PERIPHERAL MECHANISMS OF NERVE GROWTH FACTOR-INDUCED MASTICATORY MUSCLE SENSITIZATION: A MODEL OF TEMPOROMANDIBULAR DISORDERS PAIN

By

MANDEEP KAUR MANN

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ABSTRACT

It has been speculated that Nerve Growth Factor (NGF), a neurotrophic protein, may play a role in the pathophysiology of temporomandibular disorders (TMD). TMD are characterized by ongoing and activity-related pain in the jaw joint and muscles, and are more prevalent in women than in men. Indeed, intramuscular injection of human NGF into the masseter (jaw closer) muscle of healthy male subjects, though not acutely painful, does cause a prolonged period of activity-related muscle pain reminiscent of TMD symptoms [187]. The present study tested whether this human NGF-induced mechanical sensitization is mediated, in part, through a decrease in the mechanical sensitivity of primary afferent fibers that innervate the masseter muscle. In this randomized, blinded study, the effect of intramuscular injection of rat NGF (2.5 and 25 μg/ml) into non-inflamed masseter muscle on the mechanical threshold (MT) of rat masseter muscle afferent fibers was investigated for 6 hours post-injection. The level of plasma protein extravasation into the masseter muscle was also measured to determine if exogenous rat NGF causes muscle tissue inflammation. The results of the study indicated no treatment or sex differences in evoked afferent discharge, suggesting that administration of rat NGF did not excite afferent fibers in male or female rats. There was also no significant treatment, sex or time effect on the relative MT of masseter muscle afferent fibers. Intramuscular injection of rat NGF did not cause muscle inflammation. Nevertheless, some sex-related differences in the baseline properties of masseter muscle afferent fibers were observed. A significant log-linear relationship was identified between fiber CV and baseline MT for afferent fibers in males but not for fibers in females. There was also a positive correlation between baseline MT and estrogen levels for a subgroup of slow Aδ fibers (2-10 m/s) in females, but not in males. The finding that rat NGF
does not evoke significant masseter muscle afferent discharge is consistent with previous results, which indicated injections of human NGF are not painful in human subjects. The failure of intramuscular injection of rat NGF to affect the mechanical sensitivity of masseter muscle afferent fibers suggests that central mechanisms may be more important for human NGF-induced mechanical sensitization.
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<tr>
<td>Aδ</td>
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<tr>
<td>Ach</td>
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<td>Adenosine Monophosphate</td>
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<td>Brain-Derived Neurotrophic Factor</td>
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<td>Degree Celsius</td>
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<td>Ca²⁺</td>
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<td>Calcitonin Gene Related Peptide</td>
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<td>Cyclic AMP Response Element Binding</td>
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<td>Dorsal Root Ganglion</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>ER α/β</td>
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<td>Extracellular signal-regulated kinase</td>
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<td>g</td>
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<tr>
<td>GDNF</td>
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<td>Proton(s), Hydrogen ion(s)</td>
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<td>kDa</td>
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<td>LKB4</td>
<td>Leukotriene B4</td>
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<td>μl</td>
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<tr>
<td>μg</td>
<td>Microgram(s)</td>
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<tr>
<td>μg/g</td>
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<tr>
<td>μg/kg</td>
<td>Microgram(s) per kilogram</td>
</tr>
<tr>
<td>μg/ml</td>
<td>Microgram(s) per milliliter</td>
</tr>
<tr>
<td>M</td>
<td>Moles per liter</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mg/ml</td>
<td>Milligram(s) per milliliter</td>
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<tr>
<td>mm/hr</td>
<td>Millimeters per hour</td>
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<tr>
<td>Symbol</td>
<td>Term</td>
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<tr>
<td>ml</td>
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<tr>
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<td>Minute(s)</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter(s)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>m/s</td>
<td>Meters per second</td>
</tr>
<tr>
<td>ms</td>
<td>Millisecond(s)</td>
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<tr>
<td>MT</td>
<td>Mechanical Threshold</td>
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<td>Sodium</td>
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<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<tr>
<td>ng</td>
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<td>NGF</td>
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<td>Nanometer(s)</td>
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<tr>
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<td>Nanomolar(s)</td>
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<td>NMDA</td>
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<tr>
<td>NS</td>
<td>Nociceptive Specific</td>
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<tr>
<td>NSAID</td>
<td>Non-Steroidal Anti-Inflammatory Drugs</td>
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<tr>
<td>NT</td>
<td>Neurotrophin</td>
</tr>
<tr>
<td>N-type</td>
<td>Neuron-type</td>
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<td>PGE₂</td>
<td>Prostaglandin E₂</td>
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<td>PGI₂</td>
<td>Prostaglandin I₂</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PLC-γ</td>
<td>Phospholipase C gamma</td>
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<tr>
<td>PMN</td>
<td>Polymodal Nociceptor(s)</td>
</tr>
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<td>Definition</td>
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<tr>
<td>PPT</td>
<td>Pressure Pain Threshold(s)</td>
</tr>
<tr>
<td>PTOL</td>
<td>Pressure Tolerance Threshold(s)</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram(s)</td>
</tr>
<tr>
<td>pg/ml</td>
<td>Picogram per milliliter</td>
</tr>
<tr>
<td>R</td>
<td>Regression Coefficient</td>
</tr>
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<td>Radioimmunoassay</td>
</tr>
<tr>
<td>s</td>
<td>Second(s)</td>
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<tr>
<td>SE</td>
<td>Standard Error</td>
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<td>Shc</td>
<td>Src homologous and collagen-like adaptor protein</td>
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<td>Substance P</td>
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<td>Trigeminal Ganglion</td>
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<td>TMD</td>
<td>Temporomandibular Disorder(s)</td>
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<td>Temporomandibular Joint</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>Trk</td>
<td>Tropomyosin-related (or Tyrosine) kinase</td>
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<tr>
<td>TRPV1</td>
<td>Transient Receptor Potential Vanilloid 1</td>
</tr>
<tr>
<td>TSNC</td>
<td>Trigeminal Sensory Nuclear Complex</td>
</tr>
<tr>
<td>V</td>
<td>Trigeminal Nerve</td>
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<tr>
<td>V₁</td>
<td>Ophthalmic Branch of the Trigeminal Nerve</td>
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<tr>
<td>VF</td>
<td>Von Frey</td>
</tr>
<tr>
<td>VPM</td>
<td>Ventral Posterior Medial</td>
</tr>
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<td>VR1</td>
<td>Vanilloid Receptor 1</td>
</tr>
<tr>
<td>vs.</td>
<td>Versus</td>
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<tr>
<td>WDR</td>
<td>Wide Dynamic Range</td>
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Chapter 1. INTRODUCTION

Muscle Pain

Chronic muscle pain is a major health problem [64,165]. The prevalence of chronic musculoskeletal pain conditions such as low back pain, neck pain, tension-type headache, temporomandibular disorders and fibromyalgia ranges from 5 to 30% [94]. Women report chronic muscle pain more often than men [43,165]. In the Canadian population the prevalence of fibromyalgia, a musculoskeletal disorder characterized by diffuse pain originating from tender or trigger points all over the body [94], is ~3 times greater among women than men [165].

Although the reasons for these sex-related differences in the prevalence of chronic musculoskeletal pain conditions [165] are not fully understood, various factors such as physiological differences in men and women (such as differences in endocrine hormones) are thought to play a role [165]. Differences in sex hormone levels, for example, during the menstrual cycle or with the use of oral contraceptives can increase the risk for developing musculoskeletal disorders in females [165]. In addition to physiological factors, cultural differences and gender-role expectations may also contribute to gender-differences in chronic pain conditions [25,163]. Some examples of these gender-stereotyped pain expectations include a lower willingness to report pain and higher pain endurance in men as compared to women [164]. Also, there are differences in the way men and women perceive and cope with pain and other stressors [184]. Therefore, multiple factors might be responsible for the female predominance in chronic pain conditions.

Chronic pain has a profound effect on the patient’s psychological and emotional state resulting in a poor quality of life [198]. It has been shown that chronic pain patients have an
elevated level of stress, anxiety and depression and are also more likely to suffer from other psychobiological (or chronic pain) disorders [62,114,137]. Chronic pain and depression have been associated with catastrophizing (“negative emotional or cognitive response to pain involving elements of magnification, helplessness and pessimism” [80]), which is also considered one of the contributing factors for sex-differences as women report more catastrophizing than men [80]. Other factors, which include physical dysfunction and social isolation as a result of debilitating pain, can significantly decrease the health-related quality of life of chronic pain patients [20].

Craniofacial Pain

The craniofacial region (head and face) is one of the most common sites of pain in the human body [117]. For example, population studies have suggested that approximately 8% of the population may suffer from a temporomandibular disorder, which results in chronic musculoskeletal pain that can involve the temporomandibular joint and masticatory muscles [189]. Considering the role the orofacial region plays in basic activities such as eating, drinking, speech, and expression of emotions, chronic orofacial pain has a special biological, emotional and psychological significance (to the patient) [189].

Temporomandibular Disorders (TMD)

Temporomandibular disorders (TMD) are the most common chronic orofacial pain condition [128]. Temporomandibular disorders are characterized by pain in the temporomandibular joints (TMJ) and/or muscles of mastication such as the masseter (jaw-closer) muscle [20,21]. A research diagnostic criteria is used to classify TMD into three groups: masticatory muscle disorders, TMJ disc displacement and TMJ disorders (such as
arthralgia and osteoarthritis) depending on the signs and symptoms reported by the patients [78,144]. TMD patients with myofascial pain (the majority of cases [136]) experience local or regional dull, aching muscle pain (due to the presence of localized tender sites and/or trigger points) that increases during function [56,144]. Some patients also report headache and pain in the neck, teeth or ear region as a result of pain referral [151]. In addition to pain during activities involving jaw opening, other signs and symptoms of TMD include limited jaw movement (or deviation in the mandibular range of motion) and jaw-clicking/popping sounds during mandibular function [69,74,77,151,172,181,209].

Approximately 9-15% of the adult population in North America will suffer from TMD during their lifetime [59,77,129,172,183,209] and population-based studies indicate that 8-15% of women and 3-10% of men have ongoing pain in the craniofacial region [128]. Research investigating risk factors for TMD has found that both age and sex appear to strongly influence TMD prevalence [128]. Studies show that TMD is 2 to 5 times more prevalent in women as compared to men and this differential prevalence is even greater in clinical samples [151]. Studies have shown that TMD pain is rare in children prior to puberty [128,130] and the age of onset is almost always after puberty [151]. Among women, TMD prevalence is greater during the reproductive years than the postmenopausal years [129,151]. It has been reported that use of exogenous estrogen (oral contraceptives and post-menopausal hormone replacement therapy) significantly increases the risk for developing TMD [130], however, the evidence supporting the role of estrogen is not definitive at this point [129,130]. The presence of other factors such as pain in other body sites (e.g. back pain, abdominal pain), depression and bruxism (or teeth clenching) are also thought to increase the risk for developing TMD [128].
Despite considerable research, the pathophysiology of TMD-related chronic pain still remains unclear [186]. Due to the lack of any evidence of local pathology in the majority of cases, many clinicians considered TMD to be a psychosomatic disorder in the past [198]. However, it is now believed that TMD is a "multifactorial" disorder resulting from biological/physiological, psychological and social factors [96,114]. It is thought that biological factors such as modulation of endogenous pain pathways and cyclic hormonal fluctuations could be involved in pain sensitivity observed in TMD patients [85]. TMD patients are more sensitive to experimentally induced pain (thermal and ischemic stimuli) than healthy subjects [137]. This is thought to be due to alterations in the central nociceptive pathways, although the contribution of peripheral mechanisms localized to the orofacial region cannot be ruled out [137]. Also, it is known that pain thresholds of TMD patients vary across the menstrual cycle [107], therefore suggesting a role for endocrine factors in the pathophysiology of TMD. Studies have shown that TMD patients have high levels of depression and anxiety and report frequent symptoms of stress as compared to healthy subjects [19,45,62,120,153]. These psychological factors are thought to contribute to enhanced pain sensitivity [57], however, it is not clear whether these factors are a consequence or a cause of TMD-related chronic pain. Recently, it has been suggested that genetic factors may also play a role in the pathophysiology of TMD. Specifically, the three genetic variants of the catecholamine-O-methyltransferase gene are thought to contribute to variations in pain sensitivity observed in the human population [67]. It has been suggested that in humans the activity of catecholamine-O-methyltransferase inversely correlates with pain sensitivity and the risk for developing TMD [67].
At present, TMD pain is pharmacologically managed with analgesics such as non-steroidal anti-inflammatory drugs (NSAID), anti-depressants, opioids and other miscellaneous agents [56,64,69,97]. Unfortunately, most currently available analgesics have shown limited effectiveness in treating TMD-related chronic pain and many are associated with significant adverse effects [69], thus increasing the need for the development of novel analgesics. The lack of knowledge regarding the etiology and pathophysiology of TMD makes it difficult to develop new pharmacological therapies. Research intended to aid in the elucidation of neural mechanisms responsible for causing and maintaining chronic pain requires the use of animal models that mimic clinical signs and symptoms experienced by patients [64]. Unfortunately, at present there are no animal models for chronic pain conditions like fibromyalgia or TMD, and thus information related to the effectiveness of novel analgesic therapies has been limited to that which can be garnered from clinical and experimental pain studies in human subjects.

