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Title page

# The Role of Glutamate and Peripheral N-Methyl-D-Aspartate (NMDA) Receptors in Masticatory Muscle Pain Mechanisms

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†This dissertation is based on the following peer-reviewed articles referred to by their Roman number in the text.

- i. **Cairns BE**, Hu JW, Arendt-Nielsen L, Sessle BJ, Svensson P. Sex-related differences in human pain and rat afferent discharge evoked by injection of glutamate into the masseter muscle. *J. Neurophysiol* 2001; 86: 782-791. (1)
- ii. **Cairns BE**, Gambarota G, Svensson P, Arendt-Nielsen L, Berde CB. Glutamate-induced sensitization of rat masseter muscle fibers. *Neuroscience*, 2002; 109: 389-399. (2)
- iii. **Cairns BE**, Gambarota G, Dunning PS, Mulkern RV, Berde CB. Activation of peripheral excitatory amino acid receptors decreases the duration of local anesthesia. *Anesthesiology* 2003; 98: 521-529. (3)
- iv. Svensson P, **Cairns BE**, Wang K, Hu JW, Graven-Nielsen T, Arendt-Nielsen L, Sessle BJ. Glutamate-evoked pain and mechanical allodynia in the human masseter muscle. *Pain* 2003; 101: 221-227. (4)
- v. **Cairns BE**, Svensson P, Wang K, Hupfeld S, Graven-Nielsen T, Sessle BJ, Berde CB, Arendt-Nielsen L. Activation of peripheral NMDA receptors contributes to human pain and rat afferent discharges evoked by injection of glutamate into the masseter muscle. *J Neurophysiol* 2003; 90: 2098-2105. (5)

- vi. **Cairns BE**, Dong XD, Mann MK, Svensson P, Sessle BJ, Arendt-Nielsen L, McErlane KM. Systemic administration of monosodium glutamate elevates intramuscular glutamate levels and sensitizes rat masseter muscle afferent fibers. *Pain* 2007; 132: 33-41. (6)
- vii. Dong XD, Mann MK, Kumar U, Svensson P, Arendt-Nielsen L, Hu JW, Sessle BJ, **Cairns BE**. Sex-related differences in glutamate evoked rat muscle nociceptor discharge result from estrogen-mediated modulation of peripheral NMDA receptors. *Neuroscience* 2007; 146(2):822-832. (7)
- viii. Castrillon EE, **Cairns BE**, Ernberg M, Wang K, Sessle BJ, Arendt-Nielsen L, Svensson P. Effect of peripheral NMDA receptor blockade with ketamine on chronic myofascial TMD pain. *J Orofac Pain* 2008;22:122-130. (8)
- ix. Dong X-D, Svensson P, **Cairns BE**. The Analgesic Action of Topical Diclofenac May Be Mediated Through Peripheral NMDA Receptor Antagonism. *Pain* 2009;147:36–45. (9)
- x. Gazerani P, Au S, Dong X-D, Kumar U, Arendt-Nielsen L, **Cairns BE**. Botulinum neurotoxin type A (BoNTA) decreases the mechanical sensitivity of nociceptors and inhibits neurogenic vasodilation in a craniofacial muscle targeted for migraine prophylaxis. *Pain* 2010;151:606–616. (10)
- xi. Castrillon EE, Ernberg M, **Cairns BE**, Wang K, Sessle BJ, Arendt-Nielsen L, Svensson P. Interstitial glutamate concentration is elevated in the masseter muscle of myofascial temporomandibular disorder patients. *J Orofac Pain* 2010;24(4):350-360. (11)
- xii. **Cairns BE**, Dong X-D, Wong H, Svensson P. Intramuscular ketorolac inhibits activation of peripheral NMDA receptors. *J Neurophysiol* 2012;107:3308-3315. (12)
- xiii. Wong H, Dong X-D, Kang I, Christidis N, Ernberg M, Svensson P, **Cairns BE**. NGF-induced mechanical sensitization of the masseter muscle is mediated by increased expression of peripheral NMDA receptors. *Neuroscience* 2014; 269:232–244. (13)

## Preface

Research presented in this dissertation was undertaken at the Faculty of Dentistry, University of Toronto (1997-2000), the Center for Sensory-Motor Interaction (SMI), Aalborg University, Denmark (1999-2010), Department of Anaesthesiology, Harvard Medical School, USA (2000-2003), Faculty of Dentistry, Aarhus University, Denmark (2005-2010), and the Faculty of Pharmaceutical Sciences, the University of British Columbia, Canada (2003-2013).

In 1998, when this work was started, most researchers, including myself, were skeptical that primary afferent fibers expressed receptors for a major excitatory central nervous system neurotransmitter like glutamate. Indeed, one well known pain researcher (who will remain nameless) commented to me at the time that even if receptors for glutamate were present on primary afferent fibers, it was likely an epi-phenomenon and had no importance for pain transduction in the periphery. Fortunately, others could be persuaded to at least consider the possibility. In particular, I am indebted to Professors Jimmy Hu and Barry Sessle, who allowed me to pursue this research without restriction when I was a post-doctoral fellow under their supervision in Toronto. It should also be acknowledged that without the support and encouragement of Professors Lars Arendt-Nielsen and Peter Svensson, who I first met in Vienna in 1999, the translational of animal results into humans would not have been possible. Over the years, I have had the great fortune to work Charles Berde, Giulio Gambarota, Thomas Graven-Nielsen, Kelun Wang, Malin Ernberg, Eduardo Castrillon, Lene Baad-Hansen, Parisa Gazerani, Hayes Wong, Ujendra Kumar and Xu-Dong Dong, collaborators without whom the research contained in this dissertation would not have been completed.

This dissertation focuses on peer-reviewed papers I-XIII. None of these publications have been submitted previously for an academic degree.

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## **Abstract**

**Aims:** The aim of the series of studies that comprise this thesis was to determine whether activation of peripheral N-Methyl-D-Aspartate receptors (NRs) in masticatory muscle causes pain through excitation of nociceptors and plays a mechanistic role in chronic masticatory muscle pain conditions.

**Methods:** In vivo electrophysiology was performed to investigate how glutamate affects the activity of masticatory muscle nerve afferent fibers in Sprague Dawley rats.

Immunohistochemical work was done to characterize the expression of various receptors by trigeminal ganglion neurons or nerve fibers that innervate the masticatory muscles in both rats and healthy humans. Masseter muscle interstitial glutamate concentrations were measured by a glutamate biosensor and by microdialysis. Healthy human subjects were recruited for studies which involved injection of glutamate alone, or with the NR antagonist ketamine, into the masticatory muscle to assess their effects on muscle pain. Patients with myofascial temporomandibular disorders-related pain were recruited for studies to assess the analgesic effectiveness of ketamine, and to measure the interstitial concentration of glutamate in the masseter muscle.

**Results:** Electrophysiological studies conducted in rats indicated that glutamate, in a concentration-dependent manner, can both excite and mechanically sensitize masticatory muscle nociceptors and that this occurs through activation of peripheral NRs. Glutamate-evoked excitation of nociceptors was greater in female rats than in male rats; a difference mediated by estrogen levels. Immunohistochemical studies found that NRs were expressed by about half of all masticatory muscle nociceptors, and

that their expression was greater in females than in males. In humans, injection of glutamate into the masticatory muscles produced pain in a concentration related manner that was greater in women than in men. Injection of glutamate also produced mechanical sensitization of the masticatory muscle which was similar in both sexes. In rats, the non-steroidal anti-inflammatory drugs diclofenac and ketorolac, but not naproxen, were found to have NR antagonist properties when administered at concentrations achievable with topical creams or local injection. Botulinum neurotoxin A was found to decrease nociceptor mechanical sensitivity by inhibition of release of glutamate in masticatory muscle. In humans, pain and mechanical sensitization could be attenuated by local injection of ketamine, which indicates that it was mediated, in part, through activation of peripheral NRs. Masseter muscle biopsies from healthy subjects identified NR expression in a subgroup of sensory nerve fibers. In myofascial temporomandibular disorders patients, it was found that interstitial glutamate in the masseter muscle was elevated compared with healthy controls. Treatment of myofascial TMD patients with an injection of ketamine into the most painful part of the masseter muscle, could decrease pain ratings in about half of women treated, but had no effect on men.

**Conclusions:** Elevated interstitial glutamate acts on peripheral NRs on craniofacial muscle nociceptors to produce pain and mechanical sensitization in humans. In healthy humans and some myofascial temporomandibular disorders patients, the NR antagonist ketamine applied locally can attenuate pain and sensitivity that results from elevated glutamate concentrations in the muscle, which indicates that blockade of peripheral NRs is analgesic. A number of other analgesic drugs that include diclofenac, ketorolac and

botulinum neurotoxin A, have mechanisms which involve NR antagonism or reduction of interstitial glutamate concentration in the muscle, respectively. Modulation of glutamatergic tone in skeletal muscle may be a promising new approach for the treatment of myofascial pain.

## Abstrakt

**Formål:** Formålet med den række af studier, der ligger til grund for indeværende disputats, var at bestemme, om aktivering af perifere N-methyl-D-aspartat-receptorer (NRer) i tyggemuskelen forårsager smerte gennem excitation af nociceptorer og spiller en mekanistisk rolle ved kroniske smertetilstande i tyggeapparatet.

**Metoder:** In vivo blev elektrofysiologi udført for at undersøge, hvordan glutamat påvirker aktiviteten i afferente nervefibre i tyggemusklen hos Sprague Dawley rotter. Der blev udført immunhistokemiske undersøgelser for at karakterisere manifestationen af forskellige receptorer i trigeminale ganglion-neuroner eller nervefibre, som innerverer tyggemusklener hos både rotter og raske mennesker.

Interstitielle glutamat-koncentrationer i kæbemusklen blev målt ved hjælp af en glutamat-biosensor og mikrodialyse. Raske forsøgspersoner blev rekrutteret til forsøg med injektion af glutamat alene eller med NR antagonist ketamin i tyggemusklen til vurdering af virkningen på muskelsmerter. Patienter med smerter, der var relateret til myofasciale temporomandibulære lidelser, blev rekrutteret til forsøg, der skulle vurdere den analgetiske virkning af ketamin og måle den interstitielle koncentration af glutamat i kæbemusklen.

