Trigeminal Central Sensitization and Its Modulation in Acute and Chronic Orofacial Pain Models

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Faculty of Dentistry
University of Toronto

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2014

Abstract

This study aimed to examine whether trigeminal nerve injury induces chronic nociceptive behaviour and central sensitization (CS) in functionally identified medullary dorsal horn (MDH) nociceptive neurons in mice, and whether CS in acute and chronic orofacial pain models and nociceptive behaviour in the chronic model are affected by systemic administration of pregabalin. Infraorbital nerve injury induced chronic facial mechanical allodynia as well as MDH CS; acute noxious tooth pulp stimulation also induced MDS CS. Systemic administration of pregabalin attenuated the nerve injury-induced allodynia as well as the MDH CS in both the chronic and acute pain models. These findings reveal that MDH CS occurs in mouse models of acute and chronic orofacial pain and that pregabalin may prove useful clinically in acute and chronic orofacial pain states.
Acknowledgements

I would like to express my inmost gratitude to a number of individuals whose support has been crucial for the accomplishment of this work.

I would especially like to thank Dr. Barry J. Sessle, my supervisor and mentor, who taught me how to conduct scientific research and give it a concise written form.

I would also like to thank Dr. Shimon Friedman for his unstinting mentoring and help over my academic and clinical years at the University of Toronto.

I would like to thank Dr. Limor Avivi-Arber for her kind assistance in preparing this work.

Furthermore, I would like to express my appreciation to my colleague and friend at the University of Toronto, Dr. Vidya Varathan.

I wish to extend my thanks to my family for their continued understanding and support throughout these years.

Finally, my words of gratitude go to Ilona, my patient and loving wife who walked this journey with me.

This study was supported in part by grants from the American Association of Endodontists Foundation and the Canadian Academy of Endodontics Endowment Fund.
# Table of Contents

Abstract ii  
Acknowledgements iii  
Table of Contents iv  
Abbreviations vi

Chapter 1: General Introduction 1  

1.1 Introduction 1

1.2 Pain 1  
1.2.1 Multidisciplinarity and socioeconomical impact of pain 2  
1.2.2 Acute vs. chronic pain 2  
1.2.3 Classification of pain: nociceptive, inflammatory and neuropathic 3  
1.2.4 Classification and prevalence of orofacial pain 3  
1.2.5 Trigeminal neuropathic pain (atypical odontalgia and atypical facial pain) 4  

1.3 Pain management 5  
1.3.1 Non-pharmacological management of orofacial pain 5  
1.3.2 Pharmacological management of orofacial pain 6  
1.3.3 Anticonvulsant drug pregabalin 7  

1.4 Mechanisms and models of orofacial pain 8  
1.4.1 Peripheral processes of the trigeminal system 8  
1.4.2 Central processes in the trigeminal system 10  
1.4.3 Central sensitization 12
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>c-Fos</td>
<td>Cellular oncogene Fos</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CNX</td>
<td>Cervical nerve transection</td>
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<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<td>CS</td>
<td>Central sensitization</td>
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<tr>
<td>EMG</td>
<td>Electromyography</td>
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<td>GABA</td>
<td>Gamma-Aminobutyric acid</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>IAN</td>
<td>Inferior alveolar nerve</td>
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<tr>
<td>IANR</td>
<td>Inferior alveolar nerve regeneration</td>
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<tr>
<td>IANX</td>
<td>Inferior alveolar nerve injury</td>
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<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>ION-CCI</td>
<td>Infraorbital nerve ligation</td>
</tr>
<tr>
<td>IONX</td>
<td>Infraorbital nerve transection</td>
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<tr>
<td>MDH</td>
<td>Medullary dorsal horn</td>
</tr>
<tr>
<td>MO</td>
<td>Mustard oil</td>
</tr>
<tr>
<td>MWT</td>
<td>Mechanical nociceptive withdrawal thresholds</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NS</td>
<td>Nociceptive-specific</td>
</tr>
<tr>
<td>P2X</td>
<td>Purinoceptor subtype 2X</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>P38 mitogen-activated protein kinases</td>
</tr>
<tr>
<td>RCT</td>
<td>Root canal therapy</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>--------------------------------</td>
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<tr>
<td>RF</td>
<td>Mechanoreceptive field</td>
</tr>
<tr>
<td>RFm</td>
<td>Reticular formation</td>
</tr>
<tr>
<td>RM</td>
<td>Repeated measures</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>SGC</td>
<td>Satellite glial cells</td>
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<tr>
<td>TMD</td>
<td>Temporomandibular disorder</td>
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<tr>
<td>WDR</td>
<td>Wide dynamic range</td>
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Chapter 1
General Introduction

1.1 Introduction

According to the IASP, pain is an unpleasant multidimensional sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (1). Pain is the most frequent chief complaint for which patients come to a dental office. While some of the pain complaints can be easily managed by providing an adequate dental treatment usually followed by over-the-counter medications, others require comprehensive evaluation and diagnosis with long-lasting therapeutic and psychological approaches. Unrelieved pain can cause changes in the patient’s life, additional autonomic symptoms and changes in the peripheral and central nervous systems. Pain has also a motivational component that may affect the patient’s behaviour, reflexes and sleep. Understanding the physiology of pain will provide better therapeutic methods for pain management, improving the patient’s experience and preventing negative outcomes associated with the pain experience.

1.2 Pain

Being a multidimensional experience, pain is associated with actual or potential tissue damage. Pain has a sensory component that translates and transmits nociceptive signals via the peripheral and central nervous systems up to a final destination in the cortical regions such as the somatosensory cortex where the processing related to “pain sensation” (which is always a psychological state) takes place. The affective or emotional component of pain comes into play in the unpleasantness of pain. The motivational component of pain provides a conscious manifestation of a pre-conscious perception of threat to body tissues that motivates a subject to avoid or remove the threat (2). The cognitive component of pain is related to the patient’s personal experiences and beliefs. If the patient believes in a negative outcome of the treatment, he or she may catastrophize the actual experience and perceive pain to be of a higher intensity (e.g., painful experience from a dental office visit may intensify pain experience several years later).
1.2.1 Multidisciplinarity and socioeconomical impact of pain

The prevalence of chronic pain is considerably high, estimated to be within the range of 12-30% in Europe, and about 20% of the adult population in Canada, according to recent surveys (3-5). The estimated cost in lost productive time from chronic pain conditions is over US$ 60 billion annually (6). The burdens of related drug abuse, depression, and complications of opioid treatment are more difficult to quantify (6). A recent Canadian survey has demonstrated that more than 50% of patients with chronic pain have reported reduced quality of life, job loss or reduced job responsibilities and about 30% indicated increased rates of depression (7). In Canada, the personal financial costs for patients with pain is estimated around $1,500 per month (3). Chronic pain conditions carry a huge economic cost of several billion dollars per year in the United States and Canada (3, 8-10).

Also noteworthy is that more than 40 million people undergo surgical procedures every year in the United States. A high proportion of these patients undergo major surgical procedures that result in nerve damage with the potential for the subsequent development of chronic pain conditions (6). About 1.7 million people in the United States are survivors of limb loss, and each year over 130 000 new amputees are added to that number (6). There are estimates that around 50–80% of these patients experience significant, long-term phantom or residual limb pain (11). Up to 10% of these patients develop severe, life-changing, chronic pain. Analogous findings of chronic pain after trigeminal nerve damage are reviewed below.

1.2.2 Acute vs. chronic pain

Pain is commonly divided into two categories: acute and chronic pain. Acute pain usually carries a “protective” role to warn the body of potential or real tissue damage. The majority of acute pain conditions can be successfully treated and will heal uneventfully. However, there are reports indicating that about 20% of acute pain conditions can transition into a chronic state (12-18). Furthermore, chronic pain can be associated with a variety of chronic diseases and disorders such as arthritis, diabetes, cancer, HIV/AIDS or be an “independent entity” such as migraine, temporomandibular disorders (TMD), trigeminal neuralgia and fibromyalgia (12). It is generally accepted that chronic pain does not confer a positive biological role in most cases.
1.2.3 Classification of pain: nociceptive, inflammatory and neuropathic

Nociception is a process of detection and signalling the presence of a noxious stimulus. Nociceptive pain is an alarm mechanism that identifies and signals the presence of a damaging or potentially damaging stimulus. This mechanism takes place when noxious stimuli activate primary afferent neurons (nociceptors) that innervate peripheral tissues such as skin, bone, muscle, connective tissues, vessels and viscera. There are two types of primary afferent neurons that normally transmit nociceptive signals: unmyelinated (C fibres) and myelinated (Aδ fibres). The axons of these neurons carry sensory information from the peripheral tissues to the dorsal horn of the spinal cord and trigeminal brainstem sensory nuclear complex. Inflammatory pain is produced following persistent tissue injury, as occurs after trauma, surgery or during acute and chronic inflammatory diseases (19). In these conditions, damaged cells and inflammatory cells recruited to the site of damage release substances that activate, and/or sensitize, peripheral nociceptors (12, 20, 21).

Neuropathic pain is a condition caused as a direct consequence of a lesion or a disease of the parts of the nervous system that normally signal pain (22-24). This pain can be associated with such conditions as traumatic nerve injury, diabetic peripheral neuropathy, AIDS, post-herpetic neuralgia, or pain originating in the central nervous system, as in spinal cord injury and multiple sclerosis and stroke (19).

1.2.4 Classification and prevalence of orofacial pain

There is a consensus on three major steps that are used to classify orofacial pain (25, 26). During the first step, cluster analysis identifies different entities among all patients. The second step is based on diagnostic criteria for each of the previously identified groups. During the third step, a group of experts decide on unaddressed cases after the first two steps (25, 26).

The prevalence of toothache pain in the general population has been reported to be around 12% (27). Acute pain occurring within one week following root canal treatment (RCT) has been reported in 1.6% to 6.6% of all RCT-treated patients (16). Occurrence of persistent pain for up to 6 months after RCT has been reported in 3% to 12% of patients (13-16, 28). Extrapolation of this
data to an estimate for the United States (and Canadian) populations indicates that in the United States approximately 870,000 (96,000 in Canada) of the new cases of persistent pain occur following relatively common dental treatment each year, with 550,000 (61,000 in Canada) cases of such pain not having an identifiable local reason explaining why it is present (15). The prevalence of TMD pain ranges from 9% to 15% in the adult population and this pain appears to be two times as common in women than in men (29, 30). Similarly, migraine headache is more prevalent in women and affects about 20% of women and 7% of men (31, 32). The prevalence of non-migrainious headaches is relatively high and can be experienced by 60% to 80% of adults (31). The incidence of trigeminal neuralgia, a condition characterized by a sudden, brief paroxysmal stabbing pain, is about 27 patients per 100,000 persons annually (33, 34). Other pain conditions in the orofacial region (atypical odontalgia and atypical facial pain) are discussed in the next section.

