using a retrograde dye.
4. Explore the intracellular pathways involved in CGRP-mediated peripheral sensitization using immunohistochemistry.
5. Use a protein array to identify potential cytokines upregulated by CGRP-mediated pathways.

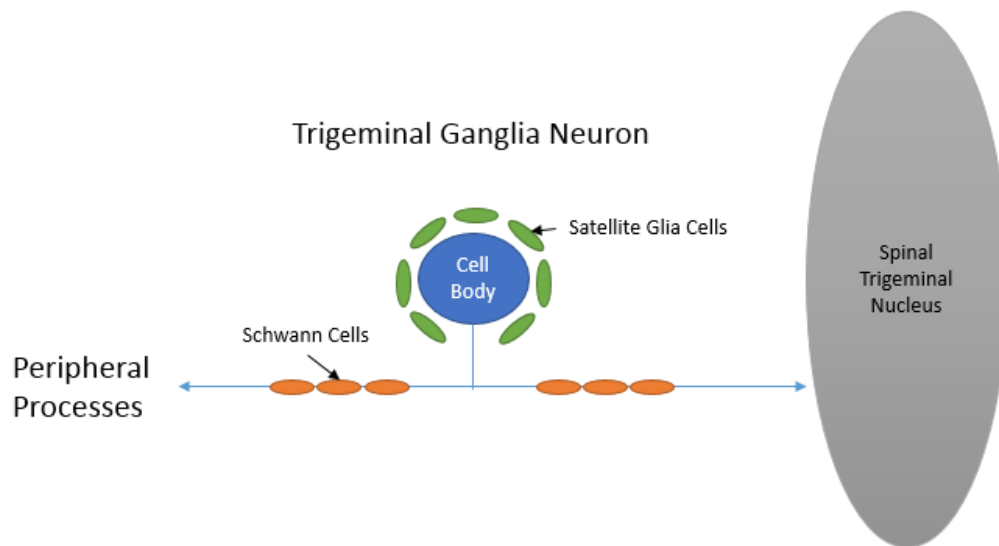


Figure 1. Schematic depiction of a pseudounipolar neuron that are found in a trigeminal ganglion. Satellite glial cells, which completely surround the neuronal cell body to create a functional unit, modulate neuronal excitability. Schwann cells are responsible for the production of myelin, which functions to speed nerve conduction. The neuron extends two processes from its cell body. One provides innervation of peripheral tissues in the head and face, while the other process projects into the upper spinal cord and synapses with second order nociceptive neurons.

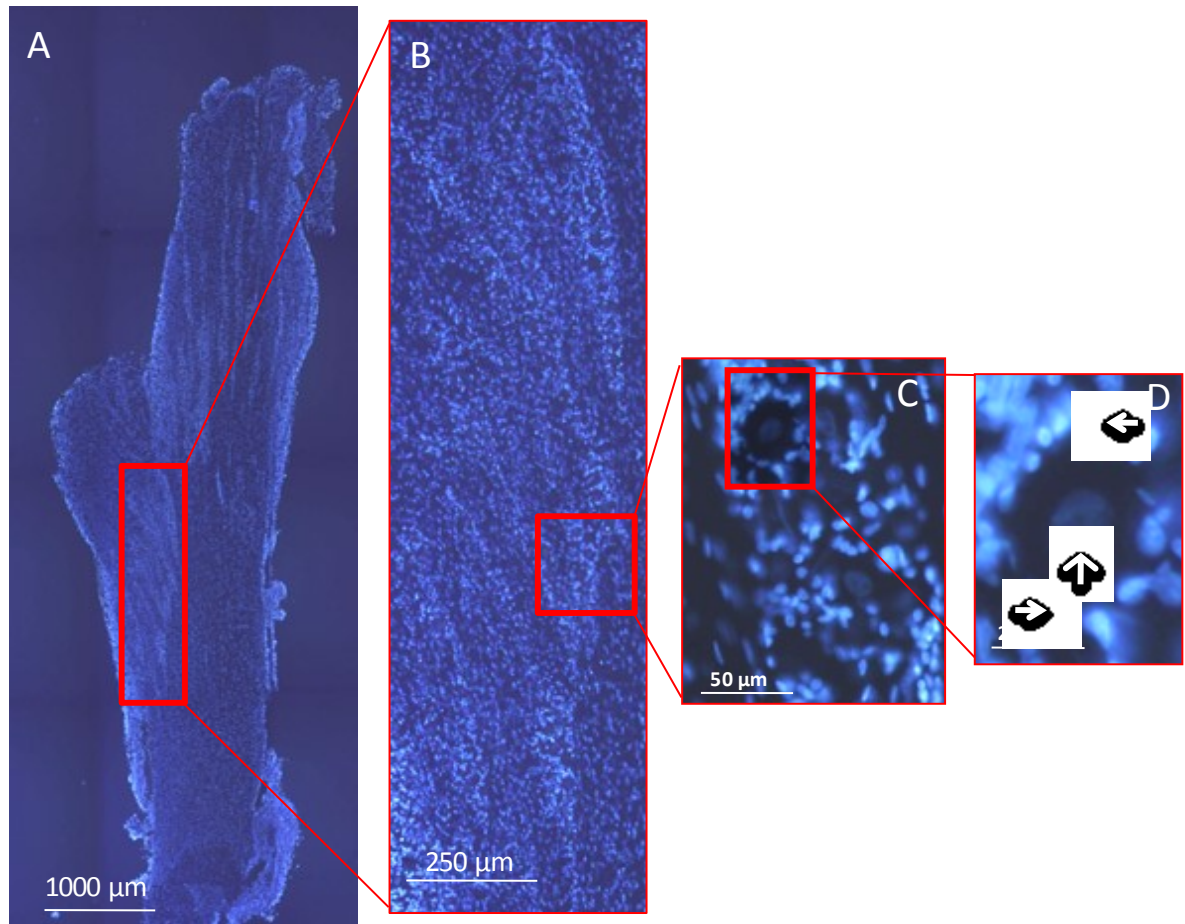


Figure 2. Identification of the neuronal and glial cell nuclei in adult rat trigeminal ganglion. (A) Longitudinal sections of the entire ganglion from an untreated animal were stained with DAPI to identify all nuclei (40x magnification). (B) Magnification (100x) of a neuron containing a section of the V3 branch of (A). (C) Magnification (400x) of a neuron-satellite glial cell cluster from (B). (D) An enlarged area from (C) showing a single neuron surrounded by satellite glial cells. The vertical arrow is pointing towards the nucleus of a single neuron while the horizontal arrows identify the nuclei of satellite glial cells.

MATERIALS AND METHODS

Animals and Reagents

Animal studies were approved by the Institutional Animal Care and Use Committee (Protocol #14-038.0 approved on August 16th, 2014) at Missouri State University. All studies were conducted in compliance with all established guidelines in the National Institutes of Health and the Animal Welfare Act. An effort was made to reduce the number of animals used in the study as well as to minimize suffering. Adult, male Sprague-Dawley rats (350-500 g) were obtained from Charles River Laboratories Inc. (Wilmington, MA) or purchased from Missouri State University (internal breeding colonies). All animals were housed in clean, plastic standard rat cages (VWR, West Chester, PA) in an animal holding room on a 12-hour light/dark cycle starting at 7 A.M. with ambient temperature maintained from 22-24°C and access to food and water *ad libitum*. Animals were acclimated to the environment for a minimum of 1 week upon arrival prior to use.

Stock solutions of CGRP or CGRP₈₋₃₇ (American Peptide Company, Sunnyvale, CA) were both prepared at a concentration of 1 mM in 0.9% saline solution (Fisher-Scientific, Fair Lawn, NJ) and frozen in aliquots of 5 µl at -20°C. The PKA inhibitor KT5720 (Tocris, Bristol, UK) was prepared at a stock concentration of 1 mM in DMSO (Sigma-Aldrich, St. Louis, MO) and frozen in aliquots of 5 µl at -20°C. On the day of the experiment, an aliquot of 1 mM CGRP was thawed and diluted in 0.9% sterile saline to a concentration of 1 µM either alone or in solution with one of the two inhibitors. The inhibitors CGRP₈₋₃₇ and KT5720 were prepared in 0.9% saline solution with CGRP at

concentrations of 5 μ M and 500 nM, respectively. The retrograde labeling dye Fast Blue (Polysciences Inc., Warrington, PA) was diluted to a concentration of 4% in sterile PBS with 1 μ M CGRP.

Behavioral Testing

Male Sprague-Dawley rats were used to determine whether injection with vehicle control or CGRP would cause the rats to develop a nocifensive response to mechanical stimulation. The procedure used was based on a study from our laboratory that validated using the Durham Animal Holder (Ugo Basile, Varese Lakes, Italy) to facilitate measurement of the nocifensive responses to mechanical stimulation in the head and face of rats by holding them still in an optimal position without exerting undue stress on the animal. (Garrett et al., 2012; Cady et al., 2014; Hawkins et al., 2015). Prior to testing, the rats were acclimated by guiding them into the holding device as shown in Figure 3. The animals were secured in the holder for 5 minutes using a plastic blockade inserted behind the hindpaws. To minimize false responses during von Frey testing, a pipette tip was used to touch the animal's head and face to acclimate the rats to having the cutaneous tissue over the masseter muscle touched with a filament. This was done for the three consecutive days prior to testing with von Frey filaments. During this acclimation period, if a rat appeared to be unwilling to go into the device or was continuously moving and shifting within the device, the animal was removed from the study.

Following acclimations, nocifensive thresholds were determined in response to a series of calibrated von Frey filaments (North Coast Medical, Inc., Gilroy, CA; 60, 100, 180, grams) applied to the cutaneous tissue over the masseter muscle. A positive response

was recorded when an animal visibly flinched away from a filament prior to it bending, while pressure was being applied. Each filament was applied five times on each side, and the data reported is the average number of withdrawals from those ten applications. If a filament failed to elicit more than three responses on a given side, a more forceful filament was applied in the same manner. The 100 gram filament was chosen for subsequent studies since baseline positive responses to this force were consistently less than one bilaterally, while the 180 gram filament regularly provoked more than three out of five nocifensive head withdrawal responses per side.

The day after establishing mechanical sensitivity baselines, the animals were anesthetized by inhalation of 5% isoflurane. Thorough anesthesia was confirmed by examining blink reflexes. The animals were then injected intrathecally using a 26 ½ gauge needle (Becton Dickinson, Franklin Lakes, NJ) and a 50 µL Hamilton syringe (Hamilton Company, Reno, NV) between the occipital bone and the first cervical vertebrae (C1) with CGRP (1 µM; 20 µl) either alone, or co-injected with CGRP₈₋₃₇ (5 µM; 20 µl) or KT 5720 (500 nM; 20 µl), a selective signaling inhibitor of PKA. Controls were injected with saline alone, and naïve controls received no treatment. These concentrations were selected based on previously published studies in which this concentration of CGRP elicited cellular changes within the trigeminal system (Cady et al., 2011). Mechanical testing for nocifensive reactions at 2 h, 24 h, 48 h, and 72 h time-points after injections was done in the same way baselines were established. A summary of my experimental design is shown in Figure 4.

For the mechanical stimulation studies, the data are reported as the mean number of withdrawal responses ± the standard error of the mean (S.E.M.) to 100 g of force at

each condition and time point. Subsequent analysis was then performed on data with $n = 6$ or greater for each experimental condition using a mixed design repeated measures ANOVA to test for general statistical significance, followed by a paired-samples t-test to find changes within subjects, and an independent samples t-test to test for differences between groups. Statistical significance was set at $P < 0.05$.

Immunohistochemistry, Cytokine Array, and Retrograde Dye Studies

To investigate changes in protein levels, injections were performed as described above with CGRP (1 μM ; 20 μl) alone or co-injected with CGRP₈₋₃₇ (5 μM ; 20 μl) or a selective signaling protein inhibitor of PKA (KT 5720). The brainstem and upper spinal cord (6 mm posterior to the obex) were removed at 2, 48, or 72 hours after injection. The spinal cord was split in half in the sagittal plane. One side was snap frozen in liquid nitrogen for protein extraction and protein array analysis. The other half was placed in 4% paraformaldehyde to be used for immunohistochemistry.

For the immunohistochemical analysis, spinal cord tissue was incubated in 4% paraformaldehyde at 4°C for approximately 24 hours. Tissues were then placed in 12.5% sucrose at 4°C, until they sank. They were then moved to 25% sucrose and left for at least 8 hours, until they sank. At this point they were stored at -20°C until they were ready for use. To section tissues, tissues were embedded in Optimal Cutting Temperature compound (OCT; Sakura Finetek, Torrance, CA). Transverse sections 14 μm in thickness were taken between 4 and 5 mm caudal to the obex of the upper spinal cord, using a cryostat set at -24°C. Sections were placed on Superfrost Plus slides (Fisher Scientific,

Pittsburg, PA) with the caudal side of the spinal cord in contact with the glass and stored at -20°C.

Slides populated with sections were rehydrated for 5 minutes in phosphate buffered saline (PBS), then permeabilized in a solution of 0.1% Triton X-100 in 5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 20 minutes at room temperature. Following this incubation, sections were thoroughly rinsed with PBS and incubated with primary antibodies for proteins of interest (Table 1) for either 3 hours at room temperature or overnight in a humidified chamber at 4°C. After this incubation was complete, the sections were incubated in solutions of secondary antibodies for one hour at room temperature, then mounted in Vectashield medium (H-1200) containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to stain cell nuclei in preparation of viewing using fluorescent microscopy.

Images were taken using a Zeiss Axiocam mRm camera mounted on a Zeiss Imager Z2 fluorescent microscope with an Apotome. Image acquisition was performed using Zeiss Zen 2012 software (Thornwood, NY). Once immunohistological images of the spinal cord were obtained, relative levels of the proteins of interest were analyzed using image J software. Fluorescent intensity was measured in ten rectangular regions in laminae I-III in the medullary horn. Average intensity from acellular regions was then measured and subtracted from the average intensity in the laminae. All data are presented as mean fold-change from the average naïve value \pm S.E.M. Values were pooled across time points for control conditions. Tissues from animals injected with CGRP were kept

separated by time point and compared to pooled control values. Analysis was performed by a one-way ANOVA with a Games-Howell post-hoc due to unequal variances.

For the cytokine arrays, tissues from the upper spinal cord were snap frozen after first being rinsed with ice-cold PBS to remove blood from neural tissue. Clean tissues were homogenized in 500 μ l of RIPA buffer (Thermo Scientific) with a plastic pestle and then allowed to incubate for 30 minutes on ice. Samples were then sonicated briefly. Finally, the tubes were centrifuged at 4° C for 20 minutes, at a relative centrifugal force of 10,000 x g to produce a pellet containing the cell membranes and debris from the cells as well as a supernatant containing the intracellular proteins of interest. The supernatant was divided into aliquots in 500 μ l tubes and stored at -20° C for array analysis. The amount of total protein was determined by the BCA method and for each experimental condition 200 μ g of protein was used for analysis. The relative levels of 19 cytokines were measured in control or CGRP-treated animals using RayBio Rat Cytokine Antibody Array 1.1 (RayBiotech, Norcross, GA). Detection of immunoreactivity was performed using a chemiluminescent peroxidase substrate (Pierce ECL Plus Western Blotting Substrate, Thermo Scientific, Waltham, MA). Silver halide film (Kodak BioMax Film, Sigma-Aldrich, St. Louis, MO) was exposed to the chemiluminescent blots for several different exposure times to provide a range of dot densities for semi-quantitative analysis.