**Resultater:** Elektrofysiologiske undersøgelser på rotter viste, at glutamat på en koncentrationsafhængig måde både kan excitere og mekanisk sensibilisere tyggemusklenes nociceptorer, og at dette sker ved aktivering af perifere NR'er. Glutamatfremkaldt excitation af nociceptorer var større hos hunrotter end hanrotter; hvilket er en forskel der er medieret af østrogen-niveauer. Immunhistokemiske undersøgelser viste, at NR'er blev udtrykt i omkring halvdelen af alle nociceptorer i

tyggemusklens, og at deres andel var større hos hunrotter end hos hanrotter. Hos mennesker fremkaldte injektioner af glutamat i tyggemusklerne smerter på en koncentrationsrelateret måde, der var højere hos kvinder end hos mænd. Injektion af glutamat producerede også mekanisk sensibilisering af tyggemusklens, hvor graden var ens hos begge køn. Hos rotter viste det sig, at de Non Steroidal Anti-Inflammatory Drug lægemidler diclofenac og ketorolac, men ikke naproxen, havde NR-antagonistegenskaber, når de blev indgivet i koncentrationer, som var opnåelige med topiske cremer eller lokal injektion. Botulinum neurotoksin A viste sig at reducere den mekaniske følsomhed af nociceptorer ved at hæmme frigivelsen af glutamat i tyggemusklens. Hos mennesker kan smerte og mekanisk sensibilisering dæmpes ved lokal injektion af ketamin, hvilket indikerer, at det delvist medieres gennem aktivering af perifere NR'er. Kæbemuskel-biopsier fra raske forsøgspersoner identificerede NR-manifestation i en undergruppe af sensoriske nervefibre. Hos patienter med myofasciale temporomandibulære lidelser blev det konstateret, at interstitiel glutamat i kæbemusklen var forhøjet sammenlignet med raske kontrolpersoner. Behandling af patienter med myofasciale temporomandibulære lidelser ved hjælp af en injektion af ketamin i den mest smertefulde del af kæbemusklen viste sig at nedsætte smerte-scoren hos omkring halvdelen af kvinderne, mens injektionen ikke havde effekt på mænd.

**Konklusioner:** Forhøjet interstitielt glutamat-niveau påvirker perifere NR'er i kraniofaciale muskelnociceptorer og forårsager smerte samt mekanisk sensibilisering hos mennesker. Hos raske personer og nogle patienter med myofasciale temporomandibulære lidelser kan NR-antagonisten ketamin indgivet lokalt dæmpe smerterne og den følsomhed, der skyldes forhøjede glutamat-koncentrationer i

musklen, hvilket indikerer, at blokaden af perifere NR'er er analgetisk. En række andre smertestillende lægemidler, der omfatter diclofenac, ketorolac og botulinum neurotoksin A, har mekanismer, der involverer henholdsvis NR-antagonisme eller reduktion af koncentrationen af interstitiel glutamat i musklen. Modulation af den glutamaterge tonus i skeletmuskulaturen kan være en lovende ny metode til behandling af myofasciale smerter.

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# Introduction

## Why glutamate?

Inflammatory tissue injury results in swelling, redness and pain sensitivity. This triad of symptoms, and in particular pain sensitivity, is due to the release of a complex cocktail of substances, collectively termed the “inflammatory soup” (14-16). It had been well established prior to late 1990’s that several components of this soup, such as histamine, serotonin, prostaglandins, neuropeptides (e.g. substance P), protons and potassium, played important roles in the initiation and maintenance of pain sensitivity during inflammation (14-16). The effectiveness of non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids as analgesics had been shown to stem from their ability lower prostaglandin tissue concentration by actions through inhibition of cyclooxygenase, the key enzyme responsible for the synthesis of these compounds. This knowledge led to a great interest in identifying other potential targets for peripheral analgesics; an area of research that has remained active for many years now. Glutamate was subsequently identified as a component of the inflammatory soup in the late 1990’s (17). This led to interest in glutamate and its receptors as potential analgesic targets.

Glutamate activates receptors that can be divided into ionotropic and metabotropic subtypes (18-23). The former are mixed cation channels, which allow the passage of  $\text{Na}^+$ ,  $\text{K}^+$  and in some cases  $\text{Ca}^{++}$  across cell membranes to depolarize plasma membranes, while the latter are G-protein coupled and alter the intracellular concentrations of second messengers such as cyclic-adenosine mono-phosphate, diacylglycerol and inositol triphosphate, that result in downstream effects on neuronal

excitability. There are 3 ionotropic glutamate receptor subtypes which have been named for the agonist first describe to be selective for them; the N-methyl-d-aspartate (NMDA) receptor (NR) and two non-NMDA glutamate receptors (GluRs) the kainate receptor and the  $\alpha$ -amino-3-hydroxy-5-methyl-5-isoxazolepropionate (AMPA) receptor (18-23). There are 8 subtypes of metabotropic receptor (mGluR), which have been grouped into 3 families on the basis of the G-proteins and downstream signaling mechanisms they activate. Group I mGluRs mediate their effects through Gq/G11 G-proteins and stimulate the activity of phospholipase C. Group II and Group III mGluRs are coupled to Gi/Go G-proteins and inhibit the activation of adenylate cyclase.

The first indication for a nociceptive effect of activation of peripheral glutamate came from a study that found that application of either glutamate or kainate to the tail skin of neonatal rats evoked a putative nociceptive ventral root reflex (24, 25). Glutamate, but not kainate, appeared to evoke ventral root reflexes of a similar magnitude over repeated trials by activating a peripheral glutamate receptor. Activation of the ventral root reflexes by application of kainate to the tail skin was competitively inhibited by the selective GluR antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) applied to the tail, which suggested that it was mediated through activation of kainate receptors in the skin. However, as this finding was made in neonatal rats, it was unclear whether the activation of these peripheral glutamate receptors was maintained into adulthood in rats.

A few years later, it was reported that intraarticular injection of a NR antagonist appeared to attenuate nociceptive reflexes in adult rats (26). In this model, mustard oil, which is inflammatory and nociceptive, is injected into the rat temporomandibular joint

and evokes a characteristic increase in the electromyographic activity of both the digastric ( jaw opener) and masseter ( jaw closer) muscles, bilaterally (26, 27). Mustard oil evoked increases in reflex jaw muscle activity were attenuated by pre-injection of the non-competitive NR antagonist MK-801 into the temporomandibular joint. This result suggested that part of the nociceptive effect of mustard oil is mediated through the activation of peripheral NR.

Finally, a study in humans with the goal of increasing the effectiveness of local anesthetic wound infiltration under conditions of inflammation, such as occurs post-surgery, suggested that peripheral NRs might play a role in nociception in humans (28). This study looked at the effectiveness of infiltration of the local anesthetic bupivacaine with and without the NR antagonist ketamine in 18 patients who had undergone hernia repair surgery. The addition of ketamine to the bupivacaine infiltration increased the duration of anesthesia by 50-100%, and also resulted in a significant increase in patient tolerance to mechanical stimulation of the wound. These results indicated that ketamine could act locally to increase the effectiveness of local anesthetics administered for pain relief post-surgery in humans. This evidence in both rats and humans that peripheral glutamate receptors could play a role in inflammatory pain mechanisms led to interest in further dissecting the mechanisms whereby glutamate might contribute to peripheral pain mechanisms.

### **Interstitial glutamate concentration and muscle pain.**

The concentration of glutamate in many peripheral tissues and in the blood is an order of magnitude greater than that found in the central nervous system. In healthy

men and women, normal blood glutamate concentrations range from 20-60  $\mu\text{M}$ , and in both men and women glutamate concentration in the blood appears to increase with age (29). Glutamate is actively transported from the blood into skeletal muscle, and the rate at which it is taken up is proportional to its concentration in the blood (30). This means that under conditions where glutamate concentration is elevated, such as consumption of a high glutamate content meal, skeletal muscle acts to rapidly remove excess glutamate from the blood and store it (30, 31). In addition, skeletal muscle contains a large storage of glutamine, which can be converted to glutamate by the enzyme glutaminase. There is also evidence that glutamate is transported anterogradely to peripheral terminals of sensory afferent fibers that innervate skeletal muscle, and is likely released from them (32). As a result, interstitial concentrations of glutamate in skeletal muscle approximate or even exceed those in the blood.

In microdialysis studies where relative recovery rate of glutamate has been determined using  $^{14}\text{C}$  labelled glutamate, mean interstitial glutamate concentrations in skeletal muscle ranged from 15-60  $\mu\text{M}$  (33, 34). This means that peripheral endings of sensory nerve fibers which innervate skeletal muscle are also exposed to resting glutamate concentrations 10-50 times higher than their endings in the central nervous system. This may explain the association that has been made between elevated interstitial concentration of glutamate and muscle pain in certain chronic musculoskeletal pain conditions, such as tendonitis and polymyalgia rheumatica (34-40).

Interstitial glutamate concentrations have been reported to increase dramatically in several non-inflammatory pain conditions involving skeletal muscle and/or tendons

(41-43). In tendons, such as the extensor carpi radialis brevis tendon of patients with tennis elbow and the patellar tendon of patients with “jumpers knee”, glutamate concentrations of greater than 200  $\mu\text{M}$  have been found (41-44). In skeletal muscle it has been reported that baseline pain pressure thresholds showed a significant negative correlation with muscle glutamate concentration in women with chronic work-related trapezius myalgia (43). During a low force exercise that resulted in muscle pain, glutamate concentrations were positively correlated to the magnitude of muscle pain reported by both healthy subjects and those with chronic trapezius myalgia (43). In other studies, no differences in interstitial glutamate concentrations in the trapezius muscle between healthy controls and pain patients have been identified (45, 46).

### **Glutamate as a peripheral neuromodulator**

*Cutaneous:* Much of the initial research to evaluate how glutamate and its receptors contribute to peripheral pain processing was done in cutaneous tissues. Behavioral investigations in adult rats found that subcutaneously-administered glutamate activates all three subtypes of ionotropic glutamate receptors as well as metabotropic glutamate receptors. Subcutaneous or intradermal injection of glutamate or selective agonists (NMDA, AMPA or kainate) as well as Group I mGluR agonists into the rat paw results in sensitization of the skin to noxious mechanical and/or thermal stimulation (47-53) and nocifensive (pain-related) behaviors, such as paw licking (54, 55). These findings indicate that there are functional ionotropic and metabotropic receptors in cutaneous tissue and that their activation is noxious.

In the skin, glutamate appears to activate both nociceptive and non-nociceptive afferent fibers. Subcutaneous injection of glutamate activates roughly 80% of

mechanoreceptive C, A $\delta$  and A $\beta$  fibers that innervate the skin of the rat's back (56, 57). In the rat paw, there has been some evidence that glutamate may selectively sensitize putatively nociceptive A $\delta$  and C fibers to mechanical and thermal stimuli (58). Glutamate also excites slowly-conducting corneal afferent fibers in vivo, whose function is principally related to nociception (59). However, the response of slowly adapting type 1 mechanoreceptive afferents of rat whiskers to sustained mechanical stimulation is attenuated by NR antagonists, which suggests that glutamate excites both fast and slowly conducting cutaneous fibers (60, 61). Subcutaneous injection of glutamate induced a significant mechanical sensitization of facial skin afferent fibers in the rat (62). Mechanical sensitization of facial cutaneous afferent fibers was attenuated by an NR antagonist (62). Taken together, these findings suggest that elevated levels of glutamate in the skin decrease the activation threshold for both low threshold mechanoreceptive afferent fibers as well as nociceptors.

Translational studies have been conducted to determine the ability of glutamate to cause pain in humans. Subcutaneous injection of glutamate into the forehead induces pain, mechanical sensitization and vasomotor responses in healthy men and women (63). Pain intensity was concentration-dependent, and greater pain responses were reported by women than men. Concentration-dependent local vasomotor responses, which are thought to result from neurogenic inflammation, were also found following the subcutaneous injection of glutamate, but there was no sex difference in this effect.

