TUMOR NECROSIS FACTOR ALPHA AND NON-INFLAMMATORY SENSITIZATION OF MASSETER MUSCLE NOCICEPTORS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in

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Abstract

Behavioral evidence in rats indicates that injection of tumor necrosis factor alpha (TNFα) into skeletal muscle results in a prolonged mechanical sensitization without gross inflammation. The present series of studies were conducted to test the idea that injection of TNFα causes mechanical sensitization of skeletal muscle through a peripheral mechanism that involves lowering of the mechanical threshold (MT) of muscle nociceptors without inflammation. In-vivo extracellular electrophysiological recording was used to assess the effect of TNFα (1 or 0.1µg) and other drugs on the excitability and MT of masseter muscle nociceptors. Expression of TNFR1 (P55) and TNFR2 (P75) receptors by the masseter muscle and trigeminal ganglion neurons that innervate that muscle was determined by Western blot and immunohistochemical methodologies, respectively. The Evans blue dye technique and thermal camera recordings were used to assess inflammation in muscle tissues. Enzyme-linked immunoassays and glutamate biosensor probes were used to measure muscle concentrations of prostaglandin (PG) E2 and nerve growth factor, and glutamate, respectively. Intramuscular injection of 1µg TNFα did not excite nociceptors, but did significantly decrease MT compared to vehicle control. There was no evidence of gross inflammation 3 hours after injection of TNFα. Co-injection of TNFα with P55 or P75 receptor antibodies attenuated TNFα-induced mechanical sensitization. P55 and P75 receptors were expressed by 29% and 62% of masseter nociceptors, respectively. PGE2 and glutamate concentrations were significantly changed 3 hours after TNFα injection into the masseter muscle. Injection of diclofenac, a cyclooxygenase inhibitor that attenuates prostaglandin synthesis, partially reversed the TNFα-induced decreases in the MT of masseter muscle nociceptors, while vehicle control, DL-2-amino-5-phophonovaleric acid, a competitive NMDA receptor antagonist, and a tyrosine kinase A receptor antibody, which blocks NGF-induced masseter muscle nociceptor sensitization, did not significantly alter nociceptor MT. These
findings indicate that TNFα-induced mechanical sensitization of masseter nociceptors is mediated, in part, by increased PGE2 levels through activation of peripheral P55 and P75 receptors. Over all, these results suggest that injection of TNFα into skeletal muscle could be used as a model of myofascial trigger points to study the peripheral pain mechanisms of masticatory muscle pain.
Preface

A version of Chapter 2 has been published: Akhlaq W. Hakim, Xudong Dong, Peter Svensson, Ujendra Kumar, Brian E. Cairns (2009). TNFα Mechanically Sensitizes Masseter Muscle Afferent Fibers of Male Rats. J Neurophysiol 102:1551-1559. I conducted all research work and wrote the manuscript. Dr. Dong assisted me in electrophysiology experiments. Dr. Kumar guided me in immunohistochemistry experiments and I used his facility to perform these experiments. Dr. Svensson edited the manuscript and Dr. Cairns supervised the project.

Chapter 3. A version of this chapter has been published: Akhlaq W. Hakim, Xudong Dong, Brian E. Cairns (2011). TNFα Mechanically Sensitizes Masseter Muscle Nociceptors by Increasing Prostaglandin E₂ Levels. J Neurophysiol 105(1):154-61. I conducted all of the research work and wrote the manuscript. Drs Dong and Cairns supervised the project.

All experiments were done in accordance with the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee (Animal Care Protocol Number: A07-0105, A08-0920).
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<td>Aβ</td>
<td>A beta fiber</td>
</tr>
<tr>
<td>Aδ</td>
<td>A delta fiber</td>
</tr>
<tr>
<td>Smase</td>
<td>Sphingomyelinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating Protein-1</td>
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<td>AMPA</td>
<td>3-Hydroxyl-5-Methyl-4-Isoxazole-Propionate</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>APV</td>
<td>DL-2-Amino-5-Phosphonovaleric acid</td>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>BDNF</td>
<td>Brain-derived Neurotrophic Factor</td>
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<td>BK</td>
<td>Bradykinin</td>
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<td>Bradykinin receptors 1</td>
</tr>
<tr>
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<td>Bradykinin receptors 1</td>
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<td>Botulinum neurotoxin type A</td>
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<td>Degrees Celsius</td>
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<td>Ca²⁺</td>
<td>Calcium</td>
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<td>Calcitonin Gene Related Peptide</td>
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<td>Cl⁻</td>
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<td>cm²</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<td>EP</td>
<td>Prostaglandin receptors</td>
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<td>Extracellular Signal-Regulated Kinase</td>
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<td>FADD</td>
<td>Fas-Associated Death Domain Protein</td>
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<tr>
<td>g</td>
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<td>GABA</td>
<td>γ-Aminobutyric Acid</td>
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<td>GDNF</td>
<td>Glial cell-Derived Neurotrophic Factor</td>
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<td>H⁺</td>
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<td>Hypothalamic Pituitary Adrenal Axis</td>
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<td>Inhibitor of Kappa B</td>
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<td>Moles per liter</td>
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<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MAP3K</td>
<td>Mitogen- Activated Protein Kinase Kinase Kinases</td>
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<td>Mg $^{2+}$</td>
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<td>mg/kg</td>
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<td>mg/ml</td>
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<td>mGluRs</td>
<td>Metabotropic Glutamate receptors</td>
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<td>ml</td>
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<td>Minute(s)</td>
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<td>Meters per Second</td>
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<td>MT</td>
<td>Mechanical Threshold</td>
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<td>MTrPs</td>
<td>Myofascial Trigger Points</td>
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<td>Sodium Chloride</td>
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<td>Nuclear Factor-Kappa B</td>
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<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
</tr>
<tr>
<td>NS</td>
<td>Nociceptive Specific</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-inflammatory Drug</td>
</tr>
<tr>
<td>ng/g</td>
<td>Nanogram/Gram</td>
</tr>
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<td>PGE2</td>
<td>Prostaglandin E2</td>
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<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<td>PKA</td>
<td>Protein Kinase A</td>
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<td>PKC</td>
<td>Protein Kinase C</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<td>RIP-1</td>
<td>Receptor-Interacting Protein</td>
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<td>s</td>
<td>Second(s)</td>
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<tr>
<td>SC</td>
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<tr>
<td>T1</td>
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<tr>
<td>T3</td>
<td>30 Minutes</td>
</tr>
<tr>
<td>TTXr Na⁺</td>
<td>Tetrodotoxin-resistant Sodium Channels</td>
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<td>Temporomandibular Disorder(s)</td>
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<td>Temporomandibular Joint</td>
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<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor Alpha</td>
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<td>TNFR1</td>
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<td>TNFsR2</td>
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<tr>
<td>Trk</td>
<td>Tyrosine Kinase</td>
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<tr>
<td>TRPV1</td>
<td>Transient Receptor Potential Vanilloid 1</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNFα Receptor Associated Factor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFα Receptor Associated Death Domain</td>
</tr>
<tr>
<td>TSNC</td>
<td>Trigeminal Sensory Nuclear Complex</td>
</tr>
<tr>
<td>V</td>
<td>Trigeminal Nerve</td>
</tr>
<tr>
<td>V₁</td>
<td>Ophthalmic Branch of the Trigeminal Nerve</td>
</tr>
<tr>
<td>( V_2 )</td>
<td>Maxillary Branch of the Trigeminal Nerve</td>
</tr>
<tr>
<td>( V_3 )</td>
<td>Mandibular Branch of the Trigeminal Nerve</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide Dynamic Range</td>
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</table>
Acknowledgments

My sincerest thanks to Dr Brain Cairns, for his able guidance, kind help, thoughtful approach and sincere suggestions. Dr Brian is the best supervisor and mentor I could have ever worked with. I appreciate your enthusiastic approach and your helpful nature, which was of great help in completing my PhD program. Thank you very much Brian for being there for me.

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Chapter 1. Background

Craniofacial Pain

The craniofacial region is one of the most common pain sites in the body (Sessle 2000). Pain in this region manifests as acute pain such as toothaches and headaches or chronic pain such as temporomandibular disorders (TMD) and trigeminal neuralgia (Sessle 2000). Acute symptomatic pain helps in diagnosis and warns the person about abnormal body function or tissue damage. Chronic pain does not have protective role and causes social stress to the patient as well as to the family (Costigan and Woolf 2000; Sessle 2000). The economic burden involved in management of pain is huge both for the patient and society (Sessle 2000).

Craniofacial Pain Mechanisms

Peripheral Sensitization

Peripheral sensitization is defined as increased sensitivity of nociceptors, which is characterized by increased excitability of nociceptors at the site of injury (Sessle 1999). The peripheral nerve endings express receptors for various algogenic substances such as adenosine triphosphate (ATP), bradykinin, nerve growth factor, glutamate and protons (H+) (Caterina et al. 1997; Chen et al. 1995; Dong et al. 2007; Gazerani et al. 2010b; Krishtal et al. 1988; Staikopoulos et al. 2007; Svensson et al. 2010). These algogenic substances released upon tissue damage or inflammation (Costigan and Woolf 2000; Couture et al. 2001; Steen et al. 1996) either activate ionotropic channels or bind to G-protein coupled receptors such as prostaglandin and bradykinin receptors expressed on nerve terminals (Dray 1997; Graven-Nielsen and Mense 2001; Mense 1981; Shinoda et al. 2008). G-protein coupled receptors activate second messengers and alter the sensitivity of ion channels through various protein kinase enzymes (Petho et al. 2001; Woolf 2004). Activation of nociceptors Aδ and C-fiber nerve endings by
these chemical mediators produce action potentials, which are conveyed to the central nervous system (CNS) resulting in acute pain (Neumann et al. 1996). Nociceptors normally respond to high threshold stimuli and are sensitized in response to various inflammatory substances released during inflammation and after tissue damage (Julius and Basbaum 2001; Petho et al. 2001). Sensitized nociceptors are characterized by spontaneous activity, lowered activation threshold and increased response to noxious stimuli that contribute to the perception of spontaneous pain, alldynia (pain in response to a normally innocuous stimuli) and hyperalgesia (an increased response to a stimulus which is normally painful) that are characteristics of various pain conditions (Basbaum and Woolf 1999; Djouhri et al. 2006; Julius and Basbaum 2001).

Furthermore, during inflammation some sensory nerve fibers release neuro-peptides such as calcitonin gene related peptide (CGRP) and substance P that cause plasma protein extravasation and vasodilatation (Beland and Fitzgerald 2001; Julius and Basbaum 2001; Neumann et al. 1996). These peptides also activate the immune system to release inflammatory substances such as histamine, serotonin, bradykinin and cytokines that cause swelling, redness and increase in temperature through a process called neurogenic inflammation (Brain and Williams 1985; Brain et al. 1985; Julius and Basbaum 2001).

Moreover, peripheral sensitization also activates silent nociceptors. Under normal conditions, silent nociceptors do not respond to high threshold noxious stimuli, however, after inflammation these nociceptors respond to noxious stimuli (Michaelis et al. 1996; Rukwied et al. 2008). In addition, inflammation also leads to increased input signal to the cell bodies of these nociceptors leading to phenotypic changes such as increased expression of receptors and ion channels of nociceptive afferent fibers. The increased expression of ion channels and neurotropins such as brain-derived nerve factor (BDNF) further augments the peripheral sensitization (Mannion et al. 1999; Woolf and Ma 2007).
Role of Second Messengers in Peripheral Sensitization

As mentioned above, sensory nerve fibers express G-protein coupled receptors for inflammatory mediators such as bradykinin, prostaglandins and metabotropic glutamate receptors. These mediators such as bradykinin and prostaglandins released during tissue damage activate second messengers via G-protein coupled receptors. Second messengers such as cyclic adenosine monophosphate (cAMP) and diacylglycerol (DAG) activate various protein kinases that phosphorylate several ion channels and alter their properties (Souza et al. 2002; Woolf 2007). For example, prostaglandins induce sensitization of nociceptors through activation of cAMP and protein kinase A (PKA). PKA phosphorylates ligand-gated ion channels such as transient receptor potential vanilloid 1 (TRPV1) and tetrodotoxin-resistant Na⁺ (TTXr Na⁺) channels resulting in increased ion channel currents, and decreased thresholds for nociceptor activation (Aley et al. 2001; Bhave et al. 2003; Fitzgerald et al. 1999; Gold et al. 1998). Further, algogenic substances such as bradykinin, epinephrine and tumor necrosis factor alpha (TNFα) activate the second messenger DAG. DAG activates protein kinase C (PKC) which plays a role in both nociception as well as in neuropathic pain (Souza et al. 2002). These studies suggest that peripheral sensitization has an important role in pain hypersensitivity. Other important protein kinases that are involved in peripheral inflammation and neuropathic pain are the mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated kinases (ERK) and p38 MAPK. Aley et al. (2001) have shown that epinephrine activates ERK1 and 2 that leads to mechanical hyperalgesia. Moreover, Jin and Gereau (2006) have shown that TNFα causes mechanical hyperalgesia by activating p38 MAPK. Taken together, these studies suggest that peripheral sensitization has an important role in pain hypersensitivity. It is essential to understand the roles of various transducer molecules, such as G-protein coupled receptors and ion channels present on the nociceptors as well as protein kinases. Blockade of peripheral targets for example, peripheral ion channels, second messengers and protein kinases involved in
Peripheral sensitization could help to develop new therapeutic agents for the treatment of pain without side effects.

**Figure 1.1** This diagram shows how peripheral sensitization is induced after tissue damage and the involvement of multiple kinases in peripheral sensitization. Hyperactivation of TRPV1 and TTXr Na⁺ channels by multiple protein kinases leads to peripheral sensitization. EP (Prostaglandin receptors), B1 and B2 (Bradykinin receptors), PLC (Phospholipase C).
Central Sensitization

Increased nociceptor activity (A\(\delta\) and C-fibers) due to peripheral sensitization sends an increased barrage of sensory inflow into the CNS resulting in increased excitability of central nociceptive neurons, known as central sensitization (Sessle 2006; Woolf 2007; 1983; 2004). Central sensitization induced by intense and repeated noxious stimuli, lasts for minutes to several hours or days independent of the persistence of noxious stimulation (Latremoliere and Woolf 2009). Sensitized neurons show an increase in spontaneous activity, increase in response to noxious stimuli, increase in receptor field size and respond to non-noxious stimuli (Woolf 1983; Woolf and King 1990; Woolf and Thompson 1991). One of the characteristic features of central sensitization is the expansion of neuron receptive fields to the areas where noxious stimuli normally do not activate these neurons. This expansion of receptive fields of nociceptive neurons beyond the site of injury has been termed secondary hyperalgesia (Torebjork et al. 1992). In addition, due to central sensitization, low threshold afferent fibers which are responsible for touch sensation start behaving like nociceptors; thus non-noxious stimuli become painful causing allodynia (Torebjork et al. 1992; Woolf 1983; Woolf and Thompson 1991).

Neuro-peptides such as substance P, CGRP and the excitatory amino-acid glutamate are believed to be involved in central sensitization (Khasabov et al. 2002; Sun et al. 2004; Woolf and Thompson 1991). Continuous noxious stimulation of afferent nociceptors release neuro-peptides from their central terminal endings. These centrally released neuro-peptides and glutamate cause excitation of second order neurons by activating post-synaptic receptors (Contractor and Heinemann 2002; Sessle 2000). Glutamate binds to post-synaptic N-methyl-D-aspartate (NMDA) and non-NMDA receptors such as 3-hydroxyl-5-methyl-4-isoxazole-propionate and metabotropic receptors (Contractor and Heinemann 2002; Nakanishi 1992). At resting membrane potential, NMDA receptors are blocked by Mg\(^{2+}\) (Mayer et al. 1984). Depolarization of membrane by neuro-peptides or other glutamate receptors such as AMPA removes Mg\(^{2+}\)
block of NMDA receptors that allows $\text{Na}^+$ and $\text{Ca}^{++}$ to pass through NMDA receptors and induce prolonged depolarization that leads to activation of various calcium-dependent protein kinases. These protein kinases phosphorylate various ion channel receptors and alter their properties, which contributes to central sensitization (Carvalho et al. 2002; Lieberman and Mody 1994; Mayer et al. 1984; Woolf 1991). For example, PKA and PKC induced phosphorylation at NR2A and NR2B subunits of NMDA receptors respectively, alters their conduction properties leading to hyperexcitability of nociceptive post-synaptic neurons (Chen and Roche 2007). Both NMDA and AMPA receptors play important roles in central sensitization. Activation of the NMDA receptor is considered essential for both induction and maintenance of central sensitization. For example, Woolf and Thompson (1991) have shown that both competitive and non-competitive antagonists of the NMDA receptor prevented mustard oil induced central nociceptive neuronal excitability. In addition, phosphorylation of the GLUR1 subunit of the AMPA receptor by protein kinases increases AMPA receptor synaptic expression that leads to increased excitability of nociceptive neurons (Carvalho et al. 2000; Fang et al. 2003). To sum up, glutamate through ionotropic receptors contributes to the development of central sensitization.

The second important mechanism involved in central sensitization is loss of inhibitory $\gamma$ aminobutyric acid (GABA) and glycine producing neurons (Sivilotti and Woolf 1994). Normally these inhibitory neurons release GABA and glycine to decrease the excitability of second order neurons (Yoshimura and Nishi 1995). Indeed, Malan et al. (2002) have shown that intrathecal injections of GABA$_A$ and GABA$_B$ antagonists, bicuculline and phaclofen, respectively, produce behavioral allodynia and hyperalgesia in rats. In another study, it has been shown that GABA$_A$ and glycine receptor antagonists decreased the activity of inhibitory neurons that could produce allodynia (Sivilotti and Woolf 1994). These studies support the role of GABA and glycine in nociception through central mechanisms.
There is growing evidence that glial cells play a role in the development of chronic pain (Tsuda et al. 2005). For example, peripheral injury induces activation of glial cells such as microglia and infiltration of immune cells such as macrophages and T-cells in the CNS (Cao and DeLeo 2008). Activated microglial cells release inflammatory substances such as cytokines and BDNF (Watkins and Maier 2002). BDNF produces central sensitization by activating protein kinases such as ERK and PKC which can then phosphorylate NMDA receptors to enhance nociceptive activity (Kerr et al. 1999; Slack et al. 2004; Thompson et al. 1999). Moreover, it has been documented that inflammation of the masseter muscle increases cytokine level of astrocytes in the trigeminal nucleus which potentiates central sensitization (Guo et al. 2007). Taken together, these studies suggest that multiple mechanisms are involved in central sensitization that contributes to the development of chronic pain.

**Tumor Necrosis Factor Alpha (TNFα) and Pain Mechanisms**

**Introduction to TNFα**

TNFα is a polypeptide that belongs to the family of cytokines (Allan and Rothwell 2001). Cytokines are soluble glycoproteins produced by host cells during immune response (Nathan and Sporn 1991). They act as intercellular signaling molecules along with neurotransmitters, endocrine hormones and autacoids (Nathan and Sporn 1991) and are released in response to inflammation or tissue damage (Leung and Cahill 2010; Wagner and Myers 1996). Cytokines that are released can exert their biological effect by binding to receptors on the same cell that releases them (autocrine effect), receptors on nearby cells (paracrine effect) or receptors distant from cell (endocrine effect) (Qin et al. 2008; Verri et al. 2006). TNFα exists in two forms, a 26 kDa membrane bound and 17 kDa soluble form, and both forms are biologically active (Beutler and Cerami 1988). The membrane bound TNFα is cleaved into soluble TNFα by
TNFα–converting enzyme and both forms are active in their trimeric forms (Black et al. 1997; McWhirter et al. 1999). TNFα is produced by different types of cells such as macrophages, monocytes, T and B-lymphocytes, astrocytes, fibroblasts, basophils, mast cells, smooth muscle cells, epidermal cells and Schwann cells upon injury or inflammation (Baud and Karin 2001; Sommer and Kress 2004; Wagner and Myers 1996). Furthermore, TNFα plays a vital role in cytotoxicity, cell death, metabolism, thrombosis and fibrinolysis (Bradley 2008; Burger and Dayer 2002; Darnay and Aggarwal 1999). For instance, the serum and tissue levels of TNFα are elevated in patients suffering from inflammatory diseases such as rheumatoid arthritis and infection (Robak et al. 1998) which suggests that TNFα could play a vital role in inflammation and nociception.

