Peripheral mechanisms of masseter muscle nociceptor sensitization by Nerve Growth Factor (NGF)

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ABSTRACT

Myofascial temporomandibular disorders (TMD) are common chronic craniofacial conditions that are characterized by pain in the masseter muscle. It has been suggested that nerve growth factor (NGF) may contribute to muscle sensitization in TMD-like pain based on various animal and human studies. Injection of NGF into the masseter muscle of healthy human subjects is not painful but does induce a localized, quick onset (~1 hour) and long lasting mechanical sensitization. It is not known how NGF causes this sensitization.

NGF binds to the p75 receptor as well as the tyrosine kinase receptor A (TrkA), both of which are expressed on nociceptive neurons and may increase excitability and neuron sensitization. NGF is also reported to enhance NMDA receptor function on ganglion excitatory synaptic transmission.

I hypothesized that human NGF mechanically sensitizes masseter muscle nociceptors by increasing the sensitivity of peripheral NMDA receptors. Co-expression of the NR2B subunit of the NMDA receptor with P75 and TrkA NGF receptors by trigeminal ganglia neurons that innervate the masseter muscle was investigated immunohistochemically. Nociceptor activity was recorded extracellularly from the

trigeminal ganglion of anaesthetized female rats. Nociceptor mechanical threshold was assessed before and every 30 minutes for 3 hours after injection of human NGF (25 μg/ml, 10 μl), and in subsequent experiments NGF with TrkA or P75 receptor antibodies. Glutamate (1 M, 10 μl), a NMDA receptor agonist, was injected at the end of each experiment. Approximately 85% of NR2B positive masseter ganglion neurons co-expressed P75 or TrkA receptors, suggesting the potential for interaction. When compared with the vehicle control, it was found that injection of NGF into the masseter muscle did not evoke significant nociceptor discharge but did significantly reduce nociceptor mechanical threshold. There was no effect of NGF on glutamate-evoked nociceptor discharge or glutamate-induced mechanical sensitization. Additional experiments indicated that NGF-induced mechanical sensitization could be partially attenuated by co-administration of TrkA receptor antibodies, but not P75 receptor antibodies. These findings indicate that human NGF-induced sensitization of masseter nociceptors results, in part, from activation of TrkA receptors but does not appear to be mediated through enhanced peripheral NMDA receptor activity.

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LIST OF ABBREVIATIONS

Aδ	A delta fiber
AChE	Acetylcholinesterase
ANOVA	Analysis of Variance
ASIC	Acid-Sensing Ion
Akt	Protein Kinase B
ATP	Adenosine Triphosphate
BDNF	Brain Derived Neurotrophic Factor
cAMP	Cyclic adenosine monophosphate
CD3+	Cluster of Differentiation 3 (T-Cell Co-receptor)
CD4+	Cluster of Differentiation 4 (T-Cell Co-receptor)
CGRP	Calcitonin Gene Related Peptide
CNS	Central Nervous System
CREB	Cyclic AMP-Response Element Binding
CV	Conduction Velocity
CY3	Cyanine
DAG	Diacylglycerol
DMEM	High glucose Dulbeco's Modified Eagle's Medium
DRG	Dorsal Root Ganglion
ECL	Enzymatic Chemiluminescence
ERα/β	Estrogen Receptor α/β
ERE	Estrogen Response Element
ERK	Extracellular Signal Regulated Kinase
FDU	5-fluorodeoxyuridylate
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
g	Gram(s)
GABA	Gamma Aminobutyric Acid
GDNF	Glial Derived Neurotrophic Factor
GnRH	Gonadotropin-releasing hormone
h	Hour(s)
H+	Proton
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer
HRP	Horseradish peroxidase
HSP	Heat shock protein
i.p.	Intraperitoneal
IgG	Immunoglobulin G

JNK	Jun N-terminal kinase
K+	Potassium
kDa	Kilodalton(s)
KF-kβ	Nuclear factor kβ
g	Microgram(s)
µg/gm	Microgram(s) per gram
µg/kg	Microgram(s) per kilogram
g/ml	Microgram(s) per milliliter
l	Microliter(s)
m	Micrometer(s)
m/s	Meter(s) per second
mg/kg	Milligram(s) per kilogram
MAP	Mitogen-activated protein
min	Minute(s)
mRNA	Messenger Ribonucleic Acid
MT	Mechanical Threshold
NADE	Neurotrophin-Associated cell death executor
NGF	Nerve Growth Factor
NGS	Normal Goat Serum
NLS	Nuclear Localization Sequences
NMDA	N-methyl-D-aspartate
NR	N-methyl-D-aspartate Receptor
NRAGE	Neurotrophin-receptor interacting MAGE homologue
NRIF	Neurotrophin-receptor interacting factor
NRS	Numerical Rating Scale
NS	Nociceptive Specific
NT	Neurotrophin
PBS	Phosphate buffered saline
pg/ml	Picogram per milliliter
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCγ	Phospholipase Cγ
pM	Picomolar(s)
PNS	Peripheral Nervous System
PPT	Pain Threshold
PTOL	Pressure Tolerance Threshold
RhoA	Ras Homolog gene family member A
RIP2	Receptor-interacting protein 2
SC1	Schwann cell 1
SDS	Sodium dodecyl sulfate

SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second(s)
Shc	Collagen-Like Adaptor Protein
SP	Substance P
Src	Sarcoma Homologous Protein
SRC-1	Steroid Receptor Coactivator 1
t1/2	Half Life
TMD	Temporomandibular Disorder
TMJ	Temporomandibular Joint
TNFα	Tumor Necrosis Factor α
Trk	Tropomyosin-related kinase
TrkA	Tyrosine Kinase Receptor A
TRPV1	Transient Receptor Potential Vanilloid Receptor
TSNC	Trigeminal Sensory Nuclear Complex
V	Trigeminal Nerve
V1	Ophthalmic Branch of the Trigeminal Nerve
V2	Maxillary Branch of the Trigeminal Nerve
V3	Mandibular Branch of the Trigeminal Nerve
VPM	Nucleus Ventralis Posteromedialis
WDR	Wide Dynamic Range

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1. INTRODUCTION

1.1 Temporomandibular Disorders (TMD)

1.1.1 Musculoskeletal Pain

Pain is an unpleasant experience that is sensory and subjective, and is caused by either actual or potential tissue damage. Excessive quantity and duration of pain may affect a person's personality, mood and behavior. Prolonged exposure to pain can lead to depression, sleep disturbance, and analgesic substance dependence. Chronic pain from the muscles, bones, ligaments and/or joints is characterized as musculoskeletal pain [1-3]. Musculoskeletal pain conditions affect a wide variety of people as well as cause a great amount of social burden [2-4]. There is a general lack of understanding of the underlying pathophysiology of these conditions. However, a greater number of women than men tend to suffer from musculoskeletal pain, and in addition, women tend to suffer more severe, frequent and prolonged sensations of pain [5-8]. Musculoskeletal pain includes conditions such as osteoarthritis, rheumatoid arthritis, osteoporosis, low back pain and craniofacial pain conditions such as TMD [1, 9-10].

1.1.2 Craniofacial Pain and TMD

Craniofacial pain refers to pain conditions that occur in the head and face region, and affect about one quarter of the population of the industrialized world [11-12]. Craniofacial dysfunction and pain includes conditions such as dental and periodontal pain,

oral soft tissue disease, orofacial neuralgia such as trigeminal neuralgia, and myalgias as a result of conditions that include fibromyalgia, and TMD.

TMD are a common chronic craniofacial condition that are characterized by pain in the temporomandibular joints (TMJs) and muscles of mastication such as the masseter (jaw-closer) muscle [13-17]. TMD are classified into three groups, masticatory muscle disorders, TMD disc displacement and TMJ disorders. TMD include symptoms such as jaw clicking, limited jaw movement and headaches [13-14]. It has been suggested from clinical research that TMD may be caused by local trauma, teeth grinding (bruxism) or teeth misalignment (malocclusion), stress, as well as other causes [10, 13-21].

TMD affect about 10% of the North American population, and there is a greater prevalence of this condition among women than men, which suggests that sex hormones may play a role in TMD-like pain, in particular menstrual cycle-related variations in sex hormone levels are thought to be associated with pain intensity in women [6-8, 22-23]. Rapid fluctuations in estrogen level during menstruations are thought to increase muscle and joint pain in TMD. TMD-related pain has been shown to be most intense around ovulation when estrogen level is at its highest, and the number of women with TMD decreases around menopause [5-8, 22]. Thus sex-related differences in pain perception may result from alterations in pain processing due to changes in estrogen levels.

Prior to the studies relating sex hormones and TMD, the disorder was traditionally thought to be caused by factors such as anatomical variation in the masticatory system, psychosocial characteristics and pain in other body regions [24]. Recently, identifying genetic markers that may contribute to TMD etiology have also come under consideration. In particular it was found that people with a genetic variant of catechol-o-methyltransferase (enzyme associated with pain responsiveness) are at greater risk for developing TMD [25]. However, the general lack of in depth knowledge with regard to the pathogenesis of TMD makes it a challenge to develop effective treatment, thus the development of animal models is brought forth in order to elucidate the mechanism behind TMD pain [18].

Several models for myofascial TMD have been developed, such as glutamate-induced sensitization of rat masseter muscle nerve fibers and the NGF-induced sensitization of the masseter muscle of human volunteers [26-27]. However, the underlying mechanisms behind TMD still remain to be explored.

1.1.3 Trigeminal Pain Pathway

Pain signals are detected by primary afferent neurons located at the site of noxious stimuli (mechanical, thermal chemical), usually generated by painful injury. The noxious stimuli will activate these sensory fibers found in the skin, joints, muscles, and other deep muscles. These primary afferent neurons, or first-order fibers, are known as nociceptors;

neurons that are activated by painful stimuli and carry pain signal to the brain. Noxious stimuli applied to the face are transmitted in the form of action potentials through two types of primary afferent fibers, Aδ and C fibers, to the trigeminal somatosensory nuclei in the brainstem [28-31]. Aδ fibers are larger, myelinated, faster conducting (conduction velocity of 2-30 m/s) afferent fibers that carry noxious stimuli from nociceptive-mechanical or mechanothermal-specific nociceptors to produce sharp, precisely localized pain sensations. C fibers are smaller, unmyelinated, slower conducting fibers (conduction velocity < 2.0 m/s) that carry mostly chemical or thermal stimuli from polymodal nociceptors to produce the dull, burning pain sensation [28-31]. C fibers are most frequently found to terminate at the junction of the dermis and the epidermis, as they make up 80% of cutaneous nerve.

Whereas noxious stimuli from the body will enter the dorsal horn of the spinal cord for transmission to the brain, noxious stimuli from face will enter the brainstem via the trigeminal nerve [32-33]. The trigeminal nerve (V), which is the fifth cranial nerve, innervates the craniofacial region and carries noxious stimuli from most of the craniofacial region to the brainstem, although some craniofacial transmissions are carried via upper cervical spinal nerves or other cranial nerves to the brain. The V nerve, which is the largest and most complex of the 12 cranial nerves, is a mixed nerve containing both sensory and motor fibers. The V ganglion (Gasserian) contains the majority of sensory neuron cell bodies that enter the pontine brainstem, and give rise to three subdivisions of

the V nerve, the ophthalmic (V1), maxillary (V2) and mandibular (V3) branches of the nerves in the periphery. The V1 branch of the nerve innervates forehead, nasal cavity, skin, cornea and the dura. The V2 branch of the nerve innervates upper lip, zygomatic area, and portions of the oral cavities including the maxillary teeth. The V3 branch of the nerve innervates lower facial regions such as the mandible with sensory afferents, and innervates the muscles of mastication such as masseter muscle with motor efferents. Thus, the V3 branch of the nerve consists of both sensory and motor fibers, in contrast to the V1 and V2 branches, which consist of only sensory nerves [32, 34-35]. The trigeminal nerves have cell bodies within the trigeminal ganglia (V ganglia), which is the equivalent of the dorsal root ganglia that receives nerve transmission from the trunk and limbs. The V ganglia are located bilaterally at the base of the brain in the posterior cranial fossa across the superior border of the poetrous temporal bone and within Meckel's cave formed by an invagination of the dura matter. Each ganglion is crescent shaped and consists of the sensory neurons, of which 60-70% are C and Aδ fibers. Cell bodies of the mechano-receptive and nociceptive afferents of the V1 branch are found medially and anteriorly, V2 branch cell bodies are caudal and lateral to V1, and V3 is caudal and lateral to V2. However, the cell bodies of muscle spindle afferents (such as found in masseter muscle) are located in the V mesencephalic nucleus located within the CNS [32, 34-38].

The primary afferent fibers (Aβ, Aδ and C) have neuronal bodies within the trigeminal

ganglion, and convey sensory information from the face, conjunctiva, oral cavity, and dura matter. Aβ fibers are large, myelinated fibers with fast conduction velocities (>35 m/s) that convey sensations such as light touch, in contrast to unmyelinated C and thinly myelinated Aδ fibers that convey nociceptive information. These larger afferent fibers are especially numerous in skin and mucosal tissues. Some proprioceptors in deep tissues such as muscles and joints are also associated with Aβ afferent fibers. Aα fibers are the largest and most heavily myelinated fibers with the fastest conduction velocities (>70 m/s). They are exclusively the axons of muscle spindles and Golgi tendon organs in masticatory system. These jaw muscle spindle afferents have primary afferent cell bodies in the V mesencephalic nucleus within the CNS, which extends from the rostral end of the principal nucleus to the superior colliculus in the midbrain, the axons from which then project to the ipsilateral trigeminal motor nucleus, and form synaptic connections with the motorneurons that innervate masticatory muscles [39-41]. All other muscle afferent fibers that have cell bodies located in the trigeminal ganglion project axons to the trigeminal sensory nuclear complex before being relayed to other parts of the CNS [32, 34-38].

The trigeminal sensory nuclear complex (TSNC), which is a key site in the brainstem, is responsible for relay of nociceptive information to cortex [38, 42-43]. The TSNC is composed of the main sensory nucleus and spinal tract nucleus, and is a bilateral and multinucleated structure that extends from the pons to the upper cervical spinal cord.