*Visceral:* Almost all information about the role of peripheral glutamate receptors in visceral pain has been derived from studies which have examined the gastrointestinal

tract. Vagal afferent fibers which innervate the duodenum and stomach have their cell bodies in the nodose ganglion, while colorectal afferent fibers of the splanchnic and pelvic nerves have their cell bodies in the dorsal root ganglion. A number of studies have demonstrated that nodose ganglion and dorsal root neurons express most if not all glutamate receptor subtypes (64-69). The ongoing discharge and mechanically evoked responses of vagal afferent fibers is enhanced by glutamate receptor agonists and decreased by their antagonists, which suggests that inotopic glutamate receptor activation, particularly NR activation, modulates vagal afferent sensitivity (64, 65, 69). The mechanical responsiveness of colorectal afferent fibers of the pelvic nerve can also be reduced by NR receptor antagonists, and this effect is mediated principally through activation of the NR2B subtype (68-71). These findings indicate that visceral afferent fiber excitability can be modulated by peripheral glutamate receptor activation. Studies to determine whether peripheral glutamate levels contribute to visceral pain in humans have not been undertaken.

*Musculoskeletal:* Functional inotropic glutamate receptors have been found in the temporomandibular (jaw) joint, where intraarticular injection of glutamate evoked a reflex jaw muscle response that could be mimicked by injection of NMDA, AMPA or kainate and attenuated by co-injection of AP5 and CNQX (72). Behavioral evidence for the involvement of mGluRs in muscle pain is derived from experiments where injection of glutamate or a group I mGluR agonist into the masseter muscle evoked nocifensive behavior and induced mechanical sensitization (73, 74). These findings indicate that there are functional peripheral inotropic and metabotropic receptors within both joints and muscles.

Elevation of glutamate concentration in joints and muscles excites slowly conducting ( $A\delta$  and C) afferent fibers with high mechanical thresholds that likely serve nociceptive roles as well as  $A\beta$  afferent fibers with lower mechanical thresholds that may be involved in proprioception (Figure 9-2) (75-77). The majority of the peripheral NRs expressed by muscle afferent fibers contain the NR2B subunit with far fewer that express the NR2A subunit and NMDA-evoked muscle afferent discharge is significantly attenuated by the NR2B selective antagonist ifenprodil (76, 78-82). Overall, NRs are expressed by more than 50% of the afferent fibers that the masticatory muscles, which implies that they must play an important role in modulating the function of these afferent fibers (7, 13, 83).

Injection of glutamate into neck and masticatory muscles reliably evokes pain and induces a period of mechanical sensitization in healthy human subjects (84-87). Both glutamate-evoked pain and glutamate-induced mechanical sensitization are reduced by local administration of the NR antagonist ketamine (5, 88). Thus, as in the skin, results from animal studies appear to be translatable into humans.

## Aim and Hypotheses

The overall aim of the series of studies that comprise this thesis was to determine whether activation of peripheral NRs in masticatory muscle tissue causes pain through excitation of nociceptors and plays a mechanistic role in chronic masticatory muscle pain conditions.

**Hypothesis 1:** Elevation of peripheral glutamate concentration will excite and sensitize nociceptors through activation of NRs.

**Hypothesis 2:** Afferent fibers that innervate the masticatory muscles express NRs.

**Hypothesis 3:** The response to glutamate in females will be greater than in males, and this difference will be due, in part, to estrogen levels.

**Hypothesis 4:** Activation of peripheral NRs in human muscle by intramuscular injection of glutamate will cause pain and induce mechanical sensitization that will be greater in women than in men.

**Hypothesis 5:** Glutamate levels will be elevated in the muscles of myofascial TMD patients and local administration of a NR antagonist will decrease pain in this condition.

**Hypothesis 6:** Co-injection of NR antagonists with local anesthetics into inflamed tissue will increase block duration compared to injection of local anesthetic alone.

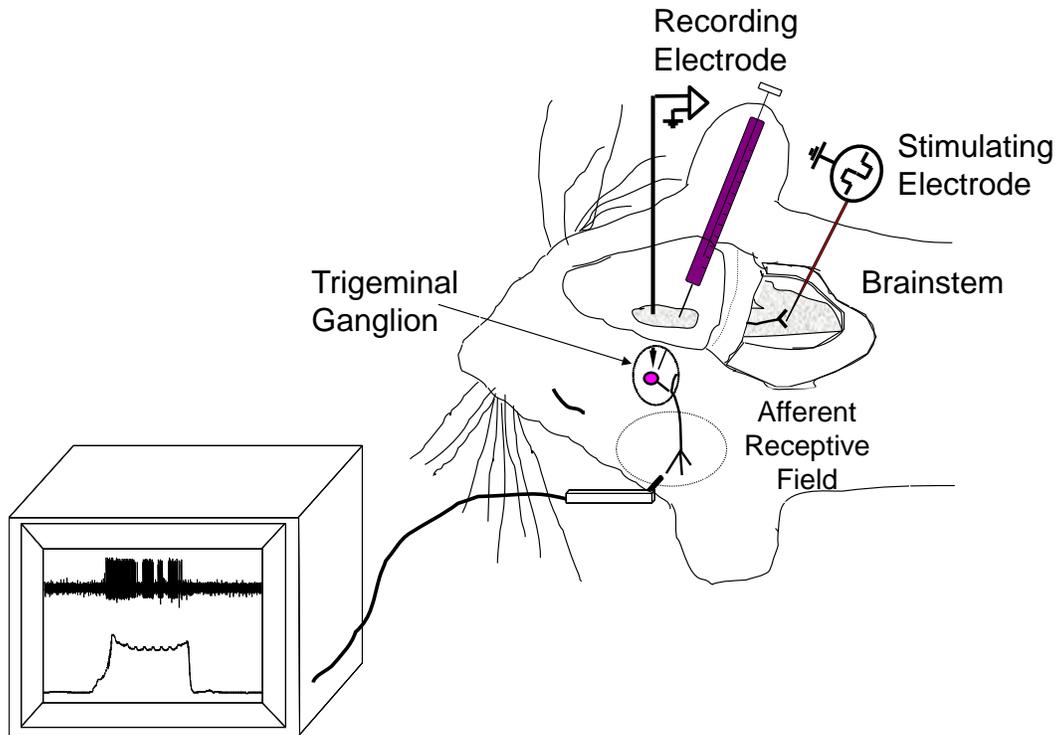
**Hypothesis 7:** Botulinum neurotoxin A increases the mechanical activation threshold of masticatory muscle afferents by decreasing interstitial glutamate concentration.

**Hypothesis 8:** The non-steroidal anti-inflammatory drugs diclofenac and ketorolac, in addition to blocking prostaglandin synthesis, will also block peripheral NRs.

## Methodologies Employed

### **Electrophysiology:**

*In vivo* electrophysiology was performed in studies (i-iii, v-vii, ix, x, xii & xiii) where the activity of masticatory muscle afferent fibers was investigated. All *in vivo* electrophysiology experiments used Sprague Dawley rats. These animals were prepared for acute recording of trigeminal primary afferent activity under isoflurane anesthesia. The basic experimental recording setup for *in vivo* recording of trigeminal nerve is illustrated in Figure 1 (ii, (2)). The recording electrode was inserted with a microdrive into the trigeminal ganglion, where the cell bodies of slowly conducting afferent fibers that innervate the masticatory muscles are located (2, 89). The output of the recording electrode was routed through an amplifier and analog-to-digital board (CED 1401, Cambridge Electronic Devices, UK) into a computer equipped with Spike 2. The output of the electronic Von Frey hair was connected to the computer to permit simultaneous recording of action potential discharge and mechanical stimulus intensity, as shown in the simulated computer monitor below. Anatomical evidence indicates that the vast majority of slowly conducting (A $\delta$  and C) afferent fibers project by way of the trigeminal ganglion to the trigeminal subnucleus caudalis (90). To help ensure that these afferent fibers were recorded from the subnucleus caudalis, a brainstem stimulating electrode was placed in contact with the caudal brainstem and was used to activate the central termination of muscle nociceptors in the trigeminal subnucleus caudalis. Except in study ii (2), all afferent fibers examined had projections to this part of the trigeminal sensory nuclear complex. In recent experiments, a Hamilton syringe



**Figure 1:** An illustration of the in vivo electrophysiology recording set up is shown.

has been inserted into the caudal trigeminal ganglion and used to micro-inject solutions containing neurotransmitters and/or their antagonists.

**Immunohistochemistry:**

In certain experiments (vii, x, xiii), immunohistochemical work was done to characterize the expression of various receptors by trigeminal ganglion neurons or nerve fibers that innervate the masticatory muscles. To identify ganglion neurons that innervated the masticatory muscles, a fluorescent tracer dye (fast blue or rhodamine) was injected into the muscle of interest. To identify nerve fibers in muscle tissue,

primary antibodies against the pan axonal marker PGP 9.5 were used. Seven days after injection, rats were euthanized and then perfused with paraformaldehyde.

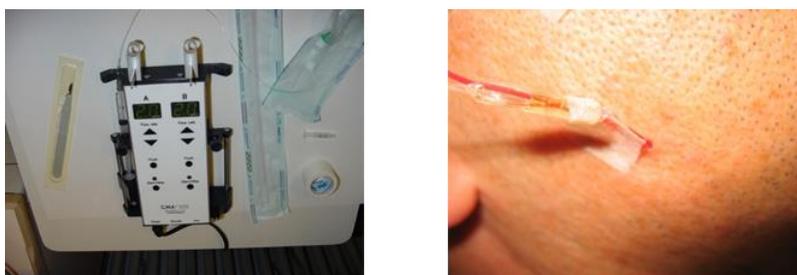
Trigeminal ganglia as well as muscle tissue in some cases, were removed and cut into sections (10-40  $\mu\text{m}$ ) with a vibratome or cryotome. Primary antibodies were obtained from commercial sources (e.g. Sigma, AbCam). Sections were treated with 5% normal goat serum (NGS) in phosphate-buffered saline for 1 h followed by overnight incubation with appropriate primary antibodies in phosphate-buffered saline containing 1% normal goat serum. The next morning sections were washed several times with phosphate-buffered saline and then incubated for 1 h at room temperature in the dark in the presence of fluorescent-conjugated secondary antibodies for antibody localization. After several washes in buffer, sections were mounted on slides with coverslips and imaged with a Leica fluorescence microscope, or in later studies, a Leica TCS SPE high resolution spectral confocal microscope.