Injury or chemical irritation of a nerve increases the concentration of TNFα (Leung and Cahill 2010; Wagner and Myers 1996). For example, nerve injury elevates the TNFα level in injured and adjacent dorsal root ganglion neurons (Schafers et al. 2003a; Schafers et al. 2003c). Schafers et al. (2003b) have shown that exogenous TNFα causes sensitization of injured and uninjured dorsal root ganglion. Moreover, in naive dorsal root ganglion TNFα is only expressed in small sized neurons, however, after chronic constriction injury, TNFα was expressed in small, medium-sized neurons and expression of TNFα was significantly increased in nearby uninjured neurons. This elevation of TNFα leading to phenotype change suggests that TNFα plays a role in the development of hypersensitivity in uninjured neurons (Schafers et al. 2003a). These studies suggest that TNFα may be involved in the development of neuropathic pain. Indeed, blockade of TNFα attenuated sciatic and spinal nerve injury-induced hyperalgesia in a neuropathic pain animal model (Sommer et al. 2001; Sommer et al. 1998a; Winkelstein et al. 2001).
Although, evidence suggests that TNFα has a role in nociception, the mechanism by which TNFα induces pain is not completely understood. Previous studies have shown that TNFα could cause sensitization of sensory neurons by acting on various ion channels such as TTXr Na⁺ and potassium (K⁺) channels. For example, Jin and Gereau (2006) have shown that TNFα produces mechanical sensitization of dorsal root ganglion neurons by enhancing a TTXr Na⁺ current which is mediated by p38 MAPK pathway. TNFα has been demonstrated to decrease outward K⁺ currents in retinal ganglion neurons (Diem et al. 2001) and inhibit K⁺ currents in small dorsal root sensory neurons (Liu et al. 2008b) that may produce increased excitability of afferent fibers. Furthermore, treatment of dorsal root ganglion with TNFα evokes neuronal discharge in both A and C-fibers and causes mechanical sensitization (Liu et al. 2002; Ozaktay et al. 2006). These results suggest that TNFα may increase the excitability of afferent fibers by directly modulating the ion channels. In contrast, other studies have shown that TNFα acts by inducing the release of other pro-inflammatory mediators such as prostaglandin E2 (PGE2), substance P, interleukins (IL), CGRP and leukotrienes as well as induces the synthesis of NGF and catecholamines (Sachs et al. 2002; Schafers et al. 2003d; Verri et al. 2006; Woolf et al. 1997). For example, TNFα increases production of phospholipase enzyme, which induces production of arachidonic acid that leads to the synthesis of prostaglandins which mediate nociceptor sensitization (Cunha et al. 1992; Dong et al. 2009; Heller and Kronke 1994; Hoeck et al. 1993). The results of these studies suggest that TNFα could play a role in nociception both by directly acting on the receptors and indirectly by inducing the release of other pro-inflammatory mediators.

In the CNS, TNFα has both neuroprotective and neurodegenerative role (Rothwell et al. 1996). Selmaj and Raine (1988) have shown that TNFα induces damage to myelin and oligodendrocytes. Moreover, Cheng et al. (1994) have shown a neuroprotective effect of TNFα
against metabolic exocitotoxicity or oxidative insult by regulating the calcium homeostasis. In addition, Dolga et al. (2008) have shown that TNFα produces its neuroprotective effect by modulating a calcium-activated potassium channel. The neuroprotective and neurodegenerative effects of TNFα are concentration dependent. For example, a low concentration of TNFα is neuroprotective and non-inflammatory, while a higher concentration of TNFα was associated with inflammation and caused death of substantia nigra neurons (Chertoff et al. 2011; McCoy et al. 2006). These studies suggest that TNFα has both neuroprotective and neurodegenerative role in the CNS in a tissue specific and concentration dependent manner.

To sum up, TNFα is involved in various inflammatory pain conditions. However, the role TNFα plays in muscle pain is largely unexplored. Further research is needed to determine the role of TNFα in muscle pain mechanisms especially in pain conditions not associated with inflammation such as masticatory muscle pain.

TNFα Receptors

TNFα produces its biological effects by binding to two different receptors, the P55 or TNFR1 and the P75 or TNFR2, which have molecular masses of 55 kDa and 75 kDa, respectively (Heller and Kronke 1994; Kriegler et al. 1988; Vandenabeele et al. 1995). The cell surface TNFα receptors are cleaved by TNFα converting enzyme into soluble receptors (TNFsR1 and TNFsR2) (Alstergren and Kopp 2006; Wallach et al. 1991). In comparison to P55 receptors, which are expressed on all cells except erythrocytes, P75 receptors are mostly expressed on epithelium cells and in cells of the immune system. P55 and P75 receptors are expressed in the brainstem, cortex, cerebellum, kidney, adipose tissue, thalamus and basal ganglia (Kinouchi et al. 1991; Li and Reid 2001; Sherry and Cerami 1988). Both P55 and P75 receptors are expressed in the skeletal muscle (Li and Reid 2001), on Schwann cells (Qin et al.
2008), dorsal root ganglion neurons (Hensellek et al. 2007; Shubayev and Myers 2001) and the P55 receptor in the trigeminal mesencephalic nucleus (Bette et al. 2003). The expression of TNFα receptors are elevated during acute and chronic inflammation (Alstergren 2009).

Inflammation or infection increases the level of soluble receptors, which are found in blood, urine, cerebral spinal and synovial fluid by the enzymatic breakdown of cell surface receptor (Alstergren and Kopp 2006; Engelmann et al. 1989). Moreover, the expression of P55 and P75 receptors is elevated in injured and nearby uninjured rat dorsal root neurons (Ohtori et al. 2004; Schafers et al. 2003c).

TNFα activates P55 receptors at a lower concentration, whereas, a higher concentration of TNFα is required to activate P75 receptors (Grell et al. 1998). Activation of P55 receptors produces biological responses at very low densities as compared to P75 receptors (Grell et al. 1998). TNFα binds to P55 receptor with a higher affinity to form a strong complex (Grell et al. 1998). In contrast, P75 receptor binding and dissociation rate with TNFα is rapid (Grell et al. 1998). Furthermore, P75 receptor passes TNFα to P55 receptor, thus functioning as a ligand (TNFα) passer that leads to increase in TNFα signaling (Aderka et al. 1992; Tartaglia et al. 1993). It is possible that due to this ligand passer function of the P75 receptor and the strong binding complex of TNFα with P55 receptors, that most of the TNFα effects are produced through the P55 receptor.

Although, TNFα receptors share similarities in the extracellular domain, their intracellular cytoplasmic domains are different (Rothe et al. 1994; Wallach et al. 1991). Activation of these receptors by TNFα activates two distinct signaling pathways and mediates different TNFα activities (Tartaglia et al. 1993; Wallach et al. 1991). The P55 receptor is considered to be responsible for pro-inflammatory, apoptotic actions of TNFα (Micheau and Tschopp 2003). In contrast, the P75 receptor plays a role in tissue repair and angiogenesis.
For example, TNFα- induced cutaneous nociceptive effects are produced through the P55 receptor (Cunha et al. 2005; Jin and Gereau 2006; Parada et al. 2003; Sommer et al. 1998b). Moreover, activation of P55 receptor initiates the secretion of IL-1β, which induces prostaglandin production (Cunha et al. 2005). However, recent studies have shown the involvement of P75 receptors in tumor-induced heat hyperalgesia by TNFα in dorsal root ganglion neurons (Constantin et al. 2008) and in the maintenance of neuropathic pain (Schafers et al. 2008). These findings suggest that P75 receptor could also be involved in TNFα–induced nociception.

Both antagonists such as Etanercept and antagonistic antibodies against TNFα inhibit TNFα-mediated effects (Rigby 2007). Infliximab and Adalimumab are the two TNFα antibodies widely used in the treatment of rheumatoid arthritis and various other inflammatory diseases (Elliott et al. 1994; Moreland et al. 1997; Rigby 2007). Etanerecept is a fusion protein of human IgG and two TNFsR2, while infliximab and adalimumab are monoclonal antibodies of TNFα (Moreland et al. 1997; Nash and Florin 2005). These antibodies bind with TNFα to prevent binding of TNFα to its receptors (Krensky Am 2006). However, these antibodies have some major disadvantages such as adverse immune effects and need for parental administration. These antibodies increase the risk of infection and relapse of tuberculosis (Baronnet et al. 2011; Lipsky et al. 2000; Listing et al. 2005) which prevents the use of these antibodies in patients with frequent chest infection. These antibodies cause allergic reactions at the injection site such as swelling, haemorrhage, erthyma, itching and pain (van de Putte et al. 2004). Moreover, the cost associated with these antibodies are very high (Tugwell 2000).

Although most TNFα effects including TNFα- induced nociception in skin are produced via P55 receptors, it is still unknown which TNFα receptor types are involved in the mediation of nociception in other tissues and their role in masseter muscle pain remains to be determined.
Signaling Pathways of TNFα

Binding of TNFα to P55 and P75 receptors causes activation of multiple pathways such as ceramide signaling and various MAPK pathways. The p38 MAPK pathway plays an important role in the regulation of inflammatory and neuropathic pain (Jin and Gereau 2006; Schafers et al. 2003e). For example, TNFα appears to cause sensitization of nociceptors by activating P55 receptors and the p38 MAPK pathway (Jin and Gereau 2006). Schafers et al. (2003e) have shown that mechanical allodynia induced by spinal nerve ligation was attenuated by pre-treatment of p38 MAPK antagonist. In addition, TNFα binds to the P55 receptor to cause activation of phospholipase C that induces the release of DAG (Schutze et al. 1991; Vilecek and Lee 1991). DAG causes Ca\(^{2+}\)-independent activation of various cellular kinases such as PKC and acidic sphingomyelinase (Smase) (Heller and Kronke 1994; Schutze et al. 1992). PKC plays various roles in TNFα actions such as activation of JUN and FOS proteins that are components of activating protein-1 (Ap-1) transcription factor (Brenner et al. 1989; Heller and Kronke 1994). The acidic Smase produces ceramides from sphingomyelin. Ceramide acts as second messenger and produces diverse cellular responses such as cell proliferation, cell differentiation as well as apoptosis (Obeid et al. 1993; Okazaki et al. 1990; Olivera et al. 1992; Schutze et al. 1992).

TNFα also activates transcription factors such as nuclear –kappaB (NF-κB) (Barbin et al. 2001; Darnay and Aggarwal 1999; Malinin et al. 1997). There are various adaptor proteins that activate these pathways (Arch et al. 1998). One of these proteins is the death domain protein, which links the P55 TNFα receptor to downstream targets and leads to apoptosis (Darnay and Aggarwal 1999; Deng 2007). Another adaptor protein associated with TNFα signaling is TNFα receptor associated factor (TRAF) which plays an important role in the activation of transcription factors and protein kinases (Hsu et al. 1996; Malinin et al. 1997). Moreover, TRAF is comprised of six different proteins, but, only TRAF2, TRAF5 and TRAF6 cause NF-κB and MAPK activation (Darnay et al. 1999). NF-κB plays a vital role in the pathogenesis of
inflammation (Darnay and Aggarwal 1999) and nociception (Kou et al. 2011). Indeed, TNFα pro-inflammatory, nociceptive and cell survival responses are mediated through NF-κB. For example, Zang et al. (2010) have described the involvement of NF-κB in TNFα induced neuropathic pain. Moreover, NF-κB is believed to play role in the pathogenesis of complex regional pain syndrome (de Mos et al. 2009).

Binding of TNFα to the P55 receptor causes attachment of TRADD (TNFα Receptor Associated Death Domain) to the receptor complex, which activates three other mediators such as receptor-interacting protein-1 (RIP-1), Fas-associated death domain protein (FADD) and TRAF2 (Hsu et al. 1996; Rothe et al. 1995; Ting et al. 1996; Vandenabeele et al. 1995). This TRADD-TRAF2 – RIP-1 complex through downstream signaling pathway leads to activation of, C-jun N-terminal kinases (JNK), p38 MAPK and NF-κB (Hsu et al. 1995; Kelliher et al. 1998). For example, TRAF2 and RIP-1 recruits MEKK-3 which activates IKK (inhibitor of κB (IκB) kinase) to cause phosphorylation of IκB proteins. Normally, IκB forms complex with NF-κB to block the activation of NF-κB. Breakdown of IκB and NF-κB complex activates NF-κB to initiate gene transcription involved in inflammation and nociception (Baud and Karin 2001; Chen and Goeddel 2002; Malinin et al. 1997). Hence, blocking of IKK may attenuate the activation of various pro-inflammatory genes that could reduce the inflammation and nociception. Indeed, Tegeder et al. (2004) have shown that inhibitor of IKK significantly reduces the heat and mechanical hyperalgesia in a zymogen induced rat hind paw inflammation model. Moreover, in the same study IKK also significantly reduced the touch and cold allodynia in a neuropathic pain animal model. In addition, the zymogen induced increased levels of cyclooxygenase (COX) enzyme in a rat spinal cord was significantly inhibited by IKK (Tegeder et al. 2004). These results suggest that TNFα activated signaling molecules are implicated in the
development of nociception. Blocking of these signaling molecules would inhibit the activation of genes responsible for TNFα induced nociception.

TNFα activated downstream signaling pathways are also implicated in the development of nociception. For example, these signaling molecules are involved in TNFα induced activation of phospholipase A2 (PLA2) and COX enzyme (Hernandez et al. 1999; Paul et al. 1999). p38 MAPK phosphorylates PLA2 leading to the activation of arachidonic acid, which is then converted into prostaglandins such as PGE2 by COX enzyme (Kramer et al. 1996; Samad et al. 2002). In addition, there is evidence that the expression of PLA2 and COX are also up-regulated by TNFα-induced release of lipid second messenger molecule ceramide which subsequently leads to the production of PGE2 (Hayakawa et al. 1996; Hoeck et al. 1993). PGE2 plays an important role in nociception and inflammation (Samad et al. 2002). Moreover, TNFα also induces release of NGF (Kuno et al. 2006; von Boyen et al. 2006; Woolf et al. 1997). NGF activates p38 MAPK and contributes to NGF- induced heat hyperalgesia by increasing the expression of TRPV1 receptors on dorsal root ganglion (Ji et al. 2002). Overall, these results suggest that TNFα activates downstream signaling molecules to release other algogenic substances that could mediate some of the effects of TNFα.
Figure 1.2  This diagram explains the peripheral mechanism of how TNFα might induce sensitization of nociceptors.
**TNFα and Craniofacial Pain**

TNFα is a pro-inflammatory polypeptide that plays an important role in inflammation, cytotoxicity and cell death (Alstergren 2009; Burger and Dayer 2002; Ksontini et al. 1998). TNFα is believed to contribute to the pathogenesis of inflammatory diseases, for example arthritis (Alstergren and Kopp 2006; Arend and Dayer 1995; Feldmann 2002). TNFα has been found in the synovial fluid of patients with rheumatoid arthritis as well as in patients suffering from other inflammatory diseases such as osteoarthritis, psoriatic arthritis and reactive arthritis (Chu et al. 1991; Feldmann et al. 1996; Francois et al. 2006; Lange et al. 2000; Nordahl et al. 2000). TNFα along with IL-1 enhances the production of interstitial collagenase, which contributes to cartilage damage associated with joint diseases such as rheumatoid arthritis and osteoarthritis (Duff 1994). Anti-TNFα drugs such as infliximab, entanercept, adalimumab are now used for the treatment of rheumatoid arthritis (Feldmann 2002; Kopp S 2008; Nash and Florin 2005).

Another condition where TNFα may play a role is in TMD, which are a group of chronic craniofacial pain conditions that affect the temporomandibular joint (TMJ) and masticatory muscles (Dimitroulis 1998). Myofascial TMD are associated with pain in masticatory muscles such as masseter muscle without signs of inflammation (Dimitroulis 1998; Fricton 2007). In contrast, rheumatoid arthritis and osteoarthritis of the TMJ are associated with inflammation (Alstergren et al. 2008; Lindblad and Hedfors 1987). Nordahl et al. (2000) showed that the synovial fluid in the TMJ of patients suffering from chronic inflammatory diseases including rheumatoid arthritis contained elevated levels of TNFα. Increased levels of TNFα contribute to allodynia and hyperalgesia in patients suffering from rheumatoid arthritis of TMJ, which indicates that either TNFα or TNFα-induced mediators are involved in the pathogenesis of arthritic TMJ pain (Nordahl et al. 2000). In various painful muscular diseases such as inflammatory myopathies and fibromyalgia, muscle cytokine levels are also increased (Lundberg
et al. 1997; Tews and Goebel 1996; Wang et al. 2008). In addition, serum TNFα levels are also elevated in patients suffering from fibromyalgia, a musculoskeletal pain syndrome characterized by widespread muscle pain (Wang et al. 2008). It is still not known whether there are also elevated levels of TNFα in the muscles of myofascial TMD patients or what role TNFα could play in the mechanisms that underlie the development and maintenance of muscle pain in this condition.

Animal Studies

Animal studies suggest that TNFα plays an important role in the development of inflammatory and neuropathic pain (Junger and Sorkin 2000; Leung and Cahill 2010; Sommer and Kress 2004). Subcutaneous injection (SC) of TNFα causes mechanical sensitization and thermal hyperalgesia which is mediated through P55 receptors (Cunha et al. 2005; Jin and Gereau 2006; Parada et al. 2003; Sommer et al. 1998b). Junger and Sorkin (2000) showed that SC injection of TNFα reduced the mechanical threshold of C-nociceptors in a dose dependent manner and caused inflammation. In the same study, TNFα evoked activity in 14% of nociceptive C-fibers but did not evoke afferent fiber discharge in mechanosensitive Aβ fibers (Junger and Sorkin 2000). These observations suggest that TNFα may be selective for slowly conducting cutaneous afferent fibers with nociceptive properties. Intraplantar injections of TNFα into the rat paw caused mechanical allodynia and hyperalgesia and were associated with increased levels of inflammatory mediators such as IL, NGF and prostaglandins (Cunha et al. 1992; Cunha et al. 2005; Woolf et al. 1997). However, previous animal studies have shown that intraplantar injection of TNFα also acts directly by exciting cutaneous afferent fibers (Junger and Sorkin 2000; Sorkin et al. 1997). These studies suggest that TNFα may cause sensitization
of nociceptors by acting directly on nociceptors or indirectly by releasing other algogenic substances.

Previous animal studies have implicated TNFα in joint inflammation. For example, intra-articular injection of TNFα caused an intense incapacitation response in carrageenan primed rat knee joints that lasted for more than 8 hours and was reversed by treating knee joints with anti-TNFα antibodies (Tonussi and Ferreira 1999). The anti-TNFα treatment reduced the joint swelling and pain in a mouse model of arthritis suggesting a role of TNFα in arthritis. Furthermore, in the complete Freunds’adjuvant (CFA)-induced model of arthritis, there was an increased expression of both TNFα receptors as well as macrophage infiltration in the dorsal root ganglion (Inglis et al. 2005). These animal studies support the role of TNFα in joint inflammation. In contrast, the levels of TNFα did not increase in rat muscle inflammation (Loram et al. 2007). Moreover, injection of TNFα into rat gastrocnemius muscle resulted in prolonged sensitization without inflammation and was associated with increased tissue levels of PGE2, CGRP and NGF (Schafers et al. 2003d). These findings suggest that TNFα may be implicated in the development of muscle hyperalgesia without inducing inflammation.

**TNFα in Muscle Pain**

In spite of the fact that TNFα plays an important role in the development of neuropathic and inflammatory pain, the role of TNFα in muscle pain is unknown. TNFα levels are significantly elevated in myofascial trigger points, areas of the muscle which are painful upon palpation (Shah and Gilliams 2008; Shah et al. 2005). Moreover, the levels of various cytokines are elevated in various muscle pain conditions such as fibromyaglia (Wang et al. 2008). Previous studies have shown that TNFα induces the release of other pro-inflammatory mediators in skeletal muscle (Alvarez et al. 2002; Zhang et al. 2000). Moreover, Schafers et al. (2003d) have
shown that IM injection of TNFα causes muscle hyperalgesia without inflammation that was associated with increased tissue level of inflammatory mediators such as PGE2, CGRP and NGF. However, in this study, the role of these pro-inflammatory mediators in TNFα induced muscle sensitization was not determined. In another study, Hoheisel et al. (2005) did not show an effect of TNFα on the mechanical sensitivity of C-fibers that innervate the rat gastrocnemius muscle. These results suggest that the role TNFα plays in non-inflammatory myofascial pain mechanisms remains to be determined. Furthermore, it is not clear whether TNFα causes mechanical sensitization by directly acting on its receptors or by inducing the release of other algogenic substances such as prostaglandins, NGF, CGRP, glutamate and substance P.

**TNFα Induced Release of Algogenic Substances**

TNFα induces the release of other cytokines and algogenic substances (Schafers et al. 2003d; Sommer and Kress 2004; Woolf et al. 1997). Previous studies have shown that *in-vitro* treatment of dorsal root ganglion cultured cells with TNFα increases the PGE2 level, and in addition, TNFα–induced cutaneous sensitization is prostaglandin dependent (Fehrenbacher et al. 2005; Russell et al. 2009). A previous behavioral animal study showed that injection of TNFα into rat gastrocnemius muscle caused prolonged mechanical sensitization of afferent fibers and was also associated with increased levels of PGE2 and NGF (Schafers et al. 2003d), which have previously been shown to be involved in muscle sensitization (Dong et al. 2009; Svensson et al. 2010). PGE2 is a pro-nociceptive substance that sensitizes muscle nociceptors in both animals and humans (Dong et al. 2009; Mense 1981; Rukwied et al. 2007). *In-vitro* studies have shown that TNFα induces the release of NGF in human intervertebral disc cells and keratinocytes (Abe et al. 2007; Takaoka et al. 2009). Moreover, Woolf et al. (1997) have shown that cutaneous injection of TNFα induces the release of NGF. In addition, previous animal and human studies...
have confirmed the role of NGF in muscle hyperalgesia (Svensson et al. 2003a; Svensson et al. 2010). Taken together, these studies suggest that TNFα could produce sensitization of muscle afferent fibers through the release of PGE2 and NGF. Further research is needed to determine the role of PGE2 and NGF in TNFα–induced masseter muscle sensitization.