The spinal tract nucleus (spinal trigeminal nucleus) is composed of oralis, interpolis and caudalis subnuclei. The caudalis is believed to be the site of trigeminal nociceptive information integration from the masticatory muscles [44-46]. Its laminated structure greatly resembles that of the dorsal horn of the spinal cord, from which nociceptive information from the rest of the body is received. Most of the primary afferent Aδ and C fibers that innervate the jaw muscles terminate in the subnucleus caudalis laminae I, II, and V and VI, in addition to caudal portions of the subnucleus interpolaris, the medullary, and upper cervical dorsal horns [47-50]. The second order neurons, which convey nociceptive information from the brainstem to higher regions of the central nervous system (e.g. the thalamus), are categorized as either nociceptive-specific (NS) or wide dynamic range (WDR) [38, 51-52]. NS neurons receive only small-diameter nociceptive Aδ and C fibers and are excited only by stimuli that are potentially painful. WDR neurons receive both large and small diameter afferent fibers, and thus can be excited by both noxious and non-noxious stimuli. From the TSNC the second order neurons (NS and WDR) will send projections to the ventrobasal complex in the thalamus called the nucleus ventralis posteromedialis (VPM), which will send the transmission to the primary somatosensory area of the cortex [32, 34-38].

1.1.4 Peripheral and Central Sensitization

Peripheral sensitization of the primary afferent fiber may be caused by tissue damage and subsequent inflammation that result in increased afferent excitability. Tissue damage

causes release of inflammatory mediators such as substance P, histamine, bradykinin, acetylcholine, ATP, ions (H^+, K^+), cholecystokinin, prostaglandin, serotonin, glutamate and NGF. These mediators are synthesized and recruited to the site of injury, and will either activate or sensitize the nociceptor for further release of neuropeptides and excitatory amino acids. Histamine, serotonin, and cytokines promote inflammation in addition to increasing the excitability of nociceptive afferent endings. K^+ ions will activate nociceptors that release substance P and CGRP that cause vasodilation and increased capillary permeability, these in turn will cause macrophages and other immune cells to release more inflammatory mediators and act on nociceptive afferent endings. Thus peripheral sensitization is caused not only by chemical mediators released at the site of injury, but also by the release of neurochemicals from the activated afferent endings. Hyperalgesia will occur as the afferent fiber lowers its activation threshold and increases its response to the noxious stimuli as well as increased background spontaneous firing [38, 42].

Inflammation or trauma of peripheral tissue may caused central sensitization; an effect intiated by repetitive activity in the primary afferent nerve fibers (Aδ and C) which leads to changes in central neurons (NS and WDR) in terms of increase of receptive field size and spontaneous firing, and decrease in threshold to noxious stimuli. This prolonged increased excitability of the second order neurons is thought to be initiated through activation of neurokinin (activated by substance P) and excitatory amino acid receptors,

in particular the NMDA receptor that is activated by glutamate. Thus the second order neurons will become sensitized to low threshold afferent input that do not usually cause painful sensation [38, 42]. This expansion of receptive fields and other functional changes in the central nociceptive processing are proposed to contribute to secondary hyperalgesia, which is a persistent pain condition displayed clinically as increased sensitization to noxious stimuli of non-inflamed tissue surrounding the site of injury, and pain referral, which is the sensation of pain in tissues not affected by the inflammation or trauma.

1.2 Nerve Growth Factor and its role in Pain Mechanism

1.2.1 Introduction to Nerve Growth Factor

Nerve Growth Factor (NGF) was discovered nearly 5 decades ago and is a peptide that regulates survival, development, function and plasticity of neurons [53-58]. Peptides that promote neuronal survival are known as neurotrophic proteins, and include brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) [59]. All these peptides are found in mammals and share similar structures and functions. Two other neurotrophin homologues NT-6 and NT-7 are found in lower vertebrates such as fish [58].

NGF is found in high concentrations in the mouse submaxillary gland protein [60-61].

It contains 3 types of subunits, α, β, and γ which form the 7S complex precursor that is cleaved at both N- and C-terminal to produce mature NGF [53, 55, 58-59]. The mature form of biologically active NGF is known as β-NGF, and can be further cleaved by submaxillary gland proteases. The β-NGF contains a core that is formed by two identical twisted β-chains which consist of 118 amino acids of 13kDa each with three intra-chain disulfide bridges, a cysteine-knot motif as well as β-hairpin loops at the ends, and is responsible for the nerve growth stimulating activity [53, 55, 58]. The coding regions of human β-NGF are located on the proximal short arm of chromosome 1 and are highly homologous to mouse β-NGF precursor sequences [62-63].

NGF is responsible for the survival and growth of sensory and sympathetic neurons in the developing nervous system, in addition to regulation of neuronal functions in the mature nervous systems such as its role in pain and inflammation, synaptic function and plasticity, and continuing modulation of neuronal survival [54-57]. NGF also regulates receptor proteins such as bradykinin, vanilloid receptors, substance P, and calcitonin-gene-related peptide (CGRP) receptors [64-69].

1.2.2 Expression of Nerve Growth Factor

Both NGF and its receptors are expressed in various cell types in the central nervous system (CNS) and peripheral nervous system (PNS), as well as immune and inflammatory systems. The level of NGF expression has been found to be elevated

upon tissue injury, inflammation and chronic pain [54-56, 58, 70].

In the PNS, various tissues such as skin (keratinocytes), vascular, endocrine and other non-neuronal targets of sympathetic and sensory neurons synthesize and produce NGF during development and maturity. Through receptor-mediated endocytosis, NGF is transported from nerve terminal to neuronal cell bodies via axons to promote neuronal growth and survival. NGF is also produced in immature Schwann cells and satellite cells during development, and its expression regulated by these glial cells during maturity [55, 58].

In the CNS, where NGF is known to play a role in neuronal growth and plasticity, the highest amount of NGF mRNA expression is found in the hippocampus and the cerebral cortex. It is produced both during development and maturity. NGF-producing cells are mostly neurons such as the pyramidal neurons, cholinergic neurons, dentate granule neurons and GABA-ergic interneurons. NGF is also produced by astrocytes and microglia in the CNS [55, 58].

In the immune system, inflammatory leukocytes such as mast cells, monocytes and macrophages, T-lymphocytes (CD3+ and CD4+ T cells), and B lymphocytes promote NGF expression, synthesis and release, suggesting a role for NGF in the inflammatory function. Leukocytes such as macrophages infiltrate the nerve following peripheral

nerve injury and release cytokines which induce synthesis of NGF in Schwann cells and fibroblasts. Mast cells also synthesize NGF and promote its release after activation [55-56, 58, 71].

Neurotrophin receptors are expressed on both sympathetic and small diameter peripheral sensory neurons, as well as leukocytes in the immune system [72-74]. NGF binds to neurotrophin receptors that are expressed on nociceptive neurons to increase excitability and neuron sensitization [55-56]

1.2.3 TrkA and p75 receptors

NGF mediates its peripheral effects through binding to and activating two different neurotrophin receptors, the high affinity tyrosine kinase receptor A (TrkA) and the low affinity p75 receptor [55, 58, 75]. In the PNS and peripheral tissues, both TrkA and p75 receptors are expressed by sympathetic and small diameter sensory neurons during development and maturity [58]. About 45% of small diameter adult dorsal root ganglia neurons express TrkA receptors, and about 60% of trigeminal ganglia neurons express both TrkA and p75 receptors [73, 76-77]. There is ample evidence to suggest that these receptors are selectively expressed by adult nociceptive afferent fibers. In addition, PNS glial cells express p75 receptors during development as well as regulate its expression during maturity. In the CNS, although neurons express both TrkA and p75 receptors, the

majority of p75 receptor expressing neurons do not also express TrkA receptors but instead TrkA receptors are expressed by nearby cells. Lastly, many types of leukocytes in the immune system display expression of TrkA receptors such as mast cells, monocytes, macrophages, and T and B lymphocytes [58].

The TrkA receptor is known as a high affinity NGF receptor because after NGF binds to TrkA, the dissociation between ligand and receptor is very slow (t1/2 ~ 10 min); whereas when NGF binds to p75, the dissociation is rapid (t1/2 ~ 3 sec) [54, 75, 78]. The Trk receptor tyrosine kinase family also includes TrkB and TrkC receptors, which bind to BDNF, NT-4, and NT-3. The TrkA receptor has a short transmembrane region and a catalytic domain that contains the tyrosine residue which upon cross phosphorylation will form a docking site for adaptor proteins and other signaling molecules. NGF binds TrkA receptors at the immunoglobulin-like domain adjacent to the membrane at the extracelluar end of the receptor through contact of its core that contains the central β-sheet to the carboxy-terminal loops of TrkA and its amino-terminal residue that forms helical shapes upon contact with TrkA [79-81].

The p75 receptor is a member of tumor necrosis factor receptor-related molecule family, which nonspecifically binds to NGF as well as other neurotrophins such as BDNF, GDNF and tumor necrosis factor α (TNFα) [71, 73, 75, 82]. It is a transmembrane glycoprotein that contains a cysteine-rich domain and binds dimeric ligands. Structural binding

studies have shown that p75 receptor binds NGF in an asymmetric fashion that allows potential for a Trk receptor to interact with NGF-bound p75 receptor and form a trimolecular signaling complex [81-82]. It has been previously shown that the p75 receptor can act as a co-receptor for Trk receptors and modulate their affinity and specificity to neurotrophins. Studies have shown that when p75 is co-expressed with TrkA, there is a 25-fold increase in NGF binding compared to low affinity Trk site, which suggests that both p75 and TrkA interact to form a high-affinity binding site [75, 83].

1.2.4 The Downstream Signaling Pathway of Nerve Growth Factor

NGF mediates its effect on neuronal growth and survival through binding to and activating TrkA receptors. Upon NGF dimer binding to TrkA, the receptors are dimerized and activated, which results in transautophosphorylation and activation of intracellular signal cascades [55, 58, 75, 78, 84]. When Src (Sarcoma) homologous and collagen-like (Shc) adaptor protein binds to the activated TrkA receptor dimer, phosphatidylinositol 3-kinase (PI3k) and Akt (protein kinase B) activities will increase to promote neuronal survival. When Shc adaptor proteins are phosphorylated by activated TrkA receptor dimer, extracellular signal regulated kinase (ERK) and Ras activities will increase to induce cyclic AMP-response element binding (CREB) transcription factor, which will in turn influence cell cycle (differentiation or proliferation), neurite outgrowth, and synaptic plasticity. Phospholipase Cγ (PLCγ) binds to activated TrkA receptor dimer to activate a signaling cascade that results in the production of inositol phosphate,

which induces release of intracellular calcium and diacylglycerol (DAG) and subsequent activation of protein kinase C (PKC), which leads to the phosphorylation of more proteins that are essential to cell survival and differentiation. PKC may also activate Ras to mediate activation of mitogen-activated protein (MAP) kinase cascade for more transcriptional regulatory activities [58, 78]. TrkA receptors are also frequently co-expressed with transient receptor potential vanilloid receptor (TRPV1) receptors, also known as capsaicin receptors, which are expressed by some nociceptors and are activated by heat and capsaicin. Other ion channels such as NMDA and acid-sensing ion (ASIC) channels are also known to be under possible modulating effects from neurotrophin receptor regulated downstream signaling pathways [78, 84].

In addition to modulating TrkA signaling, p75 receptor activation also plays a role in the signaling pathway that influences cell death and regeneration. In the absence of Trk A signaling, p75 promotes apoptosis and cell death upon NGF binding. The apoptotic event is driven by signaling components such as Jun N-terminal kinase (JNK), nuclear factor kβ (KF-kβ), and ceramide [54, 75, 78]. The p75 receptor also modulates RhoA (Ras Homolog gene family member A) activity for cytoskeletal changes and neurite outgrowth. It mediates its signaling effect through interaction with adaptor proteins that bind to its cytoplasmic domain [55, 58, 78]. The adaptor proteins include neurotrophin-receptor interacting factor (NRIF), neurotrophin-associated cell death executor (NADE), neurotrophin-receptor interacting MAGE homologue (NRAGE),

Schwann cell 1 (SC1) and receptor-interacting protein 2 (RIP2) [75, 78].

1.3 NGF in Clinical Experiments on Pain

1.3.1 Effects of increased NGF levels

Due to its role in the development, function and survival of neurons, NGF has been investigated to determine its role in neurological diseases and injury. Yet it is very difficult to measure and quantify the level of NGF expression in nervous tissues due to its low concentration (<10pM) [85]. NGF levels in the cerebrospinal fluid are also very low (9.1±4.1 pg/ml), but it was found to accumulate following acute brain injury, neurosurgery, and also in patients with fibromyalgia (41.8±12.7 pg/ml), suggesting a possible role of NGF in regeneration of nerve damage in the CNS and also chronic pain disorders [86-89]. In one study using a specific two-site enzyme immunoassay, NGF levels in serum were found to exhibit a sex-related difference in that females had significantly lower levels (112±31 pg/ml) than males (243±35 pg/ml) [90]. However, another study by the same research group showed the opposite result, females exhibited higher NGF levels (57.83±22 pg/ml) than males (37.47±12.28 pg/ml) [91], but this study was done in patients with the Alzheimer's disease, rather than normal volunteers, thus NGF levels may not be the same in healthy subjects compared to patients who are

not healthy, which may explain for the discrepancy between the NGF level measurements. Another factor to consider is that these studies were done in different parts of the world, studies focused on South American population may produced results different from studies focused on Europe population (as shown below) simply due to regional genetic variance. A third study showed no significant sex-related difference in NGF serum levels [92]. In the recent study that showed significant sex-related difference in NGF serum levels, females again had significantly lower levels than males (40.8±10. 8pg/ml), in addition, the serum NGF level in females was shown to be additionally influenced by the menstrual cycle. Serum NGF levels were significantly higher in luteal phase (14.4± 2.9 pg/ml) than in the follicular phase (8.2±1.4 pg/ml), suggesting a possible link between sex hormones such as estrogen and NGF expression[93]. Estrogen receptor mRNA was found to co-express with NGF mRNA and their receptors in the CNS, including neurons of the cerebral cortex, sensory ganglia, and cholinergic neurons of the basal forebrain [94-95], which indicates that estrogen and NGF may influence each other by reciprocal regulation at the gene transcription level or signal transduction to influence neuronal differentiation, survival and maintenance of function.