### **Tissue Glutamate Concentration**

Masseter muscle interstitial glutamate concentrations were measured by a glutamate biosensor and by microdialysis. A glutamate biosensor from Pinnacle Technology (USA) was calibrated in vitro according to the manufacturer's instructions and has the advantage of excellent time resolution (4 hz sampling speed) and real time visualization of glutamate concentration. The glutamate biosensor is a platinum–iridium electrode coated with a layer of a passive-selective membrane (Nafion and Cellulose Acetate) followed by a layer of the enzyme glutamate oxidase, which catalyzes the conversion of glutamate and oxygen to  $\alpha$ -ketoglutaric acid and hydrogen peroxide. The

hydrogen peroxide produced is detected when it diffuses through the passive-selective membrane and is oxidized at the surface of the platinum–iridium electrode (applied potential, 600 mV). The passive-selective membrane limits interference from other substances present in muscle tissue which could be oxidized at the surface of the platinum–iridium electrode at a similar applied potential. The glutamate biosensor was inserted into the masseter muscle with a catheter and used to record glutamate concentrations for periods exceeding 3 hours.

Microdialysis was performed in both humans and animals by inserting a commercially acquired microdialysis probe with a membrane exclusion of molecules greater than 10 kDaltons. In human subjects, the skin over the masseter muscle was anesthetized prior to insertion of the probe (by means of a needle catheter) with EMLA cream, a local anesthetic. The probe was connected to a microinfusion pump and perfused at 2  $\mu$ l/minute with dialysate solution (sterile, phosphate buffered saline). Collected dialysate was analyzed either by a commercial kit (Amplex Red Glutamic Acid/Glutamic Oxidase Assay Kit, Molecular Probes) or by high performance liquid chromatography (6, 11).



**Figure 2:** The pictures show the microdialysis pump (on the left) and insertion of a microdialysis probe into the masseter muscle of a human subject (on the right).

## Human Experimental Pain Studies

Healthy subjects without signs or symptoms of TMD (Dworkin and LeResche, 1992) were recruited for the various human experimental studies. The local ethics committee in Denmark (Counties of Nordjylland and Viborg) approved the study protocol and all individuals gave their informed consent in accordance with the Helsinki Declaration. Intramuscular injections into the masseter and or temporalis muscle were made manually with thin gauge (27-30) hypodermic needles and disposable syringes. All sterile solutions injected were prepared by a hospital pharmacy (glutamate, hypertonic saline) or purchased directly from the manufacturer in injectable form (ketamine). Subjects continuously rated their pain intensity evoked by intramuscular injections on an electronic 10-cm visual analogue scale. The lower endpoint of the VAS was labeled 'no pain at all' and the upper endpoint labeled 'the most pain imaginable'.

A pressure algometer (Somedic, Sweden) with a probe diameter of 1 cm was used to assess mechanical sensitivity of the masticatory muscles. The pressure pain threshold (PPT) was defined as the amount of pressure (kPa) which the subjects first perceived to be painful. The pressure pain tolerance (PPTol) was defined as the maximum amount of pressure subjects could tolerate. Subjects pushed a button to stop the pressure stimulation when the threshold or tolerance was reached. PPT and PPTol were determined by applying the algometer at a constant application rate of 30 kPa/s. Subjects were asked to keep their jaw at rest and not to clench their teeth during testing because contraction of the jaw closing muscles may influence the determination of mechanical sensitivity.

## **Microbiopsies**

Microbiopsies were obtained from healthy human subjects through the use of a disposable biopsy instrument (BARD Norden, Helsingborg, Sweden). The biopsy instrument was inserted through the skin surface overlaying the bulky part of the superficial masseter muscle under local anesthesia (5% lidocaine). The biopsy instrument was used to collect a sample of muscle tissue with a size of approximately  $\sim 0.1 \text{ cm}^3$ . The muscle section was removed from the biopsy instrument using a sterile probe, and the biopsy instrument was rinsed with isotonic saline. This procedure was repeated two more times, which resulted in a total of three micro-biopsies samples from the masseter muscle.

## **Clinical Studies**

Patients with myofascial temporomandibular disorders related pain were recruited. All patients were diagnosed with the Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD) by a qualified dental specialist. All patients were required to sign an informed consent form and were treated in accordance with the Helsinki Declaration. Patients were required to have spontaneous (ongoing) pain of at least 2/10 on the numeric rating scale as well as pain on palpation of the masseter muscle. Patients were excluded if they were pregnant, had other musculoskeletal pain disorders (fibromyalgia, arthritis, etc.), serious systemic diseases (e.g. malignancy), or were receiving medications for pain or mental health conditions.

## Results and Discussion

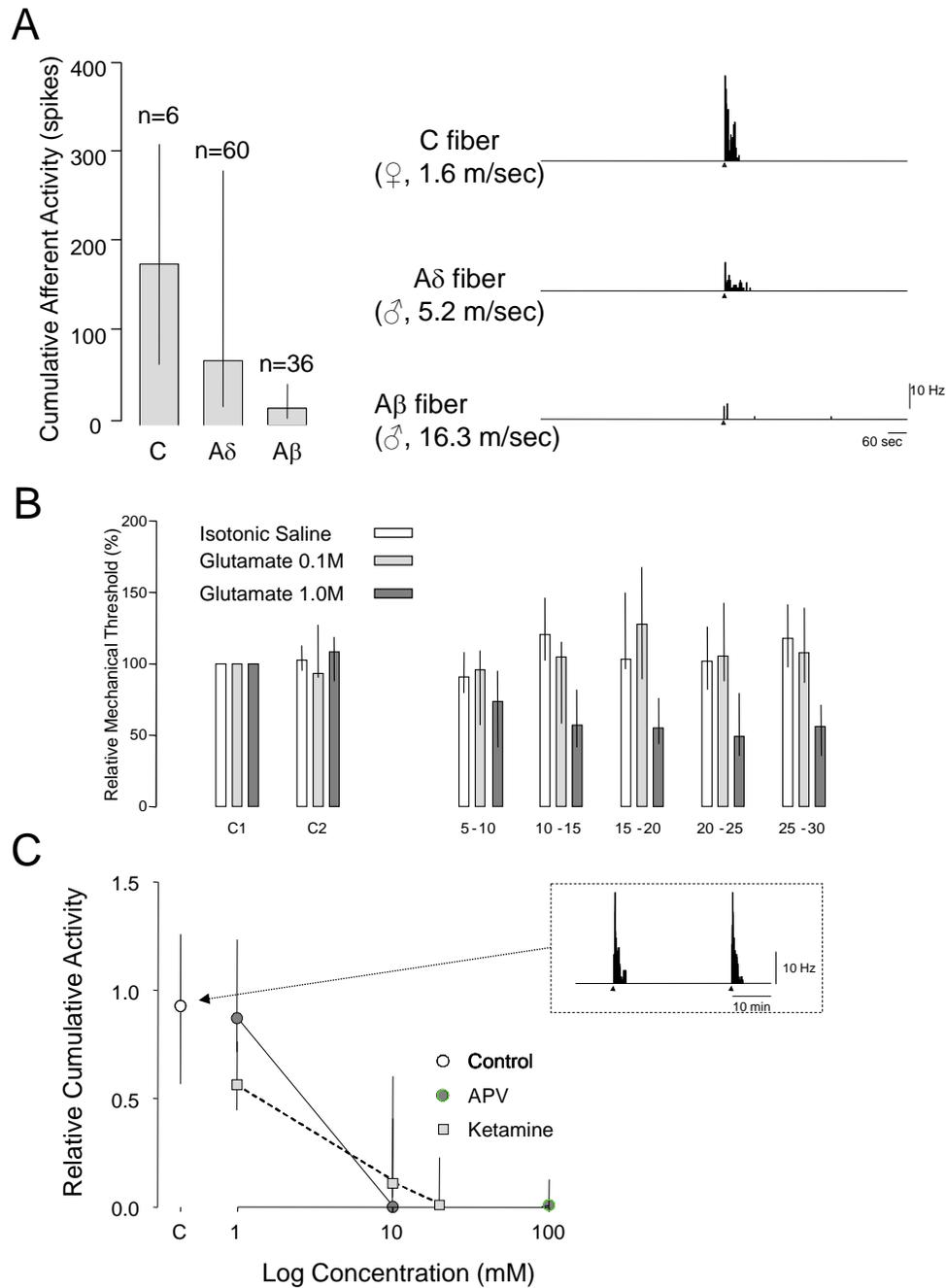
### **Masticatory Muscle Nociceptor Activity is Modulated by Glutamate Concentration**

Initial animal work to examine whether glutamate is a peripherally acting algogen involved injection of glutamate into various tissues and assessment of behavioral response to these injections in animals (27, 91, 92). These studies determined that injection of low concentrations of glutamate ( $\leq 1$  mM), did not evoke nocifensive behavior but did induce a period of localized mechanical sensitization. Most of this work was done with subcutaneous injections, often into the rat paw (92). Injection of higher concentrations of glutamate ( $> 50$  mM) produced nocifensive behavior or evoked nociceptive reflexes in a concentration related manner, when injected either subcutaneously or into deep tissues (27, 91). It was also found that both NRs and non-NRs were involved in sensitization and nocifensive behaviors induced by injection of glutamate (27, 91, 92). However, one criticism of this work was that none of these studies could directly demonstrate that the injected glutamate was having an effect on nociceptors.

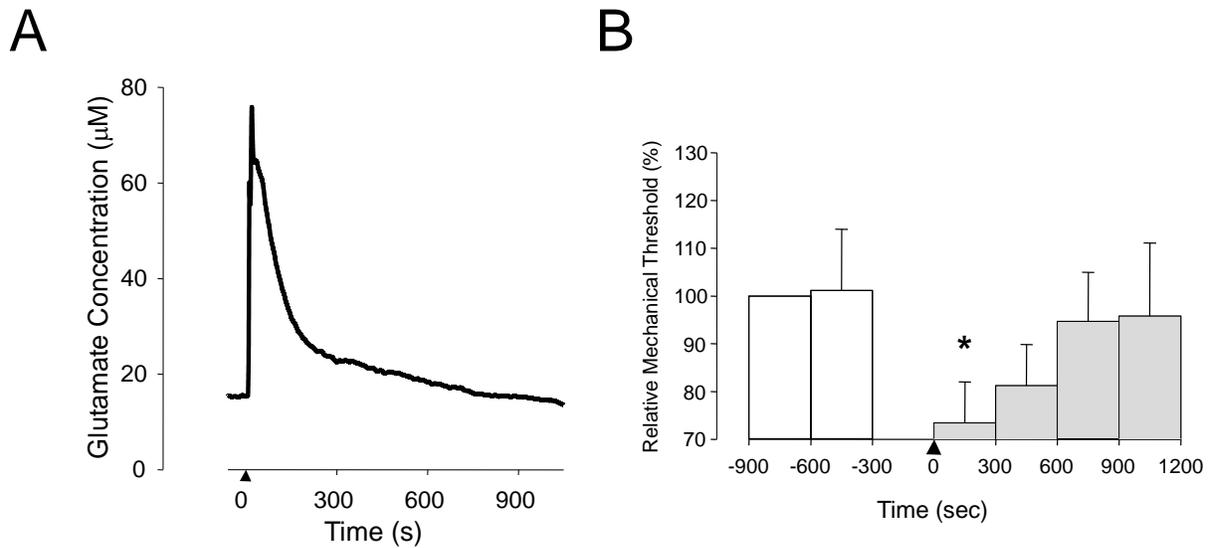
To address this concern, I developed a method for recording the activity of masticatory muscle nociceptors in vivo (Figure 1), and examined the effect of glutamate on nociceptor excitability (1, 2). Injection of glutamate into the masseter muscle evoked action potential discharge in a concentration-related manner from both slowly conducting ( $A\delta$  and C) afferent fibers as well as  $A\beta$  fibers (Figure 3A) **(i)** (1). Injection of a high concentration of glutamate (1000 mM) into the masseter muscle, also significantly decreased the mechanical threshold of afferent fibers that innervate this muscle **(ii)** (2) (Figure 3B). Glutamate-evoked afferent discharges were very sensitive

to, and discharge could be almost completely prevented by, both competitive (5-amino-phosphonovalerate (APV)) and non-competitive (ketamine) NR antagonists **(v)** (5) (Figure 3C). Glutamate-induced mechanical sensitization was sensitive to block of NRs and non-NR s (2, 10). While these findings confirmed that peripherally administered glutamate could activate and sensitize muscle nociceptors, concern that the concentrations used were far outside the normal physiological range of glutamate in tissues led to renewed skepticism as to the role of glutamate in peripheral muscle pain mechanisms.

