The serotonin receptor type 3 in chronic and experimental human muscle pain

Nikolaos Christidis
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Cover image: Pain drawings illustrating the pain induced by an intramuscular injection of hypertonic saline in the masseter muscle pre-treated with granisetron, in 30 healthy volunteers (15 women and 15 men). The red areas indicate the pain spread in women and the blue areas indicate the pain spread in men.

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Στον πατέρα μου
To my father
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Abstract

The main aim of this thesis was to gain a better understanding of the influence of serotonin on chronic craniofacial muscle pain. The thesis comprised a basic science part and a human experimental part. The aim for the basic science part was to investigate if the expression of 5-HT3 receptors in muscle tissue differ between myalgic and healthy muscles and if sex or muscle site influence this expression. The aim for the human experimental part was to investigate if the specific 5-HT3 antagonist granisetron influences pain variables in patients with chronic craniofacial myalgia and experimental pain induced by hypertonic saline, as well as the mechanical pain threshold of healthy muscles. A second aim was to investigate if any effect by granisetron was influenced by sex, muscle site or route of administration, i.e. local or systemic.

In the basic science part, patients with chronic local myalgia of the masseter muscle and age- and sex-matched healthy controls were examined clinically according to the Research Diagnostic Criteria for Temporomandibular Disorders. Traditional biopsies were taken intra-orally with a 4-mm Bergström needle from the most painful part of the masseter muscle in patients, and from a standardized point in the healthy controls. In order to compare if there were any differences in receptor expression due to sex or between muscles a microbiopsy technique was used to obtain masseter muscle and tibialis anterior muscle biopsies in healthy age-matched men and women. The patients were found to have a significantly higher amount of co-expressed 5-HT3A receptors and NaV1.8 sodium channels in the connective tissue compared to their healthy controls. This novel finding makes it presumable that in myalgic muscles proliferation of nociceptive nerve fibers occurs in the connective tissue surrounding the myocytes. Further, in healthy subjects, the co-expression of 5-HT3A receptors and NaV1.8 sodium channels in the masseter muscle was higher in women than in men. The tibialis anterior muscle expressed more 5-HT3A receptors than the masseter muscle. However, since these fibers did not co-express NaV1.8 sodium channels extensively, they are presumed being motor neurons.

The first experimental part, investigating the effect of granisetron on the mechanical pain threshold, comprised both healthy men and age-matched women. Granisetron was administrated in a randomized, placebo-controlled and double-blind manner both systemically and locally by intramuscular injection. Sugar pills and isotonic saline served as placebo. The pressure pain threshold (PPT) was examined over the masseter, temporalis anterior, trapezius and tibialis anterior muscles before and after drug administration. The PPT of the masseter muscle increased significantly after local administration of granisetron and for all muscles combined after systemic administration. However, the increase of PPT was only significant over the trapezius and tibialis anterior muscles. Further, the PPT at baseline was significantly
higher in the men and the increase of PPT after administration of granisetron was also only significant in the men. This blockage of the 5-HT3 receptors with granisetron seems to inhibit serotonin to excite and sensitize nociceptors, leading to a reduced sensitivity to mechanical stimuli.

The second experimental part used a randomized, placebo-controlled and double-blind methodology to investigate how granisetron influences masseter muscle pain variables both in patients with chronic local myalgia, and in an experimental pain situation including sex- and age-matched healthy individuals. In the experimental pain situation injections with hypertonic saline were used to induce pain, followed by injections of granisetron or placebo as pre-treatment before a second pain induction with hypertonic saline. Granisetron significantly reduced both clinical and experimentally induced pain variables. The painful area was greater in the healthy women compared to the men before pre-treatment with granisetron. However, after pre-treatment with granisetron there were no significant sex differences in any pain variable. These findings indicate that 5-HT3 receptors play a significant role in pain transmission and pain modulation in human muscles, since granisetron diminishes experimentally hypertonic saline-induced pain and chronic myalgia of the masseter muscle.

Conclusively, our novel findings that the 5-HT3 receptor is up-regulated in painful craniofacial muscles and that blocking of this receptor decreases clinical and experimental human muscle pain indicate that the 5-HT3 receptor has an important role in peripheral pain transmission in localized chronic muscle pain.

**Keywords**

5-HT3 receptor, Granisetron, Human muscles, Muscle biopsy, Pain, Pressure pain threshold, Visual Analogue Scale, Randomized Controlled Double-blind Trial.
List of publications

The present thesis is based upon the following papers, which will be referred to in the text by their Roman numerals:

I. Christidis N, Kopp S, Ernberg M.
   The effect on mechanical pain threshold over human muscles by oral administration of granisetron and diclofenac-sodium.

II. Christidis N, Nilsson A, Kopp S, Ernberg M.
    Intramuscular injection of granisetron into the masseter muscle increases the pressure pain threshold in healthy participants and patients with localized myalgia.

III. Christidis N, Ioannidou K, Milosevic M, Segerdahl M, Ernberg M.
    Changes of hypertonic saline-induced masseter muscle pain characteristics, by an infusion of the serotonin receptor type 3 antagonist granisetron.

IV. Christidis N, Cairns B, Kumar U, Dong X, Rosén A, Kopp S, Ernberg M.
    Expression of 5-HT₃ receptors and TTX insensitive sodium channels (Naᵥ1.8) by masseter muscle nerve fibers in healthy subjects compared to patients with local myalgia.
    *Manuscript*.

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Definitions

Pain terminology according to the Classification of Chronic Pain, Second Edition by the International Association for the Study of Pain® (IASP®) (Merskey and Bogduk, 1994).

Allodynia
Pain due to a stimulus which does not normally provoke pain.

Hyperalgesia
An increased response to a stimulus which is normally painful.

Nociceptor
A receptor preferentially sensitive to a noxious stimulus or to a stimulus which would become noxious if prolonged.

Noxious stimulus
A noxious stimulus is one which is damaging to normal tissues.

Pain
An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.

Pain threshold
The least experience of pain which a subject can recognize.
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, i.e. serotonin</td>
</tr>
<tr>
<td>5-HT$_3$</td>
<td>5-hydroxytryptamine type 3, i.e. serotonin type 3</td>
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<tr>
<td>AAOP</td>
<td>American Academy of Orofacial Pain</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>Da</td>
<td>Dalton (1 Da = 1.660 538 782(83)×10$^{-27}$ kg)</td>
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<tr>
<td>DNIC</td>
<td>Diffuse noxious inhibitory control</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<tr>
<td>HTM</td>
<td>High threshold mechanosensitive</td>
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<tr>
<td>IASP</td>
<td>International Association for the Study of Pain</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NMDA</td>
<td>N-M ethyl-D-A spartate</td>
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<tr>
<td>NMDA</td>
<td>N-M ethyl-D-A spartate</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
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<tr>
<td>PGE$_2$</td>
<td>Prostaglandin E$_2$</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>PPT</td>
<td>Pressure pain threshold</td>
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<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>TMD</td>
<td>Temporomandibular disorders</td>
</tr>
<tr>
<td>TMJ</td>
<td>Temporomandibular joint</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual analogue scale</td>
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<tr>
<td>WAD</td>
<td>Whiplash associated disorders</td>
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Introduction

Chronic craniofacial pain conditions with a prevalence of approximately 10–15% are increasing in the adult population (Brattberg et al., 1989; Dworkin et al., 1990; LeResche, 1997; John et al., 2005). The prevalence of these conditions is found to be 1.5 to 2 times higher among women (Dao and LeResche, 2000; Unell et al., 2006), which is in concordance with other conditions such as trigeminal neuralgia, burning mouth syndrome and atypical odontalgia (Bergdahl and Bergdahl, 1999; Muzyka and De Rossi, 1999; Kitt et al., 2000; Manzoni and Torelli, 2005).

Chronic musculoskeletal pain conditions affecting the temporomandibular joint (TMJ) or the masticatory muscles and their associated structures are commonly referred to other craniofacial structures, such as the teeth. They are accompanied by restricted mouth opening capacity, chewing difficulties, headache and neck pain (Sessle, 1999; Lund et al., 2001), i.e. being disabling for the patient.

Pain is always a subjective experience, and the impact of chronic pain is not just a sensory experience but also an emotional experience where feelings of failure, misery, guilt, alienation, and even depression may occur (Kelley and Clifford, 1997; Thomas, 2000). This is one explanation to the fact that chronic craniofacial pain conditions are accompanied by psychological suffering, impaired social relations, chronic fatigue syndrome and recurrent sick leave. Subsequently, this leads to a frequent use of health care, analgesics (Von Korff et al., 1990; Yokoyama et al., 2007) and hence to a decreased quality of life (Hallberg and Carlsson, 2000; Thomas, 2000; Ohman et al., 2003; Barros V de et al., 2009).

The pathophysiological mechanisms behind chronic craniofacial pain and the basis for the female predominance are poorly understood; thus the existing therapeutic approaches have a limited scientific basis. Increased knowledge about the peripheral mechanisms of pain mediation will provide an opportunity to improve the diagnostic procedures and lead to new therapeutic approaches. Improved treatment procedures would in turn decrease the patients’ individual pain and suffering, improve the patients’ quality of life as well as attenuate their need of health care.
Pain – definitions throughout history

Pain is a word that has been used worldwide throughout history. Both philosophers and physicians have tried to define pain. Hippocrates (460 – 370 BC), the father of medicine, described pain and alleviation of pain in the Hippocratic Corpus (Todd, 1979). During the same period the philosophers Plato and Aristotle considered that pain was nothing constant, that pain was affections or passions formed by the heart and that some people are more susceptible to pain than others (Währborg, 2001). Galenos (129 – 199 AD) was actually the first to describe how pain could affect the joints and the muscles (Waddell and Allan, 1998).

In year 1297 the word pain had the meaning of punishment, originating from the Greek word poine. However, in 1300 the meaning of the word pain came closer to today’s sense as a condition when feeling hurt, originating from the old French word peine (Longman Group, 1991).

The formal definition of pain, stated 1994 by the International Association for the Study of Pain® (IASP®) in the Classification of Chronic Pain, is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey and Bogduk, 1994).

Classifications of pain

Main categories of pain

Pain can be either acute or chronic. A cute pain is the sensory and emotional experience during the normal healing phase following an injury. This pain is also termed transient and functions in terms of protection, i.e. it warns of upcoming tissue damage. On the other hand, chronic pain is determined as a sensation of pain which persists past the normal healing phase, usually lasting between 3-6 months and therefore termed persistent. This type of pain is non-protective (Merskey and Bogduk, 1994; Lund, 1999). Pain conditions can be labeled by their spatial distribution, affected anatomical site, cause and underlying mechanisms. Spatially, they can be divided into e.g. localized or widespread pains. The anatomical site affected can be structures such as viscera, head or back, while the cause can be due to e.g. cancer or diabetes. It is common to classify pain by the underlying mechanisms, which can be e.g. nociceptive, neuropathic or idiopathic (Wall and Melzack, 1994; Apkarian et al., 2009).
**Nociceptive pain**

Nociceptive pain occurs when a noxious stimulus activates nociceptors. This stimulus can be either injurious, i.e. causing a tissue damage, or potentially injurious. Further, nociceptive pain is of acute character, i.e. a temporary pain condition (Wall and Melzack, 1994; Kidd and Urban, 2001). When this temporary pain condition is prolonged it serves instead as a warning signal to alert the organism. Nociceptive pain is divided into somatic and visceral pain (Wall and Melzack, 1994). Somatic pain results from an injury to the body and is generally localized to the affected area. Visceral pain describes a type of nociceptive pain originating from the body's internal organs, not localized to a specific area. Visceral pain is further expressed as “deep”, “squeezing” and “pressing” (Cailliet, 1993; Wall and Melzack, 1994).