The elevated level of NGF found at the nerve injury site in sensory neurons suggests a possible link between over expression of NGF and neuropathic injury [96-97]. Further studies conducted on animal models of neuropathic pain demonstrated that endoneurial administration of NGF could produce behavioral symptoms like nerve injury, such as

significant thermal hyperalgesia. Furthermore, NGF induced degeneration and demyelination of nerve fibers at the site of injection. These effects were dose-dependent and shared a similar time-course [98]. However, although these studies showed that elevated levels of NGF might be responsible for neuropathies and contribute to the pain associated with nerve injuries, NGF levels are suggested to be lowered in diabetic neuropathy [99-101]. Systemic administration of NGF as replacement therapy for diabetic neuropathy caused a number of side effects including masseter muscle pain that resembled TMD, and the pain symptoms were more prevalent in women than in men [102-103]. The fact that TMD may be associated with elevated levels of NGF led to the use of NGF in the development of an animal model for TMD-related myofascial pain.

1.3.2 NGF in animal pain studies

In animal studies, Lewin *et al* [104] showed that systemic administration of NGF (1-2μg/gm, i.p.) to neonatal rats produced earlier cutaneous thermal hyperalgesia and more prolonged mechanical hyperalgesia. Mechanical hyperalgesia in neonatal rats treated with NGF can last weeks, and is believed to be due to NGF-mediated sensitization of primary afferent nociceptors. Cutaneous mechanical hyperalgesia in adult rats treated with daily injections of NGF developed 6 hours after NGF injection, whereas cutaneous thermal hyperalgesia developed as soon as 15 minutes after injection. It was concluded that in adult rats, cutaneous thermal hyperalgesia was caused by possible peripheral mechanisms such as sensitization of peripheral receptors to heat, and that cutaneous

mechanical hyperalgesia was caused by possible changes in central mechanisms, and may involve interactions with NMDA receptors.

Rueff *et al* [105-106] showed that a single intraperitoneal (i.p.) injection of NGF (1 mg/kg) resulted in prolonged cutaneous thermal hypersensitivity which began 30 minutes after injection and lasted days. Cutaneous mechanical hyperalgesia developed within 7 hours after injection and resulted in a 70% reduction in mechanical threshold that lasted up to 4 days. The NGF induced cutaneous thermal hypersensitivity was thought to be due to upregulation and activation of bradykinin-1 receptor activity [106]. Furthermore, an *in vitro* study showed that NGF could induce cutaneous thermal hypersensitivity without changing mechnical or cold sensitivity, and that this hypersensitivity was absent in mast-cell depleted animals, suggesting mast cell involvement in NGF-induced thermal hyperalgesia [105]. Because the experiment was performed on perfusion of the receptive field of small diameter afferent neuron fibers, it was also suggested that NGF may exert its effect by acting directly on TrkA receptors located on the primary afferent fiber. In another *in vitro* study [107], NGF was found to sensitize rat dorsal root ganglion (DRG) neurons to capsaicin and noxious heat in adult but not neonatal rats, suggesting TrkA-mediated sensitization of TRPV1 as the mechanism for NGF-induced cutaneous thermal hyperalgesia during postnatal development.

Thompson *et al* [108] showed that systemic administration of NGF (1μg/kg, i.p.) resulted

in cutaneous mechanical sensitization. Cutaneous mechanical hyperalgesia was indicated by a significant drop in mechanical threshold 2 hours following NGF administration, and was maintained for up to 6 hours. In this case, thermal hyperalgesia did not develop until 4 hours after NGF injection, and was maintained for up to 6 hours. This group also believed that mechanical hyperalgesia was due to central mechanisms, and thermal hyperalgesia was due to peripheral mechanisms, in addition, that the prolonged effect of NGF sensitization could be caused by NGF binding and activation of TrkA receptor sites and subsequent signaling.

The above animal studies showed that NGF may exert its mechanical sensitizing effects as early as 1 hour post-injection, and the sensitization could last hours. These results suggested the basic timeframe over which NGF might begin to affect muscle nerve fibers.

1.3.3 NGF in human studies

In human studies, Petty *et al* [109] studied the effect of single intravenous or subcutaneous doses (0.03 to 1.0μg/kg) of recombinant human NGF in healthy human volunteers in a phase I double-blinded, randomized, placebo-controlled study. Subjects reported mild to moderate masseter muscle pain with chewing and swallowing in the 1.0μg/kg intravenous NGF dose group at 60 to 90 minutes after administration, which increased at 4 to 6 hours and took 2 to 8 days to resolve. The duration and severity of the muscle pain were found to be dose-dependent as well as sex-related, as female

subjects appeared to be more susceptible than men. The subcutaneous NGF dose group reported injection-site hyperalgesia in the form of increased tenderness to touch and heat. The duration of this effect appeared to be dose-dependent.

Dyck *et al* [110] studied the effect of intradermal injection of recombinant human NGF (1.0 or 3.0μg) in healthy human subjects and reported development of localized pressure allodynia at the NGF-injection site 3 hours to 21 days after injection. Heat-pain threshold was also significantly lowered 1, 3, and 7 days after injection. A local tissue mechanism was suggested for the cause of the pressure allodynia and the heat-pain hyperalgesia.

In a double blind, placebo controlled study, Svensson et al [27] discovered that intramuscular injection of human NGF (0.1μg/kg) into the masseter muscle of healthy human volunteers resulted in a prolonged localized mechanical sensitization that was associated with muscle pain involving chewing and yawning, symptoms that are similar to muscle pain symptoms reported by TMD patients. Changes in pressure pain threshold (PPT) and pressure tolerance threshold (PTOL) were recorded. Injection of NGF caused no more pain than injection of isotonic saline and little spontaneous pain. Almost all subjects (92%) had pain upon mouth opening and during chewing. Numerical rating scale (NRS) scores on chewing significantly increased after 1 and 7 days, yawning was also significantly influenced by NGF injection. But no significant changes in pain level during talking, swallowing, drinking, smiling or with the jaw at rest were reported.

NGF injection significantly decreased PPT in the human masseter muscle, and this decrease was greater in women [111]. In women, PPT and PPTOL were significantly decreased 3 hours, 1 and 7 days after NGF injection, and NRS scores on chewing and yawning were significantly increased for 7 days after NGF injection. In another study, sensitivity to injection of glutamate into the masseter muscle 24 hours after NGF injection was investigated, and it was reported that there was no effect of NGF on glutamate-induced decreases in PPT or glutamate-evoked pain, although there were larger pain drawing areas in NGF-pretreated masseter muscles injected with glutamate [112]. These studies confirmed that NGF injection does not produce spontaneous pain, but does produce a prolonged mechanical sensitization of the masseter muscle in humans, and that the degree of NGF-induced sensitization is sex-related.

Both animal and human studies have showed that NGF administration leads to sensitization of nociceptive neurons. It has been suggested that estrogen levels may regulate NGF receptor gene transcription and/or mRNA expression, and this interaction between the estrogen and NGF may account for the sex-related difference of the sensitization [113]. Furthermore, the sensitizing effects of NGF might be due to an interaction between NGF and peripheral NMDA receptors. The activation of peripheral NMDA receptors is known to mediate excitation and mechanical sensitization of the masseter muscle nociceptors [26, 114-115]. NGF has been shown to rapidly increase NMDA receptor currents in cultured hippocampal cells in *in vitro* studies and an increase

in NGF levels has been shown to result in phosphorylation of the NR2B subunit of the NMDA receptor, thus suggesting that NGF could exert its sensitizing effect by modifying peripheral NMDA receptor function [75, 78, 116-117]. A potential interaction between estrogen levels, NGF and NMDA receptors has not been investigated.

1.3.4 Studies on blocking NGF receptors

As previously discussed, NGF exerts its effects through interaction with high affinity TrkA receptors and the low-affinity p75 receptors. But it is not known which receptor mediates the masticatory muscle sensitization effect of NGF. Various studies have been done with blocking of either NGF receptors to evaluate which NGF-receptor interaction is responsible for the various effects of NGF. Zhang and Nicol [118] used a p75 receptor antibody (50 μg/ml) to block the NGF-induced sensitization of the excitability of rat dorsal root ganglia (DRG) neurons. They found that blocking the p75 receptor did prevent the NGF-induced increase in evoked action potentials, but it did not have an effect on a downstream signaling molecular, ceramide, to enhance or inhibit neuron excitability. Skoff and Adler [119] investigated the regulation of substance P(SP) in rat DRG neurons by NGF by exposing sensory neuron culture to TrkA and p75 blocking receptors (2 μg/ml and dilution 1:500) after incubating the cell culture with NGF, and then measuring SP release content. They found that blocking the TrkA receptor reduced NGF-induced increases in SP release by 25%, and blocking the p75 receptor reduced NGF-induced increases in SP release by 60%, thus indicating p75 and TrkA both play a

role in NGF upregulation of SP. It is already known that injection of NGF into human masseter muscle results in localized mechanical sensitization [27]. By using a rat model and blocking either of the NGF receptors the receptor which is responsible for the sensitization effect of NGF in TMD-like pain could be determined.

1.4 Glutamate, NGF and TMD

1.4.1 Introduction to Glutamate and NMDA receptors

Glutamate is an excitatory neurotransmitter involved in nociceptive transmission from trigeminal primary afferent fibers to brain stem trigeminal sensory neurons through activation of N-methyl-D-aspartate (NMDA) and non-NMDA receptors [26, 38, 120-121]. NMDA receptors (NRs) are ionotropic receptors that bind glutamate and are expressed at excitatory synapses in the mammalian central nervous system as well as peripherally on sensory afferent fibers that innervate skin, muscle and viscera [115, 122]. Functional NRs are heteromeric complexes composed of both NR1 and one or more subtypes of the NR2 subunit, of which 4 have been identified (NR2A-D) [123]. Functional NRs contain at least one NR1 subunit and either NR2A or NR2B subunits[124]. The NR1 subunit contains the glycine-binding site and the NR2 subunit contains the glutamate-binding site.

There is evidence that phosphorylation of the NR2B subunit increases NR currents and that this process is modulated by estrogen [125-126].

1.4.2 Glutamate and its role in modeling myofasical TMD

It is not known how NGF causes mechanical sensitization, whether by peripherally sensitizing muscle nociceptors to mechanical stimuli, or by altering the response of muscle nociceptors to NMDA receptor activation by glutamate. Glutamate induced sensitization of rat masseter muscle fibers has been used as a model for myofascial TMD [26, 44, 114, 121]. In previous studies, it was found that injection of glutamate into the rat masseter muscle evoked afferent discharge through activation of peripheral NRs and that this afferent discharge was greater in female than in male rats [26, 44, 46, 114]. Subsequent investigation has determined that the sex-related difference in masseter afferent discharge in response to peripheral NR activation is estrogen-dependent and due, in part, to a greater expression of NR2B subunits by masseter afferent fibers in female than in male rats [115]. In contrast, it has been found that temporalis muscle afferent fibers are less sensitive to peripheral NR activation and do not exhibit sex-related differences in afferent discharge [127]. It is not known why temporalis muscle afferent fibers respond differently than masseter muscle afferent fibers to peripheral NR activation.

1.4.3 NGF and its effect on glutamate

Recent evidence indicates that masseter muscle afferent fibers express NMDA receptors that contain the NR2B subunit [115]. It has been reported that nerve growth factor (NGF) enhances NMDA receptor function in ganglion neurons by acting on the NR2 subunit [116]. Thus NGF may also enhance NMDA receptor function on excitatory synaptic transmission, possibly either by enhancing the probability of glutamate release, or by increasing the expression of NMDA receptor subunit via TrkA receptor activation that stimulates promoter activity causes further sensitization [116].

1.5 Estrogen and its role in Pain Mechanisms

1.5.1 Introduction to Estrogen and its receptors

Estrogens are steroid hormones that are involved with the development of neurons, modulation of synaptic plasticity, neuronal excitability and neurogenesis, in addition to a possible role in sex-related differences observed in cognitive function as well as pain perception [128]. The most abundant estrogen in humans is 17β-estradiol. In the nervous system, unlike neurotransmitters such as glutamate, GABA and acetylcholine which are stored and released from presynaptic vesicles, estrogens are produced in the mitochondria and microsomes of neurons and glial cells and are released slowly via passive diffusion [129]. Both experimental and clinical data have shown estrogen involvement in various chronic pain syndromes such as TMJ pain.

The transcriptional activity of estrogen is mediated by estrogen receptor (ER)α and ERβ. The ERs are members of the nuclear hormone receptor superfamily of transcription factors. ERα is expressed highly in pituitary, kidney, epididymus, and adrenal. ERβ is expressed highly in prostate, lung, bladder and brain. Both of them are highly expressed in the ovary, testis and uterus. Both ER isoforms are also expressed at similar levels by dorsal root ganglion neurons [130-132], as well as by trigeminal ganglion neurons (unpublished data), suggesting possible involvement in the mechanisms for craniofacial pain transmission. Binding of estrogens to either of the ER isoforms can result in regulation of gene transcription via genomic actions. Estrogen can also modulate signaling cascades via non-genomic actions.

1.5.2 Genomic and non-genomic actions of estrogen and their effects on NGF signaling

The genomic effect of estrogen occurs when 17β-estradiol binds to an ER to induce a conformational change that causes the receptor to dissociate from cytoplasmic chaperone proteins such as heat shock protein 90 (HSP90) and expose nuclear localization sequences (NLS), which allow nuclear translocation and promote ER homo/heterodimerization. The receptor then binds to an estrogen response element (ERE) of the target gene and with the recruitment of specific coactivators such as steroid receptor coactivator 1 (SRC-1) and cAMP response element binding protein (CREB) either increases or decreases target gene transcription. In contrast to the slower genomic

actions of estrogens which can take hours, non-genomic actions of estrogen occur as rapid effects that take seconds to minutes. Non-genomic events occur when estrogen binds to membrane receptors coupled to ligand-gated ion channels or G-protein coupled receptors, which allow a rapid action on neuronal excitability, activation of intracellular secondary messenger systems such as cAMP/PKA and MAP/ERK kinase, as well as modulation of calcium channels and calcium flux and gonadotropin-releasing hormone (GnRH) release [129, 133-137].