Finally, TNFα also induces the release of glutamate through activation of P55 receptors in the CNS (Hermann et al. 2005; Youn et al. 2008). Glutamate is an excitatory amino acid released by both peripheral and central nerve terminals of afferent fibers (De Biasi and Rustioni 1988; deGroot et al. 2000; Omote et al. 1998). Glutamate exerts its biological effects by binding to NMDA as well as to non-NMDA receptors such as AMPA and metabotropic G-protein coupled receptors (Bhave et al. 2001; Coggeshall and Carlton 1997; Davidson et al. 1997). NMDA and AMPA receptors are non-selective cation channels widely expressed in the CNS and peripheral nervous system (Chun et al. 2008; Coggeshall and Carlton 1997). The metabotropic receptors are further divided into three groups: group I (mGluR1 and 5), group II (mGluR2 and 3) and group III (mGluR4, 6, 7 and 8). Group I receptors activate phospholipase and are excitatory and group II and III receptors inhibit adenylate cyclase (Bleakman et al. 2006; Conn and Pin 1997). Previous human and animal studies have shown that glutamate plays a role in the peripheral sensitization of masseter muscle afferent fibers (Cairns et al. 2001a; Cairns et al. 2003b; Svensson et al. 2003b). For example, injection of glutamate into healthy male volunteers produced pain and muscle sensitization which was attenuated by ketamine, a non-competitive antagonist of glutamate which suggests the role of NMDA receptors (Cairns et al. 2006). In rats, artificially elevated interstitial glutamate concentration caused sensitization of masseter muscle afferent fibers, which was mediated through NMDA receptors (Cairns et al. 2007). Further, injection of glutamate into the masseter muscle activates and causes sensitization of masseter muscle nociceptors (Cairns et al. 2003b). In the same study, DL-2-amino-5-phosphonovaleric acid (APV; 10mM), a competitive antagonist and ketamine which is a non-competitive
antagonist of glutamate at the NMDA receptor inhibited glutamate-induced sensitization, which suggests that glutamate mediates its action through peripheral NMDA receptors. These results are also supported by a study that has shown the involvement of peripheral NMDA receptor in mustard oil induced masseter muscle nociception (Ro 2003). Lee and Ro (2007) have also demonstrated that mGLuR5 receptor activation produces mechanical sensitivity in the masseter muscle. Moreover, Chun et al. (2008) have demonstrated the involvement of AMPA receptors in the mustard oil induced masseter muscle nociception. Taken together, these results suggest that both NMDA and non-NMDA receptors are involved in glutamate induced peripheral sensitization of masseter muscle afferent fibers.

To sum up, these studies support the statement that TNFα could exert indirect effects by inducing the release of other algogenic substances such as PGE2, NGF and glutamate which have been previously shown to induce sensitization of masseter muscle afferent fibers.

**Trigeminal Anatomy**

**Craniofacial Innervations**

Craniofacial tissue such as TMJ, teeth, periodontal tissues, facial skin and muscles are highly innervated (Sessle 2000). The trigeminal nerve (V) is the largest cranial nerve with both sensory and motor functions and is thus a mixed nerve similar to spinal cord nerves like the sciatic nerve (Dodd 1991). The trigeminal nerve in periphery divides into three major nerves: ophthalmic (V1), maxillary (V2) and mandibular (V3) nerves (Borsook et al. 2003; Dodd 1991). The V1 and V2 comprise only sensory nerves, while the V3 consists of both sensory and motor nerves (Borsook et al. 2003; Dodd 1991). The three trigeminal nerves leave from skull through three different openings: the superior orbital fissure, the foramen rotundum and the foramen ovale (Dodd 1991). The trigeminal nerve innervates diverse craniofacial tissues such as the oral mucosa, tongue, tooth pulps, periodontal membrane and masticatory muscles (Dodd 1991; Sessle
2000). The masseter muscle is innervated by masseteric branches of V3 (Borsook et al. 2003). The cell bodies of primary afferent fibers of the trigeminal nerve are located in the peripheral nervous system in Gasserian (trigeminal) ganglion (Borsook et al. 2003; Sessle 2000). The trigeminal ganglion is located at the base of brain in the cranial fossa across the superior border of the petrous temporal bone (Borsook et al. 2003). Afferent trigeminal nerves that terminate in the craniofacial region are activated by touch, light encode tactile (low threshold mechanoreceptors) or proprioceptive stimuli such as stretch, tension (proprioceptors), while some nerves terminate as free nerve endings (Sessle 2000). Free nerve endings that respond to noxious stimuli are termed nociceptors (Cairns 2006). These free nerve endings are present in all craniofacial tissues, including the TMJ and masticatory muscle such as masseter muscles (Cairns 2006; Cairns 2008). Afferent fibers that act as nociceptors include high threshold slowly conducting small diameter and thinly myelinated Aδ afferent fibers and unmyelinated C- afferent fibers (Cairns 2008). The conduction velocity of Aδ and C- fibers in the rat is 2.5-12 m/s and < 2.5 m/s respectively (Cairns 2008). Some Aδ fibers and C- fibers also respond to non-noxious stimuli such as cooling and warming (Sessle 2009).

Masseter Muscle Innervation and Trigeminal Sensory Nuclear Complex (TSNC)

The masseter muscle is innervated by slowly conducting afferent fibers that project to the trigeminal subnucleus interpolaris and caudalis (Capra and Wax 1989). These fibers are stimulated by noxious mechanical and chemical stimuli and thus act as nociceptors. Mechanical stimulation of rat masseter muscle activates mostly Aδ fibers and few C-fibers (Cairns 2008) which is mostly innervated with Aδ sensory fibers with conduction velocity of less than 12 m/s (Cairns 2008). Moreover, masseter muscle nociceptors are also activated by algogenic substances such as hypertonic saline (HS) and glutamate and thus are polymodal (Cairns 2008; Cairns et al. 2006). The masseter muscle is also innervated by large diameter, fast conducting
afferent fibers, which serve as proprioceptors (Sessle 2000; 2006). In contrast to slowly conducting afferent fibers whose cell bodies lie in the trigeminal ganglion, jaw muscle spindle afferent fibers and some mechanosensitive afferent fibers from periodontal tissues have their cell bodies located in CNS, within V mesencephalic nucleus (Sessle 2000). The central axons of the primary afferent cell bodies in the V mesencephalic nucleus project to and make excitatory synaptic connections with interneurons involved in craniofacial reflex function (Sessle 2000). In the brainstem, afferent nerve fibers ascend or descend in the V spinal tract and send branches that terminate in subdivisions of trigeminal sensory nuclear complex (TSNC) to activate second order neurons in the TSNC (Sessle 2000).

The TSNC is the part of brainstem, which receives and processes the craniofacial sensory information and then relays it to higher CNS parts such as thalamus and cortex (Sessle 2000). Apart from trigeminal afferent inputs, the TSNC also receives input from both cranial nerves (VII, IX, X, XII) and upper cervical nerves (Sessle 2000). The TSNC is subdivided into main or principal, sensory nucleus and the spinal tract nucleus. The spinal tract nucleus is further subdivided into oralis, interpolaris and caudalis (Dodd 1991; Sessle 2000). Caudalis, which is the most caudal, is a laminated structure that reaches to the cervical spinal cord and then connects with spinal dorsal horn (Dodd 1991; Sessle 2000). Most of nociceptive fibers synapse in subnucleus caudalis and receive sensory input from the primary afferent nociceptors (Hathaway et al. 1995; Sessle and Hu 1991). Aδ and C- fibers from craniofacial tissue terminate in laminae I, II, V, and VI of the caudal brainstem and the low threshold afferent fibers terminate in lamina III and VI of the caudalis and rostral parts of TNSC (Sessle 2000). Subnucleus caudalis is essential for conveying nociceptive information from small diameter afferent fiber endings to second order neurons in caudalis (Hathaway et al. 1995; Sessle and Hu 1991).

Cutaneous afferent fibers activate caudalis neurons that are of two types: nociceptive specific (NS) and wide dynamic range (WDR) neurons. The NS neurons receive inputs from NS
slowly conducting small diameter Aδ and C-fibers and respond only to noxious stimuli (Sessle 2008). WDR neurons which are also called convergent neurons receive input from both large diameter fast conducting afferent fibers and slow conducting small diameter afferent fibers (Sessle 2000). Most of NS and WDR neurons are activated by activation of cutaneous receptor field, however, the majority of NS and WDR neurons also receive input from deep tissues such as TMJ, muscle, and tooth pulp (Hu et al. 2005; Sessle 2000; Sessle and Hu 1991). The cutaneous receptive field of a WDR neuron is larger than that of a NS neuron and can be activated by both noxious and non-noxious stimuli. The cutaneous receptive field of WDR neurons helps to localize and discriminate superficial noxious stimuli (Sessle 2005). The presence of both cutaneous as well as deep non-cutaneous receptor field of subnucleus caudalis neurons may explain the poor localization of deep craniofacial pain such as muscle and TMJ pain (Sessle 2000). Noxious stimulation releases neuro-peptides such as substance P, CGRP and excitatory amino acids such as glutamate from the central terminals of nociceptive afferent fibers to activate NS and WDR neurons directly and/or indirectly via interneurons (Sessle 2006). NS and WDR neurons of the TSNC project to higher areas of the brain such as the ventrobasal thalamus, periaqueductal gray matter and reticular formation (Sessle 2008; Sessle 2000; 2006).
Figure 1.3 This diagram shows the pathways of sensory afferent fibers innervating the craniofacial area. The afferent fibers project to trigeminal sensory nuclear (TSNC) complex through trigeminal ganglion. Second order neurons (NS and WDR) in the TSNC project to higher levels such as thalamus and reticular formation. (Sessle 2000; Published with permission from the international and American association of dental research).
Temporomandibular Disorders (TMD)

TMD are group of musculoskeletal pain conditions that involve the craniofacial region (LeResche 1997). TMD are characterized by pain in TMJ and muscles of mastication such as masseter muscle and are considered to be the most common cause of non-dental craniofacial pain (Dworkin and Burgess 1987; List et al. 2003; McNeill 1997). Patients suffering from TMD can have limited jaw motion, jaw and muscle pain, earache, headache, facial pain and joint sounds (Dimitroulis 1998; McNeill 1997; Tenenbaum et al. 1999). Acute TMD symptoms are mild and short lasting. However, patients with chronic TMD suffer from constant pain (Dimitroulis 1998; Scrivani et al. 2008). The three most common symptoms of TMD are pain, limited jaw motion and jaw clicking sound (Celic et al. 2004; Dimitroulis 1998). Pain is the most common symptom of TMD and is dull, aching, continuous or intermittent, which worsens with jaw movement such yawning, chewing and talking (Scrivani et al. 2008). Joint sounds are not always associated with TMD as most people with joint sounds do not have signs of TMD (Dimitroulis 1998). In TMD patients, movement of the jaw is limited such that some patients are unable to open or close their jaws (jaw lock) (Scrivani et al. 2008). This restricted jaw movement leads to difficulty in talking, eating and yawning (Dimitroulis 1998).

TMD are more common in young and middle aged adults. However, they are less common in children and in the elderly population (Greene 1994; LeResche 1997; Scrivani et al. 2008; Warren and Fried 2001). About 10% of adults suffer from TMD (LeResche 1997; Warren and Fried 2001). The prevalence of TMD is two times greater in women as compared to men (LeResche 1997; Warren and Fried 2001). Eighty percent of patients treated for TMD are women (Warren and Fried 2001). This high prevalence of TMD in women could be related to behavioral, physiological and hormonal changes in women.

The research diagnostic criteria divide TMD conditions into three groups: 1. Myofascial TMD (masticatory muscle pain) with or without limited jaw opening. 2. TMJ disc displacement
(reducing or non-reducing with or without limited jaw opening). 3. Arthralgia, arthritis and arthrosis (Dworkin and LeResche 1992). Myofascial TMD and osteoarthritis are the most common TMD (Dimitroulis 1998; LeResche 1997). At least half of TMD are related to the muscles (Dimitroulis 1998; Lobbezoo et al. 2004). The pathogenesis and etiology of most TMD are not well understood (Lobbezoo et al. 2004). This could be due to the lack of understanding of peripheral mechanisms related to pathogenesis of chronic muscle pain.

Pathophysiology of TMD

The etiology of TMD is affected by several factors. These factors are grouped as predisposing factors (structural, metabolic and psychological conditions), initiating factors (injury, overloading of masticatory muscle) and aggravating parafunctional activities such as clenching of teeth and stress (Barbosa Tde et al. 2008; Greene 1995; Marklund and Wanman 2010; McNeill 1997).

Several previous studies have shown that parafunctional activities are associated with TMD pain (Dimitroulis 1998; Glaros 2008; Schiffman et al. 1992; Svensson and Arendt-Nielsen 1996; Velly et al. 2002a; Velly et al. 2002b). Parafunctional activities such as bruxism, which is defined as a motor disorder characterized by clenching and or grinding of the teeth during sleep and/or wakefulness overload the masticatory muscles (Carlsson et al. 2002; Manfredini and Lobbezoo 2010; Michelotti et al. 2010), and are considered one of the major risk factors for TMD pain. However, it is still uncertain whether bruxism plays a major role in the development of TMD pain (Alamoudi 2001; Glaros et al. 2005; Lobbezoo and Lavigne 1997). Several studies have shown the positive role of bruxism in TMD muscle and joint pain (Chen et al. 2007a; Huang et al. 2002; Michelotti et al. 2010; Miyake et al. 2004; Velly et al. 2003; Winocur et al. 2006). For example, Marklund and Wanman (2010) have recently shown that bruxism increases the incidence of myofascial pain. In addition, Michelotti et al. (2010) have also shown that day
time clenching and grinding are associated with myofascial muscle pain. In contrast, a few studies have reported no relationship between bruxism and TMD pain (Castelo et al. 2005; van der Meulen et al. 2006). The relationship between bruxism and muscle pain could be explained by decreased blood supply to the muscles (Larsson et al. 2004; Nakamura et al. 2005) and damage to the muscle fibers (Larsson et al. 1988). Repetitive strain due to bruxism results in increased oxidative metabolism that causes decrease in energy supply and induces the release of nociceptive substances such as histamine, kinins and prostaglandins in muscles that may lead to muscle tenderness and pain (Farella et al. 2010; Fricton 2007).

It is believed that psychological factors such as stress and depression may play a role in the development and maintenance of myofascial TMD (Korszun et al. 1996; Schmitter et al. 2009; Yap et al. 2002). The hypothalamic-pituitary-adrenal axis (HPA) along with sympathetic and adreno-medullary systems are implicated in stress development, and imbalance of the HPA axis leads to depression (Gameiro et al. 2006). Indeed, patients with TMD symptoms have increased activity in the HPA axis (Gameiro et al. 2006; Geissler 1985). Moreover, patients suffering from myofascial pain have elevated urinary and blood levels of catecholamines and steroids, which are correlated with stress (Evaskus and Laskin 1972; Korszun et al. 2002). In addition, several studies have reported that patients suffering from TMD have dysregulated sympathetic activity (Elam et al. 1992; Light et al. 2009; Passatore and Roatta 2007). Activation of sympathetic activity induces muscle vasoconstriction, decreases muscle contractions and muscle spindle afferent fiber activation (Matsuo et al. 1995; Roatta et al. 2002). Stress induced increase in sympathetic activity causes vasoconstriction that reduces blood flow to muscles leading to muscle damage. The subsequent release of inflammatory substances may lead to development of muscle pain (Devor and Janig 1981; Katz et al. 2007; Maekawa et al. 2002; Passatore and Roatta 2007; Thomas and Segal 2004). Ischemic muscle damage and release of other inflammatory sympathetic agonists such as epinephrine causes activation and mechanical
sensitization of nociceptors (Katz et al. 2007; Khasar et al. 1999; Passatore and Roatta 2007; Pluteanu et al. 2002). In addition, endogenous pain inhibitor systems such as serotonin and opioids are also believed to play a role in stress induced TMD symptoms (Gameiro et al. 2006). Stress leads to a decrease in the release of serotonin, which is a natural descending inhibitor of pain transmission. In general, TMD patients may have diminished endogenous pain inhibitors systems (Gameiro et al. 2006).

To reiterate, at least half of TMD are associated with muscle pain symptoms (Dimitroulis 1998). It is believed that there is a relationship between pain and muscle movement (Johansson and Sojka 1991). According to the vicious cycle theory, increased muscle activity due to stress, motion, and posture leads to masseter muscle pain causing muscle fatigue and spasm, that further increases pain in a cyclic manner (Johansson and Sojka 1991). Previous studies have shown that TMD patients have elevated resting masticatory muscle electromyographic activity, a technique used to record muscle activity (Bodere et al. 2005; Burdette and Gale 1988; Svensson et al. 1997). However, there is no strong evidence that increased muscle activity in TMD patients contributes to masticatory muscle pain (Burdette and Gale 1988; Liu et al. 1999). Moreover, pain adaptation model proposes that pain does not occur due to increased muscle activity, but due to changes in agonist and antagonist muscle activities that restrict movements of the jaw to reduce further injury, thus promoting healing (Lund et al. 1991).

Muscle trauma also has a role in the pathophysiology of myofascial TMD pain. Injuries due to wide opening of the jaw or a direct blow to the jaw can cause myofascial muscle pain and produce tender spots (Fricton 2007; Suvinen et al. 2005). Whiplash injury to neck or head is believed to induce TMD symptoms (Klobas et al. 2004; Kolbinson et al. 1998). These findings suggest the role of peripheral sensitization in TMD pain mechanisms.
TMD Conditions Associated with the Temporomandibular Joint (TMJ)

Disc displacement and joint degenerative diseases such as osteoarthritis are very common in TMD (Sato et al. 2007a; Tenenbaum et al. 1999). Disc displacement is characterized by abnormal position of disc with or without reduction (Farrar 1972; McNeill 1997; Tenenbaum et al. 1999). Disc displacement with reduction is a temporary displacement of the disc relative to mandibular condyle and moves back to normal position with clicking sounds upon jaw opening (Graff-Radford 2007; McNeill 1997). Disc displacement without reduction is also called jaw lock, in which abnormal position of the disc remains maintained upon jaw movement (Farrar 1972; McNeill 1997). The disc displacement without reduction is more severe and is associated with jaw pain, restricted jaw motion, tenderness, headache and painful chewing and may lead to degenerative changes (Graff-Radford 2007; Sharawy et al. 2000). Overloading of TMJ due to parafunctional activities such as clenching and grinding, trauma and joint friction are believed to play a role in the development of disc displacement and osteoarthritis (Gallo et al. 2006; Tanaka et al. 2008). Osteoarthritis of the TMJ is a degenerative disease associated with low levels of inflammation (Tanaka et al. 2008; Zarb and Carlsson 1999). Osteoarthritis is induced by articular cartilage abrasion and joint trauma that results in cartilage damage and release of inflammatory substances (Tanaka et al. 2008; Zarb and Carlsson 1999).

Rheumatoid arthritis of the TMJ is an autoimmune disease that causes TMJ degeneration (Okroj et al. 2007; Wordsworth 1992). Inflammation plays a vital role in the pathophysiology of rheumatoid arthritis of the TMJ (Appelgren et al. 1995; Kopp 2001; Voog et al. 2003). Indeed, levels of pro-inflammatory cytokines such as TNFα, IL-1 and -6 are elevated in patients suffering from rheumatoid arthritis of the TMJ and are believed to play a role in the development of TMJ degenerative diseases (Alstergren and Kopp 2006; Houssiau 1995; Inglis et al. 2005). For example, Alstergren and Kopp (2006) have shown that increased plasma levels of TNFα contribute to TMJ inflammation and tissue destruction in patients suffering from rheumatoid
arthritis of TMJ. In addition, elevated levels of neuro-peptides such as CGRP and substance P have been detected in patients suffering from TMJ pain (Appelgren et al. 1995; Holmlund et al. 1991; Kopp 1998; Sato et al. 2004; Sato et al. 2007b). These noxious substances activate the nociceptors innervating the TMJ which contributes to pain and inflammatory changes such as edema, increase in temperature and redness (Oliveira et al. 2005; Takeuchi et al. 2004). These inflammatory mediators sensitize TMJ nociceptors to cause allodynia and hyperalgesia (Broton et al. 1988; Takeuchi et al. 2004). These findings suggest that TNFα plays a role in peripheral pain mechanisms such as the development of arthritis of TMJ. However, the exact role that inflammatory substances such as TNFα play in myofascial TMD has yet to be determined.

Role of Peripheral Sensitization in Myofascial TMD Pain Mechanisms

Currently, the etiology and pathophysiology of myofascial TMD are not known. Patients suffering from myofascial TMD have reported lower pressure pain threshold in the masseter muscle which could be due to peripheral sensitization of nociceptors (Lobbezoo et al. 2004). The release of endogenous inflammatory substances due to noxious stimuli could cause sensitization of nociceptors innervating the muscle. Indeed, various animal studies have demonstrated that intramuscular injection of bradykinin, PGE2, glutamate and NGF decreased the mechanical threshold of muscle afferent fibers (Cairns et al. 2007; Dong et al. 2009; Mense 1993; Svensson et al. 2010). In addition, previous studies have shown expression of receptors for various algogenic substances such as ATP, PGE2, glutamate, NGF and serotonin on masticatory muscle afferent fibers (Chen et al. 1995; Dong et al. 2007 ; Patwardhan et al. 2008; Staikopoulos et al. 2007; Sung et al. 2008; Svensson et al. 2010 ). These algogenic substances released during muscle injury or fatigue activate and cause mechanical sensitization of masticatory muscle afferent fibers which supports the role of peripheral sensitization in myofascial TMD.
Glutamate is an algogenic substance that may be implicated in the development of non-inflammatory peripheral muscle pain and sensitization (Cairns et al. 2006; Cairns et al. 2003b). The masseter muscle tissue levels of glutamate in myofascial TMD patients are 2-3 times higher than in healthy subjects which could be due to increased release of glutamate by muscle nociceptors (Castrillon et al. 2010). Injection of glutamate into human masseter muscle caused short lasting pain and prolonged sensitization of masseter muscle and was associated with identical symptoms experienced by myofascial TMD patients such as restricted jaw movement, allodynia, hyperalgesia and pain referral (Castrillon et al. 2008; Svensson et al. 2003b). These results lend support to glutamate as a mediator of masticatory muscle pain and suggest the role of peripheral mechanism in the pathogenesis of myofascial TMD pain.