**Experimental Methods to Cause and Measure Muscle Pain**

Experimental pain research (basic and clinical research) often employs painful mechanical, thermal (heat/cold), electrical and chemical (hypertonic saline, glutamate, serotonin) stimuli to assess changes in pain thresholds associated with musculoskeletal disorders such as TMD [165]. In a laboratory setting, use of noxious pressure is considered a sensitive test to assess the characteristics of experimental musculoskeletal pain and to study its underlying mechanisms [165]. There is extensive evidence that suggests that the use of noxious mechanical pressure is the most useful (or sensitive) method for detecting changes in pain thresholds that occur during musculoskeletal conditions like fibromyalgia or TMD [125,126,165]. In human clinical studies, instruments such as a pressure algometer are used
to detect pressure pain threshold (PPT), which is a measure of the mechanical pressure that is first perceived as being painful by the subjects and pressure tolerance threshold (PTOL), which refers to the maximum pressure tolerated by the subject [187]. It is believed that pressure pain thresholds reveal information about the sensitivity of nociceptive fibers in superficial (skin) and deep tissues (muscles, tendons etc.) [124,165]. Indeed, animal studies have shown that the mechanical threshold of primary afferent fibers, which is usually measured with von Frey filaments or an instrument known as an electronic Von Frey hair, can be altered under experimental conditions that result in pain and mechanical sensitization in human subjects [36]. An injection of a chemical or algogenic substance such as hypertonic saline [185], glutamate [40,41,188], or capsaicin [178] has also been shown to induce experimental muscle pain or to model clinical muscle pain [43,165]. The sex-related differences present in the general population suffering from chronic pain have also been observed in animal and human studies. In animal research, glutamate injections have been shown to evoke a greater response in female rats as compared to males [38]. Similarly, in human studies female subjects have reported greater pain scores than men in response to glutamate injections into the masseter muscle [38,188].

Anatomy and Physiology of Trigeminal (Craniofacial) Pain Pathway

First-order Processing (General Pain Pathway)

Painful signals from the orofacial region are carried to the brain via the trigeminal pain pathway. Painful stimuli (Mechanical, Thermal, Chemical, Electrical) or noxious injury results in the activation of sensory fibers found in the skin, ligaments, muscles, tendons and other deep tissues called primary afferent or first-order fibers. These nerve fibers (and their endings) are known as nociceptors, as they are thought to be activated by potentially painful
stimuli and are involved in the conduction of pain signals to the brain [3,64,122]. There are two main types of cutaneous nociceptors, known as Aδ mechanical nociceptors and C-polymodal nociceptors (PMN) [122,199]. Aδ fibers have conduction velocities of 2-30 m/s [29,95], whereas C fibers have slower conduction velocities (< 2.0 m/s) [3,29,160]. It is thought that the sharp fast component of pain (acute phase) is mediated by Aδ activation and the delayed and longer phase of dull, burning pain reflects the activation of C fibers [3,115]. Aδ nociceptors are activated by strong mechanical stimuli, such as pricking or crushing of the skin and some Aδ fibers also respond to thermal and chemical stimuli [47]. C-polymodal nociceptors respond to all types of noxious stimuli such as mechanical, thermal and chemical stimuli [199]. Activation of these peripheral afferent fibers results in a sensation of pain as a consequence of transfer of nociceptive input from the periphery through spinal or trigeminothalamic pathways in the CNS to the cortex [113].

Pain from the craniofacial region is mainly transmitted to the brain via the primary afferent fibers of the fifth cranial or trigeminal nerve (V). The trigeminal nerve is the largest and most complex of the 12 cranial nerves [29]. The branches of the trigeminal nerve (V) innervate most of the craniofacial regions with the exception of some parts of the head, which are innervated by branches of the upper cervical spinal nerves or other cranial nerves [73,117]. In the periphery, the trigeminal nerve has three major divisions: the ophthalmic (V₁) branch, the maxillary (V₂) branch, and the mandibular (V₃) branch. The ophthalmic and maxillary branches are pure sensory nerves, whereas the mandibular branch consists of both sensory and motor fibers. The rat masseter muscle is innervated by the masseteric branch of the mandibular nerve (V₃ division). Thus, the trigeminal is a mixed nerve that is considered functionally equivalent to a peripheral spinal nerve [29,73]. The sensory branches of the
trigeminal nerve innervate most of the facial skin, the anterior two-thirds of the tongue, the dura mater of the anterior and middle cranial fossae, the tooth pulp and surrounding gingival, the periodontal tissues and the craniofacial muscles. Most of the sensory information from the face, conjunctiva, oral cavity, and dura mater, is conveyed by the Aβ, Aδ and C primary afferent fibers of the trigeminal nerve [29,73,117]. In contrast to Aδ and C fibers, which were discussed previously, Aβ fibers have large myelinated axons with fast conduction velocities (> 35 m/s) and convey various sensations including light touch and proprioception [29]. The motor fibers of the trigeminal nerve innervate the masticatory muscles [73].

The primary afferent sensory fibers of the trigeminal nerve have their cell bodies within the bilateral trigeminal ganglia (also called the semilunar or Gasserian ganglia) [29,73]. The trigeminal ganglion (TG) is located at the base of the skull in the posterior cranial fossa across the superior border of the petrous temporal bone [29]. The ganglion is crescent shaped and is somatotopically organized according to the afferent projections from each division of the trigeminal nerve (i.e. V₁, V₂ and V₃) [29].

Masticatory muscles are richly supplied with muscle spindle afferent fibers (Aα fibers) that function as proprioceptors [173]. The cell bodies of these jaw muscle spindle afferent fibers are located in the trigeminal mesencephalic nucleus in the CNS. All other muscle afferent fibers (i.e. non-spindle fibers) have their cell bodies in the trigeminal ganglion and send their axons to the trigeminal sensory nuclear complex [173]. However, spindle afferent fibers have a separate ganglion, the mesencephalic nucleus that extends from the rostral end of the principal nucleus to the superior colliculus in the midbrain [73,208]. The axons of the cell bodies in the mesencephalic nucleus project to the ipsilateral trigeminal motor nucleus (as well as the supra- and inter-trigeminal regions and lateral reticular
formation of the medulla) where they make synaptic connections with the motorneurons that send their efferent axons to masticatory muscles (the temporalis, the masseter, and the pterygoids) [109,173,174]. The mesencephalic nucleus is therefore involved in monosynaptic reflexes associated with control of the force of bite and chewing [208]. The anatomy of the trigeminal system is unique from that of the spinal system and the major advantage of using this system is that by recording from the TG, proprioceptive inputs can be excluded, which is not the case in the dorsal root ganglion.

Mechanical Transduction

The mechanism of mechanotransduction ("cellular signal transduction in response to a mechanical stimulus" [2]) is not known [49,201]. It is believed that like many chemical stimuli (e.g. protons, capsaicin, ATP), a mechanical stimulus results in the activation of an inward cationic current at the primary afferent terminal [2,49,201]. Some believe that this pathway may involve stretch-sensitive ion channels found on cell surface membranes [2]. As some voltage- and ligand-activated ion channels such as N-methyl-D-aspartate (NMDA), N-type Ca^{2+} channels, and Ca^{2+}-dependent K^{+} channels also exhibit mechanosensitivity, it is possible that they might also play a role in mechanotransduction [139].

Second-order Processing

The central branches of the sensory and motor fibers of the trigeminal nerve enter and exit the brain separately as the sensory (afferent) and motor (efferent) trigeminal roots [73], terminating on second-order neurons within the trigeminal sensory nuclear complex (TSNC) [196] or masticatory muscles, respectively [64,73]. The TSNC is a bilateral, multinucleated structure in the dorsolateral brainstem that extends from the pons to the upper cervical spinal
cord [73,173]. The TSNC receives afferent inputs from trigeminal as well as other cranial nerves (e.g. VII, IX, X, XII), and from upper cervical nerves [173]. Craniofacial sensory information is first processed in the TSNC and then relayed to other parts of the CNS [173]. The TSNC consists of several nuclei: principal (or main), sensory nucleus and spinal trigeminal nucleus [2]. The spinal trigeminal nucleus is further divided into three subnuclei known as oralis, interpolaris and caudalis [73,173,208]. The subnucleus caudalis (the most caudal component of the TSNC) serves as the main brainstem site for the processing and relay of nociceptive inputs from the craniofacial region [174,196,208]. The subnucleus caudalis is a laminated structure, which is continuous with and resembles the dorsal horn of the cervical spinal cord [73,173]. Although the subnucleus caudalis is considered the principal site in the brainstem responsible for the relay of trigeminal nociceptive information, the rostral components of the TSNC, subnuclei interpolaris and oralis, are also thought to contribute to craniofacial nociceptive transmission, particularly that arising from the oral cavity [173]. Aδ and C primary afferent fibers innervating the masticatory muscles send their projections primarily to the subnucleus caudalis, caudal portions of the subnucleus interpolaris and the medullary and upper cervical dorsal horns [44,174].

Based on their response to cutaneous stimulation, nociceptive neurons in the TSNC are divided into 2 main types. There are nociceptive-specific (NS) neurons that respond to noxious stimuli only (transmitted via the Aδ and C fibers) and wide-dynamic range (WDR) neurons that respond to both noxious and tactile stimuli carried by Aβ, Aδ and C fibers [173]. Synaptic transmission between primary afferent fibers and second-order neurons is mediated by a number of chemical neurotransmitters such as the amino acid glutamate, and modulated by the release of neuropeptides such as substance P (SP) and calcitonin gene-
related peptide (CGRP) [160,195,199]. From the TSNC, the second-order neurons carry trigeminal sensory information centrally and synapse on the third-order neurons in the ventral posterior medial (VPM) nucleus of the thalamus, which connect to the primary and secondary somatosensory (S-I and S-II) cortex [73,109,173].

**Peripheral and Central Sensitization**

Pain in the masticatory muscles may result from central hyperexcitability in the trigeminal pathway as well as sensitization of the primary afferent fibers peripherally. Primary nociceptive afferents can become sensitized as a result of various factors such as tissue trauma, inflammation, and ischemia [145]. Peripheral injury (tissue damage) results in the synthesis and release of various inflammatory mediators that induce inflammation and edema as part of the healing process. These mediators include bradykinin, substance P, histamine, serotonin (5-HT), glutamate, acetylcholine (Ach), ATP, ions (H\(^+\), K\(^+\)), cholecystokinin, and eicosanoids such as prostaglandin E\(_2\) (PGE\(_2\)), PGI\(_2\), and leukotriene B\(_4\) (LKB\(_4\)), and nerve growth factor (NGF) [64]. These mediators activate and sensitize nociceptors and recruit new nociceptors to enhance pain perception [64,93]. Dying/Damaged cells release K\(^+\), which activates nociceptors. Nociceptor activation also results in the release of chemicals such as substance P (SP) and calcitonin gene-related peptide (CGRP), from other nerve terminals in the periphery (through axon reflex), which augment the effects of inflammatory agents by causing vasodilation and increased capillary permeability. Bradykinin (released from blood vessels) and other chemical agents such as 5-HT released from platelets, histamine from mast cells and eicosanoids from various cellular elements contribute to sensitization of nociceptors, either by opening ion channels, or by activating second messenger systems [199].
The properties of the afferent fibers change and this process is known as peripheral sensitization [72,146]. Peripheral sensitization refers to a decrease in the activation threshold of afferent fibers (allodynia) and an increase in the response to nociceptive stimuli (hyperalgesia). Also, it has been suggested that peripheral sensitization occurs due to the recruitment of ‘silent’ nociceptors [3,72], which are unresponsive to acute thermal or mechanical stimuli in healthy tissue. Peripheral sensitization of nociceptors results in hyperalgesia, which refers to an increase in the pain produced by a noxious stimulus applied to the site of inflammation (primary hyperalgesia). [72,113,199]. In response to nociceptive stimuli, and particularly during tissue inflammation, there is a change in the properties of the primary afferent fibers and central neurons. The release of various chemical mediators in the periphery causes repetitive activity in primary afferent fibers (Aδ and C). This barrage of input results in the central release of neurotransmitters and neuropeptides that act on second-order neurons to increase neuronal excitability and expand their receptive fields. This phenomenon is called central sensitization. Central sensitization results in the amplification and persistence of nociceptive signals that eventually evoke pain [72,113,143,161,173,202]. It is theorized that central sensitization is the mechanism underlying pain referral and clinical signs of secondary hyperalgesia, which refers to the sensitization of non-inflamed tissue surrounding the site of inflammation in the periphery.