To determine whether more physiological changes in glutamate concentration would produce similar effects on muscle nociceptors, a series of experiments was conducted to measure the interstitial concentration of glutamate in the masseter muscle as well as to monitor the activity of muscle nociceptors after systemic administration of monosodium glutamate (MSG) **(vi)** (6). A dose of 50 mg/kg MSG was administered intravenously to the rats, since this dose was considered roughly equivalent to oral intake of 150 mg/kg of MSG; a dose associated with headache, facial flushing and discomfort by some humans (2). In rats, a dose of 50 mg/kg MSG given intravenously raised interstitial concentrations of glutamate in the masseter muscle from 25 to 65  $\mu\text{M}$  **(vi)** (6) (Figure 4A). This degree of elevation of interstitial glutamate in the muscle was associated with significant mechanical sensitization of nociceptors that was mediated through NR activation **(vi)** (6). Mechanical sensitization induced by intravenously administered glutamate lasted for 15 minutes, and the magnitude of the sensitization appeared related to the concentration of glutamate in the muscle (Figure 4B). Only one afferent fiber responded to glutamate with action potential discharges.



**Figure 3:** A, Glutamate (500 mM) evoked the largest afferent discharge in C fibers (1). B, Injection of 1 M glutamate induced a prolonged mechanical sensitization of masseter muscle afferent fibers (2). C, Concentration-related inhibition of glutamate-evoked afferent discharge by the NR antagonists APV and ketamine (5).



**Figure 4:** A, Intravenous injection of 50 mg/kg of MSG increased the interstitial concentration of glutamate in the rat masseter muscle and B, caused a 25% decrease in masseter afferent fiber mechanical threshold which returned to baseline after 10 min (6).

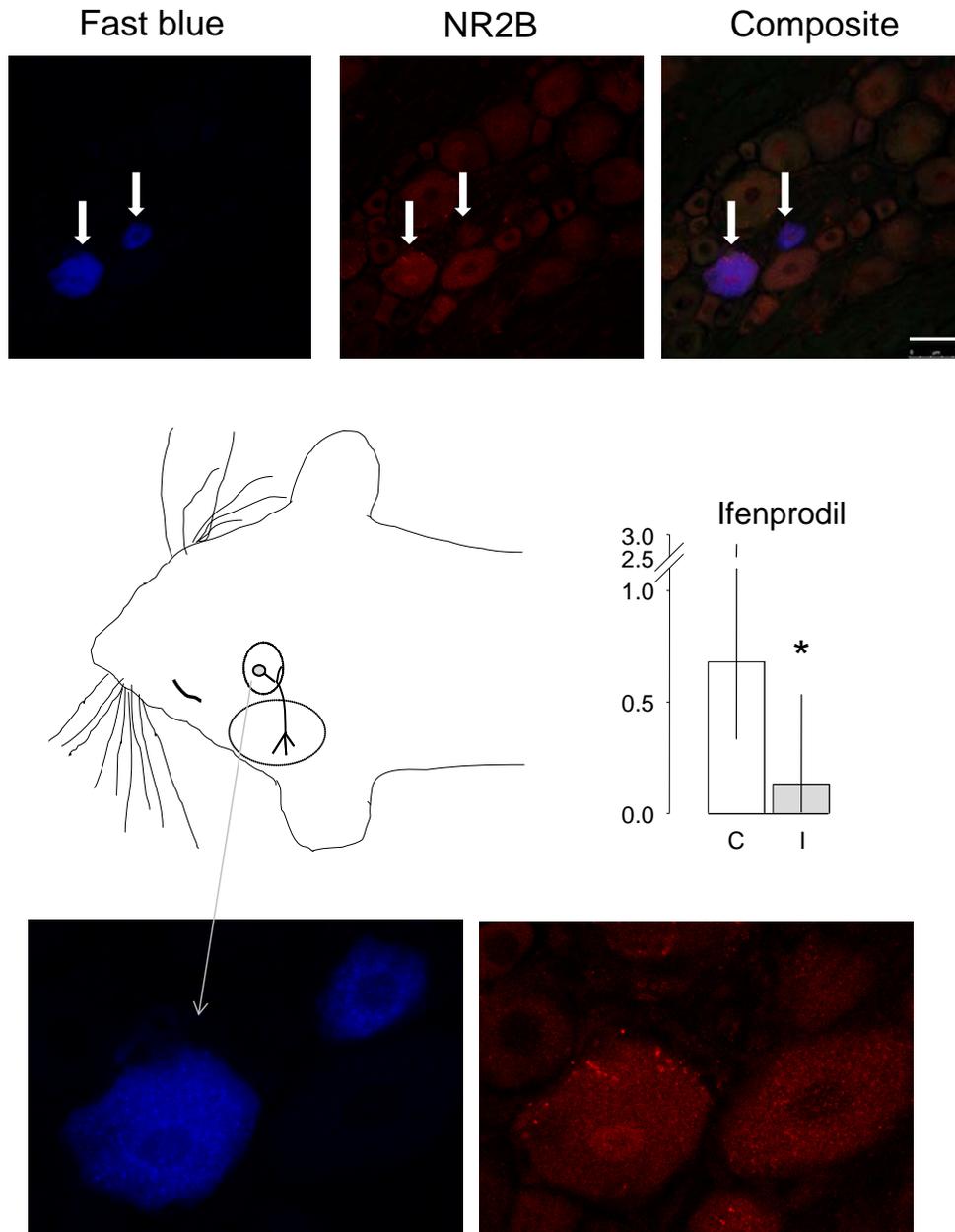
The high basal interstitial concentration of glutamate in the masseter muscle (~ 25 µM) in vivo may, in part, explain why higher concentrations of exogenously applied glutamate appear to be required to excite and sensitize afferent fibers in muscle.

These studies also indicated that the excitatory effect of elevated interstitial glutamate concentrations on muscle afferent fibers is mediated primarily through activation of peripheral NRs, since glutamate-evoked discharge and mechanical sensitization of muscle afferent fibers could be completely attenuated by local or systemic administration of NR antagonists (2, 5, 6, 10) (Figure 3C).

## **Muscle Nociceptors Express NRs of the NR2B Subtype**

Injection of glutamate into the rat masseter muscle does not evoke discharge in all afferent fibers. To determine how common the expression of the NR is in muscle afferent fibers, and whether certain NR subtypes are more common than others, immunohistochemical examination of trigeminal ganglion neurons that innervate the masseter muscle was carried out. Trigeminal ganglion neurons that innervate the masticatory muscle have a diameter between 3-70  $\mu\text{m}$  (7, 83). Roughly 10- 20% of trigeminal ganglion neurons that project to the masseter muscle expressed the NR2A subunit, while about 40-50% of ganglion neurons expressed the NR2B subunit **(vii, xiii)** (7, 13, 83). In the masseter muscle, PGP 9.5 was used to identify nerve axons and the expression of the neuropeptides CGRP or substance P to identify sensory afferent fibers that innervate the muscle **(x)** (10). In the muscle, it was found that ~ 50% of sensory afferent fibers expressed the NR2B subunit, consistent with its expression by trigeminal ganglion neurons that innervate the masseter muscle **(x)** (10).

Injection of NMDA into the masseter muscle also evokes afferent discharge and induces a period of afferent mechanical sensitization. Ifenprodil is a non-competitive antagonist selective for NR2B subunit containing NRs. It was found that local administration of ifenprodil could significantly attenuate, but did not completely block NMDA-evoked afferent discharge **(vii)** (7) (Figure 5). This suggests that in many nociceptors, it is activation of NR2B subunit containing NRs that mediates the effects of glutamate. Thus, the majority of the peripheral NRs expressed by masseter muscle afferent fibers contain the NR2B subunit with fewer that express the NR2A subunit.



**Figure 5:** The images show two trigeminal ganglion neurons labeled by injection of fast blue dye into the temporalis muscle (arrows). The larger of the two neurons expressed the NR2B subunit (magnified images at bottom). The bar chart shows how ifenprodil, an NR2B selective NR antagonist, significantly attenuated afferent discharge evoked by NMDA.

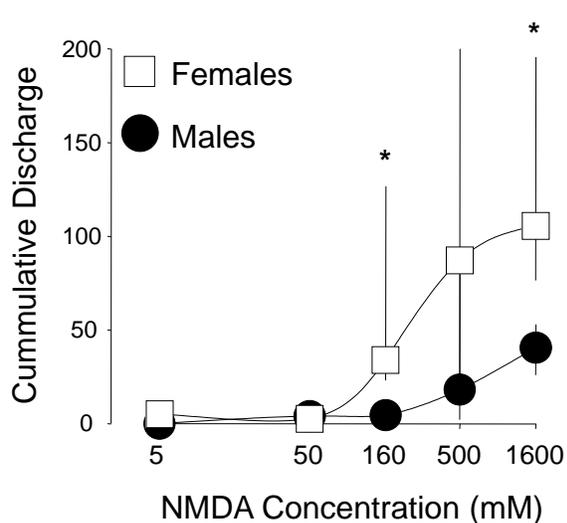
Afferent fibers that innervate the viscera also appear to express mostly NR2B subunit containing NRs, which is consistent with findings in the muscle (93). These findings suggest that NR2B selective antagonists could be effective in attenuating pain and sensitivity produced by elevated tissue concentrations of glutamate.

### **Response to glutamate is sexually dimorphic**

Masticatory muscle pain and tenderness is one of the most common symptoms reported by sufferers of chronic craniofacial pain conditions, such as temporomandibular disorders and migraine headache (94). Interestingly, about twice as many women as men suffer from these pain conditions, although the reasons for this disparity remain enigmatic (94). Although at the time most researchers were only using male rats in pain studies, both male and female rats were used to examine the effect of elevated glutamate levels on nociceptive input from the masticatory muscles. Injection of glutamate or NMDA into the masseter muscle evoked significantly greater afferent discharge in females than in males **(i, vii)** (1, 7). However, afferent discharges evoked by injection of glutamate or NMDA into the temporalis muscle were similar in male and female rats (89). There was no sex-related difference in glutamate-induced mechanical sensitization **(ii)** (2).

