NEURAL MECHANISMS OF TEMPOROMANDIBULAR JOINT AND MASTICATORY MUSCLE PAIN

By

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Abstract

The underlying nociceptive mechanisms in temporomandibular joint (TMJ) and masticatory muscles in many pain conditions are still unclear, largely due to the limited study of peripheral and central neural mechanisms affecting craniofacial musculoskeletal tissues. This study provided evidence in support of **Hypothesis 1**: ***Peripheral glutamatergic and capsaicin-sensitive mechanisms modulate the properties of primary afferents and brainstem neurons processing deep craniofacial nociceptive information.*** Effects of glutamate and capsaicin injected into the receptive field of deep craniofacial nociceptive afferents or TMJ of TMJ-responsive nociceptive neurons in trigeminal subnucleus caudalis/upper cervical cord (Vc/UCC) were studied in halothane-anesthetized rats. When injected alone, glutamate and capsaicin activated and induced peripheral sensitization in many afferents. Following glutamate injection, capsaicin-evoked activity was greater than that evoked by capsaicin alone, whereas following capsaicin injection, glutamate-evoked responses were similar to those of glutamate alone. When injected alone, glutamate and capsaicin also activated and induced central sensitization in most Vc/UCC neurons. Following glutamate injection, capsaicin evoked greater activity and less sensitization compared with capsaicin alone, whereas following capsaicin, glutamate was less effective in activating and sensitizing most Vc/UCC neurons. This apparent desensitizing effect of capsaicin on glutamate-evoked excitability of Vc/UCC neurons contrasts with the lack of capsaicin-induced modulation of glutamate-evoked afferent excitability, suggesting that peripheral and central sensitization may be

differentially involved in the nociceptive effects of glutamate and capsaicin applied to deep craniofacial tissues. Further evidence of glutamate-capsaicin interactions was documented in the attenuation by TMJ pre-injection of glutamate receptor antagonists of jaw muscle activity reflexly evoked by TMJ injection of capsaicin. Moreover, additional findings support **Hypothesis 2**: ***Surgical cutaneous incision modulates the properties of brainstem neurons processing deep craniofacial nociceptive information.*** TMJ-responsive nociceptive Vc/UCC neurons could be activated by surgical incision of the skin overlying the TMJ and this incision-induced afferent barrage caused nociceptive neurons to be temporarily refractory to further capsaicin-induced central sensitization.

These novel findings suggest that peripheral glutamate and capsaicin receptor mechanisms as well as surgical cutaneous incision may be involved in the nociceptive processing of deep craniofacial afferent inputs and may interact to modulate both activation as well as sensitization evoked from these tissues.

"If I wished to show a student the difficulties of practice, I would give him a pain in the face to treat."

— Oliver Wendell Holmes

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Toronto, June 2008 ***David King Lam.***

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ABBREVIATIONS:

ASIC: acid-sensing ion channel
CAP: capsaicin
CV: conduction velocity
EAA: excitatory amino acid
GLU: glutamate
GPCR: G protein-coupled receptor
IAA: inhibitory amino acid
IQR: interquartile range
LTM: low-threshold-mechanosensitive
MAT: mechanical activation threshold
NMDA: *N*-methyl-*D*-aspartate
NS: nociceptive-specific
Pfreq: peak frequency
RF: mechanoreceptive field
Rmag: response magnitude
Rdur: response duration
Rlat: response latency
TMJ: temporomandibular joint
TRP: Transient receptor potential
TRPA1: Transient receptor potential cation channel, subfamily A, member 1,
TRPV1: Transient receptor potential vanilloid 1
TRPV4: Transient receptor potential vanilloid 4
V: trigeminal
VBSNC: trigeminal brainstem sensory nuclear complex
Vc/UCC: trigeminal subnucleus caudalis/upper cervical spinal cord region
WDR: wide dynamic range

Chapter 1

Introduction

1.0 INTRODUCTION:

Pain is a complex multidimensional perception that integrates sensory, affective, cognitive and motivational aspects. Chronic pain is particularly challenging given that it appears to serve no clear biological purpose, yet it may be associated with immense physical, mental, emotional and social stresses. A considerable proportion of the North American population– 10-15%– suffer from chronic craniofacial pain conditions at some point in their life. Some of these pain conditions especially involve musculoskeletal tissues and the most common of these is a condition known as temporomandibular disorders (TMD) (Carlsson and LeResche 1995; LeResche 2001; Von Korff and LeResche 2005). About 70% of TMD patients are women of reproductive age as compared with postmenopausal women or men (Carlsson and LeResche 1995). The principal signs and symptoms of TMD include pain in the temporomandibular joint (TMJ) and/or the masticatory muscles, joint sounds and neuromuscular dysfunction and limited jaw function. Pain may also spread and be referred to other craniofacial sites such as the neck, face, ear as well as the temporal, occipital and frontal areas of the head (for review, see Zarb *et al.* 1994; Carlsson and LeResche 1995; Stohler 1995,1999; Sessle 1999). The characteristic pain and limitations in jaw movements associated with TMD may result from sensitization states producing allodynia and hyperalgesia and altered jaw muscle reflex activities (for review, see Sessle 1999, 2005; Svensson and Sessle 2004). It has also been reported that TMD patients frequently have lowered thresholds to experimentally induced pain (Maixner *et al*. 1995, 1998; also see Svenssen and Sessle 2004). These findings suggest a role for both peripheral and central mechanisms in TMD-associated pain. Acute pain conditions can also be manifested in the TMJ and associated musculature (*e.g.,* osteoarthritis, myositis) and again sensitization phenomena are likely involved (for review, see Sessle 2000, 2005).

The underlying nociceptive mechanisms in muscles and joints in these acute and chronic conditions are still unclear, in large part due to the limited study of peripheral

and central neural mechanisms affecting craniofacial musculoskeletal tissues, and as a consequence, diagnosis and management have not been supported by an evidence-based approach. There is emerging evidence that peripheral excitatory amino acid (EAA) and transient receptor potential vanilloid 1 (TRPV1) receptor mechanisms may play a role in nociceptive processing of deep craniofacial tissues. The following literature review outlines what is known about the peripheral mechanisms of musculoskeletal pain including TMJ and masticatory muscle pain, what is known about their properties vis-à-vis peripheral EAA and TRPV1 receptor mechanisms, and their possible role in nociceptive processing.

1.1 PAIN:

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage (IASP Task Force on Taxonomy 1994). Pain may be acute or chronic. Acute pain is of recent onset that is usually transient in nature, lasting from several minutes to several days or weeks in duration. It is usually caused by tissue damage and is often associated with some degree of inflammation. Chronic pain persists beyond the usual course of an acute illness or injury (usually beyond 3 months). It is associated with a pattern of recurrence over months or years or associated with a chronic pathological process.

Most of our knowledge of pain mechanisms comes from studies in the spinal somatosensory system. The following briefly reviews some general features of the spinal somatosensory system and findings that suggest a role for peripheral and central sensitization in acute and chronic pain mechanisms, and a more detailed review is provided of the trigeminal (V) somatosensory system and pain mechanisms.

1.2 SPINAL PAIN MECHANISMS:

1.2.1 General Gross Anatomy

Somatosensory information from the limbs and trunk is conveyed by 31 pairs of dorsal root ganglion (DRG) neurons (spinal primary afferent fibres) that are labeled by the corresponding vertebral foramen through which the root enters the spinal cord (Gardner *et al.* 2000; Willis and Coggeshall 2004; Willis 2005). The cell body of a DRG neuron is found in a ganglion on the dorsal root of a spinal nerve. The axon has 2 branches, one projecting to the periphery and one projecting to the central nervous system (CNS). There are 7 cervical, 12 thoracic, 5 lumbar, and 5 sacral roots, which are numbered rostrally to caudally for each division of the vertebral column.

The area of skin innervated by nerve fibres comprising a DRG is known as a dermatome. Upon entry to the spinal cord, the central axons of DRG neurons project to nuclei in the spinal gray matter and brainstem. The spinal gray matter is divided into 3 functionally distinct regions: the dorsal horn, the intermediate zone, and the ventral horn. In addition, the spinal gray matter is also divided into 10 laminae. Laminae I-VI correspond to the dorsal horn, lamina VII is equivalent to the intermediate zone, and laminae VIII and IX comprise the ventral horn. Lamina X is composed of the gray matter that surrounds the central canal.

1.2.2 Primary Afferent Anatomy and Physiology

The properties of the peripheral nerve terminal determine the sensory function of each DRG neuron (Gardner *et al.* 2000; Willis and Coggeshall 2004; Willis 2005; Hucho and Levine 2007; Woolf and Ma 2007). The peripheral nerve terminals may be encapsulated by a non-neural structure or a bare nerve ending. DRG neurons with encapsulated terminals mediate touch and proprioception (*e.g.,* Meissner's corpuscle, Merkel's disk, Pacinian corpuscle and Ruffini ending) by sensing stimuli that indent or physically deform the receptive surface and transmit this information via rapidly conducting large-diameter, myelinated (Aα or Aβ) axons to the spinal cord. In contrast, DRG neurons with bare nerve endings mediate painful or thermal sensations via slow conducting small-diameter, unmyelinated (C fibre afferents) or thinly myelinated (Aδ fibre afferents) axons to the spinal cord.

Harmful stimuli to peripheral tissues may activate several classes of nociceptor terminals, the peripheral endings of primary afferents (Basbaum and Jessell 2000; Willis and Coggeshall 2004; Willis 2005; Hucho and Levine 2007; Woolf and Ma 2007). Each of these afferents may have several free nerve endings, and the area of peripheral tissue from which it can be activated by stimuli applied to that area is termed the receptive field (RF) of the afferent. In peripheral tissues, many of the non-specialized endings provide the peripheral basis for pain since they act as nociceptors, that is, they are the sense organs that respond to noxious stimulation of peripheral tissues. The peripheral terminal is the site where noxious stimuli are detected and transduced. Transduction is mediated by high-threshold transducer ion channels which depolarize the peripheral terminal activating voltage-dependent Na^+ channels. The nociceptor specificity is determined by its expression of ion channels tuned to respond with a high threshold only to particular features of the mechanical, thermal, and chemical environment (Ramsey *et al.* 2006; Woolf and Ma 2007). A number of nociceptor transducers have been identified over the past decade and include transient receptor potentional (TRP) channels, acid-sensing ion channels (ASICs), and K^+ and ligand-gated ion channels (for review, see Hucho and Levine 2007; Woolf and Ma 2007). In addition to high-threshold ion channels, nociceptive transduction is also influenced by a major coupling between G-protein-coupled receptors (GPCRs) and ion channels, such as some TRP channels, in the membrane (Woolf and Ma 2007). For example, TRPA1 acts as a receptor-operated channel for bradykinin, generating Ca^{2+} influx in response to B2 receptor activation (Bautista *et al.* 2006). This ion channel-GPCR coupling may also exist with other inflammatory mediators/chemokines both with TRPA1 and other TRP channels and provides a means for GPCRs and receptor tyrosine kinases to depolarize the nociceptor terminal. As a result, although the fundamental role of nociceptors is to detect potentially tissue-damaging stimuli, when damage has already taken place, the release of bradykinin and other ligands coupled to high-threshold ion channels when may also provide a means of alerting the individual of tissue injury.

Nociceptor activation may then result in the excitation of the small-diameter Aδ and C fibre afferents with which they are associated. Once excited, the nociceptive afferent conducts impulses (*i.e.,* action potentials) into the CNS and thereby can provide

sensory-discriminative information to the brain about the location, quality, intensity and duration of the noxious stimulus. There are 3 general classes of nociceptors- thermal, mechanical, and polymodal (Burgess and Perl 1973; Price and Dubner 1977; Price *et al.* 1977). Thermal nociceptors are small-diameter, thinly myelinated Aδ fibres that are activated by extreme temperatures (*i.e.* > 45 ˚C or < 5˚C). Mechanical nociceptors are small-diameter, thinly myelinated Aδ fibres that are activated by intense pressure. Polymodal nociceptors are small-diameter, thinly myelinated Aδ fibres or non-myelinated C fibres that are activated by high-intensity mechanical, chemical, or thermal stimuli.

1.2.3 Central Organization of the Spinal System

Upon entry to the spinal cord, the central axons of DRG neurons branch extensively and project to various nuclei in the spinal gray matter (Basbaum and Jessell 2000; Gardner *et al.* 2000; Willis and Coggeshall 2004; Willis 2005). The DRG neurons mediating touch and proprioception diverge from those for pain and temperature sense. Large-diameter fibres ascend in ipsilateral dorsal columns and many terminate in brainstem cuneate and gracile nuclei, whereas small-diameter nociceptive afferent fibres as well as some large-diameter fibres terminate on second-order neurons in the dorsal horn.

Nociceptive neurons are located in the marginal layer (lamina I) and the substantia gelatinosa (lamina II) of the superficial dorsal horn (Price and Dubner 1977; Price *et al.* 1977; 2003; Basbaum and Jessell 2000). The majority of these neurons receive direct converging input from Aδ and C fibres. Many of the neurons residing in lamina I respond exclusively to noxious stimulation (nociceptive-specific or NS neurons) and project to higher brain centres, whereas some neurons in this layer, called wide-dynamic-range (WDR) neurons, respond in a graded fashion to both non-noxious and noxious stimulation. Lamina II is comprised almost exclusively of interneurons, some of which respond only to nociceptive inputs while others also respond to non-noxious inputs. Laminae III and IV especially contain neurons (low-threshold-mechanosensitive, LTM) that receive direct input from low-threshold fibres and respond to non-noxious stimuli (Gardner *et al.* 2000; Willis and Coggeshall 2004). Lamina V contains primarily WDR

and some NS neurons that project to the brainstem and to regions of the thalamus (Price and Dubner 1977; Price *et al.* 1977; 2003; Basbaum and Jessell 2000). These WDR neurons receive direct input from Aβ and Aδ fibres and also C fibre input, either directly from their dendritic extensions into lamina I/II or indirectly via excitatory interneurons that themselves receive direct C fibre input. Neurons in lamina VI receive converging non-noxious inputs from large-diameter afferents from muscles and joints. Finally, many neurons in laminae VII and VIII respond to noxious stimuli and have more complex response properties since nociceptive inputs to lamina VII neurons are polysynaptic.

1.2.4 Spinal nociceptive processing associated with deep tissue pain

Deep tissue pain is a prominent feature of a variety of disorders, including arthritis, myofascial pain, or fibromyalgia (Marchettini 1993; Mense 1993; Mense *et al.* 2000; Moldofsky and Merskey 2005; Robinson *et al*. 2005; Graven-Nielsen *et al*. 2006). The quality of pain associated with muscle or joint injury usually differs from that associated with skin injury. Injury to deep structures results in diffuse, difficult-to-localize, aching pain (Kellgren 1938; Simone *et al.* 1994; Mense *et al*. 2000; Robinson *et al.* 2005; Graven-Nielsen *et al*. 2006), whereas injury to skin produces well-localized, sharp, stabbing, or burning pain (Ochoa and Torebjork 1983; Gardner *et al.* 2000; Mense *et al.* 2000; Chang *et al.* 2004; Graven-Nielsen *et al*. 2006). In addition to differences in quality, pain associated with injury to skin may also differ in duration and radiation from pain associated with injury to deeper tissues such as muscle (Cook *et al.* 1987; Woolf and Wall 1986; Graven-Nielsen *et al*. 2006). In human subjects, intramuscular stimulation is rated as more painful than cutaneous stimulation (Svensson *et al.* 1997; Nie *et al*. 2005), the pain is longer lasting, and the evoked referred pain is more common (Witting *et al.* 2000b; Arendt-Nielsen and Svensson 2001; Graven-Nielsen and Mense 2001; Chang *et al.* 2004). Patients with fibromyalgia or knee osteoarthritis experience more pain and a larger and more diffuse area of referred pain after hypertonic saline infusion into the tibialis anterior muscle compared with normal subjects (Graven-Nielsen and Arendt-Nielsen 2002). Similarly, chronic whiplash pain patients

and chronic low-back pain patients have an extended area of referred pain after hypertonic saline infusion into the infraspinatus muscle or a distal leg muscle (tibialis anterior), suggesting changes in central processing (Graven-Nielsen and Arendt-Nielsen 2002; O'Neill *et al.* 2006).

Virtually all tissues, including deep structures, contain free nerve terminals associated with primary afferents conducting in the Aδ (myelinated, or group III) and C fibre (unmyelinated, or group IV) range. Bove and Light (1995) studied group IV afferent fibres by pressing through the skin, then dissecting the tissue to expose the specific structures innervated by the afferent fibre and found that the RF of these fibres resided in deep skin layers, fascia, muscle, tendon, periosteum and synovium. Marchettini and colleagues (1996) and Simone and colleagues (1997) studied group III and IV muscle nociceptive afferent fibres in the human common peroneal nerve and found that these afferent fibres lacked spontaneous activity and were excited by application of both noxious pressure and the inflammatory irritant capsaicin. The primary afferents supplying muscles and joints have been characterized primarily in the spinal system under both normal and inflamed conditions. Normally, these group III and IV afferents have very high mechanical thresholds that are well above their normal operating range (for review, see Mense 1993, Schaible and Grubb 1993; Ge and Khalsa 2003; Graven-Nielsen *et al.* 2004). However, following inflammation, these deep nociceptive afferents, termed 'silent nociceptors', display reductions in mechanical thresholds and can readily respond to mechanical stimuli (for review, see Mense 1993, Schaible and Grubb 1993; Schmidt *et al.* 2000; Serra *et al.* 2004; Willis 2005).

Animal ankle or knee joint inflammatory models (*e.g.,* injected with Freund's complete adjuvant or kaolin/carrageenan) have been developed to study neurotransmitter mechanisms underlying peripheral and central nociceptive processing (for review, see Schaible and Grubb 1993; Kidd *et al.* 1996; Schaible and Schmidt 1996; Ebersberger *et al.* 1999; Segond von Banchet *et al.* 2000; Schaible *et al.* 2002; Bar *et al.* 2004). When kaolin/carrageenan is injected into the knee joint, awake animals show signs of spontaneous pain and increased sensitivity to evoked pain at the site of injury (primary hyperalgesia) as well as at sites distal to the injury (secondary hyperalgesia) (Tonussi and Ferreira 1992; Sluka and Westlund 1993b; Schott *et al.* 1994; Min *et al.*

2001; Han *et al.* 2005). The primary and secondary hyperalgesia seen in the acute phase in these studies can be explained by peripheral sensitization and subsequent central sensitization caused by carrageenan-induced inflammation. After kaolin/carrageenan inflammation of the knee joint, there is an increased responsiveness of dorsal horn neurons, including spinothalamic tract neurons, to innocuous and noxious mechanical stimuli applied to the skin or joint as well as an increase in the cutaneous and/or deep RF size in rats, cats, and monkeys (Schaible *et al.* 1987, 2002; Neugebauer and Schaible 1990; Dougherty and Willis 1992; Bar *et al.* 2004).