For the cytokine arrays, densitometric analysis was performed essentially as described in previous publications from our lab with data points excluded from analysis if they were undistinguishable from background signal by the software (Vause and Durham, 2010, 2012, Cady et al., 2013, Hawkins et al., 2015). Results were analyzed using the protein array analyzer macro for Image J software (Schneider et al., 2012). A 2D rolling

ball background subtraction with a radius of 40 was used to normalize background levels. The pixel density of three pairs of dots was determined from blots with a short (10 seconds), medium (30 seconds), or long (2 minutes) exposure time based on their relative intensity. Three different exposure times were used so that the dots would be dark enough to measure density without being overexposed. Circles with a diameter of 7 pixels were placed over each dot on the protein array to determine pixel density. For analysis, the average pixel density of the background was subtracted from the pixel density for each dot. Each experimental condition was repeated in two independent experiments done in duplicate. Reported levels are the average fold-change value \pm standard error when compared to the average control level, which was made equal to one.

For the retrograde dye study, 50 μ L of Fast Blue and 1 μ M CGRP diluted in PBS, was injected into the intrathecal space between the occipital bone and C1 while the animal was anesthetized with 3.5% isoflurane in oxygen. Animals were allowed to recover as normal. Seven days later, the animals were euthanized via asphyxiation with carbon dioxide (CO₂) followed by decapitation. The brainstem and upper spinal cord (6 mm posterior to the obex) and trigeminal ganglia were removed and prepared for immunohistochemistry. Trigeminal ganglia were incubated in 4% paraformaldehyde, followed by 12.5% sucrose and 25% sucrose as described above. Following these incubations, ganglia were frozen, dorsal side up, in OCT. Longitudinal sections (14 μ m) were prepared and mounted on Superfrost Plus slides. In preparation for viewing, tissues were rehydrated with PBS and then mounted in Vectashield medium without DAPI to identify cells within the ganglion that exhibit fluorescence from retrograde transport of the dye from the spinal cord.

Table 1. Summary of Antibodies and Conditions Used for Immunohistochemistry.

Protein	Dilution	Company	Incubation Time	Incubation Temperature
GFAP	1:5,000	Abcam	3 hours	20-22°C
Iba1	1:500	Abcam	3 hours	20-22°C
NeuN	1:1,000	Millipore	3 hours	20-22°C
P-ERK	1:500	Bioworld	Overnight	20-22°C
PKA	1:500	Abcam	3 hours	4°C
Alexa 488	1:200	Life Technologies	1 hour	20-22°C
Alexa 567	1:200	Life Technologies	1 hour	20-22°C
Alexa 647	1:200	Life Technologies	1 hour	20-22°C

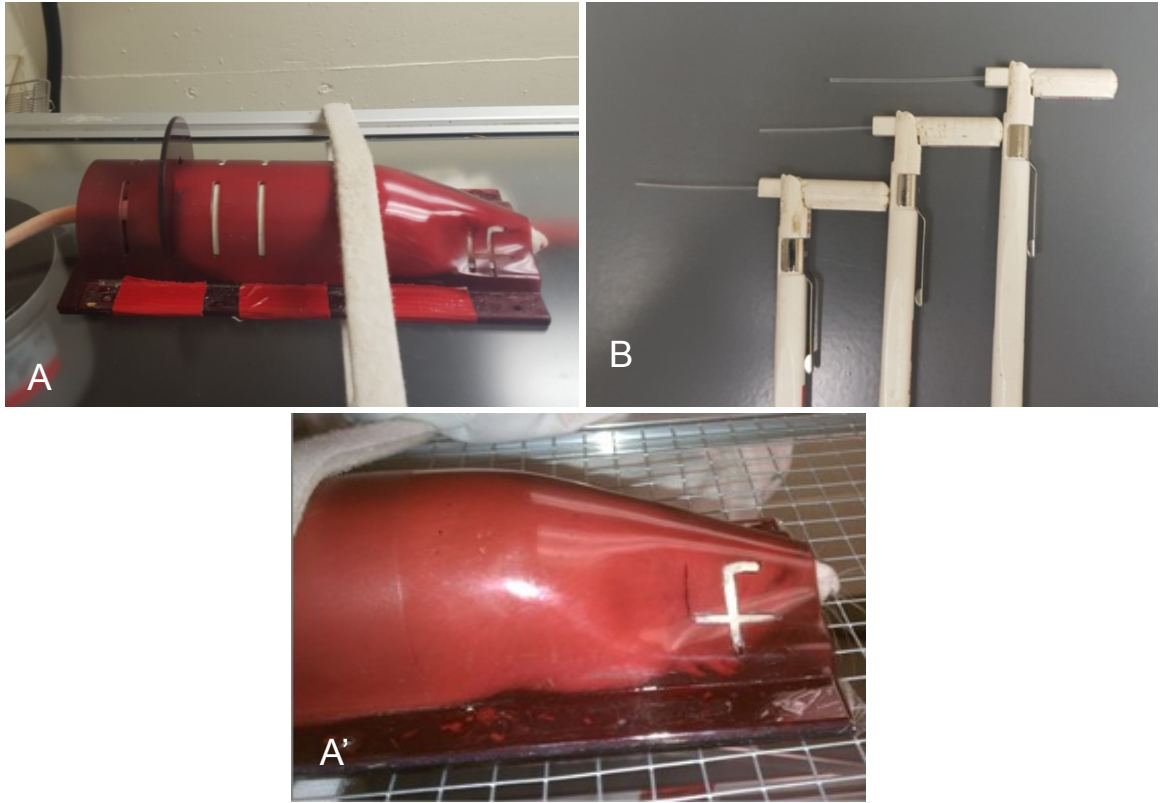


Figure 3. Devices used for behavioral testing. An animal restrained in the Durham holder (A) and an enlarged image of the holder showing cut out area that is used to gain access to specific regions in the rat's head and face (A'). Example of von Frey filaments used in my study (B). From left to right: 60 grams, 100 grams, and 180 grams.

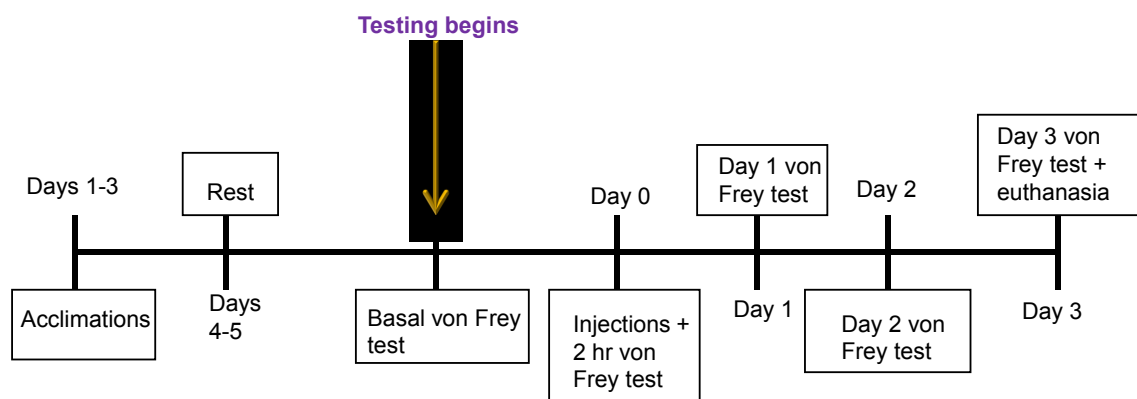


Figure 4. Schematic of experimental timeline for behavioral studies.

RESULTS

Behavioral Testing

Behavioral studies were used to determine whether CGRP injection into the cerebrospinal fluid of the upper spinal cord would produce a nocifensive response analogous with that reported in acute episodes in patients with TMD. Rats were defined as hypersensitive when their response to the filament of interest reached an average of 2.5 head withdrawals in response to mechanical stimulation over both TMJ. The filament determined to be the filament of interest had a pressure of 100 g, due to the observation that this force rarely elicited a positive response from naïve animals, while the proceeding force of 180 g almost always resulted in nocifensive reactions. The average number of withdrawals by control animals for both the left and right side to the 100 g filament 2 hours, 24 hours, and 48 hours in naïve animals and post saline injection can be seen in Figure 5. Naïve controls withdrew from the 100 g filament on average 0.7 times at baseline, 0.3 times at 2 hours, 0.9 times at 24 hours, and 1.3 times at 48 hours. Animals injected with saline alone withdrew from the 100 g filament an average of 0.7 times at baseline, 1.1 times 2 hours following injection, 1.3 times 24 hours following injection, and 0.9 times at 48 hours. The average number of withdrawal responses at baseline (basal condition) for the CGRP animals was similar to both naïve and saline control values (Fig. 6). There was no significant difference in basal responses between conditions. In contrast, increased nocifensive responses were observed in animals injected with 1 μ M CGRP at 2 hour, 24 hours, and 48 hours. However 72 hours post injection, the levels were again similar to control. Animals injected with CGRP withdrew from the 100 g filament an

average of 0.7 times at baseline, 2.7 times 2 hours following injection, 2.7 times 24 hours following injection, 3.3 times 48 hours after injection, and 1.29 times 72 hours following injections.

Statistical analysis of nocifensive withdrawals to the 100 g filament showed no difference between animals of different conditions prior to the treatment administration. Additionally, after injections, naïve and vehicle-injected controls did not show a significant difference in withdrawal rate at any time point. In contrast, the number of nocifensive withdrawals of animals injected with CGRP was significantly different from naïve ($p = 0.022$) and vehicle ($p = 0.015$) controls at 2 hours. Nocifensive withdrawal differences were still significant from vehicle ($p = 0.036$) and naïve ($p = 0.017$) at 24 hours. At 48 hours, the difference between CGRP and naïve animals remained significant ($p = 0.038$), as well as differences between animals injected with CGRP versus vehicle ($p = 0.002$). At day 3, animals injected with CGRP no longer showed differences in nocifensive responses compared to vehicle controls ($p = 0.904$) or naïve controls ($p = 0.091$). Taken together, elevated levels of CGRP in the upper spinal cord were shown to transiently increase the number of nocifensive head withdrawals in response to mechanical stimulation of trigeminal neurons.

To begin to determine the signaling pathways involved in CGRP mediated increases in nociception, animals were co-injected with CGRP and an inhibitor to the CGRP receptor or an inhibitor to the downstream signaling enzyme PKA (Fig. 7). Co-injecting CGRP with CGRP₈₋₃₇ suppressed the stimulatory effect of CGRP on the number of nociceptive withdrawals to the 100 g filament. Animals co-injected with CGRP and CGRP₈₋₃₇ responded on average 0.7 times under basal conditions, 1.3 times 2 hours post-

injection, 1.4 times at 24 hours and 0.83 times at 48 hours. Animals co-injected with CGRP and the PKA inhibitor KT5720 reacted to the 100 g filament an average of 0.5 times basally, 2.6 times 2 hours after injection, and 0.86 times at both 24 and 48 hours. Injection of KT5720 along with CGRP resulted in a time-sensitive amelioration of changes in nocifensive withdrawals. Two hours after injection, there was no significant difference between animals injected with CGRP alone and those who also received the inhibitor. However, at 24, 48, and 72 hours, animals that were simultaneously injected with CGRP and KT5720 reacted to the 100 g filament significantly less than those injected with only CGRP. Consistently, animals injected with the inhibitor exhibited a significant change from naïve and vehicle controls at the 2 hour time point, but failed to show significant difference for any subsequent time points. In addition, animals injected with both KT5720 and CGRP showed significant changes in nocifensive responses at two hours when compared back to their own baseline responses ($p = 0.012$), but failed to show significant changes at any other time point compared to basal levels.

Immunohistochemistry in the Upper Spinal Cord

Secondary antibodies were applied alone to tissues to evaluate their selectivity. The upper spinal cord tissue containing the medullary horn was dissected out and incubated with fluorescently-labeled secondary antibodies to determine the specificity of each of the antibodies used in my study. As seen in Figure 8, incubation with each of the secondary antibodies alone (without primary antibody) resulted in a diffuse staining pattern consistent with low level non-specific background staining of the upper spinal cord tissue.

PKA levels were evaluated between different treatment conditions. A summary of the fold-change \pm SEM in the relative intensity of PKA immunostaining compared to naïve levels (mean made equal to one) for each condition is shown in Table 2. In naïve animals, low levels of PKA immunostaining were detected in the upper spinal cord tissue (Fig. 9). The level of PKA staining was not significantly different from naïve controls in animals injected with sterile saline alone (0.97 fold, $p = 0.989$). At 2 hours, CGRP-injected animals exhibited an increase in PKA immunostaining intensity (2.43 fold, $p < 0.001$) in comparison with both naïve and vehicle controls. This increase was still present at 48 hours post-injection (2.43 fold, $p < 0.001$), while saline controls still showed no changes from naïve (1.15 fold, $p = 0.142$), as seen in Figure 10. At 72 hours, PKA levels in CGRP-injected animals remained significantly elevated when compared to naïve (1.82 fold, $p < 0.001$) and vehicle ($p = 0.004$) control levels (Fig. 11). Saline injection at this point still failed to elicit any significant change from naïve levels of PKA (1.04 fold, $p = 0.924$).

Levels of GFAP were compared between treatment conditions to evaluate changes in astrocyte activity in the spinal cord. A summary of the fold-change \pm SEM in the relative intensity of GFAP immunostaining compared to naïve levels (mean made equal to one) for each condition is shown in Table 3. Low levels of glial fibrillary acidic protein (GFAP) are present in upper spinal cord tissues of naïve animals. Injection of sterile saline alone was sufficient to increase expression of GFAP in astrocytes in the upper spinal cord (1.62, $p < 0.001$) two hours post-injection, as seen in Figure 12. Animals injected with CGRP were significantly different from naïve levels as well ($p <$

0.001). CGRP injected animals did not show significant changes compared with vehicle controls at 2 hours. Forty-eight hours after injection, GFAP levels in upper spinal cord from vehicle control animals were no longer significantly different from levels in naïve tissues (1.21 fold, $p = 0.538$, Fig. 13). In contrast, levels of GFAP in animals injected with CGRP were significantly higher from both naïve and vehicle controls at this time point (3.03 fold, $p < 0.001$). Seventy-two hours after injection, vehicle controls did not exhibit any significant change from naïve levels (1.23 fold, $p = 0.240$). Spinal cords from rats injected with CGRP still showed significantly different GFAP levels to those injected with saline (1.93 fold, $p < 0.001$), as seen in Figure 14.