NGF is another algogenic substance that could play an important role in peripheral mechanisms of myofascial TMD. Previous studies have demonstrated prolonged mechanical sensitization of masseter muscle afferent fibers in response to intramuscular injection of NGF. For example, Svensson et al. (2010) have shown that intramuscular administration of NGF causes sensitization of rat masseter muscle nociceptors mediated through high affinity NGF receptor, TrkA. In addition, injection of NGF into human masseter muscle caused prolonged muscle pain (Svensson et al. 2003a; Svensson et al. 2008a). These results suggest that NGF could contribute to peripheral mechanisms of myofascial TMD pain.

As mentioned already, TNFα plays an important role in inflammatory processes, as evident by elevated levels of TNFα in synovial fluid of patients suffering from various arthritic diseases such as rheumatoid arthritis and ankylosing spondylitis (Chu et al. 1991; Feldmann et al. 1996; Francois et al. 2006; Lange et al. 2000; Nordahl et al. 2000). These observations suggest that TNFα plays an important role in the peripheral mechanisms of inflammatory TMJ disease conditions. However, what role TNFα could play in masticatory muscle pain is still not known.
**Temporomandibular Joint Pain Model**

Several behavioural models of TMJ inflammation and pain have been developed. These models inject inflammatory substances such as CFA, mustard oil and formalin into TMJ (Imbe et al. 2001; Roveroni et al. 2001). Imbe et al. (2001) have demonstrated that injection of CFA into rat TMJ joint produced hyperalgesia. The behavioural thermal hyperalgesia was assessed by applying noxious heat stimuli over the orofacial area and head withdrawal latencies were measured. The head withdrawal latencies were significantly reduced at 24 hours post injection of CFA into the TMJ as compared to control. In the same study, von Frey hairs were used to determine mechanical allodynia, which was significantly decreased over 2 weeks after CFA injection. In another study injection of formalin into TMJ of rats significantly increased head flinching and face rubbing response in comparison to control. The flinching and rubbing are considered as behavioural measure of pain, which suggests that formalin injection into TMJ is painful (Roveroni et al. 2001). Furthermore, injection of 2.5% mustard oil into TMJ induced significant behavioural nociception, which was assessed by head flinching and face rubbing (Bonjardim et al. 2009). More recently, a behavioural rat model of TMJ pain and inflammation was developed by injecting CFA into TMJ and mechanical sensitivity to CFA induced inflammation in the presence and absence of NMDA receptor antagonists APV or ifenprodil was determined (Ivanusic et al. 2011). The mechanical sensitivity was determined by measuring the head withdrawal response on monofilament stimulation of TMJ. Injection of CFA significantly lowered the head withdrawal threshold from the baseline. Co-injection of CFA and APV or ifenprodil in TMJ significantly attenuated the mechanical hypersensitivity caused by CFA, which suggests the role of peripheral NMDA receptors in inflammatory TMJ pain (Ivanusic et al. 2011). Moreover, injection of CFA into rat TMJ joint produced behavioural pain response which was assessed by a digital algometer. The CFA injection significantly increased
the expression of NR1 subunit of NMDA receptor in subnucleus caudalis (Wang et al. 2009) which further supports the role of NMDA receptors in inflammatory TMJ pain.

**Myofascial TMD Models**

As mentioned already, injection of glutamate into rat masseter muscle causes peripheral sensitization of masseter muscle nociceptors. This animal model has helped to generate important information on peripheral mechanisms related to myofascial TMD. Previous studies have shown that injection of glutamate into human masseter muscle produces muscle pain and decreases the pressure pain threshold (Cairns et al. 2001a; Svensson et al. 2003b). Moreover, the glutamate induced masseter muscle pain was significantly higher in women than men and spread to TMJ area (Cairns et al. 2001a; Svensson et al. 2003b). In addition, (Svensson et al. 2003b) have demonstrated that glutamate injection into human masseter muscle produces mechanical allodynia. On the other hand, intramuscular injection of NGF in rats and humans caused prolonged sensitization of masseter muscle. For example, injection of NGF into the masseter muscle of healthy male and female volunteers resulted in prolonged muscle sensitization and pain upon jaw movement such as chewing and yawning but did not produce spontaneous pain, and these effects lasted for several days (Svensson et al. 2003a; Svensson et al. 2008a). To sum up, these models have several characteristics of myofascial TMD patients which suggest that they may serve as models of TMD related muscle pain.

**Myofascial Trigger Points**

Myofascial TMD are characterized by masticatory muscle pain and localized tenderness, which may be characterized by some clinicians as “trigger points” (Fernandez-de-Las-Penas et al. 2010; Fricton 2007). Trigger points are tender hypersensitive nodules within taut bands of skeletal muscle, which are painful upon palpation and refer pain to other body parts (Fernandez-
de-Las-Penas et al. 2010; Lavelle et al. 2007; Schneider 1995; Yunus et al. 1988). Myofascial trigger points (MTrPs) are grouped into active MTrPs and latent MTrPs (Lucas et al. 2010; Shah and Gilliams 2008; Simons 2004). Active MTrPs are characterized by local spontaneous pain and refer pain to other parts of body (Lucas et al. 2010; Simons 2004). Latent MTrPs are painless, however, produce pain on palpation that reduces the muscle movement and causes muscle weakness (Alvarez and Rockwell 2002; Lucas et al. 2010; Shah and Gilliams 2008).

Although the pathophysiology of MTrPs is not completely known, it is believed that muscle injury or micro-injury leads to the development of trigger points (Alvarez and Rockwell 2002; Lavelle et al. 2007). Abnormal posture, overload of muscle and muscle fatigue leads to the development of MTrPs (Shah and Gilliams 2008). Muscle injury induces release of various nociceptive substances such as substance P, CGRP, IL, TNFα, serotonin, bradykinin, H+ and prostaglandins (Gerwin et al. 2004; Mense 2003). These algogenic substances sensitize and activate nociceptors to induce peripheral sensitization. Sensitized nociceptors have decreased thresholds to mechanical stimuli resulting in tenderness and pain on palpation (Gerwin et al. 2004; Mense 2003).

Patients suffering from myofascial TMD show no signs of gross pathological changes in their masticatory muscles and have MTrPs areas of the muscle which when palpated reproduce their muscle pain (Clark 2008; Fernandez-de-Las-Penas et al. 2010; Fricton 2007). Shah et al. (2005, 2008) have demonstrated that the levels of inflammatory compounds such as bradykinin, ATP, substance P, CGRP, H+, IL and importantly TNFα are elevated in MTrPs, which suggests some evidence of inflammation. The nociceptive substances such as glutamate, serotonin, substance P, ATP, prostaglandins and bradykinin are released during muscle injury (Forrester and Lind 1969; Fricton 2007; Kreiner and Galbo 2011). These nociceptive substances activate muscle Aδ and C–fibers that innervate masseter muscle and induce pain as well as mechanical sensitization of muscle nociceptors (Cairns et al. 2003b; Fricton 2007; Mense 1981; Sung et al.)
These findings indicate that peripheral sensitization of muscle nociceptors could at least play a part in the development of MTrPs.

The presence of TNFα at MTrPs supports the role of TNFα in muscle nociception such as myofascial TMD. However, the mechanism by which TNFα induces muscle pain has not yet been explored. Developing a model of MTrPs by injecting TNFα into masseter muscle could help to identify mechanism involved in MTrPs pain.

Pharmacotherapy of TMD Pain

Non steroidal anti-inflammatory drugs (NSAIDs), narcotics, sedatives, centrally acting muscle relaxants, tranquilizers, corticosteroids and antidepressants are the most commonly used medicines for the treatment of TMD pain (Dionne 1997; Fricton 2007; McNeill 1997; Scrivani et al. 2008). The combination of NSAIDs and muscle relaxants such as cyclobenzaprine are used as first line therapy for myofascial TMD pain (Hersh et al. 2008). NSAIDs produce their effect by inhibiting peripheral COX enzyme involved in the synthesis of prostaglandin such as PGE2 (Cashman 1996; Gordon et al. 2002; Gotzsche 2000). Long-term use of NSAIDs are associated with hypertension, kidney damage and gastrointestinal side effects (Dionne 1997; Hersh et al. 2008). However, prolonged use of selective COX-2 inhibitors such as Celecoxib and Rofecoxib are less associated with gastrointestinal side effects (Hersh et al. 2008). Singer and Dionne (1997) have shown that the combination of ibuprofen (NSAID) and diazepam (benzodiazepine) significantly reduced the myofascial TMD pain as compared to placebo. However, ibuprofen had no effect on myofascial TMD pain when used alone, which suggests that NSAIDs alone may not be sufficient to reduce TMD pain. Cyclobenzaprine is a centrally acting muscle relaxant and is most commonly used in the treatment of myofascial pain (Dionne 1997). However, according to Cochran review (Leite et al. 2009), there is no evidence that cyclobenzaprine is effective in the treatment of myofascial TMD pain.
Benzodiazepines are also commonly used to treat patients suffering from TMD pain (Dionne 1997; Fricton 2007; McNeill 1997). However, long-term use of benzodiazepines should be weighed carefully because of the potential risk of developing dependence, abuse and depression (Dionne 1997). A double blinded clinical study reported that 30 day administration of clonazepam in TMD patients was effective as compared to placebo. Nevertheless, it was associated with depression and liver dysfunction (Harkins et al. 1991). In another double-blinded study, diazepam significantly reduced the pain in TMD patients compared to placebo (Dionne 1997). Benzodiazepines act by binding to GABA$_{A}$ receptors in the CNS to increase the affinity of GABA binding to GABA$_{A}$ receptors, resulting in enhanced GABA mediated synaptic inhibition (Knabl et al. 2009). Binding of GABA to GABA$_{A}$ receptor opens the Cl$^{-}$ channel that causes hyperpolarization of neurons and subsequently decrease their excitability (Zeilhofer et al. 2009). Hyperpolarization and decreased neuronal activity induces muscle relaxation by inhibiting the polysynaptic reflex mechanism, which may contribute to muscle pain in TMD patients (Hersh et al. 2008).

Tricyclic antidepressants are also commonly used in the treatment of chronic pain (Denucci et al. 1996; Max et al. 1992; McNeill 1997; Scrivani et al. 2008). The analgesic activity of tricyclic antidepressant is independent of its antidepressant activity and this activity is produced at a lower dose than required in the treatment of depression (McQuay et al. 1992; Sharav et al. 1987). Non-selective antidepressants, which inhibit both serotonin and norepinephrine such as amitriptyline or doxipine are more effective than selective antidepressants that block only serotonin reuptake (Dionne 1997). Despite the widespread use of antidepressants in chronic pain, there is a lack of clinical evidence in support of antidepressants for the treatment of TMD pain (Dionne 1997). Previous studies suggested that tricyclic antidepressants produce their analgesic effect through multiple mechanisms. These mechanisms include activating endogenous opioids and potentiating opioid effects (Hamon et al. 1987) as well as central
inhibition of reuptake of nor-adrenaline and serotonin (Taiwo et al. 1985). In addition, there is evidence that tricyclic antidepressants also inhibit NMDA receptors (Reynolds and Miller 1988; Sawynok 2003) and block sodium channels (Dick et al. 2007). These are the additional mechanisms that might underlie their analgesic effects. Other drugs that are also used in the treatment of TMD and orofacial pain are gabapentin, corticosteroids, opioids and carbamazepine (Brazeau et al. 1998; Kapur et al. 2003). Gabapentin, is an antiepileptic drug used for the treatment of neuropathic pain. Few studies have shown the role played by gabapentin in myogenous pain. For example, gabapentin significantly reduced the taxane- induced myalgia and arthralgia (Nguyen and Lawrence 2004). Moreover, in a single randomized clinical trial, there was a significant decrease in muscle pain and number of muscle tender spots in myofascial TMD patients who received gabapentin compared to placebo group. The effect of gabapentin became significant at week 8, but was not sustain through 12 weeks of treatment (Kimos et al. 2007), which suggests that the analgesic effect of gabapentin may be short lasting. Segerdahl (2006) have shown that gabapentin was not able to reduce the HS induced muscle pain in healthy human volunteers, which further suggests that gabapentin is not an effective analgesic for acute muscle pain. Taken together, these findings indicate that most of the current drugs used in the treatment of TMD pain lack evidence for effectiveness and are associated with serious side effects. Further research is needed to understand the basic pain mechanisms of myofascial TMD in order to develop new drugs, which could be more effective and with less side effects.
**Experimental Hypothesis**

Intramuscular injection of TNFα into rat masseter muscle will cause a decrease in mechanical threshold of masseter muscle nociceptors without causing inflammation.

**The Objectives of This Study Were:**

1. To determine the effect of injection of TNFα into rat masseter muscle on the mechanical threshold and excitability of masseter muscle nociceptors.

2. To determine the expression of P55 and P75 receptors by masseter ganglion neurons in rats.

3. To determine whether P55 and/or P75 receptor activation are responsible for the effect of TNFα injection into masseter muscle.

4. To determine whether injection of TNFα into masseter muscle causes the release of PGE2, NGF, and glutamate, which then contribute to the nociceptor sensitization.
Chapter 2. TNFα Mechanically Sensitizes Masseter Muscle Afferent Fibers of Male Rats

Introduction

TNFα is a pro-inflammatory cytokine released from different types of cells such as macrophages, monocytes, lymphocytes, neutrophils and fibroblasts (Baud and Karin 2001). Schwann cells in the peripheral nervous system also release TNFα upon injury (Wagner and Myers 1996). Although TNFα produces its biological effects by binding two receptors, the TNFR1 or P55 receptor, and the TNFR2 or P75 receptor (Vandenabeele et al. 1995), previous animal studies have suggested that P55 receptor activation was responsible for TNFα-induced cutaneous hyperalgesia (Cunha et al. 2005; Jin and Gereau 2006; Parada et al. 2003; Sommer et al. 1998b). TNFα plays an important role in inflammatory processes and for example, synovial fluid TNFα levels are elevated in patients suffering from various arthritic diseases such as rheumatoid arthritis and ankylosing spondylitis (Chu et al. 1991; Feldmann et al. 1996; Francois et al. 2006; Lange et al. 2000; Nordahl et al. 2000). Use of anti-TNFα treatment reduces joint pain in these conditions and is considered one of the major breakthroughs in the management of pain and inflammation (Boettger et al. 2008; Kopp et al. 2005; Lipsky et al. 2000; Moen et al. 2005).

In contrast, a number of chronic muscle pain conditions, such as fibromyalgia and myofascial TMD, are not associated with clinical signs and symptoms of tissue inflammation and thus it is unclear what role, if any, TNFα might play in these conditions. However, serum TNFα levels are elevated in patients suffering from fibromyalgia (Wang et al. 2008). Previous animal studies in the rat have shown that subcutaneous and intraplantar injection of TNFα caused sensitization and inflammation (Cunha et al. 1992; Junger and Sorkin 2000; Woolf et al. 1997). Moreover, subcutaneous injection of TNFα evoked activity in 14% of putative...
nociceptive C-fibers, but did not evoke afferent fiber discharge in mechanosensitive Aβ fibers (Junger and Sorkin 2000), which suggests that TNFα may be selective for slowly-conducting cutaneous afferent fibers with nociceptive properties. In a subsequent behavioural study, intramuscular injection of TNFα into the gastrocnemius muscle was also reported to induce mechanical sensitization without gross inflammation, although it did increase levels of pro-inflammatory mediators, such as PGE2, CGRP and NGF (Schafers et al. 2003d). Despite the elevation of these substances after intramuscular injection of TNFα, a previous study reported that TNFα had little effect on the excitability or mechanical sensitivity of C-fibers that innervate the gastrocnemius muscle of the rat (Hoheisel et al. 2005). The reason for these apparent contradictory findings is uncertain, however, these results suggest that the role TNFα plays in non-inflammatory myofascial pain mechanisms remains to be determined.

The purpose of this study was to elucidate the peripheral effect(s) of TNFα on a different muscle often associated with chronic muscle pain conditions, the masseter (jaw closer) muscle. To do this, the activity of individual masseter muscle afferent fibers was recorded in-vivo in the rat and the effect of intramuscular injection of TNFα on the excitability and MT of these afferent fibers was monitored. It was hypothesized that intramuscular injection of TNFα into rat masseter muscle would cause a decrease in the MT of masseter muscle afferent fibers without gross inflammation and that this effect would be mediated, at least in part, through activation of P55 receptors. To determine whether TNFα could exert direct effects on masseter muscle afferent fibers, additional immunohistochemical and Western blot experiments were undertaken to determine the extent of TNFα receptor expression by masseter muscle afferent fibers and in the masseter muscle, respectively.
Materials and Methods

Animals

A total of 80 male adult Sprague-Dawley rats (260-380g) were used in this study. All experiments were done in accordance with the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

Immunohistochemistry

Immunohistochemistry was performed in 5 male rats to determine the expression of P75 and P55 receptors on trigeminal ganglion neurons that innervate the masseter muscle. Fast blue (2%, Polyscience, USA), which is a fluorescent dye, was injected bilaterally into the masseter muscles of five male rats to identify masseter ganglion neurons. After seven days, rats were euthanized and perfused with heparinised saline followed by 4% paraformaldehyde. The right and left trigeminal ganglia were removed and each ganglion cut into 6-8 sections of 40 µm thickness with a vibratome and processed for indirect immunofluorescence immunohistochemistry as described earlier (Dong et al. 2007; Sung et al. 2008). Briefly, sections were incubated with 5% normal goat serum in phosphate buffered saline (PBS) for 1 hour and then incubated overnight with anti-rat P55 receptor antibody (1:1000; Rabbit polyclonal, Abcam, USA) or anti-rat P75 receptor antibody (1:20000; Rabbit polyclonal, Sigma-Aldrich, USA). The next morning, sections were extensively washed with PBS and then incubated with CY3 conjugated IgG (Anti-rabbit; Jackson Immunoresearch, USA) for one hour at room temperature in the dark. After several washings with PBS, sections were mounted on slides with cover slips and visualized with a Leica DM L fluorescent microscope. All sections were examined and all fast blue positive cells counted. WCIF Image J software program (National Institutes of Health Image Image, USA) was used to calculate the area of each fast blue positive cell including nucleus. In control experiments, the tissue sections were incubated without
primary antibody or with a combination of primary antibody and P55 receptor peptide (Abcam; preabsorption) to confirm specificity of receptor-like immunoreactivity, respectively.

**Electrophysiology**

Adult male Sprague Dawley rats (260-380g, n=70) were used for acute *in-vivo* extracellular recording of masseter muscle afferent fiber activity as has been previously reported in detail (Cairns et al. 2002; Dong et al. 2007; Mann et al. 2006). Rats were anesthetized with isoflurane (2-2.5%). The carotid artery was cannulated to monitor blood pressure and to administer Evans blue dye and pentobarbital (100 mg/kg) at the end of experiment to test for gross inflammation and to sacrifice the rat, respectively. Rat core body temperature, expired CO₂, heart rate and blood pressure were monitored throughout the experiment and were kept within physiological range of 36.8-37.1 °C, 20-50 mm Hg, 300-400 beats/min and 60-80 mm Hg, respectively. Depth of isoflurane anesthesia was assessed by periodically checking for the absence of a leg withdrawal reflex in response to strong pressure applied to the toes with a pair of small forceps. If this nociceptive reflex response was present, or if the mean blood pressure and or heart rate exceeded 90mm Hg or 430/min, respectively, the concentration of inhaled isoflurane was increased until the reflex disappeared and the other parameters return to their desired ranges (see preceding text). In this manner, adequate depth of anesthesia was be maintained.

A parylene-coated tungsten-recording electrode was introduced into the trigeminal ganglion to record from individual masseter afferent fibers. Masseter afferent fibers were identified by their response to blunt probing of the masseter muscle (Cairns et al. 2002; Cairns et al. 2001b). When an afferent fiber was found that appeared to respond to mechanical stimulation of the muscle, the skin overlying the muscle was pulled and pinch, and pressure stimuli were applied to the skin to check that the mechanoreceptive field of the afferent fiber was not in the
skin (Cairns et al. 2002; Cairns et al. 2001b). To confirm the projection of masseter muscle afferent fibers to the caudal brainstem, the antidromic collision technique was used (Cairns et al. 2002; Cairns et al. 2001b). Constant current electrical stimuli applied via the stimulating electrode lowered into the caudal brainstem were used to produce antidromic action potentials. Orthodromic action potentials were evoked by mechanically stimulating the masseter muscle fibers with a probe. A projection to the caudal brainstem was confirmed by a collision between the orthodromic and antidromic action potentials. At the end of the experiment, the estimated conduction velocity of each fiber was calculated by dividing the distance between stimulating and recording electrodes by the latency of the antidromic action potential. As a final confirmation that the afferent fiber was indeed in the muscle, HS (1M) was injected into the mechanoreceptive field of each fiber at the end of experiment.