In animal models of ischaemic muscle pain, certain group IV afferents are activated preferentially following limb ischaemic injury induced by an interruption of the muscle blood supply (Mense and Stahnke 1983; Mense 1993; Graven-Nielsen and Mense 2001; Graven-Nielsen *et al*. 2006). Peripheral phenomena related to the release and spread of neurochemical substances seem to be the primary basis for the excitation and sensitization of nociceptive spinal primary afferents and to be important in the spread of pain and inflammation (for review, see Handwerker 1991; LaMotte 1992; Willis 1993; Levine and Taiwo 1994; Meyer *et al*. 1994; Schmidt *et al.* 1994; Hargreaves *et al.* 1995; Graven-Nielsen and Mense 2001; Graven-Nielsen and Arendt-Nielsen 2003; Dray 2005). However, recent studies in which peripheral phenomena have been experimentally bypassed have shown inflammation or injury-induced RF expansions and heightened neuronal excitability in spinal nociceptive pathways that have been explained by unmasking or strengthening of the convergent afferent inputs to the central neurons that results in the expression of central neuroplasticity, so-called “central sensitization” (for review, see Dubner and Basbaum 1994; Woolf 1994; Coderre and Katz 1997; Willis 2002; Graven-Nielsen *et al*. 2006). Indeed, animal studies have demonstrated the sensitization of spinal dorsal horn neurons following noxious stimulation of muscles (Mense *et al.* 2000; Robinson *et al.* 2005; Graven-Nielsen *et al*. 2006).

The animal models above have suggested that a number of chemicals including bradykinin, histamine, serotonin and prostaglandins may be responsible for the increased sensitivity of primary afferent fibres (“primary hyperalgesia” or “peripheral sensitization”) under these experimental myositic or arthritic joint conditions (Hoheisel

and Mense 1989, Mense 1993, 1996, Schaible and Grubb 1993, Schaible and Schmidt 1996; Graven-Nielsen and Mense 2001; Graven-Nielsen and Arendt-Nielsen 2003; Graven-Nielsen *et al*. 2006). The EAAs, glutamate and aspartate, and their receptors, N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-5-isoxazolepropionate (AMPA), kainate (KA) and metabotropic receptors are key players in the increased sensitivity of spinal neurons ("secondary hyperalgesia" or "central sensitization") associated with deep tissue inflammation. An increased release of glutamate and aspartate, measured by microdialysis, occurs in the dorsal horn in monkeys and rats after the induction of inflammation in the knee with kaolin/carrageenan (Sluka and Westlund 1992, 1993a; Sorkin *et al*. 1992; Yang *et al.* 1996). Similarly, sensitization of dorsal horn neurons is also reduced by blockade of NMDA and non-NMDA glutamate receptors (Neugebauer *et al.* 1994; Neugebauer 2001, 2002). Thus, increased release of glutamate and aspartate activates glutamate receptors to produce central sensitization and the subsequent secondary hyperalgesia.

The following looks in more detail at these sensitization phenomena.

1.3 SENSITIZATION:

Although acute pain is typically transient, chronic pain associated either with peripheral tissue damage and inflammation or a nervous system lesion is often characterized by persistent pain hypersensitivity. This includes spontaneous pain (pain experienced in the absence of any peripheral stimulus), hyperalgesia (an increased response to noxious stimuli) and allodynia (pain in response to normally innocuous stimuli). There are two principal mechanisms that may contribute to most acute and chronic pain states: *peripheral sensitization and central sensitization.*

1.3.1 Peripheral sensitization

At the site of tissue injury or inflammation, mediators released from cell injury, degranulation of mast cells, secretion by inflammatory cells, and induction of enzymes like cyclooxygenase-2 (COX-2), sensitize nociceptors and result in a change in state

from being exclusively noxious stimulus detectors to detectors also of innocuous inputs. Various sensitizers including kinins, amines, prostanoids such as prostaglandin E_2 (PGE_2), growth factors such as nerve growth factor (NGF), chemokines, and cytokines, which with protons and ATP make up an "inflammatory soup" that sensitizes the nociceptor terminal. Recent additions to the inflammatory soup have been identified, including the TGFβ member activin, TNFα, the chemokine CCL3, prokineticins, proteases that activate protease-activated GPCRs, and GDNF (for review, see Hucho and Levine 2007; Woolf and Ma 2007). These chemicals act on GPCRs or tyrosine kinase receptors expressed on both spinal and V nociceptor terminals and increase the sensitivity and excitability of the nociceptor terminal – a phenomenon known as peripheral sensitization (Julius and Basbaum 2001; Dray 2005; Sessle 2005; Hucho and Levine 2007; Woolf and Ma 2007).

The above nociceptor sensitizers produce their effects following binding to membrane receptors by activation of multiple intracellular signal transduction pathways in the peripheral terminal that include Ca^{2+}/phospholipid-dependent kinase (PKC), cAMP-dependent protein kinase (PKA), PI3K, and the MAP kinases ERK and p38 (for review, see Hucho and Levine 2007; Woolf and Ma 2007). Downstream of these cascades, the effectors of peripheral sensitization are mainly phosphorylation of TRP and voltage-gated Na^+ channels, altering threshold and kinetics. These processes can lower the activation threshold of nociceptors to below physiological threshold and induce sustained afferent activity. For example, PGE_2 and bradykinin cause changes in TRPV1 via activation of PKA and PKC, such that the receptor can be activated by lower temperatures (< 40 °C) (Chuang *et al.* 2001; Sikand and Premkumar 2007). In addition to the acute modification of ion channels, organelles and cytoskeleton, as well as subcellular compartmentalization, are thought to be important components underlying peripheral sensitization; the surrounding environment such as sex hormones, the extracellular matrix, and neighboring cells may also play a critical role in nociceptor signaling (Hucho and Levine 2007). Afferent excitability can also be modulated by changes in the synthesis of various receptors, ion channels, and enzymes which alters gene transcription and sensory cell phenotype (Woolf and Salter 2000; Dray 2005; Hucho and Levine 2007; Woolf and Ma 2007).

Peripheral sensitization produces increases in pain sensitivity that is restricted to the site of inflammation and may manifest clinically as pain-at-rest (spontaneous afferent activity), allodynia (lowered afferent activation threshold), or primary hyperalgesia (increased afferent responsiveness to suprathreshold stimuli). A good example is heat pain sensitivity after sunburn, when a normally warm shower feels burning hot, or the increased sensitivity of a tooth with acute pulpitis.

1.3.2. Central sensitization

Central sensitization is an activity-dependent increase in the excitability of nociceptive neurons in the spinal dorsal horn or V medullary dorsal horn (Woolf 1983; Woolf and Wall 1986; Cook *et al*. 1987; Dubner 2005; Sessle 2005; Graven-Nielsen *et al*. 2006). This activity-dependent central sensitization is normally initiated only by nociceptor sensory input and results in increases in spontaneous activity, activation threshold reduction, increases in responsiveness, as well as RF enlargement of spinal dorsal horn (and V medullary dorsal horn) neurons (Cook *et al.* 1987; Cervero *et al.* 2003; Dubner 2005; Sessle 2005). Low-threshold sensory fibres activated by innocuous stimuli such as light touch can now activate normally high-threshold nociceptive neurons, contributing to a reduction in pain threshold (tactile allodynia) that is the consequence of an increased excitability of CNS neurons. This activity-dependent central facilitation manifests within seconds of an appropriate nociceptive conditioning stimulus and can outlast the stimulus for several hours (Woolf and Wall 1986; Graven-Nielsen *et al*. 2006; Treede *et al.* 2006). Activity-dependent sensitization is extremely robust and has been reported in rodent, cat and primate spinal and V neurons, including spinothalamic neurons (Kenshalo *et al.* 1982; Hylden *et al.* 1989; Schaible *et al.* 1991a; Simone *et al.* 1991; Dougherty and Willis 1992; Willis 2002; Sessle 2005). Central sensitization appears to involve excitation at receptor sites activated by EAAs and neuropeptides such as substance P (SP) and brain-derived neurotrophic factor (BDNF) (Woolf and Salter 2000; Dubner 2005). With persistent injury, the continual release of glutamate and SP from central endings of nociceptive afferents activates GPCRs that result in the activation of second messenger systems and consequent release of intracellular calcium. The initiation of synaptic depolarization

removes the voltage-dependent magnesium block of the NMDA receptor, and glutamate release results in calcium influx into the cell and the activation of calcium-dependent kinases (*e.g.,* protein kinase C and Src). BDNF is also released which phosphorylates proteins in subunits of the NMDA receptor. The NMDA receptor is a major factor in central sensitization since its phosphorylation sensitizes the receptor and enhances subsequent responsiveness to glutamate, increasing synaptic strength and allowing subthreshold inputs to reach threshold levels (Woolf and Salter 2000; Dray 2005; Dubner 2005; Sessle 2005).

The behavioural consequences of central sensitization can be readily detected in human psychophysical experiments. Intradermal injection of capsaicin, the pungent ingredient in chili peppers that activates the TRPV1 receptor, produces an intense but transient pain owing to activation of TRPV1-expressing nociceptors. This is followed by heightened sensitivity to stimulation outside the region of the capsaicin injection (secondary hyperalgesia) and to low-threshold mechanical (tactile) inputs (secondary allodynia), due to the induction of central sensitization (Simone *et al.* 1989; Koltzenburg *et al.* 1992; LaMotte *et al.* 1992; Willis 2002; Dray 2005). Clinically, central sensitization may contribute to pain hypersensitivity in the skin, muscle, joints and viscera (Sarkar *et al*. 2000; Graven-Nielsen *et al*. 2006).

Most of our knowledge of peripheral sensitization and central sensitization comes from studies in the spinal somatosensory system but recent studies in particular have provided evidence for similar processes in the V somatosensory system. The following reviews some general features of the V somatosensory system and findings of peripheral sensitization and central sensitization that suggest peripheral sensitization and central sensitization may be involved in acute and chronic craniofacial pain conditions.

1.4 V PAIN MECHANISMS:

1.4.1 General Gross Anatomy

In contrast to the spinal somatosensory system, the craniofacial complex has many specialized tissues unique to itself, such as the tooth pulp, cornea and TMJ. The craniofacial tissues are principally innervated by the fifth cranial nerve known as the V nerve (Dubner *et al.* 1978; Matthews and Sessle 2001; Sessle 2005); however, the upper cervical spinal nerves (C1 and C2) and other cranial nerves also innervate various regions of the head (Bereiter *et al.* 2000; Hu *et al.* 2005). The V nerve is a mixed nerve containing both sensory and motor fibres and is composed of three subdivisions supplying the peripheral tissues, the Gasserian (V) ganglion containing the majority of sensory neuron somata and the central root that enters the pontine brainstem. The three subdivisions of the V nerve arise from the V ganglion: the ophthalmic (V1), maxillary (V2), and mandibular (V3) nerves. V1 supplies sensory innervation to the upper third of the head including the forehead, nasal cavity and skin, cornea and dura. V2 supplies sensory innervation to the middle facial third including the zygomatic area, upper lip, nasal structures and a portion of the nasal and oral cavities including the maxillary teeth and its associated periodontium. In contrast to the first two subdivisions of the V nerve, the third subdivision of the V nerve contains both sensory and motor fibres. V3 supplies sensory afferents to the remaining extra- and intra-oral structures in the lower facial third including the mandibular teeth and periodontium, the skin covering the mandible, the chin and the TMJ, and provides motor efferents that mainly innervate the muscles of mastication.

The majority of V primary afferent cell bodies reside in the V ganglion which is organized in a somatotopic fashion (Dubner *et al.* 1978; Jacquin *et al.* 1986; Byers and Narhi 1999; Borsook *et al.* 2003; Leiser and Moxon 2006) such that the cell bodies of V1 are located anteromedially, V3 are situated posterolaterally and V2 are in between the other two subdivisions. However, the cell bodies of some periodontal afferents and muscle spindle afferents instead reside in the V mesencephalic nucleus located within the CNS (Dubner *et al*. 1978; Shigenaga *et al.* 1990; Capra and Dessem 1992; Miles *et al*. 2004). The V nerve projects to the V brainstem sensory nuclear complex (VBSNC), a bilateral, multinucleated structure in the dorsolateral part of the brainstem. The majority of craniofacial sensory information is first processed in the VBSNC before being relayed to other parts of the CNS. On the other hand, afferents with cell bodies in the V

mesencephalic nucleus project central axons to either the V motor nucleus or adjacent regions involved with craniofacial motor function (for review, see Dubner *et al.* 1978; Lund 1991; Capra and Dessem 1992; Hannam and Sessle 1994; Miles *et al.* 2004).

1.4.2 Primary Afferent Anatomy and Physiology

The V primary afferent fibres consist of the large-diameter, fast-conducting, myelinated Aβ fibres, the medium-diameter, moderate-conducting, myelinated Aδ fibres, and the small-diameter, slow-conducting, unmyelinated C fibres. All these fibres innervate a wide range of peripheral tissues located throughout the craniofacial region (for review, see Darian-Smith 1966; Dubner *et al.* 1978; Sessle 2005). Many of these V primary afferent fibres have specialized receptor organs on their peripheral terminals such as Pacinian or Meissner's corpuscles, Merkel's disks and hair follicles. These sense organs are associated with large-diameter Aβ fibres, which detect and discriminate light tactile or proprioceptive stimuli such as stretch or tension. Light tactile stimuli are also referred to as innocuous mechanical stimuli and activate receptors known as low-threshold mechanoreceptors. These specialized receptors either respond in a rapidly-adapting (Pacinian/Meissner corpuscles) or slowly-adapting manner (Merkel's disks); however, some slowly-adapting mechanoreceptors have also been shown to respond to innocuous cooling (Brown *et al.* 1981; Simone and Kajander 1997). Receptors responsive to proprioceptive stimuli such as movement of hair in a certain direction are known as proprioceptors. Proprioceptors located in deep tissues (joint and muscles) include muscle spindles and Golgi tendon organs. Activation of the above sense organs by the appropriate stimuli initiates the conduction of nerve impulses into the CNS that provide the sensory information regarding the location, intensity, modality and duration of light tactile and proprioceptive stimuli.

Other V primary afferents terminate as free nerve endings responding to noxious stimuli and are nociceptors. Aδ and C fibres transmit the sensory information of noxious stimuli. There are three classes of V nociceptive primary afferents supplying the cutaneous and mucosal craniofacial tissues (Dubner *et al.* 1978; Dubner and Bennett 1983; Hu and Sessle 1988; Cooper *et al.* 1993; Sessle 2005; Cooper 2006). The first

class are the high-threshold mechanoreceptive afferents. These are Aδ fibres which only respond to intense mechanical stimulation and their activation commonly signifies a well-localized acute mechanical tissue injury. The mechanothermal nociceptive afferents are the second class of V nociceptive primary afferents which are Aδ fibres that are activated by noxious mechanical and noxious heat stimuli (> 45°C). These Aδ nociceptive afferents have also been classified functionally in the spinal somatosensory system as type I and type II Aδ nociceptors (Meyer and Campbell 1981; Leem *et al.* 1993; Treede *et al.* 1995; Greffrath *et al.* 2003; for review, see Dubner *et al.* 1978; Sessle 2005; Cooper 2006). Type I afferents are activated by intense mechanical stimuli and by noxious heat at temperatures > 52°C. Type II afferents are activated by both mechanical and heat stimuli but at a lower thermal threshold of 43°C. The third class are the polymodal nociceptive fibres. In contrast to the other two types of nociceptive primary afferents, these are C fibres that are activated by intense mechanical, thermal and chemical stimuli. Due to its slow conduction velocity, these afferents in the spinal somatosensory system have been associated with the signaling of "second" pain that is poorly localized, dull and aching in nature (Price *et al.* 1977; Fields 1987; Narhi *et al.* 1992; Basbaum and Jessell 2000). In addition, these C fibres can be subdivided anatomically as being peptidergic or non-peptidergic (Snider and McMahon 1998; Jackson and Hargreaves 1999; Gold 2005; Sessle 2005; Cooper 2006). The former group encompasses the afferents that contain pro-inflammatory neuropeptides such as SP and CGRP, and their growth is regulated by NGF. The non-peptidergic group is identified by the presence of specific enzymes such as fluoride resistant acid phosphatase, or binding sites for the isolectin B4 (IB4). During embryogenesis, these afferents depend on NGF; however, in early postnatal life, studies have shown that the glial cell line-derive neurotrophic factor is essential. Nevertheless, this classification of IB4-positive and IB4-negative is not absolute (Petruska *et al.* 2000; Ambalavanar et al. 2005). Other peripheral receptor mechanisms have been recently discovered that may modulate V primary afferent activity such as the vanilloid TRPV1, EAA (NMDA and non-NMDA) and P2X receptors (for review, see below; also see Julius 2003; Dray 2004, 2005; Gold 2005; Cairns 2005; Lam *et al.* 2005; Sessle 2005; Cooper 2006; Staikopoulos *et al.* 2007). In particular, there may be a role

for the above peripheral receptor mechanisms in modulating nociceptive responses of primary afferents innervating deep craniofacial tissues such as the TMJ.

1.4.3 Innervation of the TMJ

Studies have demonstrated that the TMJ is innervated by free nerve endings associated with Aδ and C fibre primary sensory afferents principally from the peripheral branches of V3 (for review, see Dubner *et al.* 1978; Sessle 2000, 2005; Cairns 2005). Of the many branches involved, the auriculotemporal nerve innervates the posterior and lateral aspects of the TMJ capsule, the masseteric and deep temporal nerve supplies the anterior capsule and the medial aspect is innervated by both the auriculotemporal and masseteric nerve in monkeys and humans (Johansson *et al.* 1990; Davidson *et al.* 2003; Fernandes *et al.* 2003; Fletcher *et al.* 2004). In addition to V innervation, the rat TMJ is also innervated by peripheral endings of the upper cervical and vagus nerves (Klineberg 1971; Denny-Brown and Yanagisawa 1973; Widenfalk and Wiberg 1990; Kido *et al.* 1993, 2001; Uddman *et al.* 1998; Casatti *et al.* 1999).