1.2.5 Trigeminal neuropathic pain (atypical odontalgia and atypical facial pain)

Root canal therapy frequently involves the extirpation of pulp tissue and injury of the nerves supplying the pulp. Two persistent pain conditions in which pulp nerve injury has been implicated are atypical odontalgia and atypical facial pain. Atypical odontalgia is defined as pain in or around a tooth, which is not related to any dental cause and is often mistaken as toothache and treated with multiple dental treatments (35, 36). Atypical odontalgia has also been defined by the International Headache Society as a subgroup of persistent idiopathic facial pain, also including atypical facial pain. Atypical odontalgia and atypical facial pain share the definition of ‘persistent facial pain that does not have the characteristics of the cranial neuralgias and is not attributed to another disorder’ (37). These pains may in fact constitute a sub-set of trigeminal neuropathic pain resulting from injury of sensory fibres supplying the extirpated pulp and have been well characterized by Baad-Hansen (35) and List et al. (38). Physiological testing (38) has shown that patients with atypical odontalgia have peripheral and central sensitization changes. Characteristically, atypical odontalgia pain persists during most of the day, it is non-paroxysmal (18, 35-38) and it can affect both sexes and all adult ages, although there is a preponderance of women in their mid-40s who are affected. It has been suggested that genetic predisposition and environmental influences can contribute to the severity of the pain (18, 35-39). The diagnosis of
atypical odontalgia and atypical facial pain is often difficult, and is based on the exclusion of conditions with known pathophysiology in the teeth or adjacent structures (35, 37). Baad-Hansen et al. have suggested that the management of atypical odontalgia and atypical facial pain is primarily based on expert opinion and case reports and that these conditions are often difficult to manage effectively. Therefore, it is very important to study the mechanisms involved in the development and maintenance of atypical odontalgia and other orofacial neuropathic conditions and how they might be effectively managed.

1.3 Pain management

Based on the survey of the American Dental Association, the most common orofacial pain treatments provided by dentists are provision of occlusal appliances, occlusal adjustment (equilibration), thermal packs, medications and diet counseling (40). Historically, many of the treatment modalities have been based on personal opinions. The current clinician’s approach promotes an evidence-based concept of treatment with a focus on translation of the new scientific evidence into clinical settings. It is based on the integration of personal clinical experience with the best available evidence emerging from current research. The area of orofacial pain management includes non-pharmacological and pharmacological approaches.

1.3.1 Non-pharmacological management of orofacial pain

In the case of dental pain, management of pain associated with dentin exposure may include occlusion of dentinal tubules to prevent intra-tubular fluid flow or application of potassium ions that reduce the excitability of pulpal primary afferents (41). Treatment of reversible pulpitis due to caries or defective restoration includes removal of caries and provision of a new restoration with integral marginal seal (42). The definitive management of pain associated with irreversible pulpitis is RCT. Another approach is the extraction of the affected tooth (42). Pain associated with periodontal diseases is treated by irrigation, root planning and pocket elimination. In some periodontal situations, tooth extraction may be required (43). Pain due to the apical or periodontal abscesses is quite often treated as an emergency by surgical incision and drainage (44).
Non-pharmacological treatment options for patients with persistent pain in the orofacial region may include occlusal therapies such as the adjustment of teeth, provision of bite appliances, orthodontic and fixed prosthodontic treatments with or without surgical corrections. For long-term management, the provision of an inter-occlusal appliance can benefit the patient. Other treatment modalities for patients with persistent orofacial pain may include physiotherapy in the orofacial area (45-47).

There is some progress in management of chronic orofacial pain states such as headache TMD and trigeminal neuralgia by using the traditional acupuncture (48). Cognitive behavioural therapy is another approach that has been shown to improve outcomes for patients with TMD (49).

**1.3.2 Pharmacological management of orofacial pain**

Pharmacological management of orofacial inflammatory pain aims to block or reduce the nociceptive input from the peripheral site (e.g. tooth pulp, bone, soft tissue), block nociceptive impulse propagation along the peripheral nerve and reduce neuroplastic changes in the central nervous system. Administration of short and long-lasting local anaesthetics following tissue manipulations during dental procedures prevents generation and propagation of these nociceptive impulses along the primary afferents. Drugs that block peripheral sensitization induced by inflammatory process are used to minimize the nociceptive input from the periphery. Medications that block the synthesis of prostaglandins or cyclooxygenase (COX) enzymes perioperatively are another approach to minimize the peripheral nociceptive input. To attenuate changes that can occur following peripheral acute inflammatory processes, medications that act in the central nervous system such as acetaminophen and opioids also may be used. However, opioids have many adverse effects such as nausea, vomiting and drowsiness (50). Non-opioid analgesics include the following medications: salicylates (aspirin, diflunisal), acetaminophen, non-steroid anti-inflammatory drugs such as ibuprophen and naproxen and COX-2 inhibitors such as celecoxib (51). A combination of analgesics is another pharmacological approach to deal with inflammatory pain that does not respond to a single agent alone (52). The rationale behind this approach is to affect several mechanisms involved in the development and maintenance of inflammatory pain.
Treatment of neuropathic pain is complex and challenging, and cannot be restricted to the implicated peripheral tissue. Pharmacological treatment for neuropathic pain includes medications such as opioids, antidepressants, anticonvulsants and topical medications.

Opioids such as morphine, oxycodone, fentanyl, hydrocodone and codeine act principally via the $\mu$-receptor. However, there is a risk of drug abuse from prescribing opioids, and some degree of tolerance to opioids has been demonstrated; therefore, more attention is required from a clinician prescribing this type of medication (53).

The tricyclic antidepressants such as amitriptyline, nortriptyline, imipramine, desipramine and doxepin are often effective in the management of diabetic peripheral neuralgia, post-herpetic neuralgia and post-mastectomy chronic pain (54). The mechanism of action of these drugs is related to the blockage of reuptake of noradrenaline and serotonin in the brain that are released in response to pain. Thus, tricyclic antidepressants provide a prolonged inhibitory action via mechanisms involving noradrenaline and serotonin.

Topical drugs such as lidocaine and capsaicin act peripherally to induce analgesia and have been demonstrated to be effective in relieving pain in post-herpetic neuralgia (55).

Anticonvulsant drugs such as carbamazepine and gabapentin have been shown to be effective in treating patients with diabetic neuropathy, malignancy-related pain, post-herpetic neuralgia and any neuropathic pain states including trigeminal neuralgia (39, 55-58).

### 1.3.3 Anticonvulsant drug pregabalin

Recent studies have demonstrated that pregabalin, an anticonvulsant drug (a potent $\alpha_2\delta$-calcium channel blocker) that influences glutamatergic neurotransmission, is effective in treating neuropathic pain conditions (55, 56, 58-63). However, pregabalin still has to undergo detailed investigation in patients with trigeminal neuropathic as well as in acute inflammatory pain models. Systemic effects of pregabalin can significantly inhibit ectopic discharges from injured afferent neurons (64). Our group has demonstrated that pregabalin can decrease sensorimotor responses and glutamate release in an acute orofacial inflammatory pain model in rats (65). The effects of pregabalin on neuronal activity in an acute orofacial inflammatory pain model have not been studied yet. There is evidence indicating that intrathecal application of pregabalin reduces
the enhanced noxious stimulus-induced spinal release of glutamate seen in neuropathic rats. There are also reports indicating that pregabalin at varying doses reduces nociceptive responses in the spinal dorsal horn neurons in rat pain models (66-69). We have previously demonstrated a dose-dependent effect of pregabalin in reversing the facial mechanical allodynia and medullary dorsal horn (MDH) central sensitization present at postoperative day 7 following partial infra-orbital nerve injury (70).

Pregabalin does not appear to have been tested in acute inflammatory and long-lasting trigeminal neuropathic pain models; therefore, there is no scientific underpinning supporting or contesting its clinical use for trigeminal neuropathic pain conditions. More studies are required to document and understand the underlying mechanisms of pregabalin actions in acute inflammatory and long-lasting neuropathic pain models.

1.4. Mechanisms and models of orofacial pain

Orofacial pain is not only a “simple” transmission of the nociceptive input up to the cortical areas along the peripheral and central parts of the nervous system, but is strongly associated with cellular changes that take place along that pathway (71-74). Furthermore, not only nociceptive neurons, but also different types of neuronal and glial cells are involved in acute and chronic pain states (71, 72, 74). Peripheral and central sensitizations are contributing factors to the hyperalgesia, allodynia, spontaneous and referred pain and pain spread that may be manifested in pain conditions (71-73).

1.4.1 Peripheral processes in the trigeminal system

The orofacial region including the teeth, skin, temporomandibular joint and orofacial musculature are innervated mainly by branches of the trigeminal nerve. Among the primary afferent nerve fibres that terminate in sense organs (receptors), there is a population of slowly conducting small-diameter primary afferents with free nerve endings which are activated by noxious stimuli. Activation of these nociceptors generates action potentials in these afferents that conduct these signals into the central nervous system and that can result in pain. Damage to peripheral tissues induces the release of chemical mediators such as serotonin, histamine, and tumor necrosis factor-alpha from mast cells, macrophages and immune cells (12,
Nociceptive afferents themselves also are capable of releasing neurotransmitters such as substance P and calcitonin gene-related peptide (CGRP). Additionally, sympathetic efferents innervating peripheral blood vessels and skin can release noradrenaline and activate nociceptive afferent. These chemicals can activate certain receptors on the nociceptive afferents and result in the release of second messengers inside the cell. Another way by which nociceptive afferents can be activated is by application of inflammatory irritants such as mustard oil (MO) and capsaicin that activate TRPA1 and TRPV1 receptors respectively. Subsequently, the activated nociceptive afferents may become hyperexcitable to the noxious and non-noxious stimuli. Since damage-induced chemical mediators may spread through tissues, the changes in nociceptive afferent sensitivity can also occur in neighbouring primary nociceptive afferents. The increased excitability of the nociceptive endings can lead to spontaneous activity, lowered activation thresholds and increased responsiveness to noxious stimuli. This process of increased sensitivity of primary afferents due to changes that take place after tissue damage is called peripheral sensitization. Peripheral sensitization may contribute to such clinical conditions as allodynia, hyperalgesia and spontaneous pain.

Many primary afferents innervate the tooth pulp. What sets the tooth pulp apart from other peripheral tissues is its very low compliance due to dentinal boundaries. This factor may significantly contribute to increased sensitivity in states such as reversible and irreversible pulpitis.

The majority (exceptions are jaw muscle spindle afferents and some mechanosensitive afferents supplying periodontal tissues) of somatosensory primary afferents that innervate orofacial tissues have their cell bodies in the trigeminal ganglion. The central projections of these primary afferent cell bodies enter the brainstem and may ascend or descend in the trigeminal spinal tract from which they give off collaterals that terminate in one or more subdivisions of the trigeminal brainstem sensory nuclear complex (12, 71-73).

There is growing evidence that neurons and satellite glial cells (SGCs) in the trigeminal ganglion undergo changes following acute and chronic peripheral injury; analogous changes occur in the dorsal root ganglion in the spinal somatosensory system (75-79). Prolonged discharges of primary afferents can increase the expression of a variety of sodium channels in the trigeminal ganglion neurons that can lead to an increase in the excitability of trigeminal nerve afferents (80). The tetrodotoxin-resistant sodium channels and potassium channels are thought to be
involved in an enhancement of trigeminal neuronal activity following trigeminal nerve injury (81). Peripheral injury induces gene expression, neuropeptide generation and increased neuronal excitability in the trigeminal ganglion (12, 75, 78). Neurons and SGC may release neuropeptides such as CGRP and substance P that affect their activity (82). Novel findings from several groups indicate that gap junctions between SGCs in the trigeminal ganglion may be important in spreading excitatory signals between glial cells and neurons (78, 79). Alterations of neuropeptides, receptors, cytokines, and growth factors in trigeminal neurons are thought to be possible mechanisms that cause an increase in the excitability of trigeminal neurons following trigeminal nerve injury (83).