Relative levels of Iba1 were assessed in the spinal cord to evaluate whether there were changes in microglia activity in response to CGRP. A summary of the fold-change \pm SEM in the relative intensity of Iba1 immunostaining compared to naïve levels (mean made equal to one) for each condition is shown in Table 4. Relative levels of Iba1, a marker for active microglia, were compared between upper spinal cord tissues from rats under various treatments as described above. Injection of sterile saline alone elicited a statistically significant change from naïve levels of Iba1 (1.41 fold, $p = 0.018$). Levels of Iba1 in spinal cord taken from animals injected with CGRP at two hours post-injection (Fig. 15) showed a modest increase of 1.3-fold over naïve that was statistically different from levels in naïve ($p = 0.010$) but not saline ($p = 0.974$) controls. This increase lessened to 1.24-fold and was no longer significant from naïve controls in tissues harvested 48 hours after injections ($p = 0.184$, Fig. 16). Vehicle controls were not significantly different from naïve levels at 48 hours after injections. ($p = 0.139$). These trends persisted at the 72 hour time point, when neither animals that received saline alone

nor CGRP showed significant changes in Iba1 expression from naïve animals ($p = 0.328$, $p = 0.257$, respectively. Fig. 17).

Cytokine Arrays

To test whether intrathecal administration of CGRP could promote temporal changes in cytokine expression, tissues from the upper spinal cord (obex to a distance 6 mm posterior to the obex) were isolated from control animals and animals receiving intrathecal injection of CGRP. As seen in Figure 18, 1 μ M CGRP caused a > 2-fold increase in the relative expression levels of 19 cytokines (out of 29 total cytokines) when compared to control levels 2 hours post injection and all remained elevated above control after 24 hours. The greatest increases were seen with CNTF and Fractalkine (> 4 fold) at the 2 hour time point. These data provide evidence of coordinated regulation of cytokines by CGRP within the upper spinal cord tissue.

P-ERK

To investigate if elevated CGRP levels in the spinal cord could mediate changes in gene expression peripherally, immunohistochemistry was performed to examine the localization of the active, phosphorylated form of extracellular signal-regulated kinase (p-ERK) in the neurons. While p-ERK immunostaining was observed in the cytosol of neuronal cell bodies in naïve animals, the active form was more localized in the nuclei of trigeminal neurons in animals 2 hours post CGRP injection (Fig. 19). ERK expression in neuronal nuclei was confirmed through tissue morphology and its co-localization with the NeuN antibody (data not shown).

Fast Blue

To test whether sensitization elicited centrally could promote changes in cell bodies peripherally, Fast Blue dye was co-injected with CGRP. This structure is small enough to be endocytosed in the terminals of primary neurons. Increased signaling in the central nervous system lead to larger amounts of the dye taken up that then have the potential to be transported to the peripheral nervous system. The fluorescent dye Fast Blue was observed in the cell body of trigeminal neurons and satellite glial cells 5 days after intrathecal co-injection of Fast Blue and 1 μ M CGRP. The diffuse pattern of fluorescence is indicative of the presence of active signaling between neurons and their related satellite glia. The presence of the dye in the ganglia shows the potential for small molecules present centrally to move in a retrograde manner to the peripheral nervous system, indicating a possible mechanism by which cellular events centrally might elicit changes peripherally.

Table 2. Summary of fold changes in PKA levels in the upper spinal cord in response to CGRP. All data are reported as the average fold change when compared to levels in naïve animals, which was made equal to one \pm the standard error of the mean.

Time After Injection	Vehicle Fold Change \pm SEM	CGRP Fold Change \pm SEM
2 hr	0.97 \pm 0.06	2.43 \pm 0.11 ^{#+}
48 hr	1.16 \pm 0.04	2.43 \pm 0.15 ^{#+}
72 hr	1.05 \pm 0.05	1.82 \pm 0.14 ^{#+}

* = significantly different from naïve
= significantly different from vehicle
+ = significantly different from naïve
Significant is defined by $p < 0.05$

Table 3. Summary of fold changes in GFAP levels in the upper spinal cord in response to CGRP. All data are reported as the average fold change when compared to levels in naïve animals, which was made equal to one \pm the standard error of the mean.

Time After Injection	Vehicle Fold Change \pm SEM	CGRP Fold Change \pm SEM
2 hr	1.62 \pm 0.11*	1.88 \pm 0.13
48 hr	1.21 \pm 0.14	3.03 \pm 0.17 ^{#+}
72 hr	1.23 \pm 0.10	1.93 \pm .20 ^{#+}

* = significantly different from naïve
= significantly different from vehicle
+ = significantly different from naïve
Significant is defined by $p < 0.05$

Table 4. Summary of fold changes in Iba1 levels in the upper spinal cord in response to CGRP. All data are reported as the average fold change when compared to levels in naïve animals, which was made equal to one \pm the standard error of the mean.

Time After Injection	Vehicle Fold Change \pm SEM	CGRP Fold Change \pm SEM
2 hr	1.41 \pm 0.11*	1.33 \pm 0.09*
48 hr	0.75 \pm 0.08	1.24 \pm 0.09
72 hr	0.83 \pm 0.06	1.10 \pm .13

* = significantly different from naïve
Significant is defined by $p < 0.05$

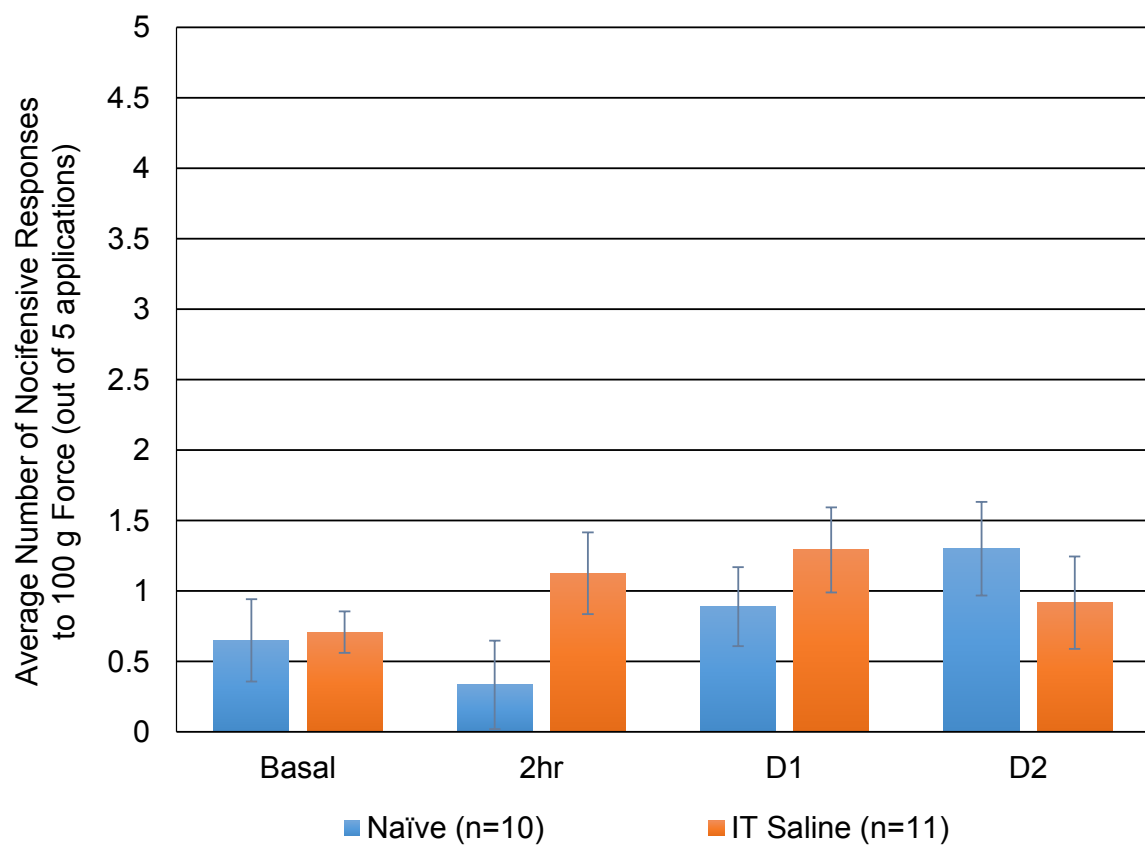


Figure 5. No significant difference in the number of nocifensive withdrawals to the 100 g filament between naïve animals and those injected intrathecally with sterile saline.

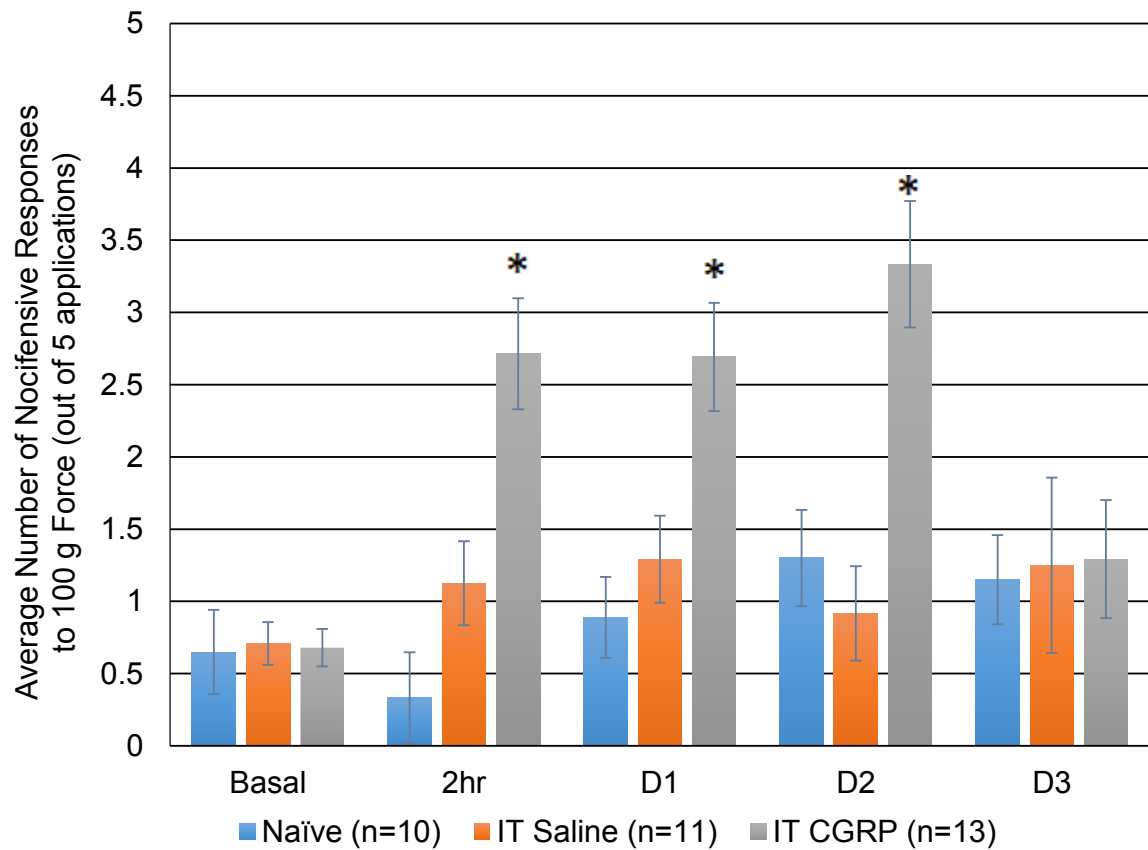


Figure 6. Increased number of nocifensive withdrawals to the 100 g filament compared with basal, naïve, and vehicle levels at 2, 24, and 48 hours after CGRP injection. Nocifensive responses returned to normal by 72 hours.

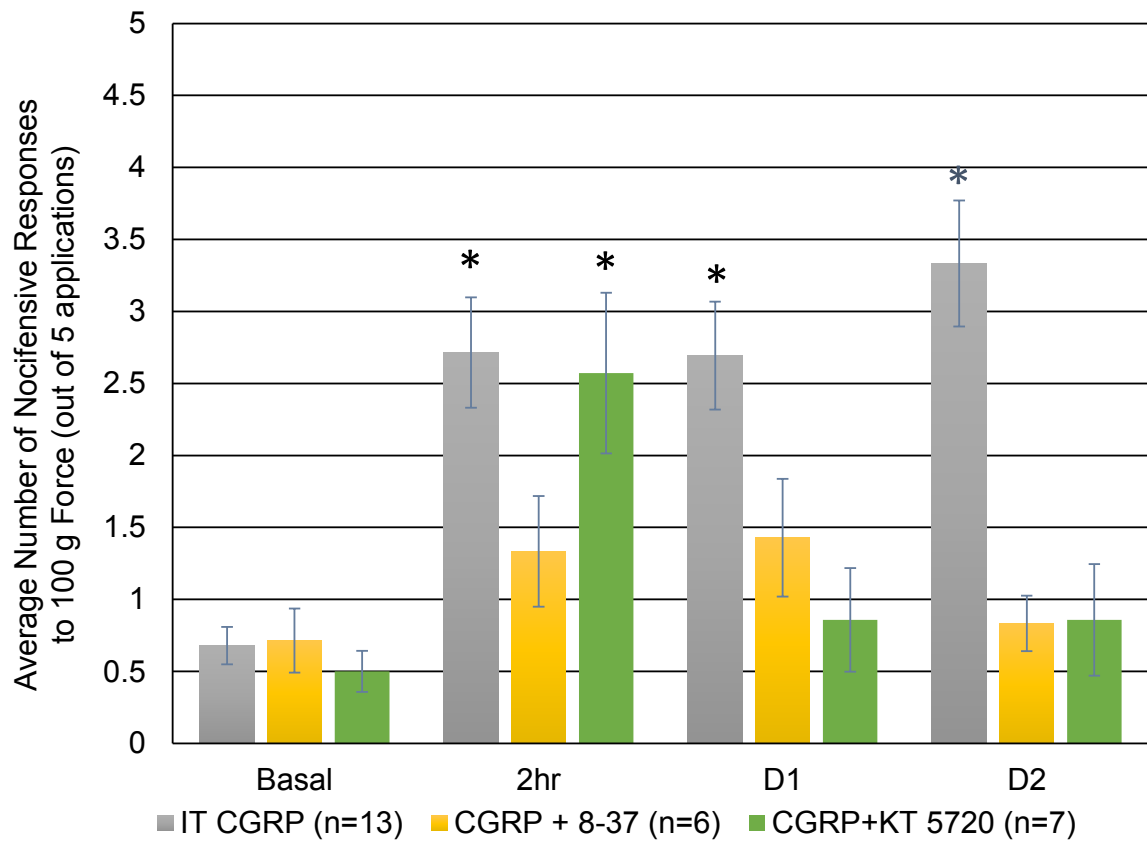


Figure 7. Decrease in number of nocifensive responses to the 100 g filament in animals that received inhibitor co-injections in a time-dependent manner. Significance from naïve and vehicle control animals is marked with an asterisk. Animals that received KT5720 responded significantly less than animals that received CGRP alone at 24 and 48 hours post-injection.