NGF has been shown to induce expression of functional follicle-stimulating hormone (FSH) and stimulate estrogen secretion in the developing rat ovary [138]. In turn, estrogen has also been shown to regulate the expression of NGF and its receptors [139-141]. Ovariectomized hamsters treated with 17β-estradiol showed stimulated expression of NGF and its two receptors in the uterus [139]. Estrogen also affects the action of NGF through its involvement in the complex signaling network of growth factors and cytokines, such as potentiating the action of NGF by activating intracellular signaling cascade (ERK, PI3K) that is also regulated by NGF. In addition, past studies on estrogen regulation of NGF receptor mRNAs in adult sensory neurons suggest that NGF receptor mRNAs were upregulated upon high estrogen level in the dorsal root ganglion, suggesting that estrogen may also regulate neuronal sensitivity to NGF and play role in mediating NGF-mediated effects[141]. Furthermore, estrogen has also been shown to modulate hippocampal NRs in the cortex and striatum of ovariectomized rats by

increasing the expression of NRs and phosphorylation of tyrosine residues on the NR2 subunits [113]. Changes in estrogen levels during the estrus cycle in female rats were also found to be associated tyrosine phosphorylation of NR2 subunit of NMDA receptors [125-126]. These findings seem to suggest that estrogen may alter primary afferent fiber sensitivity by mediating an effect on NGF and NMDA receptors. Thus, changes in estrogen level may have the capacity to modify pain.

1.5.3 Rat estrus cycle stages

Varying levels of estrogen induce changes in cell morphology that can allow the determination of estrus stage during the estrus cycle by visualizing vaginal epithelial cells under the microscope [142]. The 4-5 day rat estrous cycle is made up of four stages. These are the diestrus, proestrus, estrus and metestrus [143-145]. During diestrus, the level of estrogen is initially low but slowly increases during the later part of the stage prior to proestrus (Figure 1). The progesterone level reaches a peak during the beginning of diestrus and drops sharply during the second phase. The estrogen level peaks during the proestrus and then drops steeply to a minimum level during the latter part of estrus. The progesterone level drops at the beginning of proestrus, and reaches the lowest level at the same point where estrogen reaches its peak level, and returns to a maximum level at the end of the proestrus [142-145].

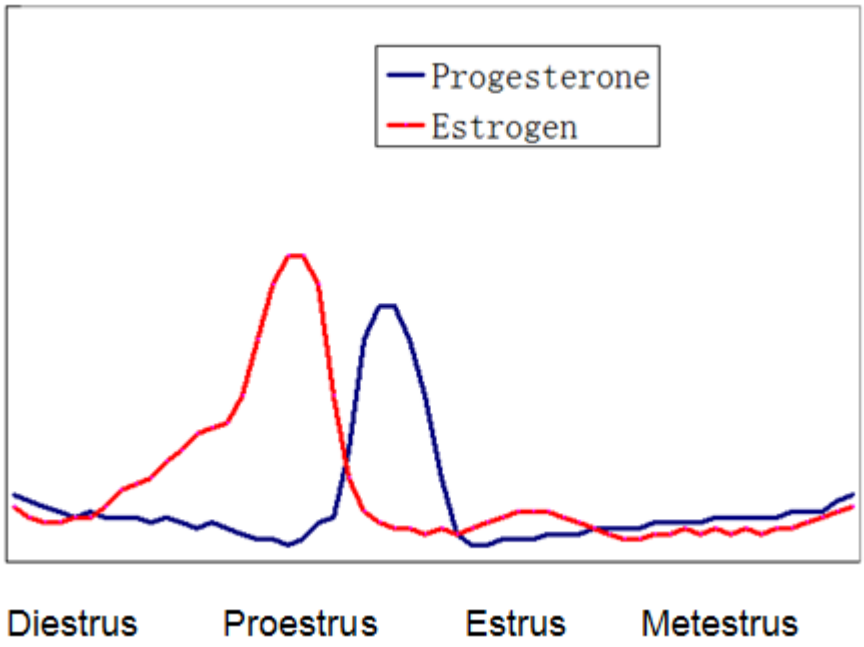

Figure 1. Sex-hormone levels during the rat estrous cycle. The red line denotes levels of estrogen, which peak during proestrus and then drop steeply to a minimum during the latter part of estrus. The blue line denotes levels of progesterone, which drop at the beginning of the proestrus, and reach their lowest level at the same point where estrogen reaches its peak level. Progesterone levels reach a maximum at the end of the proestrus.

Experimental hypothesis

It was hypothesized that NGF sensitizes primary afferent fiber in rat masseter muscle by interacting with p75 and/or TrkA receptors to increase the response of NMDA receptors to endogenous glutamate.

OBJECTIVES

The objectives of this study were:

[i] To determine the effect of intramuscular injection of human NGF on the mechanical threshold and evoked discharge of nociceptors in rat masseter muscle

[ii] To determine the expression of NGF receptors (TrkA/p75) in masseter ganglion neurons and their co-expression with the NR2B subunit of NMDA receptors in order to assess the potential for receptor interaction

[iii] To determine the effect of human NGF on glutamate-induced mechanical sensitization and glutamate-evoked nociceptor discharge, which has been shown to be mediated through activation of NMDA receptors.

[iv] To determine the effect of NGF receptor (TrkA/p75) antibodies on NGF-induced mechanical sensitization and NGF-evoked nociceptor discharge

2. MATERIALS & METHODS

2.1 Antibodies

All antibodies used in the present study were acquired from commercial sources. Antibodies included: anti-rat TrkA receptor antibody (1:40, Goat polyclonal, R&D Systems, Minneapolis, MD), anti-rat P75 receptor antibody (1:225; Rabbit polyclonal, Sigma-Aldrich, St. Louis, MO), anti-rat NR2B subunit antibodies (1:300, Mouse Monoclonal, Chemicon, Billerika, MA), anti-rat ERα receptor antibody (1:80, Rabbit polyclonal, Abcam, MA, AB21232); anti-rat ERβ receptor antibody (1:300, Rabbit polyclonal, Chemicon International, CA, AB1410). Secondary antibodies used were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, USA. Fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit IgG secondary antibodies were used for P75 and the ER receptors, FITC-conjugated donkey-anti-goat IgG secondary antibodies for TrkA receptor, cyanine (CY3)-conjugated goat anti-mouse IgG secondary antibodies for NR2B subunit.

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polyclonal, Abcam, MA, AB21232); anti-rat ERβ receptor antibody (1:300, Rabbit polyclonal, Chemicon International, CA, AB1410). Secondary antibodies used were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, USA. Fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit IgG secondary antibodies were used for P75 and the ER receptors, FITC-conjugated donkey-anti-goat IgG secondary antibodies for TrkA receptor, cyanine (CY3)-conjugated goat anti-mouse IgG secondary antibodies for NR2B subunit.

2.2 Animal Housing

Adult female Sprague-Dawley rats (n=72) weighing 225-450g (Charles River, Montreal, Quebec) were housed two per cage (same-sex) on a 12:12h light:dark cycle with ad libitum access to food and water. All procedures were performed in adherence with the principles of the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

2.3 Surgical preparation

Each Sprague-Dawley rat was weighed and then quickly anesthetized with isoflurane delivered through a facial mask, after which tracheotomy was conducted to put the rat on artificial respiration (oxygen: 97.5-98%) and under steady flow of isoflurane anesthesia

(isoflurane: 2-2.5%). In addition, mechanical ventilator rate and isoflurane levels were adjusted to keep the blood pressure between 60-80 mmHg. A temperature probe was inserted rectally to monitor core body temperature. Heart rate was monitored on electrocardiogram. Vaginal epidermal cells were collected from all female rats on glass slides and viewed under the microscope to determine the estrus stage. Fluctuations in sex hormone levels during the rat estrus cycle cause morphological changes in the vaginal epithelial cells. There are four stages in the cycle, diestrus, proestrus, estrus and metestrus. Diestrus cells include round, nucleated cells of different sizes. Proestrus cells include only large nucleated cells. Estrus cells appear to be keratinized. Metestrus cells include both nucleated cells and keratinized cells [146-147]. The femoral artery was catheterized to monitor the blood pressure and maintained to be above 60 mmHg during the recording. In addition, pentobarbital was delivered at the end of the experiment via the catheter to quickly and painlessly terminate the rat [26, 114-115, 148].

2.4 Stimulation and recording techniques

After surgery was performed on the anesthetized rats for skull trephination and exposure of caudal brainstem, a parylene-coated tungsten recording electrode (A-M Systems, Carlsborg, WA, USA) was lowered into the trigeminal ganglion to record from the cell

body of single trigeminal nociceptors (Figure 2). The output from the recording electrode was recorded on a computer. Blunt mechanical stimuli were applied to find and activate primary afferent fibers 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 produce an antidromically conducted action potential. The collision between the antidromic action potential generated from caudal brainstem stimulation, and the orthodromic action potential evoked by mechanical stimulation of the masseter muscle afferent fiber was used to confirm the projection of the fiber from the masseter muscle to the caudal brainstem, an area of the central nervous system thought to be important for higher level processing of nociceptive input from the masseter muscle [149]. The straight-line distance between the stimulating and recording electrodes was divided by the antidromic latency to permit estimation of the conduction velocity (CV) of each nociceptor [26, 114-115, 148].

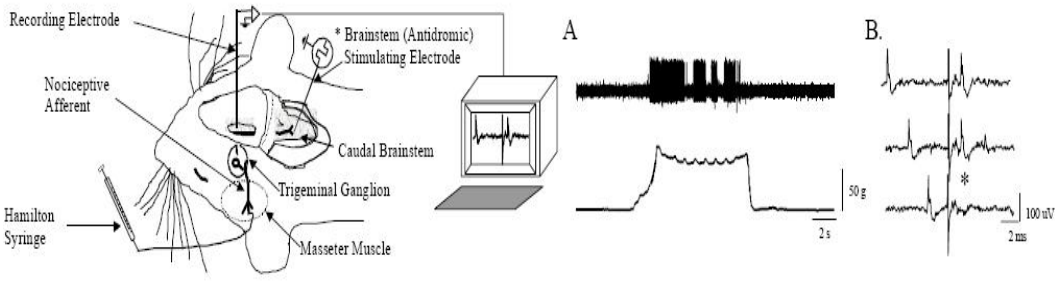

Figure 2. Electrophysiology set-up used to identify masseter nociceptors with projections to the caudal brainstem. A: The figure shows action potential firing (upper) from an activated afferent fiber by force applied with a Von Frey hair (lower). B. Afferent fiber projection into caudal brainstem was confirmed by collision (*) of the antidromic spike evoked by brainstem stimulation

with the orthodromic spike evoked by masseter muscle stimulation.

2.5 Experimental protocol

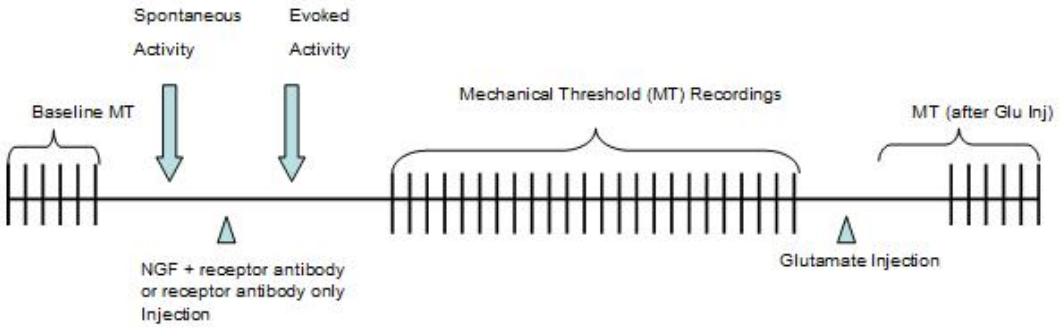

Figure 3. Experiment protocol. The arrows indicate where recording of spontaneous activity and evoked activity occurred. The small triangles indicate injections. Each vertical line represents an afferent mechanical threshold measurement. The bracket represents baseline mechanical threshold recording before injection, and mechanical recording for 10 minutes every 30 minutes after injection, as well as after glutamate injection.

Experiments were carried out to determine the effect of intramuscular injection of human NGF on masseter muscle nociceptor excitability and mechanical sensitivity. Before the injection of any substance, baseline nociceptor mechanical threshold was measured with an electronic von Frey Hair (model 160IC, IITC, California, USA) at one-minute intervals for 10 minutes (Figure 3) [26, 114-115, 148]. Each nociceptor identified was randomly assigned to injection of one of the following groups: i) vehicle control (phosphate buffered isotonic saline, n=12) or NGF (Sigma, St. Louis, MO; 25 μg/ml in phosphate buffered saline, n=12), ii) TrkA antibody alone (R&D Systems, Minneapolis, MD; 2.0 μg/ml in phosphate buffered saline, n=12) or NGF and TrkA antibody (n=12), iii) P75 antibody alone (Sigma, St. Louis, MO; 1.2 μg/ml in phosphate buffered saline,

n=12)) or NGF and P75 antibody (n=11). The concentrations of TrkA and P75 antibodies were chosen by performing western blots to determine antibody-binding saturation concentration in the masseter muscle tissue (Figure 4). The concentration of antibodies used was also in the same range as those used in previous in vitro studies to block TrkA and P75 receptors [118-119]. The investigator conducting the experiment was blinded to the content of the injections. In all experiments, the volume of each single injection was 10μl and was administered intramuscularly into mechanoreceptive field of the masseter muscle nociceptor with a 26 gauge needle connected by polyethylene tubing to a 25 μl Hamilton syringe. Prior to injection, baseline nociceptor activity was recorded for 10 minutes. After injection, evoked activity was monitored for 10 minutes, and then beginning 30 minutes post injection, mechanical threshold was assessed at one-minute intervals for 10 minutes every 30 minutes for a total of 3 hours. At the end of the 3 hour period, glutamate (10 μl, 1M, Sigma, USA) was injected into the nociceptor mechanoreceptive field. Nociceptor discharge in response to injection of glutamate was used to confirm the location of the receptive field within the masseter muscle. Ten minutes after injecting glutamate, the mechanical threshold was assessed at one-minute intervals for 10 minutes. Blood samples were taken to measure the serum sex hormone levels (estrogen, progesterones, and testosterone) with a commercially available kit. The rat was finally euthanized with an overdose of pentobarbital (100 mg/kg).