As a result of all these processes described above, the increased nociceptive activity can generate an increased afferent barrage into the central nervous system. There, additional functional changes can occur in central nociceptive processing and contribute to the pain experience.

### 1.4.2 Central processes in the trigeminal system

The trigeminal primary afferents activate second-order neurons within the trigeminal brainstem sensory nuclear complex which can be subdivided into the principal or main sensory nucleus and the spinal tract nucleus which comprises three subnuclei (oralis, interpolaris, caudalis; Fig. 1, (12, 71-73, 84)). Subnucleus caudalis extends into the cervical spinal cord where it merges with the spinal dorsal horn (12, 71-73). Due to the high functional and anatomical similarity between spinal dorsal horn and trigeminal subnucleus caudalis, latter has been designated as the ‘medullary dorsal horn’ or MDH. Many neurons in the four components of the trigeminal brainstem complex contribute to ascending nociceptive or non-nociceptive pathways involved in the somatosensory function or modulation (12, 71-73). The trigeminal brainstem complex has a somatotopic or topographic organization (12, 71-73, 85).

Many of the small-diameter primary afferents terminate in the MDH (12, 71, 73, 85). Noxious stimulation of peripheral orofacial tissues results in the release from the nociceptive central endings of substance P, CGRP, glutamate and somatostatin which act on receptors of second-order sensory neurons to produce a long-latency, sustained excitation of these neurons (12, 71, 85). Numerous findings indicate that MDH serves as the principal brainstem relay site of trigeminal nociceptive information to higher brain centres involved in the discrimination of pain and also to local brainstem neurons involved in nociceptive reflexes (72, 73).
There are anatomical and electrophysiological similarities between MDH and the spinal dorsal horn (12, 71, 73, 85). For example, MDH has a laminated structure similar to the spinal dorsal horn, and like the spinal dorsal horn, nociceptive neurons occur in the MDH and can be categorized into two main groups on the basis of their cutaneous (or mucosal) receptive field properties: nociceptive-specific (NS) neurons, which receive small-diameter afferent inputs from A-delta and/or C fibres and which respond only to noxious stimuli (e.g. pinch and heat) applied to a localized craniofacial receptive field; and wide dynamic range (WDR) or convergent neurons, which may receive large-diameter and small-diameter A-fibre inputs as well as C-fibre inputs and which are excited by non-noxious (e.g. tactile) stimuli as well as by noxious stimuli. The NS and WDR neurons are concentrated in the superficial (I/II) and deep (V/VI) laminae of MDH.

Many trigeminal brainstem neurons project to the thalamus either directly, or indirectly via polysynaptic pathways that may involve the reticular formation (12, 71, 85). These projections carry signals that reach the higher brain centres involved in the somatosensory perception (e.g. touch and pain) and other functions (e.g. emotion and motivation). The projections from the trigeminal brainstem complex to the thalamus can result in the activation of neurons in thalamus which directly transmits signals to neurons in the overlying somatosensory cerebral cortex.
Figure 1. Major trigeminal somatosensory pathways from the orofacial region. Trigeminal primary afferents project via the trigeminal ganglion to second-order neurons in the trigeminal brainstem sensory nuclear complex. These neurons may project to neurons in higher levels of the brain (for example, in the thalamus) or to neurons in brainstem regions such as the cranial nerve motor nuclei or the reticular formation (RF). Not shown are the projections of some cervical nerve and cranial nerve VII, IX, X, and XII afferents to the trigeminal complex and the projection of many V, VII, IX, and X afferents to the solitary tract nucleus (from Sessle (12)).

1.4.3 Central sensitization

There are two major means by which neuroplasticity can be induced in central somatosensory pathways; first, an increased nociceptive afferent input (e.g. by direct stimulation of peripheral nerves by an injury or by inflammation) and second, a decreased afferent input (e.g. through nerve damage resulting in deafferentation). As a result, an increased neuronal excitability may occur, accompanied by pain behaviour. Pain associated with changes in the central nervous system has been viewed as a reflection of a centrally based “functional plasticity” or “central sensitization” (12, 71, 73, 74, 85, 86).
Central sensitization of nociceptive neurons can be produced by nerve inflammation or damage, such as that associated with pulpectomy or transection of dental nerve fibres, and is reflected as an increase in nociceptive neuronal mechanoreceptive field (RF) size, a decrease in mechanical activation threshold and an increase in spontaneous activity and in responses to noxious RF stimuli. Central sensitization thus reflects a hyperexcitability of nociceptive processes in the central nervous system and has been implicated as an important mechanism in acute as well as chronic pain conditions following injury or inflammation of peripheral tissues (12, 71, 73, 74, 85, 86, 88).

Injury of primary afferents that results in central sensitization can involve several processes. For example, the generation of abnormal impulses by affected primary afferents, formation of neuroma and consequently generation of abnormal peripheral discharges, sprouting of the afferents into neighbouring tissues, abnormal expression of different receptors by primary afferents, development of physical contacts among sympathetic efferents and nociceptive afferents, sprouting of nociceptive afferents in the central nervous system can be the underlying mechanisms of induction and maintenance of central sensitization.

Central sensitization has been well documented in nociceptive neurons in MDH, but can also occur in other nociceptive neurons along the trigeminal nociceptive pathway (e.g. subnucleus oralis, ventrobasal thalamus, etc., (89, 90)). In addition, several studies have demonstrated the involvement not only of neurons but also of non-neural cells (e.g., glia, and cells of immune system) in the development and maintenance of orofacial neuropathic pain states (12, 74, 79). Furthermore, several chemical mediators such as glutamate and endogenous ATP and their receptors have been shown to be essentially involved in the initiation of central sensitization in the MDH in rodent models of acute and chronic pulpitis pain (84, 91-94) and chronic trigeminal neuropathic pain (70, 95).

1.4.4 Models of orofacial pain

The nociceptive neurons in MDH and other higher centres can be activated not only by noxious mechanical and/or thermal stimuli applied to the orofacial region, but also by application of algesic chemicals and inflammatory irritants to the orofacial tissues such as the tooth pulp of rat (84, 93, 96, 97). In the trigeminal system, application of algesic chemicals and inflammatory
irritants into orofacial tissues can markedly increase the RF and responses of NS and WDR neurons in the MDH and reduce mechanical activation threshold (MAT, (12, 71)). Injection of inflammatory irritants into cutaneous or deep orofacial tissues can induce acute nociceptive behaviour in humans and animals (12). Depending on the inflammatory irritant, the nociceptive behaviour associated with inflammation may last for hours or even months. Central sensitization in the inflammatory pain models is usually reversible, but can be associated with pain behaviour that lasts for hours or even longer.

Several studies have demonstrated that application of MO to the tooth pulp of rat induces electromyography (EMG) activity in jaw muscles, expression of c-Fos (a marker of neuronal activity) and increased neuronal excitability in the MDH (12, 65, 98). In this model increased neuronal excitability of the MDH nociceptive neurons involves activation of NMDA and P2X receptors, and several mediators such as serotonin, NMDA, and IL-1β (12, 92, 93, 97). There is also evidence that brainstem astrocytes and microglia are involved in the developing and maintenance of central sensitization in acute and chronic pulpitis models in rats (79, 94). Preemptive administration of microglial inhibitors can prevent the development of central sensitization in rats (79, 99).

Several models of trigeminal neuropathic pain have also been developed in rats and mice: the inferior alveolar nerve (IAN) injury (IANX) model, the infraorbital nerve ligation (ION-CCI) model, the infraorbital nerve transection (IONX) model (70, 100), the inferior alveolar nerve regeneration (IANR) model, and the cervical nerve transection (CNX) model (83, 101, 102). There is a modulation of spike discharges in the primary afferent neurons following such peripheral nerve injury in rats (83, 103). It has been shown in rats that primary afferent neurons are sensitized and the activation threshold in these neurons became lower following the long-lasting abnormal spike generation in injured primary afferents for more than several weeks (104). This prolonged discharge can increase expression of a variety of sodium channels in the trigeminal ganglion neurons that can lead to an increase in the excitability of trigeminal nerve afferents (80). The tetrodotoxin-resistant sodium channels and potassium channels are thought to be involved in an enhancement of trigeminal ganglion neuronal activity following trigeminal nerve injury in rats (81).
The alteration of chemical mediators including neuropeptides, receptors, cytokines, and growth factors in trigeminal ganglion neurons is thought to be a possible mechanism that causes an increase in the excitability of trigeminal ganglion neurons and increased input to the MDH nociceptive neurons following trigeminal nerve injury in rats (83). Glial fibrillary acidic protein (GFAP) expression increases in the trigeminal ganglion following nerve injury, and becomes detectable by immunocytochemistry following nerve injury in rodents (105). The increase in GFAP in SGCs in the trigeminal ganglion after nerve injury may be triggered by increased glutamate released in the sensory ganglion, resulting from increased neuronal firing in rats and mice (75, 106). It has previously been demonstrated that the number of gap junctions between SGCs in the trigeminal ganglion of rodents increases following trigeminal nerve injury, suggesting that the changes in SGC gap junctions can be a factor in generating or maintaining neuropathic pain (75, 78).

After the long-lasting hyperactivity of the primary afferent neurons, a barrage of action potentials is conveyed to the central nervous system (CNS), resulting in the production of central sensitization of the brain stem nociceptive neurons (70, 83, 94, 107). There are several reports indicating that hyperactive astroglial cells in the MDH of rats are significantly involved in the central sensitization of trigeminal nociceptive neurons (108, 109). Hyperactive astroglial cells were found in the rat MDH following IAN transection and this was also associated with an increased activity of nociceptive neurons. The proposed mechanism of astroglial involvement in the development and maintenance of trigeminal central sensitization is via excessive release of glutamine which is taken up in the primary afferent terminals via glutamate transporters, resulting in an increase in the glutamate release at the synaptic cleft.

Another important population of non-neuronal cells that has been shown to be involved in neuropathic pain mechanisms in rats is microglial cells. These cells are activated 1–3 days after IAN transection, whereas the activation of astroglial cells takes 7–14 days after that (94, 109). It has recently been reported that astroglial and microglial cells have specific interactions and communicate with each other. The astroglial and microglial cell interactions may be involved in the hyper-activation of the MDH nociceptive neurons following trigeminal nerve injury in rats (108).
1.4.5 Modulation of orofacial pain mechanisms

The central sensitization that takes place in the nociceptive neurons in the MDH following peripheral injury is a reflection of neuroplasticity. There are also other modulatory mechanisms of somatosensory transmission and these are not limited to the MDH area but can also occur in thalamic and cortical areas (12, 71, 73, 74, 89, 90). Most of the modifications of the ascending somatosensory transmission, however, take place at the level of trigeminal brainstem complex. Each subdivision of the trigeminal brainstem complex receives numerous peripheral inputs and interconnections from other parts of the brain. These interactions that are derived from the periphery are termed afferent inhibition, while those from the other brain regions are called descending modulation. There are interconnections among neurons of MDH, ascending modulatory influences of MDH on more rostral regions, and descending influences from regions such as the periaqueductal gray and cerebral cortex to the trigeminal brainstem complex (71).