Inflammatory pain is due to a cascade of complex series of biochemical and cellular events caused by tissue damage, minor infections, burns or mechanical overloading. For instance, mechanical overloading such as stress induced bruxism, ensuing in hypoxia of the muscles or impaired microcirculation, i.e. ischemia (Monteiro and Kopp, 1989; Henriksson and Bengtsson, 1991; Larsson et al., 1994), leads to a release of inflammatory mediators resulting in an inflammatory reaction, which is followed by pain (Mense, 1993; Wall and Melzack, 1994). These mediators are released from circulating leukocytes and platelets, vascular endothelial cells, immune cells or cells in the peripheral nervous system (Mense, 1993; Zeitz et al., 2002). Since 1953 when Armstrong et al. (1953) first discovered that the inflammatory mediator bradykinin had potent hyperalgesic properties, a large amount of other inflammatory mediators and cytokines have been identified with similar hyperalgesic properties. Examples of these mediators are serotonin (5-HT), prostaglandins (PG), leukotrienes, adenosine, histamine, interleukin-1, interleukin-8, nerve growth factor (NGF) and substance P (SP) (Wall and Melzack, 1994).

**Neuropathic pain**

Neuropathic pain is initiated or caused by a primary lesion or dysfunction in the nervous system (Merskey and Bogduk, 1994). In contrast to acute or nociceptive pain this type of pain is a separate pathological entity, as a result of an ongoing dysfunction in or damage to the nervous system, without any apparent biological purpose (Cailliet,
Any disorder or process that can damage the sensory pathways can cause neuropathic pain. Examples of these disorders are postherpetic neuralgia, trigeminal neuralgia, traumatic nerve injury, spinal cord injury, multiple sclerosis, diabetes and stroke (Wall and Melzack, 1994; Smith and Chong, 2000).

Idiopathic pain
Idiopathic pain is an entity of exclusion in which pain by definition persists for a longer period than 6 months and has no specific physical or mental cause. It is normally not just a condition with complaint of pain but it is also accompanied by a mixture of abnormalities e.g. in motor function, autonomic balance, neuroendocrine function and sleep. Idiopathic pain can be identified in conditions such as chronic craniofacial pain conditions, fibromyalgia, irritable bowel syndrome, burning mouth syndrome, chronic headaches and whiplash associated disorders (WAD) (Diatchenko et al., 2006; McDonald, 2007).

Musculoskeletal pain
Laborious work or unaccustomed exercise may provoke musculoskeletal pain, also termed myalgia. These pain conditions affect the quality of life considerably although they are not life threatening. They are further the major cause of non-odontogenic pain in the craniofacial region (Okeson, 1998; Lund et al., 2001). Myalgia is experienced locally in the muscles as a feeling of fatigue and soreness or even overt pain (Okeson, 1998; Graven-Nielsen et al., 2008). In the craniofacial region myalgia causes a sensation of a dull steady pain overlaying the muscles in the jaw, head and neck (Lund et al., 2001). Experimentally, muscle pain is often induced by hypertonic saline. This pain is described as aching, cramping, drilling or taut, with a dominant sensation of deep, diffusely spread pain with pain referral, thus mimicking clinical myalgia (Jensen and Norup, 1992; Vecchiet et al., 1993; Stohler and Lund, 1994; Svensson and Graven-Nielsen, 2001; Graven-Nielsen et al., 2008).

There is still limited knowledge of the peripheral and central mechanisms underlying musculoskeletal pain. Therefore, the biological mechanisms that initiate and maintain chronic myalgia as well as the basis for its higher prevalence in women (Sessle, 1999; Magnusson et al., 2005) are still poorly understood. However, psychological factors in
combination with bruxism are reported to contribute to the etiology of chronic craniofacial myalgia (Velly et al., 2003).

Chronic musculoskeletal pain is a complex condition due to plastic changes in the nervous system, and thereby of the pain transmission and modulation systems (Mense, 1993; Okeson, 1998; Graven-Nielsen and Mense, 2001) and include both inflammatory and non-inflammatory components (Graven-Nielsen et al., 2008).

It is suggested that mechanical overloading and ensuing relative hypoxia of the muscles and/or a relative blood flow insufficiency due to e.g. stress induced bruxism (Monteiro and Kopp, 1989) may lead to an increased muscle tonus (Okeson, 1998) and a cascade of biochemical events including release of inflammatory mediators such as 5-HT, PG and bradykinin (Mense, 1993). It has also been reported that patients with chronic musculoskeletal pain have higher intramuscular levels of 5-HT compared to healthy subjects (Ernberg et al., 1999; Rosendal et al., 2004; Shah et al., 2005; Gerdle et al., 2008; Larsson et al., 2008). These inflammatory mediators act as algogenic substances that may activate or sensitize nociceptive free nerve endings of slow conducting peripheral afferents, thereby causing pain (Mense, 1993; Okeson, 1998; Mense, 1999; Graven-Nielsen and Mense, 2001). Some inflammatory mediators are further reported to induce neuroplastic changes in the brainstem, i.e. central sensitization, which is proposed to contribute to muscle allodynia/hyperalgesia (Mense, 1993; Hu et al., 1997).

**Human muscles**

**Physiology and composition**

There are three types of muscle tissue in the human body. They differ in anatomy, localization and nerve supply. The skeletal muscle tissue is attached primarily to the bones and is used to move the skeleton. The cardiac muscle tissue forms the bulk of the heart-wall, while the smooth muscle tissue is located in the walls of structures such as the blood vessels, the stomach, the intestines, i.e. the hollow structures of the body (Tortora and Grabowski, 1996).

The skeletal muscle tissue is striated, i.e. the muscle fibers contain alternating dark and light bands. The dark bands are capable of slow but sustained contraction and their deeper red color is due to higher concentrations of myoglobin. They are thin and
termed slow muscle fibers or type I muscle fibers. The light bands are thicker and capable of quick contractions but fatigue more rapidly. They are subsequently termed fast muscle fibers or type II fibers. These fibers are further divided into the subgroups type IIA and type IIB. Due to their lower concentration of myoglobin they are whiter in color. Consequently, the type IIA fibers are pink and the type IIB fibers are white. The larger type IIB fibers have furthermore fewer mitochondria and rely more on anaerobic activity, while the intermediate type IIA rely on aerobic activity. Further, the muscle fibers can be divided in extrafusal and intrafusal fibers. The extrafusal fibers are contractile and form the bulk of the skeletal muscle. The intrafusal fibers are only minutely contractile and when they are bundled together, surrounded by connective tissue, they form a muscle spindle (Tortora and Grabowski, 1996; Okeson, 1998).

The skeletal muscles consist of muscle fibers, blood vessels, nerves and connective tissue that surround and protect the muscle tissue. These structures form a small cluster termed muscle fascicle. When these small clusters are bundled together in larger groups surrounded by the deep muscle fascia they form the actual muscle (Tortora and Grabowski, 1996), as shown in figure 1.
Figure 1. Illustration of the structures in a skeletal muscle. Myofilaments, the contracting element of the skeletal muscle, are arranged in small groups that together with blood vessels, nerves and connective tissue form the muscle fiber. Muscle fibers are bundled together in small clusters, also containing blood vessels, nerves and connective tissue, forming the muscle fascicle. The muscle fascicles are further gathered in larger groups containing blood vessels, nerves as well as connective tissue. The deep muscle fascia encircles these groups forming the actual muscle. The outermost layer of connective tissue that encircles the whole muscle is the epimysium. The connective tissue surrounding the bundles is the perimysium, while the connective tissue in the interior of each fascicle, i.e. separating each muscle fiber and myofibril, is the endomysium.

There are four pairs of skeletal muscles whose primary function is to move the mandible (lower jaw) and considered being the muscles of mastication as they are involved in biting and chewing. The pairs of masticatory muscles elevating the mandible are the masseter muscle, the temporalis muscle and the medial pterygoid muscle, while the pair lowering the mandible is the lateral pterygoid muscle. They are further used in speech, singing, yawning, drinking, clenching, etc. The digastric muscle and suprathyroid muscle have also an important role in the mandibular function as they participate in the lowering of the mandible even though they are not considered to be masticatory muscles (Okeson, 1998).

Masseter muscle
The masseter muscle originates from the zygomatic arch and extends downwards where it inserts on the ramus (body) and angle of the mandible. It consists of a deep portion with fibers having predominantly a vertical direction and a superficial portion with fibers that run downwards and slightly backwards. It is innervated by the mandibular branch of the trigeminal nerve (the fifth cranial nerve). By contraction of the muscle fibers the masseter muscle elevates the mandible closing the mouth and bringing the teeth together. The muscle assists in the side-to-side movement of the mandible and the superficial portion protrudes the mandible (Tortora and Grabowski, 1996; Okeson, 1998).

Temporalis muscle
The fan-shaped temporalis muscle originates from temporal and frontal bones on the lateral surface of the skull. It extends downwards where it inserts on the coronoid process and the anterior border of the ramus mandible. It consists of an anterior portion with fibers having an almost vertical direction, a middle portion with fibers running slightly forward/downward, i.e. diagonally across the lateral aspect of the skull, and a
posterior portion having almost a horizontal direction. The temporalis muscle is innervated by the mandibular branch of the trigeminal nerve. When the fibers of the anterior portion contracts the muscle elevates the mandible in a vertical direction. By contraction of the middle and posterior portions of the muscle the mandible is elevated and retruded. These portions also assist in the side-to-side movements of the mandible (Tortora and Grabowski, 1996; Okeson, 1998).

**Trapezius muscle**
The large trapezius muscle covers the back, shoulder and neck and is considered the most important muscle of the shoulder region. It originates from the occipital bone, the ligamentum nuchae and the seventh cervical as well as all thoracic vertebrae. It extends laterally to the clavicle and the acromion of scapula. The trapezius muscle is innervated by the accessory nerve (the eleventh cranial nerve) and the cervical nerves C3-C4. When the muscle fibers in its upper part contracts the muscle elevates the clavicula, i.e. the shoulders, and rotates the glenoid fossa. This rotation is assisted by the lower portion of the muscle. By contraction of the fibers in the middle portion of the muscle it adducts the scapula (Tortora and Grabowski, 1996; Okeson, 1998; Graven-Nielsen et al., 2008).

**Tibialis anterior muscle**
The tibialis anterior muscle is part of the anterior compartment of the leg and originates from the lateral condyle and body of the tibia and intraosseous membrane. It extends to the first metatarsal and first cuneiform. When the muscle fibers are contracted the muscle dorsiflexes and inverts the foot. The tibialis anterior muscle is innervated by the deep peroneal nerve (Tortora and Grabowski, 1996).

**Nerves supplying skeletal muscles**
The two types of afferent nerve fibers supplying the muscle spindles are the larger and faster thick myelinated Aα-fibers (belonging to group Ia afferents) and the smaller and slower thick myelinated Aβ-fibers (belonging to group II afferents). Their main function is to stimulate the α-efferent nerve fibers in order to contract a stretched muscle. This is further a protective monosynaptic reflex termed myotactic (stretch) reflex (Okeson, 1998).
Mainly two types of afferent sensory nerve fibers are mediating skeletal muscle pain. These are the slowly conducting thin myelinated A\(\delta\)-fibers (belonging to group III afferents) and the non-myelinated C-fibers (belonging to group IV afferents). It is believed that C-fibers may have a greater role in the nociceptive input of skeletal muscles. Though, in animal studies it has been shown that A\(\delta\)-fibers are more common. These fibers are polymodal, which means that they are sensitive to different kinds of stimuli, such as mechanical, thermal and chemical. When a noxious stimulus activates the A\(\delta\)- and C-fibers they stimulate interneurons in the brain stem that in turn activate the efferent \(\alpha\)- and \(\gamma\)-fibers. This is furthermore a protective polysynaptic reflex to noxious stimuli termed the nociceptive (flexor) reflex (Mense, 1993; Okeson, 1998; Cairns et al., 2002; Graven-Nielsen et al., 2008).

**Muscle nociceptors**

The specialized receptors on afferent sensory nerve fibers detecting and signaling actual or potential tissue damage are termed nociceptors. Their stimulation threshold is slightly lower than the threshold of actual tissue damage while their main function is to alert the central nervous system (CNS) of impending danger, not just to signal existing tissue damage (Wall and Melzack, 1994; Lund et al., 2001; Graven-Nielsen et al., 2008).