2.6 Immunohistochemistry

To identify ganglion neurons which innervate the masseter muscle, the fluorescent dye fast blue (2%, 10 μl, Polyscience, USA) was injected bilaterally into the masseter muscle of 4 isoflurane-anesthetized female rats. Seven days after the fast blue injection, the rats were euthanized (pentobarbital 100 mg/kg) and then perfused with 120 ml cold saline followed by 120 ml of paraformaldehyde (4%). The right and left trigeminal ganglia were removed and cut into sections (40 μm) with a vibratome. Free-floating sections were treated with 5% normal goat serum (NGS) in phosphate buffered saline (PBS) for 1 h at room temperature for blocking. Sections were incubated with 5% normal goat serum (NGS) in phosphate buffered saline (PBS) for 1 hour and then incubated overnight with either anti-rat TrkA receptor antibody (1:40, goat polyclonal, R&D Systems, Minneapolis, MD), anti-rat P75 receptor antibody (1:225, rabbit polyclonal, Sigma-Aldrich, St. Louis, MO), anti-rat NR2B subunit antibodies (1:300, mouse monoclonal, Chemicon, Billerika, MA), or a combination of NR2B and P75 or TrkA antibodies in PBS containing 1% NGS at 4^{o}C. The next morning sections were washed several times with PBS and then incubated for 1 h at room temperature in the dark in the presence of FITC and CY3-conjugated IgG selective for the animal species from which the primary antibodies were derived. After several washes in buffer, sections were mounted on slides and visualized with a Leica DM fluorescent microscope. In control experiments, the tissue sections were incubated without primary antibody to confirm

specificity of receptor-like immunoreactivity. Fast blue positive ganglion neurons (masseter ganglion neurons) were counted and photographed for estimation of cell area using NIH Image J freeware. Neurons were considered positive when the intensity of the fast blue and CY3 signals, respectively, exceeded the 95% confidence interval of the mean background intensity.

2.7 Western Blot

Trigeminal ganglia obtained from an adult female Sprague–Dawley rat were homogenized on ice in lysis buffer (Sigma-Aldrich), centrifuged and transferred to a fresh Eppendoff tube. The total protein content was determined by the Bradford method. Protein samples (20 μg each) were run for 90 min on 7% SDS electrophoresis polyacrylamide gel (PAGE). Proteins were then transferred onto 0.2 μm nitrocellulose membrane (GE Healthcare, Mississauga, ON, Canada), the membrane was blocked (5% non-fat dried milk) at room temperature for 1 hour and then incubated at 4 °C overnight with primary antibody. The next day, the membrane was washed and then incubated with secondary antibodies. Receptor specific bands were visualized with an ECL Western blotting detection kit (Amersham). Images were captured with an Alpha Innotech FluorChem 8800 gel-box imager (Alpha Innotech Co., San Leandro, CA).

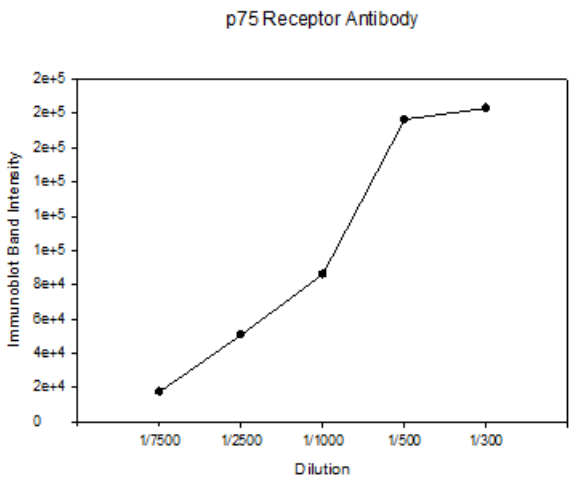

Figure 4. Concentration of p75 NGF receptor antibody graphed against band intensity. From this graph it was concluded that at the dilution of 1:500 (1.2 µg/ml) of p75 NGF receptor antibody should be used in the study, as the curve plateaus at the point where dilution is at 1:500, signifying that saturation is reached at that antibody concentration.

Western Blot was performed on rat masseter muscle protein samples by using increasing concentrations of TrkA and p75 NGF receptor antibodies to label the protein samples. The band intensity of the protein band labeled by the NGF receptors was used to construct the curve in Figure 4. The antibody concentration at which the curve plateaus was determined to be the concentration at which antibody binding reaches saturation in the muscle protein sample. The concentration for TrkA antibody used in this experiment was determined to be 2.0 µg/ml and concentration for P75 antibody was determined to be 1.2 µg/ml.

2.8 17β-Estradiol concentration

Plasma estrogen (17β-Estradiol) levels were measured with a commercially available ELISA kit (GenWay, San Diego, CA). Plasma concentration of the unconjugated form of estradiol was measured according to the protocol provided by the manufacturer. In principle, fixed amounts of estradiol labeled with horseradish peroxidase (HRP) were put

into competitive reaction with unlabelled estradiol in a 2 hour incubation for binding sites of estradiol antibody, a substrate solution (tetramethylbenzidane (TMB) –H_2O_2) was then added for the production of colorimetric compound due to substrate turnover, the reaction was then stopped with H_2SO_4, and the absorbance from the wells were read using at 450 nm using a spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Calibration curves were constructed and estradiol concentrations in the samples were calculated. The mean (±SE) estrogen concentration measured was 77±9 pg/ml.

2.9 In vitro Calcitonin gene related peptide (CGRP) release assay

The right and left trigeminal ganglia were removed from anaesthetized female rats (n=5) and transferred to ice-cold Brooks-Logan solution (5% PBS, 40mM sucrose, 30mM glucose, 10mM HEPES, pH 7.4), after which the ganglia were incubated with collagenase (5 mg/ml) for 30 minutes and then incubated with trypsin (0.05%) for 25 minutes. The resulting trigeminal ganglion neuron suspension was then centrifuged at 2000RPM for 2 minutes, vortexed and centrifuged again, after which basal culture medium DMEM (high glucose Dulbeco's Modified Eagle's Medium, with antibiotics, glutamate, and 3μg/ml 5-FDU) was added and triturated with Pasteur pipette, followed by successive triturations through 18-gauge needles. Cell suspension was then transferred

to 3 wells in 24 well plates which were pre-coated with poly-$_D$-lysine (200μg/ml) and cultivated in DMEM medium. The cell culture was maintained overnight, after which culture medium was changed. Basal medium was removed for measurement, after which NGF (50ng/ml), or NGF (50ng/ml) + TrkA antibody (2.0μg/ml), or NGF (50ng/ml) + P75 (1.2μg/ml) were added to the culture medium. Treated samples was then collected after 3 hours and stored at -20 ^{o}C.

Both basal samples and treated samples were extracted for CGRP according to enzyme immunoassay kit protocol as provided by the manufacturer (SPI Bio, France), the supernatants from each well were homogenized with 2M acetic acid, followed by 10 minute period incubation at 95^{o}C, then centrifuged for 30 minutes at 10,000g, the resultant supernatant was then lyophilized to dryness and reconstituted with EIA buffer (provided by the kit). CGRP content was determined using the enzyme immunoassay kit, samples and standards were distributed to the wells, followed by addition of anti-CGRP tracer, which is an acetylcholinesterase (AChE) Fab' conjugate that binds epitope on the CGRP molecule, concentration of CGRP is determined by measuring the enzymatic activity of the AChE using the Ellman's reagent, which forms a yellow compound, the color intensity of which was read as absorbance at 405 nm using a microplate spectrophotometer (Shimadzu Scientific Instruments, Maryland, USA). CGRP concentration was then calculated using a standard curve generated from the kit standards with absorbance on the y-axis and neuropeptide concentration (pg/ml) on the

x-axis.

2.10 Sample Size

It was determined that a difference of 25% between mean mechanical threshold of control and treatment groups was required for the result to be considered biologically meaningful. The minimum sample size estimated using an α-value of 0.05, and a power of 0.80 was 10 nociceptors per treatment group. However, it was decided to collect data from 12 nociceptors in each treatment group so a total of 72 individual nociceptors (n=72 rats) were examined, 12 each for treatment 1 (vehicle control, phosphate buffered isotonic saline), treatment 2 (25 μg/ml human NGF), treatment 3 (1.2 μg/ml p75 receptor antibody), treatment 4 (1.2 μg/ml p75 receptor antibody + 25 μg/ml human NGF), treatment 5 (2.0μg/ml TrkA receptor antibody), and treatment 6 (2.0 μg/ml TrkA receptor antibody + 25 μg/ml human NGF) groups.

2.11 Data analysis

The WCIF Image J software program (NIH Image,USA) was used to measure the area of Fast Blue positive cells. The percentage of receptor labeled temporalis ganglion neurons was calculated by dividing the number of receptor positive neurons by the total number of Fast Blue labeled neurons. Co-expression was calculated by dividing the

number of NR2B positive neurons by the number NR2B positive neurons that were also TrkA or P75 positive.

The activity of identified nociceptors was amplified and fed into a computer equipped with a 1401 Plus board and Spike 2 analysis software (Cambridge Electronic Design). Recorded nociceptor activity was stored electronically and analyzed off-line. The evoked response of each nociceptor was calculated by subtracting the total number of the spikes during the 10-minute period before the injection (baseline activity) from the total number of spikes during the 10-minute period after the injection. Mechanical threshold was determined by averaging ten consecutive mechanical stimuli at each time epoch. The percent change in mechanical threshold ($\Delta MT(\%)$) was calculated by the equation: $\Delta MT(\%) = 100 * (MT_t - MT_B)/MT_B$, where MT_t is the mechanical threshold at time t (30-180 minutes) and MT_B is the baseline mechanical threshold.

2.12 Data Statistics

The Chi Squares test was employed to assess differences in distribution of receptor expression amongst ganglion neurons of different sizes. Student t-tests were used to determine whether there are significant differences in glutamate-evoked nociceptor discharges, and glutamate-induced mechanical threshold reduction after NGF or vehicle

injection. Two-way repeated measures ANOVA and post hoc Holm-Sidak method were used to assess the effect of various substances on mechanical threshold. One-way ANOVA was used to compare the conduction velocities of masseter nociceptive fibers in the different treatment groups, in order to assess whether difference in conduction velocities has any effect on response from nociceptive fibers. Pearson product moment correlation was used to investigate the relationship between plasma estrogen levels and nociceptor excitability changes. The Fisher exact test was used to examine the frequency of sensitization between the NGF and NGF & TrkA antibody groups at one hour post injection. In all tests, the level of significance was set at $p < 0.05$.

3. RESULTS

3.1 Expression of TrkA and P75 receptors and co-expression with NMDA receptors

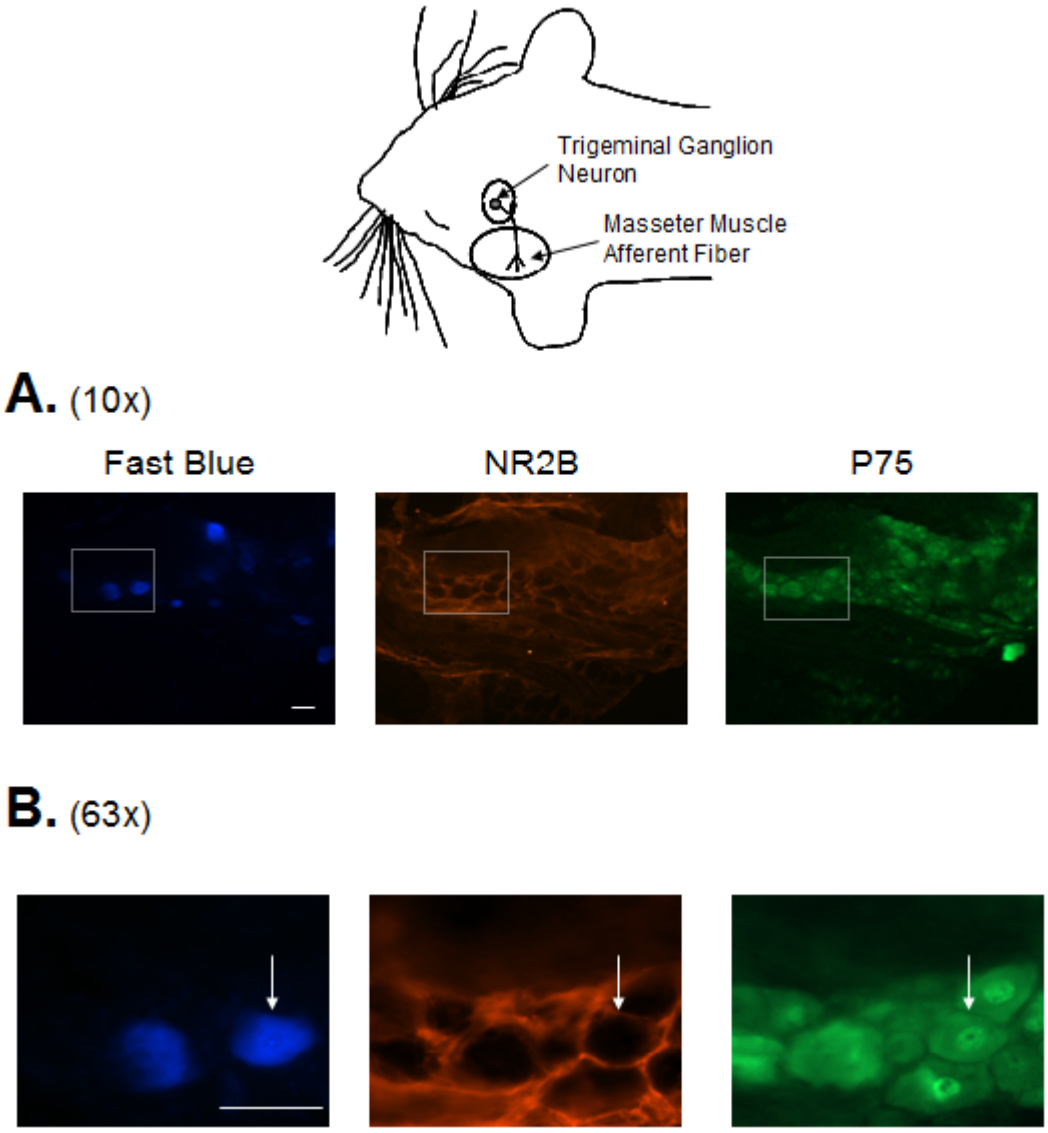

Figure 5. A. The paired low magnification images show examples of fast blue-positive trigeminal ganglion (masseter ganglion) neurons that were immunoreactive with NR2B and/or P75 receptor antibodies. B. The inset box indicates the area examined at higher magnification. The white arrow indicates a fast blue labeled neuron which was immunoreactive with both NR2B and P75 antibodies. White calibration bars indicates 50□M.