Since many of the sensory brainstem neurons are involved in somatosensory and autonomic reflexes, modification of sensory transmission can affect motor and autonomic functions. For example, our recent study has demonstrated that application of the inflammatory irritant MO to the rat’s tooth pulp induces spontaneous EMG activity in the jaw-opening and jaw-closing muscles (65).

1.5 Statement of the problem and rationale

There have been no reports in the literature documenting whether injury of the mouse ION can induce long-lasting facial nociceptive behaviour and central sensitization in functionally identified trigeminal nociceptive neurons in the MDH. There have also been no studies documenting the effect of the anticonvulsant drug pregabalin on long-lasting nociceptive behaviour and central sensitization following ION injury, and whether pregabalin is effective in attenuating central sensitization in functionally identified MDH nociceptive neurons in the trigeminal nociceptive system in an acute rodent inflammatory orofacial pain model. Thus there is a need to collect pre-clinical data on the possible therapeutic effectiveness of pregabalin in treating orofacial acute and chronic orofacial pain conditions.
Chapter 2
Project Specific Aims and Hypotheses

The specific aims of this study were to test (i) if trigeminal nerve injury in mice produces prolonged nociceptive behaviour and induces central sensitization in functionally identified nociceptive neurons in the MDH and (ii) if systemic administration of pregabalin can reverse these nociceptive changes and (iii) if MDH mustard oil-induced central sensitization in rats and mice can be attenuated by systemic administration of pregabalin.

Our two working hypotheses were:

1) injury of the ION in mice induces nociceptive behaviour and central sensitization in functionally identified nociceptive neurons in the MDH

2) Pregabalin affects central sensitization in nociceptive neurons in the MDH in acute and chronic pain models in mice and rats and reverses nociceptive behaviour in a chronic pain model in mice
Chapter 3
Articles

3.1 Article 1

(Submitted for publication)

Prolonged Nociceptive Behavior and Central Sensitization are Attenuated by Pregabalin in a Mouse Trigeminal Neuropathic Pain Model

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Funding disclosure: This research was supported in part by a Research Grant from the American Association of Endodontists Foundation, Canadian Academy of Endodontics Endowment Fund, NIH grant DE-04786 and Pfizer Canada.

Dr. Sessle was awarded a 2009-2011 research grant from Pfizer Canada.
Abstract

**Introduction:** Chronic post-endodontic pain may be generated by trigeminal nerve injury. Since central sensitization may underlie the development of chronic pain, this study aimed to examine in mice if (i) trigeminal nerve damage produces prolonged nociceptive behavior and induces central sensitization in functionally identified nociceptive neurons in the medullary dorsal horn (MDH) and (ii) if systemic administration of pregabalin can modulate these nociceptive changes.

**Methods:** Facial mechanical nociceptive withdrawal thresholds (MWT) were tested in adult A/J male mice with von Frey filaments applied to facial skin, pre-operatively and post-operatively up to day 56 following infraorbital nerve transection (IONX) or sham surgery. On post-operative days 7, 21 and 49, MWT were assessed before and after administration of pregabalin (75mg/kg i.p., n=11/group) or isotonic saline (vehicle control, n=11/group). MDH nociceptive neurons were also recorded at similar post-operative days (n=7/group), and their mechanical activation threshold (a decrease of which reflects central sensitization) was assessed before and after pregabalin administration.

**Results:** The MWT values were significantly reduced in the IONX group (p<0.05; ANOVA, followed by post-hoc tests) compared to sham, up to post-operative day 49. At post-operative days 7, 21 and 49, pregabalin (but not saline) reversed the IONX-induced reduction of MWT. A decreased mechanical activation threshold in MDH nociceptive neurons was also recorded, and this effect too was reversed by pregabalin (but not saline).

**Conclusions:** This study demonstrated that pregabalin attenuated IONX-induced mechanical allodynia and the associated MDH central sensitization in mice and indicated that pregabalin might be useful in management of orofacial neuropathic pain conditions.
Introduction

Persistent pain conditions carry a huge socioeconomic burden, at a financial cost of several billion dollars per year in both the United States and Canada (1, 2). A specific persistent pain condition, chronic post-endodontic pain may be induced by trigeminal nerve injury, associated with extirpation of pulp tissue and injury of the nerves supplying the pulp during root canal treatment (RCTx). It has been estimated that more than 22 million endodontic procedures (15.1 million RCTxs) are performed annually in the United States alone (3). Acute pain occurs within one week of RCTx in 3% to 11.6% of all treated patients (4-7); it may become chronic in 3 to 6% of patients (6-12), if persistent post-endodontic pain develops in 5% of treatments, then more than a million patients will experience a chronic neuropathic pain for a period of 6 months or longer. Post-endodontic pain is typically treated with various short-term analgesics taken orally when the pain becomes severe. This regimen is highly effective in the majority of patients (13) but not in all those experiencing post-endodontic pain, especially chronic post-endodontic pain which is often difficult to manage effectively.

Chronic pain involves a process of central sensitization which refers to an increase in the excitability of nociceptive neurons within the central nervous system (CNS) and is reflected as a decrease in mechanical activation threshold and an increase in neuronal mechanoreceptive field (RF) size and response to noxious RF stimuli. Central sensitization has been implicated as an important process in acute as well as chronic pain conditions following injury or inflammation of peripheral tissues, including trigeminal nerve injury (14, 15). We have previously demonstrated that glutamate and endogenous ATP and their receptors are essentially involved in the initiation of central sensitization in the trigeminal subnucleus caudalis (also termed the medullary dorsal horn, MDH) in rodent models of acute pulpitis pain (16-19) and chronic trigeminal neuropathic pain (20, 21).

There has been limited study of recently developed analgesic drugs in these models. Recent studies have shown that pregabalin, an anticonvulsant drug (a potent α2δ-calcium channel blocker) that influences glutamatergic neurotransmission (22, 23), is effective in treating many neuropathic and inflammatory chronic pain conditions in both patients and animals (24-26). We have demonstrated that nociceptive sensorimotor behavioral responses and the medullary release of glutamate in the acute inflammatory tooth pulp pain model can be attenuated in a dose-
dependent manner by systemic administration of pregabalin (27). To our knowledge, however, pregabalin has not been tested in a mouse trigeminal neuropathic pain model in order to provide pre-clinical data bearing on its potential use for trigeminal neuropathic pain conditions in humans. Therefore, the aims of this study were to test in mice if (i) trigeminal nerve damage produces prolonged nociceptive behavior and induces central sensitization in functionally identified nociceptive neurons in the medullary dorsal horn (MDH) and (ii) if systemic administration of pregabalin can modulate these nociceptive changes.
Materials and Methods

Animals

One hundred and twenty-six male A/J mice (Jackson laboratories, USA) aged 8-12 weeks and weighing 18-24 g, were used in this study. All experimental procedures were approved by the University of Toronto Animal Care committee in accordance with the Ontario Animal Research Act (Canada). All efforts were used to limit the number of animals required for the study.

Surgical procedure

Under isoflurane anesthesia (4% induction, 2–2.5% maintenance), an incision was made in the right maxillary gingivo-buccal groove. The infra-orbital nerve (ION), which supplies the maxillary whisker pad, mucosa and anterior teeth, was exposed and dissected free from the surrounding tissue. The ION was lifted from the maxillary bone and totally sectioned (IONX) with iridectomy scissors. In sham mice, the ION was exposed but not sectioned. Post-operatively, mice were monitored daily.

Behavioral testing

A total of 66 mice were used in the behavioral experiments [sham and IONX; IONX-pregabalin at post-operative days 7, 21 or 49 and IONX-saline (11 per group)]. Mice were initially trained for at least 3 consecutive days to stay in a plastic cage and protrude their snout through a hole in the cage wall, during mechanical stimulation of the maxillary or mandibular facial area with von Frey filaments, as previously described (28). Escape responses to the mechanical stimulation were demonstrated as a sudden backward withdrawal movement of the head from the cage opening. Baseline values of the animal’s mechanical withdrawal threshold (MWT) were obtained by slowly applying graded von Frey filaments to the contralateral maxillary or mandibular and ipsilateral mandibular areas of the face. The MWT at the maxillary or mandibular area was defined as the lowest filament intensity that evoked three or more escapes out of five stimulation trials with intervals of more than 10 seconds. Behavioral scoring was carried out at days 1-3 before IONX (baseline values), at day 0 (1 h before IONX) and up to post-operative day 56. On post-operative days 7, 21 and 49, MWT was assessed before and after intraperitoneal (i.p.) administration of pregabalin (Pfizer, Canada; 75 mg/kg i.p., n=11) or isotonic saline (vehicle
control, n=11). The pregabalin dosage chosen has been shown to be effective in other pain models (20, 27). Experiments were carried out in ‘blind’ design in all groups.

Electrophysiological recording and stimulation procedures

A total of 60 mice were used in the electrophysiological study [sham at post-operative days 7, 21 or 49 (3 per group); IONX at post-operative days 7, 21 or 49 (3 per group); IONX-pregabalin and IONX-saline at post-operative days 7, 21 or 49 (7 per group)]. Two to three neurons in each animal were studied in the IONX and sham groups, but only one neuron was studied in each animal in those IONX groups receiving pregabalin (or saline) due to the long-lasting effect of pregabalin. Evoked single neuron activity was recorded in histologically verified sites in the deep laminae of the right MDH (lateral: 0.8-1.0 mm; posterior: 0.8-1.0 mm), as previously described (17, 18, 29). Briefly, neuronal responses to stimulation of the orofacial region were amplified and displayed on an oscilloscope and computer and the data were later analyzed off-line. Mechanical (brush, pressure and pinch) and noxious thermal stimuli were applied to classify neurons recorded as nociceptive-specific (NS) if they responded exclusively to the noxious stimulation, as previously described (17, 18). Each NS neuron's cutaneous orofacial receptive field (RF) was determined with nonserrated forceps, and its activation threshold to mechanical stimulation of its RF was assessed by force-monitoring forceps. Central sensitization was reflected as a decrease in activation threshold, as previously described (17, 18). Pregabalin (75 mg/kg) or saline was administered (i.p.) after the baseline properties of each neuron were obtained in each animal group at 7, 21 or 49 days following IONX. The neuronal properties were re-assessed at 60 minutes after pregabalin or saline administration, as previously reported (20). Recording sites were marked by electrolytic lesions (anodal current of 8 µA for 13 s) and verified histologically (Fig. 3B).

Sample size calculation

Based on the results of a pilot study, we calculated the sample size required to compare populations means with 95% power, of 11 mice for behavioral testing and 6 neurons for electrophysiological experiments per group.
Analysis

Data were tested for normality (Kolmogorov–Smirnov test) and equal variance. Data are reported as mean ± SEM. For the behavioral tests, differences in MWT between the baseline (average of the 3 preoperative day values) and each postoperative day were analyzed with 1-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. The effects of pregabalin compared with saline (as vehicle control) were tested by 1-way ANOVA followed by the Bonferroni post-hoc test. Differences between the groups (sham, IONX, IONX after pregabalin and IONX after saline) were analyzed with 2-way ANOVA followed by Dunnett’s test. For neuronal recordings, differences within the same post-operative day group (7, 21 or 49 days) in neuronal mechanical activation threshold between the groups (sham, IONX, IONX after pregabalin and IONX after saline) were analyzed with 1-way ANOVA followed by the Bonferroni post-hoc test. The level of significance was set at a P < 0.05.