Sex Hormone Levels and Pain

The higher prevalence of chronic orofacial pain conditions in women is thought primarily to be a result of sex-differences in gonadal hormone levels (i.e. biological differences) and their effect on pain mechanisms and neurotransmitters [129]. However,
differences in pain modulatory pathways, receptor density/population and psychosocial factors are also thought to play a role.

*Genomic and Non-genomic Effects of Estrogen*

There is a potential for hormone levels to modulate pain mechanisms at the peripheral and central level [151]. The major female reproductive hormone, estrogen, is known to have various genomic and non-genomic effects via its actions on estrogen receptors (ER-α and ER-β) and other membrane-bound or cytoplasmic receptors/proteins, respectively [177,179]. The genomic effects of estrogen involve internalization and nuclear translocation of activated estrogen receptors that result in modulation of gene transcription and synthesis of various neuropeptides and neurotransmitters. However, various non-genomic effects of estrogen have also been identified, which involve activation of ligand-gated ion channels or G-protein coupled processes such as modulation of signal transduction pathways (mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and tyrosine kinase pathways) [26,170,177]. The non-genomic effects are thought to occur rapidly (within a few minutes) as compared to the genomic effects, which are characterized by a delayed onset [170]. The non-genomic effects of estrogen may be responsible for its role in nociceptive transmission. In the CNS, estrogen can influence craniofacial pain transmission as estrogen receptors have been found on dorsal root and trigeminal ganglion neurons [24,28,179] and trigeminal nucleus caudalis neurons [90] as well as in the TMJ region in the rat [28,151,206]. Also, a study has shown that estrogen and progesterone receptors are present in the TMJ region of men and women [1,151]. Estrogen may also alter primary afferent fiber excitability by increasing the transcription of receptors such as N-methyl-D-aspartate (NMDA) and NGF receptors or by increasing the release of inflammatory mediators at the peripheral level [138,151].
Various studies have shown that pain sensitivity (to experimentally induced pain) of healthy females changes during the menstrual cycle [163]. A study has shown that pain intensity of female TMD patients varies across the menstrual cycle with peak pain levels during the first three days of menstruation and another smaller peak after ovulation [129]. These peaks in perceived pain intensity correspond to stages in the cycle when estrogen levels are low i.e. late luteal phase (post-ovulation) and during menstruation [129]. Therefore, estrogen levels seem to be associated with (or related to) the level of pain experienced by female TMD patients. However, there are contradicting studies that show that use of exogenous estrogen (hormone replacement therapy and oral contraceptives) may increase the risk for TMD [130] and that high estrogen levels are associated with increased (although not significantly higher) pain sensitivity to thermal stimuli [84,163]. Thus, further research is required to clarify (or clearly understand) the exact role of estrogen in TMD-related pain.

The evidence from animal studies suggests that estrogen modulates pain pathways in the CNS. The trigeminal pain pathways of chronic headache patients are thought to be extremely sensitive to various factors that can act as pain triggers by causing the release of neurotransmitters that activate or sensitize the pathway [138]. It is believed that estrogen (or rapid changes in estrogen levels) may act like a trigger factor in chronic TMD patients (either acting directly or by causing an increase in neurotransmitter release), resulting in cyclic changes in TMD pain.

Sex-related differences have also been identified in experimental pain studies conducted on rats and humans. Glutamate injections into the rat TMJ and masseter muscle are known to produce a significantly greater jaw muscle reflex and afferent fiber response in
females [36,38]. Sex-related differences in glutamate-evoked jaw muscle reflexes are attenuated by gonadectomy but restored by estrogen replacement, which suggests that estrogen levels play an important role in trigeminal nociceptive processing [39]. Similarly, in the human study female subjects report glutamate injection to be more painful than males and also have a greater area of perceived pain in the glutamate injected masseter muscle [38]. Nevertheless, the role that sex hormones and in particular estrogen may play in the modulation of craniofacial pain needs to be further investigated.

**Rat Estrus Cycle Stages**

In pain research conducted on animals, estrus cycle stages (equivalent to the menstrual cycle in humans) are usually examined to investigate sex-dependent effects. The female rat estrus cycle is 4 or 5 days long and consists of four different stages: diestrus, proestrus, estrus and metestrus. Estrogen (Estradiol-17β) levels are the lowest at the end of metestrus and beginning of diestrus. Estrogen levels start to increase at the end of diestrus and peak during the mid-proestrus stage followed by a rapid decline at the beginning of estrus [32,116,180]. Progesterone concentration peaks twice during the 4-day cycle: a small peak during the metestrus stage and a larger peak during the second half of proestrus [32,116,180]. Progesterone levels are low during estrus and diestrus and drop to their lowest point at the beginning of proestrus (at the same time as the estrogen peak). These hormone fluctuations lead to changes in the cell morphology of the vaginal epithelium of the rat during different stages that can be assessed by examining a vaginal lavage [180]. Animal [91,119,140,171] and human [7,75,163] studies have shown that pain sensitivity changes during the estrus and menstrual cycle, respectively, possibly due to alterations in hormone levels. High estrogen levels have been associated with enhanced pain sensitivity and
therefore perception of pain is thought to change across the menstrual cycle [163]. In female rats, tail and hindpaw pressure pain thresholds during proestrus and estrus have been found to be lower than those during diestrus and metestrus [86,119]. Since, cyclic changes in sex hormone levels and the associated changes in estrus stages of an animal can influence pain responses, it is important to monitor these changes in an experimental pain study.

**Role of Nerve Growth Factor in Pain Mechanisms**

*Introduction to Nerve Growth Factor*

Nerve growth factor is one of the chemicals released at the site of injury. NGF is a neurotrophic protein molecule essential for the growth and survival of sympathetic and small diameter afferent neurons (nociceptive sensory neurons) [5,156] and is also involved in neuronal function and plasticity [105]. It has been suggested that NGF may also have a role in glucose and lipid metabolism and in the control of energy balance and feeding behavior [50]. NGF belongs to the neurotrophin family, which also includes the brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and neurotrophin 4/5 (NT-4/5) [4,11]. Recently, two more neurotrophins, NT-6 and NT-7 have been identified in fish but they are not present in mammals [105]. NGF was the first neurotrophic factor to be characterized [105]. The structure of NGF is comprised of 2α, 2β, and 2γ subunits and one or two zinc ions [104]. The β subunit of the complex is released as a result of autocatalytic cleavage initiated by the loss of zinc ions. The biologically active form of NGF is NGF-β which is a dimeric molecule (2β subunits) weighing 26.8 kDa with each monomer consisting of 118 amino acids and three disulfide bridges [104,142]. The neurotrophins are structurally related and have an overall sequence homology of 50% [104].
Sources of Nerve Growth Factor (Central and Peripheral)

Neurons as well as non-neuronal cells such as mast cells, fibroblasts, eosinophils, T- and B-lymphocytes, and epithelial cells synthesize NGF [30,63]. NGF and other neurotrophins can also be synthesized and secreted by sympathetic and sensory target organs [105]. There are high levels of NGF present in the CNS where it is known to play a crucial role in growth and plasticity [4]. During development, NGF is secreted by target tissues of sensory axons/neurons [105]. Also, during peripheral nerve injury, NGF synthesis is initiated in Schwann cells and fibroblasts within the injured nerve by cytokines released in response to the injury (for survival and regeneration of the injured nerve) [105].

NGF Receptors

NGF exerts its biological effects by acting on two different receptors found on target cells: tyrosine kinase receptor A (TrkA; tropomyosin related kinase A) and p75 receptor [4]. The TrkA receptor is a tyrosine kinase consisting of intracellular, transmembrane and extracellular domains and NGF binding results in phosphorylation of key transduction-related proteins or ion channels causing downstream receptor modulation [4,63]. The TrkA receptor has a very slow dissociation rate for NGF and is therefore called a high-affinity NGF receptor [52]. In the periphery, TrkA receptors are expressed on primary sensory neurons, inflammatory cells and sympathetic neurons [11,204]. It is believed that the cell bodies of 60% of the primary afferent fibers in the adult rat trigeminal ganglion express the TrkA receptor [110,146]. There are two more types of Trk receptors, TrkB and TrkC, which bind to other neurotrophins. Trk receptor subtypes have a different amino acid sequence in the extracellular domains that affects their ligand interactions [105]. NGF is a selective
agonist for TrkA receptors, BDNF and NT-4 are selective agonists for TrkB receptors and NT-3 activates TrkC receptors but also has some affinity for the other subtypes [105].

The p75 receptor is a member of the superfamily of TNF (tumor necrosis factor) receptor-related molecules that has extracellular and transmembrane domains. The p75 receptor can bind all neurotrophins and thus is not only activated by NGF but also by BDNF, GDNF, NT-3 and NT-4/5 [4]. The p75 receptor is known as the low-affinity NGF receptor because it has fast association and dissociation rates for NGF binding [52]. The binding of NGF to TrkA and p75 receptors results in the activation of downstream signal transduction pathways that mediate the physiological effects of NGF [51].

**Downstream Signaling Pathways**

NGF binding to the TrkA receptors causes receptor activation and dimerization, which results in transautophosphorylation and activation of intracellular signaling cascades like extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLC-γ) pathways [51,105]. The Src homologous and collagen-like (She) adaptor protein binds to the activated Trk receptor and its phosphorylation results in an increase in the activities of PI3K and Akt (protein kinase B), which are involved in neuronal survival [51]. The She binding also increases the activities of Ras and ERK, which can influence transcriptional events such as induction of cyclic AMP-response element binding (CREB) transcription factor that is involved in neurite outgrowth, cell cycle and synaptic plasticity [51]. It is thought that sustained activation of the ERK pathway requires the internalization of the NGF-TrkA receptor complex into membrane vesicles [105,207]. The PLC-γ signaling cascade is initiated by direct PLC-γ binding to the activated Trk receptor and results in protein kinase C activation and the release of inositol phosphates and calcium
It has been suggested that activation of these signaling pathways may result in the modulation of ion channels such as the capsaicin receptor channel known as TRPV1 or VR1 channel [51,54]. The p75 receptor also activates signaling components such as Jun N-terminal kinase (JNK), Nuclear factor-κB (NF-κB) and ceramide to mediate processes such as apoptosis or cell death [51].

NGF Levels

As compared to neurotransmitters, neurotrophic factors such as NGF exist in very low concentrations, therefore their detection is very difficult [106]. The maximum peak level of NGF in the interstitial fluid of the brain of patients with severe head injury was found to be 1100 pg/ml [200]. There are sex-related differences in the levels of serum NGF in men and women [141]. A study using an enzyme-linked immunosorbent assay (ELISA) (measuring unbound β-NGF concentration) has shown that men have significantly higher serum NGF concentration (40.8 ± 10.8 pg/ml) than women (8.2 ± 1.4 pg/ml in the follicular phase and 14.4 ± 2.9 pg/ml in the luteal phase) [141]. The differences in the NGF concentrations in both phases of the menstrual cycle were reported to be statistically significant. Therefore, it has been suggested that female sex hormones (and androgens) influence circulating NGF levels [141]. There is a growing body of evidence that suggests that NGF may have a role in the neuroendocrine system, in particular the hypothalamic-pituitary-adrenal axis [141]. NGF and its receptors have been identified in the hypothalamus and glands such as pituitary, gonads, thyroid, pancreas etc.

It has been shown that estrogen receptors colocalize with NGF receptors (both TrkA, B and p75) in the neurons of the dorsal root ganglion, cerebral cortex, striatum and hippocampus [179]. In fact, it was found that estrogen receptor and NGF mRNA expression
is differentially regulated by the ovarian status of female rats [179]. It has been suggested that estrogen sensitivity may be a general feature of central and peripheral targets of NGF [72,179]. Since changes in endogenous NGF levels have been associated with various pain conditions (discussed below), the association between NGF and estrogen receptors may be one of the factors responsible for the higher prevalence of these conditions in the female population.

Clinical Consequences of Elevated NGF

Effects of an Increase in Exogenous NGF Levels

NGF levels change during some neurodegenerative disease states and other pathological conditions [50,100]. NGF levels have been increased exogenously in experiments investigating diabetic peripheral neuropathies. There is a loss of dorsal root ganglion neurons and degeneration of Schwann cells and small diameter sensory neuronal fibers during diabetic peripheral neuropathies [127]. Experimentally, it has been shown that induction of diabetes with streptozotocin causes a decrease in NGF levels in the sensory neurons and target tissues of rats [31,82] and also human diabetic patients have low NGF levels in the skin [10]. Also, studies have shown that NGF uptake and retrograde transport mechanisms are impaired in animal models of diabetes [101,111]. Therefore, it was thought that NGF might have a beneficial role in the treatment of this condition as it is involved in promoting neuronal growth and survival and preventing neuronal damage of small diameter sensory and autonomic neurons [14].