It had been found that female rats have greater nociceptive reflex responses in the jaw muscles to injection of glutamate into the temporomandibular joint than male rats (95). The increased reflex jaw muscle response of female rats to glutamate was eliminated by ovariectomy, but restored when ovariectomized female rats were treated with estrogen, but not progesterone (95). Nociceptor discharge was also greater in

females than in males, when either glutamate or NMDA was injected into the masseter muscle (Figure 6) **(i, ii, vii)** (1, 2, 7). In ovariectomized female rats, pre-treatment with estrogen to achieve levels similar to those seen at ovulation or during pregnancy, also significantly increased NMDA-evoked masseter muscle afferent discharges **(vii)** (7). This effect was associated with an increase in the expression of NR2B subunit containing NRs by trigeminal ganglion neurons that innervate the masseter muscle in female rats treated with estrogen **(vii)** (7). Subsequently, it has been found that both estrogen  $\alpha$  and  $\beta$  receptors are expressed by trigeminal ganglion neurons in female rats, and that more than 80% of NR2B expression masticatory muscle ganglion neurons also express estrogen receptors (83). This suggests that expression of NRs by masticatory muscle afferents is directly controlled by estrogen levels in female rats, and may explain why glutamate-evoked afferent discharge is sexually dimorphic.

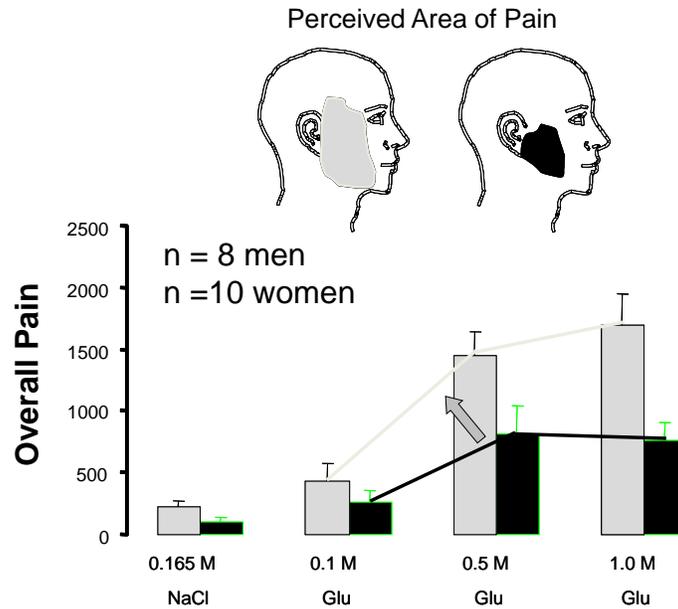


**Figure 6:** The semi-log line and scatter plot illustrates the concentration-response relationship for NMDA-evoked masseter nociceptor discharge in male and female rats. Nociceptors in male rats were less sensitive to NMDA than those in female rats. Data points indicate median and interquartile range. \* $p < 0.05$ , Mann-Whitney test.

## **Elevation of glutamate in the human masseter muscle is painful and induces mechanical sensitization**

To determine whether results obtained in rats were translatable into humans, human experimental pain studies where glutamate was injected into the masseter muscle of healthy human volunteers have been conducted (**i, iv, v**). In healthy subjects, injection of glutamate [100-1000 mM, 0.2 ml] into the masseter or temporalis muscles produces short-lasting muscle pain with a peak intensity of around 4-6/10 on the numeric rating scale, and a duration of about 5-10 minutes (**i, iv, v**) (1, 4, 5, 84, 86, 96-98) (Figure 7). Assessment of pain pressure threshold over the site of injection in the muscle revealed a significant decrease could be induced by high concentration (1 M) glutamate (4, 88). This glutamate-induced mechanical sensitization began within 15 minutes of injection and lasted for 90 minutes or longer in some individuals (**iv**) (4, 88). It was further noted that injection of glutamate (500 or 1000 mM, 0.2 ml) into the masseter muscle evoked significantly greater pain responses in women than in men (**i, iv**) (1, 4, 84). Glutamate-induced mechanical sensitization was similar in men and women (**iv**) (4).

Glutamate-evoked pain and glutamate-induced mechanical sensitization were attenuated by co-injection of the NR antagonist ketamine (10 mM) in men (**v**) (5, 88) (Figure 8). Interestingly, this same concentration of ketamine was found to be ineffective in attenuating glutamate evoked pain in a subsequent study in women (97). A more recent study found that a higher concentration of ketamine (20 mM) was needed to reduce glutamate-evoked muscle pain in healthy women (84).

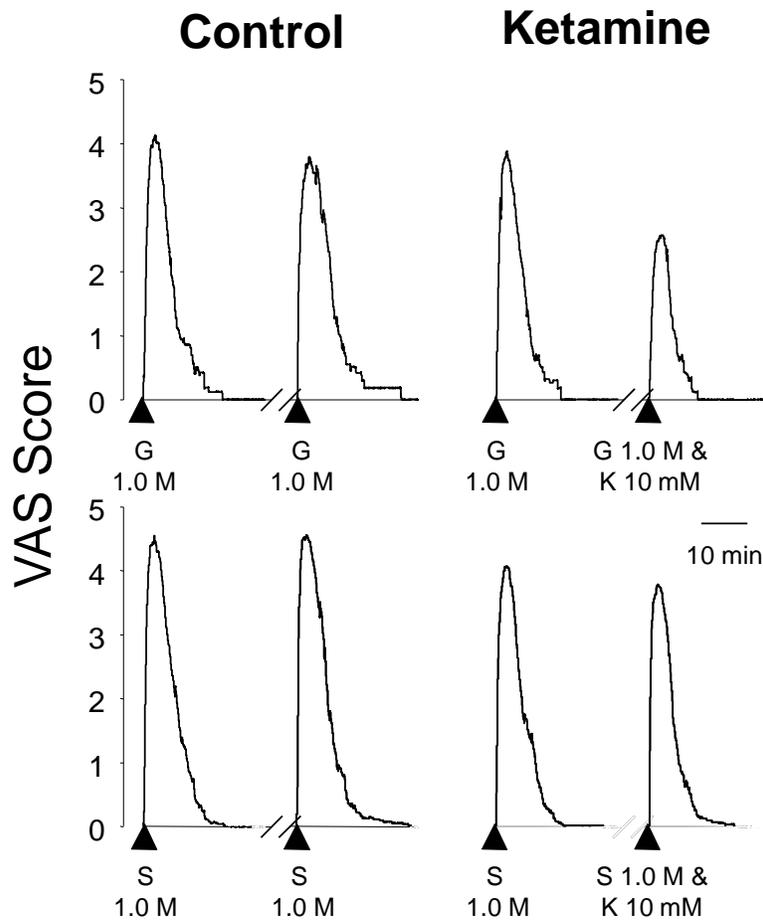


**Figure 7:** The concentration response relationship for glutamate-evoked masseter muscle pain in healthy men and women is illustrated. Injection of glutamate into the masseter muscle produced significantly greater pain and pain area drawings in women than in men.

Masseter muscle biopsies from healthy men and women were examined for expression of NR2B subunits and substance P by nerve fibers **(xiii)**. In healthy men and women, comparable basal expression levels of NR2B and SP were found in PGP9.5 identified nerve fibers (13). There was a tendency towards higher expression of NR2B by putative sensory nerve fibers (identified by their expression of substance P receptors) in women than in men (13).

These findings indicate that exogenous increases in masticatory muscle glutamate result in pain and mechanical sensitization, similar to that seen in rats. In humans, both glutamate evoked pain and mechanical sensitization could be attenuated

by ketamine, and NRs have been shown to be expressed by putative sensory fibers that innervate the human masseter muscle. Taken together, these findings are consistent with the idea that peripheral NRs are activated by elevated glutamate concentrations in human masticatory muscle to cause pain and mechanical sensitization.



**Figure 8:** The drawing shows mean electronically recorded visual analogue scale (VAS) responses from 14 healthy men given two injections of glutamate (G) or hypertonic saline with and without the NR antagonist ketamine (K). Injection of either G or S evoked reproducible pain responses. K significantly decreased responses to G but not S.

## Interactions between glutamate receptors and analgesic drugs

Infiltration of local anesthetics into inflamed tissue can result in a failed local anesthetic block. The reasons for this failure are complex, and may involve sensitization of afferent fibers as well as pharmacokinetic factors, which include tissue edema and increased blood flow. Glutamate is found in increased concentrations in inflamed tissues and, as has been discussed above, could contribute to sensitization of nociceptive afferent fibers that is characteristic of inflammation. A study was undertaken to determine if elevated tissue glutamate concentrations can shorten the duration of local anesthesia induced by injection of lidocaine into the rat masseter muscle (iii). Pre-injection of glutamate (1000 mM) was found to significantly decrease the mechanical threshold of masseter muscle nociceptors and shorten the duration of their block by lidocaine compared to isotonic saline (3). Glutamate-induced sensitization and glutamate-related shortening of the duration of lidocaine block were significantly attenuated when the broad-spectrum excitatory amino acid receptor antagonist kynurenate was co-injected with glutamate (3). Thus, activation of NRs and non-NRs on masseter nociceptors was contributing to the ability of high concentrations of glutamate to shorten lidocaine anesthetic blocks. Hypertonic dextrose, which has approximately the same osmotic strength as 1,000 mM glutamate, had no effect on the nociceptor mechanical threshold but did significantly shorten the duration of lidocaine block compared with isotonic saline. The duration of lidocaine block after glutamate was, however, significantly shorter than after hypertonic dextrose. These findings indicate that under conditions, such as inflammation, which lead to tissue concentrations of glutamate becoming sufficiently elevated to sensitize nociceptors,