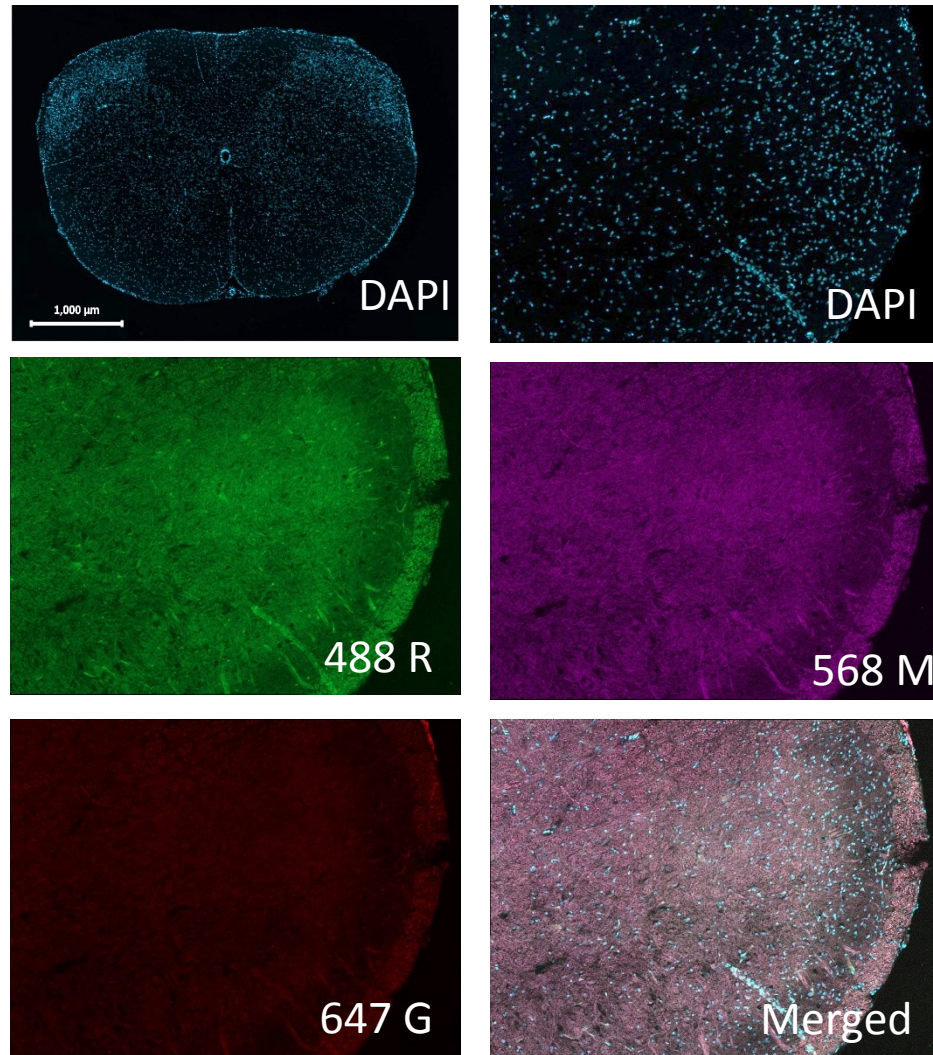


Figure 8. No detectable staining pattern observed in tissues that were incubated with secondary antibodies only. A representative spinal cord tissue was stained with the nuclear dye DAPI and a 50x multi-image alignment photograph was taken of the whole tissue to show morphology (top left). A representative DAPI-stained 200x image of the medullary horn of the upper spinal cord tissue is shown in the top right panel. The same section was stained with the Life Technologies AlexaFluor secondaries specified in Table 2. A merged image of secondary antibodies and DAPI staining is shown in the bottom right panel.

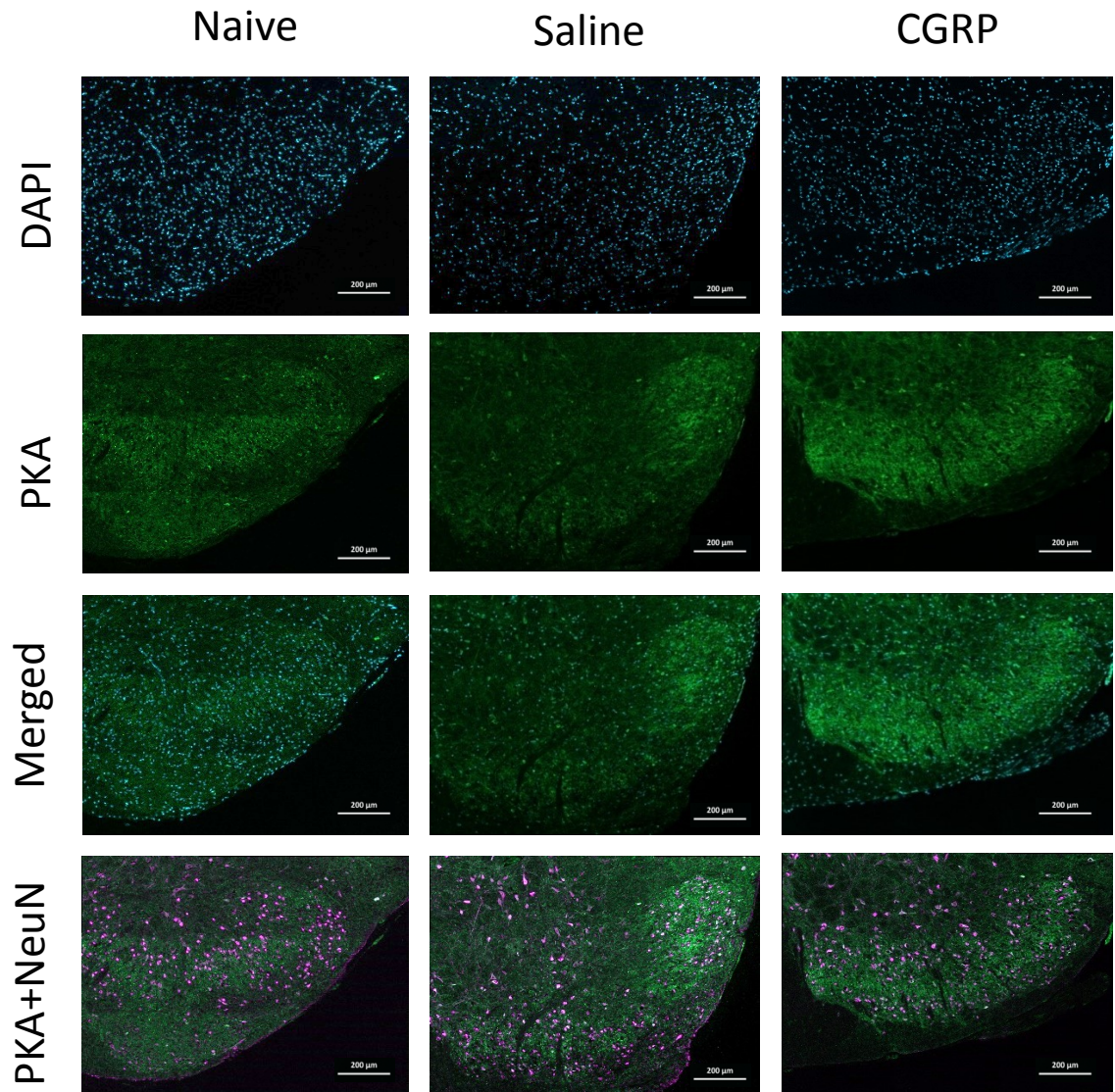


Figure 9. Increased expression of PKA in upper spinal cord in response to CGRP injection two hours prior. Images (200x) from a representative region of the medullary horn stained with DAPI is shown in the top panels, while PKA immunostaining in naïve animals (left), vehicle controls (center) and treated animals (right) is seen in the second row panels. A merged image of the same sections co-stained with PKA and DAPI are shown in the third row. The bottom panels are a merged image of PKA and NeuN staining, showing the localization of PKA expression relative to neuronal cell bodies.

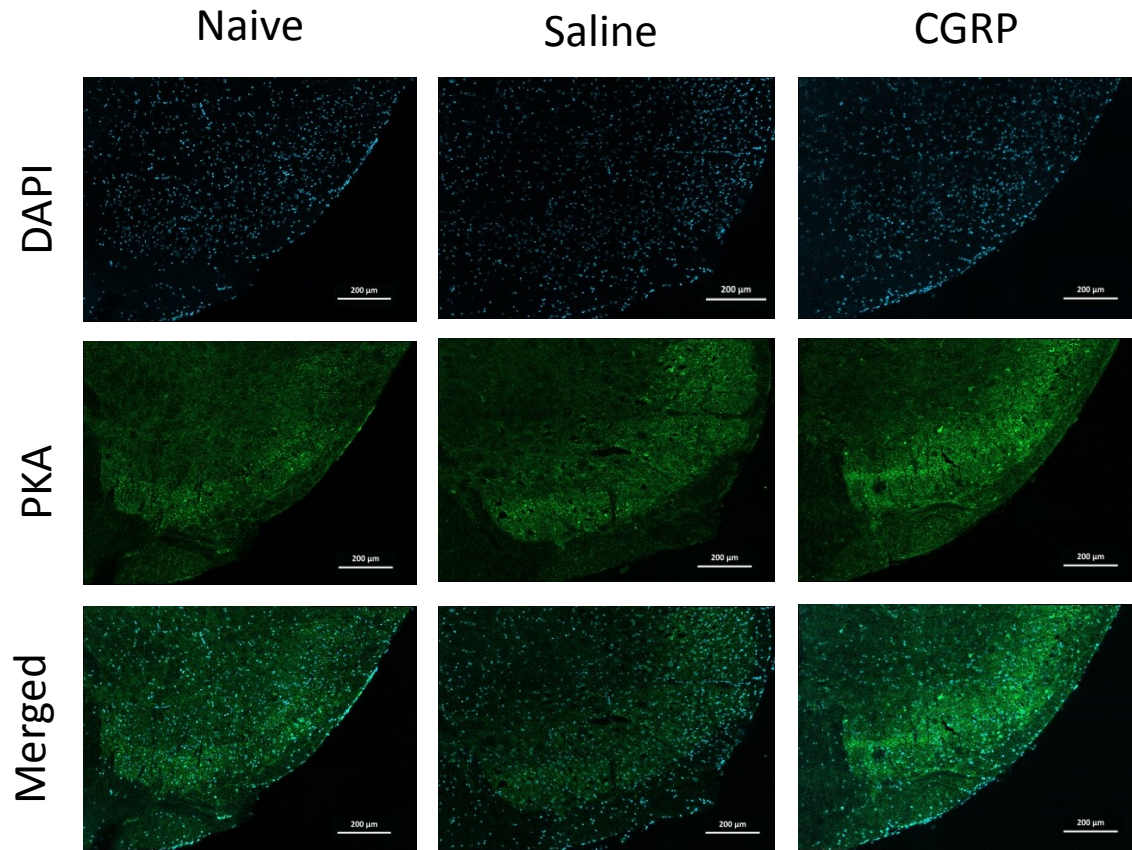


Figure 10. Increased expression of PKA in upper spinal cord in response to CGRP injection 48 hours prior. Images (200x) from a representative region of the medullary horn stained for expression of PKA in naïve animals (left), vehicle controls (center) and treated animals (right). The same sections shown in the top panels were stained with the nuclear dye DAPI. A merged image of the PKA and DAPI staining is shown in the bottom panels.

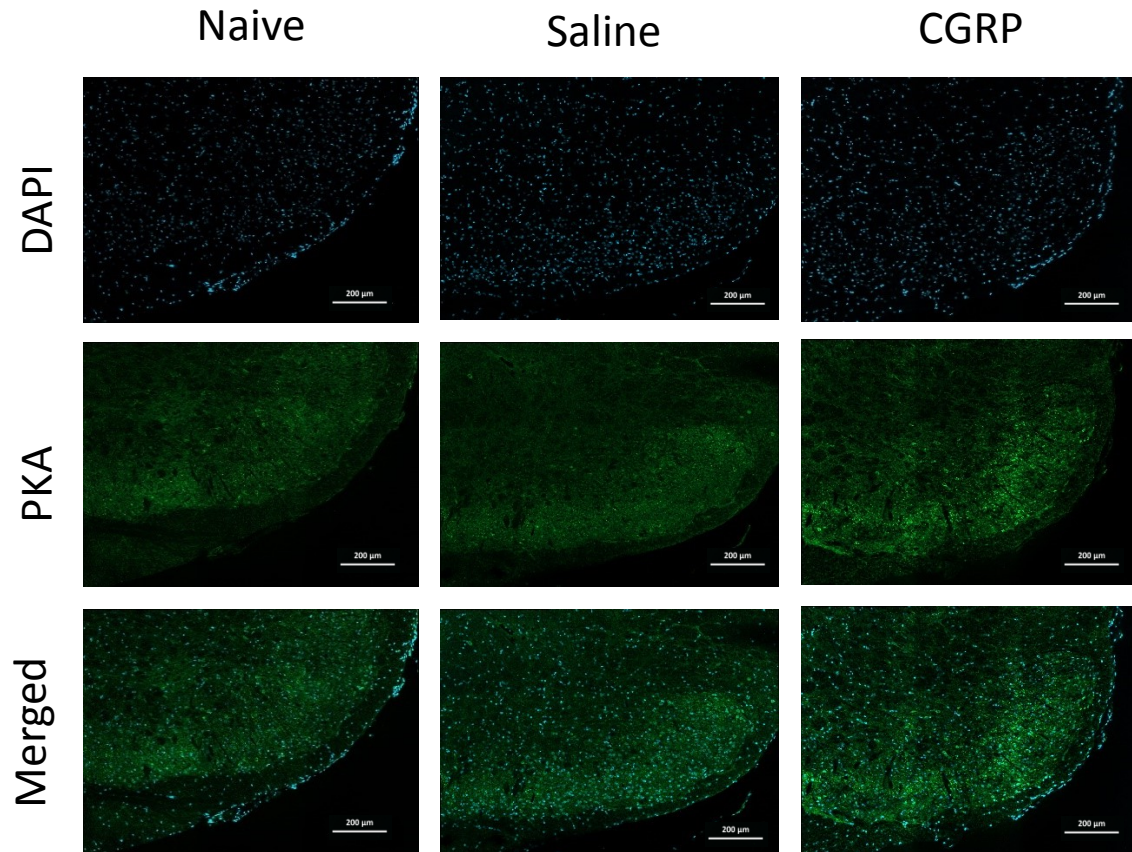


Figure 11. Increased expression of PKA in upper spinal cord in response to CGRP injection 72 hours prior. Images (200x) from a representative region of the medullary horn stained for expression of PKA in naïve animals (left), vehicle controls (center) and treated animals (right). The same sections shown in the middle panels were co-stained with the nuclear dye DAPI. A merged image of the PKA and DAPI staining is shown in the bottom panels