Initial animal studies showed that increasing NGF levels in experimental models of peripheral neuropathy had beneficial effects (prevented the decrease in SP and CGRP levels in the sensory ganglia and prevented the loss of sensitivity to thermal noxious stimuli i.e.
prevented the onset of symptoms associated with polyneuropathy) [14,15,83]. This led to phase I (0.03-1 μg/kg human NGF) and II (0.1 or 0.3 μg/kg human NGF three times a week for 6 months) clinical trials that involved subcutaneous administration of human NGF into healthy human volunteers and diabetic patients suffering from polyneuropathy [16,157]. In addition to demonstrating the beneficial effects of human NGF, these trials also revealed that human NGF administration results in serious dose-limiting, pain-related systemic side effects such as hyperalgesia at the site of injection, diffuse myalgias or arthralgias and leg cramps (more frequent and severe with higher doses) [16,157]. The positive results from phase II clinical trials resulted in phase III clinical trial (0.1 μg/kg human NGF three times a week for 48 weeks) that also reported pain-related side effects in response to human NGF administration but failed to show the efficacy of human NGF in treating diabetic polyneuropathy [17]. Although, initially NGF appeared to be an ideal therapeutic agent for the treatment of diabetic neuropathy the failure of these subsequent clinical trials has shown that administration of human NGF results in various undesirable effects such as muscle pain, the mechanism for which is not understood.

Effects of an Increase in Endogenous NGF Levels

NGF is also considered to be a novel therapeutic target for the treatment of neuropathic pain, which refers to “pain initiated or caused by a primary lesion or dysfunction in the nervous system” [169]. Studies conducted on animal models of neuropathic pain suggest that there might be a relationship between neuropathic pain and NGF [65,134,159,168]. Procedures used to induce neuropathic pain such as sciatic nerve constriction [159] or spinal nerve ligation [154] result in pain behaviors such as allodynia, mechanical and thermal hyperalgesia [65,159,168] and lead to increases in the levels of NGF.
Administration of NGF anti-serum has been shown to inhibit mechanical and thermal hyperalgesia caused by elevated endogenous NGF levels [134,190], suggesting that NGF is a mediator of mechanical sensitivity. Also, neuropathic patients with chronic hyperalgesia and allodynia have increased levels of NGF in the area of peripheral neuropathy [9]. However, some studies conducted on animal models of nerve constriction injury have reported contradictory results. For example, exogenous NGF administration (chronic infusion of 10μl of 0.5 mg/ml NGF) has been shown to abolish behavioral hyperalgesia (decrease in mechanical threshold) caused by chronic constriction injury of the rat sciatic nerve [162]. Also, a study has shown that intrathecal rat NGF infusion restores the anti-allodynic and anti-hyperalgesic effects of morphine in a rat model of sciatic nerve constriction injury [33]. Overall, these studies suggest that there might be a correlation between NGF levels in the periphery and pain behaviors such as mechanical allodynia, thermal hyperalgesia etc.

Tissue inflammation is known to result in an increase in the production and release of NGF [27,63,70,205]. Studies have shown that inflammatory mediators such as interleukin-1 (IL-1), IL-4, IL-5, tumor necrosis factor-α and interferon-γ induce NGF release. In turn, NGF can augment neurogenic inflammation by promoting the release of inflammatory mediators from basophils, mast cells, macrophages, and T- and B-lymphocytes [63,204]. Various inflammatory diseases such as rheumatoid arthritis [6], multiple sclerosis [30] and systemic lupus erythematosus [27,30] lead to up-regulation of NGF synthesis. Anti-NGF antibodies have been shown to significantly reduce inflammatory hypersensitivity caused by high levels of NGF [203]. Therefore, NGF is considered to be a key mediator in the production of inflammatory pain. It has been suggested that NGF influences inflammatory responses by affecting immune cell function; altering neuropeptide (SP and CGRP) levels in sensory fibers.
or sensitizing nerve terminals through receptor phosphorylation [204]. Therefore, elevated endogenous levels of NGF seem to be associated with neuropathic and inflammatory pain.

**NGF and Experimental Muscle Pain**

*Animal Studies*

It is thought that in the periphery NGF is responsible for maintaining the sensitivity of primary afferent fibers and that up regulation of NGF can result in alterations in pain related behavior [63,156]. Studies in adult rats [132,205] and in humans [79,157] have shown that NGF administration leads to hyperalgesia and sensitization of nociceptive neurons [70,72,166,167,191].

Lewin et al [132,133] have shown that a single systemic (intraperitoneal injection) dose of mouse submaxillary NGF (2.5S) in adult male and female rats results in two phases of hyperalgesia (in response to thermal and mechanical stimuli): an early phase starting 30 min after NGF administration and a late, but longer-lasting phase starting several hours after NGF administration. The authors reported thermal hyperalgesia during both phases but mechanical hyperalgesia only during the late phase. They suggested that the first phase of the hyperalgesia was due to the peripheral effects of NGF whereas the second phase was a result of central mechanisms. They found that the late phase consisting of mechanical hyperalgesia was impaired when NMDA receptors were blocked [133]. Rueff and Mendell [166] have reported that 1 mg/kg intraperitoneal injection of NGF results in thermal hypersensitivity that begins 30 min post-injection and lasts for several days. They reported that rats developed mechanical hyperalgesia one hour post-injection that persisted for up to 7 days [166]. In an *in vitro* study, Rueff and Mendell [167] have shown that human NGF applied directly to the
receptive fields of primary afferent fibers of the rat saphenous nerve decreased the thermal or heat threshold without changing the mechanical or cold threshold [167]. These results suggest that NGF may have short- and long-term effects that could be mediated by different mechanisms. Therefore, the timing of recording after NGF administration is of critical importance as it may explain the response observed.

Other studies have shown that intradermal administration of human NGF (50 µl subcutaneous injections of 1-10 µg/ml) into the adult rat hindpaw skin activates and sensitizes cutaneous primary afferent fibers [11]. It was found that human NGF did not result in any overt pain behavior but produced cutaneous thermal hyperalgesia [11]. The authors also reported that human NGF administration resulted in plasma protein extravasation (indicating tissue inflammation) in rat skin around the injection site [11]. Hyperalgesia was observed only ipsilaterally (at the site of injection) and therefore the authors concluded that human NGF probably acts at the site of injection and not systemically [11]. The peripheral mechanism of action was supported by the results that human NGF-induced extravasation of Evans’ blue dye occurred only around the injection site [11]. These results were consistent with a previous report [155] that investigated the effect of bovine and murine NGF on plasma extravasation in rat skin. More recently, another study has reported that subcutaneous mouse submaxillary gland-derived NGF (2.5S) injection (0.75 nM) into the mouse hind paw resulted in thermal hypersensitivity within 1 hour of injection [54]. Overall, these studies suggest that NGF’s effects involve a peripheral mechanism of action and these effects may occur as soon as 30-60 min after NGF injection and can last for hours. These results also suggest that there is a possibility that NGF (dose as low as 50 ng) may induce rapid tissue inflammation (within 1 hour after the injection) localized to the injection site.
**Human Studies**

In 1994, a phase I, randomized, placebo-controlled study was conducted in healthy human male and female subjects to determine the effects of human NGF [157]. It was found that single intravenous or subcutaneous doses of human NGF (0.03 to 1 μg/kg) produced mild to moderate muscle pain, especially pain in the masseter muscle that increased with chewing, beginning 1-1.5 hour post-injection. Beginning at 60-90 min after human NGF administration, subjects reported muscle pain that increased at 4 to 6 hours and then slowly decreased over 2 to 8 days. Female subjects reported greater muscle pain as compared to male subjects and the effect was found to be dose-dependent, which suggests that human NGF may have sex-dependent effects [157]. Subcutaneous injection of human NGF resulted in hyperalgesia to touch and heat at the site of the injection (in addition to mild, diffuse myalgias), which lasted for 7 weeks and was also found to be dose-dependent [157]. This study demonstrated that human NGF administration results in diffuse muscle pain (including pain in the orofacial region) that peaks hours after injection and increases with muscle use. Another study conducted by Dyck et al [79] had reported that healthy human subjects injected with intradermal injection of human NGF in the forearm (1 or 3 μg) developed pressure allodynia and heat-pain hyperalgesia within 3 hours on the NGF-injected side. The authors suggested that local tissue mechanisms might be responsible for the rapid onset of these processes [79].

A relationship between NGF and masticatory muscle pain was first proposed by Stohler in 1997 [182]. Until recently, however, no one had looked at the effects of NGF injection on human masticatory muscles. Svensson et al (2003) conducted a double-blinded, placebo-controlled study on the masseter muscle of healthy human male subjects to
determine the effect and duration of a local injection of human NGF [187]. They recorded changes in pressure pain thresholds (PPT) and pressure tolerance thresholds (PTOL) to indicate mechanical allodynia and hyperalgesia in the masseter muscle in response to human NGF injection. Results from this study showed that a single intramuscular injection of human NGF (0.1 μg/kg) into the masseter muscle resulted in mechanical sensitization (mechanical allodynia) localized to the injection site for at least 7 days in addition to hyperalgesia [187]. Only a few subjects reported pain during human NGF injection. One and 7 days after human NGF injection, subjects reported pain during activities involving jaw movement such as chewing and yawning [187]. This study demonstrated that human NGF injection in the masseter muscle produces symptoms similar to those experienced by TMD patients and therefore this method could be used to model TMD pain in humans.

TMD and NGF

It has been speculated that masseter muscle pain associated with temporomandibular disorders could be, in part, due to a local elevation of nerve growth factor [187]. However, the mechanism of action of the intramuscularly injected human NGF is still not clear. It is not known whether human NGF causes mechanical sensitization by directly acting on the primary afferent fibers in the masseter muscle or if it induces some other process such as muscle inflammation, which then results in mechanical sensitization. Also, it is not known if the effects of human NGF on masseter muscle are sex-dependent. There is a higher prevalence of TMD in the young female population [77,129,172,183,209], which suggests that sex hormone levels may play a role in the pathophysiology of TMD. In fact, various studies have suggested that reproductive hormones especially estrogen are involved in TMD-related pain [39,77,129,130,172]. It has been suggested that there might be an interaction
between NGF and estrogen levels. Estrogen receptor mRNA colocalizes with NGF receptor (TrkA) mRNA in some targets of NGF and it has been speculated that estrogen levels may influence NGF receptor gene transcription or increase NGF receptor mRNA levels [179]. Also, there seems to be a cross talk between NGF and excitatory amino acid receptors, in particular the NMDA receptor. Studies have suggested that NGF may enhance NMDA receptor expression and increase NMDA receptor activity (by tyrosine phosphorylation of NMDA receptor subunits) in striatal, hippocampal and spinal cord neurons [66,112]. Therefore, it is possible that an interaction between estrogen levels, NGF and NMDA receptors may result in sex-related differences in the sensitivity of primary afferent fibers to exogenous NGF.

In order to assess the interaction between NGF and muscle pain, changes in the mechanical response properties of primary afferent fibers in the masseter muscle of both male and female rats were recorded after an injection of rat NGF. This study was conducted in the masseter muscle as it is the major jaw-closing (masticatory) muscle in a rat [152]. Experiments were conducted in both male and female rats in order to determine if there were any sex-related differences in the effects of rat NGF. As estrus cycle stage could influence the effect of rat NGF on the sensitivity of primary afferent fibers, any correlation between the estrogen levels during different estrus stages and the changes in mechanical threshold of masseter muscle fibers was also determined [39]. In addition, it was determined if muscle inflammation occurs in response to rat NGF injection. To exclude the possibility of rat NGF-induced inflammation resulting in afferent fiber mechanical sensitization and to be a good model of TMD-related muscle pain, it was hypothesized that rat NGF injection would not cause muscle inflammation because TMD-related muscle pain is non-inflammatory.
Experimental Hypothesis

It was hypothesized that an intramuscular injection of nerve growth factor would cause a decrease in the mechanical threshold of primary afferent fibers in the masseter muscle in a sex-dependent manner (females showing a greater degree and duration of sensitization than males), without inducing muscle inflammation.

Research Objectives

The objectives of this study were:

(i) To determine the effect of an intramuscular injection of rat NGF on the mechanical threshold of primary afferent fibers in the masseter muscle of anesthetized rats.

(ii) To determine whether evoked afferent activity or changes in mechanical threshold and the duration of changes in mechanical threshold (i.e. duration of sensitization) differ in male and female rats, and if these differences are related to plasma estrogen levels.