It has been shown that approximately 40\% of all thin myelinated A\(\delta\)-fibers and non-myelinated C-fibers have the function of muscle nociceptors. They are free nerve endings found in the connective tissue of the muscle and muscle tendons. These afferent fibers are equipped with tetrodotoxin (TTX) insensitive sodium channels which make them resistant to the neurotoxin TTX that normally blocks the conduction in nerve fibers (Sarnat and Laskin, 1992; Wall and Melzack, 1994; Tortora and Grabowski, 1996; Graven-Nielsen et al., 2008). The TTX insensitive sodium channels containing the \(\alpha\)-subunit Na\(_{\text{V}}\)1.8 are strictly limited to nociceptors and play a significant role in the nociceptive signaling. The \(\alpha\)-subunit Na\(_{\text{V}}\)1.8 is further present in most nociceptive neurons of the A\(\delta\)- and C-fibers (Djouhri et al., 2003) and involved in peripheral sensitization (Cesare and McNaughton, 1997).
There is not yet any generally accepted classification of nociceptors but they may be distinguished as high-threshold mechanosensitive (HTM) receptors, chemonociceptors and polymodal nociceptors (Graven-Nielsen et al., 2008).

**High-threshold mechanosensitive (HTM) receptors**
When pressure is applied to muscle tissue, HTM receptors are activated (Marchettini et al., 1996). In order to reach excitation of the HTM receptors a tissue-threatening pressure stimulus is required due to their high stimulation threshold. If the stimulus is of sufficient intensity, pain is evoked when the mechanical pain threshold is reached. However, everyday stimuli such as physiological movements do not activate the HTM receptors (Graven-Nielsen et al., 2008).

The HTM receptors are mainly supplied by the myelinated Aδ-fibers (group III) and the non-myelinated C-fibers (group IV).

**Chemonociceptors**
Receptors responding to algesic agents but not to mechanical stimuli are termed chemonociceptors. These receptors respond strongly to e.g. ischemic contractions, but not contractions alone, and are only located on the non-myelinated C-fibers (group IV) (Graven-Nielsen et al., 2008). Examples of chemical or algesic agents that may activate chemonociceptors are bradykinin, PG, 5-HT, capsaicin, glutamate and SP (Wall and Melzack, 1994).

**Polymodal nociceptors**
Some of the nociceptors respond both to high-intensity pressure stimulation and to chemical stimuli (Marchettini et al., 1996; Graven-Nielsen and Mense, 2001). Due to this mechanism even a relatively light pressure can be experienced as painful, if a sufficient amount of the chemical stimulus is present to activate this subgroup of polymodal nociceptors (Marchettini et al., 1996). It has been argued that all muscle nociceptors are polymodal (Graven-Nielsen et al., 2008).

**Craniofacial pain mediation**
Craniofacial muscle pain is mediated by inputs from the trigeminal nerve. This nerve carries neural information from the craniofacial tissues. The primary afferent fibers
from the trigeminal nerve are located in the trigeminal ganglion from which neural information is passed to the sensory complex of the brain stem to terminate in the subnucleus caudalis. The subnucleus caudalis is considered to be the main site of dispatch of craniofacial nociceptive information and from here the information is transmitted to second order neurons. These neurons relay the nociceptive information, directly or indirectly, to higher brain centers situated in the thalamus. Finally, via third order neurons, they reach the cerebral cortex, where pain is perceived (Sarnat and Laskin, 1992).

**Inflammatory mediators**

The five cardinal signs of inflammation, dolor (pain), calor (heat), rubor (redness), tumor (swelling) and functio laesa (loss of function) are, at least partly, explained by the action of specific inflammatory mediators. These inflammatory mediators are released due to e.g. injury (tissue trauma) and act as algogenic substances that may activate or sensitize nociceptive free nerve endings of slow conducting peripheral afferents, thereby causing pain (Mense, 1993; Wall and Melzack, 1994; Okeson, 1998; Mense, 1999; Graven-Nielsen and Mense, 2001). Further, the inflammatory mediators evoke peripheral release of neuropeptides by the axon reflex such as SP and calcitonin gene-related peptide (CGRP) which in turn can modulate inflammation by altering the release, metabolism or actions of the inflammatory mediators (Sessle et al., 1995).

Bradykinin was the first inflammatory mediator that was recognized to have hyperalgesic properties (Armstrong et al., 1953). Since then other mediators such as PGs, 5-HT, leukotrienes, adenosine, histamine, NGF, interleukin-1 and -8 as well as SP have been identified to have hyperalgesic properties. It is assumed that some of these mediators, such as PGs, 5-HT and adenosine, act directly on the nociceptor, while other, such as bradykinin, interleukin-8, are believed to act indirectly. The mediators acting indirectly induce hyperalgesia by first acting on other cells such as postganglionic neurons or neutrophils (Wall and Melzack, 1994).

**Serotonin**

The neurotransmitter 5-HT is a small molecule with a molecular weight of 176.2 Da. It functions as a mediator with several roles in the human body. 5-HT is a major neurotransmitter component of the chemical milieu of inflammation and an important
mediator of pain both peripherally and centrally (Zeitz et al., 2002). In an area of a tissue damage and subsequent inflammatory ischemia, 5-HT may be released from platelets, mast cells or basophils that infiltrate this damaged area (Dray, 1995).

5-HT has further been emerged as a regulator of mood, appetite, hemodynamics, gastrointestinal motility, secretion and sensation. Therefore, it is widely spread in the human body and found in the CNS, the peripheral nervous system (PNS), the enterochromaffin cells of the intestines (where 90% of the 5-HT is located and produced) as well as in platelets, mast cells and certain immune cells (Hindle, 1994; Rapport, 1997; Gershon and Tack, 2007; Lesurtel et al., 2008).

5-HT is synthesized peripherally in the enterochromaffin cells and certain regions of the CNS from the essential amino acid tryptophan. Tryptophan is first converted to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. The second step in this reaction is the synthesis of 5-HT by the enzyme L-amino acid decarboxylase. 5-HT is further converted to metabolites. The main metabolite 5-hydroxyindoleacetic acid is synthesized by the enzyme monoamine oxidase and finally excreted by the urine (Russell, 1993; Gyermek, 1996). Only 1-2% of the dietary tryptophan is converted to 5-HT (Bender, 1983; Hindle, 1994). Examples of food containing high levels of tryptophan are egg, milk, meat, potatoes, eggplants, broccoli, tomatoes, plums, kiwi, bananas, fish, seafood, chocolate and walnuts.

**Effects of serotonin on pain**

It is well known that 5-HT participates in pain processing. Depending on the site of action, the effects of 5-HT are diverse. In the periphery, 5-HT seems to be involved in the pain transmission, i.e. causing pain, while it centrally seems to be part of the descending endogenous pain inhibitory system, i.e. reducing pain, illustrated in figure 2.
Figure 2: The upper chart (A) illustrates the peripheral role of 5-HT in the pain transmission, while the lower (B) illustrates the central role of 5-HT as a pain reducer. (A) As a result of a tissue trauma 5-HT is released that excites and sensitizes nociceptors leading to a sensation of pain. This activation of peripheral afferents may be due to either a direct activation of pro-nociceptors, such as the 5-HT$_3$ receptor, or an indirect activation by promoting the release of SP and glutamate. (B) When the periaqueductal gray (PAG) and the nucleus raphe magnum (NRM) are stimulated, they promote a release of 5-HT that in turn activates inhibitory interneurons. These interneurons release endogenous opioids and gamma-aminobutyric acid (GABA), thus acting as a pain inhibitor (Saria et al., 1990; Giordano and Schultea, 2004; Sommer, 2006).

The serotonin receptors

5-HT was discovered in the late 1940s, and in the late 1950s Gaddum and Piccarelli (1957) identified two types of 5-HT receptors, the D- and the M-receptors. Since then the classification has been revised and today there are seven receptor classes (5-HT$_1$ to
5-HT) and at least fifteen different 5-HT receptors (Bradley et al., 1986; Glennon and Dukat, 1991; Ernberg, 2009).

**The 5-HT1 receptor**
Six 5-HT1 receptors have been identified (5-HT1A-5-HT1F) and are mainly found in the CNS. They are involved in the cerebral circulation, in feelings of anxiety, depression and in sleep. They can further attenuate migraine, act as vasodilators, while they also seem to be involved in pain processing (Buzzi et al., 1991; Hindle, 1994).

**The 5-HT2 receptor**
Three 5-HT2 receptors have been identified (5-HT2A-5-HT2C) and are mainly found in the CNS. They have a role in anxiety and depression and seem to be involved in cerebrospinal fluid production, obsessive-compulsive disorders and anorexia nervosa (Hindle, 1994; Mackie et al., 2000). The 5-HT2 receptors are also found in the periphery where they both act as vasoconstrictors on large arterial vessels (5-HT2A) and vasodilators on meningeal blood vessels (5-HT2B) (Alsip and Harris, 1991; Leonard, 1992; Schmuck et al., 1996; Kurita et al., 1999).

**The 5-HT3 receptor**
The 5-HT3 receptor is declared to be the phylogenetically oldest 5-HT receptor (Rajkumar and Mahesh, 2010). It was first discovered in the ileum of the guinea pig and was the former named “M -receptor” since morphine blocked 5-HT induced contractions (Gaddum and Piccarelli, 1957). In humans the 5-HT3 receptor gene maps to chromosome 11 (Barnes and Sharp, 1999). Further, the 5-HT3 receptor is the only ligand-gated ion channel among the 5-HT receptors and the activation of it facilitates the influx of sodium, potassium and calcium ions due to the channel opening (Jackson and Yakel, 1995; Brown et al., 1998; Livesey et al., 2008). This influx is followed by a rapid desensitization, characteristic for the 5-HT3 receptor (Jackson and Yakel, 1995). The 5-HT3 receptor is found in neural tissue in both the CNS and PNS. The presynaptic and somatodendritic 5-HT3 receptors control the neurotransmitter release while the postsynaptic 5-HT3 receptors mediate rapid ionotropic neurotransmission (Peters et al., 1992). The 5-HT3 receptors are involved in many events in the human body. They have a well known role in emetic pathways (Haus et al., 2004). Centrally they play a great role in psychosis, anxiety, cognition and eating disorders (De Ponti, 2004). In the
periphery they cause vasodilation due to their major effects on the heart and blood vessels. Furthermore, they have a role in the intestinal tone and are probably the most important 5-HT receptor for pain transmission in the periphery (Blauw et al., 1988; Mackie et al., 2000). Over the past few years, five subunits of the 5-HT₃ receptor have been identified (5-HT₃A-5-HT₃E). Until now, the 5-HT₃A-C subunits have been identified in muscles but only the 5-HT₃A subunit can alone form a complete functional receptor. The other subunits require the 5-HT₃A subunit in order to form functional receptors, such as the 5-HT₃A/B complex (Niesler et al., 2007). However, the functional properties of the newer subunits (5-HT₃C-E) is not yet determined and neither are the implications of which role the different subunits play for pain transmission (Jensen et al., 2008; Rajkumar and Mahesh, 2010).

The 5-HT₄ receptor
The 5-HT₄ receptor is found in many tissues, both centrally and in the periphery. Centrally it has a role in affective disorders and psychoses, while it peripherally appears to evoke tachycardia and mediate gastric motility (Boess and Martin, 1994; Mackie et al., 2000). Further it seems to modulate the pain signal in visceral hypersensitivity (De Ponti, 2004; Bueno et al., 2007).

The 5-HT₅ receptor
Two 5-HT₅ receptors have been identified (5-HT₅A-5-HT₅B). They are found in the CNS but the knowledge of their function is largely limited. They seem to be involved in mental disorders (5-HT₅A) (Mackie et al., 2000; Glennon, 2003).

The 5-HT₆ receptor
The 5-HT₆ receptor is mainly found in the CNS and is involved in mental disorders as well as cognitive and memory dysfunctions (Mackie et al., 2000; Glennon, 2003).