In the 1960s, light microscope studies demonstrated the general innervation of the mouse and monkey TMJ consisted of neurons terminating in the synovial lining layer of the TMJ (Frommer and Monroe 1966; Keller and Moffett 1968). Sensory afferents along with CGRP- and SP-like immunoreactivity were later demonstrated in and around the synovial lining of the rat and monkey TMJ (Johansson *et al.* 1986; Ichikawa *et al.* 1989; Kido *et al.* 1993; Shinoda *et al.* 2003; Ichikawa *et al*. 2004; Ioi *et al.* 2006). Heym and colleagues revealed that CGRP and SP-like immunoreactivity was not restricted to sensory primary afferents and included small-diameter sympathetic fibres supplying craniofacial (Heym *et al.* 1993ab) and spinal tissues (Heym *et al.* 1993c). This finding is problematic since autonomic efferents were also identified in the rat TMJ (Widenfalk and Wiberg 1990; Kido *et al.* 2001). Therefore, the specific type of nerves innervating the TMJ cannot be distinguished based on immunohistochemical studies as previously thought.

The introduction of anatomical tracing techniques such as neuronal labeling with horseradish peroxidase (HRP) allowed Kido and colleagues (1991, 1995) to transport

HRP in an anterograde direction from the V ganglion to the neural terminal. This protocol demonstrated the fine anatomical distribution of sensory afferents in the TMJ. The nerve bundles entered the joint anteriorly, laterally and posteriorly, where branches were mainly distributed in the peripheral portion of the disc with the lateral aspect being the most densely innervated. No fibres were observed in the central region of the disc and only a few fibres were located in the periosteum of the mandibular condyle and temporal bone. Nerve fibres were demonstrated in the synovial and subsynovial layer of the membrane lining the joint compartment and overlying the cartilage of the condyle. The most prominent nerve fibre network was in the anterior aspect of the TMJ. Moreover, electron microscope findings revealed that sensory afferent terminals were located in the superficial synovial layer near the joint cavity and close to synovial cells (Kido *et al.* 1991). Therefore, it was suggested that the close approximation of afferent terminals to the synovial lining of the TMJ could be responsible for monitoring the intra-articular environment (Kido *et al.* 1993) and their intimate association with blood vessels favour a neurogenic component in the inflammatory process in the TMJ (Widenfalk and Wiberg 1990; Kido *et al.* 1995; for review, see Imbe 2001; Kopp 2001).

The majority of anatomical studies demonstrated that the TMJ is supplied by nerve fibres originating mainly from the V ganglion (Widenfalk and Wiberg 1990; Kido *et al.* 1991, 1995; Ichikawa *et al*. 2004; Shinoda *et al.* 2005); however, the involvement of fibres from the V mesencephalic nucleus and parasympathetic ganglia was unclear. The retrograde immunocytochemical rat TMJ study by Uddman and colleagues (1998) confirmed the majority of fibres originate from the V ganglion, although the otic, sphenopalatine, superior cervical, stellate, nodose and dorsal root ganglia at levels C2–C5 all contribute to the nerve fibres in the rat TMJ. Among the autonomic ganglia, the parasympathetic fibres have their primary afferent cell bodies in the otic and sphenopalatine ganglia, and the sympathetic fibres have their primary afferent cell bodies in the superior cervical and stellate ganglia. Similarly, the retrograde axonal tracing rat TMJ study by Casatti and colleagues (1999) demonstrated that 44% of the labeled perikarya were of sensory and 56% were autonomic origin. Within the sensory ganglia, 88% of the labeling was localized to the posterolateral aspect of the V ganglion and 12% were found in C2-C5 dorsal root ganglia. Among the autonomic ganglia, 66%

were located in the superior cervical ganglia, 19% in the stellate ganglion and 15% in the otic ganglia. There were neither profiles found in the V mesencephalic nucleus nor large-diameter perikarya observed, indicating the lack of large-diameter afferents such as the Aα and Aβ fibres. Therefore, there is an approximately equal distribution of nerve fibres of sensory and autonomic origin (predominantly sympathetic) innervating the rat TMJ.

1.4.4 Peripheral sensitization

A number of factors and chemicals are involved in the activation of the V nociceptive afferent by noxious stimulation of the peripheral endings within the RF of the afferent (for review, see Lund and Sessle 1994; Matthews and Sessle 2001; Dray 2004; Robinson *et al*. 2004; Sessle 2005). The tissue damage produced by the noxious stimulus causes the release of chemical mediators from surrounding tissues (*e.g.,* prostaglandins, bradykinins). These substances can activate the free nerve endings and this then can result in the excitation of the afferent and the production of action potentials in the afferent. These action potentials are conveyed into the CNS and may elicit the perception of transient or acute pain. The excitability of the nociceptive afferent endings can also be influenced by several other factors and chemical mediators. Damage to peripheral tissues often results in inflammation which involves products released from blood vessels or from other tissue elements including immune cells. Some of these substances (*e.g.,* histamine, 5-hydroxytryptamine [5-HT], cytokines) promote inflammation but also may act on the nociceptive afferent endings in the vicinity of the injured site to increase their excitability. In addition, substances synthesized in and released from the afferents themselves may influence the excitability of their endings. The effect of many of the chemical mediators is to produce peripheral sensitization.

The processes and chemical mediators involved in producing peripheral sensitization include not only the chemical products of tissue injury but also, as noted above, the release of neurochemicals from the V afferent endings themselves. V studies using *in vivo* or *in vitro* recordings from these cells as well as molecular and

immunocytochemical techniques have shown that these cell bodies synthesize a vast array of chemicals that help define the role that the primary nociceptive afferents play in encoding pain (for review, see Dray 2004, 2005; Robinson 2004; Svensson and Sessle 2004; Gold 2005; Cooper 2006). These include CGRP, SP, somatostatin, and nerve growth factors, and the afferents may express serotonergic, cholinergic, opiate, purinergic, bradykinin, histamine, anandamide, prostaglandin and acid-sensitive receptors and ion channels, adrenoreceptors as well as the capsaicin-sensitive or capsaicin-insensitive vanilloid receptors. The chemicals also act on the nociceptive afferent endings and contribute to the peripheral sensitization which is reflected in enhanced spontaneous afferent firing, an increase in their responsiveness to noxious stimuli, and a decrease in their activation threshold.

Other receptor mechanisms have been recently discovered that are involved in the peripheral processes contributing to craniofacial pain (for review, see Julius 2003; Dray 2004, 2005; Gold 2005; Sessle 2005). They include the vanilloid TRPV1 receptor that responds to protons (H+), heat, and algesic chemicals such as capsaicin which is the ingredient in hot chilli peppers. Furthermore, chemical mediators that have long thought to be involved in nociceptive transmission or modulation within the CNS can also act peripherally on the nociceptive afferents. For example, glutamate is synthesized by primary afferent cell bodies, and some afferent endings in peripheral craniofacial tissues have receptors (NMDA and non-NMDA receptors) for this EAA. Glutamate can excite nociceptive afferents supplying craniofacial musculoskeletal tissues and when injected into human jaw muscles produces a transient pain by activating the glutamate receptors located on the afferent endings (Cairns *et al.* 2001ab, 2002a, 2003b; Svensson *et al.* 2003, 2005). The excitatory and pain-producing effects of glutamate can be blocked by peripheral injection within the TMJ or muscle of a NMDA receptor antagonist (Cairns *et al*. 2003a, 2006).

The physiological and chemical responses to tissue damage and inflammation of craniofacial tissues are important factors in many painful conditions such as arthritis and mucositis. Peripheral processes involving peripheral sensitization of nociceptive afferent endings at the injury site are associated with a decreased activation threshold and increased responsiveness to subsequent stimulation of the site of the nociceptive

afferent endings; this can account for the acute pain sensitivity that occurs at the site of an injury (primary hyperalgesia). In addition, the chemicals may also diffuse through the peripheral tissues and act on the endings of adjacent nociceptive afferents, and thus contribute to the spread of the painful area. Moreover, since activated and sensitized nociceptive afferents may also exhibit spontaneous activity as well as lowered activation thresholds and increased responsiveness to subsequent noxious stimuli, these changes are thought to contribute, respectively, to the spontaneous pain, allodynia and hyperalgesia that are features of many chronic or persistent craniofacial pain conditions.

1.4.5 Central Organization of the V System

The majority of cell bodies of V primary afferents are located in the V ganglion with the exception of some periodontal and muscle spindle afferents (which are in the V mesencephalic nucleus). The somata of the V ganglion send their axons to the CNS via the V nerve root and ascend or descend along the V spinal tract. The central axons of these afferent fibres project to the VBSNC and synapse with second-order neurons within this complex or other parts of the brainstem (*e.g.,* the solitary tract nucleus, reticular formation, supratrigeminal nucleus) (for review, see Darian-Smith 1966; Dubner *et al.* 1978; Capra and Dessem 1992; Sessle 1996, 2005). The organization of the VBSNC is comparable to that of the spinal system whereby primary afferent cell bodies in the dorsal root ganglion give off central collaterals and synapse onto second-order neurons in the spinal dorsal horn. In addition to receiving primary afferents from the V nerve, the VBSNC also processes sensory input from other cranial nerves (*e.g.,* VII, IX, X, XII) and from upper cervical nerves. This extensive convergence pattern is one of the features that distinguishes the VBSNC from the spinal dorsal horn (for review, see Sessle 2000, 2005).

The morphology of central terminals of V primary afferents has been extensively studied with anatomical tracing techniques such as neuronal labeling with HRP and localization of neurochemicals with immunohistochemistry (Marfurt and Turner 1983, 1984; Jacquin *et al.* 1993; Takemura *et al.* 1991; for review, see Capra and Dessem 1992; Sessle 2005). The VBSNC is subdivided into the main (principal) sensory nucleus

and the spinal tract nucleus. The latter nucleus consists of three subnuclei: oralis (Vo), interpolaris (Vi) and caudalis (Vc). The main sensory nucleus and the two rostral subnuclei (Vo, Vi) consists of relatively uniform heterogeneous populations of second-order neurons of various sizes and projection targets. Some are short-range projection neurons which serve as connections to other brainstem nuclei (*e.g.,* cranial nerve V motor nuclei, cranial nerve VII and cranial nerve XII) and subnuclei, whereas neurons projecting to the thalamus or cerebellum are long-range (Falls and Alban 1986; Jacquin *et al.* 1986; Jacquin and Rhoades 1990; Shigenaga *et al.* 2000; Gojyo *et al.* 2002; Craig 2004; Guy *et al.* 2005; Yoshida *et al.* 2005; for review, see Sessle 2000, 2005). In contrast, Vc has been described as being analogous to the dorsal horn of the spinal cord in which the neurons are organized in a laminated structure (Gobel *et al.* 1981; Dubner and Bennett 1983; Sessle 2000, 2005; also see below). While this homology has been useful for us to better understand craniofacial pain mechanisms, it may need revision since evidence suggests that select portions of Vc are organized differently from spinal systems (for review, see Bereiter *et al.* 2000; Sessle 2000, 2005; Dubner and Ren 2004; also see below). For example, compared to the spinal dorsal horn, the caudal Vc displays a broader density of IB4-positive fibres which crosses laminae I, IIo, and IIi and overlaps that for SP and CGRP (Bereiter *et al.* 2000). The wider distribution of IB4-positive fibres in the superficial laminae of caudal Vc compared to the spinal dorsal horn may be related to the extensive convergence from cervical and multiple cranial nerves received by the caudal Vc. Also, the ventral Vi/Vc transition region displays dense immunoreactivity for SP and CGRP and lacks staining for IB4. These findings suggest that the ventral Vi/Vc transition region and caudal Vc receive a different complement of small-diameter fibres.

The neurons comprising the VBSNC are arranged in a somatotopic organization described as an inverted medially directed face (Kruger and Michel 1962; Nord 1968; for review, see also Dubner *et al.* 1978; Bereiter *et al.* 2000; Sessle 2000, 2005; DaSilva *et al.* 2002; Hu and Woda 2006). V1 primary afferents synapse with second-order neurons located ventrally, V2 primary afferents terminate centrally, and V3 primary afferents terminate in the dorsal part of each of its nuclei or subnuclei. In addition, neurons with an oral RF are located medially and those with a facial RF are situated laterally. In

addition to the above somatotopic organization, the oral and peri-oral sensory inputs in Vc project to the rostral region of Vc whereas the caudal portion represents the more lateral regions of the face. This somatotopic organization of neurons in Vc has been referred to as the 'onion skin' arrangement. Moreover, unlike the spinal dorsal horn, some craniofacial tissues (*e.g.,* cornea) are dually represented in the rostral and caudal regions of Vc. Also, these two regions may play different roles in both autonomic and muscle reflex responses to noxious stimulation applied to craniofacial tissues (Bereiter et al. 2000; Sessle 2000, 2005; Dubner and Ren 2004).

There are five major types of V brainstem neurons that are classified based on their functional and cutaneous RF properties (for review, see Bereiter *et al.* 2000; Woda 2003; Sessle 2000, 2005; Dubner and Ren 2004; Hu and Woda 2006). The first type is the LTM neuron that receives sensory information from Aβ and/or Aδ fibres and responds to innocuous mechanical stimuli. The second type is the WDR neuron responding to both innocuous and noxious stimuli in a graded manner and receives extensive convergent input from Aβ, Aδ and/or C fibre afferents. The third type termed NS neurons respond only to noxious mechanical, thermal or chemical stimuli conveyed by Aδ and/or C fibre afferents. The fourth type is the polymodal nociceptive neuron that conveys noxious heat, pinch or cold stimuli from C-fibre afferents (Craig and Dostrovsky 2001). The fifth type is the thermoreceptive-specific neuron (WARM or COOL, formerly "COLD") that is excited by innocuous cutaneous warming (WARM) or cutaneous cooling (COOL) (Craig and Dostrovsky 2001). These second-order neurons in the VBSNC contribute to the transmission of somatosensory information to higher brain centres such as the thalamus. Some of the projections may be direct (*e.g.,* from the V main sensory nucleus to the thalamus), others may synapse with the reticular formation or other parts of the brainstem before traveling to higher brain centres whereas a few may bypass the thalamus and project to the cerebellum, cranial nerve motor nuclei, superior colliculus, pontine parabrachial nucleus and periaqueductal gray. Moreover, there are intrinsic connections between subnuclei within the VBSNC, which is termed the deep bundle system. Somatosensory transmission in the V system is also subject to segmental and descending modulatory influences that may be inhibitory or facilitatory (for review, see Chiang *et al.* 2003; Woda 2003; Dubner and Ren 2004; Sessle 2005).

The periaqueductal gray matter, rostroventral medulla/nucleus raphe magnus, locus ceruleus, pontine parabrachial area, anterior pretectal nucleus, thalamic nucleus submedius, and cerebral cortex have all been demonstrated to inhibit V brainstem neuronal, reflex and behavioural responses to noxious craniofacial stimulation in animals via descending pathways that project to the VBSNC.

Craniofacial somatosensory information from the VBSNC is relayed to thalamic regions that include the ventrobasal complex (or ventroposterior nucleus in humans) and the posterior group of nuclei and the medial thalamus (Sessle and Iwata 2001; DaSilva *et al.* 2002; Craig 2004; Guy *et al.* 2005; Dostrovsky and Craig 2006). In the thalamus, glutamate is also important in the transmission of the somatosensory signals. It is released from the thalamic terminals of the axons of the VBSNC neurons and acts through glutamate receptors on the thalamic neurons to activate the neurons. The ventrobasal thalamus is somatotopically organized, and neurons receiving and relaying tactile information from the face and mouth are concentrated in the medial portion of the ventrobasal thalamus (the nucleus ventralis posteromedialis, VPM). The lateral part (VPL) of the ventrobasal thalamus receives somatosensory information from the limbs, trunk and neck, principally via the dorsal column-medial lemniscal system and the spinothalamic tract. Most of the VPM LTM neurons faithfully relay the detailed somatosensory information they receive via the brainstem (or spinal cord in the case of VPL neurons) to the overlying somatosensory areas of the cerebral cortex.

Compared with V primary afferent and VBSNC mechanisms, there has been much less research focus on higher brain processing of craniofacial nociceptive processing. Neurons in the posterior group and medial thalamic regions also contain nociceptive neurons (DaSilva *et al.* 2002; Craig 2004; Willis and Coggeshall 2004; Sessle 2005; Dostrovsky and Craig 2006; Tracey and Mantyh 2007). In general, the properties of these neurons in experimental animals are similar to those described for NS and WDR neurons in the VBSNC such as Vc. Recordings in the human thalamus also reveal analogous neurons. Some of these neurons, like their VBSNC counterparts, respond to musculoskeletal, cerebrovascular or tooth pulp stimuli as well as cutaneous stimulation. Nonetheless, many VPM nociceptive neurons have RF and response properties and connections with the overlying somatosensory cerebral cortex that indicate a role in the

localization and discrimination of noxious stimuli. In contrast, nociceptive neurons in the more medial nuclei of the thalamus appear to be involved more in the affective or motivational dimensions of pain, and are connected with other nociceptive neurons in higher brain areas such as the hypothalamus and anterior cingulate cortex that participate in these functions or in neuroendocrine responses related to pain. There is also evidence suggesting a sensory-discriminative role for the somatosensory cortex in pain since NS and WDR neurons that respond to noxious craniofacial stimuli have been demonstrated in the primary face somatosensory cortex (DaSilva *et al.* 2002; for review, see Bushnell 2005).