(iii) To investigate whether exogenous rat NGF causes muscle tissue inflammation.
Chapter 2. METHODS

Surgical Procedures

*In vivo* single unit recordings were conducted on anesthetized adult male and female Sprague-Dawley rats using electrophysiological recording techniques and set-up. For each experiment, a rat was weighed and then anesthetized with isoflurane using a facemask. Following tracheotomy, the rat was mechanically ventilated with a mixture of isoflurane (2-2.5%) and oxygen (98-97.5%) at a steady rate. A rectal temperature probe was used to monitor the core body temperature of the rat. Electrocardiogram leads were used to monitor the heart rate of the animal. In female rats, a vaginal lavage was performed and a sample of epidermal cells was collected on a glass slide to determine the estrus stage using a microscope (*details below*). The femoral artery was catheterized to monitor the mean arterial blood pressure of the rat throughout the experiment. The blood pressure was maintained above 60 mmHg throughout the experiment and during recordings. The mechanical ventilator rate and isoflurane levels were adjusted accordingly to keep the blood pressure between 60-80 mmHg and end-tidal CO$_2$ between 20-50 mmHg. The femoral vein was also catheterized to deliver pentobarbital at the end of the experiment to sacrifice the rat. The rat’s head was positioned in a stereotaxic frame and a trephination was made on the right side of the skull so that an electrode could be lowered into the right trigeminal ganglion. Also, blunt dissection was conducted to expose the right subnucleus caudalis in the brainstem. The dura overlying the brainstem was cut with small surgical scissors.

*Determination of Estrus Cycle Stage*: The rat estrus cycle is divided into four stages: diestrus, proestrus, estrus and metestrus. During the estrus cycle, sex hormone fluctuations cause morphological changes in the vaginal epithelial cells that are shed into the vagina
The estrus stage of female rats was determined by conducting a vaginal lavage and examining the shape of the epithelial cells under a microscope. The diestrus stage of the estrus cycle is characterized by the presence of round, nucleated cells of different sizes, whereas only big cells are present during the proestrus stage [48]. The epithelial cells lose their nucleus and become keratinized (stratified squamous) during the estrus stage, which is the most distinctive stage of the cycle [46,48,192]. The presence of both keratinized and round, nucleated cells characterizes the metestrus stage of the estrus cycle [48].

**Recording Procedures**

A parylene-coated tungsten-recording electrode was lowered into the trigeminal ganglion to record from the cell body of a single primary afferent fiber. The output from the recording electrode was fed into a computer (Spike 2 software, Cambridge Electronic Design, Cambridge, UK) (Figure 1). A blunt mechanical search stimulus was used to find and activate a primary afferent fiber in the masseter muscle. To confirm the projection of the primary afferent fiber to the subnucleus caudalis, a stimulating electrode was lowered into the caudal brainstem to stimulate an antidromic action potential. The projection of the fiber to the brainstem was confirmed by collision of the orthodromic action potential (evoked by mechanically stimulating the receptive field of the fiber in the masseter muscle) with the antidromic action potential (generated by directly stimulating the caudal brainstem) (Figure 1B).

The receptive field of the primary afferent fiber was marked with a permanent marker to make sure the same area in the muscle was used to obtain mechanical threshold values. The baseline mechanical threshold (minimum force required to evoke primary afferent fiber
Figure 1: The methodology developed to identify masticatory muscle nociceptors with projections to the caudal brainstem is illustrated. A: Mechanical distension of the masseter muscle with an electronic Von Frey hair (lower trace) activates a nociceptor (upper trace). B: Stimulation of the caudal brainstem evoked an antidromic spike in this nociceptor, which could be collided with spikes evoked by electrical (or mechanical) stimulation of the masseter muscle (*), confirming its projection to the caudal brainstem.
response) of the fiber was recorded with an electronic Von Frey hair (VF hair; blunt polypropylene tip, diameter 0.5 mm, Model 1601C, IITC Inc., Woodland Hills, CA, USA) every min for 10 min. The jaw was opened to determine if the primary afferent fiber responded to jaw opening and the distance at which the fiber responded was recorded and called the effective jaw opening distance. For the fibers that did not respond to jaw opening the maximal jaw opening distance was recorded.

**Experimental Protocol**

Each primary afferent fiber was randomly assigned to one of the following three groups: vehicle control (10 μl of phosphate buffered isotonic saline and albumin, n = 20), 2.5 and 25 μg/ml rat NGF (~ 0.1 and 1 μg/kg respectively, 10 μl, n = 20 each; Sigma Chemical Company, St. Louis, MO, USA) dissolved in phosphate buffered isotonic saline and albumin. The concentration of rat NGF was matched to the dose and concentration of human NGF used in human experiments [187]. All solutions were at physiological pH (pH 7.3 ± 0.1). The investigator conducting the mechanical threshold recordings was blinded to the contents of the solution. A catheter (a 27-gauge needle connected to a Hamilton syringe with polyethylene tubing) was used to inject the solution into the receptive field of the fiber. After inserting the needle into the muscle, a 10-min baseline recording was taken to assess any spontaneous firing activity before the injection. At 10 min, the solution was injected and the evoked response was recorded for 10 min. Thirty min after the injection, 10 consecutive mechanical threshold recordings were conducted at 1 min intervals. At the end of the 10-min recording period the jaw was opened to determine if the primary afferent fiber responded to jaw opening and the distance at which it responded. The response to jaw-opening and mechanical threshold recordings was assessed every 30 min for a total of 6 hours (Figure 2).
**Figure 2:** The illustration is of the experimental protocol mentioned in the methods section. The arrows represent the events labeled and the bracket represents 10 mechanical threshold recordings conducted every 30 mins for 6 hours.
At the end of the recordings, a blood sample was collected to measure plasma estrogen levels with a commercially available radioimmunoassay kit (see below for protocol). After blood sample collection, Evans’ blue dye (6 mg/kg) was injected into the left femoral vein (via the venous catheter) and the catheter was flushed with normal saline. After 15-20 min, the rat was euthanized with an overdose of pentobarbital (100 mg/kg) and immediately perfused with 120 ml of normal saline using an infusion pump (KD Scientific, Model 200 Series, Holliston, MA). Following saline infusion, the part of the masseter muscle around the injection site was isolated, weighed and stored in the freezer for Evans’ blue dye analysis. At the end of each experiment, the distance between the recording and the stimulating electrode was measured and divided by the latency of the antidromically stimulated action potential to estimate the central conduction velocity of the primary afferent fiber.

**Evans’ Blue Dye Absorbance Analysis**

Each muscle tissue sample was placed in a test tube and immersed in 2 ml of formamide. The test tube was placed in a water bath (Isotemp 205 Fisher Scientific, Augusta, GA) at 60°C for 24 hours [87]. The amount of dye extracted from the muscle tissue sample was determined by measuring the absorbance of the supernatant at 620 nm using a spectrophotometer (8452A Diode Array Spectrophotometer) [87]. A standard curve was generated with the absorbance values plotted on the y-axis and a range of Evans’ Blue Dye concentrations from 0 – 10 µg/ml on the x-axis (Figure 13). The Evans’ blue dye concentration in each muscle sample (µg/g) was calculated by multiplying the concentration (µg/ml) obtained from the standard curve by 2 ml and dividing the value by the weight of the muscle sample (g).
**17β-Estradiol Radioimmunoassay Protocol**

Plasma estrogen (17β-Estradiol) levels were measured with a commercially available kit (ImmuChem™ Double Antibody 17β-Estradiol \(^{125}\)I RIA Kit). A radioimmunoassay was conducted to determine the plasma concentration of the unconjugated form of Estradiol according to the protocol provided by the supplier (ICN Biomedical, Inc., Costa Mesa, CA). Briefly, anti-17β-Estradiol antibody was added to a mixture of plasma samples (unlabelled antigen) and estradiol \(^{125}\)I samples (radioactive antigen). A precipitant solution (mixture of polyethyleneglycol and Goat anti-rabbit gamma globulins contained in a tris buffer) was added and the samples were centrifuged. A gamma counter (Cobra II Auto-Gamma, Canberra Packard Canada) was used to count the radioactivity in the precipitate and a standard curve was generated with percent bound \(^{125}\)I antigen on the y-axis and estradiol concentration in pg/ml on the x-axis (log scale) (Figure 3). This standard curve was used to determine the concentration of estrogen in the plasma samples.

This experimental protocol was approved by the UBC Animal Care Committee.

**Sample Size**

A difference of at least 25% between the mean mechanical threshold of control and treatment groups was required for the results to be considered biologically meaningful [188]. It was calculated (using a 1-way ANOVA sample size estimation, SigmaStat 3.0 software, \(\alpha = 0.05\), Power = 0.80) that a minimum sample size of 10 was required in each group to see a difference of 25% (Standard Deviation = 18%) between the means of the three groups. Therefore, a total of 60 individual afferent fibers (total \(n = 60\) rats) were examined: 20 each (10 males, 10 females) for saline control, 2.5 \(\mu g/ml\) rat NGF and 25 \(\mu g/ml\) rat NGF.
Figure 3: The graph shows the standard curve for the 17β-Estradiol radioimmunoassay. The y-axis represents the percentage of radioactively labeled antigen (17β-Estradiol-\(^{125}\)) bound to the anti-17β-Estradiol antibody and the x-axis represents the log concentration of 17β-Estradiol-\(^{125}\).
Data Analysis

The 10 mechanical threshold (MT) values for each time point were averaged. In order to account for inter-fiber variability (i.e. differences in baseline MT) and compare different populations of fibers, the mean MT for each time point was divided by the baseline mean MT of each fiber to calculate the relative MT. The evoked response of each fiber was calculated by subtracting the total number of the spikes during the 10-min period before the injection from the total number of spikes during the 10-min period after the injection. The evoked response of fibers in each group (vehicle control and two treatment groups) was averaged to calculate the mean evoked response. The firing rate (in Hz) of spontaneously active fibers was determined by dividing the number of spikes fired prior to the injection by the time period (600 s). The concentration of the Evans’ blue dye (used as an indicator of plasma protein extravasation) was calculated per gram weight of muscle tissue (discussed above).

Statistical Analysis

A General Linear Model (GLM) repeated measures three-way ANOVA (Covariates: baseline MT, conduction velocity, estrogen levels, Evans’ blue dye levels) was used to assess treatment, sex and time (repeated) effects on the relative mechanical threshold. A log transformation was carried out to normalize the distribution of baseline mechanical threshold data. A log-linear regression analysis was used to determine the relationship between conduction velocity and baseline mechanical threshold. A log-linear regression analysis was also used to determine the relationship between plasma estrogen levels and baseline MT for slow Aδ fibers. A one-way Analysis of Variance (ANOVA) was conducted to determine estrus-stage effects on baseline mechanical threshold. An inverse transformation of rat body
weight, a square root transformation of estrogen levels and a log transformation of conduction velocity was required to create a normal distribution of the data for assessment of sex-differences. Student t-tests were conducted to determine sex-differences in body weight, plasma estrogen levels, baseline mechanical threshold and conduction velocity. The fiber conduction velocity before and after the experiment was compared using a Wilcoxon signed rank test. Fisher Exact tests were carried out to determine differences in the frequency of A\(\delta\) and C fiber types, and of fibers exhibiting spontaneous activity between male and female rats. Also, the frequency of fibers demonstrating injection-evoked afferent discharge was compared between vehicle control and rat NGF groups with a Fisher Exact test. A one-way ANOVA was used to determine any treatment effect on the evoked response of fibers. The fiber conduction velocity was compared between treatment and sex with a two-way ANOVA. A linear regression analysis was used to determine the correlation between plasma estrogen levels and the variability in MT recording (square root transformation of the standard deviation). The mean post-injection mechanical threshold data was log transformed to determine treatment effects with a one-way ANOVA. A square-root transformation was conducted on the Evans' blue dye concentration data and the effect of treatment and sex was determined with a two-way ANOVA.

The level of significance of all statistical tests was set at \(P < 0.05\). All values are reported as mean ± SE.
Chapter 3. RESULTS

A total of 71 masseter muscle afferent fibers were recorded from 62 rats and baseline properties of the fibers, which included conduction velocity, mechanical threshold, and response to jaw opening, collected. The vast majority of these fibers had conduction velocities in the Aδ range (2-30 m/s); however, recordings from a total of 3 C fibers (< 2.0 m/s; 4.2%) were also made. This percentage of mechanically activated C fibers is similar to that reported previously for uninflamed masseter muscle [40]. Given the small number of C fibers, the response properties of all recorded fibers were considered together and no attempt was made to examine the property of the C fibers independently. Sixty of these fibers (30 in males, 30 in females) were also examined for their response to injection of rat NGF or vehicle control into the masseter muscle.