The 5-HT₇ receptor
The 5-HT₇ receptor is mainly found in the periphery and seems to mediate relaxation of the smooth muscle cells. It is also suggested that it has a role in the pain processing while it is found in the axons of the peripherals nerves (Prins et al., 1999; Mackie et al., 2000; Hedlund and Sutcliffe, 2004; Rocha-Gonzalez et al., 2005).
**Granisetron**

Granisetron is among the drugs that exhibit the most selective binding to the 5-HT₃ receptors, with an affinity to the 5-HT₃ receptor that is between 4 000- and 40 000-fold of any other receptor. It has been shown that granisetron binds competitively to the 5-HT₃ receptors and that it is slowly released from these receptors (Wong et al., 1995; Gan, 2005). Ondansetron, dolasetron, tropisetron and granisetron seem to be equal in their efficacy and safety, all expressing very few side-effects. But granisetron has a higher affinity to the 5-HT₃ receptor and is not dependent on the Cytochrome P450 2D6 (CY P2D6) route of metabolism, in contrast to the other antagonists. The CY P2D6 is one of the most important enzymes involved in the metabolism of many therapeutic drugs, i.e. xenobiotics which are chemicals not normally produced or found in the human body. This dependence on metabolism via CY P2D6 may decrease the efficacy in patients considered to be ultrarapid metabolisers (Gan, 2005; Neafsey et al., 2009).

With all this in mind, one can wonder which role the 5-HT₃ receptor has in chronic and experimental human muscle pain.
Aims

General aim
The general aim for the research project was to gain better understanding of the influence of 5-HT on localized chronic craniofacial muscle pain. Special emphasis is directed towards sex differences in the response to 5-HT receptor-blocking and differences between muscles in the trigeminal and spinal regions.

Specific aims
The specific aims for the research project were:

- To investigate if the mechanical pain threshold of healthy muscles is influenced by local and systemic administration of the specific 5-HT$_3$ antagonist granisetron.
- To investigate if a possible effect by 5-HT on mechanical pain threshold in healthy muscles depends on sex differences or muscle site.
- To investigate if local treatment with granisetron influences pain variables in patients with chronic craniofacial myalgia.
- To investigate if local treatment with granisetron influences the pain variables in an experimental pain situation with hypertonic saline-induced muscle pain.
- To investigate if the expression of 5-HT$_3$ receptors in muscle tissue differ between myalgic and healthy muscles and is influenced by sex or muscle site.
Materials and Methods

The methods and selection of subjects were approved by the local ethics committee at Karolinska University Hospital, Karolinska Institutet, Huddinge, Sweden, (Study I: 110/00; Study II: 110/00 and 457/01; Study III: 2006/1174-32; Study IV: 2007/2:6) and by the Medical Products Agency in Uppsala, Sweden (Study I-II: 151:2002/18994; Study III: 151:2003/22767).

All subjects were above 18 years of age and the studies followed the principles for medical research according to the guidelines of the Declaration of Helsinki and the Good Clinical Practice guidelines. The subjects received verbal as well as written information and gave their verbal (Study I-II) and written consent (Study III-IV).

Healthy volunteers

A group of 97 healthy individuals, 47 men and 50 women, participated on a voluntary basis. The participants were age- and sex-matched. The distribution is shown in table 1.

Table 1. The distribution of the number of the participating healthy volunteers and their age (years).

<table>
<thead>
<tr>
<th>Study</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>20</td>
<td>24</td>
<td>30</td>
<td>23</td>
</tr>
<tr>
<td>Men</td>
<td>10</td>
<td>13</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Women</td>
<td>10</td>
<td>11</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>38.0 (±14.6)</td>
<td>31.9 (±12.1)</td>
<td>25.8 (±3.8)</td>
<td>41.8 (±13.4)</td>
</tr>
<tr>
<td>Men</td>
<td>39.5 (±15.6)</td>
<td>29.4 (±11.1)</td>
<td>25.8 (±3.1)</td>
<td>38.7 (±14.1)</td>
</tr>
<tr>
<td>Women</td>
<td>36.4 (±14.3)</td>
<td>34.2 (±13.2)</td>
<td>25.9 (±4.6)</td>
<td>42.6 (±13.3)</td>
</tr>
</tbody>
</table>

Values are expressed as number of participants, and as mean (±SD) for age.

The healthy volunteers underwent a clinical examination in order to evaluate if they fulfilled the inclusion criteria that were: age over 18, good general health, no pain from craniofacial region, no known previous allergic reactions to the substances used as well as no use of analgesics for at least one week (Study I-II) or at least 24 hours (Study III-
prior to the trial. The participating individuals were further informed about the study protocol.

**Patients**

A number of 18 patients (4 men and 14 women) with craniofacial pain of muscular origin participated in *Study II*, and a number of five patients all women with localized craniofacial pain participated in *Study IV*. They were referred to the division of Clinical Oral Physiology, Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden. The distribution of age, sex and pain duration of the participating patients is shown in table 2.

**Table 2.** The distribution of the number of the participating patients, their age (years) and the duration of local pain (years).

<table>
<thead>
<tr>
<th>Study</th>
<th>II</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>Men</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Women</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>36.9 (±12.0)</td>
<td>50.4 (±8.2)</td>
</tr>
<tr>
<td>Men</td>
<td>38.5 (±10.5)</td>
<td>-</td>
</tr>
<tr>
<td>Women</td>
<td>36.4 (±12.7)</td>
<td>50.4 (±8.2)</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>5.7 (±4.7)</td>
<td>3.0 (±2.6)</td>
</tr>
<tr>
<td>Men</td>
<td>5.0 (±3.7)</td>
<td>-</td>
</tr>
<tr>
<td>Women</td>
<td>5.9 (±5.3)</td>
<td>3.0 (±2.6)</td>
</tr>
</tbody>
</table>

Values are expressed as number of participants, and as mean (±SD) for age and duration.

The patients underwent a clinical examination in order to evaluate if they fulfilled the inclusion criteria and in the purpose of being informed about the study protocol. The clinical inclusion criteria for the patients were as follows: pain from the masseter muscle region for more than 3 months and a diagnosis of local myalgia-unclassified according to the American Academy of Orofacial Pain (AAOP) (Okeson, 1996) (*Study II*) or a diagnosis of myofascial pain according to the Research Diagnostic Criteria for
Temporomandibular Disorders (RDC/TMD) (Dworkin and LeResche, 1992) and pain upon digital palpation of the masseter muscle (Study IV). Exclusion criteria were systemic connective tissue disease (e.g. rheumatoid arthritis) and a previous history of allergic reactions to granisetron (Study II), systemic inflammatory connective tissue diseases (e.g. rheumatoid arthritis) (Study II; IV), local temporomandibular joint disease (e.g. synovitis/capsulitis or osteoarthrosis) (Study II), local skin infection over the injection site (Study II), whiplash associated disorder (WAD) (Study IV), fibromyalgia (Study IV), neuropathic pain or neurological disorders (e.g. myasthenia gravis, craniomandibular dystonia) (Study IV), pain of dental origin (Study IV), pregnancy (Study IV), frequent use of muscle relaxants and analgesic or anti-inflammatory medication during 24 hours preceding the procedure (Study II; IV).

Methods

Assessment of pain

Visual analogue scale

Both the patients (Study II) and the healthy volunteers (Study III) were asked to assess their present pain intensity in the masseter muscle on a 100-mm Visual analogue Scale (VAS), for the right and left side respectively. The scale was anchored at each end by the words “No pain” and “Worst pain ever experienced”.

Pain drawings

The participants were asked to make a drawing of the maximum distribution of pain induced (Study III) by the injection (painful area) on two lateral views of the head for each side separately, one for extra-oral assessments and one high-lightening the teeth and jaws for intra-oral assessments. For the analysis of pain drawings a transparency with 1.5*1.5 mm squares was placed over the pain drawing (50*50 mm) and the number of full squares inside the border of the pain drawing was counted. Squares that were partly inside the border were added to full squares, i.e. two half squares or three 1/3 squares were considered as one full square. The area of the pain drawings were expressed in arbitrary units (au).

Assessment of pressure pain threshold

PPT was assessed with an electronic pressure algometer (Somedic Sales AB, Hörby, Sweden). The probe tip of the algometer was 1 cm² and covered with a 1 mm thick
rubber pad to minimize the risk of irritation of the skin. The algometer was held perpendicular to the skin surface over the muscles and the reference point, and the pressure was increased at a standardized rate of 50 kPa/s. A digital screen on top of the algometer was used to control the pressure increasing rate. The instrument was calibrated at the start of the series and the zero level was balanced before each measuring session. The participants were instructed to press a signal button when the sensation of pressure changed into pain. This was first performed over the soft tissue close to the base of the thumb on the dorsal side of the right hand, in order to accustom the participant to the procedure. The PPT was recorded bilaterally over the superficial masseter muscles (Study I-III), temporalis anterior muscles (Study I), trapezius muscles (Study I) and tibialis anterior muscles (Study I) as well as over a reference point which was either the glabella on the forehead (Study I-II) or the tip of the right index finger (Study III). The extra-cranial reference point was chosen to investigate any possible systemic effects by the study treatments (Study III).

Administration of substances

Oral administration - Study I

Due to the diverse appearance of the pills the pharmacy at the Karolinska University Hospital in Huddinge, Sweden, manufactured red, opaque capsules of granisetron (Kytril® 1 mg, Roche, Stockholm, Sweden) and placebo (lactose monohydrate) with identical appearance in order to blind them. Six capsules of each substance were allocated in separate plastics bag, provided at different visits marked with the order of administration, i.e. capsule A or capsule B, by one of the investigators not participating in the clinical examinations and data collection.

Injection - Study II

The substances were administrated with bilateral injections of active substance (granisetron; Kytril®, 1 mg/mL, Roche, Stockholm, Sweden) on one side and placebo (isotonic saline; Natriumklorid, 9 mg/mL, Fresenius Kabi, Uppsala, Sweden) on the other side. The injection points were marked with a felt pen on the skin overlaying the masseter muscles. For the healthy individuals a standardized point (the most prominent point of the muscle during contraction, i.e. in the midline and approximately 2 cm superior to the lower mandibular border) was used, while for the patients the most painful point to digital palpation on each side was used.
Infusion - Study III

To administrate the substances by infusion, standardized points (same as Study II) were used. They were marked with a felt pen on the skin overlaying the masseter muscle on both sides. No kind of anesthetics were used for the skin. The 19 mm needles (diameter 0.4 mm) were inserted and fixed perpendicular to the skin surface at a depth of approximately 15 mm (figure 3). The needles were not removed between the infusions in order to ensure that exactly the same site was used. The first substance infused was hypertonic saline (Natriumklorid, 58.5 mg/mL, Karolinska University Hospital Pharmacy), followed 30 min later, by active substance (granisetron; Kytril®, 1 mg/mL, Roche, Stockholm, Sweden) on one side and placebo on the contralateral side (isotonic saline; Natriumklorid, 9 mg/mL, Fresenius Kabi, Uppsala, Sweden). Finally, hypertonic saline (Natriumklorid, 58.5 mg/mL, Karolinska University Hospital Pharmacy) was infused. For all infusions the needles were connected to an infusion pump (infusion rate 1200 μL/min; Harvard Infusion Pump 22, Harvard Apparatus, Great Britain) in order to ensure that the bilateral infusions occurred simultaneously and with the determined rate, shown in figure 3.

Biopsies

Microbiopsies

Microbiopsies were taken through the skin from the most prominent part of the superficial masseter muscle and from the most prominent part of the tibialis anterior muscle approximately 12 cm below the knee, under skin surface anesthesia (5% lidocaine). A disposable Monopty®Bard® biopsy instrument with a penetration depth of 11 mm and a diameter of 18G was used for the masseter muscle, while a diameter of
16G was used for the tibialis anterior muscle. The biopsy instrument, which was guided with a Bard®TruGuide™ coaxial needle (BARD Norden, Helsingborg, Sweden), was inserted to a depth of 10 mm, as shown in figure 4. This biopsy system is a current version of an automated biopsy system that has been proven to be effective i.e. for the diagnosis of musculoskeletal sarcomas and valid for muscle biopsies in larger muscles (Welker et al., 2000; Hayot et al., 2005).