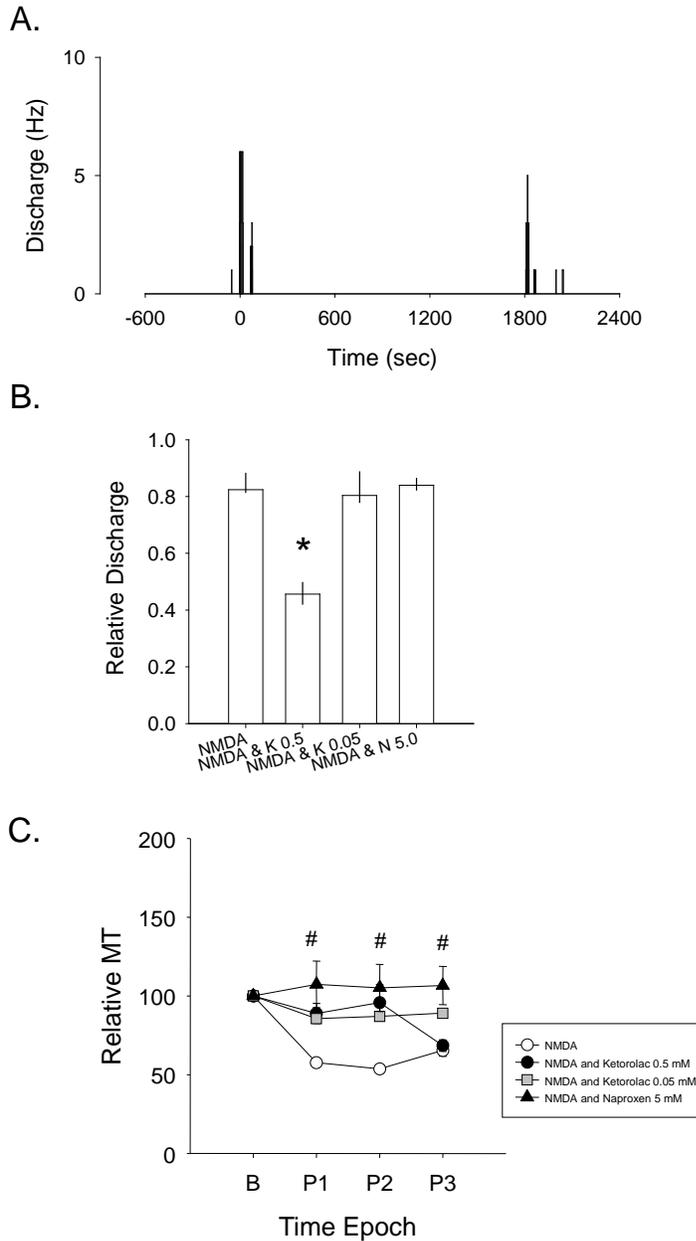
local anesthetic blocks will be shortened. It may be possible to avoid this effect by administering the local anesthetic with a glutamate receptor antagonist, such as kynurenate or ketamine.

Botulinum neurotoxin A (BoNTA) is used to decrease the frequency of migraine headaches. BoNTA is injected into several craniofacial muscles that include the temporalis muscle about every 3 months. It remains unclear how BoNTA decreases the frequency of migraine headaches, however, there is evidence that BoNTA may be effective in other pain conditions, such as neuropathic pain, which suggests that it is capable of exerting a local analgesic action. To investigate this idea, the effect of BoNTA on the mechanical and chemical responsiveness of individual temporalis muscle nociceptors and muscle neurogenic vasodilation was undertaken in female rats (x) (10). Three hours after injection of BoNTA into the temporalis muscle, there was a significant increase in the mechanical threshold of muscle nociceptors. BoNTA treatment also prevented injection of glutamate from inducing mechanical sensitization or neurogenic vasodilation. These effects of BoNTA did not appear due to muscle paralysis, since systemic administration of the neuromuscular blocker pancuronium had no effect on nociceptor mechanical threshold or on the ability of glutamate to induce mechanical sensitization or neurogenic vasodilation. Measurement of interstitial glutamate concentration with a glutamate biosensor indicated that BoNTA significantly reduced glutamate concentrations in the temporalis muscle over the same time frame as mechanical sensitivity changes (10). These findings suggest that injection of BoNTA into craniofacial muscles decreases the mechanical sensitivity of temporalis muscle nociceptors through inhibition of glutamate release.

Topical non-steroidal anti-inflammatory drugs (NSAIDs) containing diclofenac are not only effective analgesics in inflammatory pain conditions like arthritis, but also appear to be useful in reducing muscle pain in conditions that are not associated with frank inflammation, such as myofascial temporomandibular disorders (94). The analgesic effect of NSAIDs is thought to be due to their ability to inhibit prostaglandin synthesis, which is significantly increased by tissue inflammation. Topical application of NSAIDs results in local tissue concentrations that greatly exceed those achieved by systemic administration of these drugs. This may mean that additional mechanisms contribute to their analgesic actions. Indeed, the analgesic effect of topical diclofenac in non-inflammatory pain conditions, such as temporomandibular disorders (94), where inflammatory mediators would not be expected to contribute as greatly to pain, suggests that other mechanisms contribute to the analgesic efficacy of this NSAID when used topically.

Human experimental pain research suggests that elevation of serotonin (5-HT) and/or glutamate concentrations in skeletal muscle tissue is associated with muscle pain and sensitization (11, 99). An in vivo study in anesthetized rats was conducted to determine if diclofenac, an NSAID commonly used in topical analgesic products for muscle pain, might also be able to affect 5-HT<sub>3</sub> and/or NMDA or non-NMDA glutamate receptors as part of its analgesic mechanism **(ix)** (9). Diclofenac had no effect of 5-HT or AMPA (GluR selective agonist) mediated temporalis and masseter muscle nociceptor discharges, however, it significantly attenuated NMDA-evoked nociceptor discharges from this muscle (9). Diclofenac appeared to exert a competitive antagonism of the NR,

as its inhibitory effects could be overcome by increasing the concentration of NMDA injected into the muscle. Diclofenac also completely inhibited NMDA-induced mechanical sensitization of masseter muscle nociceptors (9).



**Figure 9:** **A.** The peristimulus histogram illustrates NMDA-evoked afferent discharge. **B.** The vertical bar chart compares the median (lines: interquartile range) relative cumulative discharge evoked by repeated injection of NMDA alone or when ketorolac (K0.5 = 0.5mM, K0.05 = 0.05mM) or naproxen (N = 5mM) were added to the second injection of NMDA (n=5) \*:p < 0.05, Kruskal-Wallis One Way ANOVA on Ranks, Dunn's post-hoc test. **C.** The line and scatter plot shows mean ( $\pm$ SE) relative MT. # p < 0.05, two-way repeated measures ANOVA and Holm-Sidak post hoc test.

Subsequent work was undertaken to determine whether the NR antagonism was a unique property of diclofenac, or if other NSAIDs might share this property **(xii)** (12). It was found that ketorolac, an injectable NSAID, also attenuates NMDA-evoked rat masseter muscle nociceptor discharges (Figure 9). Ketorolac had no significant effect on masticatory muscle nociceptor discharge evoked by  $\alpha\beta$ -methylene ATP, AMPA, or 5-HT, which suggests that it had no effect on the  $P_2X_3$ , GluR or 5-HT<sub>3</sub> receptors (12). In contrast, naproxen, another commonly used NSAID analgesic, had no effect on NMDA-evoked nociceptor discharges (12). It was suggested that internal structures of diclofenac and ketorolac, which resemble glutamate and NMDA, respectively, are responsible for the ability of these NSAIDs to block peripheral NRs (12). This feature may add to the effectiveness of these compounds when used as topical analgesics, particularly for non-inflammatory pain conditions such as myofascial TMD.

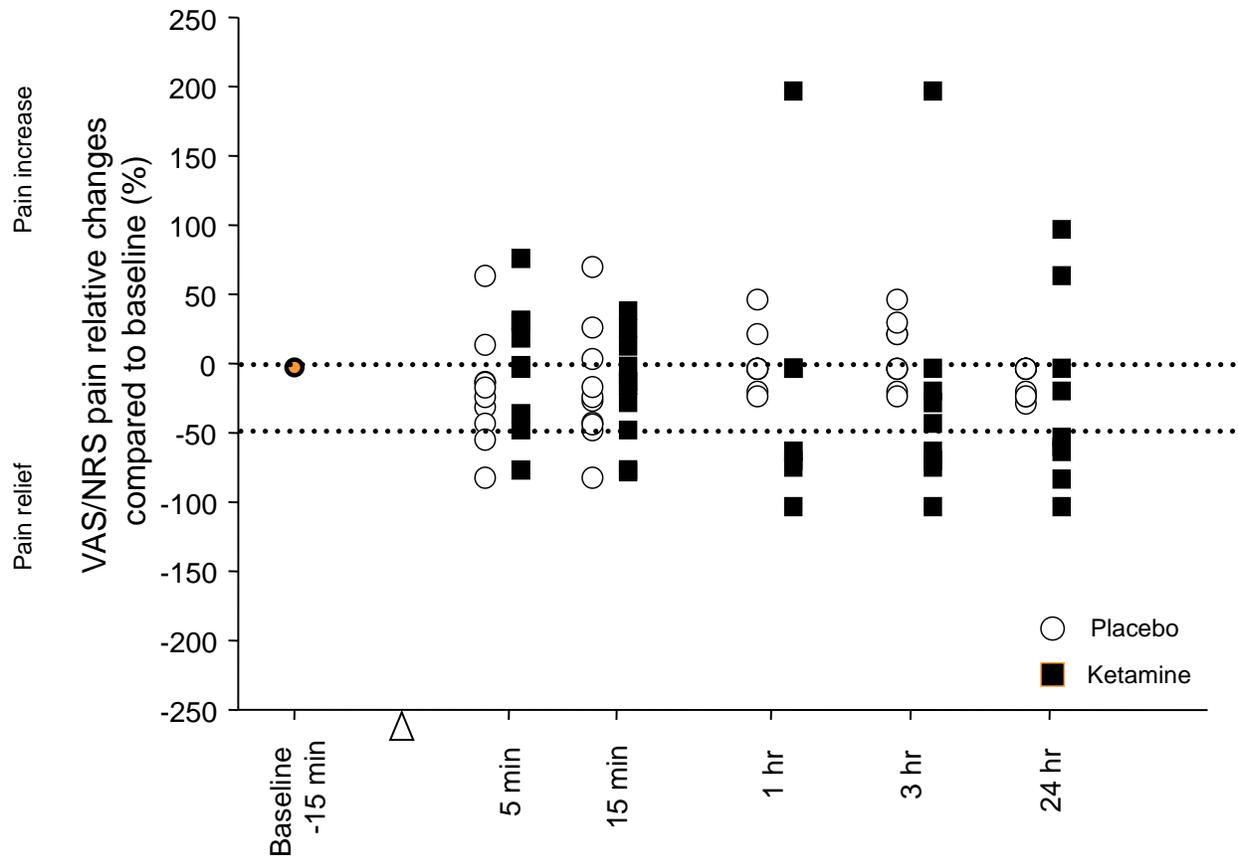
### **Role of glutamate in chronic masticatory muscle pain conditions**

The extent to which changes in extracellular glutamate contribute to pain and sensitivity in the craniofacial region remains uncertain. One of the most common chronic pain conditions that involve the masticatory muscles is myofascial TMD. This condition is characterized by ongoing pain in the masseter and/or temporalis muscles, that is often exacerbated by activity, such as eating, speaking and yawning, and can be often be provoked by palpation of specific points of the affected muscle(s) (94). To ascertain whether activation of peripheral NRs might contribute to muscle pain in this condition, 14 patients (10 female, 4 male) with myofascial TMD were recruited for a randomized double blind crossover study to compare the effect of injection of ketamine

(10 mM, 0.2 ml) and saline (0.2 ml) into the most painful point in the masseter muscle on ongoing and provoked pain **(viii)** (8). The concentration of ketamine employed was based on results discussed previously, which indicated that a concentration of 10 mM ketamine significantly attenuated glutamate-evoked masseter muscle pain in healthy humans and nociceptor discharges in rats (5). Patients were asked to rate their pain on a 0-10 numeric rating scale and were assessed for pressure pain threshold over the injection site just prior to and at regular intervals (10, 15, 60, 180 min, 24 hours) after injection of ketamine or saline. A decrease in pain rating of 50% or more was considered clinically meaningful. One hour after injection, 4 women, and at 3 hours (and 24 hours) 5 women of the 10, respectively, reported a more than 50% decline in their pain intensity. This decrease in pain was maintained for 24 hours. Unfortunately, two subjects reported a substantial increase in pain at various times after ketamine injection. In contrast, none of the 4 men in the study reported a similar decrease 50% decrease in pain intensity after ketamine. Injection of saline did not improve pain ratings in any of the subjects for more than 15 min post injection. There was no overall effect of ketamine injections of pressure pain threshold (8).