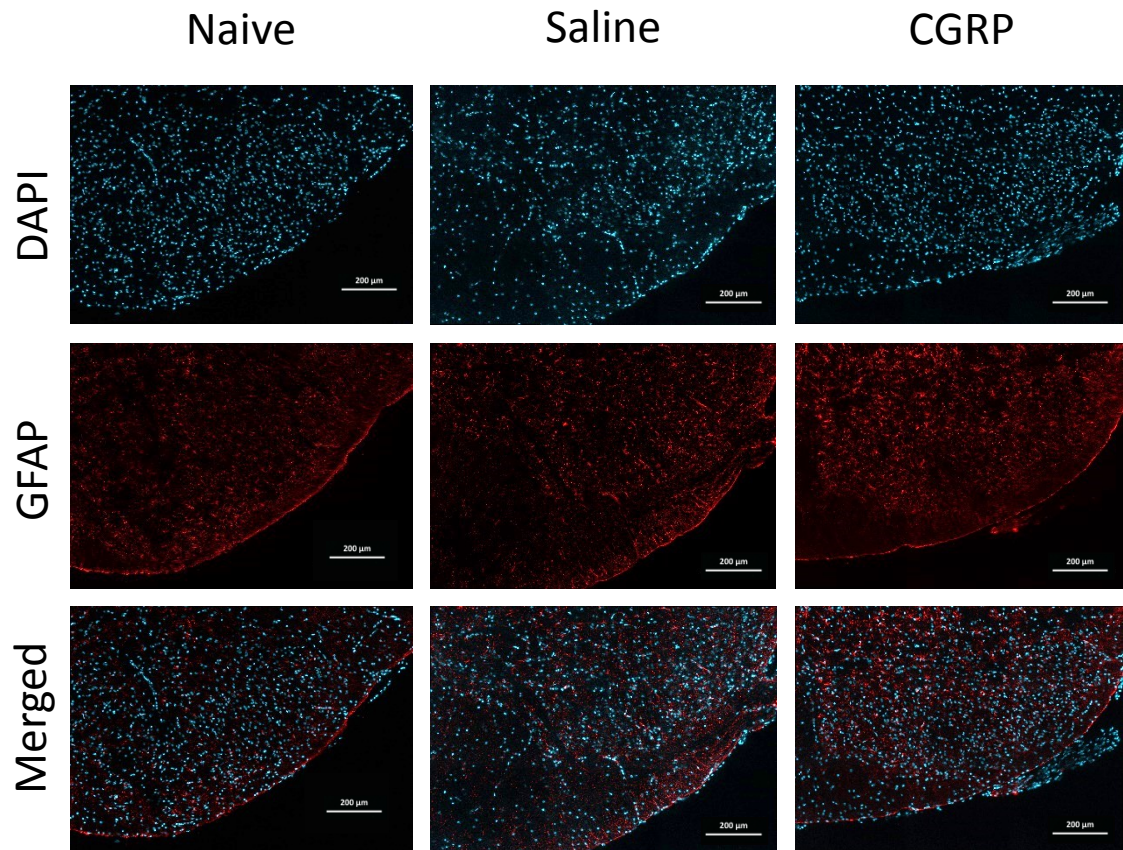


Figure 12. Increased expression of GFAP in upper spinal cord compared to naïve control in response to CGRP injection two hours post injection. Images (200x) from a representative region of the medullary horn stained for expression of GFAP in naïve animals (left), vehicle controls (center) and treated animals (right). The same sections shown in the middle panels were co-stained with the nuclear dye DAPI. A merged image of the GFAP and DAPI staining is shown in the bottom panels.

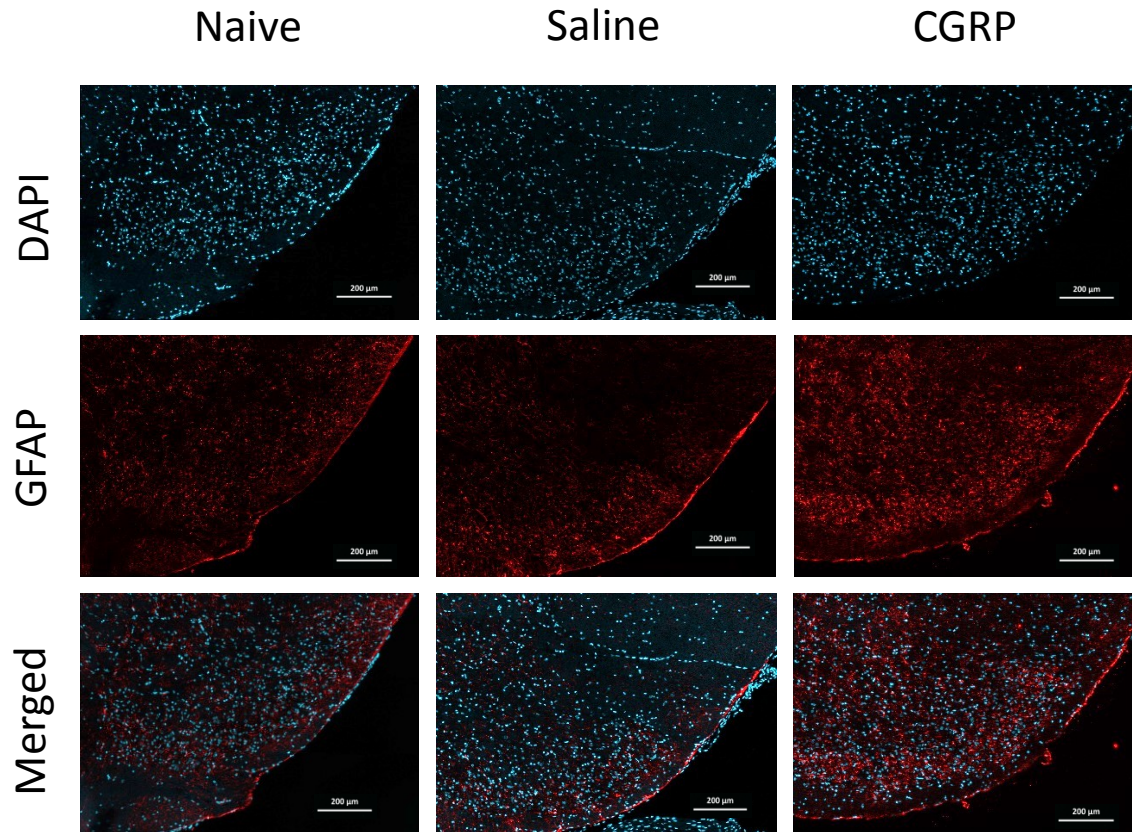


Figure 13. Increased expression of GFAP in upper spinal cord in response to CGRP injection 48 hours prior. Images (200x) from a representative region of the medullary horn stained for expression of GFAP in naïve animals (left), vehicle controls (center) and treated animals (right). The same sections shown in the middle panels were co-stained with the nuclear dye DAPI. A merged image of the GFAP and DAPI staining is shown in the bottom panels.

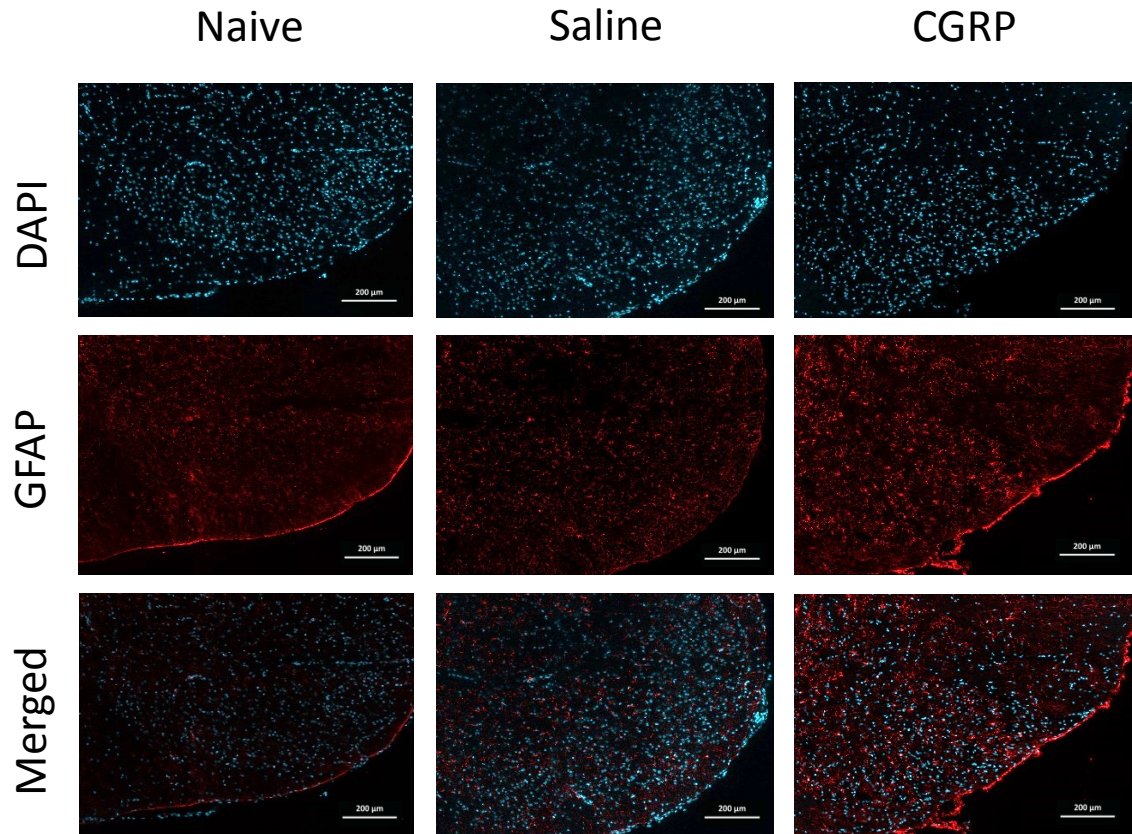


Figure 14. Increased expression of GFAP in upper spinal cord in response to CGRP injection 72 hours prior. Images (200x) from a representative region of the medullary horn stained for expression of GFAP in naïve animals (left), vehicle controls (center) and treated animals (right). The same sections shown in the middle panels were co-stained with the nuclear dye DAPI. A merged image of the GFAP and DAPI staining is shown in the bottom panels

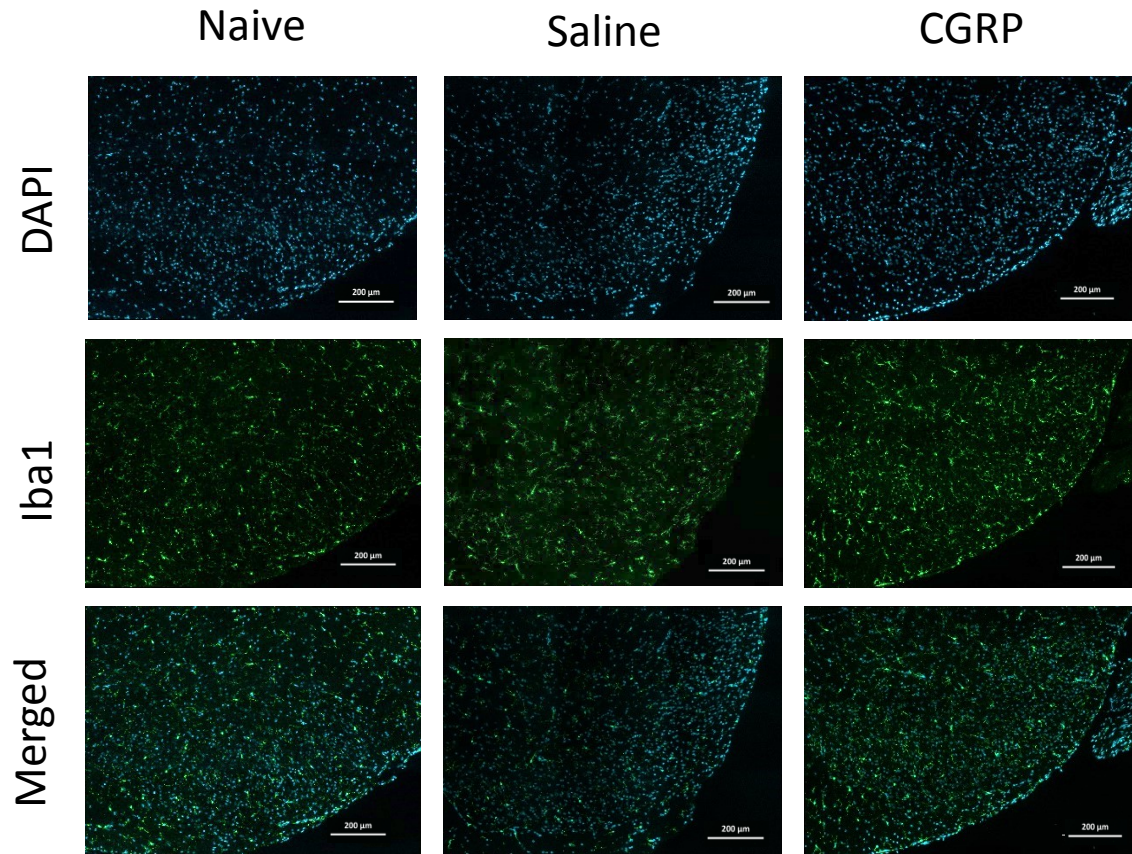


Figure 15. Increased expression of Iba1 in upper spinal cord in response to CGRP or saline injection two hours prior. Images (200x) from a representative region of the medullary horn stained for expression of Iba1 in naïve animals (left), vehicle controls (center) and treated animals (right). The same sections shown in the top panels were co-stained with the nuclear dye DAPI. A merged image of the Iba1 and DAPI staining is shown in the bottom panels.