Baseline Properties of Afferent Fibers

Males: Thirty-three of 71 afferent fiber recordings were made in male rats (weight: 301.1 ± 4.9g; n = 33). The mean baseline mechanical threshold (MT) and mean conduction velocity (CV) of these afferent fibers was 23.9 ± 6.0 g and 7.3 ± 0.6 m/s respectively. Analysis of this data revealed a significant inverse log linear correlation between CV and baseline MT (P = 0.005, R = 0.473, Figure 4 Top). The mean plasma estrogen levels of male rats were found to be 22.8 ± 3.1 pg/ml (n = 28). Since slowly conducting Aδ fibers (CVs of < 10 m/s) are more likely to be nociceptive, the relationship between plasma estrogen levels and baseline MT of only this subgroup of fibers was examined. There was no significant correlation between the baseline MT and plasma estrogen levels for slow Aδ fibers (P = 0.43, R = 0.187, Figure 4 Bottom). During baseline recording, none of the fibers responded to maximal jaw opening.
Figure 4: *Top* - The top graph shows the relationship between conduction velocity and baseline mechanical threshold for fibers recorded in male rats. The log-linear correlation coefficient was calculated to be $R = 0.473$. *Bottom* - The relationship between plasma estrogen levels and baseline mechanical threshold for slow $A\delta$ fibers recorded in male rats is shown. The log-linear correlation coefficient was calculated to be $R = 0.187$. 
Approximately 85% of the fibers (28 out of 33 fibers) were slowly adapting fibers (firing rate remained constant or decreased slowly when sustained pressure was applied to the receptive field). Eight fibers fired spontaneously during the baseline recording before the injection (0.1 ± 31.6 Hz.).

**Females:** Thirty-eight of 71 afferent fiber recordings were made in female rats (weight: 257.5 ± 3.1 g; \( n = 32 \)). The mean baseline MT and mean CV of all afferent fibers was 17.0 ± 2.3 g and 7.2 ± 0.6 m/s respectively. Unlike in males, the fiber CV and baseline MT of afferent fibers in females were not log linearly correlated (\( P = 0.688, R = 0.07 \), Figure 5 *Top*). Of these 38 afferent fiber recordings, 6 were made during diestrus, 3 during proestrus, 11 during estrus and 18 during metestrus. There was no significant difference between the mean baseline MT of fibers recorded during the various estrus stages (\( P = 0.124 \)). The baseline MT was not correlated with CV in any of the individual estrus cycle stages (Figure 5 *Top*). The plasma estrogen levels of female rats were found to be 59.0 ± 7.2 pg/ml (\( n = 26 \)). There was a significant correlation between the baseline MT and plasma estrogen levels for slow Aδ fibers (\( P = 0.044, R = 0.444 \), Figure 5 *Bottom*). During baseline recording, none of the fibers responded to maximum jaw opening. Approximately 89.5% of the fibers (34 out of 38 fibers) were slowly adapting fibers. Six fibers fired spontaneously during the baseline recording before the injection (0.2 ± 60.0 Hz.).

**Sex Comparisons:** Male rats were significantly larger than female rats (\( P < 0.001 \)). However, there was no difference in the baseline mechanical threshold (\( P = 0.42 \)) or conduction velocity (\( P = 0.95 \)) of fibers recorded from male and female rats (Table 1), which is
Figure 5: Top - The top graph shows the relationship between conduction velocity and baseline mechanical threshold for fibers recorded in metestrus (○), diestrus (○), estrus (▽) and proestrus (▽) stages of female rats. The log-linear correlation coefficient was calculated to be R = 0.07 for all fibers recorded in female rats. Bottom - The relationship between plasma estrogen levels and baseline mechanical threshold for slow Aδ fibers recorded in female rats is shown. The log-linear correlation coefficient was calculated to be R = 0.444.
Table 1: The baseline properties of all afferent fibers recorded from male and female rats are given. The values are reported as mean ± SE.
consistent with previous reports [36,40]. There was a significant log-linear relationship between baseline MT and fiber CV for male rats but not for female rats. The plasma estrogen levels of female rats were significantly greater than those of male rats (P < 0.001). There was a significant log-linear correlation between baseline MT and plasma estrogen levels for female rats but not for male rats. Fibers from both male and female rats did not respond to jaw opening during baseline recording and the majority of the fibers were slowly adapting in both groups, which is consistent with previous reports [36,40]. Also, the majority of the fibers were Aδ fibers in both male and female groups. There was no difference in the frequency of Aδ and C (P = 0.594) recorded in male and female groups.

Evoked Response (Action Potential Discharge)

Sixty afferent fibers were also examined for their response to injection of vehicle (phosphate-buffered saline) or rat NGF (2.5 or 25 μg/ml) into their masseter muscle receptive fields. There was no significant treatment effect on the evoked response of fibers (P = 0.076). Also, there was no obvious change in the mean arterial blood pressure in response to the injection (Figure 6).

In males, relatively few fibers responded to injection of vehicle (40%), 2.5 μg/ml rat NGF (40%) or 25 μg/ml rat NGF (10%) (Table 2). Similarly, in females only 20% of the fibers in each group (vehicle, 2.5 or 25 μg/ml rat NGF) responded to the injection (Table 2). There was no difference in the proportion of fibers responding to vehicle or rat NGF injection in male (P = 0.431) and female (P = 0.100) rats. There was no sex-difference in the frequency of fibers exhibiting spontaneous activity before the injection (P = 0.390).
Figure 6: The *bottom* trace represents the afferent fiber activity (bottom) of a slow Aδ fiber before and after a 10-μl injection of 25 μg/ml rat NGF (at time 0), recorded from a female rat. The *middle* and *top* trace represent the mean arterial blood pressure and heart rate of the rat before and after injection. Note the injection evoked only a transient afferent discharge and did not alter blood pressure or heart rate.
<table>
<thead>
<tr>
<th>EVOLED ACTIVITY</th>
<th>Vehicle Control</th>
<th>2.5 μg/ml NGF</th>
<th>25 μg/ml NGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of fibers that responded to the injection</td>
<td>Male: 4</td>
<td>Male: 4</td>
<td>Male: 1</td>
</tr>
<tr>
<td></td>
<td>Female: 2</td>
<td>Female: 2</td>
<td>Female: 2</td>
</tr>
<tr>
<td>Mean Sum Evoked Response (Spikes)</td>
<td>Male: 25.3 ± 12.5</td>
<td>Male: 16.5 ± 5.2</td>
<td>Male: 7.0</td>
</tr>
<tr>
<td></td>
<td>Female: 61.0</td>
<td>Female: 2.0</td>
<td>Female: 9.0</td>
</tr>
</tbody>
</table>

Table 2: The table contains the number and mean (± SE) sum evoked response of fibers in response to vehicle control, 2.5 and 25 μg/ml rat NGF for male and female rats.
Comparison with Glutamate: As a positive control, the effect of a 10-μl injection of 1M glutamate on the evoked response of two masseter muscle afferent fibers was tested in two female rats. Injection of 1M-glutamate into the masseter muscle evoked considerably greater afferent discharge than did vehicle or 2.5 or 25 μg/ml rat NGF (Figure 7, 8 and 12 insets). Since previous studies have identified significant sex-related differences in the chemically evoked activity of slowly conducting Aδ fibers (CVs of < 10 m/s), the data was reanalyzed to examine only the response properties of this subgroup of fibers for both male and female rats together [36,38,40]. There was, however, no significant difference between vehicle (n = 4 fibers), 2.5 μg/ml rat NGF (n = 5 fibers) and 25 μg/ml rat NGF (n = 3 fibers) groups (P = 0.083).

Conduction Velocity: For all fibers, there was no significant difference in the central CV between control and treatment groups (P = 0.516) or male and female groups (P = 0.465) and there was no significant treatment and sex interaction (P = 0.762). Also, there was no obvious change in the central CV of fibers during the experiment (P = 0.470).

Mechanical Properties

There was no treatment, sex or time (repeated) effect on the relative MT and there was no significant interaction between any of these factors (Figure 9). However, a significant interaction between time and estrogen levels (added as a covariate) was identified. To determine if this interaction was due to the variability in MT recording, the relationship between the standard deviation of the MT recording (for the vehicle control group only) and plasma estrogen levels was investigated (Figure 10). It was found that the plasma estrogen
Figure 7: The mean evoked response of afferent fibers that fired in response to an injection of phosphate-buffered saline vehicle (n = 6), 2.5 μg/ml rat NGF (n = 6), 25 μg/ml rat NGF (n = 3) or 1M glutamate (n = 2) is illustrated.
Figure 8: Top – The bar graph illustrates the jaw opening distance measured during baseline and at various time points after a 10-μl injection of 25 μg/ml rat NGF (at time 0) into the masseter muscle receptive field of an Aδ fiber (CV = 4.29 m/s) in a male rat weighing 328g. The black bars represent afferent response to jaw opening whereas the white bars represent no response to jaw opening. Bottom – The bar graph shows the relative mechanical threshold of the same fiber during baseline and at various time points post-injection. Inset – A histogram of the baseline activity during the 10 min period before injection and the evoked activity of the fiber in response to the rat NGF injection made at time 0 sec is shown.
Figure 9: Line and scatter plots illustrate the relative mechanical thresholds of fibers from male (Top) and female (Bottom) rats at various time points after a 10-μl injection of phosphate-buffered saline vehicle (●, n = 10), 2.5 μg/ml rat NGF (○, n = 10) and 25 μg/ml rat NGF (▼, n = 10) into the masseter muscle receptive field. The error bars represent the standard error of the mean.
levels were significantly correlated with the standard deviation of MT recordings for fibers recorded in female rats \((P = 0.037, R = 0.698, n = 9)\), but not in male rats \((P = 0.422, R = 0.287, n = 10)\). This suggested that estrogen levels influenced the variability in mechanical threshold measure in female rats.

In order to assess only the treatment effect on relative MT, the relative MT values for all time points after injection were averaged to calculate mean post-injection MT. The comparison of mean post-injection MT of all fibers (both male and female groups combined) showed no difference between vehicle and rat NGF groups \((P = 0.952)\) (Figure 11). Only 1 fiber in the male group (3.3%) responded to jaw opening \((25 \mu g/ml\) rat NGF injection group) (Figure 8 Top). At 1.5 hours post-injection, the fiber became responsive to 15 mm of jaw opening and this effective jaw-opening distance dropped to 14 mm at 2.5 hours and 13 mm at 3 hours post-injection (~24% decrease in effective jaw opening distance). After 3 hours, the fiber was no longer responsive to maximal jaw opening (Figure 8 Top). None of the fibers in the female group responded to jaw opening. Also, it was found that there was no correlation between fiber CV or baseline MT and mean post-injection MT of 2.5 \(\mu g/ml\) and 25 \(\mu g/ml\) rat NGF-injected fibers. This suggested that the fiber CV or baseline MT did not influence the response to rat NGF (i.e. rat NGF's effect on MT).

Comparison with Glutamate: The effect of a 10-\(\mu l\) injection of 1M glutamate on the evoked response and mechanical threshold of two masseter muscle afferent fibers was tested in two female rats. Consistent with the published data, it was found that glutamate caused a considerable decrease in the relative MT starting 30-min post-injection [36] and this mechanical sensitization lasted for ~3 hours post-injection (Figure 12). In addition, the fiber
Figure 10: Scatter plots exhibit the linear relationship between plasma estrogen levels and the standard deviation in the mechanical threshold recording for fibers in the vehicle control group in male (Top, $R = 0.287$) and female rats (Bottom, $R = 0.698$).
Figure 11: The bar graph shows the mean post-injection mechanical threshold of all masseter muscle afferent fibers injected with 10-μl solution of phosphate-buffered saline vehicle (n = 20), 2.5 μg/ml rat NGF (n = 20), 25 μg/ml rat NGF (n = 20) or 1M glutamate (n = 2).
Figure 12: Top – The graph shows the jaw opening distance measured during baseline and at various time points after a 10-μl injection of 1M glutamate (at time 0) into the masseter muscle receptive field of an Aδ fiber (CV = 2.53 m/s) in a female rat weighing 270g. The black bars represent afferent response to jaw opening whereas the white bars represent no response to jaw opening. Bottom – The bar graph shows the relative mechanical threshold of the same fiber during baseline and at various time points post-injection. Inset – The histogram illustrates the baseline activity during the 10 min period before injection and the evoked activity of the fiber in response to the glutamate injection made at time 0 sec.
became responsive to jaw opening at 2.5 and 3 hours post-injection. After 3 hours the fiber became unresponsive to maximal jaw opening (18 mm). The decrease in relative MT of glutamate-injected fibers was greater (~60% decrease) than that of rat NGF-injected fibers (~7% decrease for 2.5 µg/ml rat NGF group) (Figure 11).