**TNFα–Induced Mechanical Sensitization**

Experiments were carried out to determine the effect of intramuscular injection of two different doses (0.1µg and 1µg) of TNFα on masseter muscle afferent fiber excitability (ability of afferent fibers to respond to stimuli) and mechanical sensitivity. Before the injection of any substance, baseline afferent fiber MT was measured with an electronic von Frey Hair at one-minute intervals for 10 minutes (Cairns et al. 2007; Cairns et al. 2002; Mann et al. 2006). The baseline MT (MT was defined as the minimum force required to evoke a single action potential) was determined by averaging ten consecutive mechanical stimuli. Each afferent fiber identified was randomly assigned to injection of one of the following groups: vehicle control (10µL PBS, n = 10), 1µg TNFα (Sigma, USA; 1µg in 10µl PBS, n = 10) and 0.1µg TNFα (Sigma, USA; 0.1µg in 10µl PBS, n = 10). Only one afferent fiber was recorded per rat. The investigator (AH) was blinded to the content of injections. The dose of TNFα was the same as in a previous rat
behavioral study (Schafers et al. 2003d). Solutions were injected intramuscularly with a 26 gauge needle connected by polyethylene tubing to a 25µl Hamilton syringe. The needle was inserted into mechanoreceptive field of the masseter muscle afferent fiber, and then baseline afferent fiber activity was recorded for 10 minutes prior to the first injection to record any spontaneous fiber activity before the injection. At the end of the 10-minute baseline recording, a 10µl of solution was injected into masseter muscle and evoked activity was monitored for 10 minutes. MT was recorded every hour for 10 minutes for a total of 3 hours after substance injection. At the end of MT recording, HS was injected into the mechanoreceptive field of the masseter afferent fiber as positive control. Afferent discharge in response to injection of HS confirmed that the afferent fiber being recorded innervated the masseter muscle and could respond to a noxious stimulus, thus identifying it as a putative masseter nociceptor. Finally, to assess whether injection of TNFα resulted in gross inflammation of the masseter muscle, Evans blue dye (6 mg/kg) was injected via carotid artery (Mann et al. 2006). Approximately 15 minutes after Evans blue dye injection, the rat was perfused and the part of the masseter muscle around the site of injection was removed for Evans blue dye analysis.
Figure 2.1. Illustration of TNFα experimental protocol mentioned in the methods section. The arrows represent the events labeled and the bracket represents mechanical threshold recordings conducted every hour for 3 hours.
**Formalin Injection**

Formalin (37%, 10 µl) was injected into the masseter muscle as positive control. Three hours after injection, the Evans blue dye method as described in the previous section was used to assess plasma protein extravasation.

**Evans Blue Dye Analysis**

After removal, muscle tissues were weighed and placed in test tubes containing 2 ml of formamide. Test tubes were incubated at 60 °C for 24 hours. After 24 hours, the supernatant was collected and the amount of dye extracted from the muscle was determined by measuring the absorbance of supernatant at 620 nm using a spectrophotometer (Fiorentino et al. 1999). The concentration of dye was calculated from per gram weight of muscle tissue using a concentration versus absorbance standard curve for Evans blue dye (Fig 2.6A).

**Muscle Blood Flow Change Experiments**

An infra-red camera (ThermaCAM EX320, FLIR Systems AB, Danderyd, Sweden) was used to measure the change in local surface temperature of the rat temporalis muscle as an indirect measure of change in muscle blood flow (Gazerani et al. 2010a; Hassan et al. 2004). TNFα was injected into the temporalis muscle instead of masseter muscle, as it was impossible to focus the camera on the masseter muscle using our experimental setup. The camera was focused towards the injection site, which was marked with the silver pen. Baseline temperature of the muscle was recorded before injection of TNFα or vehicle control by taking the pictures of muscle every minute for 10 minutes. After injection of TNFα (1µg; 10µl) or vehicle control (PBS; 10µl) temperature was recorded hourly for 10 minutes for a total of 3 hours. Pictures were analysed by using ThermaCAM software.
**Receptor Pharmacology**

Additional experiments were carried out to determine the effect of P55 and P75 receptor antagonism on the effect of TNFα. In these experiments, 1µg of P55 receptor antibody (Abcam, USA) was injected alone (control) or co-injected with 1µg TNFα into the mechanoreceptive field of the masseter afferent fiber. Similarly, 1.2 µg of P75 receptor antibody (Sigma-Aldrich, USA) was injected alone or co-injected with 1µg TNFα. The investigator (AH) was blinded to the content of injections. Baseline and post injection MTs were recorded as described above in the TNFα experiments. The dose of antibodies was determined *in-vitro* by using the Western blot technique to determine antibody-binding saturation in the masseter muscle.

**Western Blot Analysis**

Western blot experiments were carried out as previously described (Billova et al. 2007). Briefly, rat masseter muscles were taken and weighed. Muscles were homogenized on ice using lysis buffer. Homogenised muscle was centrifuged at 14000 rpm for 30 min at 4 °C, the supernatant was collected and its total protein content was determined using the Bradford method (Bradford 1976). Protein samples (20µg) were separated by electrophoresis using 7% SDS polyacrylamide gel and were transferred out to 0.2µm nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk and was cut and treated with different concentrations of P55 receptor antibody or P75 receptor antibody at 4 °C overnight. The next morning membranes were washed and treated with the secondary antibody (Goat anti-rabbit peroxide conjugated; Jackson Immunoresearch,USA) for 1 hour at room temperature. After washing, the bands were detected with ECL western blotting detection chemiluminescence reagent (Amersham™; GE Health Care,UK) and analysed by Alpha Innotech Imager FluorChem.
Data Analysis

Immunohistochemistry

Fast blue positive masseter ganglion neurons were examined under the Leica DML fluorescent microscope. P55 and P75 receptor-like immunoreactivity was examined in fast blue positive cells. The WCIF Image J software program (NIH Image, USA) was used to measure the area of the fast blue positive cells. The percentage of P55 and P75 labeled masseter ganglion neurons was calculated.

Electrophysiological Experiments

The average of ten afferent fiber MTs at each time point was calculated. Relative MTs were then calculated by dividing mean MT at each time point by the mean baseline threshold. Cumulative discharge was defined as the difference between the number of action potentials recorded before and after injection of substances into the masseter muscle. Cumulative discharge was calculated by subtracting the number of action potentials recorded for 10 minutes before injection (baseline) from the number recorded for 10 minutes after injection.

Statistics

Significant differences in the mean expression of P75 and P55 receptors by masseter ganglion neurons in the 5 male rats were assessed with a paired t-test. The distribution of expression frequencies amongst the various sizes of masseter ganglion neurons for P75 and P55 receptors was compared with a Chi-square test.
A sample size estimate employing analysis of variance suggested that samples of 10 afferent fibers per group would permit detection of a 50% difference in afferent fiber MT between the TNFα and control groups with α=0.05 and a power of 0.80. A 2-way, repeated measures ANOVA test was used to determine the effect of all treatments (vehicle, TNFα (1µg, 0.1µg), TNFα (1µg) coinjected with P55 receptor antibody, TNFα (1µg) coinjected with P75 receptor antibody or P55 or P75 receptor antibody alone) and time (repeated) on relative MTs. The data was normally distributed and a logarithmic transformation of the MT data was performed to produce equal variance prior to analysis with ANOVA. Post-hoc comparisons were undertaken with the Holm-Sidak test. Since the afferent discharge data was not normally distributed, Kruskal Wallis ANOVA on ranks was employed to determine whether there were significant differences in the median afferent discharge evoked by vehicle control, TNFα (1µg,0.1µg), TNFα (1µg) coinjected with either P55 or P75 receptor antibody, or P55 or P75 receptor antibody alone (control). Alpha < 0.05 was considered significant.
Results

Immunohistochemistry

Masseter muscle ganglion neurons were examined for expression of P55 (n=688 cells) and P75 (n=503 cells) receptor-like immunoreactivity in five male rats (Figure 2.2A). The mean percentage expression of P75 receptors was significantly higher (62 ± 1%) than P55 receptors (29 ± 2%) in masseter ganglion neurons. Receptor-like immunoreactivity was uniformly distributed amongst small, medium and large masseter muscle ganglion neurons without any discernable differences in fluorescence intensity in both P75 and P55 receptors (Figure 2.2B). There was no labelling in the preabsorption studies (P55 receptor antibody) or in the absence of the primary antibody. Masseter muscle ganglion neurons were characterized as small- (<600 μm²), medium- (600-1200 μm²) and large- (>1200 μm²) size neurons according to their area (Fukuoka et al. 2002; Schafers et al. 2003a).
Figure 2.2. (A) The arrows on the photographs indicate examples of masseter muscle ganglion neurons positive for Fast blue (left) and P55 or P75 receptor-like immunoreactivity (right). (B) The graph indicates the frequency (%) of P75 and P55 receptor labeling in masseter ganglion neurons of different sizes. Although the distribution of P55 or P75 receptor-like immunoreactivity was similar in small, medium and large neurons, twice as many masseter ganglion neurons expressed the P75 receptor as expressed the P55 receptor. (n=5 rats; P >0.05, Chi-square test).
Afferent Fiber Properties

Single unit recordings from 70 masseter muscle afferent fibers (n=70 rats) that projected to the caudal brainstem were made (Figure 2.3A). The conduction velocity (CV) of 66 of these afferent fibers was in the Aδ (CV = 2-12 m/s) range, 1 in the Aβ (CV > 12 m/s) range and 3 in the C-fiber (CV < 2 m/s) range. The percentage of C-fiber mechanoreceptors recorded (4%), though relatively low, is consistent with previous studies (Cairns et al. 2003b). An example of MT determination in an individual masseter afferent fiber is shown in Figure 2.3B. The mean baseline MT of these fibers was 35±3 g. HS was used as positive control to confirm that the mechanoreceptive field of each afferent fiber was in masseter muscle (Figure 2.4). HS evoked afferent discharge from all the fibers included in the study. The combination of slow conduction velocity, relatively high MT and response to a noxious chemical stimulus indicate that all recorded afferent fibers could be classified as putative muscle nociceptors.
Figure 2.3. (A) An example of a collision between an orthodromic and antidromic action potential is shown. The collision (*) indicated that this masseter afferent fiber (CV=8.5 m/s) projected to the caudal brainstem. (B). The tracings illustrate examples of afferent fiber (CV = 4.3 m/s) mechanical threshold (MT) assessment before (baseline) and at 3 h after the injection of 1 µg tumor necrosis factor alpha (TNFα) into the masseter muscle. The mean MT of this afferent fiber was 34 ± 5 before injection of TNFα, and 8.8 ± 1 g 3 h later.
Figure 2.4. The histogram shows discharge evoked by injection of hypertonic saline (HS) into the mechanoreceptive field of the masseter muscle afferent fiber in figure (2.2 B). Responses to HS injection indicated that the recorded afferent fibers were putative nociceptors.
Effect of TNFα on Evoked Activity and MT

The median [interquartile range] cumulative discharge evoked by injection of TNFα (1µg: 0-1.5 spikes, 0.1µg: 0-0.75 spikes) was not significantly different from the control (0 [0-3.5] spikes). Mean baseline MT was 24±5 g, 26±5 g, and 19±6 g, respectively for the 1µg TNFα, 0.1 µg TNFα and control group. Two-way repeated measures ANOVA on all the MT data indicated a significant effect of treatment and time and a significant interaction between time and treatment. Post-hoc evaluation revealed that there was a significantly greater effect of treatment with 1µg TNFα than vehicle on MT, and that a significant difference between the treatment and control group occurred at 180 minutes post-injection (Figure 2.5B). These results indicate that there was a time-related mechanical sensitization of masseter nociceptors that occurred independent of treatment, but that treatment with TNFα produced a significantly greater mechanical sensitization than vehicle or 0.1µg TNFα at 180 minutes post-injection.
Figure 2.5. (A) The bar graph shows median evoked activity produced by 0.1 µg or 1 µg TNFα, vehicle control, co-injection of 1 µg TNFα and P55 or P75 receptor antibody and P55 or P75 antibody alone (control). 0.1 µg or 1 µg TNFα did not evoke significantly more activity than vehicle control. Similarly, co-injection of 1 µg TNFα and P55 receptor antibody or P75 receptor antibody did not evoke significantly more activity than P55 receptor antibody or P75 receptor antibody alone (control). The error bars indicate the interquartile range (n=10; P > 0.05, Kruskal Wallis ANOVA on ranks) (B). The line and scatter plot illustrates the effect of intramuscular injection of TNFα (0.1 µg, 1 µg) on the mean relative MT of rat masseter muscle nociceptors at various time points. Overall, the MT of nociceptors was significantly decreased by 1 µg TNFα but not by 0.1 µg TNFα or vehicle control (n=10; P < 0.05, two-way repeated measures ANOVA). The error bars indicate standard error of mean. *P < 0.05, Holm Sidak test 1 µg TNFα versus vehicle.
Evans Blue Dye Analysis

Evans blue dye was used to assess the extent of plasma protein extravasation into the masseter muscle 3 hours after TNFα (0.1 or 1µg) or vehicle control injections. There was no statistically significant difference between the amount of Evans blue dye in the masseter muscle when the vehicle control and TNFα (0.1 or 1µg) group were compared (Figure 2.6B). In contrast, the amount of dye absorbed in the masseter muscle 3 hours after 37% formalin injection was 22±1µg/g. These results indicate that TNFα did not cause greater inflammation than vehicle control when injected into the masseter muscle. In the present study, a higher concentration of formalin was used as a positive control, because a previous animal study showed that injection of formalin (2.5%) into the rat masseter muscle did not produce any behavioral evidence of nociception (Roveroni et al. 2001).
Figure 2.6. (A) This graph shows the standard curve used to calculate the concentration of Evans blue dye in muscle extract (B). The bar graph shows Evans blue dye accumulation in the masseter muscle 3 hours after injection of TNFα (0.1µg, 1µg) or vehicle control (n=6 rats per group). The concentration of Evans blue dye in the TNFα groups was not significantly different from the concentration of Evans blue dye in the control group (P >0.05, one way ANOVA). As a positive control, 37% formalin was also injected into the masseter muscle (n=3 rats) and Evans blue dye accumulation assessed after 3 hours. A substantially greater concentration of Evans blue dye after formalin was found. The error bars represent the standard error of mean.
**Muscle Blood Flow Analysis**

Muscle temperature experiments were done to determine whether TNFα alter the blood flow at three hours after its injection. The change in muscle temperature after injection of TNFα at three hours was not different from the vehicle control (Figure 2.7B). A previous study done in our lab has shown that glutamate, which increases masseter muscle blood flow (Cairns et al. 2003a), produced significant increase in surface muscle temperature (Fig 2.7C). This result suggests that the thermal camera used in our study is sensitive to temperature change and that temperature changes can be used as indirect measure of increase in blood flow which would occur during inflammation. Overall, these results suggest that TNFα– induced sensitization of masseter muscle nociceptor is not associated with vasodilation. These findings are consistent with Evans blue dye analysis data that indicated that TNFα did not induce gross inflammation of the masseter muscle.
Figure 2.7 (A). An example of a thermal camera image of the temporalis muscle is shown. (B). The bar chart shows the relative (%) change in temperature after injection of PBS or TNFα at various time points. Injection of TNFα did not change the temperature in the temporalis muscle.
Figure 2.7(C). Intramuscular injection of glutamate increases masticatory muscle blood flow (Cairns et al. 2003a). The graph above constructed from data collected in our laboratory (Gazerani et al. 2010a), shows that injection of glutamate into masticatory muscle also significantly increases muscle surface temperature. This control data shows that temperature changes can be used as an indirect measure of increased muscle blood flow. Interestingly, injection of botulinum neurotoxin A (BoNTA), inhibits glutamate-induced changes in muscle surface temperature, which indicates that they are not produced by a direct effect of glutamate on blood vessels.
Determination of P55 and P75 Receptor Antibody Concentration

To determine an appropriate concentration of P75 and P55 receptor antibodies with which to test the involvement of these receptors in TNFα-induced mechanical sensitization. Western blot experiments were undertaken to assess the concentration at which the antibody binding was saturated in masseter muscle homogenate. Bands were detected at 55 kDa and 75 kDa, which correspond to P55 receptor and P75 receptors, respectively (Figure 2.8A,2.9A). It was found that at concentrations 1µg/ml (Houzen et al. 1997) and 1.2µg/ml, the binding of the P55 receptor (Figure 2.8B,2.9B) and P75 receptor antibodies, respectively, to the masseter muscle homogenate were effectively saturated.

Determination of P55 and P75 Receptor Expression on Masseter Muscle

Western blot analyses were also performed to determine the expression of P75 and P55 receptors in the masseter muscle. The antibodies used against P55 and P75 receptor interacted with the muscle homogenate proteins of 55 kDa and 75 kDa, respectively. The presence of bands at 55 kDa and 75 kDa confirms the expression of P55 and P75 receptors in the masseter muscle tissue (Figure 2.8A, 2.9A). Multiple bands were observed in P55 and P75 receptor antibody Western blot experiments which suggest that these antibodies were not absolutely selective to their receptors.
Figure 2.8. (A) The Western blot shows the example of labeling of P55 receptor with P55 receptor antibody at 55 kDa (1 µg/ml) in rat masseter muscle. (B) The graph illustrates the saturation of P55 receptor binding to masseter muscle homogenate by increasing concentrations of P55 receptor antibody. P55 receptor antibody binding to the muscle homogenate appeared to be saturated at concentration of 1 µg/ml.
Figure 2.9. (A) The Western blot shows an example of labeling of P75 receptor with P75 receptor antibody (1.2 µg/ml) in muscle homogenate. B) The graph illustrates the saturation of P75 receptor binding to masseter muscle homogenate by increasing concentrations of P75 receptor antibody. P75 receptor antibody binding to the muscle homogenate appeared to be saturated at concentration of 1.2 µg/ml.
Effect of P55 and P75 Receptor Antibodies on Evoked Activity and TNFα-Induced Mechanical Sensitization

TNFα receptor antibodies were used to confirm the involvement of P55 receptors or P75 receptor in TNFα-induced mechanical sensitization of masseter muscle nociceptors. Co-injection of P55 receptor antibody and 1µg TNFα into the masseter muscle evoked median [Interquartile range] cumulative nociceptor discharge (0 [0-2.25] spikes; n=10) that was not significantly different from that evoked by P55 receptor antibody alone (control; 1[0-16.5] spikes, n = 10; P > 0.05). Similarly, co-injection of P75 receptor antibody and 1µg TNFα into the masseter muscle evoked median [Interquartile range] cumulative nociceptor discharge (0[0-104.5] spikes; n = 10) that was not significantly different from that evoked by P75 receptor antibody alone (control; 0[0-4.5] spikes, n = 10; P > 0.05). Mechanical sensitization induced by 1µg TNFα with P55 receptor antibody was not significantly different from vehicle control (P > 0.05) (Figure 2.10A). However, when 1µg TNFα with P75 receptor antibody was injected, relative MT was significantly increased compared to the vehicle control and 1µg TNFα alone (P< 0.05) (Figure 2.10B). These results indicate that both P55 and P75 receptors play a role in TNFα–induced mechanical sensitization of masseter muscle nociceptors.
Figure 2.10(A) The line and scatter plot illustrates the effect of 1µg TNFα, vehicle control (PBS), co-injection of 1µg TNFα and P55 receptor antibody on the masseter muscle nociceptors. P55 receptor antibody attenuated the development of TNFα-induced nociceptor mechanical sensitization. (B) The line and scatter plot illustrates the effect of 1µg TNFα, vehicle control (PBS), of co-injection of 1µg TNFα and P75 receptor antibody on the masseter muscle nociceptors. Co-injection of TNFα and P75 receptor antibody significantly increased the mechanical threshold of nociceptors compared with 1µg TNFα alone or vehicle control. The error bars represent the standard error of mean. (*P < 0.05 compared to vehicle control, two-way repeated measures ANOVA and Holm-Sidak test).
Discussion

Injection of TNFα into the masseter muscle did not excite nociceptors but 1µg TNFα did induce a delayed mechanical sensitization that was mediated through activation of both P55 and P75 receptors. Subsequent injection of the algogen HS into the masseter muscle evoked discharge in all nociceptors. Thus, TNFα appears to induce mechanical sensitization in masseter muscle nociceptors. Immunohistochemical and Western blot results confirmed that P55 and P75 receptors are expressed by masseter ganglion neurons and in the masseter muscle, respectively. Together, these results suggest that TNFα-induced changes in muscle sensitivity previously reported in behaving rats (Schafers et al. 2003d) occur, in part, due to mechanical sensitization of muscle nociceptors through activation of peripheral P55 and P75 receptors.

Injection of 1µg TNFα into the masseter muscle induced a slowly developing mechanical sensitization of nociceptors that became significant 3 hours post-TNFα injection. This finding is consistent with previous animal studies that have demonstrated that subcutaneous injection of TNFα produced behavioural evidence of mechanical and thermal hyperalgesia in the rat paw within a few hours (Cunha et al. 1992; Jin and Gereau 2006; Woolf et al. 1997). Subcutaneous injection of 5 ng TNFα has been shown to result in the mechanical sensitization of C-fibers as well as to induce cutaneous inflammation, which suggests that part of the sensitizing effect of TNFα in the skin could be due to the release of other inflammatory mediators (Junger and Sorkin 2000) Behavioural studies in rats also indicated that intramuscular injection of 10µg TNFα could induce a prolonged mechanical sensitization of the gastrocnemius muscle (Schafers et al. 2003d). However, intramuscular injection of 0.25µg TNFα was not demonstrated to increase the response of gastrocnemius muscle C-fibers to mechanical stimulation (Hoheisel et al. 2005). This apparent discrepancy between the results obtained in behavioural and afferent fiber recording studies is likely due to the substantially smaller dose of TNFα employed in the
C-fiber recording experiments and to the fact that afferent fiber mechanical response was only assessed for 10 minutes after the injection of TNFα into the gastrocnemius muscle (Hoheisel et al. 2005). In the current study, it was found that several hours were required for significant mechanical sensitization of masseter nociceptors to become apparent.