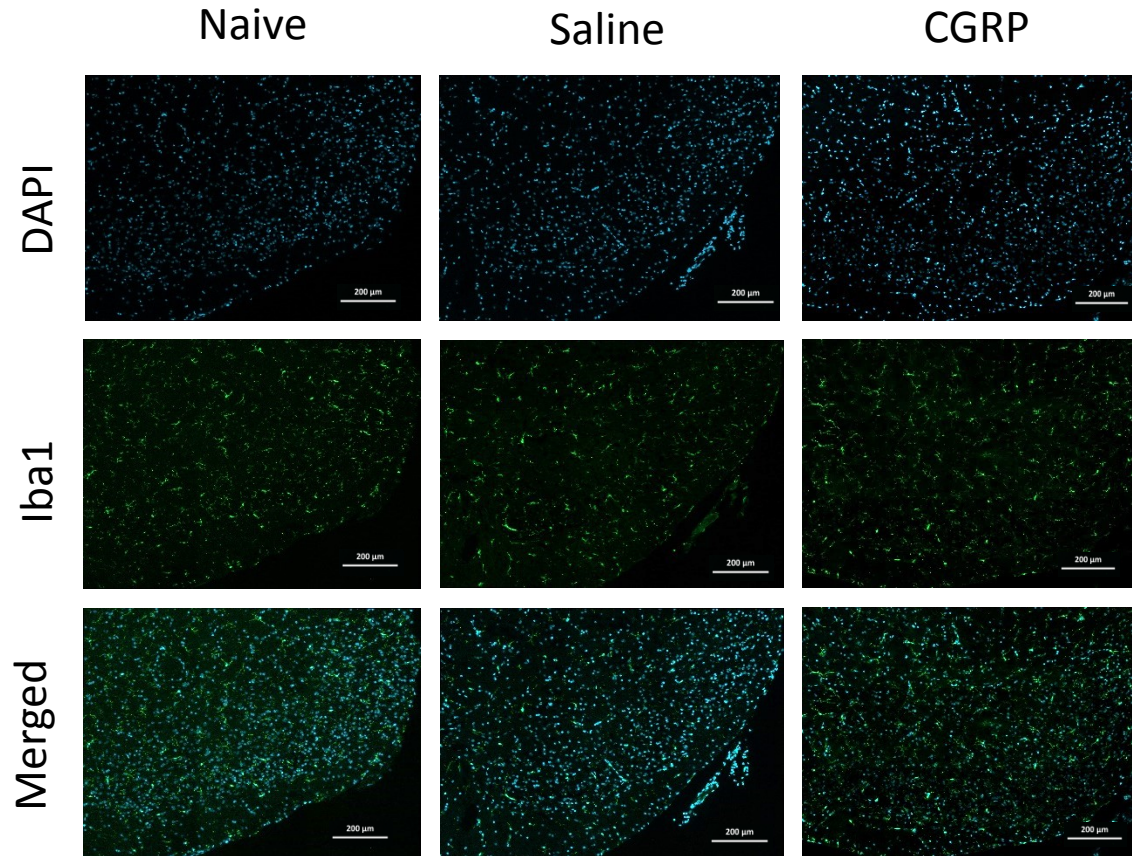


Figure 16. No change in expression of Iba1 in upper spinal cord in response to CGRP injection 48 hours prior. Images (200x) from a representative region of the medullary horn stained for expression of Iba1 in naïve animals (left), vehicle controls (center) and treated animals (right). The same sections shown in the top panels were co-stained with the nuclear dye DAPI. A merged image of the Iba1 and DAPI staining is shown in the bottom panels.

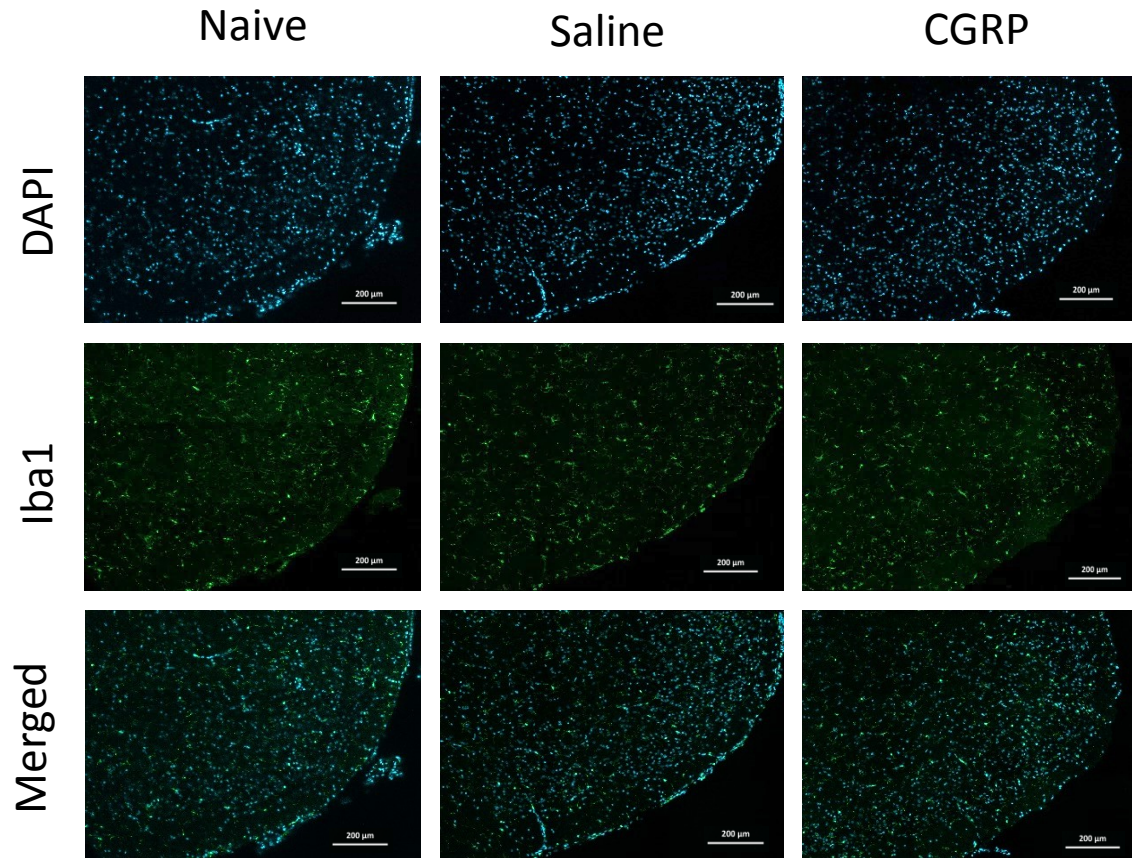


Figure 17. No significant change in expression of Iba1 in upper spinal cord in response to CGRP injection 72 hours prior. Images (200x) from a representative region of the medullary horn stained for expression of Iba1 in naïve animals (left), vehicle controls (center) and treated animals (right). The same sections shown in the top panels were co-stained with the nuclear dye DAPI. A merged image of the Iba1 and DAPI staining is shown in the bottom panels.

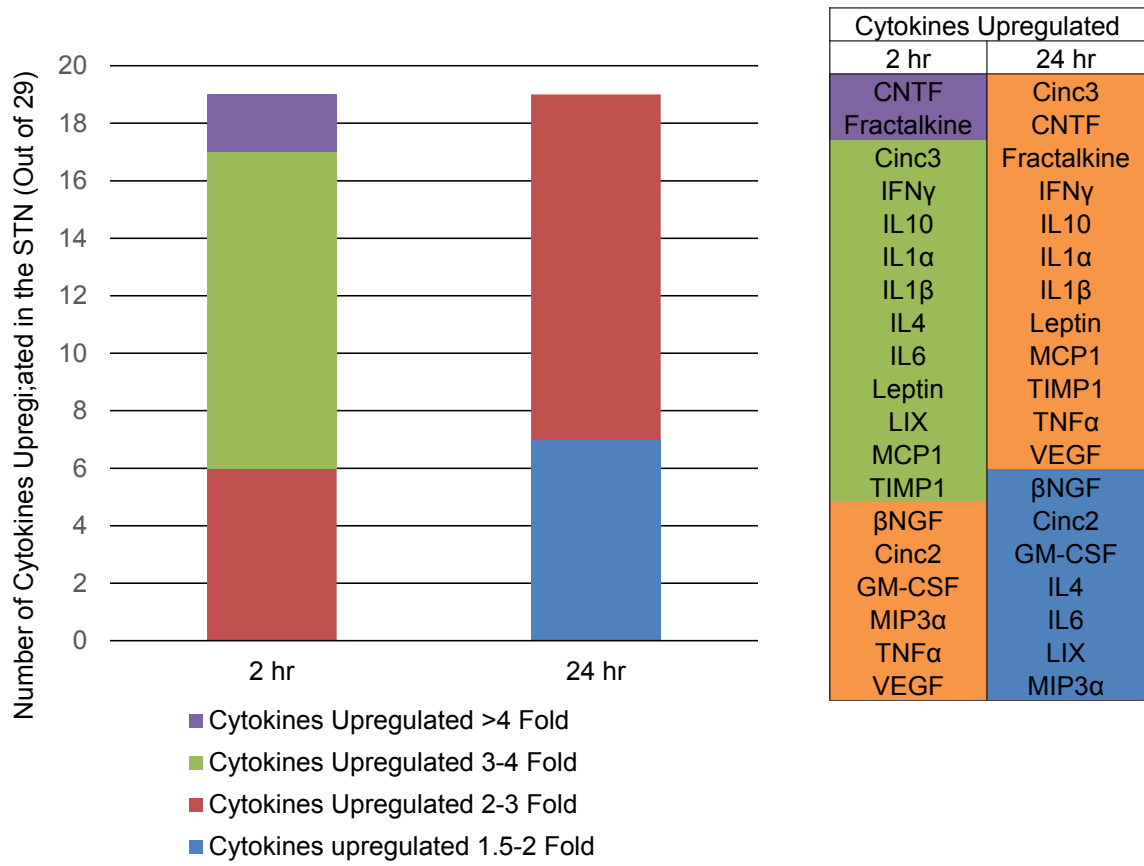


Figure 18. Intrathecal injection of CGRP caused prolonged elevation of cytokine expression in the upper spinal cord tissue. CGRP was administered intrathecally rostral to C1 and spinal cord tissues were isolated at 2 or 24 hours for analysis using a rat cytokine antibody array. (n = 2 independent experiments done in duplicate)

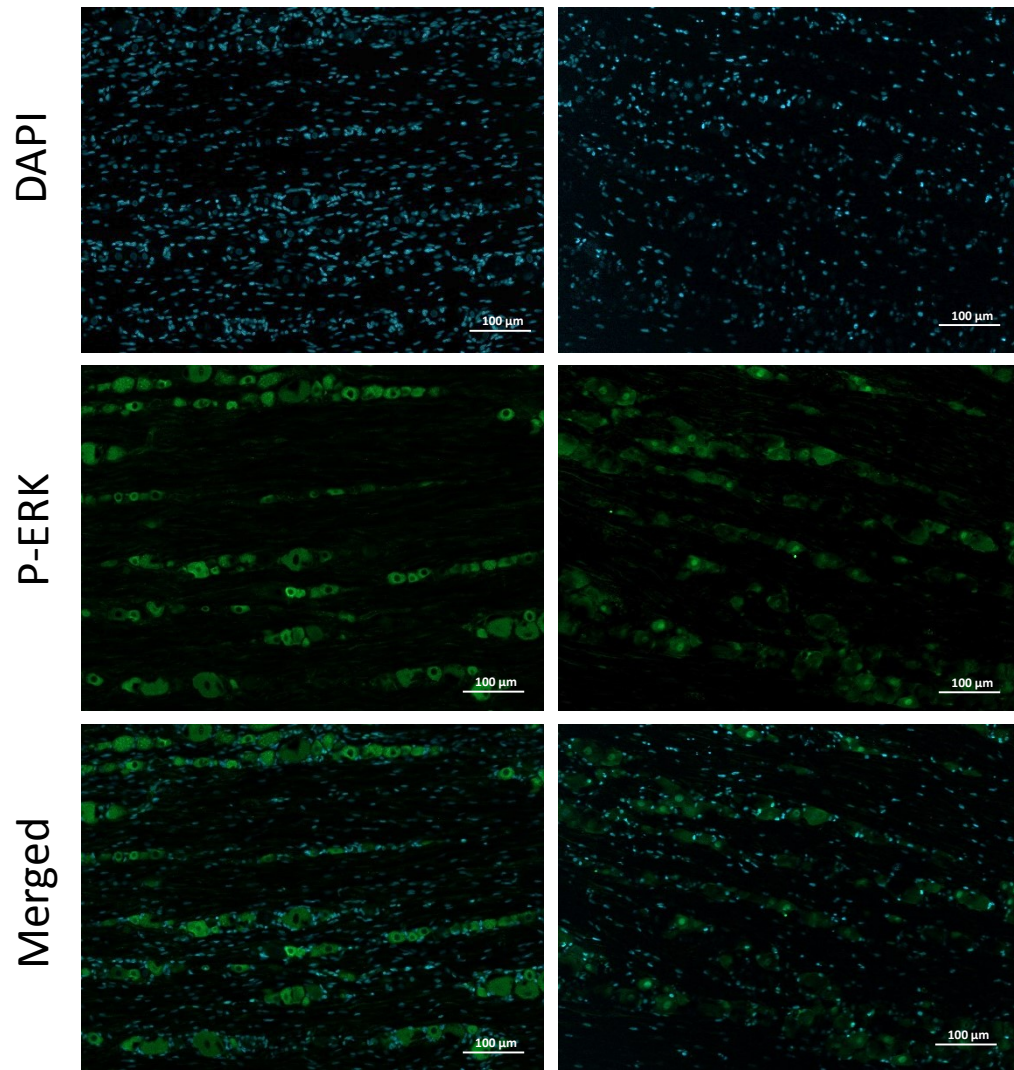


Figure 19. Elevated levels of CGRP stimulate increased expression of p-ERK in trigeminal ganglion neurons. The panels on the left are representative images from naïve animals while the ones on the right are from CGRP treated animals. The number of neurons exhibiting nuclear localization of the active form of ERK was increased compared to naïve controls 2 hours post intrathecal injection of CGRP.

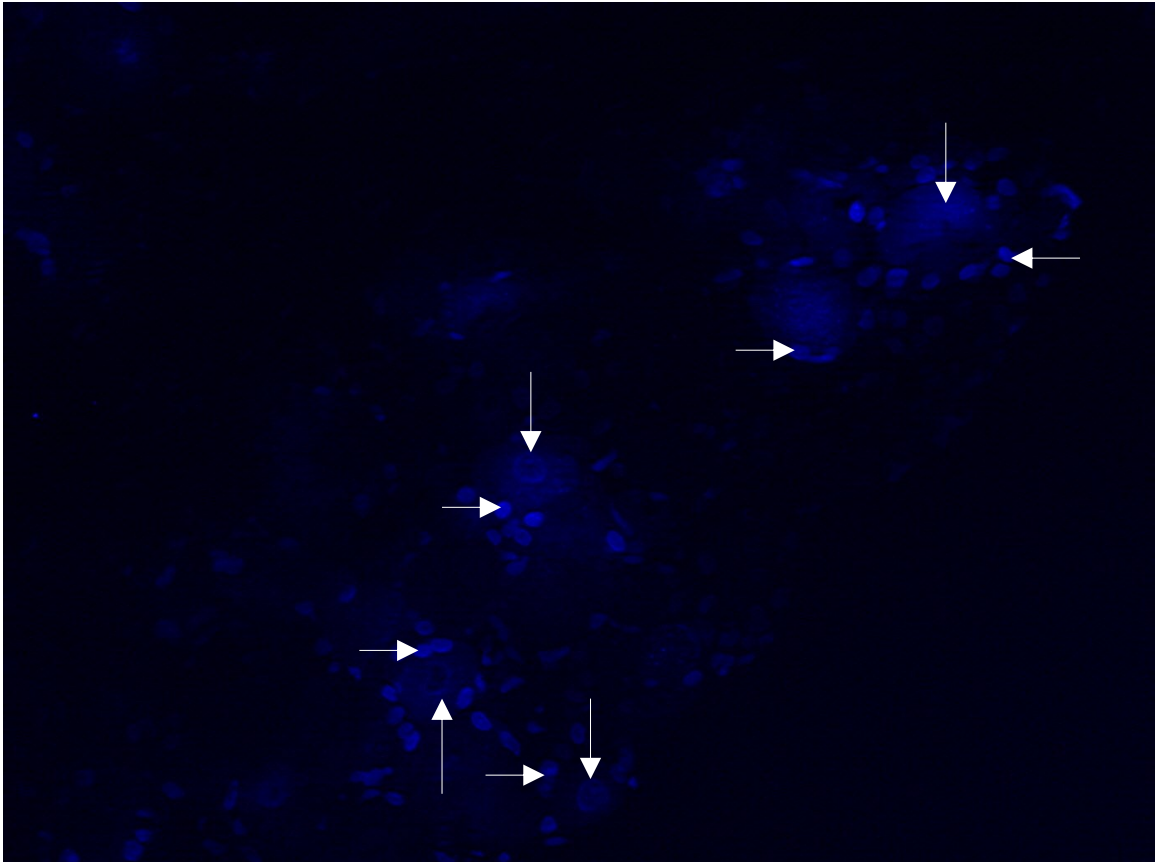


Figure 20. Evidence of bidirectional signaling within the trigeminal system: central to peripheral retrograde transport. The fluorescent dye Fast Blue was localized in the cell body of neurons and satellite glial cells 5 days after co-injection of CGRP and Fast Blue in the upper spinal cord. Horizontal arrows identify neuronal cell bodies while vertical arrows identify satellite glial cells.