1.4.6 VBSNC Involvement in Craniofacial Nociceptive Processing

Many studies using morphological, clinical, behavioural, and electrophysiological approaches have verified the involvement of VBSNC in the processing of craniofacial nociceptive information. The VBSNC is the site where second-order neurons receive V primary afferent input from most craniofacial tissues. The Vc has been especially implicated in V nociceptive mechanisms and has morphological similarities with the spinal dorsal horn, which is the fundamental component of spinal nociceptive processing, including analogous cell types, laminae and thalamic projections (for review, see Dubner *et al.* 1978; Gobel *et al.* 1981; Renehan *et al.* 1986; Hannam and Sessle 1994; Sessle 2000, 2005; Woda 2003; Dostrovsky and Craig 2006; Hu and Woda 2006) although some differences do exist (see above). A common feature shared with the spinal dorsal horn is the presence of the substantia gelatinosa layer (lamina II) where Aδ and C fibre primary afferents terminate. In addition to lamina II, Vc neurons receive small-diameter (Aδ and C fibre) afferent convergence in lamina I, V and VI. Many of the small-diameter V primary afferents that terminate almost exclusively in Vc contain neuropeptides such as SP and CGRP as well as EAAs implicated in nociceptive transmission, and Vc neurons are subject to segmental or descending modulatory influences (for review, see Dubner and Bennett 1983; Sessle 1987, 1996, 1998, 2005; Chiang *et al.* 2003; Woda 2003; Dubner and Ren 2004). In accordance with analogous findings in the spinal dorsal horn, noxious craniofacial stimulation evokes release of

putative neurotransmitters from Vc (Jessel and Iversen 1977; Suarez-Roca and Maixner 1993; Bereiter and Benetti 1996; Sessle 2005) and excites Vc nociceptive neurons (for review, see Dubner and Bennett 1983; Sessle 1987, 1996, 1998, 2000, 2005; Sessle and Hu 1991; Cooper and Sessle 1992; Bereiter *et al.* 2000; Dubner and Ren 2004). Immunocytochemical techniques have allowed the examination for proto-oncogene expression (*e.g., c-fos*) as a marker for the identification of neurons responding to peripheral noxious stimulation (for review, see Sessle 2000; Imbe *et al.* 2001; Mitsikostas and Sanchez del Rio 2001). An increased expression of *c-fos* in the Vc region was demonstrated when noxious stimuli were applied to different types of craniofacial tissues (Nozaki *et al.* 1992; Wakisaka *et al.* 1992; Coimbra and Coimbra 1994; Strassman *et al.* 1994; Ebersberger *et al.* 1995; Hathaway *et al.* 1995; Lu and Bereiter 1995; Martinez and Belmonte 1996; Imbe *et al.* 2001; Mitsikostas and Sanchez del Rio 2001; Bereiter *et al.* 2005). Additional evidence confirming the involvement of Vc in nociceptive processing was obtained when significantly greater *c-fos* labeling in laminae I-II and III-IV of Vc was revealed when compared with that induced by innocuous stimuli (Strassman and Vos 1993). Immunocytochemical and various anatomical studies indicate a strong role played by Vc in craniofacial nociceptive processing. Although there may be some differences between Vc and the spinal dorsal horn (Bereiter *et al.* 2000; Sessle 2005; and see above), the Vc has been designated as the medullary dorsal horn due to its close morphological and functional similarities with the spinal dorsal horn, a critical site for spinal and spinothalamic nociceptive mechanisms (for review, see Hu *et al.* 1981; Dubner and Bennett 1983; Sessle 1987, 1996, 2000, 2005).

Clinical and behavioural investigations have found that by disrupting the components of VBSNC, facial pain and nociceptive behavior may be altered in both animals and humans. Animal studies demonstrate that lesions or chemical injections to Vc interfere with the perception of noxious stimulation to the craniofacial region and attenuates nociceptive behavior (Broton *et al.* 1988; Duale *et al.* 1996; for review, see Dubner *et al.* 1978; Sessle 2000, 2005; Woda 2003). The inactivation of Vc has also been shown to attenuate jaw muscle activity reflexly evoked by injection of mustard oil or glutamate into the TMJ capsule (Hu *et al.* 1997; Cairns *et al.* 1998, 2001b; Tsai *et al.* 1999). In

humans, V tractotomy, in which the V spinal tract is transected at the rostral pole of the Vc, reduces the perception of facial noxious stimuli while preserving considerable tactile sensation (Sjoqvist 1938; Kanpolat *et al.* 2005; for review, see Dubner *et al.* 1978; Fromm and Sessle 1991; Woda 2003). Thus, both behavioural and clinical studies support the involvement of Vc in nociceptive processing of craniofacial tissues.

Electrophysiological recordings in Vc have provided further support for the involvement of Vc in craniofacial nociceptive mechanisms. They have demonstrated the presence of NS and WDR neurons and their activation by cutaneous noxious stimuli (for review, see Sessle 2000, 2005; Woda 2003). Peripheral afferent inputs from deep craniofacial tissues, such as those supplying the TMJ and masticatory muscles, can also converge onto a majority of these neurons and activate them. For example, most neurons activated by electrical or chemical stimulation of TMJ afferent inputs have deep and cutaneous RFs (Broton *et al.* 1988; Sessle and Hu 1991; Takeshita *et al.* 2001; Okamoto *et al.* 2003, 2005b; Hu *et al.* 2005b; Mørch *et al.* 2007). This convergence of input may contribute to the poor localization of deep pain and the phenomenon of referred pain. However, central sensitization of Vc neurons may allow the "unmasking" or "strengthening" of convergent afferent inputs (for review, see Hu *et al*. 1997; Sessle 2000, 2005; Dubner and Ren 2004) and also contribute to the diffuse nature and referral of pain.

However, Vc is not the only component of the VBSNC with a role in craniofacial nociceptive processing. Several lines of evidence point to the involvement of the rostral components, such as Vo and Vi (for review, see Sessle 2000, 2005; Woda 2003; Dubner and Ren 2004). Briefly, these include findings that lesions of rostral components may disrupt some craniofacial pain behaviours, and that many neurons in Vo and Vi project to brainstem or higher brain centres involved in reflex or perceptual aspects of craniofacial pain. Furthermore, NS and WDR neurons occur in these rostral subnuclei and have cutaneous RFs that are usually localized to perioral or intraoral areas, and many can be activated by stimulation of tooth pulp or other sites (*e.g.,* muscle, cerebrovasculature). These neuronal features, plus the effects of rostral lesions, suggest that the more rostral components of the VBSNC may play a role especially in perioral and intraoral nociceptive processing.

1.4.7 Central nociceptive processing for the TMJ and masticatory muscles

In addition to its importance in craniofacial nociceptive mechanisms, as noted above, there is also considerable evidence to indicate the caudal Vc is an important site of nociceptive processing specifically for the TMJ and masticatory muscles. An abundance of Aδ and C fibre primary afferents innervate the TMJ and masticatory muscles, and these afferents have been shown to primarily project directly to Vc and caudal parts of Vi (Capra 1987; Broton *et al.* 1988; Hayashi *et al.* 1984; Jacquin *et al*. 1983, 1986; Pfaller and Arvidsson 1988; McNeill *et al.* 1991; Sugiyo 2005; Wang *et al.* 2006; for review, see Sessle and Hu 1991; Imbe *et al.* 2001). Several studies have shown that SP and CGRP-containing small-diameter afferents (Ichikawa *et al.* 1990, Kido *et al.* 1995, Lundeberg *et al.* 1996; Loughner *et al.* 1997; Shinoda *et al.* 2003; Ichikawa *et al*. 2004; Ioi *et al.* 2006), innervating the TMJ and masticatory muscles terminate in laminae I and II, and V and VI of Vc (Capra and Dessem 1992; Hannam and Sessle 1994; Sessle 1999; Imbe *et al.* 2001; Sugiyo 2005). HRP tracing studies (Nishimori *et al.* 1986; Shigenaga *et al.* 1988; for review, see Capra and Dessem 1992) indicate that deep input afferent terminals are distributed in the caudal Vc. Moreover, *c-fos* immunocytochemistry following acute TMJ injury yields a high density of *fos*-positive neurons in the caudal Vc that is proportional to stimulus intensity and inhibited by analgesics (Hathaway *et al.* 1995; Bereiter and Bereiter 2000; Bereiter 2001; Takeshita *et al.* 2001; Bereiter *et al.* 2005). Selective destruction of the caudal Vc, but not of more rostral areas of the VBSNC, has been shown to block the increase in activity in masseter muscle caused by inflammation of the TMJ region (Hu *et al.* 1997; Cairns *et al.* 1998, 2001c; Tsai *et al.*1999). Injection of either mustard oil or glutamate into the TMJ also evokes activity in Vc neurons, which are thought to relay nociceptive information from the craniofacial region to higher centres (Broton *et al.* 1988; Kojima 1990; Sessle and Hu 1991; Hathaway *et al.* 1995; Kishimoto *et al.* 1999; Sessle 1999; Zhou *et al.* 1999; Cairns 2005; Lam *et al.* 2005). Other studies have induced inflammation in the TMJ region and adjacent muscles and reported increased excitability of deep afferents (Harriott *et al.* 2006) and enlargement of cutaneous RF size

in Vc neurons (Hu *et al.* 1992; Iwata *et al.* 1999; Imbe *et al.* 2001; Lam *et al.* 2005). Moreover, there are sex differences in TMJ-responsive Vc neurons to the peripheral application of inflammatory mediators (Okamoto *et al.* 2003, 2005b; Bereiter *et al.* 2005). With the exception of a few recent studies (Takeshita *et al.* 2001; Okamoto *et al.* 2003, 2005ab; Bereiter *et al.* 2005) in which they recorded nociceptive activity from TMJ-responsive neurons in the superficial laminae of the caudal Vc, the majority of electrophysiological studies to date have studied more rostral components of Vc. Thus there is clearly a need to study TMJ-responsive neurons in the caudal Vc/Upper cervical cord (UCC) region as well due to the limited studies to date and the importance of the caudal Vc/UCC region in TMJ and masticatory muscle nociceptive processing (see above; Hu *et al.* 2005b; Mørch *et al.* 2007).

Nociceptive transmission in the V somatosensory system (and spinal system) can also be enhanced by craniofacial afferent inputs into the VBSNC that are induced by inflammation or trauma of peripheral tissues and nerves (Ren and Dubner 1999; Sessle 2000; Woda 2001; Dubner and Ren 2004). For example, injection of an inflammatory agent and small-fibre excitant such as formalin, capsaicin or mustard oil into deep tissues such as the TMJ or masticatory muscles evokes nociceptive afferent inputs into the brainstem that can lead to several changes in the properties of Vc NS and WDR neurons. These neuronal changes can include expansion of the cutaneous RF, enhancement of spontaneous firing, increased responses to noxious craniofacial noxious stimuli and lowered activation threshold to craniofacial stimuli. In contrast, comparable stimulation of cutaneous nociceptive inputs by these inflammatory agents is much less effective. This enhanced and prolonged excitability of neurons in V nociceptive pathways appears to be due to mechanisms that reflect a *central sensitization* of V nociceptive neurons, and may involve an “unmasking” of some of the extensive convergent afferent inputs to these neurons and other processes such as disinhibition (see below). The resulting increased excitability that characterizes the “sensitized” neuron underlies the increased responsivity to noxious stimuli and lowered activation threshold and the increase in spontaneous firing and RF size that can occur in nociceptive neurons following craniofacial injury or inflammation. The following

describes in more detail some of the essential features and mechanisms of central sensitization, especially as they apply to the V system.

1.4.8 Central sensitization

As noted above, tissue inflammation or nerve injury can lead to an enhanced excitability of nociceptive neurons in the VBSNC and spinal system. This central sensitization may enhance the responses of nociceptive neurons to noxious stimuli and their sensitivity to low-threshold mechanosensitive afferent inputs in conditions associated with peripheral injury or inflammation, and thus could contribute to the hyperalgesia and allodynia that often are associated with pain conditions (for review, see Ren and Dubner 1999; Sessle 2000, 2005; Woda 2001; Dubner and Ren 2004). The increased spontaneous activity and RF size of the nociceptive neurons also appears to represent a central factor contributing to spontaneous pain and pain spread and referral. It is important to note that central sensitization appears to be a normal physiological reaction to sustained noxious stimulation, and in most situations is reversible. Its prolongation is thought to underlie chronic or persistent pain, but the factors predisposing to its prolongation are not yet well understood, and likely include genetic as well as environmental influences and psychophysiological factors (Dworkin 2001; Feinmann and Newton-John 2004; Salter 2004; Seltzer and Dorfman 2004).

The neurochemical processes involved in central sensitization in the VBSNC have been the subject of recent studies. Like their counterparts in the spinal dorsal horn, the V nociceptive primary afferent endings in Vc release EAAs (*e.g.,* glutamate) and neuropeptides (*e.g,.* SP, CGRP) in Vc (Bereiter *et al.* 2000; Sessle 2000, 2005; Salter 2004; Dubner 2005). Some of these afferents stain positive for SP, CGRP and neurotrophins, others stain negative for these neuropeptides but positive for IB4; these IB4-positive afferents have a different distribution in Vc compared with the spinal dorsal horn. Several receptor types associated with nociceptive processing are localized in the afferent endings or neurons in Vc (Sessle 2000, 2005; Dubner and Ren 2004; Salter 2004; Dubner 2005) *e.g.,* EAA, neurokinin, purinergic, and TRPV1. Some of these receptors (*e.g.,* NMDA, non-NMDA, neurokinin) occuring on the membrane of the

second-order neurons are involved in the processes by which glutamate or SP excites nociceptive Vc neurons. The release of glutamate leads to the excitation of nociceptive Vc neurons by processes involving different ionotropic receptors for glutamate, namely NMDA, KA and AMPA receptor subtypes, as well as metabotropic glutamate receptors. The former process involves the direct gating of ion channels on the membrane of the neuron, but the latter process gates ion channels indirectly through the action of GPCRs which utilize intracellular second messengers. The different subtypes of glutamate receptors also have different physiological characteristics and actions. For example, activation of the AMPA/KA subtypes is usually rapid and short-lived, and typically is involved in the neuronal responses to brief, mild noxious stimulation. In contrast, the NMDA receptor subtype has a longer period of activation and is important in "wind-up" and "central sensitization" that typically occur in response to more prolonged, intense noxious stimuli. Activation of the NMDA receptors seems to be particularly important in the development of central sensitization, and is associated with removal of the voltage-dependent magnesium block of the NMDA receptor, the entry of calcium into the neurons, phosphorylation of the NMDA receptor, and changes in neuronal kinetics. In addition, several second messenger systems such as nitric oxide and protein kinase C as well as purinergic receptors may also be involved in the V central sensitization process, as outlined in recent reviews (Chiang *et al.* 2003; Woda 2003; Salter 2004; Sessle 2005). NMDA receptor antagonists in particular can block these nociceptive phenomena in Vc or analogous processes in the spinal dorsal horn, suggesting that centrally acting NMDA antagonists might be useful analgesics in certain pain conditions.

The central sensitization may also involve disinhibition, which refers to a loss of some of the central inhibitory processes that were mentioned above in terms of segmental and descending modulatory influences. For example, chronic inflammation of craniofacial musculoskeletal tissues produces enhancement of pain behaviour and nociceptive neuronal activity through, at least in part, an alteration in the descending inhibitory or facilitatory influences from structures such as the rostral ventromedial medulla (for review, see Ren and Dubner 1999; Dubner and Ren 2004; Dubner 2005; Sessle 2005). Moreover, a central opioid depressive effect may be "triggered" into action by peripheral injury or inflammation since the intrathecal or systemic

administration of the opioid antagonist naloxone can “re-kindle” the enhanced jaw muscle EMG activity and increased Vc neuronal excitability that can be induced by mustard oil application to orofacial tissues (Sessle 2000, 2005). These findings suggest that a nociceptive V afferent input to the brain, triggered in this case by the inflammatory irritant mustard oil, evokes nociceptive neuronal activity in Vc and associated neuromuscular changes, but these neural changes are limited by the recruitment of a central opioid inhibitory mechanism. It is also noteworthy that central sensitization may not be solely due to changes in neuronal circuits in the CNS. Recent research also suggests that glia may play a role in the development of central sensitization in Vc (Chiang *et al.* 2007; Guo *et al.* 2007; Xie *et al.* 2007; for review, see Salter 2004; Watkins and Maier 2005).

Although the above findings indicate that the Vc is crucial in both nociceptive processing and central sensitization, more studies are required on the functional roles of the different regions of the Vc, especially its transition zone with the upper cervical dorsal horn (UCC). Furthermore, V central sensitization is not limited to nociceptive neurons in Vc. Noxious craniofacial stimulation can also produce central sensitization of nociceptive neurons in Vo and in higher brain regions such as VPM thalamus, although Vc (and not Vo) is responsible for the expression of central sensitization by way of its projections to both structures (Chiang *et al.* 2003; Sessle 2005; Zhang *et al.* 2006; Park *et al.* 2007). The net result of these neuroplastic changes reflecting central sensitization is an increased central excitatory state that is dependent on peripheral nociceptive afferent input for its initiation, but may not be fully dependent on peripheral afferent drive for its maintenance.

1.5 PERIPHERAL MECHANISMS IN DEEP CRANIOFACIAL PAIN:

Despite many breakthroughs in pain research, our current understanding of the neural mechanisms of articular pain still derive mainly from knee or ankle joint models (Schaible and Grubb 1993; Kidd *et al.* 1996; Schaible and Schmidt 1996; Ebersberger 1999; Segond von Banchet *et al.* 2000; Schaible *et al.* 2002; Bar *et al.* 2004; Henry

2004; Sharif *et al.* 2005). Most of these experimental studies have suggested that a number of chemicals such as bradykinin, histamine, serotonin and prostaglandins may be responsible for peripheral and central sensitization under experimental myositic or arthritic joint conditions (Hoheisel and Mense 1989; Mense 1993, 1996; Schaible and Grubb 1993; Schaible and Schmidt 1996; Ebersberger 1999; Heppelmann and Pawlak 1999; Mense *et al.* 2000; Segond von Banchet et al. 2000; Schaible *et al.* 2002; Bar *et al.* 2004). It is noteworthy that these chemicals are inflammatory irritants and ligands for GPCR types which were applied peripherally to excite and sensitize primary afferents and spinal dorsal horn neurons. While injury, inflammation or degeneration of the TMJ or muscle are often conceptualized as important in the pathophysiology of TMD, the majority of TMD do not appear to be associated with gross indications of inflammatory changes (Zarb *et al.* 1994; Stohler 1995; Singer and Dionne 1997). This suggests that different receptor mechanisms may underlie the development of TMD and inflammatory pain. Since it has been demonstrated that activation of peripheral EAA receptors within the TMJ region evokes jaw muscle activity similar to that produced by the injection of algesic chemicals, *e.g.,* mustard oil, but glutamate application to the TMJ does not result in significant inflammation (Yu *et al.* 1996; Cairns *et al.* 1998, 2001b, 2002a; Fiorentino *et al.* 1999; Cairns 2005), it has thus been suggested (Cairns *et al.* 1998) that glutamate may be a better candidate than traditional algesic/inflammatory mediators (*e.g.,* bradykinin, serotonin, histamine or prostaglandins) for studying musculoskeletal pain mechanisms that may be operative in pain conditions such as TMD.

Another peripheral receptor that may play an important role in craniofacial nociceptive mechanisms is the TRPV1 receptor. Capsaicin-induced Ca^{2+} influx into peripheral nerve terminals through the TRPV1 receptor has been shown to result in the release of neuropeptides and EAAs (Bevan and Szolcsyanyi 1990; Sikand and Premkumar 2007). It is also possible that plasma extravasation and/or a neurogenic release secondary to micro-trauma or TRPV1 receptor activation could play a role in changing the local glutamate concentration. These findings suggest the potential involvement and interaction of peripheral EAA and TRPV1 receptor mechanisms in nociceptive processing. It is currently unclear, however, what role peripheral EAA and TRPV1 receptor mechanisms might play in peripheral and central nociceptive

processing in TMJ and muscle pain. The following will review peripheral EAA and TRPV1 receptor mechanisms in craniofacial pain in the context of findings in both spinal and V systems.