The findings of the present study demonstrate the expression of both P55 and P75 receptors on masseter muscle ganglion neurons and within the masseter muscle. Previous studies have shown the expression of both P55 and P75 receptors in the skeletal muscle (Li and Reid 2001), on Schwann cells (Qin et al. 2008), dorsal root ganglion neurons (Hensellek et al. 2007, Shubayev and Myers 2001) and the P55 receptor in the trigeminal mesencephalic nucleus (Bette et al. 2003). Taken together, these findings suggest that TNFα could potentially induce some of its effects through a direct action on masseter nociceptors. Animal studies have shown a role of P55 receptors in TNFα-induced cutaneous hyperalgesia (Cunha et al. 2005; Jin and Gereau 2006; Parada et al. 2003; Sommer et al. 1998b). However, recent studies have shown the involvement of P75 receptors in tumor-induced heat hyperalgesia by TNFα in dorsal root ganglion neurons (Constantin et al. 2008) and in the maintenance of neuropathic pain (Schafers et al. 2008). While activation of either P55 or P75 receptors appears sufficient to induce mechanical sensitization of masseter muscle nociceptors, the expression of P55 receptors was only observed in about one third of masseter ganglion neurons. In contrast, the expression of P75 receptors was much higher (62%). This higher expression level of P75 receptors by masseter muscle ganglion neurons suggests that any direct action of TNFα on masseter muscle nociceptors to induced mechanical sensitization was more likely due to activation of P75 receptors.

The low level of P55 receptor expression by masseter ganglion neurons and presence of both receptors in masseter muscle tissue suggests that TNFα could also operate indirectly
through activation of non-neuronal P55 and/or P75 receptors to induce the release of other sensitizing mediators (Schafers et al. 2003d; Woolf et al. 1997). This concept is supported by the finding that in the current as well as a previous study (Schafers et al. 2003d), TNFα-induced mechanical sensitization of skeletal muscle required several hours to develop. The expression of TNFα receptors by non-neuronal cells such as muscle cells (Li and Reid 2001) and previous findings that TNFα induced the release of various sensitizing substances such as PGE2, NGF and CGRP (Schafers et al. 2003d; Woolf et al. 1997) further support the hypothesis that TNFα could act indirectly to mechanically sensitize masseter nociceptors. For example, recent evidence indicates that TNFα indirectly suppresses potassium currents to increase the excitability of rat dorsal root ganglion neurons through an increase in PGE2 (Liu et al. 2008a). Intramuscular injection PGE2 has been shown to enhance the response of cat gastrocnemius muscle C-fibers to the action of bradykinin (Mense 1981) and to mechanically sensitize rat cutaneous nociceptors (Chen et al. 1999) and masseter muscle nociceptors (Dong et al. 2009). PGE2-induced sensitization is mediated in part through EP2 and EP3 receptors that have recently been shown to be expressed by trigeminal nociceptors (Patwardhan et al. 2008), which suggests that TNFα-induced release of PGE2 could have sensitized masseter nociceptors to mechanical stimulation in the present study. Intradermal and intramuscular injection of TNFα also elevates tissue concentrations of NGF, however, in one study injection of rat NGF into rat masseter muscle did not appear to induce significant afferent fiber mechanical sensitization (Mann et al. 2006). On the other hand, injection of human NGF has been shown to mediate a prolonged mechanical sensitization of the human masseter muscle and appears to also mechanically sensitize rat masseter muscle nociceptors (Svensson et al. 2003a; Svensson et al. 2008b; Svensson et al. 2010). In addition, TNFα modulates the release of glutamate in the central nervous system through activation of P55 receptors (Hermann et al. 2005; Youn et al. 2008). Increased
interstitial concentrations of glutamate in the masseter muscle have been shown to induce a long lasting mechanical sensitization of masseter muscle nociceptors through activation of peripheral NMDA receptors (Cairns et al. 2007; Cairns et al. 2002; Mann et al. 2006). Finally, although TNFα stimulates the synthesis and release of CGRP in the trigeminal ganglion through activation of P55 receptors (Bowen et al. 2006), there is only indirect evidence for a role of CGRP in mechanical sensitization of the masseter muscle nociceptors at present (Ambalavanar et al. 2006). The aforementioned studies support the concept that TNFα may act indirectly to induce mechanical sensitization through a P55 and/or P75 receptor-mediated increase of various sensitizing substances in the masseter muscle.

It was observed that injection of vehicle control also caused a decrease in the MT of masseter muscle nociceptors over time in the present study, although this effect was significantly less pronounced than the effect of TNFα injections. Similar results have been found in some (Sung et al. 2008), but not all studies examining the MT of masseter afferent fibers over prolonged time periods (Cairns et al. 2002; Mann et al. 2006). It is possible that insertion of the catheter needle used to inject substances into the masseter muscle results in a variable degree of tissue injury that leads to afferent fiber mechanical sensitization in some cases. Indeed, TNFα is released after muscle injury (Chen et al. 2007b; Warren et al. 2002). We speculate that endogenous release of TNFα and other sensitizing substances from muscle cells upon insertion of the catheter needle may have contributed to the observation of a decrease in afferent MT post-vehicle injection.

Tissue accumulation of Evans blue dye is an indication of plasma protein extravasation (Fiorentino et al. 1999). In the present study, no difference in plasma protein extravasation between the TNFα and vehicle injected muscles was found, which suggests that intramuscular injection of TNFα does not induce plasma protein extravasation and thus is not grossly
inflammatory. However, while the amount of Evans blue dye found in the masseter muscle of the vehicle group was substantially lower than that produced in the present study by formalin, or in a previous study by mustard oil (Fiorentino et al. 1999), it was about twice as high as the concentration of Evans blue dye previously found in the masseter muscle after a similar vehicle injection (2.2µg/g) (Mann et al. 2006). The increased Evans blue tissue concentrations in the present study are consistent with our speculation of modest tissue trauma after vehicle injection.

The masseter muscles are a common site of pain in patients suffering from either fibromyalgia or myofascial TMD (Dworkin and LeResche 1992; Hedenberg-Magnusson et al. 1997; Leblebici et al. 2007). Plasma and serum levels of TNFα are elevated in certain fibromyalgia patients (Bazzichi et al. 2007; Wang et al. 2008) and there is some evidence that TNFα levels are also elevated at myofascial trigger points (Shah et al. 2008). Although the exact pathophysiology of trigger points remains speculative, increased mechanical sensitivity is a characteristic feature of these myofascial trigger points. The present study suggests that when TNFα levels in skeletal muscle are elevated, mechanical sensitization occurs. Together, these findings indicate a potential role for TNFα in the pathophysiology of fibromyalgia and myofascial TMD. Future experiments to study the effect of TNFα on human muscle pain will be required to further bolster this concept.
Chapter 3. TNFα Mechanically Sensitizes Masseter Muscle Nociceptors by Increasing Prostaglandin E2 Levels

Introduction

The mechanisms responsible for chronic muscle pain in conditions such as myofascial TMD and fibromyalgia are not known (Cairns 2010; Dworkin and LeResche 1992; Hedenberg-Magnusson et al. 1997; Leblebici et al. 2007). However, an association between elevated serum and plasma levels of TNFα, a pro-inflammatory cytokine, and chronic muscle and joint pain has been previously reported (Bazzichi et al. 2007; Nordahl et al. 2000; Shah et al. 2008; Wang et al. 2008). In addition, many patients with chronic muscle pain have so called myofascial “trigger points” which are areas of focal muscle hypertonicities that when palpated reproduce their muscle pain, and TNFα levels appear to be significantly elevated in these regions (Shah et al. 2008). It is thought that TNFα acts on P55 (TNFR1) and P75 (TNFR2) receptors to produce its pro-nociceptive effects in various tissues (Vandenabeele et al. 1995). Both P55 and P75 receptors are expressed by trigeminal ganglion neurons that innervate the masseter muscle as well as in the masseter muscle itself (Hakim et al. 2009). We have recently shown that intramuscular injection of TNFα causes a prolonged mechanical sensitization of masseter muscle nociceptors which takes 2-3 hours to manifest and is mediated via activation of peripheral P55 and P75 receptors (Hakim et al. 2009). However, it is not known whether some of the sensitizing effects of TNFα are mediated indirectly through the release of other nociceptive substances such as NGF, PGE2 and glutamate that have been shown to induce mechanical sensitization of muscle nociceptors (Cairns et al. 2002; Dong et al. 2009; Mense 1981; Murase et al. 2010; Svensson et al. 2010).

In other tissues, such as the skin, animal studies have shown that intraplantar injection of TNFα acts directly by exciting cutaneous afferent fibers (Junger and Sorkin 2000; Sorkin et al. 1997) as well as indirectly by inducing the release of other sensitizing substances (Cunha et al.
Behavioral studies have shown that intramuscular injection of TNF\(\alpha\) causes a delayed mechanical sensitization of skeletal muscle that appeared to be associated with elevated levels of PGE2, NGF, and neuro-peptides, although a cause-effect relationship was not demonstrated in these studies (Schafers et al. 2003d). In the central nervous system, TNF\(\alpha\) also induces release of glutamate through activation of P55 receptors (Hermann et al. 2005; Youn et al. 2008) and enhances AMPA and NMDA receptor currents as well as phosphorylation of the NR1 subunit of NMDA receptor (Kawasaki et al. 2008; Wei et al. 2008). A 2-3 times increase muscle interstitial glutamate concentration results in significant sensitization of masseter muscle nociceptors (Cairns et al. 2007). These results support the concept that TNF\(\alpha\) could be mediating sensitization of masseter muscle nociceptors by inducing release of other algogenic substances.

The purpose of this study was to further explore the mechanism of TNF\(\alpha\)-induced delayed mechanical sensitization of masseter muscle nociceptors. It was hypothesized that TNF\(\alpha\) acts indirectly by inducing the release of NGF, PGE2 and/or glutamate and that these nociceptive mediators are responsible for TNF\(\alpha\)-induced masseter muscle sensitization. To test this hypothesis, glutamate concentration was measured by using glutamate-selective biosensor probes, and NGF and PGE2 levels were measured by Enzyme-linked immunosorbent assay (ELISA) after injection of a mechanically sensitizing dose of TNF\(\alpha\) (1\(\mu\)g). Electrophysiology experiments were performed to assess whether these substances play a functional role in TNF\(\alpha\)-induced mechanical sensitization of muscle nociceptors. Diclofenac, a cycloxygenase inhibitor that blocks synthesis of PGE2 (Cashman 1996; Gotzsche 2000), DL-2-amino-5-phophonovaleric acid (APV), a competitive NMDA receptor antagonist, and a tyrosine kinase A (TrkA) receptor antibody, which blocks NGF-induced masseter muscle nociceptor sensitization, were injected 3
hours after intramuscular injection of TNFα to determine whether these antagonists could reverse TNFα-induced mechanical sensitization.
**Material and Methods**

**Animals**

A total of 70 adult male Sprague-Dawley rats (300-400 g) were used in this study. All experiments were done in accordance with the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

**Enzyme-linked Immunosorbent Assay**

These experiments were done to determine masseter muscle concentration of PGE2 and NGF. TNFα (1µg) or vehicle control (n=5 in each group) was injected into rat masseter muscle and after 3 hours the rat was terminated with a high dose of pentobarbital (120 mg/kg). Approximately 1 cm² of masseter muscle tissue was harvested from the injection site, which was marked on the overlying skin with a black marker. Muscle tissue was placed on dry ice and stored at –70 °C. Tissues were weighed and homogenised using homogenization buffer (50 mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton, 0.1% Sodium Dodecyl sulphate (SDS), 0.5% Sodium deoxycholate). The volume of the muscle harvested was estimated on the basis of average weight of muscles. The average weight of muscle tissue in TNFα and PBS group was 0.20±0.03g and 0.25±0.05g respectively. Samples were centrifuged at 4 °C for 15 minutes at 15000 rmp. The supernatant of the homogenate was collected and muscle protein concentration was determined using Bradford method (Bradford 1976).

**PGE2 Concentrations**

PGE2 level in muscle tissue homogenate was measured by enzyme immunoassay (Assay Design, Ann Arbor, USA) according to manufacturers’ instructions. The sensitivity of kit was 8.26 pg /ml. Samples and standards were run in duplicate and were averaged. The concentration of PGE2 was determined per gram of muscle.
**NGF Concentrations**

NGF level in the muscle homogenate was measured by ELISA (Promega, Madison, USA) according to manufacturers’ instructions. The minimum sensitivity of kit was 7.8 pg/ml. Both samples and standards were run in duplicate. The concentration of NGF was determined per mg of muscle protein.

**Glutamate Concentration**

Glutamate biosensor probes (Pinnacle Technology Inc., USA) were used to measure interstitial glutamate concentration in the masseter muscle (Cairns et al. 2007). Glutamate probes were calibrated *in-vitro* according to the manufacturers’ instructions. The glutamate biosensor probe was inserted through a guide cannula, which was affixed to a catheter needle (3 mm between probe and needle tip), into the masseter muscle of isoflurane anesthetized adult male Sprague Dawley rats (n=8). Blood pressure, core body temperature, heart rate were continuously monitored throughout the experiment. After a 60-90 minute stabilization period, the baseline glutamate concentration was measured over 10 minutes and subsequently TNFα or vehicle control (10µl, n=4) was injected into the masseter muscle near to glutamate biosensor probe. Masseter muscle glutamate concentration was measured each hour for 3 hours.

**Electrophysiological Recording of Muscle Nociceptors**

Adult male Sprague Dawley rats (300-400g, n =45) were used for acute *in-vivo* recording of masseter muscle afferent fibers (Cairns et al. 2002; Dong et al. 2007; Hakim et al. 2009). Isoflurane (2-2.5%) was used to anesthetize rats. A rectal thermometer was used to measure temperature. The trachea was cannulated and the rat was given artificial respiration using rodent ventilator. The carotid artery was cannulated to monitor blood pressure and to inject
pentobarbital (100 mg/kg) to terminate the rat at the end of the experiment. Rat core body
temperature, expired CO₂, heart rate, and blood pressure were monitored throughout the
experiment and were kept within the range of 36.8–37.1°C, 20–50 mmHg, 300–400 beat/min,
and 60–80 mmHg, respectively. The rat’s head was placed into a streotaxic frame. The skin,
muscle and dura overlying the caudal brainstem was reflected to allow stimulation of brainstem
with stimulating electrode. A parylene-coated tungsten-recording electrode was lowered into the
trigeminal ganglion to record action potentials from single masseter afferent fibers. A brush and
a probe were used to search for masseter muscle afferent fibers (Cairns et al. 2002; Cairns et al.
2001b). The skin overlying the masseter muscle was pulled and pinch and pressure was applied
to confirm the afferent fiber did not innervate the skin (Cairns et al. 2002; Cairns et al. 2001b).
Masseter afferent fibers projecting to caudal brainstem were identified by the antidromic
collision technique. Antidromic action potentials were evoked by stimulation of caudal
brainstem with constant electric current. Masseter afferent fibers were mechanically stimulated
to evoke orthodromic action potentials. Collision of antidromic and orthodromic action
potentials was used to confirm the projection of masseter muscle afferent fibers to the caudal
brainstem (Cairns et al. 2002; Cairns et al. 2001b). The conduction velocity of afferent fibers
was calculated by dividing the distance between stimulating and recording electrodes by the
latency of the antidromic action potential. To confirm that the mechanoreceptive field of each
fiber was in masseter muscle, fibers were required to discharge in response to either injection of
TNFα or HS (1M), which was injected into the masseter muscle at the end of the experiment.
Afferent fibers were excluded from further analysis if they failed to discharge in response to
injection of at least one of the substances injected into the masseter muscle. The relatively high
MT of most afferent fibers coupled to their response to HS, led us to identify these afferent fibers
as putative nociceptors.
Electrophysiology Experiments

These experiments were carried out to investigate the role of glutamate, NGF and PGE2 in TNFα-induced masseter muscle nociceptor sensitization. Nociceptors were assigned randomly to receive an injection of either vehicle control (10 µl phosphate buffered saline, n=10), the competitive NMDA receptor antagonist APV (10µl, 10mM or 50mM; n=10 per concentration), the non-steroidal anti-inflammatory drug (NSAID) diclofenac (0.1mg/ml, 10µl; n=7) or an antibody against the high affinity NGF TrkA receptor (2µg/ml, 10µl; n=8) 3 hours after TNFα injection. The investigator (AH) was unaware of the content of the injections. Baseline line mechanical threshold (MT) of nociceptors was measured using an electronic von Frey hair (model 160IC, IITC) every minute for 10 minutes prior to insertion of the catheter needle that contained TNFα (Hakim et al. 2009). The baseline MT was determined by averaging 10 consecutive mechanical stimuli. TNFα (Sigma; 1µg in 10µl phosphate buffered saline (PBS), n =10) was injected into the receptor field of the identified fiber and MT was measured after every hour for 10 minutes over 3 hours. Ongoing discharge was measured by counting the number of action potentials over 1 minute immediately before the baseline MT assessment, the injection of TNFα and each hour for 3 hours 1 minute before each MT assessment (Bove and Dilley 2010). Three hours after injection of TNFα, one of the test substances was injected and MT was measured once each minute for 30 minutes. At the end of MT recoding, HS was injected into the mechanoreceptive field of masseter nociceptor. Rats were then terminated by injection of pentobarbital.

Data Analysis

The glutamate biosensor probe was connected to a 4-channel potentiostat (model 3104) and the input signal from the probe was analysed with pinnacle Acquisition Laboratory software
(Pinnacle Technology Inc., USA). The change in glutamate concentration after injection of TNFα or PBS was determined by subtracting baseline glutamate concentration from the concentration of glutamate obtained after injection of TNFα or PBS.

The average nociceptor MTs at each time point was calculated. Relative MTs were then calculated by dividing the mean MT at each time point by the mean baseline MT to permit assessment of change in MT. To determine whether TNFα injections increased nociceptor discharge, cumulative discharge was calculated by subtracting the number of action potentials recorded for 10 minutes before injection from the number recorded for 10 minutes after injection. Change in ongoing discharge was calculated by subtracting the number of action potentials recorded before injection of TNFα from the number recorded after injection at each hour. Positive numbers indicated an increase in ongoing discharge, and negative numbers indicated a decrease in ongoing discharge.

**Statistical Analysis**

The Pearson product moment test was used to determine whether correlations were significant. Two way repeated measures ANOVA and Holm-Sidak post hoc test were used to determine the differences in interstitial glutamate concentration over time after injection of TNFα and PBS. A Students t-test was used to compare mean levels of NGF between vehicle control and TNFα. One-way ANOVA was used to compare mean levels of PGE2 between TNFα, vehicle control and diclofenac injections. Data was normally distributed and a logarithmic transformation of PGE2 concentrations was necessary to produce equal variance before running the ANOVA. The MTs after injection of various treatments (PBS, APV, NGF antibodies or diclofenac) were compared with the MT 3 hours after injection of TNFα through the use of one-way repeated measures ANOVA and Holm- Sidak post hoc test. Differences
were considered statistically significant when $p < 0.05$. Unless otherwise indicated, data in the
text are given as a mean ± standard error of the mean.
Results

Effect of TNFα

In-vivo single unit extracellular recording of 52 nociceptors was made. Seven nociceptors, which were not mechanically sensitized by TNFα three hours after injection, were excluded from further analysis. The CV of the remaining 45 nociceptors was in the Aδ (CV=2-12m/s) range. The mean baseline MT of these nociceptors was 38 ± 5 g. HS was injected as positive control to confirm that the receptor field of the afferent fiber recorded was in masseter muscle and that all afferent fibers were nociceptors (Cairns et al. 2003b; Kumazawa and Mizumura 1977; Mense 1977; Paintal 1960). TNFα evoked discharge in 12 out of 45 recorded masseter muscle nociceptors (Fig 3.1). Eight out of 45 nociceptors had ongoing activity prior to baseline MT assessment. Insertion of the needle into the masseter muscle caused 4 additional nociceptors to begin ongoing discharge, thus a total of 12 nociceptors exhibited some degree of ongoing activity (average 0.23 Hz, range 0.02-1.58 Hz) prior to TNFα injection. Three hours post injection, only 2 of the original 12 nociceptors having ongoing discharge had increased their discharge rate, while the rate of discharge had decreased or stopped altogether in the other 10 nociceptors. However, 6 previously non-discharging nociceptors developed ongoing discharge by 3 hours post TNFα injection. Therefore, over the 3 hour period after injection, TNFα increased ongoing or induced novel ongoing discharge in a total of 8 of 45 nociceptors recorded. Three hours after injection, TNFα (1µg) significantly decreased the MT of masseter muscle nociceptors as compared with the pre-injection baseline MT. The mean relative MT (%) of the 45 nociceptors examined 3 hours after TNFα injection was 39 ±3%. There was no significant correlation between baseline MT or CV and TNFα– induced mechanical sensitization.
Figure 3.1. The peri-stimulus histogram shows an example of TNFα evoked Aδ fiber (CV = 3.4 m/s, MT = 24g) masseter muscle afferent discharge. Baseline activity was recorded for 600 seconds and at 600 seconds TNFα was injected into the mechanoreceptive field of masseter muscle afferent fiber (arrow), which evoked a brief afferent fiber discharge. The trace above the peristimulus histogram shows TNFα evoked masseter muscle nociceptor discharge activity. The size of the action potential decreased shortly after injection of TNFα possibly due to sustained depolarization and rapid inactivation of sodium channels.
Role of PGE2 in TNFα-Induced Mechanical Sensitization

To investigate whether TNFα might be acting to increase prostaglandin levels or alter NMDA receptor activation, we tested the effect of the non-steroidal anti-inflammatory drug diclofenac on TNFα-induced mechanical sensitization at a concentration 0.1 mg/ml; a concentration which also competitively inhibits peripheral NMDA receptors (Dong et al. 2009) and prostaglandin synthesis (Cashman 1996; Gotzsche 2000). This concentration of diclofenac also inhibits prostaglandin synthesis, as demonstrated by our finding that TNFα injection significantly elevated levels of PGE2 as compared to vehicle control and that this TNFα-induced elevation in muscle PGE2 level was attenuated by diclofenac (Fig.3.2A).