![Figure 4. Illustration of the microbiopsy technique. A disposable Monopty®Bard® biopsy instrument with a penetration depth of 11 mm and a diameter of 18G (masseter) or 16G (tibialis anterior) was used. The biopsy instrument, which was guided with a Bard®TruGuide™ coaxial needle (BARD Norden, Helsingborg, Sweden), was inserted to a depth of 10 mm. The muscle section was removed from the biopsy instrument with a sterile, blunt probe and placed in 4% paraformaldehyde.](image)

**Traditional open biopsies**

Intra-oral open biopsies were taken from the masseter muscle with a 4 mm Bergström needle, as shown in figure 5. In the patients the most painful point of the masseter muscle was chosen, while in the healthy subjects the point selected coincided with the most painful point in the patients that they were to be compared with. After incision with a scalpel the muscle section was cut with a Bergström needle and removed from the muscle with a forceps. Two vicryl-sutures were placed over the incision and removed after one week. These biopsies were used to determine if the expression of 5-HT3 receptors differed between patients with craniofacial myalgia and healthy volunteers. The biopsies from the healthy subjects were also used to analyze the validity and reliability of the microbiopsy technique, by comparing them with the microbiopsies from the healthy masseter muscles.
Figure 5. Illustration of the open biopsy technique. After anesthesia with 1.8 mL xylocain-adrenalin (10 mg/mL + 5 μg/mL) an incision with a scalpel was performed to bring out the masseter muscle. A muscle section was cut with a 4 mm Bergström needle and removed from the muscle with a forceps. Two vicryl-sutures were placed over the incision and removed after one week. The muscle section was removed from the biopsy instrument with a sterile, blunt probe and placed in 4% paraformaldehyde.

Analysis of biopsies

Both the microbiopsies and the open biopsies were fixed at 4°C over night, with 4% paraformaldehyde, rinsed in phosphate-buffered saline (PBS), dehydrated, embedded in paraffin and sectioned at a thickness of 10 μm. The sections were mounted on glass slides, stored at 37°C over night, dipped into xylene to remove the paraffin and rehydrated by being rinsed in ethanol (100%, 90% and 70%). The sections were then boiled in 10mM (pH 6.0) citrate buffer for 10 minutes and rinsed as well as stored in PBS. Subsequently, the sections were incubated with monoclonal antibodies against the 5-HT\textsubscript{3A} receptors and Na\textsubscript{v} 1.8 sodium channels, and the specific axonal marker PGP 9.5, all raised against human proteins. Finally, the sections were incubated with fluorescent secondary antibodies, and examined in a Leica TCS SPE Confocal Microscope and photographs were captured using a Leica scanner attached to the microscope. The expression of the receptors was then analyzed by counting the number of labeled PGP 9.5 nerve fibers co-expressing the 5-HT\textsubscript{3A} receptor alone or the 5-HT\textsubscript{3A} receptor in combination with the Na\textsubscript{v} 1.8 sodium channel.

Antibodies

The sections were incubated with mouse monoclonal anti-human PGP 9.5 antibody (Abcam Inc, Cambridge, England; ab75447), goat anti-human polyclonal antibody against the 5-HT\textsubscript{3A} receptor (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA; sc-
19150) and rabbit polyclonal antibody against the Na\textsubscript{v}1.8 sodium channels (Abcam Inc, Cambridge, England; ab66743).

**Experimental protocol**

A randomized, placebo-controlled, double blind study design was used for all experiments (*Study I-III*) and all participants received both granisetron and placebo. In *Study I* half of the volunteers received granisetron first and the other volunteers received placebo first and in *Study II-III* the sides for a of each substance were randomized in a balanced manner, i.e. half of the volunteers received granisetron on the right side and the other half received granisetron on the left side. The randomization was performed by computer at www.randomization.com.

The participants were seated in a relaxed position in a conventional dental chair during the experiments.

**Study I**

After a clinical examination and triplicate recordings of PPT the participants were instructed to take one drug capsule every morning and evening for three days, beginning the next morning (day 1). They were also informed about the importance of compliance and were asked about the compliance at the following visit. The PPT over the selected sites was again recorded at day 4. This was followed by a 7 day-washout period, after which the participants crossed over to receive the alternative substance for three days (2 capsules daily). The PPT was finally assessed again (day 15).

**Study II**

A clinical examination that included tenderness to digital palpation of the TMJ and masticatory muscles, range of active jaw movements and presence of jaw sounds was first performed. Tenderness to palpation was determined with a 4-graded scale, where 0 = no tenderness, 1 = mild tenderness, 2 = moderate tenderness with pain reflex, 3 = marked tenderness with a defensive withdrawal reflex (Russell et al., 1986).

The injection points of the masseter muscles were then marked with a felt pen and baseline recording of pain intensity (patients only) and PPT (triplicate recordings) were performed. No anesthetics were used for the skin.
Hereafter, the participants received injections with test substance on one side and placebo on the other side. Pain intensity (patients only) and PPT (single recording) were recorded immediately after injection and then every second minute during the first 10 minutes, followed by recordings every 5 minutes during the next 20 minutes.

**Study III**

Baseline recording of pain intensity and PPT (triplicate recordings) were first performed. No kind of anesthetics were used for the skin.

Simultaneous bilateral pain was then induced by injection of 0.2 mL of sterile hypertonic saline into the masseter muscles during 10 s with an infusion rate of 0.02 mL/s (first injection). This was done as an internal control to ensure that pain of similar intensity was induced on both sides. The pain intensity was assessed immediately and every fifteenth second during the first five minutes after injection. The pain spread was recorded five minutes after injection and the PPT (duplicate recordings) was recorded five minutes after injection and every fifth minute during the following 25 minutes.

Thirty minutes after the first injections 0.5 mL of granisetron was injected into one of the masseter muscles and simultaneously 0.5 mL of placebo was injected into the contra lateral masseter muscle during 25 s, also with an infusion rate of 0.02 mL/s. Two minutes later the hypertonic saline injections were repeated (second injection) and the recordings were repeated in the same manner as after the first injection.

**Study IV**

At a separate visit the participants were screened for trial suitability with a clinical examination, performed in a conventional dental chair, in accordance with the RDC/TMD examination.

At a second visit, the healthy volunteers in the microbiopsy group were pre-treated with skin surface anesthesia (Emla; 5% lidocain) for 30 min before three biopsies were taken from each of the left masseter muscle and the right tibialis anterior muscle. In the traditional biopsy group, both healthy volunteers and patients were anesthetized with a
local injection of xylocain-adrenalin (20 mg/ml+5 μg/ml) before the biopsy was taken from the masseter muscle.

**Statistics**

For data and statistical analyses the SigmaStat software (Systat software Inc., San Jose, CA, USA) was used (version 2.0 in Study I and version 3.10 in Study II-IV). In Study III the Statistica software (version 7.0, StatSoft Scandinavia AB, Uppsala, Sweden) was used for the non-parametric Friedman’s analysis of variance on ranks for repeated measures.

In order to evaluate the normality of the data the Kolmogorov-Smirnov test for continuous variables was used (Study I-II) and the Shapiro-Wilk’s test (Study III). In Study I-II all variables were normally distributed and parametrical statistical analyses were used, while in Study III the pain variables were not normally distributed and consequently non-parametric statistics were used. For the descriptive statistics the mean and standard deviation (SD) was used. A significance level of $P < 0.05$ was used.

**Changes in pain variables**

In Study II parametric statistics were used and two-factor repeated measures analysis of variance (RM ANOVA) with time as the repeated factor was used to test the significance of the differences in pain intensity after injections. The Bonferroni corrected $t$-test for multiple comparisons versus a control group was used as posthoc test. This was performed after RM ANOVA revealed a significant difference. The raw values were used for pain intensity.

In Study III the non-parametric Friedman’s analysis of variance on ranks for repeated measures and the associated multiple comparison tests for two-tailed values (Siegel and Castellan, 1988) was used to test the statistical differences in pain intensity between sides (Gra-side and placebo-side) after the first injection and between treatments (Gra and placebo) as well as after the second injection of hypertonic saline. The significance of the difference between sides (first injection) and treatments (second injection) in pain duration, peak pain and painful area and after injections was tested with Wilcoxon signed rank test.
Changes in pressure pain threshold

In Study I the Friedman repeated analysis of variance on ranks was used to test the difference between sites regarding basal PPT values and Dunn’s method to test for all pair-wise multiple comparison procedures was used as posthoc test. The paired t-test was used in order to test the significance of the difference regarding the changes of PPT and PPT_{SUM} for the different sites after administration of the substances as well as the difference between the administrated substances regarding changes of PPT.

In Study II, after normalization of the PPT values, i.e. the relative change in percent from the pre-injection values, parametrical statistics were used. The RM ANOVA, with time as the repeated factor, was used to test the significance of the differences in PPT after injections. The Bonferroni corrected t-test for multiple comparisons versus a control group was used as posthoc test, when RM ANOVA revealed a significant difference. The Friedman repeated measures ANOVA on ranks, with time as the repeated factor, was used to test for differences in PPT over the reference point in the healthy subjects and in the patients, since the variables were not normalized.

In Study III the difference between sides in baseline PPT was analyzed with paired t-test. The two-way repeated measures ANOVA with the Holm-Sidak test for multiple comparisons versus a control group (baseline) as posthoc test was used for the changes in PPT between sides (Gra-side and placebo-side) after the first injection and between treatments (Gra and placebo) as well as after the second injection of hypertonic saline. These tests were performed for all subjects combined, but also for each sex separately. One-way repeated measures ANOVA was used to test the significance of the changes in PPT over the reference point with time.

Sex differences

In Study I-II the unpaired t-test was used to evaluate the difference between sexes regarding basal PPT values and the changes of PPT values.

In Study III the unpaired t-test was used to test the significance of the differences between sexes in age and baseline PPT. In order to test the significance of the difference between sexes in pain duration, peak pain and painful area after injections the Mann-Whitney U-test was used.
In Study IV the Mann-Whitney U-test was used to test for significant sex-related differences regarding the expression of the 5-HT$_{3A}$ receptors and the Na$_v$1.8 sodium channels in the healthy masseter muscle.

**Immunohistochemistry**

In Study IV the Mann-Whitney U-test was employed to test for significant differences regarding the expression of the 5-HT$_{3A}$ receptors and the Na$_v$1.8 sodium channels between healthy and painful masseter muscles. Further, the Mann-Whitney U-test was used to test if the expression patterns of the 5-HT$_{3A}$ receptors and the Na$_v$1.8 sodium channels differed significantly between the masseter and tibialis anterior muscle and also in order to compare a minimal invasive technique, i.e. microbiopsies, with the traditional open biopsies.
**Results and Discussion**

**Changes in pain variables after administration of granisetron**

In patients (*Study II*), the pain intensity decreased after administration of granisetron, while it increased immediately after administration of placebo. The peak pain intensity (VAS peak) was significantly lower after administration of granisetron compared to placebo, as shown in figure 6. The difference between substances was only significant during the first 2 minutes after injection (*P* < 0.05).

When pain was induced experimentally with hypertonic saline, in the masseter muscles of healthy volunteers (*Study III*), the pain was localized to the area over the masseter muscle but also spread to adjacent regions. The induced pain was of similar intensity and duration on both sides (figure 6 and 7) and there were no further differences in VAS peak or painful area between the sides (figure 7).

![Figure 6](image-url) **Figure 6.** Graph showing the mean (SD) peak pain intensity (VAS peak) in the masseter muscle of patients with local myalgia after treatment with granisetron or placebo and in the masseter muscle of healthy volunteers after experimentally induced pain with hypertonic saline alone or after pre-treatment with either granisetron or placebo. In the patients the VAS peak decreased on the side that was pre-treated with granisetron, compared to placebo. In the healthy volunteers the induced pain was of similar intensity on both sides but when the masseter muscle was pre-treated with granisetron the VAS peak was lower on the side that was pre-treated with granisetron, compared to placebo. * = Significant difference between substances (granisetron and placebo; *P* < 0.05)
After pre-treatment with granisetron the pain intensity was significantly lower ($P < 0.001$), the pain duration shorter (44.1% ; $P < 0.001$), the VAS peak lower (62.5% ; $P < 0.001$) and the painful area smaller (77.4% ; $P = 0.003$) compared to placebo (figure 6 and 7). However, the second injection of hypertonic saline induced less pain than the first ($P < 0.001$).