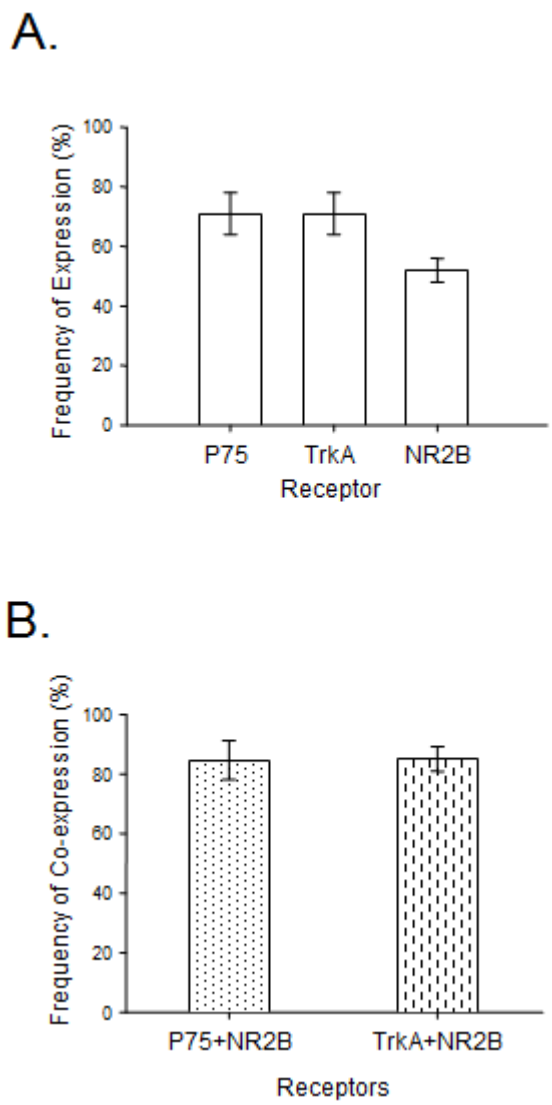

Figure 6. A. The bar graph illustrates the mean frequency of expression of P75, TrkA and NR2B receptors in 4 female rats. B. A large number of NR2B positive masseter ganglion neurons co-expressed P75 (85%) or TrkA (85%) receptors, which confirm the potential for a receptor interaction. Error bars indicate standard error of the mean (SEM).

A total of 882 fast blue positive neurons were examined for TrkA expression, and 783 neurons for P75 expression from the 4 rats. More than 70% of masseter ganglion neurons examined expressed TrkA or P75 receptors, respectively (Figure 5 & 6A). These receptors were uniformly distributed amongst small, medium and large ganglion

neurons (Figure 7).

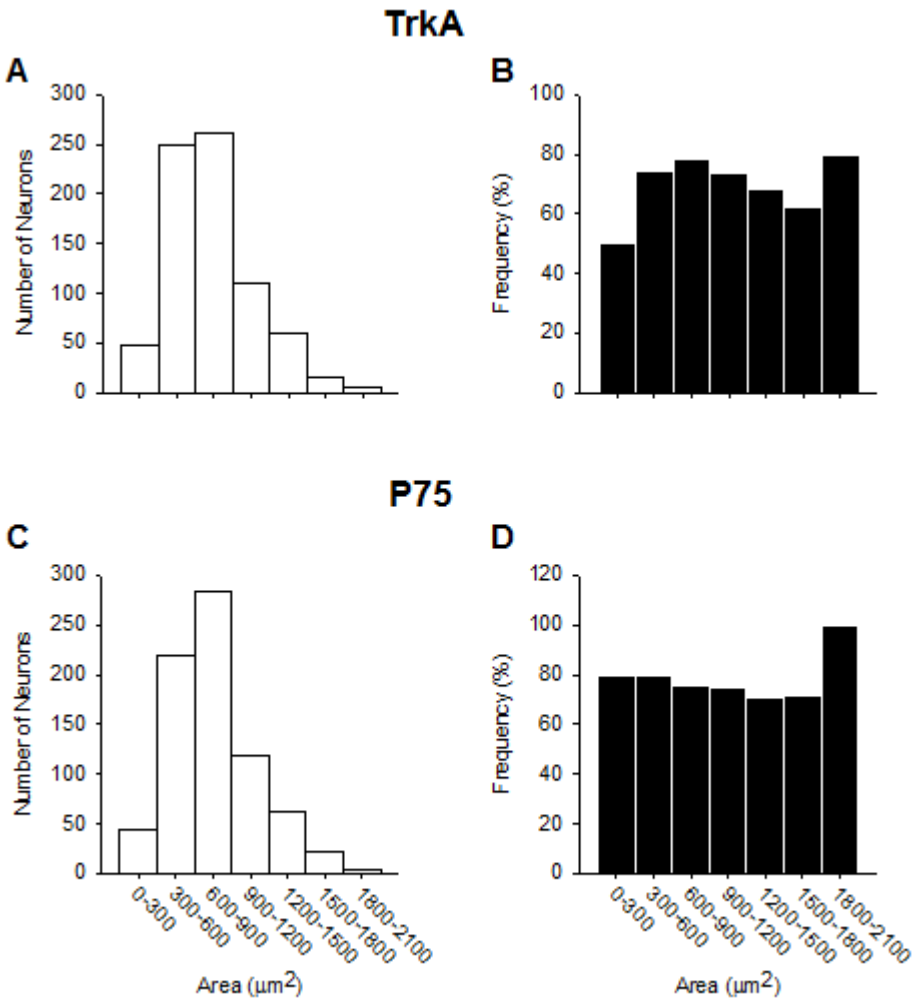

Figure 7. The histograms in A. and C. show the total number of masseter ganglion neurons assessed in each size category for TrkA and P75 receptor expression, respectively. The histograms in B. and D. illustrate the frequency of TrkA and P75 receptor expression, respectively, in these masseter ganglion neurons. Both receptors were uniformly distributed amongst small, medium and large ganglion neurons.

3.2 Effect of NGF on masseter nociceptors

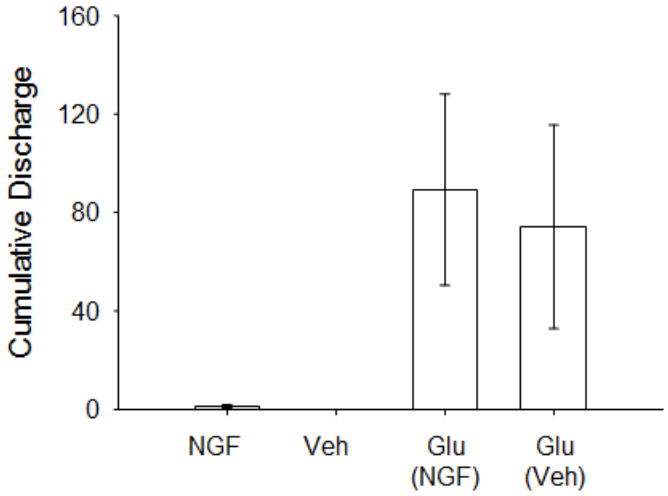

Figure 8. The bar graph shows nociceptor discharge evoked by the initial injection of NGF or vehicle (veh) and also by the subsequent injection of glutamate. No significant differences between NGF and vehicle evoked discharge or effects of treatment on glutamate-evoked nociceptor discharge were found.

Injection of NGF did not evoke significantly more discharge than injection of the vehicle (Figure 8). Injection of glutamate resulted in substantial nociceptor discharges, however, the magnitude of cumulative discharge evoked by glutamate was similar in the NGF and vehicle groups.

Injection of NGF significantly decreased the mechanical threshold of masseter nociceptors compared to vehicle (Figure 9A). Two-way repeated measures ANOVA revealed a significant effect of treatment and time on the relative mechanical threshold, but no significant interaction between treatment and time. Post-hoc tests indicated that

overall NGF significantly decreased nociceptor mechanical threshold compared with vehicle.

The change in mechanical threshold relative to the pre-glutamate injection mechanical threshold (at 3 hours post NGF) revealed no significant difference in the magnitude of glutamate-induced mechanical sensitization between the two groups (Figure 9B). Taken together, these results indicate that NGF did not increase glutamate-evoked nociceptor discharge or glutamate-induced mechanical sensitization.

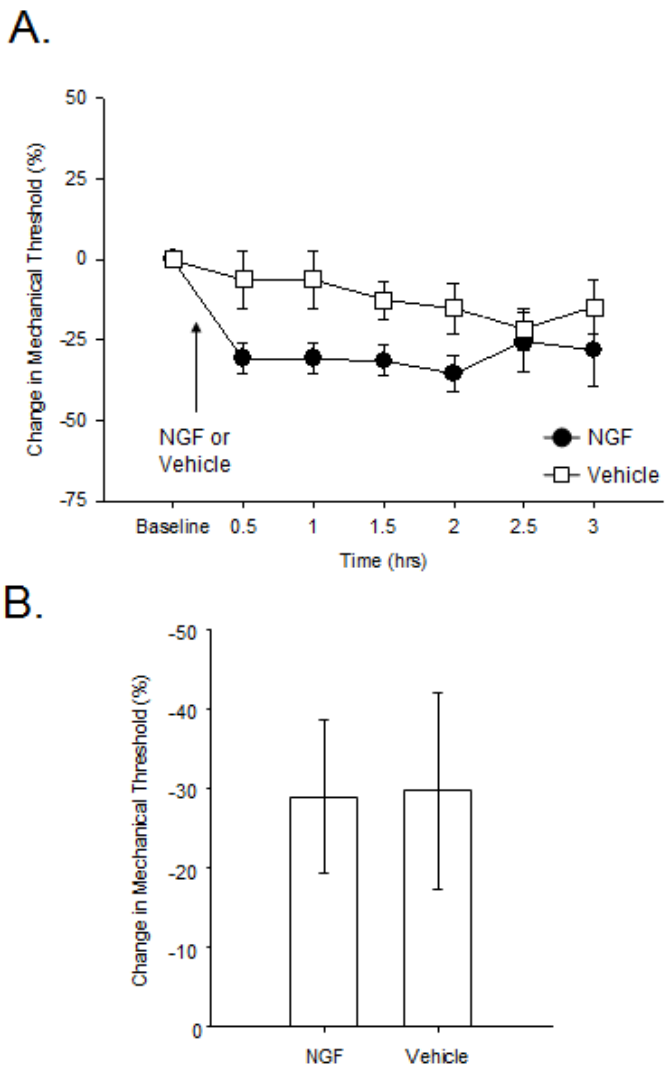

Figure 9. A: The line and scatter plot illustrates changes in the mechanical threshold of nociceptors after injection of NGF or vehicle into the masseter muscle. B: Relative mechanical threshold calculated by dividing the mechanical threshold after glutamate injection by the mechanical threshold recorded 3 hours after injection of NGF or vehicle. There was no significant difference in the magnitude of glutamate-induced mechanical sensitization between the two groups.

3.3 Effect of TrkA and P75 receptor antibodies

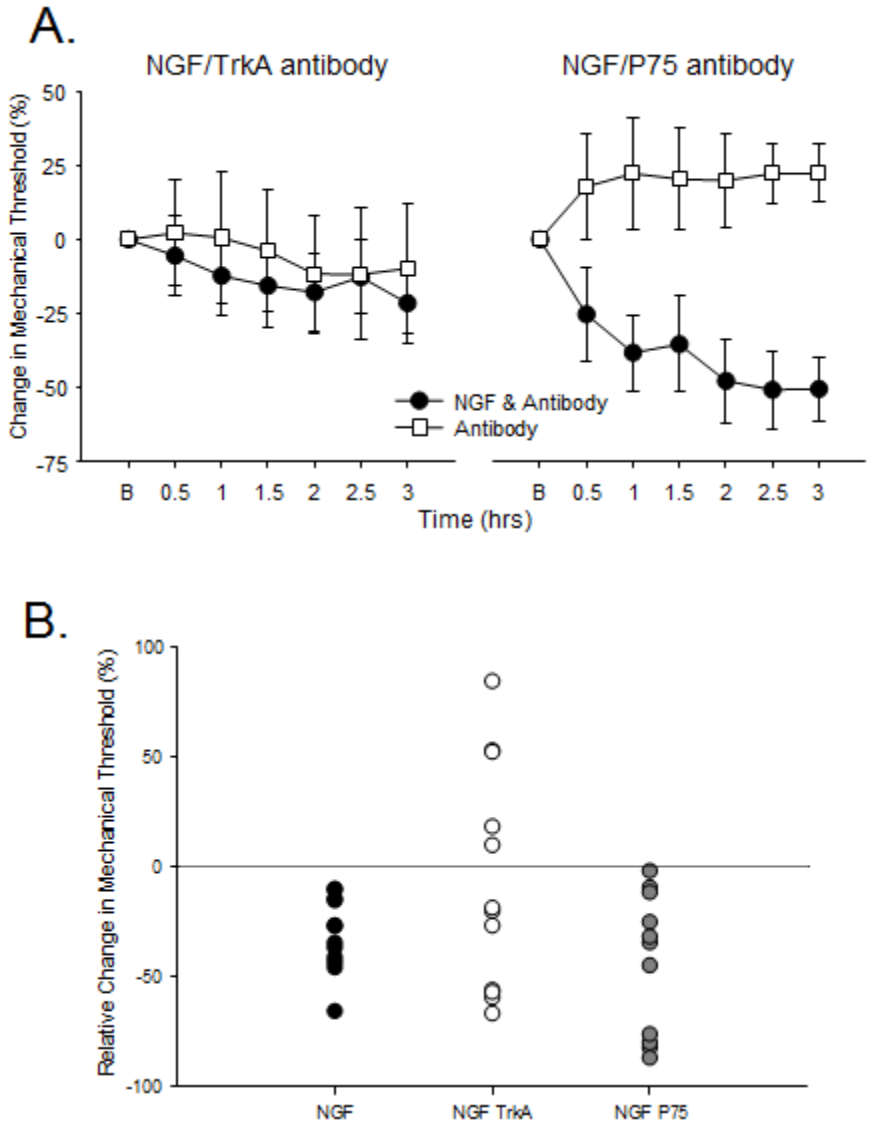

Figure 10. A: The line and scatter plots illustrate changes in the mechanical threshold of nociceptors after injection of NGF with TrkA antibody or NGF with P75 antibody. Co-injection of NGF and TrkA antibody attenuated NGF-induced mechanical sensitization whereas co-injection NGF and P75 antibody appeared to enhance NGF-induced mechanical sensitization. B: The scatter plot illustrates the effect of NGF, NGF with TrkA antibody and NGF with P75 antibody on the mechanical threshold of individual nociceptors 1 hour post injection. Injection of NGF decreased the mechanical threshold of all 12 nociceptors tested at this time point, whereas only 7 of 12 nociceptors in the NGF and TrkA antibody group had a decreased mechanical threshold after 1 hour ($p<0.05$, Fisher Exact test).