1.5.1 Peripheral EAA receptors

Glutamate is the endogenous agonist for EAA receptors. There are two broad groups of EAA receptors, namely the ionotropic EAA receptors and the metabotropic EAA receptors (Collingridge and Lester 1989; Monaghan *et al.* 1989; Bockaert *et al.* 1993; Schoepp and Conn 1993; Gold 2005). The former are mixed cation channels which allow the passage of Na^+, K^+ and in some cases Ca^{2+} across cell membranes, while the latter are G-protein linked. Ionotropic EAA receptors subtypes are named for the agonist that activates them and include NMDA receptors, and the non-NMDA AMPA and KA receptors (Collingridge and Lester 1989; Monaghan *et al.* 1989; Coderre *et al.* 1997; Gold 2005). Glutamate is a well-documented central excitatory neurotransmitter but a number of studies have provided evidence in support of a role for *peripheral* glutamate receptors in the transduction of nociceptive information. EAA receptors are found on both V and DRG neurons (Sato *et al*. 1993; Sahara *et al.* 1997; Gold 2005) and unmyelinated terminals of cutaneous afferents (Carlton *et al.* 1995; Coggeshall and Carlton 1998; Du *et al.* 2006). Glutamate is present in both V and DRG neurons (Battaglia and Rustioni 1988; Kai-Kai 1989; Clements *et al*. 1991; Clements and Beitz 1991; Kai-Kai and Howe 1991; Azerad *et al.* 1992; Keast and Stephensen 2000) as well as their central (Westlund *et al.* 1989; Keast and Stephensen 2000) and peripheral (Westlund *et al.* 1992; Keast and Stephensen 2000) terminals (for review, see Carlton 2001; Carlton *et al.* 2003). Nociceptive stimulation of primary afferents results in the release of EAAs centrally in V and spinal afferents (Wanaka *et al.* 1987; Battaglia and Rustioni 1988; Skilling *et al.* 1988; Kai-Kai 1989; Westlund *et al.* 1989; Clements *et al.* 1991; Clements and Beitz 1991; Kai-Kai and Howe 1991; Azerad *et al*. 1992; Boucher *et al.* 1993; Bereiter and Benetti 1996; Bereiter *et al.* 2002; Fujita *et al.* 2004). It is not known whether glutamate is also released from the peripheral endings of V afferent fibres but nociceptive stimulation of spinal afferents has been shown to result in the

neurogenic release of glutamate from peripheral terminals of spinal afferents (Omote *et al.* 1998; deGroot *et al.* 2000). Since sensory nerve terminals contain glutamate receptors and release glutamate, it has been suggested that there is a possible role for autocrine and/or paracrine regulation of spinal nociceptor excitability (Carlton 2001). Similarly, since NMDA and non-NMDA receptors are found on V ganglion neurons and can be activated by glutamate to depolarize V ganglion neurons *in vitro* (Puil and Spigelman 1988; Sahara *et al.* 1997; Pelkey and Marshall 1998) and peripherally applied glutamate can excite V nociceptive afferents *in vivo* by NMDA and non-NMDA receptor mechanisms (Cairns *et al.* 2001ab, 2002a, 2003a; Cairns 2005; also see below), this may suggest a similar role for peripheral glutamate receptors to modulate V nociceptive mechanisms.

Peripheral EAA receptor mechanisms may have an important role in inflammatory pain since peripheral glutamate levels are elevated during cutaneous or deep tissue inflammation and the number of EAA receptors in cutaneous tissues increases during inflammation (Omote *et al.* 1998; Carlton and Coggeshall 1999; Lawand *et al.* 2000; McNearny *et al.* 2000; Dray 2005). A number of studies have provided evidence in support of a role for peripheral glutamate receptors in the transduction of nociceptive information. Subcutaneous or intra-articular glutamate injection decreases mechanical paw withdrawal thresholds in rats through activation of peripheral NMDA, KA and AMPA receptors (Carlton *et al.* 1995; Zhou *et al.* 1996; Lawand *et al.* 1997; Leem *et al.* 2001; Du *et al*. 2006). In rats, the development of thermal and/or mechanical hyperalgesia observed after intraplantar or intra-articular (knee) application of irritant chemicals can be mimicked by application of EAA receptor agonists to these sites (Zhou *et al.* 1996; Davidson *et al.* 1997; Lawand *et al.* 1997; Du *et al*. 2006). It has been reported that peripheral application of selective EAA receptor antagonists may attenuate behavioural signs of irritant-chemical or peripheral nerve injury-induced hyperalgesia (Jackson *et al.* 1995; Davidson *et al.* 1997; Lawand *et al.* 1997; Davidson and Carlton 1998; Jang *et al.* 2004; Du *et al*. 2006). Intraplantar injection of NMDA results in a dose-dependent increase in *c-fos* expression in the ipsilateral dorsal horn and the co-injection of an NMDA receptor antagonist with formalin suppresses formalin-induced *c-fos* expression

(Wang *et al.* 1997). Similarly, glutamate injection into the human trapezius muscle causes pain and mechanical hyperalgesia (Ge *et al.* 2005).

Animal studies have suggested that tissue inflammation or injury–related increased levels of peripheral glutamate might be involved in nociceptive mechanisms in deep craniofacial tissues. Glutamate has been shown to excite V ganglion neurons through activation of NMDA and non-NMDA receptors (Pelkey and Marshall 1998; Puil and Spiegelman 1988; Jackson and Hargreaves 1999; Cairns *et al.* 2001ab, 2002a, 2003a; Cairns 2005). Sessle and colleagues have recently identified a novel peripheral nociceptive role for glutamate within the TMJ and masticatory muscles. In their acute rat model of TMJ injury, glutamate injection into the TMJ evokes a concentration-related reflex increase in jaw muscle EMG activity (Cairns *et al.* 1998) similar to that evoked by the inflammatory irritants and algogenic compounds mustard oil (Yu et al. 1996) or capsaicin (Tang *et al*. 2004). This glutamate-evoked reflex response appears to reflect the integration of primary afferent drives with the central sensitization of central neurons involved in the TMJ reflex pathway since it can be abolished by brainstem lesions of Vc (Tsai *et al*. 1999; Cairns *et al.* 2001c). This reflex response results in the co-contraction of jaw-opening and jaw-closing muscles which is thought to represent a physiological “splinting” action to limit movement and prevent further injury (Hu *et al*. 1997; Sessle 1999, 2000, 2005). Moreover, this glutamate-evoked jaw muscle activity can be significantly attenuated by co-injection of NMDA and non-NMDA receptor antagonists into the TMJ (Cairns *et al.* 1998). Intramuscular (masseter) or TMJ injection of glutamate can also preferentially evoke activity in small-diameter, mechanosensitive afferent fibres and sensitize muscle and TMJ afferent fibres through activation of peripheral EAA receptors (Cairns *et al.* 2001ab, 2002a, 2003a; Gambarota *et al.* 2005). In particular, peripheral NMDA receptors may play an important role in glutamate-induced effects on nociceptive afferents since NMDA application to the masseter or temporalis muscles excites nociceptive afferents and NMDA receptor antagonists applied locally into these same sites significantly decrease glutamate-evoked afferent discharges (Cairns *et al.* 2003a; Dong *et al.* 2006, 2007).

Thus, these results suggest that peripheral ionotropic glutamate receptors are present in deep craniofacial tissues and when activated may contribute to the primary

hyperalgesia or allodynic states characteristic of craniofacial pain conditions such as TMD. Moreover, the concentration of glutamate injected into the masseter muscle to evoke afferent discharges through peripheral NMDA receptor activation approximates the concentration of glutamate that could be released on afferent excitation from presynaptic vesicles in masseter muscle afferent fibre terminals (Riveros *et al.* 1986; Gambarota *et al.* 2005). This increase in peripheral levels of glutamate may activate peripheral EAA receptors, a process that can modify the excitability of afferent fibres, lead to sensitization of cutaneous and joint tissues, and evoke nociceptive responses (Ault and Hildebrand, 1993ab; Carlton *et al.* 1995; Jackson *et al.* 1995; Yu *et al.* 1996; Davidson *et al.* 1997; Lawand *et al.* 1997; Cairns *et al.* 1998, 2001abc, 2002ab, 2003ab, 2006). The mechanisms responsible for increased peripheral levels of glutamate have not been investigated; however, since trauma has long been implicated in the aetiology and pathogenesis of TMD, it is possible that injury-induced tissue cell damage excites and sensitizes nociceptors through the release of cytosolic glutamate from affected neurons, dermal/epidermal cells (Nordlind *et al.* 1993), macrophages (Piani *et al.* 1991), released blood serum (McAdoo *et al.* 1997) or Schwann cells (Parpura *et al.* 1995). Sensory endings of nociceptors are unencapsulated so they are readily susceptible to cytosolic contents released from nearby damaged cells (Keele and Armstrong 1964; Fields 1987; Belmonte and Cervero 1996; see Cook and McCleskey 2002). Cytosol has a high concentration of glutamate, which if released in the extracellular medium, has been shown to activate KA channels on sensory neurons (Huettner 1990). Cutaneous nociceptors have also been shown to be excited and sensitized by exogenous application of glutamate (Du *et al.* 2000).

Peripheral metabotropic glutamate receptors also cannot be overlooked but their relative contribution to peripheral nociceptive responses has not been studied in detail in the V system. However, they may contribute to craniofacial nociception since they have been shown to modulate both peripheral and central spinal nociceptive processes (Jang *et al.* 2004; Lee *et al.* 2007; for review, see Karim *et al.* 2001, Neugebauer 2001, 2002; Varney and Gereau 2002). More recently, peripheral metabotropic receptors have been shown to have a role in masster muscle inflammation and behavioural mechanical

hypersensitivity in rats (Ahn *et al.* 2005; Lee *et al.* 2006; Lee and Ro 2007a,b), however, more studies are required.

The novel nociceptive role for peripheral glutamate receptors in animal models can also be demonstrated in humans. Glutamate injection into the masseter muscle induced pain in male and female human volunteers and caused significantly higher levels of peak pain, duration of pain, overall pain, and pain spread than injection of isotonic saline in both men and women (Cairns *et al.* 2001a, 2003b, Svensson *et al.* 2003, 2005). This evidence suggests that activation of peripheral EAA receptors may excite masseter muscle nociceptors that contribute to pain responses in humans and is consistent with the association between the development of hyperalgesia and elevated tissue levels of glutamate elsewhere in the body (McNearney *et al.* 2000). Furthermore, consistent with findings in rats, peripheral NMDA receptors may play a role in these effects of glutamate since ketamine, an NMDA antagonist, applied in combination with glutamate, decreases glutamate-evoked muscle pain in human males (Cairns *et al.* 2003a, 2006). Moreover, there is a sex-related difference in ketamine-mediated analgesia since peripherally administered ketamine has no effect on glutamate-evoked masseter muscle pain and sensitization in women (Castrillon *et al.* 2007). The glutamate injection also demonstrated signs of both peripheral and central sensitization, not unlike that found in TMD and related craniofacial pain conditions, since masseter muscle pressure pain thresholds were reduced, a sign of allodynia (Svensson *et al.* 2003), and muscle pain also spread to involve the TMJ, the temporal regions as well as the teeth in many of the volunteers (Cairns *et al.* 2001a).

1.5.2 Peripheral TRPV1 receptor

Another novel peripheral receptor that appears to be involved in nociceptive mechanisms is the vanilloid type 1 receptor (TRPV1). This non-selective cation channel receptor is activated by application to peripheral tissues of the inflammatory irritant capsaicin, protons (pH<6) or noxious heat (>43°C) (Caterina *et al.* 1997; Tominaga *et al.* 1998; Benham *et al.* 2003; Jordt *et al.* 2003; Dray 2005; Gold 2005; Woolf and Ma 2007) and results in the neurogenic release of EAAs into peripheral tissues (deGroot *et*

al. 2000). Capsaicin is a lipophillic vanilloid compound that renders hot chili peppers pungent. Capsaicin and the ultrapotent irritant resiniferatoxin bind to TRPV1 receptors on the peripheral terminals of nociceptors, mainly polymodal nociceptors. Capsaicin or its analogues trigger cation influx and, in repeat applications, desensitizes receptors, while at the same time its lower pH levels enhance receptor activation efficacy. Moreover, the release of high cytosolic concentrations of H^+ that may occur following cell damage can also depolarize nociceptors through proton-gated ion channels such as the TRPV1 receptor (Caterina *et al.* 1997; Tominaga *et al.* 1998; Caterina and Julius 1999; Benham *et al.* 2003; Jordt *et al.* 2003; Dray 2005; Gold 2005; Woolf and Ma 2007). Protons can interact directly with the vanilloid receptor to allosterically modulate channel function and have two main effects: the TRPV1 receptor can be activated at room temperature when the extracellular pH drops below 6 and protons can potentiate responses to capsaicin or heat, and do so over pH 6-8 (Julius and Basbaum 2001; Dray 2005; Gold 2005).

Capsaicin is a hydrophobic molecule that bears structural similarity to several lipid second messengers (Julius and Basbaum 2001). It has been proposed that anandamide may be the endogenous ligand for the TRPV1 receptor (Szolcsanyi 2000; van der Stelt *et al.* 2005; Woolf and Ma 2007) and it may also strengthen the effects of other TRPV1 receptor activators such as capsaicin (Di Marzo *et al.* 2001; van der Stelt *et al.* 2005). TRPV1-immunoreactive sensory neurons have been demonstrated in the rat and human V ganglion (Helliwell *et al.* 1998; Guo *et al.* 1999; Ichikawa and Sugimoto 2001, 2004; Hou *et al.* 2002; Stenholm *et al.* 2002; Renton *et al.* 2003; Ioi *et al.* 2006). Intense expression of both TRPV1-like immunoreactivity and TRPV1 mRNA in A-δ and C primary afferents (Caterina *et al.* 1997; Tominaga *et al.* 1998; Dray 2005; Gold 2005) is consistent with the selective action of capsaicin on small-diameter sensory neurons. Capsaicin application to the cutaneous RF has been shown to activate and sensitize nociceptive afferents such as C-polymodal, A-δ mechanical and heat nociceptor II and chemical specific nociceptors as well as dorsal horn neurons (Baumann *et al.* 1991; Peterson and LaMotte 1991; Simone *et al.* 1991, 1997; LaMotte *et al.* 1992; Marchettini 1996; Dray 2005; Gold 2005) and evoke intense pain and both primary and secondary hyperalgesia in humans (LaMotte *et al.* 1991; see Raja *et al.* 1999; Witting *et al.* 2000a;

Gottrup *et al.* 2000, 2004; Gazerani and Arendt-Nielsen 2005; Gazerani *et al.* 2006; Poyhia and Vainio 2006) and pain-related nocifensive behaviours in animals (Ko *et al.* 2000; Laird *et al.* 2001; Neubert *et al.* 2006).

To date, however, studies of capsaicin effects on deep nociceptive afferents and how they are modulated by central neural mechanisms are sparse. Pain and hyperalgesia from deep somatic tissue (*i.e.,* muscle and joint) have been shown to be processed differently than that from skin. Injection of capsaicin into deep tissues results in longer-lasting mechanical allodynia in rats (Sluka 2002) as well as more frequent referred pain in humans (Witting *et al.* 2000b) compared with injection into skin. When injected into the rat knee, capsaicin has been demonstrated to induce mechanical hyperalgesia whereas blockade of NK1, bradykinin and IL-1ß receptors can substantially modulate this hyperalgesia (Davis and Perkins 1996). In the craniofacial region, TRPV1 receptors have also been demonstrated on V afferents innervating the human tooth pulp (Renton *et al.* 2003; Morgan *et al.* 2005) and the rat TMJ (Ichikawa *et al.* 2004; Ioi *et al.* 2006). Capsaicin can activate and sensitize V afferents (Liu and Simon 1994, 1996, 2003; Strassman *et al.* 1996) and brainstem nociceptive neurons (Carstens *et al.* 1998; Zanotto *et al.* 2007). Hu and colleagues have recently documented that capsaicin injection into the rat TMJ also reflexly evokes a dose-dependent increase in jaw muscle EMG activity (Tang *et al.* 2004), and produces an inflammatory response within these tissues that can be blocked by TRPV1 receptor antagonists (Hu *et al.* 2005a). In addition, several studies have shown that capsaicin-induced jaw muscle pain is associated with changes in jaw motor function in humans (*e.g.,* Sohn *et al.* 2000, 2004; Wang *et al.* 2002). Taken together, the above findings suggest a role for peripheral TRPV1 receptors in both peripheral and central sensitization of V neurons involved in deep pain processing. However, the above underscores the limited information of capsaicin effects on deep tissues or afferents, and there has been no study at all of its effects on identified deep craniofacial afferents or TMJ-responsive nociceptive neurons. Also, while it is known that repeated capsaicin application produces desensitization (Baumann *et al.* 1991; Craft and Porreca, 1992; Liu and Simon 1996; Caterina *et al.* 1997; Szolcsanyi 2004; Gold 2005; Sikand and

Premkumar 2007), we know little about its interactions with other receptor agonists, such as glutamate.

1.5.3 Potential interactions between peripheral EAA and TRPV1 receptors

Previous studies examining the activity of knee joint and muscle small-diameter afferent fibres have reported that up to 75% of afferents respond to application of algesic compounds to their RFs (Mense 1993, 1996; Schaible and Grubb 1993; Reinohl *et al*. 2003; Hoheisel *et al.* 2004, 2005). It is not yet known what proportion of craniofacial joint and muscle afferents respond to capsaicin or whether there is overlap in the response incidence of two different chemicals within a subpopulation of deep afferents- namely glutamate and capsaicin. The above data (see section 1.5.1) suggest that changes in peripheral glutamate levels may play an important role in modulating the sensitivity of cutaneous and deep tissues, although it is still unclear what types of interactions exist between peripheral EAA and other peripheral receptor mechanisms, such as TRPV1, and how this nociceptive information is modulated by central neural mechanisms. This is of particular interest since in addition to direct activation and modulation of nociceptive neuronal responses, evidence suggests that EAA receptors may also indirectly modulate nociception via interactions with other nociceptive receptors (Mitsikostas *et al.* 1998; Afrah *et al.* 2001; Palazzo *et al.* 2002). There is evidence suggesting NMDA receptor mechanisms may modulate TRPV1 activity in the CNS. For example, studies have demonstrated that both capsaicin-evoked release of spinal SP (Afrah *et al.* 2001) as well as capsaicin-evoked activity at the periaqueductal grey level (Palazzo *et al.* 2002; Xing and Li 2007) may be dependent on the release of glutamate acting on NMDA receptors. A role for NMDA receptor modulation of capsaicin-induced *c-fos* expression within the rat Vc has also been demonstrated (Mitsikostas *et al.* 1998). Sessle and colleagues (Yu *et al*. 1996) have also demonstrated a role for peripheral EAA receptors in mediating the nociceptive responses elicited by mustard oil-evoked activation of the peripheral TRPA1 (ANKTM1, see Dray 2005, Gold 2005) receptor, also a member of the TRP family. Peripheral NMDA receptors, in particular, may play a role in mediating increases in jaw muscle activity resulting from application of mustard oil to

the TMJ region since application of the non-competitive NMDA receptor antagonist MK-801 into the TMJ region attenuates mustard oil-evoked jaw muscle activity (Yu *et al*. 1996).