**Figure 7.** Graph showing the mean (SD) pain duration (sec) and painful area (au) in the masseter muscle of healthy volunteers after experimentally induced pain with hypertonic saline alone or after pre-treatment with either granisetron or placebo. The induced pain was of similar duration and area on both sides, but when the masseter muscle was pre-treated with granisetron the pain duration was shorter and the painful area smaller on the side that was pre-treated with granisetron, compared to placebo. * = Significant difference between substances (granisetron and placebo; $P < 0.05$)

The results of these studies where granisetron significantly reduced the pain are in agreement with the results from previous studies in patients, although they suffered from other types of localized muscle pain such as low back pain and neck pain (Ettlin, 2004; Stratz and Müller, 2004), or generalized pain conditions such as fibromyalgia (Färber et al., 2001). This effect was immediate and seemingly short-lasting, which is contrary to the previous mentioned studies where the effect was long-lasting. One explanation to this inconsistency can be that this thesis only used a single injection of granisetron, whereas the other studies were clinical and used repeated injections (Färber et al., 2001; Ettlin, 2004; Stratz and Müller, 2004). Further, these studies used tropisetron, another 5-HT₃ receptor antagonist, but this is probably of minor importance, since tropisetron has a much lower affinity to the 5-HT₃ receptors (Wong et al., 1995; Gan, 2005) and also has an affinity to 5-HT₄ receptors (Haus et al., 2004). However, this immediate and short-lasting effect on pain is in concordance with a study in patients with TMJ arthritis where granisetron had an immediate, specific and short-
lasting effect on pain (Voog et al., 2000) and one study where a single injection of ondansetron had a short-lasting effect on neuropathic pain (McCleane et al., 2003). Nevertheless, no conclusions about the duration can be drawn because the effect on pain intensity was just recorded for 30 minutes.

The hypertonic saline induced pain in the masseter muscle (Study III) had a quality that is comparable to previously described acute clinical muscle pain (Graven-Nielsen, 2006). The results from this study are similar to the results of previous studies with a sensation of deep pain of moderate intensity (64-65/100 mm on VAS), a distribution and spread of pain to adjacent regions as well as pain referral to other structures, such as the molar teeth (Svensson and Graven-Nielsen, 2001; Svensson et al., 2003a; Graven-Nielsen, 2006). No side-effects occurred which is in line with the numerous intramuscular injections of hypertonic saline that have been given in previous studies with no reported side-effects (Graven-Nielsen, 2006). Thereby this method can be considered as safe.

It has been shown, in animal studies, that myelinated Aδ-fibers (belonging to group III afferents) and non-myelinated C-fibers (belonging to group IV afferents) are excited by hypertonic saline (Paintal, 1960; Iggo, 1961; Cairns et al., 2003). This excitation of afferent fibers has been suggested to occur both directly or indirectly. Directly through an opening of stretch-insensitive sodium channels or indirectly through local release of excitatory mediators (Cairns et al., 2003). It has further been shown that hypertonic saline causes a pain reaction by a nonspecific activation of the sodium channels on the group III and group IV afferents (Graven-Nielsen and Mense, 2001), and by activation of central neurons encoding nociceptive information (Svensson and Graven-Nielsen, 2001). In a microdialysis study, with hypertonic saline induced pain, increased levels of glutamate were found (Tegeder et al., 2002) and in another study a release of SP was registered (Garland et al., 1995). These findings could indicate that other pain mediators such as 5-HT also might be released after intramuscular injections of hypertonic saline. Hence, the nociceptive effect of hypertonic saline might be contributed either by a direct activation of sodium channels and 5-HT₃ receptors or indirectly by a release of 5-HT acting on 5-HT₃ receptors.
The potential influence on the results by the activation of the diffuse noxious inhibitory control (DNIC) was minimized by the used study design with simultaneous bilateral injections. As supposed, the second injection of hypertonic saline induced less pain on both sides than the first, which indeed indicates an activation of the DNIC, but since this effect was the same on both sides it did not influence the results as the treatments were compared between sides.

**Changes in pressure pain threshold after administration of granisetron**

The PPT increased significantly \( (P < 0.05) \) after administration of granisetron, both in the healthy volunteers and in the patients \( (Study\ I-III) \), as shown in figure 8. In \( Study\ II \) this increase was significant \( (P < 0.05) \) only the first 2 minutes after injection while the increase in \( Study\ III \) was significant \( (P < 0.05) \) the first 5 minutes. In \( Study\ II-III \) there was a decrease in PPT after injection of placebo but this change was non-significant.

The difference between granisetron and placebo was significant \( (P < 0.05) \) in all studies \( (I-III) \), figure 8.

![Figure 8](image-url)

*Figure 8.* Graph showing the mean percentage difference of pressure pain thresholds (PPT; kPa) compared with baseline (0) and between substances, after administration of granisetron and placebo. In healthy volunteers and in patients the PPT increased with a difference between substances both after oral administration and local intramuscular injection. In healthy volunteers the PPT increased on the side pre-treated with granisetron with a difference between substances.

* = Significant difference between substances (granisetron and placebo; \( P < 0.05 \))

# = Significant change compared with baseline \( (P < 0.05) \)
When the muscle sites were analyzed separately (Study I) the difference compared to placebo was only found over the larger trunk and limb muscles but not over the smaller jaw muscles, as shown in figure 9.

**Figure 9.** Graph showing the mean percentage difference of pressure pain thresholds (PPT; kPa) compared with baseline (0) and between substances, in healthy volunteers after oral administration of granisetron and placebo. The PPT increased with a difference between substances in the trapezius and tibialis anterior muscles, while no significant change was found in the masseter and temporalis anterior muscles.

* = Significant difference between substances (granisetron and placebo; *P* < 0.05)
# = Significant change compared with baseline (*P* < 0.05)

The results of the studies where granisetron significantly increased the PPT over both healthy and painful muscles are in concordance with a previous study where PPT increased in fibromyalgia patients after an injection of granisetron (Ernberg et al., 2003). A similar finding was noticed in a study regarding patients with systemic joint inflammatory disorders suffering from TMJ pain in which PPT also increased after an injection of granisetron (Voog et al., 2000).

The increase of PPT after administration of the 5-HT3 antagonist granisetron indicates that granisetron suppresses the 5-HT binding to 5-HT3 receptors. Intramuscular injections of 5-HT have been reported to decrease the PPT in healthy individuals by activation of nociceptors (Ernberg et al., 2000a), which is further supported by other studies indicating that 5-HT activates or sensitizes nociceptive free nerve endings of
slow conducting peripheral afferents (Mense, 1993; Graven-Nielsen and Mense, 2001). In addition, other studies have reported increased levels of 5-HT in patients with chronic masseter myalgia (Ernberg et al., 1999) and trapezius myalgia (Rosendal et al., 2004; Shah et al., 2005; Gerdle et al., 2008). Hence, granisetron seems to inhibit 5-HT to excite and sensitize nociceptors by binding to these receptors.

**Expression of receptors**

In healthy volunteers the PGP 9.5 immunoreactive fibers were mainly found in association with the myocytes. This pattern was seen both in the microbiopsies from the masseter and tibialis anterior muscles and in the traditional biopsies from the masseter muscle. However, in contrast to the healthy volunteers, the PGP 9.5 immunoreactive fibers were mainly found in the connective tissue of the masseter muscle in patients with local myalgia, as shown in table 3.

<table>
<thead>
<tr>
<th></th>
<th>Myocytes</th>
<th>Connective tissue</th>
<th>Blood vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traditional biopsy group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Masseter</td>
<td>88.89</td>
<td>55.56</td>
<td>22.22</td>
</tr>
<tr>
<td>Microbiopsy group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Masseter</td>
<td>72.22</td>
<td>41.67</td>
<td>19.44</td>
</tr>
<tr>
<td>Tibialis anterior</td>
<td>85.71</td>
<td>14.29</td>
<td>23.81</td>
</tr>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traditional biopsy group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Masseter</td>
<td>70.59</td>
<td>76.47</td>
<td>17.65</td>
</tr>
</tbody>
</table>

Values are expressed in per cent (%).

When the co-expression of the PGP 9.5, 5-HT\textsubscript{3A} and Na\textsubscript{v}1.8 was analyzed, only few nerve fibers showed a co-expression in healthy volunteers, as shown in figure 10 and 11. In the microbiopsy group, the majority of these nerve fibers, with an expression of 28.0\%, were found in the tibialis anterior muscle in association with myocytes. However, in the masseter muscle the majority of these nerve fibers were found in the connective tissue, with an expression of 28.6\%, figure 10. An equivalent pattern was also found in the healthy masseter muscle in the traditional biopsy group where 87.5\%
of the PGP 9.5 positive fibers co-expressed 5-HT_{3A} and Na_{1.8} in the connective tissue, figure 11.

**Figure 10.** Graph showing the number of PGP 9.5 positive fibers that are co-expressed with 5-HT_{3A} alone or with both 5-HT_{3A} and Na_{1.8}, in the masseter muscle and tibialis anterior muscle of 18 healthy volunteers. There were more positive fibers in association with the myocytes in the tibialis anterior muscle than in the masseter muscle (P < 0.001) and in comparison with the connective tissue and blood vessels in the same muscle (P < 0.001). The same pattern, with more positive fibers in association with the myocytes than in the connective tissue and blood vessels, was also found in the masseter muscle (P < 0.001). However, in the connective tissue there were more positive fibers in the masseter muscle than in the tibialis anterior muscle (P = 0.002). There were more 5-HT_{3A} positive fibers than when 5-HT_{3A} co-expressed with Na_{1.8} in all tissues (P < 0.05).

* = Significant difference of PGP 9.5 co-expression with of 5-HT_{3A} alone or with both 5-HT_{3A} and Na_{1.8} (P < 0.05)

# = Significant difference between different tissues (P < 0.001)

¤ = Significant difference between the masseter and tibialis anterior muscles (P < 0.001)

In patients, the amount of positive nerve fibers was significantly increased, as shown in figure 11. The co-expression of the PGP 9.5, 5-HT_{3A} and Na_{1.8} was mainly found in the connective tissue with an expression of 77.9%. The co-expression of the PGP 9.5, 5-HT_{3A} and Na_{1.8} was only 41.9% in association with the myocytes and lower compared to the connective tissue.
**Figure 11.** Graph showing the number of PGP 9.5 positive fibers that are co-expressed with 5-HT$_{3A}$ alone or with both 5-HT$_{3A}$ and Na$_v$1.8, in five patients with local myalgia and five healthy controls. There was a larger amount of positive fibers in the patients than in the controls, both in association with the myocytes and in the connective tissue. The co-expression of PGP 9.5, 5-HT$_{3A}$ and Na$_v$1.8 in the patients was larger in the connective tissue than in association with the myocytes.

* = Significant difference between patients and healthy controls ($P < 0.001$)

# = Significant difference between different tissues in patients ($P = 0.034$)

Both in healthy volunteers and in patients the PGP 9.5 positive fibers in blood vessels were 5-HT$_{3A}$ positive, but these fibers were not positive to the Na$_v$1.8 sodium channels, as shown in figure 12. Further, no significant differences could be found regarding different tissues, muscle sites or when comparing patients with healthy volunteers.

**Figure 12.** Two photomicrograph showing, in A, a PGP 9.5 (red) and, in B, a 5-HT$_{3A}$ (green) positive nerve fiber in a blood vessel of the masseter muscle.

The immunohistochemical analysis of the muscle sections indicate that the 5-HT$_{3A}$ receptor is highly expressed in human skeletal muscle tissue, which is in similarity with previous animal studies where more than half of all trigeminal ganglion neurons in the rat expressed the 5-HT$_3$ receptor (Pierce et al., 1997; Sung et al., 2008).
The results indicate that there is an increase of muscle nerve fibers that co-express the 5-HT$_{3A}$ receptors and Na$_v$1.8 sodium channels in the connective tissue of patients with local myalgia. The TTX insensitive sodium channels have been shown to be involved in peripheral sensitization (Cesare and McNaughton, 1997) since they contribute to action potentials (Tripathi et al., 2006) and are limited to small diameter nerve fibers (Cairns, 2009). This indicates that the Na$_v$1.8 positive fibers are sensory neurons with a potential nociceptive function. Further, 5-HT is known to participate in pain mediation acting on the 5-HT$_3$ receptors which are known to mainly appear on nociceptive sensory, autonomic and enteric neurons in the peripheral nervous system (Hoyer et al., 1994). Consequently, one can assume that when the 5-HT$_3$ receptors are co-expressed with the Na$_v$1.8 sodium channel, they are likely being expressed on nociceptive nerve fibers.