Results

Behavioral testing

Nociceptive MWT to mechanical stimulation of the contralateral maxillary and ipsilateral and contralateral mandibular areas were tested in the IONX and sham-operated animals (Fig. 1). When compared with sham animals, the IONX group demonstrated a significant (2-way ANOVA, p< 0.05) decrease in MWT in the ipsilateral mandibular, contralateral maxillary and mandibular area. The decrease in MWT, considered to reflect mechanical alldynia, lasted up to day 42 following IONX for all three facial sites and up to day 49 for the contralateral maxillary and ipsilateral mandibular areas (2-way ANOVA, p< 0.05).

The effects on nociceptive behavior of pregabalin or vehicle (control) were studied in 3 different groups of IONX animals tested at 7, 21 or 49 days following IONX (Fig. 2). Administration of pregabalin significantly reversed the IONX-induced decreased MWT values to control levels for two hours at days 7, 21 and 49 (2-way ANOVA, p<0.05). Administration of vehicle (control) did not reversed the IONX-induced decreased MWT to control levels (2-way ANOVA, p<0.05).
**Electrophysiological recording**

Eighty-four functionally identified NS neurons were studied in the sham (control) and in the IONX groups at days 7, 21 and 49. All neurons were located in the deep laminae of the MDH (Fig 3, B). The mechanical activation threshold of single nociceptive MDH neurons in sham animals at days 7, 21 and 49 did not change significantly (1-way ANOVA followed by Bonferroni post-hoc test, Fig.3 A). The IONX produced significant decrease in mechanical activation threshold at days 7, 21 and 49 (1-way ANOVA followed by Bonferroni post-hoc test), indicative of central sensitization in the neurons. Administration of pregabalin 60 minutes before neuronal evaluation significantly reversed the IONX-induced central sensitization at day 7, 21 and 49 (1-way ANOVA followed by Bonferroni post-hoc test, p>0.05). Administration of saline (vehicle control) did not significantly impact on the IONX-induced decreased mechanical activation threshold (1-way ANOVA followed by Bonferroni post-hoc test, p>0.05).

**Discussion**

This study has demonstrated for the first time that injury of the mouse ION produced long-lasting facial nociceptive behavior, reflected as a decrease in MWT, and also induces central sensitization in trigeminal nociceptive neurons in the MDH, reflected as a decrease in mechanical activation threshold. This is the first report to document that administration of pregabalin (but not saline as vehicle control) reduced the long-lasting nociceptive behavior and central sensitization at days 7, 21 and 49 after IONX. These findings indicated that pregabalin might be effective in treating orofacial neuropathic pain conditions, which could be of significant clinical importance.

Central sensitization of central nociceptive neurons, reflected as a decrease in mechanical activation threshold and increases in their responses to noxious RF stimuli, has been implicated as an important process in acute and chronic orofacial pain conditions following injury or inflammation of peripheral tissues (16, 17, 30, 31). The present findings were consistent with our recent results (20) indicating that pregabalin is effective in attenuation of facial mechanical hypersensitivity and central sensitization in a rat model of trigeminal neuropathic pain (20). Also, in another recent study, we have documented in a model of acute inflammatory dental pain that pregabalin is effective in a dose-dependent manner in significantly attenuating the
nociceptive sensorimotor behavioral responses and medullary release of glutamate evoked by application of the inflammatory irritant mustard oil to the rat tooth pulp (27). Reports have demonstrated the effectiveness of pregabalin in the spinal nociceptive system since it can reduce nociceptive behavioral responses in rat models of neuropathic pain (22, 32). Our previous study (20) demonstrates in the rat trigeminal neuropathic pain model a dose-dependent effect of pregabalin in reversing the facial mechanical allodynia and MDH central sensitization at an early post-operative stage (day 7) but the present study demonstrated that pregabalin might be effective at longer post-operative times, even when the nociceptive behavior and central sensitization have become well maintained.

One limitation of this work is the unknown site and mode of action of pregabalin (33) that were not addressed in this study, although it has been proposed that pregabalin can reduce the influx of calcium by binding to the α2δ protein of calcium channels. This action reduces the release of neurotransmitters such as glutamate, norepinephrine and substance P (34-37). Another limitation of this study was use of general anaesthesia for the MDH study and use of awake animals for the testing of nociceptive behaviour. General anaesthesia may have an effect on neuronal properties by blocking normal conductivity and altering membrane permeability. Despite this, the MDH and behavioural data as well as effects of pregabalin on them were nonetheless complementary.

Systemic administration of pregabalin can significantly inhibit ectopic discharges from injured peripheral sensory neurons (38), this could partially explain the effect of pregabalin in the present study with the drug reducing or preventing afferent input to the MDH NS neurons. In a previous study, we have demonstrated that administration of pregabalin abolishes spontaneous activity in NS MDH neurons in a trigeminal neuropathic pain model in rats (20) which is consistent with this possibility. Nevertheless, there is an additional possibility of direct pregabalin action in the central nervous system since intrathecal application of pregabalin reduces the enhanced noxious stimulus-induced spinal release of glutamate seen in neuropathic rats (22). Pregabalin has affinity to receptors in the cortex, olfactory bulb, hypothalamus, amygdala, hippocampus, cerebellum and dorsal horn of the spinal cord (39).

Detailed clinical studies of the potential use of pregabalin in orofacial pain states are limited (40). However, several reports have demonstrated the analgesic success of pregabalin in the treatment of paresthesia following inferior alveolar nerve damage (41), glossopharyngeal (42) and lacrimal (43) neuralgias as well as post-traumatic neuropathic facial pain (44). The current
study provided pre-clinical data suggesting that pregabalin might be effective in the management of trigeminal neuropathic pain conditions, including chronic post-endodontic pain.

**Conclusions**

This study demonstrated for the first time that pregabalin attenuated the long-lasting IONX-induced mechanical allodynia and associated MDH central sensitization induced by trigeminal nerve injury. It provided pre-clinical evidence to support the use of pregabalin in treating orofacial neuropathic pain conditions.
References.


32. Hurley RW, Chatterjea D, Rose Feng M, Taylor CP, Hammond DL. Gabapentin and pregabalin can interact synergistically with naproxen to produce antihyperalgesia. Anesthesiology 2002;97:1263-1273.


Figure legends

**Figure 1.** Time course of the MWT in bilateral maxillary and mandibular facial areas after IONX. In the control (sham) group (n=11), the thresholds did not differ from pre-operative values bilaterally in mandibular area (yellow line). In the IONX groups (n =11), bilateral mechanical allodynia was established at day 1 after surgery. The MWT to mechanical stimulation of the ipsilateral mandibular (red line), contralateral maxillary (grey line) and mandibular (black) area were significantly (n=11, 2-way ANOVA, p<0.05) different from the control (sham) values and lasted for up to day 42 following IONX. The MWT to mechanical stimulation of the ipsilateral mandibular (red line) and contralateral maxillary (grey line) were significantly different from the control (sham) values for up to 49 days post-operatively. *P < .05 for comparison between sham group values and values at the different time points after IONX (2-way ANOVA). All values are shown as mean ± SEM.

**Figure 2.** The effects on the nociceptive behavior of pregabalin (PG, 75 mg/kg, i.p., B) or saline (vehicle control, A) were determined at 7, 21 and 49 days post-operatively. Administration of pregabalin (75 mg/kg, i.p., B) but not saline (vehicle control, A) significantly reversed the IONX-induced decreased MWT values to control levels for two hours at days 7, 21 and 49 (n=11, 2-way ANOVA followed by Dunnett's test, p<0.05). *P < .05 for comparison between sham group values and values at the different time points after IONX (2-way ANOVA followed by Dunnett's test). #P < .05 for comparison between the pre-treatment (pre-tx) IONX values and values at the different time points after pregabalin administration (1-way ANOVA followed by Bonferroni post-hoc test). All values are shown as mean ± SEM.

**Figure 3.** (A) Changes in mechanical activation threshold (MAT) of MDH nociceptive neurons at 7, 21 and 49 days following IONX. Each group consists of 7 NS neurons. The IONX significantly (1-way ANOVA followed by Bonferroni post-hoc test) decreased the MAT compared to control values at all tested days (red bars). Administration of pregabalin (75 mg/kg, i.p., PG, green bars) significantly (1-way ANOVA followed by Bonferroni post-hoc test) reversed the IONX-induced decreased MAT values at days 7, 21 and 49. Saline (vehicle control, blue bars) did not affect the IONX-induced decreased MAT values at days 7, 21 and 49 following IONX (1-way ANOVA followed by Bonferroni post-hoc test). (B) Histologically confirmed neuronal recording sites in sham and experimental groups. The sites were plotted onto
a section of the caudal medulla (~4.4 mm behind interaural line). Sp 5, trigeminal spinal tract; MDH, medullary dorsal horn (trigeminal subnucleus caudalis). All values are shown as mean ± SEM. *P < .05.
Fig. 2

A

- Head withdrawal threshold (g)
- Sham
- IONX pre-op
- IONX + saline
- 60 min
- 120 min
- 180 min
- 24 hr

B

- Head withdrawal threshold (g)
- Sham
- IONX pre-op
- IONX + PG
- 60 min
- 120 min
- 180 min
- 24 hr
Fig. 3

A

![Bar chart showing MAT (µg) for different groups over 7, 21, 49 days.](chart.png)

- **IONX**
- **IONX + PG**
- **IONX + saline**
- **sham**

B

- **Sp5**
- **MDH**

- **7 Days**
- **14 Days**
- **49 Days**

Legend:
- Red: IONX
- Orange: sham
- Green: IONX + PG
- Blue: IONX + saline
3.2 Article 2

(Submitted for publication)

**Pregabalin Blocks Central Sensitization in Medullary Dorsal Horn in a Rodent Model of Acute Tooth Pulp Inflammatory Pain.**

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Funding disclosure: This study was supported by NIH grant DE-04786 and Pfizer Canada.
Abstract
Acute inflammation of the tooth pulp can induce nociceptive sensorimotor behavioural responses as well as glutamate release in the rat medullary dorsal horn (MDH) and central sensitization reflected as increases in receptive field (RF) size and responses to noxious stimuli and a decrease in mechanical activation threshold in functionally identified MDH nociceptive neurons. The aim of this study was to test if MDH central sensitization in this acute inflammatory pain model in rats and mice can be attenuated by systemic administration of pregabalin, a potent α2δ-calcium channel agent that is used for the management of especially neuropathic pain. Male adult rats and mice were anaesthetized with α-chloralose (50-75 mg/kg)/urethane (1-1.125 g/kg) i.p.. The right maxillary first molar pulp and the dorsal surface of the caudal medulla were surgically exposed. The activity of single neurons was recorded at histologically verified sites in the MDH, and mechanical (brush, pressure, and pinch) stimuli were applied to functionally identified nociceptive-specific (NS) neurons. Pulp application of mineral oil (vehicle) produced no evidence of central sensitization in NS neurons, compared to their baseline properties. However, application of mustard oil (MO) to the pulp induced MDH central sensitization in rats and mice, as reflected in significant increases in RF size (40-130%, n= 6/group, repeated measures ANOVA, p<0.05) and responses to noxious stimuli (80-200%, n=6/group, p<0.05) and decreased mechanical activation threshold (45-75%, N=6/group, p<0.05). Compared to vehicle control treatment, administration of pregabalin (100 mg/kg, i.p.) markedly reduced the MO-induced central sensitization in rats and mice to baseline levels in all measured parameters: RF size (n=6/group, 2-way ANOVA, p<0.05), responses to noxious stimuli (n=6/group, 2-way ANOVA, p<0.05) and mechanical activation threshold (n=6/group, 2-way ANOVA, p<0.05). These results indicate that pregabalin can block central sensitization in the MDH in a rodent acute inflammatory pain model. Further understanding of the mechanisms involved in pregabalin-induced reduction in MDH central sensitization in inflammatory, as well as neuropathic, orofacial pain models may lead to broader clinical applications of pregabalin and other anticonvulsant drugs in pathological pain states.