Vehicle control injected at 3 hours after TNFα injection had no significant effect on TNFα-induced mechanical sensitization at 10, 20 or 30 minutes post injection (Fig.3.2B). Diclofenac, however, when injected 3 hours after TNFα, partially reversed TNFα-induced mechanical sensitization of masseter muscle nociceptors. A significant effect of diclofenac on relative MT was seen at 10, 20 and 30 minutes after its injection (Fig.3.2C). These results suggested that an increase in PGE2 levels contributes to TNFα-induced mechanical sensitization, however, as the concentration of diclofenac employed also inhibits NMDA receptor activation, additional experiments were undertaken to examine the role of glutamate and peripheral NMDA receptor activation in TNFα-induced mechanical sensitization.
Figure 3.2. (A) The bar graph shows the levels of PGE2 after injection of phosphate buffered saline (vehicle control), TNFα and TNFα with diclofenac (0.1 mg/ml). Injection of TNFα significantly elevated the levels of PGE2 as compared to vehicle control (n=5). Injection of diclofenac at 3 hours after TNFα injection significantly decreased the levels of PGE2 (*p < 0.05 one-way ANOVA, Holm-Sidak post-hoc test).
Figure 3.2. (B). The vertical bar chart illustrates the change in relative MT after injection of PBS at 10(T10), 20(T20) and 30(T30) minutes after its injection. TNFα significantly decreased the MT 3 hours after injection and this TNFα-induced decrease in MT was not significantly changed by injection of PBS. (C). The line and scatter plot illustrates the change in MT after injection of diclofenac (0.1mg/ml). TNFα significantly decreased the MT at 3 hours after injection and this decrease in MT was partially reversed by injection of diclofenac (* p<0.05 one-way repeated measures ANOVA, n=7). The error bars indicate SEM.
Role of Glutamate and Peripheral NMDA Receptors in TNFα-Induced Mechanical Sensitization

The mean baseline concentration of glutamate in the masseter muscle was 44 ± 16µM (n= 8). Two way repeated measures ANOVA revealed a significant effect of treatment (p<0.05) but not time on glutamate concentration, and there was no significant interaction between time and treatment (p=0.1). Post hoc test revealed that TNFα significantly elevated the concentration of glutamate compared to vehicle control (Fig.3.3). However, as can be seen in Figure 3.3, the source of the difference between the two treatment groups was principally due to a decline in interstitial glutamate concentration after injection of vehicle, rather than an increase in glutamate after injection of TNFα.

Injection of the competitive NMDA receptor antagonist APV (10mM or 50mM) 3 hours after injection of TNFα did not significantly change the MT of masseter muscle nociceptors at 10, 20 and 30 minutes after its injection (Fig.3.4 A,B). NMDA receptor activation did not appear to contribute significantly to the mechanism of TNFα-induced mechanical sensitization.
Figure 3.3. The histogram shows the mean change in rat masseter muscle glutamate concentration at various time points after injection of TNFα (n= 4) or vehicle control (n= 4). Overall, the change in glutamate concentration after injection of TNFα was significantly higher than after injection of vehicle control. (*P < 0.05 two way repeated measures ANOVA). The error bars indicate SEM.
Figure 3.4. The vertical bar charts illustrate the change in relative MT 10(T1), 20(T2) and 30(T3) minutes after injection of APV (A. 10mM, B. 50 mM). The TNFα-induced decrease in relative MT was not significantly altered by injection of either concentration of APV. (One way repeated measures ANOVA, n=10). The error bars indicate SEM.
Role of NGF and TrkA Receptors in TNFα-Induced Mechanical Sensitization

TNFα injection did not significantly elevate the level of NGF as compared to vehicle control (Fig.3.5A). Injection of TrkA antibody 3 hours after TNFα injection did not significantly reverse TNFα-induced masseter muscle nociceptor sensitization at 10, 20 or 30 minutes after its injection (Fig.3.5B). This result shows that NGF is not playing an important role in TNFα– induced masseter muscle nociceptor sensitization.
Figure 3.5(A). The vertical Bar chart shows the level of NGF in the masseter muscle after injection of TNFα or vehicle control. Injection of TNFα did not significantly elevate NGF level compared to vehicle control (t-test, n=5) (B). The vertical bar chart illustrates the change in MT 10(T1), 20(T2) and 30(T3) minutes after injection of TrkA antibody. TNFα significantly decreased the MT at 3 hours of its injection and this decrease in MT was not significantly reversed by injection of TrkA antibody (one way repeated measures ANOVA, n=8).
**Discussion**

Injection of TNFα had no effect on ongoing discharge, but did produce mechanical sensitization of masseter muscle nociceptors that was associated with an increase in muscle concentrations of PGE2 and glutamate. Diclofenac, but not the NMDA receptor antagonist APV, partially reversed TNFα-induced sensitization of masseter muscle nociceptors. TrkA receptor antibody, which binds to the high affinity NGF receptor and has been previously demonstrated to attenuate masseter muscle nociceptor mechanical sensitization induced by exogenously administered NGF (Svensson et al. 2010) did not reverse TNFα− induced mechanical sensitization of masseter muscle nociceptors. These results suggest that TNFα-induced sensitisation of masseter muscle nociceptors is mediated, at least in part, by PGE2.

PGE2 is a pro-nociceptive cytokine that sensitizes muscle nociceptors in both animals and humans (Dong et al. 2009; Mense 1981; Rukwied et al. 2007). In humans, PGE2 potentiates acid-induced muscle pain (Rukwied et al. 2007) and has been suggested to contribute to the development of masseter muscle pain in fibromyalgia patients (Hedenberg-Magnusson et al. 2001). Further, PGE2 in combination with bradykinin, histamine and serotonin has a sensitizing effect on human muscle (Mork et al. 2003). In animals, PGE2 injection has been demonstrated to cause mechanical sensitization of rat gastrocnemius and masseter muscle nociceptors (Dina et al. 2008; Dong et al. 2009) and enhances the sensitizing response of bradykinin on cat gastrocnemius muscle nociceptors (Mense 1981). The concentration of PGE2 measured in rat masseter muscle in the present study after vehicle control is similar to that previously reported for human skeletal muscle (Trappe et al. 2001). These findings indicate that an increase in the concentration of PGE2, for example due to tissue injury, contributes to the development of muscle pain through nociceptor sensitization (Graven-Nielsen and Mense 2001; Mense 1981; Tegeder et al. 2002). PGE2 induced sensitization is mediated by PGE2 receptors EP2 and EP3.
These receptors are expressed on muscle tissue and trigeminal ganglion neurons (Graven-Nielsen and Mense 2001; Patwardhan et al. 2008). Studies have shown that TNFα treatment of dorsal root ganglion cell culture or injection into rat gastrocnemius muscle significantly elevated the level of PGE2 (Fehrenbacher et al. 2005; Schafers et al. 2003d). Our results are consistent with previous cutaneous animal experiments that have demonstrated that TNFα-induced sensitization is mediated through prostaglandins (Cunha et al. 2005; Russell et al. 2009).

We have shown that TNFα induces delayed mechanical sensitization of masseter nociceptors via activation of peripheral P55 and P75 receptors (Hakim et al. 2009). Activation of either TNFα receptor (P55 or P75) stimulated the release of PGE2 in synovial and gingival fibroblasts (Butler et al. 1994; Taylor 1993). In-vitro, activation of P55 and P75 receptors by TNFα leads to the activation of p38 MAPK which in turn activate phospholipase A2 to liberate arachidonic acid, the precursor for prostaglandin synthesis (Ji and Woolf 2001). In the current study, PGE2 concentration was significantly elevated 3 hours post TNFα injection and diclofenac reversed the elevated level of PGE2, which supports the concept that TNFα acts through P55/P75 receptor mechanisms to increase the synthesis of PGE2 in-vivo. However, since diclofenac only partially attenuated the effect of TNFα, although it completely reversed the TNFα-induced increase in PGE2 levels, it is conceivable that other algogenic substances also contribute to TNFα-induced mechanical sensitization of masseter muscle nociceptors.

Dong et al. (2009) have shown that intramuscular injection of 5-hydroxytryptamine, NMDA and AMPA caused sensitization of masseter muscle nociceptors. The NMDA, but not AMPA or 5-hydroxytryptamine, induced sensitization of masseter muscle nociceptors was significantly reversed by diclofenac (0.1 mg/ml). In the present study, this concentration of diclofenac also reversed the TNFα-induced mechanical sensitization of masseter muscle.
nociceptors. Dong et al. (2009) have also demonstrated that injection of diclofenac (0.1 mg/ml) as well as APV alone could increase the mechanical threshold of masseter muscle nociceptors, which was interpreted to suggest that the mechanical sensitivity of these nociceptors is modulated, in part, by endogenous glutamate activation of peripheral NMDA receptors. However, Dong et al. (2009) also demonstrated that PGE2 could decrease the mechanical threshold of masseter muscle nociceptors. Although TNFα, modestly increased the interstitial concentration of glutamate in the masseter muscle, injection of APV 3 hours after TNFα did not significantly alter the mechanical threshold of masseter muscle nociceptors. In contrast, TNFα dramatically increased levels of PGE2 in the masseter muscle 3 hours after injection, and pretreatment with diclofenac attenuated the increase in PGE2 in conjunction with an increase in the mechanical threshold of masseter muscle nociceptors, which suggested that inhibition of prostaglandin synthesis and not NMDA receptor antagonism was responsible for the effect of diclofenac on TNFα induced mechanical sensitization.

Glutamate is an excitatory amino acid that causes sensitization of rat masseter muscle nociceptors through activation of peripheral NMDA receptor (Cairns et al. 2007; Cairns et al. 2003b). A 200-300% increase in interstitial glutamate concentration of rat masseter muscle from baseline concentration is required to induce sensitization of masseter muscle nociceptors (Cairns et al. 2007). In the present study, TNFα increased glutamate concentrations on average by about 6 μM (~15%), which suggests that the increase in glutamate concentration induced by TNFα was likely not great enough to contribute to mechanical sensitization, at least through activation of peripheral NMDA receptors. It should be noted that the significant difference in glutamate concentration between TNFα and PBS injections mostly reflected a decrease in glutamate concentration after PBS injection, which we suggest was a result of local dilution of glutamate concentrations by the injectate (Cairns et al. 2003a). Nevertheless, it is possible that
these small increases in interstitial glutamate concentrations could have activated non-NMDA receptors such as metabotropic glutamate receptors (mGluRs) or AMPA-activated ionotropic glutamate receptors (GluRs), which have been shown to contribute to the development of nociception after inflammatory injury (Bhave et al. 2001; Dogrul et al. 2000; Walker et al. 2001). mGluR5 protein is expressed in the masseter nerve and in trigeminal ganglion and activation of mGluR5 induces mechanical sensitivity in the masseter muscle (Lee and Ro 2007). In addition, both GluR1 and GluR2 subtypes are expressed in trigeminal ganglion neurons (Chun et al. 2008) and injection of AMPA into masseter muscle both excites and mechanically sensitizes masseter muscle nociceptors (Dong et al. 2009). Although there is a potential that these other glutamate receptors might have been activated by the TNFα-mediated increase in interstitial glutamate concentration, previous findings that glutamate-induced mechanical sensitization of masseter muscle nociceptors can be completely reversed by NMDA receptor antagonists (Cairns et al. 2007) suggest that mechanisms other than activation of glutamate receptors are more important for TNFα-induced nociceptor sensitization. The other possibility is that actual glutamate concentration at the site of injection could have been higher than detected by the probe, as injecting needle was around 3 mm away from the probe. There is no easy way to prove what the actual concentration of glutamate was at the center of the injected TNFα and thus we cannot be certain that glutamate concentration obtained with these experiments was accurate.

NGF has also been shown to induce mechanical sensitization when injected into muscles (Andersen et al. 2008; Mann et al. 2006; Nie et al. 2009; Svensson et al. 2003a; Svensson et al. 2008a; Svensson et al. 2010). Injection of NGF into the masseter muscle causes prolonged (~14 day) mechanical sensitization in healthy human subjects (Svensson et al. 2003a; Svensson et al. 2008a; Svensson et al. 2008b). In rats, the onset of mechanical sensitization of masseter muscle nociceptors by exogenously administered NGF into rat masseter muscle occurs within 30 minutes of injection and is mediated through activation of the TrkA receptors (Svensson et al.
In the present study, TNFα (1µg) injection into the masseter muscle did not significantly elevate the level of NGF, although in previous study TNFα injection into rat gastrocnemius muscle did significantly elevate the level of NGF as compared to vehicle control (Schafers et al. 2003d). This discrepancy in results could be due to higher dose of TNFα (10µg) injected into rat gastrocnemius muscle in the Schafers et al. (2003d) study. In addition, the concentration of NGF in our vehicle control treated masseter muscle was higher than baseline concentrations of NGF in other rat skeletal muscles (Wu et al. 2009), which may indicate that there are higher basal concentrations of NGF in the masseter muscle. These high baseline concentrations may have made it more difficult to detect a significant change in NGF levels by TNFα in the masseter muscle. However, even with this caveat, we also found no significant attenuation of TNFα-induced mechanical sensitization of masseter muscle nociceptors with the same concentration of a TrkA receptor antibody that we have previously demonstrated inhibits NGF-induced mechanical sensitization (Svensson et al. 2010). Taken together, these results suggest that NGF is also not playing important role in the mechanism of TNFα-induced mechanical sensitization of the masseter muscle.

TNFα modulates variety of ion channels. For example, TNFα enhances TTXr Na⁺ currents, an effect which is mediated by the p38 MAPK pathway through P55 receptor activation (Jin and Gereau 2006). TNFα has also been shown to reduce outward potassium currents in retinal ganglion neurons (Diem et al. 2001) and inhibits potassium currents in small dorsal root sensory neurons (Liu et al. 2008b). Further, increases in PGE2 levels act to further enhance the inhibitory effects of TNFα on potassium currents (Liu et al. 2008b). These results suggest that downstream modulation of sodium and/or potassium channel function by TNFα could also contribute to TNFα-induced mechanical sensitization of masseter muscle nociceptors. These
additional mechanisms of nociceptor sensitization may explain why we were only able to partially attenuate TNFα-induced mechanical sensitization with diclofenac.

In the present experiment, the insertion of the needle used for injection into the masseter muscle increased the ongoing discharge of a subpopulation of nociceptors and that ongoing discharge had decreased or stopped in the majority of these nociceptors 3 hours after injection of TNFα. It is speculated that insertion of the needle into the masseter muscle could have released various algogenic substances such as ATP and bradykinins which might have induced nociceptor activity. Injection of TNFα decreased this ongoing activity which could be due to dilution effect of PBS used as vehicle, on the concentration of these algogenic substances. It is also conceivable that TNFα could have interfered with the release of algogenic substances caused by insertion of the catheter needle into the muscle, however, there is no evidence in the literature which suggests that TNFα blocks the release of algogenic substances.

Clinical Relevance

Myofascial TMD are characterized by masticatory muscle pain and localized muscle tenderness, which some clinicians call “trigger points” but are more accurately described by the term “focal muscle hypertonicities” (Cairns 2010; Fricton 2007). Focal muscle hypertonicities are tender nodules within taut bands of skeletal muscle, which are painful upon palpation and refer pain to other body parts (Lavelle et al. 2007). Although, focal muscle hypertonicities are not associated with tissue damage or inflammation (Cairns 2010) elevated levels of a number of pro-inflammatory compounds such as bradykinin, ATP, substance P, CGRP, protons, IL and, importantly TNFα have been found in them (Shah et al. 2008), which suggests that some degree of tissue injury and/or inflammation could be occurring at these sites. Although, NSAIDs are a drug of choice for the treatment of myofascial TMD-related pain conditions, in the small number
of clinical studies undertaken to date, it has been difficult to demonstrate the efficacy of these agents for the treatment of muscle pain in these conditions (Fricton, 2007; Cairns 2010). We previously found that TNFα mechanically sensitizes masseter muscle nociceptors without gross inflammation and in the present study, that TNFα induces mechanical sensitization of muscle nociceptors that was partially attenuated by the NSAID diclofenac. We propose that injection of TNFα into skeletal muscles could be useful to model focal muscle hypertonicities and may prove useful to study mechanisms of NSAIDs for the treatment of masticatory muscle pain.
Chapter 4. Conclusion

Results of this study show that TNFα receptors are expressed on rat trigeminal masseter muscle ganglion neurons as well as in the masseter muscle. In accordance with our hypothesis, TNFα injection caused mechanical sensitization of rat masseter muscle nociceptors through peripheral TNFα receptors (P55 or P75) without inflammation. Blocking of either P75 or P55 receptor attenuated the effect of TNFα. Further, injection of TNFα caused sensitization of masseter muscle nociceptors by increasing the levels of PGE2. TNFα-induced mechanical sensitization was partially attenuated by diclofenac, a NSAID that blocks the synthesis of prostaglandins such as PGE2 (Cashman 1996; Gotzsche 2000) but not by NMDA or TrkA receptor antagonism. Thus, it was concluded that sensitizing substances other than glutamate and NGF are also involved in TNFα-induced masseter muscle sensitization of nociceptors. Further research is needed to determine what other sensitizing substances are contributing to TNFα-induced rat masseter muscle nociceptor sensitization.

In summary, these results suggest that TNFα might sensitize masseter muscle nociceptors through indirect mechanism by acting on peripheral TNFα receptors expressed by non-neuronal cells to induce the release of other sensitizing substances such as PGE2. PGE2 produces sensitization of masseter muscle nociceptors by binding to its target receptors expressed on masseter muscle nociceptors (Dong et al., 2009; Patwardhan et al., 2008). The other possibility is that TNFα acts directly on its receptors expressed on the masseter muscle nociceptors and modulates various ion channels such as Na⁺ and K⁺ channels. For example, activation of various MAP kinases such p38 MAPK by TNFα causes phosphorylation of ion channels such as TTXr Na⁺ channels that could lead to the sensitization of masseter muscle afferent fibers Fig (4.1).
Figure 4.1. This diagram shows how TNFα could produce sensitization of masseter muscle nociceptors. TNFα could activate P55 and P75 receptor expressed on non-neuronal cells and releases PGE2. PGE2 in turn activates prostaglandin receptors (EP 2-3) expressed on trigeminal ganglion and causes sensitization of masseter muscle nociceptors. TNFα could also directly act on receptors expressed on masseter muscle nociceptors and activate various signaling molecules such as MAP kinases. Phosphorylation of ion channels such as TTXr Na\(^+\) channels by p38 MAPK increases the Na\(^+\) current which could cause sensitization of masseter muscle nociceptors. TG (Trigeminal ganglion).
The pathogenesis of myofascial TMD is not known (Cairns 2010; Fricton 2007). The current randomized blinded studies were undertaken to determine whether TNFα causes sensitization of rat masseter muscle nociceptors so that it could be used as a model of myofascial TMD. Myofascial TMD are characterized by the presence of myofascial trigger points (MTrPs), which upon palpation produce pain (Cairns 2010; Fricton 2007). Previous studies have shown that MTrPs have elevated levels of sensitizing substances, which sensitize muscle afferent fibers and lower pain thresholds (Shah et al. 2008; Shah et al. 2005). Elevated levels of TNFα at the MTrPs suggest that TNFα could contribute to pain and sensitivity associated with MTrPs. In the present study, injection of TNFα into rat masseter muscle did cause sensitization of masseter muscle nociceptors without gross inflammation, which is a characteristic of MTrPs (Cairns 2010; Ge et al. 2008). These findings support the argument that TNFα injection into masseter muscle could be used as model of MTrPs.

Presently, drugs used in the treatment of myofascial TMD pain conditions are not very efficacious and are associated with side effects. However, recently topical diclofenac, has been shown to reduce pain in patients suffering from TMD disorders without producing adverse effects (Di Rienzo Businco et al. 2004). Dong et al. (2009) have shown that local injection of diclofenac into the masseter muscle blocks peripheral NMDA receptors, a type of glutamate receptor. These findings suggest that further research should be undertaken to determine the effectiveness of topical NSAIDs for the treatment of myofascial TMD pain. In the present study, TNFα mechanically sensitized masseter muscle nociceptors by increasing COX enzyme, therefore, it could be used as an animal model of MTrPs to study the effectiveness of topical NSAIDs in these conditions. Overall, this animal model could help us to understand the basic
mechanism of myofascial TMD pain that could lead to the development of new drugs to treat this condition.