DISCUSSION

CGRP Promotes Trigeminal Nociceptor Sensitization

Elevated levels of CGRP are implicated in the development and maintenance of central sensitization and an enhanced pain state characterized by hyperalgesia and allodynia in diseases involving trigeminal nerves including TMD and migraine (Sun et al., 2003; Sun et al., 2004; Seybold, 2009). However, whether elevated CGRP levels in the upper spinal cord could cause peripheral sensitization of primary nociceptive trigeminal neurons that provide sensory innervation to tissues in the head and face had not been investigated. I found that administration of CGRP in the upper spinal cord resulted in a significant increase in the number of nocifensive head withdrawals in response to mechanical stimulation of the trigeminal nerve. Importantly, this finding is in agreement with data from human studies in which both TMD and migraine patients report increased sensitivity to mechanical pressure applied to the head and face during an attack (Burstein et al., 2015; Dahan et al., 2015; Furquim et al., 2015). Under normal physiological conditions, the increase in pain sensitivity provides a protective mechanism that helps to prevent the individual from doing further damage to the tissue. As shown in my study, the enhanced nocifensive response was seen as early as 2 hours post administration and this sensitized state was maintained for at least 48 hours with resolution by 72 hours post intrathecal CGRP injection. This finding is in agreement with the time course reported by migraine and TMD patients during an acute attack in which the severe pain and enhanced sensitivity is rarely sustained past 72 hours. My finding that elevated levels of CGRP within the upper spinal cord can lower the activation

threshold of trigeminal primary sensory neurons to mechanical stimulation provide the first direct evidence, to my knowledge, to help explain the association of CGRP and the enhanced pain states reported by TMD and migraine patients.

CGRP-Mediated Sensitization Involves PKA

The physiological and cellular effects of CGRP are mediated via activation of the CGRP receptor, which is expressed on primary trigeminal neurons that synapse in the outer lamina of the spinal cord and the associated glial cells, astrocytes and microglia (Edvinsson et al., 1997; Durham, 2008; Durham and Vause, 2010). To demonstrate the specificity of the CGRP-mediated response, I co-administered CGRP with the truncated CGRP molecule (CGRP₈₋₃₇), which acts as a competitive inhibitor of the CGRP receptor (Chiba et al., 1989; Recker and Russo, 2009). I found that CGRP₈₋₃₇ could inhibit the stimulatory effect of CGRP on nociception at each of the time points included in my study. These results provide evidence that blocking the CGRP receptor within the spinal cord is sufficient to suppress the initiation and maintenance of peripheral sensitization of trigeminal nociceptive neurons. Following binding of CGRP to its G-protein-coupled receptor, there is an increase in adenylate cyclase activity within those cells leading to an increase in the intracellular level of the secondary messenger cyclic adenosine monophosphate (cAMP). Elevated levels of cAMP then cause an increase in expression of the active form of the signaling kinase PKA via binding to an allosteric site in the protein. Once activated, PKA promotes cellular changes via phosphorylation of proteins that function to regulate the excitability state of neurons and glial cells, and the synthesis and release of pro-inflammatory molecules that sustain central sensitization. Somewhat

surprisingly, I found that co-injection of the PKA inhibitor (KT 5720) with CGRP did not inhibit trigeminal sensitization to mechanical stimulation at the 2 hour time point but did suppress mechanical sensitivity 24 and 48 hours post injection. While these results appear contradictory at first, a possible explanation is that CGRP in the spinal cord elicits an immediate increase in nuclear p-ERK in primary neurons, independent of PKA activity in the central nervous system. The maintenance of the sensitization, however, is likely mediated by central PKA expression at least partially in astrocytes. Taken together, my data support the notion that the initial increase in neuronal sensitivity is due to cellular changes within the primary neurons while the more sustained sensitized state is attributable, at least in part, to activation of glial cells.

Data from my behavioral studies suggest the involvement of PKA activation in mediating the downstream stimulatory effects of CGRP. To determine if elevated CGRP levels in the spinal cord could stimulate increased expression of PKA in neurons and glial cells within the medullary horn, I used immunohistochemistry to directly study changes in PKA levels. The rationale for investigating PKA is based on evidence that PKA activation is associated with development of central sensitization (Levy and Strassman, 2002; Hu et al., 2003; Kohno et al., 2008). Based on colocalization of PKA with both NeuN and GFAP, I found that PKA levels were significantly increased in both second order neurons and glial cells in response to CGRP at 2 hours post injection when compared to levels in naïve and vehicle treated animals. PKA levels remained significantly elevated at 48 and 72 hours post injection. This finding is suggestive that, although a CGRP-mediated enhancement in nociceptive sensitivity had resolved by 72 hours, the neurons and glia within the upper spinal cord retained a level of sensitization

referred to as cellular memory. In this state, a lower level of inflammatory stimuli is required to cause activation of those cells. My finding is in agreement with other studies that have shown that elevated levels of PKA are involved in the initiation and maintenance of peripheral and central sensitization (Aley and Levine, 1999; Hu et al., 2003; Hucho and Levine, 2007; Seybold, 2009). 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). Furthermore, activation of the PKA pathway within the spinal cord has been shown to lead to central sensitization of second order nociceptive neurons and can promote bidirectional sensitization of primary nociceptors (Kohn et al., 2008; Cady et al., 2011; Hawkins et al., 2015). More specifically, increased levels of PKA in the spinal cord are involved in the development of central sensitization by increasing the activity of glutamate receptors expressed on second order neurons that facilitate pain transmission (Aley and Levine, 1999; Hucho and Levine, 2007; Latremoliere and Woolf, 2009). In addition, activation of intracellular signaling pathways involving PKA are reported to promote the induction and maintenance of central sensitization and persistent pain by phosphorylation of ion channels (Fitzgerald et al., 1999; Bhawe et al., 2002; Han et al., 2005), and increasing expression of pro-inflammatory and pro-nociceptive cytokine genes that contain CRE regulatory sites within their promoters (Kawasaki et al., 2004). PKA activity also leads to increased phosphorylation and hence activation of ERK in nociceptive neurons (Kohn et al., 2008). Furthermore, blocking PKA signaling results in reduction of inflammation-induced hyperalgesic behaviors (Malmberg et al., 1997;

Aley and Levine, 1999). Taken together, my findings provide evidence to further support the notion that PKA signaling plays a central role in mediating the stimulatory effects of CGRP, and thus is likely to be an important signaling pathway in promoting central and peripheral sensitization associated with TMD and migraine.

CGRP Induces Astrocyte Activation and Increased Cytokine Expression

Activation of the glial cells, including astrocytes and microglia, within the spinal cord is known to contribute to prolonged sensitization of nociceptive neurons and development of chronic pain states (Ren and Dubner, 1999; DeLeo and Yeziarski, 2001; Watkins et al., 2001; Wieseler-Frank et al., 2004; Guo et al., 2007; Ren and Dubner, 2008; Gosselin et al., 2010). In support of this notion, I observed increased levels of immunoreactivity of GFAP in spinal cord sections of CGRP injected animals, indicating the activation of astrocytes. The observed increase in GFAP occurred at the 2 hour time point with levels greatest after 48 hours post injection, and remained significantly elevated even at 72 hours, a finding similar to my PKA results. In contrast, CGRP did not cause an increase in the expression of Iba1 in microglia when compared to control levels at any of the time points. It is now generally accepted that hyperactivation of spinal glia, in particular astrocytes, contribute to the development and maintenance of inflammatory pain (Aschner, 2000; Svensson et al., 2003; Raghavendra et al., 2004) and the development of central sensitization. Astrocytes are thought to promote and sustain sensitization of peripheral and central neurons through the release of cytokines. Interestingly, activation of the PKA signaling pathway is known to promote synthesis and secretion of cytokines from activated glia cells (Miller et al., 2009). Cytokines can act

directly to sensitize nociceptors and increase neuronal sensitivity to chemical, thermal, and mechanical stimuli by increasing the expression of receptors and ion channels involved in pain and analgesia (Ren and Torres, 2009; Uceyler et al., 2009). Towards this end, in preliminary studies, I found that CGRP could induce the expression of almost 20 different cytokines in the upper spinal cord. The observed increased expression level of several cytokines would further suggest that elevated levels of CGRP leads to sensitization of the trigeminal system by increasing neuron-glia cell interactions in the spinal cord. Based on my data, I propose that elevated CGRP levels in the spinal cord functions to facilitate enhanced central neuron-glia interactions and cytokine release and therefore stimulate key cellular events known to promote and maintain central sensitization and peripheral sensitization.

CGRP Promotes Bidirectional Signaling within the Trigeminal System

To investigate a possible mechanism by which elevated CGRP levels in the spinal cord could lead to an increased nocifensive response in primary trigeminal nociceptive neurons indicative of peripheral sensitization, I determined changes in the level of the MAP kinase ERK in trigeminal neurons. Intrathecal CGRP caused a large increase in the nuclear localization of active, phosphorylated form of ERK (P-ERK) in the cell bodies of trigeminal neurons throughout the entire ganglion 2 hours post injection. In contrast in naïve control ganglion, P-ERK was mostly localized in the cytosol of neurons. Increased levels of the P- ERK are associated with a sensitized state of primary nociceptive neurons by upregulating ion channel expression and activity of membrane receptor expression (Cheng and Ji, 2008a; Cheng and Ji, 2008b; Ji et al., 2009; Takeda et al., 2009). Further

evidence of the importance of MAP kinases in the induction of peripheral sensitization and persistent pain is provided by results from studies in which blocking MAP kinase activity with specific inhibitors was reported to suppress nociceptive responses and sensitization (Milligan et al., 2003; Tsuda et al., 2004; Ji et al., 2009). Results from my study provide evidence that CGRP promotes changes in neurons within the trigeminal ganglia that are consistent with development and maintenance of peripheral sensitization of primary nociceptors. These data are in agreement with a previous study from our laboratory that demonstrated that nicotine could mediate an increase in neuron-glia signaling within the upper spinal cord leading to peripheral sensitization of primary trigeminal nociceptive neurons (Hawkins et al., 2015). Taken together, these findings provide evidence to support the notion of bidirectional signaling with the trigeminal system.

To demonstrate that CGRP can promote retrograde signal transduction from the spinal cord to neuronal cell bodies located in the trigeminal ganglion, the retrograde dye Fast Blue was co-injected in the upper spinal cord and 5 days later its location observed using immunofluorescent microscopy. I found that Fast Blue was not only detected in the cell bodies of neurons but was also observed at higher levels in satellite glial cells. These data for the first time provide direct evidence of bidirectional signaling from the cerebrospinal fluid to neuronal cell bodies and then movement through gap junctions to satellite glial cells. We can only speculate on the mechanism by which Fast Blue is retrogradely delivered to the cell body but is likely to involve endocytosis and the formation of transport vesicles. Taken together, my results support the idea that elevated

levels of CGRP within the spinal cord can facilitate sensitization of primary trigeminal neurons by mediating direct changes in trigeminal nociceptive neurons.

Summary and Future Directions

I propose the following model to explain how elevated levels of CGRP within the upper spinal cord could cause a decrease in the activation threshold to mechanical stimuli in primary trigeminal neurons. CGRP would promote a rapid change in primary trigeminal neurons that are associated with initiation of peripheral sensitization via activation of the ERK signaling pathway and increased neuron-satellite glial cell interactions. The ability of the PKA inhibitor to ameliorate latent, but not immediate hyperalgesia indicates that although the upregulation of PKA production is acute, its function is not critical for the initiation of a sensitized state. It appears likely that PKA instead acts to maintain sensitization through a downstream target. The more sustained neuronal sensitization is likely due to CGRP-mediated activation of astrocytes and the increased expression of numerous cytokines that are known to promote a prolonged state of sensitization of both primary and second order neurons in the pain transmission pathway. My novel results provide direct evidence of bidirectional signaling within the trigeminal system, and hence challenge one of the long-standing dogmas in the pain field that peripheral sensitization promotes central sensitization. However, this narrow view fails to explain how trigeminal nociceptive neurons become activated in the absence of any physical trauma or chemical-mediated inflammation in the peripheral tissues as is commonly reported in more chronic pain patients (often referred to as idiopathic pain).

As with most studies, there are many unanswered questions that could be addressed to better understand the mechanism of action involved in CGRP-mediated sensitization of trigeminal neurons. For example, does CGRP receptor activation couple to other intracellular pathways such as PKC and other MAP kinases such as p38 and JNK, which are associated with glial activation? An obvious series of experiments would be to investigate the cellular changes within the spinal cord tissue and trigeminal ganglion in response to the PKA selective inhibitor KT 5720. To test my hypothesis that glia are involved in the maintenance of sensitization, application of a glial inhibitor such as fluorocitrate might be used to observe changes in the length of sensitivity behaviorally. Another line of research could focus on using ELISAs to confirm the CGRP-mediated upregulation of individual cytokines such as IL-1 β , IL-6, TNF- α , and Fractalkine, which are all known to play important roles in glial-maintained central sensitization (Ji et al., 2013; Souza et al., 2013). Initially I would study changes in the levels the pro-inflammatory cytokine TNF- α since our laboratory recently has shown that intrathecal delivery of nicotine caused the greatest increase in the expression of the pro-inflammatory cytokine TNF- α (Leung and Cahill, 2010). Elevated levels of TNF- α are associated with central sensitization and prolonged pain states (Kawasaki et al., 2008; Miller et al., 2009) and similar to CGRP, elevated cytokine levels have been reported in serum and cerebrospinal fluid in migraine patients (Rozen and Swidan, 2007; Uzar et al., 2011). In the central nervous system, activation of p38 in glia due to TNF- α can lead to increased secretion of the pro-inflammatory cytokines IL-1 and IL-6 as well as ATP (Leung and Cahill, 2010). Additionally, TNF- α stimulates its own production in astrocytes via the CXCR4 receptor. This self-regulation leads to production of IL-1, IL-6,

ATP and nitric oxide, which are factors that contribute to enhanced neuronal activity and thus pathological pain. Yet another possible avenue to pursue in future studies would be to determine if monoclonal antibodies directed against either CGRP or its receptor, which are currently being tested in human clinical trials, would block CGRP-mediated changes in peripheral trigeminal neurons and glia. This is an interesting and important question since monoclonal antibodies cannot cross the blood brain barrier and hence could not directly block the stimulatory effect of CGRP within the upper spinal cord on primary neurons and glial cells. Finally, having demonstrated that elevated levels of CGRP centrally can cause an increase in the coupling of neuronal cell bodies with satellite glial cells, which is implicated in the development of more chronic states (Durham and Garrett, 2010b), it will be of interest to identify the connexin proteins mediating this cellular communication via gap junctions.