Overall, co-administration of TrkA antibody with NGF appeared to attenuate the sensitizing effect of NGF (Figure 10A). In contrast, co-administration of P75 antibody appeared to enhance the sensitizing effect of NGF (Figure 10A). The scatter plot of Figure 9B illustrates the effect of NGF, NGF with TrkA antibody and NGF with P75 antibody on the mechanical threshold of individual fibers 1 hour post injection. Injection of NGF decreased the mechanical threshold of all 12 nociceptors tested at this time point, whereas only 7 of 12 nociceptors in the NGF and TrkA antibody group had a decreased mechanical threshold after 1 hour ($p<0.05$, Fisher Exact test). The addition of P75 antibody to NGF did not attenuate the sensitizing effect of NGF on individual fibers. There was no significant difference in the conduction velocities of the nociceptors treated by NGF, NGF+TrkA, or NGF+P75 injections, which suggests that the differences in sensitization were not due to differences in nociceptor properties between the groups.

3.4 Effect of estrogen on masseter nociceptors

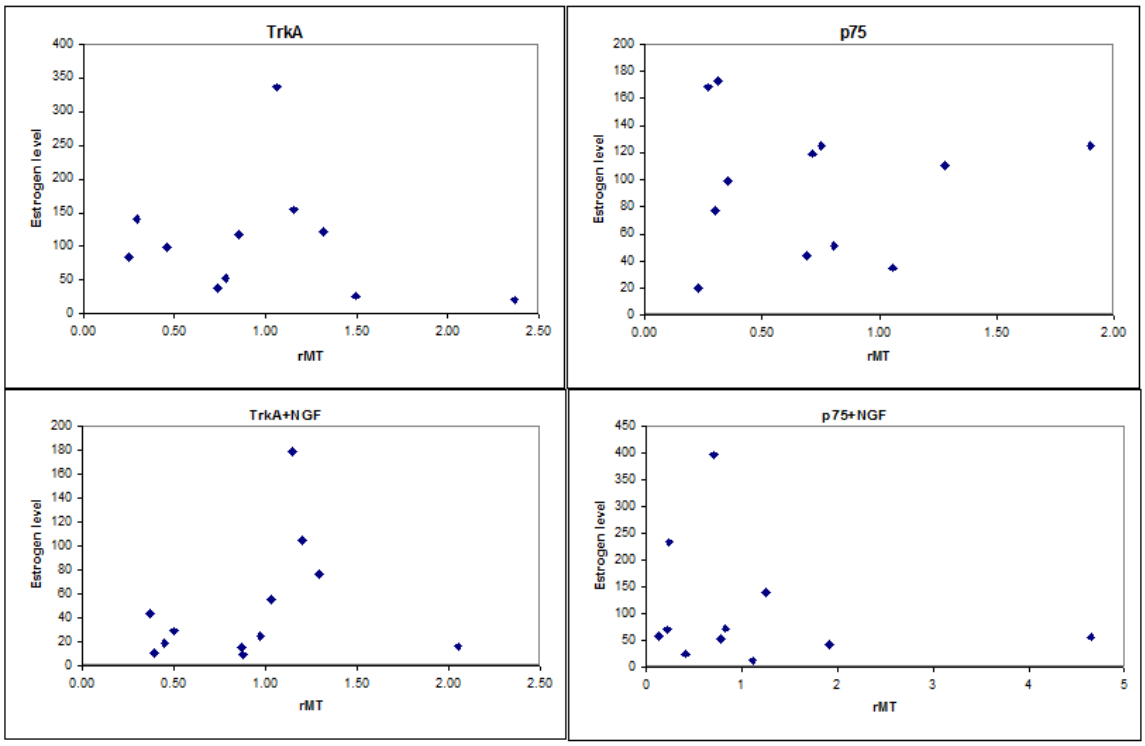

Figure 11. Scatter plots illustrate the relationship between plasma estrogen levels and relative mechanical threshold (rMT) of nociceptors 30 minutes after injection of TrkA antibody (Top left, R = 0.038), after injection of P75 antibody (Top right, R =0.000), after injection of NGF with TrkA antibody (Bottom left, R = 0.062), and after injection of NGF with P75 antibody (Bottom right, R = 0.0378).

Estrous cycle was monitored to assess possible effect of estrogen on NGF activity for the correlation between estrogen level during different estrus stages in the cycle and afferent fiber mechanical threshold changes. The mean (±SE) estrogen concentration measured was 77±9 pg/ml. There was no significant correlation between estrogen concentration and the change in mechanical threshold in the NGF+TrkA or NGF+P75 groups (Figure 11).

3.5 Expression of estrogen receptors

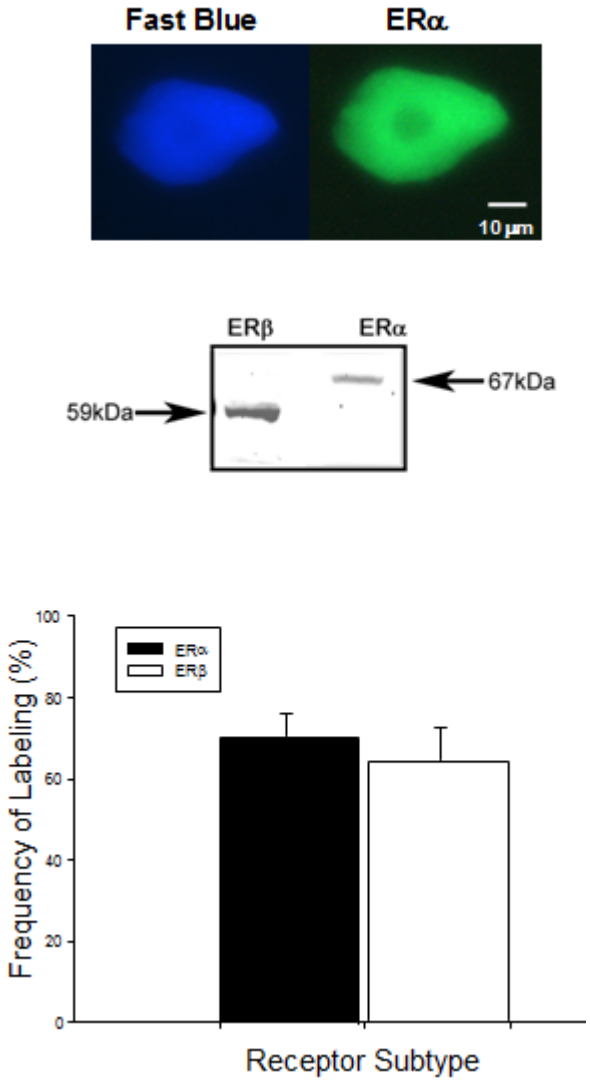

Figure 12. A. The pictures show an example of a fast blue-labeled (left) trigeminal ganglion neuron that also expressed the ER□ receptors in a female rat. ER□ receptor labeling was obvious in the cell cytoplasm. B. Western blots for ERα, and ERβ receptors in the rat trigeminal ganglion neuron (n=4) are shown. The position of the molecular weight standard is indicated in kilodaltons (kDa). The asterisks denote the single bands that were labeled by the receptor specific antibodies (ERα: 67 kDa, ERβ: 59 kDa). C. The bar graph illustrates the expression frequency of estrogen receptors (ERα and ERβ) in masseter muscle ganglion neurons of female rats (n=4). There was a high frequency of expression of both ERs by trigeminal ganglion neurons (70% and 62% respectively).

In order to further study the effects of estrogen on primary afferent fiber sensitivity, ER expression in the trigeminal ganglion neurons was assessed. A total of eight ganglia from four female rats were examined for ER expression (Figure 12). ERα expression was found to be 71±6 % and ERβ expression was found to be 64±8 % in the trigeminal

ganglion neurons. Western blot technique was undertaken to confirm that the antibodies used were selective [150]. Western blots confirmed that the ERα and ERβ antibodies labeled single bands at 67 and 59 kDa, respectively (Fig. 10B). These bands correspond to the expected molecular weights for the two receptors [151]. Both ER receptors were evenly distributed amongst trigeminal ganglion neurons of various sizes (Figure 13).

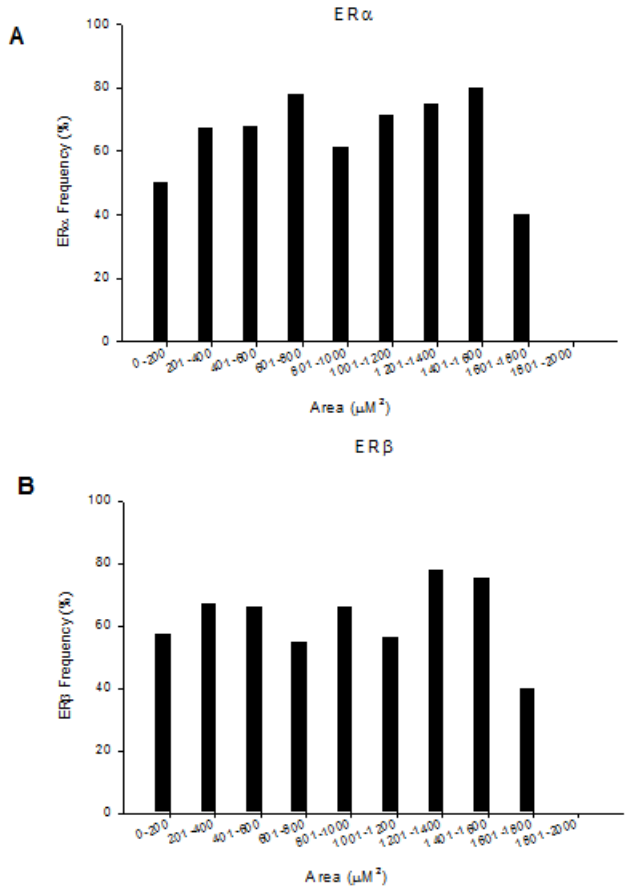

Figure 13. The histograms of A and B illustrate ER□□and ER□□expression, respectively, in trigeminal ganglion neurons of different sizes in female rats (n=4). There was a uniform expression of both ERs amongst trigeminal ganglion neurons of different sizes.

3.6 In Vitro Blocking of NGF-induced CGRP release

NGF receptor antibodies were used to block NGF-induced CGRP release from rat trigeminal ganglion neurons in order to conduct an *in vitro* study that could provide confirmatory evidence for the efficacy of the NGF receptor antibodies. Trigeminal ganglion cells were cultured and incubated in medium containing NGF to induced CGRP release. From the 5 assays that were performed, only 1 assay resulted in NGF-induced CGRP release. It was observed from that one assay that when NGF did induce CGRP release, wells that were incubated in culture medium that contained both NGF and NGF receptor antibodies yielded less CGRP content than wells that were incubated in culture medium that contained NGF only. It was concluded that when CGRP release was induced by NGF, the effect could be blocked by using TrkA or P75 antibodies *in vitro*.

3.7 Summary

More than 70% of masseter ganglion neurons examined expressed TrkA or P75 receptors, respectively. The expression of these receptors was uniformly distributed amongst small, medium and large ganglion neurons. About 85% of the NR2B expressing ganglion neurons co-expressed either P75 or TrkA receptors, which confirmed the potential for a receptor interaction. Injection of NGF did not evoke significantly more discharge than

injection of vehicle, but did significantly decrease the mechanical threshold of masseter nociceptors compared to vehicle. Furthermore, injection of NGF did not affect glutamate-evoked discharge, as well as glutamate-evoked decrease in mechanical threshold, indicating that NGF did not enhance glutamate-induced mechanical sensitization. There were no significant correlations between estrogen concentration, baseline mechanical threshold or conduction velocity and glutamate-evoked nociceptor discharge. In addition, co-administration of TrkA antibody with NGF appeared to attenuate the sensitizing effect of NGF，as blocking TrkA receptor resulted in only 7 of 12 nociceptors to have a decreased mechanical threshold after 1-hr. But co-administration of P75 antibody with NGF did not appear to attenuate the sensitizing effect of NGF. There was no significant correlation between estrogen concentration and the change in mechanical threshold in either the NGF + TrkA antibody or NGF + P75 antibody groups.

4.1 NGF-induced sensitization and blocking of NGF-induced sensitization

The expression of the NGF receptors was determined, since the expression of TrkA and P75 indicates the ability of primary afferent fibers to respond to NGF. Various studies have shown that NGF receptor expression varies between species, by the type of nociceptor, and by the type of tissue innervated. In humans the TrkA receptor, identified as a 140kDa protein tyrosine kinase, has been found in 65% of primary sensory neuron (adult human dorsal root) cell bodies and 82% of sympathetic ganglion neuron cell bodies that innervate the skin as well as non-neural cutaneous tissues such as the epidermis, sweat glands, arterial blood vessels and Schwann cells [152]. Studies in rats have shown TrkA receptor expression to be less in adults (40%) than in neonatal (80%) dorsal root ganglion neurons, which shows that TrkA expression is reduced with age [153-156]. TrkA expression was also found to be greater in visceral afferent fibers (75%) than in cutaneous afferent fibers (43%), which suggests that it is greater in deep tissues than in the skin [157]. Furthermore, TrkA receptors in the DRG were found to be selectively expressed in nociceptive neurons, and there was a positive correlation with CGRP expression, which suggests that TrkA-expressing primary afferent fibers are part of the CGRP expressing population of putative nociceptors [72]. In comparison to the TrkA

expression found in DRG nociceptors, trigeminal ganglion neurons from the mandibular incisor showed low levels of TrkA expression, less than 10% in neurons innervating the incisor and around 15% innervating the cornea [156]. However, other studies have found expression of NGF receptors (P75 and TrkA) to be more than 60% in the trigeminal ganglion, and 86-91% in the mesencephalic nucleus and trigeminal motor nucleus [73]. Reports differ from study to study depending on selected tissues. A very recent study confirmed the high expression of TrkA receptors in the mesencephalic nucleus and trigeminal motor nucleus of the rat trigeminal system [158]. Since trigeminal mesencelphalic nucleus and motor nucleus neurons innervate masseter muscle spindle afferent fibers, this result is consistent with our finding that more than 70% of masseter afferent fibers express both TrkA and P75 receptors. NGF may act on its receptors to evoke the process of mechanical sensitization as found in both human and rat studies, especially studies involving mechanical sensitization induced in the muscles of mastication and jaw movements of human subjects. In addition, the high co-expression of NMDA receptor NR2B subunit with the NGF receptors suggests the machinery for receptor interaction is in place to allow NGF to produce sensitizing effects through modification of NMDA receptor activity.