Nevertheless, while animal models could be useful to study the basic mechanisms of myofascial TMD pain, they don’t mimic the myofascial TMD pain conditions seen in patients. They need to be translated into human models, which are more relevant to clinical myofascial TMD pain. Although levels of TNFα are elevated in certain muscle pain conditions such as fibromyalgia and inflammatory myopathies (Wallace et al. 2001; Wang et al. 2008), little is known about the role of TNFα in human muscle pain. Future studies where the effects of TNFα injection into human masseter muscle are investigated will help to better understand the role of TNFα in the mechanisms of TMD conditions seen in patients. Thus, development of human myofascial TMD models could provide more relevant clinical information about myofascial TMD pain conditions.
Study Limitations

Electrophysiological recordings of masseter muscle afferent fibers were carried out under inhalation anesthesia. Isoflurane, which was used as an anesthetic agent, may have possibly interfered with our experiments. Several previous in-vitro and in-vivo studies have shown that isoflurane inhibits the release of TNFα and decreases the cytokine concentration (Mitsuhata et al. 1995; Plachinta et al. 2003; Sato et al. 1995; Vaneker et al. 2009). As already mentioned in chapter 1, TNFα activates MAP kinases, and it is believed that isoflurane induces activation of MAP kinases and enhances the TNFα–induced activation of MAP kinases (Itoh et al. 2004). However, in another study, isoflurane inhibited the lipopolysaccharide–induced activation of NFκB in monocytes, which is downstream of MAPK activation (de Rossi et al. 2004). The results of these studies suggest that isoflurane could have interfered with the downstream signaling pathway of TNFα and may have altered the effect of TNFα. It is possible that under the deep anesthesia conditions necessary for my studies, the effect of TNFα could have decreased due to inhibition of NFκB, which is considered responsible for TNFα-induced biological activities (Bowen et al. 2006; Ksontini et al. 1998). This could also be one of the reasons that TNFα took three-hours to produce significant mechanical sensitization after injection. Moreover, Plachinta et al. (2003) have shown that isoflurane pretreatment is protective against the lipopolysaccharide-induced inflammation in rats. In the present study, TNFα did not cause gross inflammation of masseter muscle, it is possible that isoflurane could have decreased the TNFα–induced masseter muscle inflammation.

Results of the present study have shown that prostaglandins are not completely responsible for TNFα–induced masseter muscle nociceptor mechanical sensitization. Moreover, glutamate and NGF are not playing a role in TNFα induced sensitization. Previous studies have
shown several algogenic substances are elevated after injection of TNFα into rat gastrocnemius muscle (Schafers et al. 2003d). In the present study experimental design, it was not possible to find all mechanisms responsible for TNFα–induced masseter muscle nociceptor sensitization. Future experiments could be carried out to determine other mechanisms responsible for TNFα-induced masseter muscle nociceptor sensitization.

In the current work, it was found that TNFα receptors (P55 and P75 receptors) are expressed on the trigeminal masseter muscle ganglion neurons as well as on non-neuronal masseter muscle cells. In the present study experimental design, it was not possible to determine whether TNFα acts directly on TNFα receptors (P75 and P55) or on non-neuronal receptors expressed on masseter muscle cells to induce the release of other algogenic mediators that cause masseter muscle nociceptor mechanical sensitization. In-vitro studies could be done to determine whether TNFα acts directly on trigeminal ganglion afferent fibers or on non-neuronal masseter muscle cells to induce the release of other algogenic substances such as PGE2.

In the present study, an infra-red thermography camera was used to determine the change in the surface temperature of rat temporalis muscle after injection of TNFα or vehicle control as an indirect measure of change in the muscle blood flow. One of the limitations of this study was that the temperature change was measured on temporalis muscle, a muscle similar to masseter muscle. It was not possible to focus the camera towards the masseter muscle and was difficult to remove the skin to record the temperature changes directly from the masseter muscle surface. Infra-red thermography is a non-invasive procedure which captures and processes infra-red radiation released from the body surface. It is possible that reflection of infra-red radiation from non-muscle surfaces could have contributed to the images obtained (Ferreira et al. 2008). Further, to record muscle surface temperature, the skin has to be removed to gain access to the surface of temporalis muscle. It is possible that removal of skin induces mild inflammation that
could increase the background signal making it difficult to detect small increases in surface temperature induced by injection of TNFα.

The signs of inflammation are pain, redness, heat and swelling. Swelling is caused by increased permeability of blood vessels that leads to plasma protein and fluid extravasation (Haas et al. 1992). In the present study, Evans blue dye was used to determine the plasma protein extravasation as a marker of inflammation (Haas et al. 1992). Inflammation increases vascular permeability that leads to leakage of plasma proteins into tissues. Evans blue dye binds with high affinity to plasma proteins which are normally present within the vasculature (Kerezoudis et al. 1993). However, increased vascular permeability due to inflammation causes plasma proteins leak out of the blood vessels into tissue (McMahon et al. 1989). This results in Evans blue dye being deposited into inflamed tissues. However, this method of inflammation measurement has some limitations that could affect the deposition of Evans blue dye into tissues, for example, increased blood flow could enhance capillary filtration by changing the surface area for exchange. This could lead to higher deposition of Evans blue dye in tissues. Another factor that could affect the deposition of Evans blue dye into tissues is vascular leakiness. Increased vascular leakiness is accompanied with increased extravasation of plasma proteins and fluid into tissue and could result in increased tissue deposition of Evans blue dye (Baluna and Vitetta 1997). In addition, the time between injection and measurement of dye concentration could have also affected our results. A previous study has shown that injection of mustard oil into TMJ induced plasma protein extravasation which was determined by Evans blue dye analysis. The concentration of dye in TMJ increased to highest level at 30 minutes post its injection, which then decreased after 60 minutes and remained at constant level for 4 hours (Fiorentino et al. 1999). The results of this study suggest that differences in time course of muscle sample collection could have produced variations in the Evans blue dye analysis results.
In the present study, Western blot analyses were done to determine the expression of P55 and P75 TNFα receptors on the masseter muscle and the dose of P55 and P75 receptor antibodies. The protein samples must be loaded accurately, and inaccurate loading of proteins will give false results or high background signal. Furthermore, detergents used in the experiment may also affect the binding of some antibodies resulting in weak signal (Wisdom 1994). Since this method also requires the labeling and detection of protein of interest with antibody, the results of Western blot are limited by the quality and specificity of antibody used to detect the particular protein. Multiple bands observed on the Western blots due to non-specificity of the antibody, could pose a problem in detection of the protein.

As there were no small molecule TNFα receptor inhibitors available for these experiments, antibodies against the TNFα receptors were used to antagonize the effect of TNFα. Polyclonal antibodies used to block the TNFα receptors are large molecules that could bind with several epitopes of the antigen of interest. In addition to binding with the native antigen, these antibodies could also cross-react with non-specific antigens. In the present study, several bands were observed in the Western blot experiments, which suggest that the antibodies used, did not selectively bind to the target proteins. These multiple bands could be fragments of receptor proteins or represent non-target proteins, the identity of which is not known. It is possible that the concentration at which the antibodies were used in the experiment might have produced effects which were not specific to blocking the intended receptor.

**Localization of Receptors**

It is not possible to determine the expression of receptors selectively on the sensory afferent fiber nerve endings innervating the masseter muscle, as sympathetic as well as motor neurons also innervate the masseter muscle. Thus the receptor expression was determined on the cell bodies of afferent fibers which lie in the trigeminal ganglion neurons. It is believed that
receptors are synthesized in the cell bodies and are transported to afferent nerve fiber endings (Laduron 1987), thus receptor expression of the trigeminal ganglion are considered representative of receptor expression at the nerve terminals.

**TNFα and Apoptosis**

As mentioned already, TNFα activates NF-κB which induces cell proliferation, cell differentiation and cell survival as well as cell death. However, TNFα also activates caspases and ceramides which are downstream of TNFα signaling pathways. There is evidence that binding of TNFα to P55 receptor, which is linked to a death domain, mediates the activation of caspases to induce apoptosis (Micheau and Tschopp 2003). Blockade of both caspases and ceramides inhibits TNFα− induced neuropathic pain (Joseph and Levine 2004), which suggests the involvement of pro-apoptotic proteins in peripheral neuropathic pain. Moreover, recently Chen et al. (2011) have shown that TNFα inhibitor, entanercept blocks TNFα-induced neuronal and glial cell apoptosis after spinal injury. In addition, Stewart et al. (2004) have shown that TNFα exposure causes apoptosis of skeletal muscle cells. The expression of TNFα death receptor (P55 receptor) on both masseter muscle and trigeminal ganglion neurons and the results of previous studies suggest the possible involvement of caspases and ceramides in TNFα-induced sensitization of masseter muscle afferent fibers. However, the present series of experiments lasted only for 3 hours, and in order to induce apoptosis, the signal from afferent fiber endings must travel to nucleus of their cell bodies. A previous in-vitro study has shown that TNFα induces apoptosis of olfactory epithelium cells in six hours (Suzuki and Farbman 2000). Moreover, in-vitro treatment of cultured cortical cells with TNFα for 24 hours reduced cell viability by 20% (Badiola et al. 2009). Cell bodies of the masseter muscle afferent fibers lie
in the trigeminal ganglion, and accordingly, it is unlikely that the apoptotic signal will travel to nucleus in 3 hours.

**Use of Male Rats**

Although TMD are more common in females, this study was carried on male rats to determine the mechanisms involved in TNFα induced sensitization of masseter muscle afferent fibers. In female rats, menstrual cycle related hormonal changes could alter the nociceptive response of afferent fibers to algogenic substances, a potential confounder. Indeed, previous studies have shown that nociceptive response varies in female animals during different stages of menstrual cycle (Cairns 2007; Cairns et al. 2001a; Drury and Gold 1978; Frye et al. 1993; Martinez-Gomez et al. 1994). Thus it was not possible to determine the mechanism of TNFα induced sensitization of masseter muscle afferent fibers in female rats due to variation in responses during different stages of menstrual cycle. Further, by examining only male rats, it is not possible to determine the sex differences in the effect of TNFα that would be consistent with the sex differences that are observed in myofascial TMD.
Future Studies

Role of other Sensitizing Substances in TNFα–induced Masseter Muscle Nociceptor Sensitization

The results of the present study demonstrate that TNFα– induced masseter muscle sensitization was partially mediated by PGE2. TNFα could also cause the release of other algogenic substances such as bradykinin, CGRP, substance P, interleukins and sympathetic amines. TNFα increases the expression of bradykinin B1 and B2 receptors (Couture et al. 2001) and previous studies have shown that bradykinin has a sensitizing effect on muscle nociceptors and excites group Aδ and C-fiber muscle afferents (Mense 1981). In addition, the concentration of bradykinin is also elevated at myofascial trigger points (Shah et al. 2005). These studies suggest that TNFα–induced release of bradykinin could have sensitizing effect on masseter muscle nociceptors. Previous animal studies have shown that injection of TNFα into rat gastrocnemius muscle induces the release of CGRP, PGE2, NGF and substance P (Schafers et al. 2003d). Furthermore, SC co-injection of indomethacin (COX inhibitor) and atenolol (β1–adrenergic receptor antagonist) completely inhibited the TNFα–induced nociceptive activity, which suggests a role of sympathetic amines as well as prostaglandins in TNFα–induced subcutaneous nociceptor sensitization (Verri et al. 2006). Future studies could be conducted to determine whether these algogenic substances are elevated by TNFα and what roles other algogenic substances play in TNFα–induced masseter muscle nociceptor sensitization.

Receptors Responsible for TNFα–Induced Increase in PGE2

The results of this study confirm the involvement of both TNFα receptors (P55 and P75) in TNFα induced mechanical sensitization of masseter muscle nociceptors. However, it is not
known which TNFα receptor is responsible for elevated PGE2 levels. Previous in-vitro studies have shown that both P55 and P75 receptors promote the release of prostanoids in rheumatoid synovial fibroblasts (Alsalameh et al. 2003; Taylor 1993). Future in-vitro studies could be conducted to determine which TNFα receptors are involved in TNFα− induced release of PGE2 in masseter muscle trigeminal ganglion neurons. This study would involve treatment of trigeminal ganglion culture with TNFα and co-injection of TNFα and P55 or P75 receptor antibodies, and PGE2 levels of the trigeminal culture supernatant will be determined. If the results of this study provide evidence of TNFα receptors mediated release of PGE2, then the following study could be conducted.

**Determine the Signaling Pathway Responsible for TNFα-Induced Elevation of PGE2**

The signaling pathway by which TNFα increases PGE2 synthesis in trigeminal ganglion is not known. As already mentioned, TNFα activates several signaling cascades such as MAPK pathways (Baud and Karin 2001; Bradley 2008). TNFα causes activation of ERK, JNK and p38 MAPK (Barbin et al. 2001; Schafers et al. 2003e). An animal study has shown that inhibition of p38 MAPK attenuates TNFα− induced cutaneous nociception (Jin and Gereau 2006). Future in-vitro studies could be conducted to determine the pathways activated by TNFα in trigeminal ganglion. This study would involve treatment of trigeminal ganglion culture with TNFα and followed by measuring PGE2 concentration in the presence and absence of MAPK inhibitors. The possible role of MAPK involved in the TNFα-induced release of PGE2, if discovered, would be further explored as a potential therapeutic target in clinical conditions in which TNFα is elevated.
Clinical Studies

Very little is known about the role of TNFα in human muscle pain. In one clinical study, SC injection of TNFα antagonist, entanercept did not improve muscle soreness, which suggests that TNFα is not involved in pathophysiology of delayed muscle soreness (Rice et al. 2008). However, TNFα is elevated in various idiopathic inflammatory myopathies (Lundberg 2000) and fibromyalgia (Wang et al. 2008). These findings suggest that future clinical studies should be conducted to determine the role of TNFα in human muscle pain conditions. If it is found that increased levels of TNFα in human masseter muscle cause a decrease in pressure pain thresholds and produce similar symptoms as those reported by myofascial TMD patients, then it could be used as a human model of myofascial TMD pain conditions.
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Appendix A

Detailed Methodologies

Immunohistochemistry Method

Immunohistochemistry experiments were done to determine P75 and P55 TNFα receptor expression on masseter muscle trigeminal ganglion neurons. Fast blue (2%, Polyscience, USA), a retrograde fluorescent dye was injected bilaterally into the masseter muscle of 5 male rats. Rats were monitored for 7 days and were euthanized with a high dose of pentobarbital. Rat’s chest was opened to locate the heart. The right atrium was opened and 240 ml solution of each heparinized saline and 4% paraformaldehyde was perfused into left ventricle. After perfusion, the trigeminal ganglia were removed from the head of rat and were stored in 20-40% sucrose solution. Vibratome was used to cut the trigeminal ganglion into 40µm thick sections in PBS at room temperature. Free floating sections were blocked with 5% normal goat serum for one hour to prevent the non-specific binding of antibodies followed by overnight treatment with P55 (1:1000; Rabbit polyclonal, Abcam, USA) or P75 receptor primary antibodies (1:20000; Rabbit polyclonal, Sigma-Aldrich, USA) at 4 °C. Next morning, after three washes in PBS, sections were incubated with CY3-conjugated goat anti-rabbit secondary antibody (Anti-rabbit; Jackson Immunoresearch, USA) for one hour at room temperature to amplify the signal. Sections were then mounted on the slides using GelTol aqueous mounting medium and observed under Leica DMLB fluorescent microscope. All fast blue positive cells (two standard deviations above the average baseline labeling intensity) were counted and their area was estimated using WCIF Image J software program (National Institutes of Health, USA). The masseter muscle ganglion neuron cell bodies were considered positively labeled for P55 or P75 receptor when the intensity of fast blue and CY3 signals of the cell bodies was two times the standard deviation of the mean background intensity. To determine the specificity of the primary antibodies used, preabsorption
(P55 receptor antibody) or omission of primary antibody experiments were done. Absence of any
signal is a criterion for specificity of the antibody to the targeted receptor.

**Bradford Method for the Determination of Protein Concentration**

The protein concentration of the muscle homogenate was determined by the Bradford
method (Bradford 1976). This method is based on the formation of a complex between the dye,
Brilliant Blue G and proteins present in the muscle homogenate. The absorption of the protein-
dye complex was measured at 595 nm. The intensity of absorption is directly proportional to the
amount of protein present. A standard curve was plotted by using standard protein
concentrations and the concentration of the sample protein was determined from the slope of the
standard curve. The samples were diluted to bring them under linear range (0.1-1.4 mg/ml). The
final concentration of sample protein in the muscle homogenate was determined by multiplying
by the dilution factor.
Figure A.1 This graph shows the example of standard curve (Bradford method) used to calculate the protein concentration in the muscle homogenate.

\[ y = 0.2229x - 0.0027 \]

\[ R^2 = 0.9948 \]
Western Blot Analysis

Western blot experiments were performed to determine the dose of P55 and P75 receptor antibodies and expression of P55 and P75 receptors on the masseter muscle. Masseter muscle was harvested from male rats and weighed. Muscle samples were cut into small pieces and were homogenized on ice using homogenizing buffer. The muscle homogenate was centrifuged at 14000 rpm for 30 minutes at 4 °C. The supernatant was collected and immediately protease inhibitor (1:100 dilution) was added to prevent the degradation of proteins. The protein concentration of the supernatant was determined by the Bradford method using standard curve (Fig.A1). Loading buffer (Laemmli sample buffer + 5% 2-mercaptoethanol) was added to the protein sample. Samples were then boiled at 99 °C for 5 minutes. Protein samples (20 µg) were subjected to electrophoresis using 7% SDS polyacrylamide gel along with standard protein markers to separate the proteins according to their size. The samples were run for 90 minutes at 100 V and transferred out to 0.2 µm nitrocellulose Hy-Bond ECL membrane for 90 minutes at 100 V in transfer buffer with ice bucket. Membrane was blocked with 5% non-fat dried milk at room temperature for 1 hour to prevent the non-specific binding. The membrane was incubated with P55 receptor primary antibody (Rabbit polyclonal, Abcam, USA) or P75 receptor primary antibody (Rabbit polyclonal, Sigma-Aldrich, USA) in 8-10 ml 5% Bovine serum albumin in Tris-Buffered Saline with 0.2% Tween 20 (TBST) at 4 °C overnight on a rocking platform. Next morning, the membrane was given three washes in TBST and subsequently treated with secondary antibody (Goat anti-rabbit peroxidase-conjugated; Jackson Immunoresearch, USA) for 1 hour at room temperature. After washing, the bands were detected with ECL Western blotting detection chemiluminescence reagent (Amersham™; GE Health Care, UK) and analysed by Alpha Innotech Imager FluorChem 8800 (Alpha Innotech Co; San Leandro, CA) gel box imager. Band density was measured by using FluorChem software (Alpha Innotech Co.).
Composition of Buffers used in Western Blot

1. Homogenisation Buffer

50 mM Tris-HCl pH 7.5
150 mM NaCl
1% Triton X-100
0.1% SDS
0.5% Sodium deoxycholate

2. Sample Buffer

De-ionized water
0.5 M Tris-HCl pH 6.8
Glycerol
1% Bromophenol blue

3. TBST Wash Buffer

20 mM Tris-HCl pH 7.6
137 mM NaCl
0.2% (v/v) Tween 20

4. Transfer Buffer

10% Methanol
25 mM Tris Base
192 mM Glycine
Electrophysiology Techniques

Rat Surgery

Adult male Sprague Dawley rats were used for acute in-vivo extracellular recording of trigeminal primary afferent fiber using isoflurane as an anesthetic agent (Isoflurane 2.5-3%). The trachea was cannulated and the rat was put on artificial respiration using ventilator. The carotid artery was cannulated to monitor blood pressure and to administer Evans blue dye and pentobarbital. The rat’s head was placed over a stereotaxic frame (KoPf) and a trephination on the left side of the skull bone was made and the recording electrode was lowered into the trigeminal ganglion through brain. The skin and muscle over the brainstem was removed and cervical C1 laminectomy was done. The brainstem was exposed by removing the dura overlying the brainstem with a pair of small scissors to facilitate the placement of a recording electrode into the caudal brainstem. Rat core body temperature, expired CO₂, heart rate and blood pressure were monitored throughout the experiment and were kept within physiological range of 36.8-37.1 °C, 20-50 mm Hg, 300-400 beats/min and 60-80 mm Hg, respectively. The depth of isoflurane anesthesia was assessed by periodically checking for the absence of a leg withdrawal reflex in response to strong pressure applied to the toes with a pair of small forceps. If this nociceptive reflex response was present, or if the mean blood pressure and or heart rate exceeded 90 mm Hg or 430 beats/min respectively, the concentration of inhaled isoflurane was increased until the reflex disappeared and the other parameters returned to their desired ranges. In this manner, adequate depth of anesthesia was maintained.
Extracellular Electrophysiological Recording Techniques

*In-vivo* extracellular recording of single trigeminal ganglion afferent fiber was done using a parylene coated tungsten electrode. The trigeminal ganglion is found 7-8 mm below the surface of cerebral cortex. The electrode was lowered slowly into the trigeminal ganglion through brain to record action potentials from single masseter muscle afferent fibers. A brush and blunt probe technique was used to search for masseter muscle afferent fibers. The skin overlying the masseter muscle was pulled, and pinch and pressure stimuli were applied to confirm that the receptor field of the afferent fiber was lying in the muscle. A stimulating electrode (2MΩ, parylene-coated tungsten) was lowered into the brainstem and electrical stimuli (50µs biphasic) were applied to record the antidromic action potentials. The orthodromic action potential evoked by mechanical stimulation of the masseter muscle was collided with the antidromic action potential to confirm the projection of a masseter muscle afferent fiber to the caudal brainstem. Conduction velocity was calculated by dividing the conduction distance (the distance between recording and stimulating electrodes) by the antidromic latency. The recorded masseter muscle afferent fiber activity was amplified (Afferent gain X1000; bandwidth 30-1,000 Hz) and data was analyzed by using off line software (Spike 2; Cambridge Electronic Design, Cambrige, UK).
Figure A.2. This diagram shows the methodology used to record and identify the masticatory muscle nociceptors that project to the caudal brainstem. The recording electrode connected to the computer through amplifier was used to record extracellular action potentials from the rat trigeminal ganglion neurons innervating the rat masseter muscle. A stimulating electrode was inserted into the caudal brainstem to evoke antidromic action potentials. The electronic von Frey hair connected to the computer was used to measure the mechanical threshold of masseter muscle afferent fibers.