Highlights
• Application of mustard oil to the tooth pulp induces trigeminal central sensitization
• Pregabalin blocks central sensitization in trigeminal nociceptive neurons
Abbreviations: MDH, medullary dorsal horn; NS, nociceptive-specific
Keywords: pregabalin, central sensitization, plasticity, trigeminal, dorsal horn, pain

Introduction
We have previously shown in an acute tooth pulp inflammatory pain model that application of the inflammatory irritant mustard oil (MO) to the rodent molar tooth pulp induces central sensitization in trigeminal subnucleus caudalis (also termed the medullary dorsal horn, MDH) and involves N-methyl-D-aspartate (NMDA) glutamatergic receptor mechanisms [10]. Central sensitization of central nociceptive neurons is reflected as an increase in their mechanoreceptive field (RF) size, a decrease in mechanical activation threshold and an increase in responses to noxious RF stimuli, and has been implicated as an important process in acute and chronic orofacial pain conditions following injury or inflammation of peripheral tissues [15, 32-34]. Pregabalin is an anticonvulsant drug that is known to decrease glutamate release by binding to the α2δ subunit of voltage-dependent calcium channels [26, 30]. Recent evidence indicates that pregabalin is effective in treating many chronic inflammatory and especially neuropathic pain conditions in patients and reducing nociceptive behaviour in animal pain models [21, 23-26, 28, 31, 35-38]. Recently, we have shown that nociceptive sensorimotor behavioral responses and the medullary release of glutamate evoked by MO application to the rodent tooth pulp can be attenuated in a dose-dependent manner by systemic administration of pregabalin [28]. Also, we have documented that pregabalin attenuates the mechanical allodynia and MDH central sensitization in a rat trigeminal neuropathic pain model [4]. However, it is unclear whether pregabalin interferes with central sensitization in functionally identified MDH nociceptive neurons in conditions of acute inflammatory pain. Therefore, the aim of this study was to test if MDH central sensitization in this acute inflammatory pain model in rats and mice can be attenuated by systemic administration of pregabalin. Some of the data has been presented in abstract form [6-8].

Material and Methods
All procedures and surgeries were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Canada).
**Animal preparation**

Adult male Sprague-Dawley rats (280-400g, n=18) were initially anesthetized by intraperitoneal α-chloralose (50 mg/kg)/urethane (1g/kg) and adult male C57BL6 mice (18-24g, n=18) by α-chloralose (75 mg/kg)/urethane (1.125 g/kg). The animals were prepared as previously described in detail [10, 12]. Briefly, the coronal pulp of the right maxillary first molar was exposed and covered with a saline-soaked cotton pellet, and then the dorsal surface of the caudal medulla was surgically exposed. Each animal then received a continuous intravenous infusion of a mixture of 70% α-chloralose/urethane solution (0.2 g/ml) and 30% pancuronium solution (1mg/ml in rats and 0.1mg/ml in mice) at a rate of 0.4 or 0.04 ml/h, respectively. The animal was artificially ventilated throughout the whole experiment and heart rate, expired CO2 level, and rectal temperature were continuously monitored and maintained at physiological levels.

**Electrophysiological recording and stimulation procedures**

Spontaneous and evoked single neuron activity was recorded in histologically verified sites in the deep laminae of the right MDH (lateral: 1.4-2.0 mm; posterior: 1.5-2.0 mm referred to the obex in rats and lateral: 0.8-1.0 mm; posterior: 0.8-1.0 mm in mice), as previously described in detail [10, 12, 20]. Briefly, neuronal responses to stimulation of the orofacial region were amplified and displayed on an oscilloscope and computer and the data were later analyzed off-line with Spike 2 software (Cambridge Electronic Designh, Science Park, Milton Road, Cambridge, UK). Mechanical (brush, pressure and pinch) and noxious thermal (radiant heat 51–53°C) stimuli were applied to classify neurons recorded in the deep laminae of MDH as nociceptive-specific (NS) if they responded exclusively to the noxious stimulation, as previously described [10, 12]. Each NS neuron's cutaneous orofacial RF was determined with nonserrated forceps, and its activation threshold to mechanical stimulation of its RF was assessed by force-monitoring forceps. Its responses were also recorded to graded noxious pressure applied to the RF by the forceps (50 g, 75 g and 100 g in rats and 10 g, 15 g and 26 g in mice applied in ascending order, each for 5 s at an interval of >45 s), and the number of spikes evoked by each of these graded stimuli were summed. The level of any spontaneous activity of a NS neuron was determined over an initial 1-min recording period. As previously documented [10, 12], central sensitization was reflected as an increase in spontaneous activity, RF size and responses to
noxious stimuli, and a decrease in activation threshold. Recording sites were marked by electrolytic lesions (anodal current of 8 µA for 13 s) and verified histologically.

**Experimental paradigm**

Only one neuron was studied in each experiment due to the long-lasting effects of MO and pregabalin. Two assessments of neuronal properties were carried out before each animal received a bolus (1 ml in rats, 0.5 ml in mice, i.p.) of isotonic saline or pregabalin (100 mg/kg). The dose of pregabalin was chosen on the basis of that used in the early studies [4, 37] and on our recent sensorimotor behavioral study defining the dose-response relationship of pregabalin on the behaviour [28]. Then, 30 min later, MO (n=6) or its vehicle (mineral oil, n=6) was applied in each animal (at room temperature) to the exposed pulp which was then sealed with CAVIT (ESPE, Seefeld/Oberbayren, Germany). Starting at three minutes after the solution was applied to the pulp, neuronal properties were assessed at 10 min intervals over the next 50 min.

**Statistical analyses**

Statistical analyses were based on normalized data (in percentages) of orofacial RF size, responses to graded pressure or pinch stimuli and mechanical activation threshold and spontaneous activity. Differences between baseline values and values at different time-points after MO or vehicle (mineral oil) application and after pregabalin or vehicle and MO application were treated by repeated measures (RM) analysis of variance (ANOVA) or ANOVA on ranks, followed by Dunnett's test. Differences between the groups of the same species were treated by 2-way ANOVA followed by Dunnett's test. The level of significance was set at a P value of less than 0.05. All values are presented as mean ± SEM.

**Results**

A total of 36 functionally identified NS neurons in rats (n=18) and mice (n=18) were studied. In both rats and mice, one group of neurons (n=6) was tested with MO application to the pulp preceded by saline (i.p.), another (n=6) with MO to the pulp preceded by pregabalin, and another (n=6) with mineral oil to the pulp preceded by saline. The recording sites of all NS neurons were histologically verified and were located in the deep laminae of MDH (see Fig. 1G, H). The baseline level of spontaneous activity was low (0–2 spikes/min) in all NS neurons in both rats
and mice. Application of mineral oil (vehicle) to the tooth pulp in rats and mice produced no evidence of central sensitization in any of the NS neurons (data not shown), compared to their baseline properties. Only one neuron in rats and two in mice had baseline activity (0.03-0.05 Hz) before and after mineral oil application. In contrast to the lack of the effect of mineral oil, MO application to the pulp produced an immediate response in 50% (rats) and 83% (mice) of the NS neurons tested. In these neurons, there was a brief burst (latency 10–30 s; duration 3–4 min) of discharges followed by a long-lasting (20–30 min) firing (3–4 spikes/min) that was significantly (RM ANOVA, p<0.05) higher than baseline level. In 34% (rats) and 17% (mice) of the neurons, MO induced no immediate burst of firing and only a mild (1–3 spikes/min) long-lasting firing; in the remaining neurons it produced no changes in background activity. Administration (i.p.) of saline control had no effect on baseline activity of the NS neurons prior and after MO application to the tooth pulp in both rats and mice. Administration (i.p.) of pregabalin had also no effect on baseline activity of the NS neurons prior to MO application, but significantly (2-way ANOVA, p<0.05) reduced all MO-induced immediate and long-lasting background activity in both rats and mice.

**Orofacial RF size**

Application of MO to the pulp induced significant increases in RF size in the NS neurons in rats (130%, RM ANOVA, p<0.05) and in mice (40%, RM ANOVA, p<0.05). Application of mineral oil to the pulp did not induce any significant increases in RF size in the NS neurons in either rats or mice (RM ANOVA, p<0.05). The effect of MO on orofacial RF size was significantly larger in rats than in mice (Fig.1 A, B). In addition, a transient novel tactile RF appeared in two of the six neurons following MO application in both rats and mice (data not shown), consistent with our earlier findings in rats [4, 10]. Compared to saline (control) treatment, administration of pregabalin significantly (2-way ANOVA, p<0.05) reversed the MO-induced increased RF size to baseline levels in both rats and mice (Fig. 1 A, B).

**Mechanical activation threshold**

Application of MO to the pulp significantly (RM ANOVA, p<0.05) decreased mechanical activation threshold in rats (45%, Fig. 1C) and in mice (75%, Fig. 1D). Application of mineral oil to the pulp did not decrease mechanical activation threshold in the NS neurons in either rats
or mice (RM ANOVA, p>0.05). The effect of MO on mechanical activation threshold lasted significantly longer in mice than in rats (Fig.1 C, D; 2-way ANOVA, p<0.05). Administration of pregabalin (but not saline control) significantly reversed the MO-induced decreased mechanical activation threshold to baseline levels in both rats and mice (p<0.05, 2-way ANOVA, Figs. 1 C, D).

Responses to noxious stimuli
Application of MO to the pulp significantly (RM ANOVA, p<0.05) increased responses to the noxious stimuli in rats (200%, Fig. 1E) and in mice (80%, Fig. 1F). Application of mineral oil to the pulp did not induce any significant increases in responses to the noxious stimuli in the NS neurons in either rats or mice (RM ANOVA, p>0.05). The effect of MO on responses to the noxious stimuli lasted significantly longer in mice than in rats (Fig.1 E, F; 2-way ANOVA, p<0.05). Compared to saline (control) treatment, administration of pregabalin significantly reversed the MO-induced increased responses to the noxious stimuli to baseline levels in both rats and mice (p<0.05, 2-way ANOVA, Fig. 1 E, F).