As noted above, Hu and colleagues have recently shown that TRPV1 is a novel peripheral receptor in the TMJ region since capsaicin injected into the rat TMJ produces an inflammatory response (Hu *et al.* 2005a) and induces a dose-dependent increase in jaw muscle EMG activity (Tang *et al*. 2004). It is not yet known whether peripheral TRPV1 receptors in craniofacial tissues can be modulated by peripheral EAA receptor mechanisms and vice versa but it is possible EAA receptors co-exist with TRPV1 receptors in some single primary afferent fibres since EAA receptors are widely distributed. Such a relationship would suggest that EAA receptors may play an important modulatory function, such as sensitization of V nociceptors to subsequent TRPV1 receptor activation of V nociceptors as suggested from limb afferents (Lawand *et al.* 2000). Moreover, there is evidence of a major coupling between GPCRs and some TRP channels in the membrane such as TRPA1 and TRPV1 (Sikand and Premkumar 2007; Woolf and Ma 2007). For example, bradykinin can significantly potentiate TRPV1 activity by activating the PLC-PKC pathway (Sikand and Premkumar 2007). Activation of the PKC pathway has also been shown to lower the heat threshold of TRPV1 below body temperature and sensitize TRPV1 responses to capsaicin (Premkumar and Ahern 2000; Crandall *et al.* 2002). The mechanism behind this effect is thought to involve direct phosphorylation resulting in PKC-dependent insertion of TRPV1 receptors into the neuronal membrane (for review, see Hucho and Levine 2007). This type of coupling may also exist between TRPV1 and other GPCRs, including the metabotropic EAA receptors, and provide a means for potential interactions between EAA and TRPV1 receptors. It is well known that capsaicin can desensitize (Baumann *et al.* 1991; Craft and Porreca, 1992; Caterina *et al.* 1997; Serra *et al.* 2004; Szolcsanyi 2004; Gold 2005) afferent fibres, thus TRPV1 receptor activation of V nociceptors may render subsequent glutamate activation of EAA receptors ineffective in exciting V nociceptors.

1.5.4 Surgical trauma

As stated above (see section 1.5-1.5.2), injury-induced tissue cell damage and its consequent activation and sensitization of nociceptors (*e.g.* via peripheral chemical mediators such as glutamate) may play an important role in mediating many pain states. The following will review what is known about the effect of surgical trauma on nociceptive processing.

Although there has been much research on the effect of peripheral chemical receptor mechanisms on nociceptive activation and peripheral sensitization, little attention has been paid to the effects of direct mechanical tissue trauma on central nociceptive processing. This is surprising since direct tissue trauma, be it accidentally or surgically-induced, is a frequent contributor to acute pain and may predispose patients to chronic pain states (Coderre *et al.* 1993; Kalso 1997; Katz 1997, 2001; Goldman 2002; American Pain Society 2003; Watt-Watson *et al.* 2004ab; ANZCA 2005). Unencapsulated sensory endings of nociceptors are readily susceptible to cytosolic contents released from nearby damaged cells (Keele and Armstrong 1964; Fields 1987; Belmonte and Cervero 1996; see Cook and McCleskey 2002). In addition, although the mechanisms behind noxious mechanical transduction remain relatively unknown, there are several candidates for high-threshold mechanotransducers such as TRPs, ASICs, and K^+ channels (Hu *et al.* 2006; Woolf and Ma 2007). As a result, surgical incision may produce activation and peripheral sensitization of nociceptive afferents (Omote *et al.* 2001; Dalle and Eisenach 2005). This, in turn, could lead to effects on central nociceptive processing. Rat hindpaw (Brennan *et al.* 1996; Vandermeulen and Brennan 2000; Pogatzki *et al*. 2002a; Kawamata *et al.* 2005a) and human forearm (Kawamata *et al.* 2002) models of postoperative pain have demonstrated central sensitization following surgical incision. Evidence from the rat incision models above have suggested similar nociceptive responses between animals with skin and fascia incisions and skin, fascia, and muscle incisions (Brennan *et al.* 1996; Vandermeulen and Brennan 2000). However, the effect of cutaneous incision on central nociceptive neurons with deep afferent inputs has not been investigated since the above studies did not test for the presence of a deep afferent RF in the spinal dorsal horn neurons examined.

Previous studies on sensitization suggest that activation of muscle or deep tissue afferents produces more powerful sensitization than cutaneous afferents in both spinal

(Cook *et al.* 1987; Woolf and Wall 1986; Graven-Nielsen *et al.* 2006) and V systems (Yu *et al.* 1993). In addition, injection of capsaicin into deep tissues results in longer-lasting secondary mechanical allodynia in rats (Sluka 2002) as well as more frequent referred pain in humans (Witting *et al.* 2000b) compared with injection into skin. These findings raise the possibility that cutaneous incision-induced changes in central neuronal excitability may modulate central processing of subsequent noxious stimulation of deep tissues. However, there have been no studies of the effects of such peripheral injury on central neuronal processing of deep afferent inputs in spinal dorsal horn or Vc/UCC, including afferent inputs evoked by noxious stimuli such as capsaicin.

Evidence in animals and humans have suggested that pre-emptive analgesia may reduce the surgical incision-induced afferent barrage and may reduce postoperative pain states. For example, local anaesthetic pretreatment in rat hindpaw incision models prevented spinal incision-induced *c-fos* expression (Sun *et al.* 2004) and rat pain behaviors for several hours after incision (Pogatzki *et al.* 2002a; Sun *et al.* 2004). However, the promising results from animal models have often not been translated into clinical practice. Many clinical studies failed to demonstrate major benefits of pre-emptive analgesia and recent systematic reviews indicate that the effects on postoperative pain are equivocal (Kaufman *et al.* 2005; Pogatzki-Zahn and Zahn 2006; Grape and Tramèr 2007).

1.6 SPECIFIC HYPOTHESES AND STUDY AIMS:

1.6.1 Issues and Hypotheses

The above review reveals several unresolved issues that raise several questions related to the peripheral and central mechanisms involved in nociceptive processing of deep craniofacial afferent inputs to the CNS: 1) Are identified TMJ and jaw muscle-responsive nociceptive inputs modulated by peripheral glutamatergic mechanisms? 2) Are identified TMJ and jaw muscle-responsive nociceptive inputs modulated by peripheral capsaicin-sensitive mechanisms? 3) Are there interactions between these peripheral

glutamatergic and capsaicin-sensitive mechanisms? and 4) Are there differences in central nociceptive processing of peripheral glutamatergic and capsaicin-sensitive afferent inputs? This doctoral study addresses these questions by testing the following hypothesis:

Hypothesis 1: ***Peripheral glutamatergic and capsaicin-sensitive mechanisms modulate the properties of primary afferents and brainstem neurons processing deep craniofacial nociceptive information.***

It was also noted that tissue trauma (microtrauma or macrotrauma) has long been implicated in the aetiology and pathogenesis of TMD. However, there have been no studies of the effects of direct mechanical tissue injury on central neuronal processing of deep afferent inputs in the Vc/UCC, including afferent inputs evoked by noxious stimuli such as capsaicin. Questions related to the effect of tissue injury on V nociceptive processing include: 1) Are TMJ-responsive nociceptive brainstem neurons modulated by surgical incision? and 2) Does surgical incision modulate central nociceptive processing of peripheral capsaicin-sensitive mechanisms? This doctoral study addresses these questions by testing the following hypothesis:

Hypothesis 2: ***Surgical cutaneous incision modulates the properties of brainstem neurons processing deep craniofacial nociceptive information.***

1.6.2 Aims and Significance

Since we are interested primarily in studying the existence of, and potential interaction between peripheral glutamatergic and capsaicin-sensitive mechanisms in nociceptive processes involving deep craniofacial tissues, an acute rat TMJ injury model has been used in this study. As reviewed above, the common signs and symptoms of TMD are suggestive of a role for both peripheral and central sensitization in TMD-associated

pain. It is therefore important to study both peripheral as well as central nociceptive processes. The model employs single unit recording of primary afferents and nociceptive neurons in caudal Vc/UCC to study peripheral and central nociceptive processes, respectively, in the TMJ and associated musculature. Moreover, the study incorporates recording of reflexly evoked jaw muscle EMG activity as well as single unit recording to investigate the integrative effects of peripheral and central nociceptive processes.

To address ***Hypothesis 1*** with respect to ***peripheral nociceptive processing***, this doctoral study has the following aims:

AIM 1-1: To determine if nociceptive afferents supplying TMJ or associated musculature and projecting to the caudal Vc/UCC can be modulated by peripheral glutamatergic mechanisms.

AIM 1-2: To determine if nociceptive afferents supplying TMJ or associated musculature and projecting to the caudal Vc/UCC can be modulated by peripheral capsaicin-sensitive mechanisms.

AIM 1-3: To determine if there are interactions between peripheral glutamatergic and capsaicin-sensitive mechanisms that modulate the properties of nociceptive afferents supplying TMJ or associated musculature and projecting to the caudal Vc/UCC.

To address the ***Hypothesis 1*** with respect to ***central nociceptive processing***, this doctoral study has the following aims:

AIM 1-4: To determine if TMJ-responsive nociceptive neurons in the caudal Vc/UCC can be modulated by peripheral glutamatergic mechanisms.

AIM 1-5: To determine if TMJ-responsive nociceptive neurons in the caudal Vc/UCC can be modulated by peripheral capsaicin-sensitive mechanisms.

AIM 1-6: To determine if there are interactions between peripheral glutamatergic and capsaicin-sensitive mechanisms that modulate the properties of TMJ-responsive nociceptive neurons in the caudal Vc/UCC.

Significance. This is the first study to investigate if peripheral glutamatergic and capsaicin-sensitive mechanisms do indeed influence both peripheral and central nociceptive processing in deep craniofacial tissues. Moreover, by using the same experimental paradigm in both peripheral and central processing studies on glutamate and capsaicin-evoked responses in nociceptive afferents supplying deep craniofacial tissues, this is the first study to provide direct comparisons of peripheral versus central nociceptive neural responses to peripherally applied glutamate and capsaicin and determine if there are differences in peripheral compared to central nociceptive processing of these peripheral receptor mechanisms.

The results of this doctoral study will contribute further to our understanding of peripheral influences on peripheral and central sensitization. The demonstration of a relationship between peripheral glutamate and/ or capsaicin receptor mechanisms and craniofacial pain may lead to the development of novel diagnostic and therapeutic approaches for TMD and other craniofacial pain conditions.

To address ***Hypothesis 2*** with respect to the effects of ***surgical cutaneous incision***, this doctoral study has the following aims:

AIM 2-1: To determine if a surgical incision of the skin and fascia overlying the TMJ can induce central sensitization in TMJ-responsive nociceptive neurons in the caudal Vc/UCC.

AIM 2-2: To determine if the surgical incision can influence the central sensitization induced by the subsequent injection of capsaicin into the TMJ in TMJ-responsive nociceptive neurons in the caudal Vc/UCC.

Significance. This is the first study to detail the effect of surgical cutaneous incision on V nociceptive neurons and the expression of central sensitization in Vc/UCC. The demonstration of surgical cutaneous incision-induced central sensitization may contribute to our understanding of the pain spread and referral and to the development of allodynia that have been documented in postoperative pain states (see Melzack *et al.* 2001; Kawamata *et al.* 2002). Moreover, this doctoral study may also provide support for the use of pre-emptive regional analgesia in addition to general anaesthesia prior to surgery in order to attenuate or prevent incision-induced activation and central sensitization in V nociceptive pathways.

Chapter 2

Glutamate and capsaicin-induced sensitizing effects on nociceptive trigeminal primary afferents and brainstem neurons activated by deep craniofacial stimulation

2.0 ABSTRACT:

Effects of peripheral application of glutamate and capsaicin were tested on functionally identified deep craniofacial afferents and trigeminal subnucleus caudalis/upper cervical cord (Vc/UCC) neurons. The activity of single trigeminal nociceptive afferents or Vc/UCC neurons with mechanoreceptive fields (RF) in deep craniofacial tissues were recorded in 89 halothane-anesthetized rats. The mechanical activation threshold (MAT) of each afferent and neuron was assessed before and after injection of 0.5M glutamate and 1% capsaicin (or vehicle) into the RF. When injected alone, glutamate and capsaicin activated and induced peripheral sensitization reflected as MAT reduction in many afferents. Following glutamate injection, capsaicin-evoked activity was greater than that evoked by capsaicin alone, whereas following capsaicin injection, glutamate-evoked responses were similar to glutamate alone. When injected alone, glutamate and capsaicin also activated and induced central sensitization (reflected in cutaneous RF expansion and cutaneous and/or temporomandibular joint (TMJ) MAT reduction) in most Vc/UCC neurons. Following glutamate injection, capsaicin evoked greater activity and less cutaneous or TMJ MAT reduction compared with capsaicin alone, whereas capsaicin abolished all subsequent glutamate-evoked activity and depressed cutaneous RF expansion in most neurons. Glutamate effects on deep afferents and Vc/UCC neurons were analogous since glutamate sensitized afferent and neuronal responses to capsaicin. However, the desensitizing effects of capsaicin on glutamate-evoked excitability of Vc/UCC neurons contrast with the lack of capsaicin-induced modulation of glutamate-evoked afferent excitability, suggesting that peripheral

and central sensitization may be differentially involved in the nociceptive effects of glutamate and capsaicin applied to deep craniofacial tissues.

2.1 INTRODUCTION:

There is evidence that both peripheral excitatory amino acid (EAA) and vanilloid (TRPV1) receptor mechanisms may modulate nociceptive processing of input from deep craniofacial tissues. Glutamate, the endogenous agonist for EAA receptors, is a well-documented central excitatory neurotransmitter but a number of studies indicate a role for *peripheral* glutamate receptors in the transduction of nociceptive information (Yu *et al.* 1996; Cairns *et al.* 1998; Lawand *et al.* 2000; McNearney *et al.* 2000, 2004; Carlton 2001; Carlton *et al.* 2003; Lam *et al.* 2005). A novel *peripheral* nociceptive role for glutamate in the craniofacial region has been identified. Intramuscular (masseter) or temporomandibular joint (TMJ) injection of glutamate reflexly evokes a dose-dependent increase in rat jaw muscle activity (Cairns *et al.* 1998), and activates and sensitizes mechanosensitive nociceptive afferents through the activation of peripheral EAA receptors (Cairns *et al.* 2001a,b, 2002a, 2003a; Dong *et al.* 2006, 2007). The peripheral EAA receptor N-methyl-D-aspartate (NMDA) in particular may play an important role in glutamate-induced effects on nociceptive afferents since NMDA receptor antagonists applied locally into masticatory muscles significantly decrease glutamate-evoked afferent activation and sensitization as well as jaw muscle activity (Cairns *et al.* 1998, 2003a, 2007; Dong *et al.* 2006, 2007). Similarly, glutamate injection into the human masseter muscle causes pain that may be attenuated by co-injection of an NMDA receptor antagonist (Cairns *et al.* 2001a, 2003a,b, 2006; Svensson *et al.* 2003, 2005).

Another peripheral receptor involved in craniofacial nociceptive mechanisms is the TRPV1 receptor. This receptor is activated by application to peripheral tissues of the inflammatory irritant capsaicin, protons or noxious heat (Caterina *et al.* 1997; Tominaga *et al.* 1998; Benham *et al.* 2003; Jordt *et al.* 2003; Dray 2005; Gold 2005). Capsaicin can activate (Liu and Simon 1994, 1996, 2003) and sensitize trigeminal afferents (Strassman *et al.* 1996) and also activate brainstem nociceptive neurons (Carstens *et al.* 1998; Zanotto *et al.* 2007). Furthermore, capsaicin injection into the rat TMJ reflexly evokes a dose-dependent increase in

jaw muscle activity (Tang *et al.* 2004; Chapter 3), and produces an inflammatory response within these tissues that can be blocked by TRPV1 receptor antagonists (Hu *et al.* 2005a). Capsaicin injected into human craniofacial regions can also induce secondary hyperalgesia, allodynia and jaw muscle pain associated with changes in jaw motor function (Sohn *et al.* 2000, 2004; Wang *et al.* 2002; Gazerani and Arendt-Nielsen 2005; Gazerani *et al.* 2006).

However, there has been little investigation of possible interactions between peripheral EAA and TRPV1 receptors and their effect on nociceptive processing. There is evidence that EAA receptors may indirectly modulate nociception via interactions with TRPV1 in the central nervous system. Both capsaicin-evoked release of spinal substance P (Afrah *et al.* 2001) and capsaicin-evoked activity at the periaqueductal grey level (Palazzo *et al.* 2002; Xing and Li 2007) may be dependent on glutamate-evoked activation of NMDA receptors. A role for NMDA receptor modulation of capsaicin-induced *c-fos* expression within the rat trigeminal subnucleus caudalis (Vc) has also been demonstrated (Mitsikostas *et al.* 1998). Similarly, the presence of EAA and TRPV1 receptors on primary afferents and findings that their agonists have effects when injected into peripheral tissues raise the possibility that the actions of peripherally applied glutamate and capsaicin could involve interactions between EAA and TRPV1 receptors in these tissues. Indeed, jaw muscle activity reflexly evoked by the TMJ application of capsaicin can be attenuated by pre-injection into the TMJ of NMDA receptor antagonists (Chapter 3), suggesting that peripheral EAA receptors may interact with peripheral TRPV1 receptor mechanisms. While it is also well known that capsaicin can sensitize or desensitize nociceptive primary afferents (Szolcsanyi *et al.* 1975; Baumann *et al.* 1991; Craft and Porreca 1992; Dray 1992; LaMotte *et al.* 1992; Liu and Simon 1996; Caterina *et al.* 1997; Szolcsanyi 2004; Gold 2005; Sikand and Premkumar 2007), it is not known whether glutamate or capsaicin would sensitize or desensitize each other's effects on afferent responses. In addition, there is no knowledge of how these peripheral glutamate and capsaicin effects might influence central neurons involved in processing of deep nociceptive information such as those found in the Vc/upper

cervical spinal cord (Vc/UCC), an important site for processing of nociceptive afferent inputs from TMJ and other deep craniofacial tissues (for review, see Bereiter *et al.* 2000; Sessle 2005; Hu *et al.* 2005b). Thus, the aim of the present study was to examine the effects of peripheral application of glutamate and capsaicin on functionally identified deep craniofacial afferents and TMJ-responsive nociceptive neurons in the Vc/UCC. The data have been briefly presented in abstract form (Lam *et al.* 2003a,b, 2004a,b).