The main experiments in this thesis were designed to determine whether intramuscular injection of human NGF would lower mechanical threshold and evoked discharge of nociceptors in rat masseter muscle, and whether it would alter glutamate evoked

mechanical sensitization and nociceptor discharge. Injection of human NGF into the masseter muscle did cause a significant reduction in mechanical threshold 30 minutes post injection without significant evoked activity, but had no effect on glutamate evoked responses. This is consistent with the results from the human studies [27, 111-112]. Intramuscular injection of human NGF into female and male human subjects produced a prolonged increase in mechanical sensitivity associated with jaw movement, but did not produced spontaneous pain upon injection or when the jaw was at rest [27, 111-112]. The decrease in pressure pain threshold was significant at 1 and 3 hours after injection for female and male subjects, respectively [27, 111-112]. The quick onset of the NGF-induced mechanical sensitization suggests that a peripheral mechanism is likely responsible. This is consistent with the quick onset of significant reduction in afferent mechanical threshold 30 minutes after injection in the current study.

The effect of NGF on glutamate induced sensitivity was also assessed in the human studies by injection of glutamate into the masseter muscles 24 hours after NGF injection. Glutamate injection induced a decrease in pressure pain threshold that was found to be lower in the NGF treated masseter muscles compared to the control group, but there was no significant difference in perceive pain intensity between the two groups [112]. This is more or less consistent with the result of the current study that NGF had no effect on glutamate evoked response. The human studies [111] also showed that NGF-induced mechanical sensitization was greater in women than in men, however, studies on

sex-related differences in NGF-induced mechanical sensitization in rats have yet to be performed.

To determine which of the two NGF receptors was responsible for mediating the NGF-induced mechanical sensitization, the effect of intramuscular injection of NGF receptor (TrkA/p75) antibodies with NGF on the mechanical threshold and evoked discharge of primary afferent fibers in the rat masseter muscle was examined The results obtained indicate that NGF-induced mechanical sensitization of masseter muscle afferent fibers was mediated through activation of Trk A receptors. Past studies [159-160] have shown that NGF bound TrkA receptor complexes can be internalized and transported back to the cell bodies to activate gene transcription, NGF binding to TrkA or p75 receptors can also activate signaling pathways that alter gene expression [161]. However, the retrograde transport of NGF-TrkA complex cannot account for the rapid onset of the mechanical sensitization induced by the intramuscular injection of NGF, because of the delay in time it takes for the complex to be transported. TrkA activated signaling pathways have been shown to be involved in alteration of phosphorylation of proteins and sensory neuron excitability [71]. Injection of PI3K and ERK inhibitors into rat hindpaw were found to attenuate NGF-evoked heat hyperalgesia in primary sensory DRG neurons [162]. Potentiation of capsaicin-induced TRPV1 currents were also blocked by PI3K inhibitors and suppressed by ERK inhibitors. Additionally, NGF-induced mechanical hyperalgesia in nociceptive primary afferent fibers has been shown to be attenuated by using intrathecal antisense to reduce TrkA expression in the saphenous nerve [163]. The same researchers also found using specific inhibitors to

block TrkA activated signalling pathways such as ERK/MAP, PI3K and PLC also attenuated NGF-induced mechanical hyperalgesia, but PKC blocking did not produce this attenuation. Furthermore, blocking ERK/MAP also blocked activated PI3K induced hyperalgesia, suggesting interaction and cross-talk between the two signalling pathways. Using antisense against p75 to reduce mechanical hyperalgesia was found to be much less effective. Taken together, these studies seem to suggest that the TrkA receptor could be responsible for mediating sensitizing effects of NGF.

It has also been shown that there is a significant interaction between the TrkA and the p75 receptors, as p75 receptors have been known to increase binding affinity of TrkA receptor and enhances TrkA-mediated phosphorylation [164-165], which seems to suggest that blocking the p75 would affect both TrkA and p75 induced activities. NGF-induced enhancement of TTX-resistant sodium currents and suppression of potassium currents in the membrane of capsaicin-sensitive small-diameter rat sensory neurons were found to be blocked by p75 receptor antibodies, suggesting the p75 receptor could mediate NGF-induced sensitization of rat nociceptive sensory neurons [118, 166]. Moreover, NGF was found to affect excitability of mesencencephalic trigeminal nucleus [154], this effect was not blocked by TrkA receptor inhibitors, and p75 receptor antibodies were found to block NGF-induced excitations of forebrain cholinergic neurons in the CNS grafts of septum, spinal cord, and hippocampus [167], suggesting p75 blocking may also mediate the neuron excitability enhancing effects of NGF. The results of the current study indicated that blocking TrkA, but not P75 receptors was effective in attenuating mechanical sensitization induced by NGF injection into the masseter muscle in a

subpopulation of masseter nociceptors. However, since blocking TrkA receptor was observed to be not entirely effective at attenuating the NGF-induced mechanical sensitization, additional mechanisms other than activation of TrkA and p75 receptors seem to be at work.

4.2 Estrogen expression and lack of estrogen mediated effect on NGF-induced sensitization

Studies have shown that ERα/β receptors seem to be expressed at similar levels in the dorsal root ganglion [130-132]. In contrast, an earlier study suggested that between 8 and 12% of trigeminal ganglion neurons in female rats expressed ERα but very few trigeminal ganglion neurons expressed ERβ [150]. In the present study, we found the expression of both ERs to be relatively similar at around 60-70% in trigeminal ganglion neurons. Subsequent Western blot experiments indicated that the commercially acquired antibodies employed were selective for the ERα and ERβ receptors, respectively, and that both receptors were expressed in the trigeminal ganglion. Our finding may suggest that masticatory muscle afferent fibers have a much higher expression of ERs than nerve fibers which innervate other craniofacial tissues. However, estrogen levels were found not to have any effect on NGF-induced sensitization. Human studies [111] have shown the sensitization effect to be greater in female subjects. The lack of estrogen effect on NGF-induced sensitization in rat models may be due to the fact that although estrogen is

suspected to alter primary afferent fiber sensitivity by mediating effect on NGF and NMDA receptors, modulation activities such as regulation of NGF receptor mRNAs and phosphorylation of NMDA receptor subunits may not exhibit direct consequential influence on peripheral sensitization.

4.3 Using NGF to induced release of CGRP in trigeminal ganglion cell culture

The in vitro study to measure CGRP release from trigeminal ganglion neurons was undertaken to confirm the effectiveness of the TrkA and P75 antibody concentrations used in the in vivo studies. Unfortunately, it was challenging to get consistent results from this arm of the study. One complication that rose from performing the *in vivo* study was that, since sympathetic and sensory neurons are dependent on NGF for survival, differentiation, and axonal growth, incubating the trigeminal cell culture with NGF and NGF receptor antibodies at the same time appeared to affect maintenance of the cell culture health . Another complication that came up was the great variation in the basal CGRP measured from well to well, and from day to day, which created great variations in the NGF-induced CGRP to basal CGRP release ratio values. In addition, because the cells were left without NGF for the overnight period in order to obtain a basal release of CGRP without NGF induction, changing the medium the next day may have delivered

additional stress to the cell culture that promoted undesired release of CGRP. This may have lead to greater CGRP release in the basal level than that induced by subsequent incubation with NGF. In future experiments, a neurosupportive medium such as an astrocyte feeder layer could be considered, as astrocyte conditioned medium has been studied to improve neuronal attachment and growth for low-density populations of pure neurons.

4.4 Experimental Caveats

The present study suggests that intramuscular injection of human NGF into female rats can be used as an animal model of myofascial TMD-like mechanical sensitization. One may question whether blocking of TrkA receptors contributes to mechanical sensitization in human subjects given that TrkA antibodies were effective in attenuating NGF-induced mechanical sensitization in only a subpopulation of masseter muscle afferent fibers. Incomplete blocking of the NGF-induced sensitization may be due to genetic and species differences in the interaction between NGF and its receptors. Human, mouse and rat β-NGF share approximately 90% sequence homology that allows full cross reactivity in their biological actions. Human NGF is effective at producing peripheral sensitizing effects in rat models as well as in humans, however, it may be considered that due to the species difference, human NGF causes greater effects in humans than in rats. The effect

of NGF may be harder to block in rats due to a possible interaction between human NGF and other rat signaling proteins. Human NGF could potentially exert part of its sensitizing effect through mechanisms other than NGF receptor activation in the rat, since it is a non-rat protein. This would not occur when human NGF is injected into the human masseter muscle. It could be hypothesized that using human NGF receptor antibodies would be more effective to block human NGF-mediated sensitization in human subjects than using rat NGF receptor antibodies to block human NGF-mediated sensitization in rats.

Another confounding factor to consider is the use of the general anesthetic isoflurane. It is important to distinguish, whether results obtained from experiments performed on anesthetized animals are truly physiological responses or artificial responses due to use of anesthetic agents. It has been suggested that isoflurane produces general anesthesia by enhancing neural inhibition mediated by $GABA_A$ receptors, leading to decrease in neuronal excitability. Isoflurane also depresses excitatory transmission by decreasing NMDA receptor mediated synaptic signaling and reducing basal release of glutamate at nerve terminals in the CNS [168-169]. Studies of the effect of isoflurane on the rat thalamic somatosensory information transfer show that whisker vibration signals can still reach the thalamic neurons under high isoflurane concentration [170]. Other studies in dogs have shown that inhaled anesthetics sensitize cutaneous nociceptors, but to heat, not mechanical stimuli [171]. It was concluded from this study that the peripheral effects of

isoflurane do not influence the response of nociceptors to a noxious stimulus. Studies of membrane properties of trigeminal root ganglion neurons of guinea pigs under the effect of isoflurane did not elicit large changes in membrane potentials, only reduced repetitive neuron firing [172]. Our laboratory has previously found that only very high concentrations of inhaled isofluorane that result in a drop in mean blood pressure below 40 mm Hg affect nociceptor MT, and that this effect results in an increase in MT by only 5-10% [173]. Taken together, these various findings suggest that the use of isoflurane in this study would have had little or no effect on the results obtained.

A potential pitfall in the study is the usage of widefield fluorescence microscope to capture the neuron cell images for the immunohistochemistry study instead of the confocal microscopy technique. Counting of neurons cells using images captured in widefield microscope cannot account for cells that are overlapped and appear as a single cell in the captured image, a confocal microscope may offer 3D dimension view of the neuron cell to be captured in the image to differentiate between single cell and overlapped cell [174], and thus would provide more accurate calculation of cell expression.

Another potential pitfall is the lack of analysis for the co-expression of TrkA and P75 receptors, as it is well known that when the two receptors are co-expressed together, the binding of affinity to the ligands is much stronger [165], thus investigating the co-expression of the two NGF receptors may offer insight into the extent of which they

may interact in the masseter nociceptors and the trigeminal ganglion, and can be used to elucidate possible mechanisms involved in the NGF induced sensitization due to the interaction between the two receptors and crosstalk between convergent signaling pathways.

4.5 Future Directions

To evaluate the extent of sex-related differences in NGF-induced sensitization, intramuscular injection of human NGF in male rats would provide additional insights, in addition, as human study results have shown that men also exhibit mechanical sensitivity after NGF injection into the masseter muscle, it would be interesting to see whether intramuscular injection of TrkA/p75 receptor antagonists in male rats would affect this mechanical sensitivity to the same extent as displayed in female rats. Furthermore, since blocking TrkA receptors was observed to be not entirely effective at attenuating the NGF-induced mechanical sensitization, it could be hypothesized that NGF exerts effects by a downstream signaling pathway activation that leads to primary afferent sensitization. To investigate the extent to which the downstream signaling pathway is involved in NGF-induced mechanical sensitization, select protein kinase inhibitors could be used to target specific intracellular signaling cascades, such as inhibiting the PI3K/Akt pathway activity. However, there is significant overlap between the signaling cascades activated

by both NGF receptors, which may make it difficult to use selective inhibitors to knock out enzymes in specific pathways.

5. CONCLUSIONS

Injection of human NGF into the rat masseter muscle caused a significant reduction in mechanical threshold 30 minutes post injection without evoking afferent discharge, consistent with results in human studies where NGF injection into the masseter muscle produced mechanical sensitization without causing muscle pain [27]. Blocking TrkA but not P75 receptors was effective in attenuating mechanical sensitization induced by NGF injection into the rat masseter muscle in a subpopulation of masseter nociceptors. However, additional mechanisms may contribute to the sensitizing effects of NGF, as blocking TrkA receptor was observed to be not entirely effective at attenuating the NGF-induced mechanical sensitization. Evidence for estrogen modulation of NGF-induced sensitization was not found, which suggests estrogen may not be directly involved in the mechanisms contributing to sensitizing effects of NGF in female rats. In addition, despite high co-expression of NGF receptors with the NR2B subunit of the NMDA receptor, NGF injection was found to have no effect on glutamate-induced sensitization and evoked activities, which indicates that NGF does not induced its sensitizing effects on masseter muscles by altering sensitivity of peripheral NMDA receptors to glutamate. Whether sex-related differences in NGF-induced mechanical sensitization observed in humans also can be demonstrated in rats will require future studies of the effect of NGF on male rats.

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