Discussion
This is the first report to document that pregabalin is effective in attenuating central sensitization of functionally identified dorsal horn nociceptive neurons in either spinal or trigeminal nociceptive systems in an acute rodent inflammatory pain model. Consistent with our previous findings in rats, we found that application of the inflammatory irritant MO to the rat molar tooth pulp could induce MDH central sensitization of NS neurons that was reflected as increases in RF size, spontaneous activity and responses to noxious RF stimuli and a decrease in mechanical activation threshold [9-12, 22], but we have also shown for the first time that MO application to the molar pulp also induces MDH central sensitization in mice. The effects of pregabalin that were documented are also consistent with our recent study in this model of acute inflammatory pain [28] where we found that pregabalin was effective in a dose-dependent manner in significantly attenuating the nociceptive sensorimotor behavioural responses and medullary release of glutamate evoked by the application of MO to the rat tooth pulp. In the current study, we have shown that administration of pregabalin was effective in significantly reducing all the parameters of central sensitization tested in the MDH nociceptive neurons in this model as well
as in the analogous acute tooth pulp inflammatory pain model in mice. Also, in another recent study [4], we demonstrated that pregabalin effectively attenuated facial mechanical hypersensitivity and MDH central sensitization in a rat model of trigeminal neuropathic pain. Although delineation of the mechanisms and sites of action of pregabalin was not an aim of this study, it is known that pregabalin is a structural analog of gamma-aminobutyric acid (GABA), but it is inactive at GABA-A or -B receptors, is not converted into a GABA or a GABA antagonist, and it does not affect GABA uptake [17]. Indeed, the exact mechanism of action of pregabalin is not completely clear. One explanation is that by tightly binding to the α2δ protein, pregabalin reduces the influx of calcium, thereby reducing the release of neurotransmitters, including glutamate, norepinephrine, and substance P [13, 14, 16]. Systemic effects of pregabalin can significantly inhibit ectopic discharges from injured afferent neurons, which suggests that the analgesic effect of pregabalin on neuropathic pain is potentially mediated, at least in part, by a peripheral inhibitory action on the generation of ectopic discharges caused by nerve injury [5]. In our acute inflammatory model, we have shown that application of MO to the tooth pulp can induce spontaneous activity in some NS MDH neurons, which could conceivably be due to ectopic afferent discharges, and that administration of pregabalin completely prevented the MO-induced spontaneous responses. Therefore, it is possible that the effects of pregabalin observed in the present study were at least partly mediated by a reduction of ectopic afferent activity and direct reduction or elimination of the nociceptive afferent input to the MDH, thereby reducing the central sensitization of the MDH neurons. Our findings that pregabalin reduced MO-induced activity of the MDH nociceptive neurons is consistent with this possibility, although it is likely pregabalin also acts in the central neural system to produce this effect. There is previous evidence indicating that intrathecal application of pregabalin reduces the enhanced noxious stimulus-induced spinal release of glutamate seen in neuropathic rats [26], supporting the possibility of a central action of pregabalin. In addition, identification of pregabalin binding sites in mice has revealed that the highest concentration occurred in the cortex, olfactory bulb, hypothalamus, amygdala, hippocampus, cerebellum and dorsal horn of the spinal cord [3], further supporting a central action of pregabalin. There are reports indicating that pregabalin at varying doses reduces nociceptive behavioural responses in the spinal nociceptive system in rat pain models [2, 18, 21, 26]. We have previously demonstrated a dose-dependent effect of pregabalin in reversing the facial mechanical
allodynia and MDH central sensitization present at postoperative day 7 following partial infraorbital nerve injury [4]. A notable finding of the present study was that pregabalin had no effect on the baseline neuronal properties of the MDH nociceptive neurons. This suggests that pregabalin can attenuate central sensitization in MDH nociceptive neurons without affecting normal nociceptive processing, consistent with our previous findings in the trigeminal neuropathic pain model [4].

Detailed clinical studies of the potential use of pregabalin in orofacial pain states are limited, but there are reports of clinical analgesic effects of pregabalin for acute pain following third molar extraction [19] and for paresthesia following inferior alveolar nerve damage [27], and that pregabalin can be successfully used to manage glossopharyngeal [23], lacrimal [29] and postherpetic [1] neuralgias as well as post-traumatic facial pain due to a peripheral nerve injury [35]. Also, there is an expert recommendation for management of orofacial neuropathic pain conditions by pregabalin [38]. However, no randomized clinical trials have been conducted to address the effects of pregabalin in orofacial neuropathic or acute inflammatory conditions. Nevertheless, in animal models, we have shown it is effective in a trigeminal neuropathic pain model as noted above [4], and that pregabalin can also prevent both the medullary release of glutamate and prolonged jaw and tongue muscle activity produced by application of MO to the pulp [28]. Furthermore, our current findings demonstrate that pregabalin effectively attenuates nociceptive neuronal hyperexcitability reflecting central sensitization in the rodent model of acute inflammatory tooth pain, and provides pre-clinical data supporting its potential clinical use in the treatment of orofacial inflammatory pain states as well as neuropathic pain conditions in humans; further clinically based studies are needed to test its usefulness in such inflammatory pain states.

Acknowledgements
The authors acknowledge Ms. Susan Carter and Mr. Yathavan Varathan for their technical assistance. This work was supported by grants from Pfizer Canada and US National Institutes of Health DE04786. We thank Pfizer Canada for providing the pregabalin used in this study. BJS is the holder of a Canada Research Chair.


F. Bian, Z. Li, J. Offord, M.D. Davis, J. McCormick, C.P. Taylor, L.C. Walker, Calcium channel alpha2-delta type 1 subunit is the major binding protein for pregabalin in neocortex, hippocampus, amygdala, and spinal cord: an ex vivo autoradiographic study in alpha2-delta type 1 genetically modified mice, Brain research 1075 (2006) 68-80.


Legend to Figures

Figure 1. Blockade by pregabalin of the MO-induced MDH central sensitization in rats (A, C, E) and mice (B, D, F).

(A, B) Changes in NS neuronal RF size. Application of MO to the tooth pulp in both rats (A) and mice (B) produced significant increases in pinch RF size (p<0.05, RM ANOVA; * p<0.05, Dunnett's test, n=6). Administration of pregabalin (but not saline) reversed the MO-induced increases in neuronal RF size (p>0.05, RM ANOVA, n=6). (C, D) Changes in mechanical activation threshold of NS neurons. Application of MO to the tooth pulp in both rats (C) and mice (D) produced significant decreases in mechanical activation threshold (p<0.05, RM ANOVA; * p<0.05, Dunnett's test, n=6). Administration of pregabalin (but not saline) significantly blocked the MO-induced decrease in threshold and increases in responses (p>0.5, RM ANOVA, n=6). (E, F) Changes in neuronal responses to noxious stimuli. Application of MO to the tooth pulp in both rats (E) and mice (F) produced significant increases in neuronal responses to noxious stimuli (p<0.05, RM ANOVA; * p<0.05, Dunnett's test, n=6). Administration of pregabalin (but not saline) significantly blocked the MO-induced increases in responses (p>0.5, RM ANOVA, n=6). Note that the baseline RF size, activation threshold and responses to noxious stimuli did not change significantly following pregabalin administration in both rats and mice (p>0.5, RM ANOVA). Post-hoc analysis indicated that there were significant differences in values at post-MO time-points between the saline (control) and pregabalin administrations (# p<0.05, 2-way ANOVA followed by Dunnett test, n=6). (G, H) The recording sites of the NS neurons were histologically verified in the deep laminae of MDH in rats (G) and mice (H); Sp 5, trigeminal spinal tract.
Chapter 4
Discussion

This is the first documentation of long-lasting facial nociceptive behaviour associated with central sensitization in functionally identified trigeminal nociceptive neurons in the MDH following injury of the mouse ION. The study also demonstrates for the first time that administration of pregabalin (but not saline as vehicle control) reduces the long-lasting nociceptive behaviour and central sensitization at days 7, 21 and 49 following ION injury. This is also the first report to document that the anticonvulsant drug pregabalin is effective in attenuating central sensitization of functionally identified MDH nociceptive neurons in the trigeminal nociceptive system in an acute rodent inflammatory pain model. These findings provide pre-clinical evidence suggesting that pregabalin may be effective in treating orofacial acute and chronic pain conditions, which is of significant clinical importance as noted below.

4.1 Central sensitization and nociceptive behaviour

The present study is a continuation of an ongoing series of experiments conducted in our laboratory that has explored the effects of the inflammatory irritant MO on nociceptive processes in the rodent MDH. Consistent with our previous findings in rats, we found that application of MO to the rat molar tooth pulp could induce MDH central sensitization of NS neurons that was reflected as increases in neuronal RF size, spontaneous activity and responses to noxious RF stimuli and a decrease in mechanical activation threshold (84, 92, 96, 99, 110). In addition, we have also shown for the first time that MO application to the molar pulp similarly induces MDH central sensitization in mice. Central sensitization has been implicated as an important process in acute and chronic orofacial pain conditions following injury or inflammation of peripheral tissues (12, 71, 73, 74).

The current findings from the neuropathic pain model experiments are also consistent with previous studies of our laboratory that have documented the occurrence of central sensitization of NS neurons following partial ION injury in rats (70). In this study we found that damage of the ION induces long-lasting bilateral facial mechanical allodynia and central sensitization in functionally identified NS nociceptive neurons in the medullary dorsal horn in mice.
4.2 Nociceptive behaviour: contralateral effects

In the present study we demonstrated that injury of the mouse ION produces long-lasting facial nociceptive behaviour evoked not only by stimulation of the ipsilateral mandibular skin but also by stimulation of the contralateral maxillary and mandibular skin. Contralateral as well as ipsilateral nociceptive behaviour experienced as hyperalgesia and allodynia may reflect neuroplastic changes manifested as central sensitization of nociceptive neurons in MDH and other central nervous system areas (12, 71, 73, 74). The scope of the current work was not intended to determine the mechanisms underlying these neuroplastic changes but one study has suggested that the development of contralateral orofacial hyperalgesia in a chronic orofacial pain model is mediated through descending facilitatory influences of the rostral ventromedial medulla on the spinal trigeminal nucleus (111). Another study has demonstrated that trigeminal subnuclei interpolaris and caudalis play a unique role in the development of contralateral allodynia in the deep orofacial tissues and that it involves glial cells and inflammatory cytokines (112). These mechanisms would explain the contralateral behaviour in our present experiments where unilateral injury to ION produced long-lasting facial nociceptive behaviour also on the contralateral side, but further studies are required to identify the mechanisms involved in the development and maintenance of contralateral hyperalgesia and allodynia in the current IONX model.

4.3 Effects of pregabalin

There are reports indicating that pregabalin at varying doses reduces nociceptive behavioural responses in the spinal nociceptive system in rat pain models (66-69). The effects of pregabalin that were documented in the present study are also consistent with findings in our recent study in an acute inflammatory pain model (65) where we found that pregabalin was effective in a dose-dependent manner in significantly attenuating the nociceptive sensorimotor behavioural responses and medullary release of glutamate evoked by the application of MO to the rat tooth pulp. In the current study, we have shown that administration of pregabalin was effective in significantly reducing the effect of central sensitization in MDH nociceptive neurons in this model in rats as well as in the acute tooth pulp inflammatory pain model in mice.
A notable finding of the present study was that pregabalin had no effect on the baseline neuronal properties of the MDH nociceptive neurons. This suggests that pregabalin can attenuate central sensitization in MDH nociceptive neurons without affecting normal nociceptive processing, consistent with our previous findings in the trigeminal neuropathic pain model in rats (70). In that study we also demonstrated a dose-dependent effect of pregabalin in reversing the facial mechanical allodynia and MDH central sensitization at an early post-operative stage (day 7). The present data are consistent with this finding but the study in mice has additionally revealed that pregabalin may be effective at longer post-operative times when the nociceptive behaviour and central sensitization have become well maintained.