REFERENCES

- Aaron LA, Burke MM, Buchwald D (2000) Overlapping conditions among patients with chronic fatigue syndrome, fibromyalgia, and temporomandibular disorder. *Archives of internal medicine* 160:221-227.
- Aley KO, Levine JD (1999) Role of protein kinase A in the maintenance of inflammatory pain. *J Neurosci* 19:2181-2186.
- Aschner M (2000) Neuron-astrocyte interactions: implications for cellular energetics and antioxidant levels. *Neurotoxicology* 21:1101-1107.
- Bellamy JL, Cady RK, Durham PL (2006) Salivary levels of CGRP and VIP in rhinosinusitis and migraine patients. *Headache* 46:24-33.
- Benemei S, Nicoletti P, Capone JA, Geppetti P (2007) Pain pharmacology in migraine: focus on CGRP and CGRP receptors. *Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology* 28 Suppl 2:S89-93.
- Benemei S, Nicoletti P, Capone JG, Geppetti P (2009) CGRP receptors in the control of pain and inflammation. *Curr Opin Pharmacol* 9:9-14.
- Bereiter DA, Hirata H, Hu JW (2000) Trigeminal subnucleus caudalis: beyond homologies with the spinal dorsal horn. *Pain* 88:221-224.
- Bernstein C, Burstein R (2012) Sensitization of the trigeminovascular pathway: perspective and implications to migraine pathophysiology. *Journal of clinical neurology* 8:89-99.
- Bevilaqua Grossi D, Lipton RB, Bigal ME (2009) Temporomandibular disorders and migraine chronification. *Current pain and headache reports* 13:314-318.
- Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RWt (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* 35:721-731.
- Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I (1985) Calcitonin gene-related peptide is a potent vasodilator. *Nature* 313:54-56.
- Burstein R, Nosedà R, Borsook D (2015) Migraine: multiple processes, complex pathophysiology. *J Neurosci* 35:6619-6629.

- 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, 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 RK, Vause CV, Ho TW, Bigal ME, Durham PL (2009) Elevated saliva calcitonin gene-related peptide levels during acute migraine predict therapeutic response to rizatriptan. *Headache* 49:1258-1266.
- Cheng JK, Ji RR (2008a) Intracellular signaling in primary sensory neurons and persistent pain. *Neurochem Res* 33:1970-1978.
- Cheng JK, Ji RR (2008b) Intracellular signaling in primary sensory neurons and persistent pain. *Neurochemical research* 33:1970-1978.
- Chiba T, Yamaguchi A, Yamatani T, Nakamura A, Morishita T, Inui T, Fukase M, Noda T, Fujita T (1989) Calcitonin gene-related peptide receptor antagonist human CGRP-(8-37). *Am J Physiol* 256:E331-335.
- Dahan H, Shir Y, Velly A, Allison P (2015) Specific and number of comorbidities are associated with increased levels of temporomandibular pain intensity and duration. *The journal of headache and pain* 16:528.
- 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.
- DeLeo JA, Yezierski RP (2001) The role of neuroinflammation and neuroimmune activation in persistent pain. *Pain* 90:1-6.
- Devor M (1999) Unexplained peculiarities of the dorsal root ganglion. *Pain Suppl* 6:S27-35.
- Dodick D, Silberstein S (2006) Central sensitization theory of migraine: clinical implications. *Headache* 46 Suppl 4:S182-191.

- Durham PL (2008) Inhibition of calcitonin gene-related peptide function: a promising strategy for treating migraine. *Headache* 48:1269-1275.
- Durham PL, Garrett FG (2010a) Development of functional units within trigeminal ganglia correlates with increased expression of proteins involved in neuron-glia interactions. *Neuron glia biology* 6:171-181.
- Durham PL, Garrett FG (2010b) 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.
- Dworkin SF, Huggins KH, LeResche L, Von Korff M, Howard J, Truelove E, Sommers E (1990) Epidemiology of signs and symptoms in temporomandibular disorders: clinical signs in cases and controls. *J Am Dent Assoc* 120:273-281.
- Edvinsson L, Cantera L, Jansen-Olesen I, Uddman R (1997) Expression of calcitonin gene-related peptide1 receptor mRNA in human trigeminal ganglia and cerebral arteries. *Neurosci Lett* 229:209-211.
- Fischer JA, Born W (1985) Novel peptides from the calcitonin gene: expression, receptors and biological function. *Peptides* 6 Suppl 3:265-271.
- Fitzgerald EM, Okuse K, Wood JN, Dolphin AC, Moss SJ (1999) cAMP-dependent phosphorylation of the tetrodotoxin-resistant voltage-dependent sodium channel SNS. *J Physiol* 516 (Pt 2):433-446.
- Furquim BD, Flamengui LM, Conti PC (2015) TMD and chronic pain: a current view. *Dental press journal of orthodontics* 20:127-133.
- 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.
- 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.
- Han JS, Li W, Neugebauer V (2005) Critical role of calcitonin gene-related peptide 1 receptors in the amygdala in synaptic plasticity and pain behavior. *J Neurosci* 25:10717-10728.

- Hanani M (2005) Satellite glial cells in sensory ganglia: from form to function. *Brain Res Brain Res Rev* 48:457-476.
- Hawkins JL, Denson JE, Miley DR, Durham PL (2015) Nicotine stimulates expression of proteins implicated in peripheral and central sensitization. *Neuroscience* 290:115-125.
- Holmlund A, Ekblom A, Hansson P, Lind J, Lundeberg T, Theodorsson E (1991) Concentrations of neuropeptides substance P, neurokinin A, calcitonin gene-related peptide, neuropeptide Y and vasoactive intestinal polypeptide in synovial fluid of the human temporomandibular joint. A correlation with symptoms, signs and arthroscopic findings. *Int J Oral Maxillofac Surg* 20:228-231.
- 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.
- 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.
- Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ, Ji RR (2004) Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *J Neurosci* 24:8310-8321.
- Kawasaki Y, Zhang L, Cheng JK, Ji RR (2008) Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1 β , interleukin-6, and tumor necrosis factor- α in regulating synaptic and neuronal activity in the superficial spinal cord. *J Neurosci* 28:5189-5194.
- 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.
- Kress M (2010) Nociceptor Sensitization by Proinflammatory Cytokines and Chemokines. *The Open Pain Journal* 3:97-101.
- Latremoliere A, Woolf CJ (2009) Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* 10:895-926.
- Lee M (2013) Neurotransmitters and microglial-mediated neuroinflammation. *Current protein & peptide science* 14:21-32.
- Leung L, Cahill CM (2010) TNF-alpha and neuropathic pain--a review. *Journal of neuroinflammation* 7:27.
- Levy D, Strassman AM (2002) Distinct sensitizing effects of the cAMP-PKA second messenger cascade on rat dural mechanonociceptors. *J Physiol* 538:483-493.
- Light A, Vierck C, Light K Myalgia and Fatigue: Translation from Mouse Sensory Neurons to Fibromyalgia and Chronic Fatigue Syndromes. In: *Translational Pain Research: From Mouse to Man*, pp 252-282.
- Malmberg AB, Brandon EP, Idzerda RL, Liu H, McKnight GS, Basbaum AI (1997) Diminished inflammation and nociceptive pain with preservation of neuropathic pain in mice with a targeted mutation of the type I regulatory subunit of cAMP-dependent protein kinase. *J Neurosci* 17:7462-7470.
- Manfredini D, Guarda-Nardini L, Winocur E, Piccotti F, Ahlberg J, Lobbezoo F (2011) Research diagnostic criteria for temporomandibular disorders: a systematic review of axis I epidemiologic findings. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics* 112:453-462.
- Miller RJ, Jung H, Bhangoo SK, White FA (2009) Cytokine and chemokine regulation of sensory neuron function. *Handb Exp Pharmacol* 417-449.
- Milligan ED, Twining C, Chacur M, Biedenkapp J, O'Connor K, Poole S, Tracey K, Martin D, Maier SF, Watkins LR (2003) Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. *J Neurosci* 23:1026-1040.
- Milligan ED, Watkins LR (2009) Pathological and protective roles of glia in chronic pain. *Nature reviews Neuroscience* 10:23-36.
- Mujakperuo HR, Watson M, Morrison R, Macfarlane TV (2010) Pharmacological interventions for pain in patients with temporomandibular disorders. *The Cochrane database of systematic reviews* CD004715.

- Ohrbach R, Fillingim RB, Mulkey F, Gonzalez Y, Gordon S, Gremillion H, Lim PF, Ribeiro-Dasilva M, Greenspan JD, Knott C, Maixner W, Slade G (2011) Clinical findings and pain symptoms as potential risk factors for chronic TMD: descriptive data and empirically identified domains from the OPPERA case-control study. *The journal of pain : official journal of the American Pain Society* 12:T27-45.
- Poveda Roda R, Bagan JV, Diaz Fernandez JM, Hernandez Bazan S, Jimenez Soriano Y (2007) Review of temporomandibular joint pathology. Part I: classification, epidemiology and risk factors. *Med Oral Patol Oral Cir Bucal* 12:E292-298.
- Raghavendra V, Tanga FY, DeLeo JA (2004) Complete Freund's adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. *Eur J Neurosci* 20:467-473.
- Raivich G (2005) Like cops on the beat: the active role of resting microglia. *Trends Neurosci* 28:571-573.
- Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang SY, Nau C, Kress M (2002) PKA/AKAP/VR-1 module: A common link of Gs-mediated signaling to thermal hyperalgesia. *J Neurosci* 22:4740-4745.
- Recober A, Russo AF (2009) Calcitonin gene-related peptide: an update on the biology. *Curr Opin Neurol* 22:241-246.
- 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.
- Ren K, Torres R (2009) Role of interleukin-1beta during pain and inflammation. *Brain research reviews* 60:57-64.
- Rosenfeld MG, Mermel JJ, Amara SG, Swanson LW, Sawchenko PE, Rivier J, Vale WW, Evans RM (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* 304:129-135.
- Rozen T, Swidan SZ (2007) Elevation of CSF tumor necrosis factor alpha levels in new daily persistent headache and treatment refractory chronic migraine. *Headache* 47:1050-1055.
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature methods* 9:671-675.
- Sessle BJ (2011) Peripheral and central mechanisms of orofacial inflammatory pain. *International review of neurobiology* 97:179-206.

- Seybold VS (2009) The role of peptides in central sensitization. *Handb Exp Pharmacol* 451-491.
- Shankland W (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.
- Song DD, Li Y, Tang D, Huang LY, Yuan YZ (2014) Neuron-glia communication mediated by TNF-alpha and glial activation in dorsal root ganglia in visceral inflammatory hypersensitivity. *Am J Physiol Gastrointest Liver Physiol* 306:G788-795.
- Souza GR, Talbot J, Lotufo CM, Cunha FQ, Cunha TM, Ferreira SH (2013) Fractalkine mediates inflammatory pain through activation of satellite glial cells. *Proc Natl Acad Sci U S A* 110:11193-11198.
- Spears R, Dees LA, Sapozhnikov M, Bellinger LL, Hutchins B (2005) Temporal changes in inflammatory mediator concentrations in an adjuvant model of temporomandibular joint inflammation. *J Orofac Pain* 19:34-40.
- Sun RQ, Lawand NB, Willis WD (2003) The role of calcitonin gene-related peptide (CGRP) in the generation and maintenance of mechanical allodynia and hyperalgesia in rats after intradermal injection of capsaicin. *Pain* 104:201-208.
- 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.
- Svensson CI, Marsala M, Westerlund A, Calcutt NA, Campana WM, Freshwater JD, Catalano R, Feng Y, Protter AA, Scott B, Yaksh TL (2003) Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. *J Neurochem* 86:1534-1544.
- Takeda M, Takahashi M, Matsumoto S (2009) Contribution of the activation of satellite glia in sensory ganglia to pathological pain. *Neurosci Biobehav Rev* 33:784-792.
- Takeda M, Tanimoto T, Kadoi J, Nasu M, Takahashi M, Kitagawa J, Matsumoto S (2007) Enhanced excitability of nociceptive trigeminal ganglion neurons by

- satellite glial cytokine following peripheral inflammation. *Pain* 129:155-166.
- Takeshita S, Hirata H, Bereiter DA (2001) Intensity coding by TMJ-responsive neurons in superficial laminae of caudal medullary dorsal horn of the rat. *J Neurophysiol* 86:2393-2404.
- Taylor BK, Corder G (2014) Endogenous analgesia, dependence, and latent pain sensitization. *Current topics in behavioral neurosciences* 20:283-325.
- Tsuda M, Mizokoshi A, Shigemoto-Mogami Y, Koizumi S, Inoue K (2004) Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. *Glia* 45:89-95.
- Uceyler N, Schafer M, Sommer C (2009) Mode of action of cytokines on nociceptive neurons. *Exp Brain Res* 196:67-78.
- Uzar E, Evliyaoglu O, Yucel Y, Ugur Cevik M, Acar A, Guzel I, Islamoglu Y, Colpan L, Tasdemir N (2011) Serum cytokine and pro-brain natriuretic peptide (BNP) levels in patients with migraine. *European review for medical and pharmacological sciences* 15:1111-1116.
- Vause CV, Durham PL (2010) Calcitonin gene-related peptide differentially regulates gene and protein expression in trigeminal glia cells: findings from array analysis. *Neurosci Lett* 473:163-167.
- Vause CV, Durham PL (2012) Identification of cytokines and signaling proteins differentially regulated by sumatriptan/naproxen. *Headache* 52:80-89.
- 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.
- Watkins LR, Milligan ED, Maier SF (2001) Glial activation: a driving force for pathological pain. *Trends Neurosci* 24:450-455.
- Wieseler-Frank J, Maier SF, Watkins LR (2004) Glial activation and pathological pain. *Neurochem Int* 45:389-395.
- Wilcox SL, Gustin SM, Macey PM, Peck CC, Murray GM, Henderson LA (2015) Anatomical changes within the medullary dorsal horn in chronic temporomandibular disorder pain. *NeuroImage* 117:258-266.
- Woolf CJ (2011) Central sensitization: implications for the diagnosis and treatment of pain. *Pain* 152:S2-15.