**Evans’ Blue Dye Analysis**

There was no significant difference in the amount of Evans’ blue dye between vehicle and rat NGF treatment groups for both male and female rats and there was no significant interaction between treatment and sex ($P = 0.643$ for treatment, $P = 0.886$ for sex and $P = 0.165$ for interaction between treatment and sex) (Figure 13). The concentration of Evans’ blue dye in vehicle injected muscles was $2.2 \pm 0.4 \, \mu g/g$, which was lower than the values reported previously for normal saline and glutamate injections into the temporomandibular joint (4 hours post-injection) [87]. The concentration of Evans’ blue dye in 2.5 µg/ml and 25 µg/ml rat NGF injected muscles was $2.1 \pm 0.4 \, \mu g/g$ and $1.6 \pm 0.3 \, \mu g/g$ respectively.
Figure 13: Top – The graph shows the standard curve for Evans Blue Dye absorbance analysis. Bottom - The bar graph shows the concentration of Evans’ blue dye in the masseter muscle receptive fields of afferent fibers injected with phosphate-buffered saline vehicle ($n = 18$), 2.5 µg/ml rat NGF ($n = 16$) and 25 µg/ml rat NGF ($n = 18$).
Chapter 4. DISCUSSION

The principal purpose of this study was to determine whether rat NGF injection significantly alters masseter muscle afferent fiber mechanical threshold as part of the mechanism whereby it decreases PPTs in human subjects. It was hoped that if rat NGF injection induced masseter afferent sensitization in rats, then this might lead to the development of a useful animal model to study mechanisms of masseter muscle pain related to the TMDs. It was found that rat NGF injection (2.5 and 25 μg/ml) had no effect on the mechanical threshold of afferent fibers within 6 hours post-injection. This result suggests that rat NGF does not cause any mechanical sensitization in the rat masseter muscle. The rat NGF injection did not evoke any significant afferent fiber activity in the rat masseter muscle (as compared to vehicle control), suggesting that rat NGF injection is not noxious. Also, it was found that the rat NGF injection does not induce any muscle inflammation. In addition to these results some interesting sex-related differences in the baseline properties of the afferent fibers were also identified. In agreement with previous studies [36,40], it was found that there was an inverse relationship between baseline MT and CV. However, this relationship was only found in the fibers recorded from male rats and not from female rats. The plasma estrogen levels of female rats (but not male rats) were found to be positively correlated with the baseline MT.

Baseline Properties

The majority of afferent fibers recorded in this study were slow Aδ and only 4.2% of the fibers were classified as C fibers. This percentage is similar to what has been reported previously for mechanically activated afferent fibers recorded from an uninflamed rat masseter muscle [40]. Also, consistent with previous reports [36,40], the majority of the
fibers had slowly adapting properties. The baseline MT and CV of fibers recorded from male and female rats was not significantly different, which agrees with previous reports [36,37,40]. For female rats, the baseline MT of fibers recorded during all four stages of the estrus cycle was not different. A previous study has shown that the baseline MT of putative nociceptors (CV < 10 m/s) during proestrus is significantly lower as compared to other stages; however, a large sample size (> 70 fibers) was required to observe this difference [35]. It is possible that the small number of fibers recorded during each stage (especially during proestrus) limited the power of the statistical test to identify stage-related differences in baseline MT. A larger fiber number in each estrus stage group may be required to examine the effect of estrus stage on the baseline MT.

Human studies have shown that pain perception is altered through the menstrual cycle, although the results of such studies are often contradictory with regard to the stage of the cycle where women are more sensitive to pain stimuli [55,99,107]. There have been contradictory reports on the effect of menstrual cycle on PPTs in healthy women as well [75]. Most of these studies relied on self-report by subjects for determining the stage of the menstrual cycle rather than measurement of plasma hormone levels. The contradictory findings could be a result of inconsistencies in self-reports by subjects and different methodologies used in different studies. It has been reported that the masseter and temporalis muscle PPTs of female TMD patients are lowest during the perimenstrual phase [107], which is equivalent to the rat metestrus/diestrus stage of the estrus cycle when estrogen levels are low. Similarly, it has been reported that chronic low pain patients rate pain higher during the menstrual and premenstrual phases as compared to the ovulatory phase [99]. These studies
suggest that in humans low estrogen levels are correlated with higher pain sensitivity to mechanical stimulation.

Some novel sex-related differences in the baseline characteristics of masseter muscle nociceptors were identified in this study. The baseline MT and CV of nociceptors recorded from the male rats was found to be inversely correlated, however, nociceptors recorded from the female rats did not show this relationship. Previous studies conducted on male mice and rat cutaneous afferent fibers [34,76] and female guinea pig dorsal root and sural nerve afferents [71] have reported an inverse relationship between baseline MT and CV, however these studies did not investigate any sex-related differences. A study conducted on dural afferents has shown that fibers with CV > 5m/s had higher MT than fibers with CV < 5m/s [131]. Very few studies on muscle afferent fibers have been conducted and these studies have also shown a similar trend toward an inverse correlation in the relationship between MT and CV [36,92,95]. The results of this study agree with these previous reports and also indicate that there are sex-related differences in the relationship between baseline MT and CV.

Another novel finding was that the plasma estrogen levels of female rats were positively correlated with the baseline MT of slow Aδ fibers. In contrast, a previous study conducted on ovariectomized female rats has shown that exogenous estrogen administration causes an increase in the facial receptive field area and decreases the threshold of trigeminal cutaneous afferent fibers, which has been interpreted to mean that elevated estrogen levels cause peripheral sensitization [23]. Thus, the result of the present study appears to contradict the commonly held opinion that high estrogen levels are sensitizing or pro-nociceptive [39]. It was speculated that the relationship between baseline MT and CV is related to plasma estrogen levels. For female rats, the baseline MT (17.3 ± 3.3 g ~ 230 kPa) of slowly
conducting fibers (CV < 10 m/s) was similar to that reported previously (median MT >200 kPa) [35]. The slowly conducting fibers recorded from female rats with high plasma estrogen levels (>60 pg/ml) had higher mean baseline MT as compared to those recorded from females with low estrogen levels. It is possible that high estrogen levels in female rats masked the relationship between CV and baseline MT. In other words, it is possible that during periods of high estrogen levels, the baseline MT of rapidly conducting afferent fibers (CV > 10 m/s) of female rats is increased. Due to the very small number of rapidly conducting fibers (n = 2) recorded from female rats with high estrogen levels, a statistical comparison of baseline MT between females with low and high estrogen levels was not possible in this study. Unlike females, the male rats never experienced high plasma estrogen levels and this might explain why the relationship between CV and baseline MT was never masked by estrogen in the males. Different levels of estrogen may have different effects on MT i.e. low estrogen levels may be pro-nociceptive but when estrogen increases above a certain level it may have protective or anti-nociceptive effects. As discussed earlier, the mechanisms involved in mechanotransduction are not known, however, it is thought that opening of cation channels might be responsible for activating afferent fibers in response to mechanical stimulation [2,49,201]. It is possible that estrogen can modulate the MT of afferent fibers through its non-genomic effects. For example, it can be speculated that estrogen could alter the activity of other membrane-bound receptors (via intracellular proteins) that are involved in mechanotransduction. Estrogen could also have a direct effect on other receptor channels if these receptors have allosteric binding sites for estrogen.

Afferent fibers examined in this study appear to be involved in somatosensory function rather than proprioceptive function. None of the fibers responded to jaw opening
during the baseline recording, which suggests that they are not muscle spindle (proprioceptive) fibers. The anatomy of the trigeminal system is such that by recording from the trigeminal ganglion the activity of only somatosensory afferent fibers was likely recorded. Since, muscle spindle fibers have their cell bodies at a different anatomical site (mesencephalic nucleus), the involvement of these fibers (in response to jaw opening) can be excluded.

Relationship of MT to PPT

Previous experimental pain studies that have examined the effect of intramuscular algesic chemicals like glutamate and capsaicin in both animals and humans have reported similar results, i.e. activation and sensitization of nociceptors in animals and pain and sensitivity responses in humans [36,40]. The assessment of afferent fiber mechanical thresholds in animal models can be likened to the measurement of PPT in human subjects. Although there are some inconsistencies in the reports, the majority of the human studies have shown that the baseline masseter muscle PPTs (~200 kPa) in humans are similar in men and women [108,188]. Likewise, previous animal studies [36,40] have shown that there are no sex-related differences in baseline MT of masseter muscle afferent fibers. The majority of fibers recorded in this study had a baseline MT of greater than 15g, which is equivalent to the human pressure pain threshold of ~200 kPa. This suggests that the afferent fibers examined in this study were most likely to be involved in nociceptive transmission. Taken together, these studies show that there is generally a strong correlation between results that can be obtained with the animal model employed in the current study and human experimental pain.
Evoked Afferent Response

The rat NGF injection did not evoke any significant afferent fiber response, which suggests that rat NGF injection was not a noxious stimulus. Noxious stimuli are also known to increase blood pressure [8], so the lack of change in mean arterial blood pressure in response to intramuscular injection of rat NGF is consistent with the suggestion that rat NGF is not a noxious chemical. Human subjects did not find injection of human NGF into the masseter muscle painful [187]. Animal and human studies on evoked responses and pain perception are well correlated as the effective doses of algesic chemicals such as bradykinin, histamine etc. are similar to those that cause pain in humans [95]. In addition, previous studies have shown that the duration and sum of evoked afferent response in rats to injection of the algogen glutamate into the masseter muscle correlates well with the duration and magnitude of glutamate-evoked masseter muscle pain reported by human subjects. Therefore, intramuscular injection of glutamate into the rat masseter muscle has been used to model glutamate-induced pain in human subjects. Slow $A\delta$ and C fibers have been found to be more responsive to glutamate injections than faster conducting $A\beta$ and $A\alpha$ fibers [36,38,40]. The evoked afferent response of these slower conducting fibers is considered to be a good model for chemically induced pain in humans. Therefore, the lack of response of slow $A\delta$ and C fibers in this study to rat NGF injection is consistent with the interpretation that intramuscular injection of rat NGF does not evoke muscle pain. However, in contrast to this interpretation, a recent study conducted on rat gastrocnemius-soleus muscle afferent fibers has reported that ~62% of high mechanical threshold C-fibers responded to the human NGF injection. In the present study, however, none of the C-fibers responded to the rat NGF injection. As the number of C-fibers ($n = 2$ in the rat NGF group) recorded was very small, it
is not possible to make any conclusions about the response properties of C fibers to rat NGF. Nevertheless, rat NGF-induced response (or lack of response) is in agreement with the pain scores reported by humans injected with human NGF in the masseter muscle. Also, a behavioral study has shown that intradermal injection of human NGF does not cause any overt pain behavior in rats [11], which agrees with the results of this study.

The central CV of masseter muscle afferent fibers did not change during the experiment. This result was not surprising as the surgical and recording procedures used in this study did not interfere with the factors that would be expected to cause a change in the central CV such as myelin thickness, internodal distance, axon diameter or changes in ionic currents (for e.g. voltage-gated Na channel currents) [71]. Also, the time course of the experiment may not be long enough to cause and thus allow observation of any changes in axon properties.

**Mechanical Properties**

This study was undertaken to investigate whether intramuscular rat NGF injection significantly alters masseter muscle afferent fiber mechanical threshold as part of the mechanism whereby it decreases PPTs in human subjects. It has been reported that intravenous and subcutaneous/intradermal administration of human NGF results in (dose-dependent) muscle pain symptoms in the bulbar and truncal musculature as well as the masseter muscle that increased with function [79,157]. Women were reported to be more susceptible to the effects of human NGF than men [157]. Further, men have reported sensitization in the human NGF-injected masseter muscle 1 day after injection and this sensitization lasted for 7-14 days [187]. Preliminary data from an ongoing study on women suggests that human NGF-induced mechanical sensitization of the masseter muscle can be
observed as early as 3 hours post-injection and also lasts for 7-14 days (Svensson et al, Unpublished Observations). The failure of intramuscular injection of rat NGF to induce rat masseter afferent fiber sensitization in a manner similar to the effect of intramuscular injection of human NGF on human subjects suggests the following possible conclusions could be drawn: 1) NGF acts in the central rather than the peripheral nervous system to cause mechanical sensitization, and/or 2) there are differences in the effective concentration of rat vs. human NGF to induce mechanical sensitization.

Peripheral vs. Central Mechanisms: Behavioral studies conducted on adult rats have shown that systemic NGF administration causes rapid sensitization to cutaneous thermal (within 15 min) stimuli and a slower onset of (starting 6.5 hours after injection) cutaneous mechanical sensitization that may last for up to 7 days [132,133,166]. Cutaneous thermal sensitization after systemic NGF administration appears to be mediated through sensitization of afferent fibers, perhaps secondary to an increased sensitivity of the vanilloid TRPV1 receptor, which is the receptor activated by capsaicin (the substance that makes peppers taste hot) [175,176]. In contrast, NGF-induced decreases in cutaneous mechanical sensitivity do not appear to be associated with a decrease in the mechanical threshold of cutaneous Aδ mechano-nociceptors [132]. The authors suggested that the mechanical sensitization in the absence of injury or inflammation was more likely due to central processes, i.e. sensitization of second-order sensory neurons in the spinal cord. In vitro experiments on isolated cutaneous tissue have also failed to induce mechanical sensitization (only thermal) in response to NGF [167]. In comparison with the in vivo recording techniques, the in vitro skin nerve preparation model is limited in its ability to detect chemically induced mechanical sensitization [12,53,60,121,123]. A recent in vivo study by Mense and his colleagues suggests that
intramuscular injection of human NGF also does not cause any significant mechanical sensitization in muscle C afferents 30 min post-injection [103], which is consistent with the results of the present study. However, an indirect assessment of MT was used in this study therefore the results need to be interpreted cautiously. The lack of any effect of NGF on the mechanical sensitivity of Aδ cutaneous afferents agrees with the results of the present study. Taken together, these animal studies support the idea that central mechanisms may underlie the development of mechanical sensitization after injection of NGF into the masseter muscle.