The significant co-expression of 5-HT$_{3A}$ receptors and Na$_v$1.8 sodium channels that was found in the connective tissue of the patients, was not found in the healthy volunteers, which indicates a proliferation of nociceptive nerve fibers in the muscle connective tissue of patients with local myalgia and also that pain modulation seems to mainly take part in the connective tissue.

In contrast to the patients, the majority of muscle nerve fibers in the healthy volunteers are only 5-HT$_{3A}$ positive and located in association with the myocytes. Since PGP 9.5 labels both small and large diameter myelinated fibers and Na$_v$1.8 is a marker of small diameter, thinly myelinated fibers, this finding suggests that the 5-HT$_{3A}$ positive fibers in association with the myocytes are likely either proprioceptive muscle spindle afferent fibers or motor axons (i.e. the efferent axons of motor neurons that mediate muscle contraction) (Okeson, 1998). This leads to the conclusion that since the majority of muscle nerve fibers were located around the myocytes and only a few nerve fibers co-expressed 5-HT$_{3A}$ and Na$_v$1.8 they are most likely motor axons.

It has previously been shown and suggested that the 5-HT$_3$ receptor mediates vasodilation in the human forearm muscular blood vessels and that it lowers the heart rate in animals (Blauw et al., 1988; Saxena and Villalon, 1990). 5-HT$_3$ receptor
antagonists have also been shown to block venum induced hypertension (Singh and Deshpande, 2009). Therefore, the presence of 5-HT$_{3A}$ receptors in blood vessels is not surprising and is an indication that 5-HT may alter the vascular tone in the masseter muscle in part through activation of 5-HT$_{3}$ receptors on sympathetic afferents.

**Biopsy technique differences**

The muscle tissue provided with the microbiopsy technique had a weight of 19.8 (±2.5) mg and an average volume of 13 mm$^3$, while the weight of the muscle tissue from the traditional biopsies was 58.4 (±9.6) mg and the average volume was 64 mm$^3$. All microbiopsies provided sufficient muscle tissue for immunohistochemical analysis and were more accurate than traditional open biopsies, since they contained less of the surrounding tissues, such as parts of the salivary glands. When comparing the size of the injury it was approximately 1 mm$^2$ in diameter in the microbiopsy group, while it was approximately 10 mm$^2$ in the traditional biopsy group. Only a slight tenderness to palpation, which lasted for an average of 3.6 (±1.6) days, occurred in all volunteers from the area where the microbiopsy was taken. In contrast to the microbiopsies, most volunteers experienced a slight decrease in mouth opening capacity, chewing difficulties due to intraoral swelling and irritation from the suture after the traditional open biopsies. An additional visit was further needed for removal of the sutures after 7-10 days, which the participants experienced as negative.

When the different techniques were compared it was shown that the microbiopsy technique is a valid biopsy method for immunohistochemical analyses of muscle tissue, and may be preferable to traditional open biopsies. This statement is based on the results indicating that the microbiopsy technique provides sufficient muscle tissue for immunohistochemistry, induced less injury, caused only minor local pain that quickly resolved, and less postoperative complaints than traditional open biopsies. This is in agreement with a previous study comparing the microbiopsy technique with traditional open biopsy technique in the vastus lateralis of the quadriceps femoris muscle (Hayot et al., 2005). Further, the microbiopsy technique seems to be more precise by using the Bard®TruGuide™ coaxial needle since the surgeon is certain to
remove tissue from the exact region of interest without damaging the surrounding tissue, which is in concordance with a previous study (Welker et al., 2000).

**Sex differences**

When pain was induced experimentally with hypertonic saline, in the masseter muscles of healthy volunteers (*Study III*), the painful area was significantly larger in women than in men, but there were no sex differences regarding pain intensity or duration. However, when the masseter muscle was pre-treated with granisetron, no significant sex differences were found in any pain variable, as shown in figure 13.

![Figure 13. Graph showing the mean (SD) pain intensity (0-100 mm), pain duration (sec) as well as painful area (au) in the masseter muscle of 30 healthy volunteers (15 men and 15 women). On the right side after experimentally induced pain with hypertonic saline alone and on the left side after pre-treatment with granisetron. The painful area was larger in the women after experimentally induced pain, but there were no other significant sex differences. All pain variables were lower after pre-treatment with granisetron, both in men and women (*P* < 0.05). * = Significant difference between sexes (*P* = 0.017)]

In general, there were no sex differences in the basal PPT over the examined muscles. However, in *Study I* the PPT was significantly higher in men than in women in the masseter muscle and in the temporalis anterior muscle (*P* < 0.05). There were further no sex differences in PPT in the masseter muscle after experimentally induced pain with hypertonic saline (*Study III*).

After oral administration of granisetron there were no differences between sexes regarding changes in PPT, except for in the tibialis anterior muscle that showed a significant difference with higher PPT in men after administration of granisetron compared to placebo (*P* = 0.012).
When granisetron was injected into the healthy masseter muscle (Study II) the PPT increased significantly, but there were no sex differences. However, when pain was induced experimentally with hypertonic saline after pre-treatment with granisetron (Study III) there was a significant increase in PPT compared to placebo only in the men (P = 0.002).

The result with larger painful area in women is in concordance with several other studies measuring pain response to experimentally induced pain, with different kinds of pain stimuli such as glutamate (Cairns et al., 2001; Svensson et al., 2003b) or electrical and pressure stimuli (Dawson and List, 2009). Further, it was concluded in a review article that women rate analogous pain stimuli as more painful than men (Berkley, 1997).

Nonetheless, the results regarding sex differences in PPT were incongruent, which is in agreement with several previous studies, both in our group and in others. The result with higher basal PPT values in men is in accordance with previous studies (Fredriksson et al., 2000; Chesterton et al., 2003). However, it was reported in one study that an intramuscular injection of 5-HT decreased the PPT in the women only and that when 5-HT was co-injected with granisetron the PPT increased only in the women (Ernberg et al., 2000b). A similar pattern of disparate findings have been shown in studies conducted on the effects of opioid analgesia in combination to sex differences. For instance, morphine and pentazocine have shown to have a better pain-relieving effect in women (Gear et al., 1996; Fillingim and Gear, 2004), while other studies have not shown any sex differences in opioid potency and efficacy (Lomas and Picker, 2005). It has further been suggested that the incongruent results are influenced by the drug dose, pharmacokinetics, hormonal, situational and motivational factors (Berkley, 1997).

When the expression of positive PGP 9.5 immunoreactive fibers was analyzed, a significant sex difference was found in the healthy volunteers, where the women showed a significantly larger number of PGP 9.5 positive fibers close to myocytes that co-expressed 5-HT3A receptors alone, or in combination with NaV1.8 sodium channels, as shown in figure 14.
Figure 14. Graph showing the number of PGP 9.5 positive fibers that are co-expressed with 5-HT₃A alone or with both 5-HT₃A and Naᵥ1.8, in men and women for the masseter and tibialis anterior muscles. In women, there was a larger amount of PGP 9.5 positive fibers that co-expressed with 5-HT₃A alone or with both 5-HT₃A and Naᵥ1.8, in association with the myocytes in the masseter muscle.

* = Significant difference between sexes (P < 0.002)

This finding is in concordance with a previous study (Sung et al., 2008) that showed that the expression of 5-HT₃ receptors in the trigeminal ganglion was higher in female than in male rats. A higher expression of 5-HT₃ receptors in women may also explain the better effect of granisetron in men, found in the experimental part of this thesis, since the 5-HT₃ receptor antagonist has fewer receptors to bind on. However, in the healthy volunteers the expression of 5-HT₃ receptors on sensory nerves in general was very low and there were no men in the patient group, why these results must be interpreted with caution.
General Discussion

The main findings of this thesis were that patients with local myalgia have a significantly higher amount of co-expressed 5-HT$_3$A receptors and Na$_V$1.8 sodium channels, especially in the connective tissue surrounding the myocytes, compared to healthy participants. Also that a single intramuscular injection of granisetron attenuates experimentally induced muscle pain in healthy masseter muscles and provides a small pain relief in patients with chronic craniofacial masseter muscle pain. Further, that granisetron increases the PPT both in healthy participants as well as in patients with local myalgia, and with a greater effect in men.

It is well known that acute pain arises due to activation of nociceptors by mechanical, chemical or thermal stimuli, and that chronic pain also involves production and release of inflammatory mediators, such as neurotransmitters (Kidd and Urban, 2001; Zeitz et al., 2002). Indeed, several human and animal studies have suggested that 5-HT activates peripheral nociceptors via the 5-HT$_3$ receptor (Ernberg et al., 2000a; Graven-Nielsen and Mense, 2001; Zeitz et al., 2002; Sung et al., 2008). 5-HT and the other neurotransmitters do not only directly activate the primary nociceptors and sustain pain but they also act indirectly via inflammatory cells in order to stimulate the release of algesic agents. Further, these neurotransmitters act to modify and increase the nociceptors' sensitivity, causing a peripheral sensitization (Kidd and Urban, 2001; Zeitz et al., 2002).

The role of 5-HT$_3$ receptors in pain transmission and pain modulation is well documented from animal studies (Martin et al., 1998; Zeitz et al., 2002). Also in humans they seem to mediate pain, since in a human experimental study 5-HT-induced pain was attenuated by the 5-HT$_3$ receptor antagonist granisetron (Ernberg et al., 2000b). This is further supported by the finding in this thesis that granisetron reduced experimentally induced pain by hypertonic saline injection. This role is also supported by a few clinical trials showing that 5-HT$_3$ receptor antagonists reduce pain in patients suffering from both local (Stratz and Müller, 2004) and generalized pain conditions such as fibromyalgia (Färber et al., 2001; Stratz et al., 2001). The novel finding in the present thesis, that patients with local myalgia have a significantly
higher amount of co-expressed 5-HT\textsubscript{3A} receptors and Na\textsubscript{v}1.8 sodium channels in the connective tissue compared to healthy participants, strengthens the role of the 5-HT\textsubscript{3} receptors in pain transmission and pain modulation, and makes it presumable that a proliferation of nociceptive nerve fibers occur in myalgic states and that this mainly takes part in the connective tissue surrounding the myocytes.

Different 5-HT\textsubscript{3} receptor antagonists have successfully been used in both clinical and experimental trials, reducing the pain sensation and increasing the PPT (Ernberg et al., 2000a; Voog et al., 2000; Graven-Nielsen and Mense, 2001; Zeitz et al., 2002; Färber et al., 2004). In concordance to other studies the effect of granisetron on pain was short-lasted in the present thesis. This could be either due to the dosage or to the use of a single injection instead of repeated injections that were used in the clinical trials (Ettlin, 2004; Stratz and Müller, 2004). By blocking the 5-HT\textsubscript{3} receptors with granisetron the PPT increased over human muscles. The blockage of the 5-HT\textsubscript{3} receptors seems to inhibit 5-HT to excite and sensitize nociceptors, leading to a reduced sensitivity to mechanical stimuli. Some of the HTM receptors can act as nociceptors (Graven-Nielsen and Mense, 2001). A possible tissue damage may cause a release of 5-HT activating the HTM receptors leading to a pain response. However, it is not probable that the pressure applied on the muscles while measuring the PPT affected the results in this thesis. This is also supported by one study that showed that topical anesthesia of the skin overlaying the muscle did not influence the PPT (Kosek et al., 1995), indicating that the release of 5-HT probably takes part in the deeper part of the muscle. This further indicates that 5-HT\textsubscript{3} receptors, that are present in the human masseter muscle, play a role in muscle sensitization.

In addition, the effect of granisetron could be influenced by the TTX insensitive sodium channels by a possible dual or nonspecific blocking effect. The TTX insensitive sodium channels are known to take part in the peripheral sensitization (Cesare and McNaughton, 1997) and are mainly found in the peripheral nociceptors. The Na\textsubscript{v}1.8 sodium channel has been shown to increase with increased age and to participate in normal pain function as well as to play a role in inflammatory pain (Cairns, 2009). The increased amount of Na\textsubscript{v}1.8 could therefore be one reason for the increase of musculoskeletal pain conditions with age. Further, they have been found
to be expressed in the trigeminal ganglion neurons of the rat masseter muscle (Connor et al., 2005).