2.2 MATERIALS AND METHODS:

ANIMAL PREPARATION.

Adult male (*n*=89, 250-400 g) Sprague-Dawley rats were prepared for acute *in vivo* recording activity of trigeminal afferents or Vc/UCC neurons under surgical anesthesia (O_2: 1 L/min; halothane: 1.5–2.5%; Cairns *et al.* 2001a,b). A tracheal cannula was inserted and artificial ventilation initiated. The rat's head was then placed in a stereotaxic frame and the skin over the dorsal surface of the skull was reflected. For afferent recordings, a trephination was made on the left side of the skull to allow a recording microelectrode to be lowered through the brain and into the trigeminal ganglion. An incision was also made in the skin overlying the Vc/UCC region, a C1 laminectomy was performed, and the dura was reflected to expose the Vc/UCC and facilitate placement of a stimulating microelectrode in the left Vc/UCC (Hu 1990; Cairns *et al.* 2001a,b; Hu *et al.* 2005b).

After completion of all surgical procedures, the halothane level was slowly reduced to a level (1–1.3%) that was just sufficient to produce reflex suppression of the hindlimb to noxious pressure applied to the hindpaw to ensure that an adequate level of anesthesia was maintained for the duration of the experiment. Heart rate and body core temperature were continuously monitored throughout the experiment and kept within the physiological range of 330–430/min and 37–37.5°C, respectively. All procedures were approved by the University of Toronto

Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Canada).

RECORDING AND STIMULATING PROCEDURES.

Deep craniofacial afferents. Extracellular activity of single trigeminal nociceptive primary afferents with receptive fields (RFs) in deep craniofacial tissues (masseter or temporalis muscles, or TMJ) was recorded with an epoxy-resin-coated tungsten microelectrode. One hour after completion of surgery, the microelectrode was slowly lowered into the brain under stereotaxic guidance (anterior 3.5-4 mm, lateral 3-4 mm) until afferent discharges were observed in response to light brush stimuli applied to the craniofacial region; these discharges were usually found 7-8 mm below the cortical surface. A round dental burnisher (1-mm diameter) was then applied as a noxious mechanical search stimulus (~100 g) over the craniofacial cutaneous tissues while the microelectrode was slowly lowered in an attempt to identify trigeminal afferents with deep craniofacial nociceptive RFs (Cairns *et al.* 2001b).

When an afferent that responded to direct, blunt noxious mechanical stimuli was found, a careful assessment of its RF was made to ascertain that the afferent was responding to deep as opposed to cutaneous stimulation. The skin overlying the RF was pulled gently away from contact with the deep tissue, and brush, pinch, and pressure stimuli were applied directly to the skin surface. If the afferent did not respond to any of these cutaneous stimuli, then the afferent was considered to have a deep mechanonociceptive RF. The RF and mechanical activation threshold (MAT) of the afferent was assessed at the time intervals specified under the experimental paradigm (see below). The deep RF of each afferent was determined through the use of a round dental burnisher. The size and location of the afferent's deep RF was also outlined on a life-size drawing of the rat's head. The MAT of the afferent's deep RF was determined with an electronic von Frey device (SENSELab Model 735, 1.0-mm diameter probe tip, Somedic Sales AB, Sweden) applied to the center of the RF and was defined as the force (g) required to evoke the first spike, or a firing rate of greater than 2

standard deviations above baseline afferent activity when the afferent was spontaneously active, measured at the afferent's deep RF site with a ramp of gradually increasing force.

To test whether an afferent with a deep craniofacial RF projected to the caudal brainstem, electrical stimuli (50 μs biphasic pulse, range 10-80 μA, 0.5 Hz) were applied to a stimulating electrode lowered into the left Vc/UCC (0-6 mm caudal to obex). The stimulating electrode was moved mediolaterally (0.1-mm steps) and rostrocaudally (0.5-mm steps) in the Vc/UCC until electrical stimulation evoked antidromic responses as determined by classical criteria for antidromic activation (all-or-none at threshold, invariant latency, high-frequency following (≥ 100 Hz), and collision) (Darian-Smith 1960; Price *et al.* 1976; Cairns *et al.* 1996, 2001a,b). The initial electrical stimuli were applied 6 mm caudal to the obex. If stimulation at this location did not evoke an antidromic action potential, the stimulating electrode was moved rostrally toward the obex until either an antidromic action potential was evoked or electrical stimuli had been applied unsuccessfully up to the level of the obex. Antidromic action potentials were collided with the orthodromic action potentials evoked by mechanical stimulation of the deep craniofacial RF, to confirm the projection of the deep nociceptive afferent to the caudal brainstem. At the end of the study, the antidromic conduction velocity (CV) of the afferent was determined by calculating the straight-line distance between the stimulating microelectrode placed in the Vc/UCC and the recording microelectrode, divided by the antidromic latency. The skin overlying the deep craniofacial RF of the afferent was also surgically excised and it was confirmed that mechanical and/or electrical (50-100 μs biphasic pulse, range 10-80 μA, 0.5 Hz) stimuli applied directly to the deep tissue could also evoke activity in the afferent. If electrical stimulation applied to the deep tissue RF evoked an orthodromic action potential of invariant latency (<0.2 ms variability) with the ability to follow high-frequency electrical stimuli (≥ 100 Hz), then the conduction distance between the stimulation location and the trigeminal ganglion was estimated and an orthodromic CV calculated. For afferents that could not be demonstrated to project to the Vc/UCC, only orthodromic CVs were determined.

TMJ-responsive Vc/UCC neurons. Using a similar search procedure as above, a microelectrode was slowly lowered into the exposed Vc/UCC region under stereotaxic control (3-6 mm caudal to obex, 0.5–3 mm lateral to the midline) until the extracellular activity of a single TMJ-responsive nociceptive neuron was isolated and identified (Hu 1990). When a neuron was found that responded to direct, blunt noxious mechanical stimulation of the TMJ, the nociceptive neuron was considered *TMJ-responsive*. Mechanical (brush, pressure, and pinch) stimuli were applied to the craniofacial cutaneous tissues to classify each TMJ-responsive nociceptive neuron as wide dynamic range (WDR) or nociceptive-specific (NS). Mechanical (pinch) stimuli were also applied to contralateral skin as well as to selected spinally innervated tissues (e.g. paws). Baseline spontaneous neuronal activity (spikes/second) was recorded for a 10-minute period prior to mechanical and chemical stimulation. The cutaneous RF size and MAT of the TMJ-responsive nociceptive neurons were assessed at the time intervals specified under the experimental paradigm (see below). The cutaneous RF of each neuron was determined through the use of a brush, blunt probe, and a pair of nonserrated forceps. The extent of the neuron's cutaneous RF was also outlined on a life-size drawing of the rat's head. The MAT of the neuronal RF was determined with an electronic von Frey device applied to the center of the cutaneous RF or the TMJ region (similar as above with deep craniofacial afferents).

RECEPTOR AGONISTS.

Glutamate (0.5M; 10 µL; Sigma Chemical Company, St. Louis, MO), 1% capsaicin (10% capsaicin in ethanol:Tween-80: sterile normal saline in a 1:1:8 ratio by volume; 10 µL; Calbiochem, La Jolla, CA) or vehicle (isotonic saline as control for glutamate or ethanol:Tween 80:sterile normal saline in a 1:1:8 ratio by volume as control for capsaicin; 10 µL) was injected into the deep craniofacial afferent RF or TMJ for TMJ-responsive Vc/UCC neurons at 30 minute intervals in both experimental subgroups of rats. The concentrations of glutamate and capsaicin were chosen on the basis of their efficacy in evoking jaw muscle

activity and inflammation and we have previously documented that such injected solutions are localized to the site of injection (Cairns *et al.* 1998, 2001a,b, 2002a, 2003a; Tang *et al.* 2004; Hu *et al.* 2005a). All solutions were adjusted to approximate physiologic pH (7.2- 7.6).

EXPERIMENTAL PARADIGM.

Experimental animals were divided into subgroups according to the sequence of injection of receptor agonists: afferent response properties of rats with glutamate (or vehicle) injection followed by capsaicin (or vehicle) injection in one subgroup of rats were compared with properties of afferents in a second subgroup of rats with capsaicin (or vehicle) injection followed by glutamate (or vehicle) injection. To control for possible activation or sensitization effects from the repeated needle insertion or mechanical distension of the deep tissues during the second agonist injection, afferent response properties of additional subgroups of rats with a second injection of glutamate vehicle control or capsaicin vehicle control following injection of the first agonist were also studied. Analogous subgroups were used for a similar comparison of Vc/UCC neuronal properties.

Deep craniofacial afferents. The following experimental paradigm was applied to the subgroups in the afferent study: 10 minutes after a nociceptive afferent was classified on the basis of deep RF, antidromic CV and response properties as an Aδ (CV ≥ 2.5-30 m/s) or C (CV < 2.5 m/s) fiber nociceptive afferent (Price *et al.* 1976; Cairns *et al.* 2001a,b), the baseline MAT (in g) was determined by averaging the threshold for three consecutive mechanical stimuli applied at 1-minute intervals. The needle tip of a catheter (a 27-gauge needle connected by polyethylene tubing to a Hamilton syringe, 100 µL) was carefully inserted into the deep tissue RF of the afferent. It was observed that insertion of the catheter used to inject the receptor agonist evoked a spike discharge in all nociceptive afferents identified in order to confirm that the injection site was within the afferent RF. Baseline afferent activity was recorded for 10 minutes prior to injection of the first receptor agonist or vehicle control into the deep tissue RF. The receptor agonist or vehicle control was then slowly injected into the deep tissue (over a 5-second

period). The following four response properties were assessed over the next 10 minute period: (1) *Response magnitude (Rmag)*: the total number of evoked spikes, or a firing rate of greater than 2 standard deviations above baseline afferent activity when the afferent was spontaneously active, following agonist or vehicle injection, (2) *Response duration (Rdur)*: the total time (seconds) from the first spike following agonist or vehicle injection to the last spike, (3) *Response latency (Rlat)*: the total time (seconds) from agonist or vehicle injection to the first spike following agonist injection, and (4) *Peak frequency (Pfreq)*: the highest firing rate in a one second period (Hz) during the Rdur. The needle was then withdrawn at the end of the 10 minute period and an assessment was made at this 10 minute time point and again at 20 minute after the injection of the receptor agonist or vehicle control to determine if any MAT changes from baseline had occurred. *MAT reduction* was defined as ≥50% threshold reduction from baseline MAT score measured at the center of the RF site. Raw MAT threshold values measured post-injection were normalized to the initial baseline pre-injection value to illustrate population responses. The same protocol described above was used for injection of the second receptor agonist (or vehicle) 30 minutes post-injection of the first receptor agonist (or vehicle).

In 13 rats, it was possible to examine the effect of injected receptor agonists on more than one afferent on the ipsilateral left side because the RFs of the afferents were at different deep tissue sites or opposite ends of the same muscle. In these cases, which helped minimize the total number of animals used in the present study, a minimum of 2 hours elapsed between injections into the RFs.

TMJ-responsive Vc/UCC neurons. Similar to above, the following experimental paradigm was applied to the subgroups in the Vc/UCC neuron study: 10 minutes after a TMJ-responsive nociceptive neuron was identified as a WDR or NS neuron, the neuronal cutaneous RF and cutaneous MAT were determined and served as baseline values. A short-acting local anesthetic (2% Lidocaine in 10 μL) was infiltrated subcutaneously 15 minutes prior to incision of the skin overlying the left TMJ to reduce the effects of the incision-induced afferent barrage on neuronal excitability (Chapter 4). The skin overlying the TMJ was

then raised with tissue forceps to avoid damage to the TMJ and deep tissues, and a surgical incision (<2 mm long) was made with a 16-gauge needle through skin and fascia overlying the left TMJ to facilitate later injection of receptor agonists (or vehicle) into the TMJ and allow direct assessment of the TMJ MAT before and after receptor agonist injection. Neuronal cutaneous RF and MAT were reassessed at 10 and 20 minutes post-incision to ensure there were no changes from baseline values (Chapter 4). In addition, the TMJ MAT was determined at 10 and 20 minutes post-incision. Thirty minutes after incision, the tip of a catheter was carefully inserted into the left TMJ through the incision site. It was observed that subsequent insertion of the catheter used to inject receptor agonists or vehicle control into the TMJ evoked a spike discharge in all TMJ-responsive neurons identified in order to confirm that the injection site was within the neuronal RF. The same protocol described above for craniofacial afferents was used for injection of receptor agonists (or vehicle) as well as assessment of its effects on TMJ-responsive Vc/UCC neurons. In each animal, only one TMJ-responsive neuron was tested with the above protocol.

Cutaneous RF expansion was defined as expansion in RF size to include a predetermined point lying 5 mm outside the perimeter of the baseline cutaneous RF. This methodology for assessment of RF reduces the possibility of iatrogenically induced sensitization by avoiding multiple sites of noxious stimulation. In a small number of neurons, the border of the cutaneous RF was mapped in detail for illustration purposes (e.g. Figs. 3 and 4). *Cutaneous MAT reduction* was defined as ≥ 50% threshold reduction from baseline MAT score measured at the center of the cutaneous RF site. *TMJ MAT reduction* was defined as ≥ 50% threshold reduction from baseline MAT score measured at TMJ RF sites. Raw MAT threshold values measured post-injection were normalized to the initial baseline pre-injection value to illustrate population responses.

TERMINAL PROCEDURES.

At the end of each experiment recording deep craniofacial afferents, rats were euthanized with the agent T61 (Hoechst, Canada). The brain was removed and it

was confirmed that microelectrode tracks were visible on the surface of the trigeminal ganglion. Whereas at the end of each experiment recording TMJ-responsive Vc/UCC neurons, electrolytic lesions were made in the Vc/UCC recording site of rats by applying a monopolar, monophasic current pulse of 10 μA for 10 seconds to identify the recording site. Rats were then euthanized with the agent T61 (Hoechst, Canada). The unit recording sites confirmed histologically by hematoxylin and eosin staining were subsequently reconstructed and plotted on standardized diagrams of the brainstem (Paxinos and Watson 1997).

DATA ANALYSIS.

Recorded afferent or neuronal activity was stored electronically and analyzed off-line (Hu 1990). Most of the population data are reported as mean±SE. However, if not normally distributed, population data are reported as median values with interquartile ranges indicated in square brackets; median [IQR]. Mann-Whitney U test, t-test, Fisher exact test, and RM ANOVA-on-ranks were used as appropriate ($p<0.05$ considered to reflect statistical significance).

2.3 RESULTS:

NEURONAL PROPERTIES:

***Deep craniofacial afferents*.** A total of 68 trigeminal nociceptive primary afferents with RFs in deep craniofacial tissues were recorded: 54 Aδ-fiber and 14 C-fiber afferents. The estimated antidromic (mean±SE= 9.8±0.8 m/s) and orthodromic (8.6±2.0 m/s) CVs were not statistically different ($p>0.05$, Mann-Whitney U test). The majority of afferents could be activated antidromically and orthodromically (60/68); the remainder were activated only orthodromically (8/68) (mean pooled antidromic and orthodromic CV= 7.7±0.8 m/s). A small proportion (10%, 7/68, 6 Aδ-fiber, 1 C-fiber) were spontaneously active (7/68; 0.38±0.12

spikes/second) prior to injection of receptor agonists. Table 1 displays the baseline mean CV and MAT for afferents with RFs in the TMJ (n=40), masseter (n=23) and temporalis (n=5).

There were no differences between the Aδ-fiber and C-fiber afferents in baseline MAT ($p>0.05$, t-test) (Table 1) or in responses (Rmag, Rdur, Rlat and Pfreq) to glutamate and capsaicin ($p>0.05$, Mann-Whitney U test; $p>0.05$, Fisher exact test) (data not shown) and as a result, the data were pooled together for analysis of glutamate and capsaicin-induced activation and sensitization. Examples of typical afferent RF and response properties are shown in Fig. 1.

TMJ-responsive Vc/UCC neurons. The properties of 49 nociceptive neurons (37 NS, 12 WDR) that responded to noxious mechanical stimulation of the TMJ region and that were recorded primarily in the deep laminae (III-V) of the Vc/UCC region were studied (Fig. 2). Examples of typical neuronal RF and response properties are shown in Fig. 3 and 4 and their histologically confirmed recording sites are shown in Fig. 2. Less than 5% (2/49, 1 NS, 1 WDR) of these neurons were spontaneously active (0.22±0.15 spikes/second) prior to injection of receptor agonists. All 37 NS neurons and 12 WDR neurons had at baseline (i.e. before agonist injection) an ipsilateral cutaneous RF involving the skin overlying the TMJ and extending into the maxillary and mandibular divisions as well as an ipsilateral deep RF involving the TMJ region. There were no differences in baseline spontaneous activity, laminae location (i.e. superficial vs. deep), or responses to glutamate and capsaicin (i.e. Rmag, Rdur, Rlat and Pfreq; cutaneous RF expansion and cutaneous/TMJ MAT reduction) between the WDR and NS neurons ($p>0.05$, Mann-Whitney U test; $p>0.05$, Fisher's exact test) (data not shown); thus, the data from NS and WDR neurons were pooled together for analysis of glutamate and capsaicin-induced activation and sensitization.

GLUTAMATE EFFECTS:

ACTIVATION.

***Deep craniofacial afferents*.** Injection of glutamate alone (but not vehicle alone, 0/8) activated 43% (12/28) of the afferents tested (9/23 Aδ-fibers, CV= 8.2±1.5 m/s; 3/5 C-fibers, CV= 1.6±0.3 m/s) ($p<0.001$, Fisher exact test), with the activation response properties outlined in Table 2. Glutamate-evoked afferent activation began approximately 6 seconds from injection (Rlat = 5.8 [8.2] seconds) and lasted over 2 minutes (Rdur = 144 [138] seconds). There was no difference in mean CV between glutamate-sensitive (n=12, CV=6.3±1.4 m/s) and glutamate-insensitive afferents (n=16, CV=9.4±1.4 m/s, $p>0.05$, t-test).

TMJ-responsive Vc/UCC neurons. Injection of glutamate alone (but not vehicle alone, 0/9) activated 86% (12/14) of the neurons tested ($p<0.001$, Fisher exact test), with the activation response properties outlined in Table 3. Glutamate-evoked neuronal activation began approximately 5 seconds from injection (Rlat = 4.3 [2.6] seconds) and lasted about 2 minutes (Rdur = 98.6 [49.1] seconds).