The site and mode of action of pregabalin are still not fully explained (113) and we did not address them in this study. Several mechanisms of action have been proposed. One is that pregabalin binds to the α2δ protein of calcium channels and reduces the influx of calcium. This action reduces the release of neurotransmitters, such as glutamate, norepinephrine, and substance P (114-117). Systemic administration of pregabalin can significantly inhibit ectopic discharges from injured peripheral sensory neurons (64) which could at least partially explain the effect of pregabalin in the present study. Systemic administration of pregabalin could have reduced or prevented afferent input to the MDH NS neurons, which is consistent with current findings in the acute inflammation model and our group’s demonstration that administration of pregabalin abolished spontaneous activity in NS MDH neurons in a trigeminal neuropathic pain model in rats (70). In addition, there is a possibility of direct action of pregabalin in the central nervous system. It has been demonstrated that intrathecal application of pregabalin reduces the enhanced noxious stimulus-induced spinal and MDH release of glutamate seen in neuropathic rats (69, 118). Also, pregabalin has affinity to its receptors in the cortex, olfactory bulb, hypothalamus, amygdala, hippocampus, cerebellum and dorsal horn of the spinal cord (119). In addition, pregabalin may also affect glial cell function since it has been reported to suppress proliferation of microglia and astrocytes observed in a spinal cord injury model in rats (120). All these findings strongly suggest that pregabalin could be effective in reducing behavioural allodynia and central sensitization in MDH NS neurons in the current trigeminal neuropathic pain model in mice via a central mode of action.
4.4 Clinical implications

Our findings that acute inflammation of the tooth pulp and chronic injury of the ION induce nociceptive behaviour and central sensitization in NS neurons in MDH are of significant clinical importance. Untreated pulp inflammation or TMDs, injury to the trigeminal nerve during RCT or apical surgery, periodontal surgery, microsurgery, orthodontic tooth movement, tooth extraction, implant placement or orthognathic surgery may induce long-lasting chronic pain states which are frequently very difficult to manage (14, 15, 17, 28, 121). The current findings provide, at least partial, insights into of mechanisms that take place in the trigeminal nociceptive system following acute or chronic nerve injury.

The current work has demonstrated that injury of the mouse ION produces long-lasting facial nociceptive behaviour not only ipsilaterally but also contralaterally. These finding are important for understanding of certain clinical situations that may manifest contralateral pain spread. For example, persistent orofacial pain following dental treatment or tooth extraction, implant placement, persistent pain associated with TMD and other chronic clinical conditions in the orofacial region may induce contralateral pain spread (122-124). These clinical conditions are extremely difficult to treat. Understanding the mechanisms of development and maintenance of contralateral pain spread in trigeminal pain states may provide a significant improvement in clinical management of such pain states.

Not only has this study produces insights into the mechanisms underlying the development and maintenance of acute and chronic pain states, but it also suggests a possible management approach to such conditions. There are numerous reports in the literature demonstrating the use of pregabalin in management of several chronic pain states involving the spinal somatosensory system. Pregabalin has been found effective in treating neuropathic conditions associated with cancer (57, 125), management of fibromyalgia (126) and post-herpetic neuralgia (55, 56). Also pregabalin has been found effective in management of central neuropathic pain (127), following spinal cord injury (128) and diabetic peripheral neuropathy (62). There are however only a limited number of clinical studies of the use of pregabalin in orofacial pain states, but there are some reports of analgesic effects of pregabalin for acute pain following third molar extraction (129) and for paresthesia following inferior alveolar nerve damage (130). Some studies have demonstrated successful use of pregabalin in management of glossopharyngeal (58), lacrimal
(131) and post-herpetic (56) neuralgias as well as post-traumatic facial pain due to a peripheral nerve injury (60). Additionally, there is an expert recommendation for management of orofacial neuropathic pain conditions by pregabalin (63). Up to now, no randomized clinical trials have been conducted to address the effects of pregabalin in orofacial neuropathic or acute inflammatory conditions. The current study provides further pre-clinical data suggesting that pregabalin may be effective in the management of acute inflammatory tooth pain as well as trigeminal neuropathic pain conditions, including chronic post-endodontic pain. In animal models, we have shown that pregabalin is effective in a trigeminal neuropathic pain model as noted above (70), and that pregabalin can also prevent the medullary release and MDH central sensitization as well as prolonged jaw and tongue muscle activity produced by application of MO to the pulp (65). These pre-clinical findings collectively also point to the need for further clinically based studies to test its effectiveness in acute and chronic orofacial pain states.

4.5 Study strengths and limitations

The novel findings of this study that demonstrate a long-lasting facial nociceptive behaviour and central sensitization in functionally identified trigeminal nociceptive neurons in the MDH following injury of the mouse ION is a strength of this work. The demonstration of therapeutic effects of pregabalin in this chronic model of a long-lasting nociceptive behaviour and central sensitization is another strength of this work.

The confounding factors that could have altered the observed nociceptive behaviour and central sensitization in the MDH nociceptive neurons in this study (e.g. exposure to oral operatory procedures, pain induced by soft tissue trauma, general anaesthesia) were controlled by the use of a Sham group. The Sham group experienced the general anaesthesia and all the operatory procedures performed on the IONX group except for the ION transection and showed no significant changes in the animal behaviour or electrophysiological properties of the NS neurons. In addition, application of mineral oil to the tooth pulp served as a vehicle control in the acute inflammatory model. Administration of saline (vehicle control) controlled for the systemic effects of pregabalin. The experimental vehicle control group demonstrated no significant changes in the nociceptive behaviour or electrophysiological properties of the NS neurons. Another strength was the correlation between nociceptive behaviour and neuronal changes in
MDH, emphasizing a potential underlying mechanism contributing to nociceptive neuropathic pain behaviour.

Despite these strengths, this study had some limitations. These include the unknown site of action of the systemically administered pregabalin and the uncertainty whether the effect was due to its direct peripheral action or an analgesic effect in the central nervous system, and exactly what central nervous system sites were affected by pregabalin. This limitation was discussed previously in sub-section 4.3.

Another limitation of this study was use of general anaesthesia for the MDH study and use of awake animals for the testing of nociceptive behaviour. General anaesthesia may have an effect on neuronal properties by blocking normal conductivity and altering membrane permeability. Despite this, the MDH and behavioural data as well as effects of pregabalin on them were nonetheless complementary.

An additional limitation of this study that restricts the generalizability of the findings was the use of male animals in all experiments. This was done deliberately since fluctuations in female sex hormones may cause variability in nociceptive responses and thus introduce confounding effects (87). Studies in female animals analogous to those conducted in male animals in this project represent a future research direction that may provide insights into effects of pregabalin in females and into possible sex difference in these effects.

Another limitation is that this study evaluated the effects of inflammation or trigeminal injury only on nociceptive neurons in the MDH and only on NS neurons in the deep MDH laminae. Therefore, it cannot provide a complete picture of the central sensitization that occurs at the higher brain levels (e.g., thalamus, somatosensory cortex, etc.) and whether wide dynamic range and NS neurons in superficial laminae of the MDH are affected in these models. Evaluation of the central sensitization in these brain sites is necessary to provide a more comprehensive picture of the neuroplastic changes that take place in acute or chronic orofacial pain states and the potential effects of pregabalin at these sites.
4.6 Future research directions

The novel findings in this study provide valuable information on the potential clinical applications of pregabalin in pathological pain states. However, more studies are needed to understand the mechanism of action and whether the effects of pregabalin are due to central or peripheral actions, or both, in acute and chronic orofacial pain states, and as noted above, and whether there are sex differences in its effects.

Another approach in management of acute and chronic orofacial pain is identification of new drugs and their potential sites of action. Other future directions could test the effects of agents that might potentially influence nociceptive behaviour and MDH central sensitization in the acute and chronic orofacial pain models. For example, acetaminophen is a non-steroidal anti-inflammatory drug that is widely used over-the-counter as analgesic and antipyretic. It could be used to see if it reduces acute and chronic nociceptive pain behaviour and MDH central sensitization. Other candidate agents are antibiotics. There are several review articles indicating that there is no benefit of using antibiotics in acute inflammatory conditions in endodontics in cases without systemic complications (132, 133), but the emphasis has been on the antimicrobial properties of the antibiotics. Non-microbial properties of these substances associated with pain mechanisms have never yet been studied. One of the antibiotics used in endodontics, minocycline, inhibits microglial function via p38 MAPK and produces analgesia in neuropathic pain models (134-137). While minocycline has yet to undergo detailed investigation in animal models of chronic or acute orofacial pain, studies have recently demonstrated that it attenuates the MDH central sensitization induced in the acute pulp inflammatory and chronic neuropathic pain models in rats (99, 138) Therefore, studying the effects of minocycline on sensorimotor behaviour and trigeminal central sensitization in a dose-dependent manner in an acute inflammatory pain model may be of interest to support its clinical use for treatment of trigeminal pain, including endodontic pain.

An additional gap in knowledge of trigeminal pain mechanisms is the role of genetic factors. Humans display highly variable sensitivity to pain, including variable responses in developing a chronic pain state to identical injuries or pathologies, including those related to endodontic therapy or trigeminal nerve injury. Also, humans may vary in their response to drugs used to treat
their pain conditions, e.g., due to individual differences in absorption and processing of chemicals. These individual differences in developing pain conditions as well as in responses to analgesic drug may depend at least in part on individual’s genotype. The current literature on mouse pain models indicates the importance of the mouse genotype in mediating nociceptive sensitivity and establishing a predisposition to neuropathic pain following neural injury (139-142). In preliminary experiments we have found differences between two genetically different mouse strains (C57BL/6 and A/J) in orofacial nociceptive behaviour and MDH central sensitization induced by trigeminal nerve injury (143), which suggests genetic factors may be involved in the predisposition to developing orofacial nociceptive behaviour and MDH central sensitization. The findings might possibly also account for differences between patients in the efficacy of current treatment regimens for trigeminal neuropathic pain conditions. Therefore, pre-clinical studies to identify genetic factors in establishing and maintenance of acute and chronic pain and the effects of pain-relieving agents are of great importance.
Chapter 5
Conclusions

The current work has demonstrated that application of the inflammatory irritant MO to the molar tooth pulp induces central sensitization in trigeminal MDH neurons in rats and mice. This work also shows that injury of the mouse ION produces long-lasting facial nociceptive behaviour and central sensitization in trigeminal MDH neurons in mice. The results of this study also indicate that administration of pregabalin can block central sensitization in the MDH in a rodent acute inflammatory pain model as well as attenuate the long-lasting IONX-induced mechanical allodynia and associated MDH central sensitization. These findings provide insights into mechanisms that may contribute to acute and chronic orofacial pain states, and also pre-clinical data supporting the potential clinical use of pregabalin in the treatment of orofacial inflammatory pain states as well as neuropathic pain conditions in humans.
References


64. Chen SR, Xu Z, Pan HL. Stereospecific effect of pregabalin on ectopic afferent discharges and neuropathic pain induced by sciatic nerve ligation in rats. Anesthesiology 2001;95:1473-1479.


136. Ma F, Zhang L, Lyons D, Westlund KN. Orofacial neuropathic pain mouse model induced by Trigeminal Inflammatory Compression (TIC) of the infraorbital nerve. Mol Brain 2012;5:44.