One can only speculate about why the effect of granisetron on PPT was greater on the larger trunk and limb muscles compared to the smaller craniofacial muscles. One explanation could be that a larger muscle has more 5-HT₃ receptors to bind on. This is in agreement with the results from Study IV where the majority of the co-expressed 5-HT₃ positive fibers and TTX insensitive sodium channels (Naᵥ1.8) in healthy subjects were found in the tibialis anterior muscle in association with the myocytes. Further, the trunk and limb muscles are reported to be less densely vascularized compared to the masseter muscle (Stal et al., 1996). This implies that the administrated granisetron has relatively less 5-HT to compete with for the binding on the 5-HT₃ receptors. On the other hand, a larger amount of receptors provides more binding sites for the antagonists to block. This indicates that the effect in this case would be greater in a smaller muscle, containing less receptors and therefore offering a greater possibility to block the majority of the receptors, i.e. that a relatively smaller amount of 5-HT₃ receptors need to be blocked. Future studies are needed to elucidate these tentative different hypotheses.

The larger amount of co-expressed 5-HT₃A receptors and Naᵥ1.8 sodium channels found in the masseter muscle of women compared to men might explain the better effect the 5-HT₃ antagonists seem to have in men, as the men seem to have fewer receptors to block. This larger amount of nociceptive nerve fibers in women might also be one of the explanations of the predominance of women in musculoskeletal pain conditions. On the other hand, the tibialis anterior muscle showed a larger amount of positive 5-HT₃A receptors than the masseter muscle. However, our results also showed that the PPT increased more over the larger trunk and limb muscles than over the smaller craniofacial muscles. These findings seem contradictory, but one explanation might be that the fibers expressing the 5-HT₃ receptor in the tibialis muscle are located on the myocytes and on fibers not co-expressing Naᵥ1.8 sodium channels. Therefore they are probably motor neurons not playing any significant role in pain transmission.
Taken together, the results from the present thesis indicate that 5-HT is involved in the peripheral pain transmission and pain modulation via the 5-HT$_3$ receptor, mainly located on nociceptive fibers in the connective tissue surrounding the myocytes in human muscles. The results further indicate that the 5-HT$_3$ receptor has a role in chronic and experimental human muscle pain, since granisetron has a potent pain reducing effect in both experimental and clinical pain situations.

However, it must be taken into consideration that 5-HT, which was focused on in this thesis, is one of several mediators such as bradykinin, glutamate, PGs and SP acting all together in a complex manner, causing an excitation and sensitization of peripheral nociceptors.

**Methodological considerations and confounding factors**

**Pain and pressure pain threshold measurements**

Since pain always is a subjective experience, a subjective method to assess and reflect the patient’s perception of pain was used. VAS was chosen for pain assessments in the present thesis, since the essential advantage over the descriptive scales and numeric rating scales is the increased sensitivity with an infinite number of points between the ends of the scale. Further, the VAS is widely used both in the clinical situation as well as in clinical and experimental trials (Bird and Dixon, 1987).

In order to assess the PPT an electronic pressure algometer was used. The algometer was early validated to have an acceptable intra- and inter-observer reliability (Ohrbach and Gale, 1989a). Further, the PPTs are regarded to be an important tool in clinical studies of pain both in healthy participants and in patients (Ohrbach and Gale, 1989b). However, it was reported that pressure rate influences the results and that PPT increases linearly with increased pressure rate (List et al., 1991), therefore the same pressure rate was used in all studies. Further, both duplicate and triplicate recordings were used in the present thesis since duplicate recordings are shown to be more reliable than single recordings (Ohrbach and Gale, 1989a; Isselee et al., 1997).

**Substances**

The used methodology does not allow a conclusion regarding the exact mechanism behind the positive effect of granisetron in this experimental pain model, since a non-
specific blocking of, for instance, the TTX-insensitive sodium channels might occur as well.

Repeated injections of algesic substances such as hypertonic saline are known to activate DNIC. However, due to the study design (Study III) the DNIC effect was the same on both sides and consequently should not influence the results.

**Phase of the menstrual cycle**
The phase of the menstrual cycle and the use of contraceptives during the experiments were not taken into consideration in the present thesis. This might be regarded as a limitation or a confounding factor. However, this aspect is considered to be without any major importance for the results since it has been shown that the intra-individual variability in pain response is greater than the influence of estrogen (Sherman and LeResche, 2006). The results from other studies regarding the influence of estrogen levels are contradictory (Warren and Fried, 2001) and since the women in the present thesis most probably were in different phases of the menstrual cycle this aspect should only have very limited or no influence on the results.

**Future research**
The results from this thesis indicate that granisetron might offer a new treatment approach for local myalgia. However, the efficacy must be determined with clinical trials, measuring the long term effect on pain relief. One study, where patients with chronic myalgia in the masseter muscle and/or the temporalis anterior muscle are treated with repeated intramuscular injections with granisetron, is already ongoing.

To further improve the diagnostic procedure and to enhance the treatment, studies analyzing the release of inflammatory mediators, estrogen and cortisol in different experimental pain models, both in healthy participants and in patients, are warranted.
Conclusions

The combined results of this thesis indicate that there is a proliferation of nociceptive nerve fibers expressing the 5-HT$_3$ receptor in the interstitial connective tissue of the muscles in patients with chronic craniofacial myalgia. The expression of 5-HT$_3$ receptors is higher in the masseter muscle of healthy women than in healthy men as well as higher in the tibialis anterior muscle than in the masseter muscle. Further, blocking of the 5-HT$_3$ receptor with the antagonist granisetron increases the mechanical pain threshold, while it decreases the intensity and duration of pain, as well as the pain spread both in patients with chronic craniofacial myalgia and in subjects with experimentally induced pain. However, these effects of granisetron are more pronounced in men.

In conclusion, our novel findings that 5-HT$_3$ receptors are up-regulated in painful craniofacial muscles and that blocking of this receptor decreases clinical and experimental human muscle pain indicate that the 5-HT$_3$ receptor has an important role in peripheral pain transmission in localized chronic muscle pain.
Populärvetenskaplig sammanfattning

En stor del av den vuxna befolkningen i världen lider av långvarig muskelsmärta. Hos ungefär hälften av dessa individer är smärtan lokaliserad till ansiktet och käkarna, vilket innebär att så mycket som ca 10-15% av den vuxna befolkningen är drabbade av detta. En majoritet av dessa individer är kvinnor. Förutom smärta och ömhet i käkmusklen besvärar dessa individer ofta med nedsatt gapförmåga samt huvudvärk. Förutom det individuella lidandet är detta ett stort problem för samhället med ökad sjukfrånvaro och ökade sjukvårdskostnader som följd. Än så länge är inte orsakerna och mekanismerna som ligger bakom långvarig käkmuskelsmärta fullständigt upptäckta, inte heller varför kvinnor drabbas i högre utsträckning än män. Ökad kunskap om de bakomliggande orsakerna och mekanismerna kommer att hjälpa oss att förbättra diagnostiken och leda till nya och mer effektiva behandlingsmetoder. Detta kan på sikt leda till minskat behov av sjukvårdsresurser och vårdkostnader, och inte minst leda till minskat lidande för den enskilda individen.

De nuvarande teorierna angående långvarig käkmuskelsmärta innefattar flera bakomliggande orsaker där exempelvis psykosociala faktorer såsom stress i kombination med tandnissling (bruxism) är bidragande faktorer. Bruxism leder till en överbelastning av muskelvävnaden som i sin tur misstänks orsaka syrebrist till följd av otillräcklig blodcirkulation. Detta leder till frigörande av inflammationsfrankallande substanser (mediatorer), som exempelvis serotonin (5-HT). Dessa substanser fäster i sin tur på mottagare (receptorer) på musklernas nervfibrer (smärtafferenter) och tolkas som smärta när smärtsignalen når hjärnan. Tidigare forskning har visat att 5-HT deltar i smärtfortledningen genom aktivering av 5-HT₃ receptorn, liksom vid inflammation. En blockering av dessa receptorer har i tidigare studier visats reducera smärta och ömhet hos patienter med fibromyalgi.

Denna avhandling består av en basvetenskaplig del och en experimentell del. Målet med den basvetenskapliga delen var att undersöka huruvida antalet 5-HT₃ receptorer skiljer sig mellan patienter med långvarig käkmuskelsmärta och friska kontroller, men även att undersöka eventuella könsskillnader eller skillnader mellan musklar. Målet för den experimentella delen var att undersöka hur en blockering av 5-HT₃ receptorer med läkemedlet granisetron påverkar smärta och smärttröskeln för tryck (PPT; ett mått
på ömhet) både hos patienter och hos friska försökspersoner. Dessutom undersöktes eventuella skillnader i effekt avseende kön, muskler och läkemedelsadministrering (lokal i injektionsform och systemisk i tablettform).


Den första experimentella delen syftade till att undersöka vilken effekt granisetron har på PPT. Granisetron gavs till friska män och åldersmatchade friska kvinnor på ett randomiserat och dubbelblint sätt, både genom en lokal intramuskulär injektion och i tablettform. Som placebo användes fysiologisk koksaltlösning (0,9 %) och sockerpiller. Resultaten visade i samtliga studier att PPT ökade efter administration av granisetron, oberoende av administrationssätt. Effekten av granisetron var större i tibialis och kappmuskeln (trapezius) än i masseter och den främre tinningmuskeln (temporalis). Effekten var dessutom större hos män än hos kvinnor. Genom att blockera 5-HT$_3$ receptorerna med granisetron dämpas serotoninets effekt vid smärtfortledningen.

Den andra experimentella delen syftade till att undersöka hur granisetron påverkar olika smärtvariabler hos patienter med långvarig käkmuskelsmärta och friska försökspersoner. Studierna var randomiserade, dubbelblinda och placebokontrollerade. Patienterna erhöll en injektion av granisetron i den ena masseter och placebo i den andra. De friska försökspersonerna injicerades först med hyperton koksalt (5,8 %) i båda massetermusklerna för att inducera smärta. Trettio minuter efter injektionen injicerades granisetron i ena massetern och placebo i den andra, följt av injektion med
hyperton koksalt 2 minuter senare i båda musklerna. Resultaten visade att granisetron reducerade samtliga smärtvariabler både hos patienterna och hos de friska försökspersonerna. Smärtutbredningen var större hos kvinnorna efter den första smärtframkallande injektionen, men denna skillnad försvann när muskeln hade förbehandlats med granisetron. Dessa resultat tyder vidare på att 5-HT₃ receptorer spelar en stor roll i smärtfortledningen och att granisetron tycks vara ett potent smärtreducerande ämne.

Sammanfattningsvis tyder resultaten från denna avhandling på att antalet 5-HT₃ receptorer är förhöjt i smärtande käkmusklar och att en blockering av dessa receptorer minskar både klinisk och experimentell muskelsmärta. Detta antyder att perifera 5-HT₃ receptorer spelar en väsentlig roll i smärtfortledningen vid långvarig muskelsmärta.
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Ούκ ἔστιν ὁ θεός αἴτιος.
God is not the cause (of any illness).

Εἰδέναι τί ἔστιν ἄνθρωπος.
To know what the human being is.

Φύσες πάντων ἀδίδακται.
The natures of all are untaught.

Ὅ βίος βραχύς, ἢ δὲ τέχνη μακρή, ὥ δὲ καιρός ὀξύς, ἢ δὲ πείρα σφαλερή, ἢ δὲ κρίσις χαλεπή.
Life is short, art is long, time is swift, enterprise dangerous, and judgment difficult.

Ἀγνὸς δὲ καὶ ὁσίος διατηρήσω βίον τὸν ἐμὸν καὶ τέχνην τὴν ἐμὴν.
I will preserve the purity of my life and my arts.

Τὸ προλαμβάνειν μεῖζὸν ἔστι τοῦ θεραπεύειν.
Prediction exceeds treatment.

Ὡφελέσειν ἢ μὴ βλάπτειν.
To help or to do no harm.