Nevertheless, the present study does contain some evidence that peripheral mechanisms may also contribute to the mechanical sensitizing effect of NGF. In particular, one Aδ fiber was found to become responsive to jaw opening post-rat NGF injection. Approximately 90% of the human subjects reported pain upon chewing and yawning one day after human NGF injection into the masseter muscle. Thus, it is conceivable that NGF alters the mechanical sensitivity of a subgroup of masseter muscle afferent fibers and this effect underlies some of the features of NGF induced mechanical sensitization observed in humans after human NGF injection.

**NGF and Myositis**

The rat NGF did not cause any significant plasma protein extravasation around the injection site, suggesting that rat NGF does not induce muscle inflammation. Some previous studies have reported inflammation in response to intradermal injection of NGF [11,155]. One study has reported that saline injection causes extravasation of >40 μg/g Evans’ blue dye at the injection site and human NGF injection causes a significantly higher Evans’ blue dye extravasation in rat skin (~ 40 min post-human NGF injection) that is dose-dependent [11]. The authors suggested that human NGF-induced extravasation might be due to an indirect
effect of human NGF on sympathetic nerve terminals. It is possible that human NGF may be inflammatory in rat skin; however, another study that used murine or bovine NGF has also reported that NGF induces plasma extravasation in rat skin 30 min after injection [155]. The results of the present study differ from the results reported previously, however, it should be stressed that in the present study rat NGF, and not human, murine or bovine NGF, was injected into the masseter muscle. Although the dose of Evans' blue dye used in the present study (6 mg/kg) was lower than the dose used in the previous studies (20 and 50 mg/kg) [11,155], other studies that have used 6 mg/kg Evans’ blue dye have shown marked plasma protein extravasation in response to inflammatory substances like mustard oil [87,98]. Thus, differences in Evans’ blue dye dose do not appear likely to explain our result. The previous studies that have reported NGF-induced inflammation were conducted in rat skin and not muscle tissue, which may be one of the factors responsible for inconsistent results. As muscle inflammation would be expected to cause afferent fiber sensitization, the lack of inflammation in rat NGF-injected masseter muscle agrees with the lack of sensitization in response to rat NGF.

Study Limitations

Effect of Anesthetic

One of the limitations of the present study is the requirement for experimentation to be conducted under general anesthesia, which could be speculated to alter the response characteristics of muscle afferent fibers. As this study was conducted under the influence of anesthesia there is a possibility that the anesthetic agent, isoflurane, may have altered the properties of the afferent fibers under study or interfered with the effects of rat NGF. Few studies have been conducted to test the effects of general anesthetics on primary afferent
fibers [13,42,61,158,193] and most of these studies have reported inconsistent or contradictory results. It has been reported that the inhalational anesthetic halothane can sensitize cutaneous primary afferent fibers in the monkey to heat, but not mechanical stimuli [42]. Isoflurane has been shown to have no effect on the membrane potential and impedance of trigeminal ganglion neurons in decerebrate guinea pigs [158]. However, the effect of isoflurane on the mechanical response properties of rat primary afferent fibers has not been investigated.

Since, general anesthetics slow down the metabolism it is possible that isoflurane could have influenced the results of this study. Isoflurane could have interfered with the downstream targets involved in rat NGF’s effect on nociception for example voltage-gated Na$^+$ and Ca$^{2+}$ channels [68,88,89]. In other words, the anesthetic could have decreased the ability of rat NGF to affect these targets. Anesthesia may delay the effects of rat NGF by influencing the rate of retrograde axonal transport. A study conducted on anesthetized (sodium pentobarbital and chloral hydrate) male rats has reported that retrograde transport of radiolabeled NGF from tooth pulp to the trigeminal ganglion requires ~15 hours (retrograde transport rate ~3 mm/hr) following tooth injury [197]. Since, retrograde transport of neurotrophins is known to increase after injury [58] it is possible that the rate of retrograde transport from an uninjured or uninflamed muscle may be even slower and possibly more susceptible to the effects of an anesthetic.

**Human vs. Rat NGF**

So far, all of the animal studies conducted with NGF have used either human or mouse submaxillary NGF in rats, with the exception of one study that has reported that intrathecal rat NGF administration has no effect on thermal (heat) and mechanical thresholds
in rats [33]. As discussed above, most of these studies (conducted on cutaneous tissues) using human or mouse NGF have observed thermal and mechanical hyperalgesia in response to the NGF injection. Although mouse and human NGF are thought to be quite similar, rat and human NGF share only 90% amino acid sequence homology. The differences in rat and human NGF sequence may be crucial in determining their effects. It is possible that there are differences in the potency of rat and human NGF. Rats may be less sensitive to their own NGF as compared to the NGF of another species such as humans. The rat NGF concentrations used in this study were dose and concentration matched with the human NGF used in the Svensson et al study [187]. However, it is possible that a higher dose or concentration of rat NGF is required to observe a similar response in rats as in humans. It can also be argued that human NGF might be more efficacious in causing mechanical sensitivity in rats than rat NGF, possibly due to a non-specific mechanism (such as a foreign protein reaction, decreased degradation of human NGF by rat proteases, slowed clearance from the muscle etc.). However, due to the lack of detailed information regarding the differences between rat and human NGF these statements cannot be supported by any experimental evidence and are merely speculations at this point.

NGF Receptor Expression/Density

The biological actions of NGF are mediated by TrkA and p75 receptors. The expression of these receptors determines the responsiveness of afferent fibers to NGF. It is possible that there might be differences in the expression or density of NGF receptors on masseter muscle afferent fibers in rats and humans. Immunohistochemical study conducted on human peripheral ganglia (sympathetic and dorsal root) and skin has shown that only 65% of the cell bodies of primary sensory neurons express the high affinity NGF receptor (gp140-
TrkA) [194]. Human non-nervous cutaneous tissues such as epidermis and sweat glands etc. also express NGF receptors [194]. However, various animal studies have shown that only a small percentage of myelinated DRG neurons express the TrkA receptor. In adult rats, only 40% and 50% of the DRG neurons (small and medium sized cell bodies) express the high affinity TrkA and low affinity p75 receptors, respectively [18,21,81,149,150]. As compared to the DRG neurons, the expression of TrkA receptors in nociceptive trigeminal ganglion neurons has been reported to be even lower [150]. Only 10% of neurons innervating the incisor and 15% of corneal neurons were found to be TrkA positive in adult and neonatal mice [150]. Another study has suggested that a higher percentage of rat visceral afferents express the TrkA receptor than cutaneous afferents, suggesting that NGF receptor expression may be tissue-dependent [18,22,150]. Interestingly, the majority of the TrkA-expressing primary afferents are also CGRP and SP positive [18,22,118,150,182]. These studies clearly suggest that there are differences in the expression of NGF receptors (and therefore NGF-responsiveness) between spinal and trigeminal nociceptors in addition to tissue and species-related differences in NGF receptor expression. In addition, neurotrophin responsiveness may be dependent on other factors such as the presence of second messengers such as cAMP and calcium, which are responsible for the insertion of NGF receptors into the cell membrane of some neurons [105].

**MT vs. PPT**

Despite the similarities between the results from animal and human studies (*discussed above*) there are some differences between the PPT and MT recordings. The PPT recording is a subjective measurement that is dependent on the subject’s perception and report of pain. Therefore, it represents a response of a population of fibers and involves both the peripheral
and central processes (higher brain centers). On the other hand, the mechanical threshold recording represents the response of a single primary afferent fiber and does not involve higher brain centers. This study was limited to examining the effect of rat NGF on only one mechanically sensitive fiber at a time. It is possible that rat NGF does not alter the mechanical sensitivity of mechanically sensitive fibers, but instead causes mechanically insensitive fibers (silent nociceptors) to become sensitive to mechanical stimulation [147,148]. If this were the mechanism, we would not have detected this change because our technique does not allow us to identify and record from mechanically insensitive afferent fibers, i.e. we are using a mechanical search stimulus. However, this (recruitment of silent nociceptors) would be detected by PPT measurements in humans for the reasons mentioned above. Also, the different diameters of probes used for VF hair stimulation and PPT recordings could be responsible for the differences in MT and PPT. A larger probe diameter would result in the application of a mechanical stimulus to a larger area of the receptive field resulting in spatial summation, which would be assessed with a PPT recording but not with the MT recording technique. Therefore, if the mechanism of human NGF-induced mechanical sensitization involves recruitment of other afferent fibers in the masseter muscle, then this would not have been detected by the single fiber MT recording technique used in this study.

**Future Directions**

As discussed above, a number of factors could explain the lack of effect of rat NGF on rat masseter muscle afferent fiber sensitivity. In order to exclude some of these factors/possibilities, the following studies could be conducted.
(i) **Use of Human NGF in Rats:** As mentioned previously, it is possible that human NGF may be more potent in causing mechanical sensitization and other TMD-like symptoms in rats. Therefore, one possible future project could be to determine the peripheral effects of human NGF (25 µg/ml) injection on the mechanical threshold of rat masseter muscle afferent fibers, using the same protocol as the present study. If this study confirms that human NGF (is different from rat NGF and) causes mechanical sensitization similar (in intensity and duration) to that observed in humans than human NGF could be used to model TMD-like symptoms in rats. However, if the results show that human and rat NGF are not different in terms of their effects on afferent fiber sensitivity, then the following study could be conducted to determine if rat masseter muscle afferent fibers are responsive to rat NGF, i.e. express NGF receptors.

(ii) **Histological Study:** In order to exclude the possibility of species differences in the expression of NGF receptors a histological study could be conducted to determine if the cell bodies of rat masseter muscle afferent fibers express NGF receptors, especially the TrkA receptor. This study would involve identifying TrkA receptors with a specific TrkA receptor-antibody and then staining the antibody-bound receptors with a dye. The trigeminal ganglion could then be sliced to examine the expression/density of dye-stained receptors. If the results of this study show that rat masseter muscle afferent fibers express TrkA receptors, then the following study could be conducted.
(iii) **Peripheral Study over a Longer Time Period:** In order to account for a possible effect of the anesthetic in slowing down the effect of NGF (human or rat depending on the results of study (i)), a similar study could be carried out over a longer period of time. The study design could involve injecting rats with vehicle or NGF and the next day (~24 hours later) anesthetizing the rat to record the post-injection MT, fiber CV, response to jaw-opening etc. This study would allow NGF to have an effect in the absence of any anesthetic for approximately 24 hours, therefore excluding any possibility of anesthetic interfering with the actions of NGF. A much larger sample size would be needed for this study to account for the inter-animal and inter-fiber variability in MT and to see differences between vehicle and NGF groups. If the results of this study are negative then it will be concluded that NGF does not have a peripheral effect on the mechanical threshold of masseter muscle afferent fibers. Although, at present, the results of the human NGF study strongly suggest a peripheral mechanism of action it is possible that NGF's effect on the mechanical sensitivity of afferent fibers may have a central component. To investigate the central mechanism, further studies could be conducted on second-order neurons by recording from the subnucleus caudalis in the brainstem.

(iv) **Influence of Rat NGF on the Response to Glutamate:** Another possible study could be conducted to determine if NGF alters the response of afferent fibers to other chemicals such as glutamate. The experimental protocol could involve injecting rat NGF or vehicle into the RF of masseter muscle afferent fibers 20 min before injecting glutamate solution and conducting evoked response and
mechanical threshold recordings. This study may help determine if rat NGF increases glutamate-induced sensitization, possibly due to phosphorylation of NMDA receptors by NGF.
Chapter 5. SUMMARY OF CONCLUSIONS

Overall, the results of this study suggest that intramuscular injection of rat NGF does not cause any significant evoked response or mechanical sensitization in rat masseter muscle afferent fibers. As hypothesized, rat NGF injection does not cause any muscle inflammation. It was concluded that the injection of rat NGF into the rat masseter muscle is not a good method to model human NGF-induced sensitization or human NGF-induced TMD-like symptoms observed in the human study. It is possible that there might be species differences in the effect of NGF. On a positive note, the results show some interesting sex-related differences in the baseline properties of masseter muscle afferent fibers that need to be further investigated.
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