This study found that in half of the female myofascial TMD patients, ketamine injection into the most painful part of the muscle was effective in providing a clinically meaningful reduction of pain for up to 24 hours (8). Unfortunately, this study was underpowered due to difficulties in recruiting patients given the invasive nature of the procedure, so it is unclear whether the results obtained would be applicable to a larger population. In addition, patients who did not respond to a single site injection of

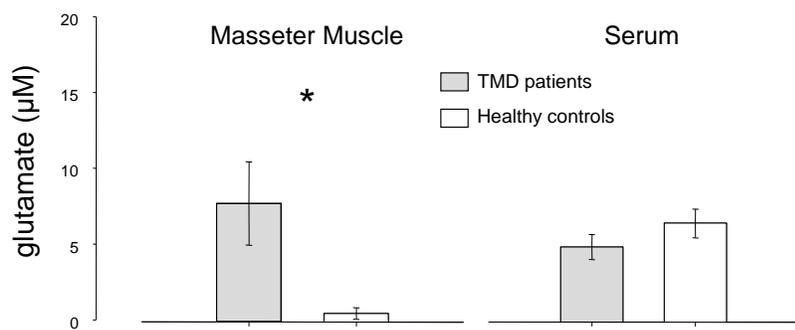
ketamine may have had more diffusely distributed pain in their muscles, making them less likely to respond to a single injection in a small area of the masseter muscle.



**Figure 10:** The result of intramuscular injection of ketamine into the most painful area of the masseter muscle of 10 female myofascial TMD patients on pain scores is show. One hour after injection, half the patients reported pain relief in excess of 50% (lower dotted line). Interestingly, 6 reported a greater than 50% reduction in pain 24 hours after the single injection of ketamine.

Finally, at the time the clinical trial of ketamine was conducted, it was unclear whether myofascial TMD patients have elevated masseter muscle interstitial glutamate concentrations. To try to answer that latter question, a microdialysis study was performed on myofascial TMD patients (xi).

Microdialysis probes were inserted into the masseter muscle of 13 (10 women, 3 men) myofascial TMD patients and 10 (8 women, 2 men) age-matched healthy controls and used to measure baseline interstitial glutamate concentrations (xi) (11). In TMD patients, the probe was inserted into the most painful spot in the muscle. The median concentration of glutamate in the masseter muscle was significantly greater in TMD patients than in the health control subjects. However, there was no significant relationship between glutamate concentration and pain intensity in TMD patients. In women, interstitial concentrations of glutamate in TMD patients were about 4-7 times higher than those in healthy controls (Figure 11). As discussed above, significant mechanical sensitization of muscle nociceptors has been demonstrated when interstitial glutamate concentrations in the masseter muscle are increased 2-3 times above their baseline (6). Serum glutamate concentrations were similar in patients and healthy control subjects (Figure 11), suggesting that the increased glutamate concentration measured in the masseter muscle was a local phenomenon (11). These findings support a potential role for glutamate in the mechanisms that result in muscle pain and sensitivity in myofascial TMD. The lack of relationship between glutamate concentration and pain intensity suggests that many other factors are likely important in determining the nature of pain from the masticatory muscles in this chronic pain condition.



**Figure 11:** The bar graph shows the concentration of glutamate measured in the interstitial fluid of the masseter muscle and serum.

## Summary and Conclusions

The research presented in this thesis indicates that elevated interstitial glutamate acts on NRs to alter the response properties of craniofacial muscle nociceptors. When interstitial concentrations of glutamate in the muscle are elevated within a “physiological range” ( $\leq 200 \mu\text{M}$ ), the principal effect of NR activation was found to be mechanical sensitization of the muscle nociceptor. However, when pharmacological doses of high concentration glutamate (0.1-1 M) are administered intramuscularly, these both evoke action potential discharge and sensitize the nociceptor, also through activation of the NR. Injection of 1 M glutamate into the masseter muscle produces local interstitial concentrations of glutamate that exceed 100 mM but decline below 10 mM in less than 10 minutes (100). These concentrations are in the range of the concentrations of glutamate estimated to be contained within synaptic vesicles. It is thought that vesicular release of glutamate can occur from the endings of sensory nerve fibers, including those that innervate skeletal muscle (10, 101). This suggests that local vesicular release of glutamate, such as may occur after depolarization of the peripheral ending of the afferent fiber, is better modelled by injection of a high concentration of glutamate into the muscle. However, afferent fibers that innervate the masseter muscle also contain the neuropeptides calcitonin gene related peptide and substance P (10, 13). Interestingly, although these neuropeptides are released by injection of high concentration glutamate into the muscle, they do not appear to contribute to glutamate-induced nociceptor mechanical sensitization (10). It seems that some other factor of NR activation contributes to the prolonged mechanical sensitization that is seen when

high concentrations of glutamate are injected into the masticatory muscles. One possibility is that since the NR is permeable to calcium, it is the elevated calcium levels within the nociceptor ending that, by activation of downstream cascade pathways, initiate and maintain glutamate-induced nociceptor mechanical. This concept has not yet been subjected to scientific investigation.

Results presented in this thesis indicate that the mechanical sensitivity of masticatory muscle nociceptors is modulated by the concentration of glutamate in the interstitium. Specifically, increasing interstitial glutamate concentrations by ~300% (to ~65  $\mu$ M) after systemic administration of MSG resulted in a decrease in the mechanical threshold of masseter muscle nociceptors by ~25%, while decreasing interstitial glutamate concentrations by ~75% by intramuscular injection of botulinum neurotoxin A, resulted in ~20% increase in nociceptor mechanical threshold (6, 10). As mentioned in the Introduction, glutamate concentration in the muscle and tendon has been shown to be increased in a number of chronic and acute muscle pain conditions, including myofascial temporomandibular disorders (11, 35-38). This leads to the question of whether modulation of glutamate concentration might be a means to control pain in conditions such as temporomandibular disorders. Glutamate transporters may play a significant role in the rapid clearance of glutamate from the muscle interstitial fluid. The glutamate transporter 1 (GLT-1) also known as the excitatory amino acid transporter 2 (EAAT2) has been reported to be expressed peripherally in tissues such as skeletal muscle and is thought to play an important role in clearance of glutamate (102, 103). A number of inhibitors and activators are available that modulate the activity of glutamate transporters (104). Kainic acid, and an analogue, dihydrokainic acid, have selectivity

for EAAT2, and dihydrokainic acid may be a useful probe to investigate the role of this transporter, as it is a poor activator of inotropic glutamate receptors, unlike kainic acid (104). In addition, a number of highly potent and selective EAAT2 inhibitors have been recently synthesized (104). In research looking at the role of glutamate and glutamate receptor activation in the trigeminal ganglion, it was reported that TFB/TBOA, a potent non-selective inhibitor of EAAT1-3, increases ganglion neuron action potential discharge evoked by intraganglionic injection of glutamate (105). Additional research is required to better understand the role of EAATs in the modulation of glutamate concentrations outside of the central nervous system. Future work may look at the possibility of enhancing transporter function and/or altering dietary intake of foods that may inhibit transporter function, as treatments for chronic craniofacial muscle pain.

Vesicular glutamate transporters (VGlut), which are required for the uptake of glutamate into synaptic vesicles, have also been identified in skeletal muscle tissue (40). Inhibitors include azo dyes such as Evans blue dye which binds with 90 nM affinity (106). Additional inhibitors of VGluts are being developed, and based on the finding that botulinum neurotoxin A, which acts to inhibit vesicular release by cleaving the docking protein SNAP 25, has a pronounced effect on nociceptor mechanical sensitivity in muscle (10), these agents may also be effective analgesics for musculoskeletal pain conditions that are associated with elevated tissue glutamate concentrations.

The non-steroidal anti-inflammatory drugs (NSAIDs) are extensively used as analgesics for pain conditions that involve the masticatory muscles (94). Unfortunately, NSAIDs have the potential to cause serious side effects that include gastrointestinal

ulceration, as well as altered renal function and blood pressure control. Application of topical NSAID containing creams and ointments offer the possibility directing therapy to the source of pain with lessened systemic exposure. Limited evidence suggests that topical may be as effective as oral diclofenac for the treatment of myofascial TMD-related masticatory muscle pain (94). However, topical NSAIDs achieve local tissue concentrations that are substantially higher than those achieved by systemic administration. The findings presented in this thesis indicate that at high concentration, both diclofenac and ketorolac have NR antagonist activity, and it is proposed that this effect enhances their analgesic effect when they are administered topically or injected locally. It has been determined that ketorolac, at a concentration that has been shown to exert both NR antagonist activity and local anesthetic like effects, significantly reduced pain produced by injection of hypertonic saline into the masseter muscle of healthy women (12, 107). It would be of interest to determine if lower concentrations of ketorolac or diclofenac are selective NR antagonists when injected into the human masseter muscle, as an unpublished pilot study conducted a few years ago suggested that ketorolac is very effective in reducing glutamate-evoked muscle pain in healthy subjects.

It now appears that one of the reasons for a lack of effect of ketamine in this study may have been that women are less sensitive than men to the local effects of ketamine (108). Despite this, the results of a clinical trial with ketamine were disappointing. Does this mean that peripheral glutamate receptors are not a good target for the development of useful peripherally-acting analgesic agents? It is important to consider that only one small clinical trial has been completed with ketamine. This, of

course, reflects an important limitation to studies of peripheral glutamate receptor function in humans, namely that there are very few glutamate receptor antagonists approved for use in human subjects, despite interest in the potential of these compounds for analgesia. Of the drugs that are currently available for use in humans, all have been designed for actions in the central nervous system, including the NSAIDs diclofenac and ketorolac. Indeed, one of the problems with using ketamine to investigate the role of peripheral NRs, is that the drug is rapidly cleared from skeletal muscle into the systemic circulation, and thus it is difficult in vivo to maintain for any length of time the elevated tissue concentrations that appear to be necessary to block peripheral NRs. Indeed, some positive results have been reported for local ketamine analgesia when it is delivered topically in an ointment, a situation where sustained elevated concentrations of ketamine in the skin may be maintained. Clearly, though, additional research that examines other NR receptor antagonists, particularly those with NR subunit selectivity, is required before the potential utility of peripherally-acting glutamate receptor antagonists for pain treatment will be well understood.

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