The incidence of activation ($p<0.01$, Fisher exact test) and Rmag ($p<0.05$, Mann-Whitney U test) evoked by glutamate alone was significantly greater in Vc/UCC neurons compared to the afferents tested. Similarities and differences in response properties of afferents and Vc/UCC neurons to glutamate and capsaicin injection are summarized in Table 4.

MAT REDUCTION.

***Deep craniofacial afferents*.** The spontaneous afferent activity returned to baseline level prior to determination of MAT post-injection of glutamate in all 27 afferents tested. Injection of glutamate alone (but not vehicle alone, 0/8) induced a marked MAT reduction (≥50% threshold reduction from baseline MAT score) at 10-20 minutes post-injection in 48% (13/27) of the afferents (11/22 Aδ-fibers; 2/5 C-fibers; $p<0.05$, Fisher exact test). Similarly, for the afferent population tested as a whole, the mean baseline MAT value was 33.2±3.6 g, and injection of glutamate alone (but not vehicle alone, n=8, $p>0.05$, RM ANOVA-on-ranks) produced a significant reduction in MAT values relative to their pre-injection baseline ($p<0.05$, RM ANOVA-on-ranks, Dunn's method) (Fig. 5a). Many afferents activated by glutamate [50% (6/12)] injection alone did not show MAT

reduction, whereas some afferents displaying MAT reduction following glutamate [47% (7/15)] injection alone showed no prior activation by glutamate ($p>0.05$, Fisher exact test).

TMJ-responsive Vc/UCC neurons. Consistent with previous findings, the post-incision cutaneous MAT values remained unchanged from baseline and remained stable prior to glutamate (or vehicle) injection ($p>0.05$, RM ANOVA-on-ranks) (Chapter 4). The spontaneous neuronal activity returned to baseline level prior to determination of MAT post-injection of glutamate in all 10 neurons tested. Injection of glutamate alone (but not vehicle alone, 0/9) induced TMJ MAT reduction (≥ 50% threshold reduction from baseline MAT score) in 56% (5/9) and cutaneous MAT reduction in 60% (6/10) of the neurons tested ($p<0.05$, Fisher exact test) at 10-20 minutes post-injection. However, for the neuronal population as whole, the baseline median TMJ and cutaneous MAT values were 67.9 [56.1] g and 63.0 [53.0] g, respectively, and injection of glutamate alone (or vehicle alone, n=9) produced a non-significant reduction in TMJ and cutaneous MAT values at 10-20 minutes post-injection relative to their pre-injection baseline ($p>0.05$, RM ANOVA-on-ranks) (Figs. 6a and 6b). Some neurons activated by glutamate injection alone did not show cutaneous [33% (3/9)] or TMJ [44% (4/9)] MAT reduction, and the one neuron not activated by glutamate injection alone also did not display cutaneous or TMJ MAT reduction.

CUTANEOUS RF EXPANSION.

TMJ-responsive Vc/UCC neurons. Consistent with previous findings, the post-incision cutaneous RF size remained unchanged from baseline and remained stable prior to glutamate (or vehicle) injection ($p>0.05$, Fisher exact test) (Chapter 4). Injection of glutamate alone (but not vehicle alone, 0/9) induced cutaneous RF expansion at 10-20 minutes post-injection in 90% (9/10) of the neurons tested ($p<0.05$, Fisher exact test). All 9 neurons activated by glutamate injection alone showed cutaneous RF expansion, whereas there was no cutaneous RF expansion following glutamate injection alone in the one neuron that showed no prior activation by glutamate.

CAPSAICIN EFFECTS:

ACTIVATION.

***Deep craniofacial afferents*.** Injection of capsaicin alone (but not vehicle alone, 0/9) activated 24% (6/25) of the afferents (2/18 Aδ-fibers, CV= 7.5±0.3 m/s; 4/7 C-fibers, CV= 1.7±0.2 m/s) tested ($p<0.01$, Fisher exact test), with the activation response properties outlined in Table 2. Capsaicin-evoked afferent activation began approximately 10 seconds from injection (Rlat = 9.8 [20] seconds) and lasted about half a minute (Rdur = 30 [50] seconds). The median CV for capsaicin-sensitive (n=6, CV=1.7[0.5] m/s) afferents was significantly smaller than that for capsaicin-insensitive afferents (n=19, CV=9.0[10.3] m/s, $p<0.05$, Mann-Whitney U test) reflecting the difference in the properties of Aδ- and C-fibers activated.

The incidence of afferent activation induced by injection of capsaicin alone was similar to that induced by glutamate alone ($p>0.05$, Fisher exact test). However, capsaicin alone produced lower responses in Rmag, Rdur and Pfreq compared with glutamate alone ($p<0.05$, Mann-Whitney U test) (Table 2).

TMJ-responsive Vc/UCC neurons. Injection of capsaicin alone (but not vehicle alone, 0/10) activated 88% (14/16) of the neurons tested ($p<0.001$, Fisher exact test), with the activation response properties outlined in Table 3. Capsaicin-evoked neuronal activation began approximately 20 seconds from injection (Rlat = 16.4 [10.5] seconds) and lasted about 2-3 minutes (Rdur = 112 [201] seconds). The incidence of Vc/UCC neuronal activation ($p>0.05$, Fisher exact test) and response properties (Rmag, Rlat, Rdur and Pfreq) ($p>0.05$, Mann-Whitney U test) induced by injection of capsaicin alone was similar to that induced by glutamate alone (Table 3).

The incidence of activation ($p<0.01$, Fisher exact test) and Rdur ($p<0.05$, Mann-Whitney U test) evoked by capsaicin alone was significantly greater in Vc/UCC neurons compared to the afferents tested (Table 4).

MAT REDUCTION.

***Deep craniofacial afferents*.** The spontaneous afferent activity returned to baseline level prior to determination of MAT post-injection of capsaicin in all 22 afferents tested. Injection of capsaicin alone (but not vehicle alone, 0/9) induced a marked MAT reduction (≥50% threshold reduction from baseline MAT score) at 10-20 minutes post-injection in 41% (9/22) of the afferents (6/15 Aδ-fibers; 3/7 C-fibers; $p<0.05$, Fisher exact test). However, for the afferent population tested as a whole, the median baseline MAT value was 20.2[29.7] g, and injection of capsaicin alone (or vehicle alone, n=9) did not significantly alter the MAT values compared with their pre-injection baseline ($p>0.05$, RM ANOVA-on-ranks) (Fig. 5b). Many afferents activated by capsaicin [83% (5/6)] injection alone did not show MAT reduction, whereas some afferents displaying MAT reduction following capsaicin [36% (8/16)] injection alone showed no prior activation by capsaicin ($p>0.05$, Fisher exact test).

TMJ-responsive Vc/UCC neurons. Consistent with previous findings, the post-incision cutaneous MAT values remained unchanged from baseline and remained stable prior to capsaicin (or vehicle) injection ($p>0.05$, RM ANOVA-on-ranks) (Chapter 4). The spontaneous neuronal activity returned to baseline level prior to determination of MAT post-injection of capsaicin in all 14 neurons tested. Injection of capsaicin alone (but not vehicle alone, 0/10) induced TMJ MAT reduction (≥ 50% threshold reduction from baseline MAT score) in 64% (9/14) and cutaneous MAT reduction in 53% (8/15) of the neurons tested ($p<0.05$, Fisher exact test) at 10-20 minutes post-injection. Similarly, for the neuronal population as whole, the baseline median TMJ and cutaneous MAT values were 80.5 [63.1] g and 74.6 [56.9] g, respectively, and capsaicin alone (but not vehicle alone, n=10) injected into the TMJ produced a significant reduction in TMJ ($p<0.01$) and cutaneous ($p<0.05$) MAT values relative to their pre-injection baseline (RM ANOVA-on-ranks, Dunn's method) at 10-20 minutes post-injection (Figs. 6c and 6d). However, some neurons activated by capsaicin injection alone did not show cutaneous [36% (5/14)] or TMJ [36% (5/14)] MAT reduction,

whereas the two neurons not activated by capsaicin injection alone displayed both cutaneous and TMJ MAT reduction ($p>0.05$, Fisher exact test).

There was no significant difference between the incidence of reductions in TMJ MAT and cutaneous MAT induced by injection of capsaicin alone compared to those induced by glutamate alone ($p>0.05$, Fisher exact test).

An example of a neuron manifesting capsaicin-induced RF expansion and MAT reduction is shown in Fig. 4.

CUTANEOUS RF EXPANSION.

TMJ-responsive Vc/UCC neurons. Consistent with previous findings, the post-incision cutaneous RF size remained unchanged from baseline and remained stable prior to capsaicin (or vehicle) injection ($p>0.05$, Fisher exact test) (Chapter 4). Injection of capsaicin alone (but not vehicle alone, 0/10) induced cutaneous RF expansion at 10-20 minutes post-injection in 60% (9/15) of the neurons tested ($p<0.05$, Fisher exact test). However, some neurons activated by capsaicin [36% (5/14)] injection alone did not show cutaneous RF expansion, whereas one neuron displaying cutaneous RF expansion following capsaicin injection alone showed no prior activation by capsaicin.

The incidence of cutaneous RF expansion induced by injection of capsaicin alone was similar to the incidence of cutaneous RF expansion induced by glutamate alone ($p>0.05$, Fisher exact test).

GLUTAMATE AND CAPSAICIN INTERACTIONS:

ACTIVATION.

***Deep craniofacial afferents*.** There were four types of agonist-responsive deep craniofacial afferents found in the glutamate followed by capsaicin subgroup: (1) glutamate-sensitive and capsaicin-sensitive [15% (4/27)]; (2) glutamate-sensitive and capsaicin-insensitive [30% (8/27)]; (3) glutamate-insensitive and capsaicin-sensitive [18% (5/27)]; and (4) glutamate-insensitive and capsaicin-insensitive

[37% (10/27)]. Following glutamate injection, capsaicin (but not vehicle, 0/8) evoked responses in 32% (9/28) of the afferents tested and produced greater increases in Rmag and Pfreq ($p<0.05$, Mann-Whitney U test) but no change in Rlat and Rdur compared with capsaicin alone ($p>0.05$, Mann-Whitney U test) (Table 2). There was no significant difference in the incidence of capsaicin-induced activation with capsaicin alone, compared with capsaicin following glutamate injection ($p>0.05$, Fisher exact test).

There were also four types of agonist-responsive afferents found in the capsaicin followed by glutamate subgroup: (1) capsaicin-sensitive and glutamate-sensitive [11% (2/19)]; (2) capsaicin-sensitive and glutamate-insensitive [16% (3/19)]; (3) capsaicin-insensitive and glutamate-sensitive [26% (5/19)]; and (4) capsaicin-insensitive and glutamate-insensitive [47% (9/19)]. Following capsaicin injection, glutamate (but not vehicle, 0/9) evoked responses in 32% (7/22) of the afferents tested that were not significantly different in Rmag, Rlat, Rdur and Pfreq compared to glutamate alone (Table 2). Similarly, there was no significant difference in the incidence of glutamate-induced activation with glutamate alone, compared with glutamate following capsaicin injection ($p>0.05$, Fisher exact test).

TMJ-responsive Vc/UCC neurons. There were only two types of agonist-responsive Vc/UCC neurons found in the glutamate followed by capsaicin subgroup, as summarized in Fig. 7: (1) glutamate-sensitive and capsaicin-sensitive [92% (12/13)] and (2) glutamate-insensitive and capsaicin-sensitive [8% (1/13)]. Following glutamate injection, capsaicin (but not vehicle, 0/10) evoked responses in all 13 neurons tested and neuronal activation occurred approximately 5-6 seconds from injection (Rlat = 5.3 [2.5] seconds) and lasted about 6-7 minutes (Rdur = 377 [283] seconds). Moreover, following glutamate injection, capsaicin produced significantly greater (2-18 fold) increases in Rmag, Pfreq, and Rdur and a decrease in Rlat compared with capsaicin alone ($p<0.05$, Mann-Whitney U tests) (Table 3). The incidence of activation evoked by capsaicin following glutamate was markedly greater in Vc/UCC neurons [100% (13/13)] compared to the afferents [33% (9/27)] tested ($p<0.001$, Fisher exact test). Injection of capsaicin following glutamate also resulted in significantly

greater Rmag and Rdur in Vc/UCC neurons relative to the afferents tested ($p<0.01$, Mann-Whitney U test).

There were also only two types of agonist-responsive Vc/UCC neurons found in the capsaicin followed by glutamate subgroup (see Fig. 7): (1) capsaicin-sensitive and glutamate-insensitive [90% (9/10)] and (2) capsaicin-insensitive and glutamate-insensitive [10% (1/10)]. Injection of glutamate following capsaicin evoked a moderate incidence of activation in the afferents [37% (7/19)], whereas it failed to evoke any activity in the Vc/UCC neurons [0% (0/10)] tested ($p=0.063$, Fisher exact test). Moreover, in contrast to the findings in afferents, following capsaicin injection, glutamate evoked no responses (Rmag, Rlat, Rdur and Pfreq) in any of the 10 Vc/UCC neurons tested ($p<0.05$, Mann-Whitney U tests) (Table 3).

MAT REDUCTION.

Deep craniofacial afferents. Compared to the incidence of capsaicin-induced MAT reduction with capsaicin alone, capsaicin following glutamate injection induced MAT reduction in only 12% (3/25) of the afferents ($p<0.05$, Fisher exact test). Furthermore, when injected following glutamate, capsaicin (or vehicle, 0/8) produced a non-significant increase in the median MAT from pre-injection baseline, indicating that capsaicin induced no further MAT reduction than that induced by the preceding glutamate injection ($p<0.05$, RM ANOVA-on-ranks, Dunn's method) (Fig. 5a).

There was no significant difference in the incidence of glutamate-induced MAT reduction with glutamate alone compared with glutamate following capsaicin injection [45% (9/20)] ($p>0.05$, Fisher exact test). Furthermore, when injected following capsaicin, glutamate (but not vehicle, 0/9) produced a significant reduction in MAT values relative to their pre-injection baseline ($p<0.01$, RM ANOVA-on-ranks, Dunn's method) (Fig. 5b).

TMJ-responsive Vc/UCC neurons. Capsaicin injected into the TMJ following glutamate produced a non-significant reduction in TMJ MAT ($p>0.05$) (Fig. 6b) but did produce a significant reduction in cutaneous MAT ($p<0.05$) (Fig. 6a)

values post-injection relative to the pre-injection baseline (RM ANOVA-on-ranks). The incidence of capsaicin-induced TMJ MAT reduction following glutamate (11%) was significantly less than that with capsaicin alone (64%) ($p<0.05$, Fisher exact test). The incidence of cutaneous MAT reduction induced by capsaicin following glutamate was not statistically different compared to that induced by capsaicin alone ($p>0.05$, Fisher exact test) at 10-20 minutes post-injection.

Glutamate injected into the TMJ following capsaicin produced no significant reduction in TMJ MAT ($p>0.05$) (Fig. 6d) but there was a significant reduction in cutaneous MAT values at 10-20 minutes post-injection relative to the pre-injection baseline ($p<0.05$, RM ANOVA-on-ranks, Dunn's method) (Fig. 6c). The incidence of glutamate-induced reduction in TMJ MAT and cutaneous MAT following capsaicin was not statistically different compared to that induced by glutamate alone ($p>0.05$, Fisher exact test).

CUTANEOUS RF EXPANSION.

TMJ-responsive Vc/UCC neurons. There was no significant difference in the incidence of cutaneous RF expansion induced by injection of capsaicin alone [60% (9/15)] and capsaicin following glutamate [22% (2/9)] ($p>0.05$, Fisher exact test) at 10-20 minutes post-injection. However, following capsaicin injection, the incidence of glutamate-induced cutaneous RF expansion [15% (2/13)] was significantly less than that with glutamate alone [90% (9/10)] ($p<0.001$, Fisher exact test) at 10-20 minutes post-injection.

2.4 DISCUSSION:

This is the first study to document that a considerable proportion of deep craniofacial nociceptive afferents as well as neurons in the Vc/UCC may be activated or sensitized by the peripheral application of glutamate, capsaicin, or both, and that these receptor agonists may interact to modulate activation as well as peripheral and central sensitization evoked from deep craniofacial tissues.

Glutamate sensitized afferent responses to subsequent noxious stimulation of the deep craniofacial tissues by capsaicin, whereas capsaicin neither sensitized nor desensitized afferent responses to subsequent noxious stimulation by glutamate. Similarly, the nociceptive afferent inputs activated by glutamate applied to the TMJ sensitized Vc/UCC neurons, and produced more immediate, larger and more prolonged responses to noxious stimulation of the TMJ by capsaicin. However, in contrast to its effects on afferents, capsaicin applied to the TMJ desensitized the Vc/UCC neurons to subsequent noxious stimulation of the TMJ by glutamate. As depicted in Fig. 7, these findings suggest that convergent signaling pathways are involved in glutamate and capsaicin-evoked activation of deep craniofacial afferent inputs to Vc/UCC nociceptive neurons. Taken together, these findings suggest that both peripheral EAA and TRPV1 receptor mechanisms may be involved in the nociceptive processing of deep craniofacial afferent inputs and may interact to modulate both activation as well as peripheral and central sensitization evoked from these tissues.

Properties of deep craniofacial afferents projecting to Vc/UCC and their CVs are consistent with previous findings (Cairns *et al.* 2001a,b, 2002a, 2003a; Dong *et al.* 2006, 2007). Similarly, the RF properties, lamina loci and convergence of TMJ and cutaneous afferent inputs of TMJ-responsive neurons are consistent with previous findings (Yu *et al.* 1993; Hu *et al.* 2005b; Mørch *et al.* 2007), The present study employed glutamate concentrations that approximate the concentration that could be released from presynaptic vesicles in masseter muscle afferents (Riveros *et al.* 1986; Gambarota *et al.* 2005). However, although a contribution of metabotropic EAA receptor mechanisms to the present findings cannot be ruled out (Ahn *et al.* 2005; Lee and Ro 2007a,b), as little as a 2-3 times elevation in interstitial glutamate levels in the rat masseter muscle is sufficient to excite and induce afferent mechanical sensitization through NMDA receptor activation (Cairns *et al.* 2007). Glutamate concentrations are 3–4 times greater in the tendons of ‘‘jumper’s knee’’ and ‘‘tennis elbow’’ patients (Alfredson *et al.* 2000, 2001; Alfredson and Lorentzon 2002) and 54 times greater in the synovial fluid of arthritic patients (McNearney *et al.* 2000, 2004) compared to

controls. Elevated glutamate tissue concentrations have also been associated with increased pain intensity and mechanical sensitivity in chronic myalgia sufferers (Rosendal *et al.* 2004).

Our findings of glutamate-induced activation and peripheral sensitization, a process reflected in enhanced evoked activity (e.g. increase in Rmag and Pfreq) and MAT reduction, in trigeminal nociceptive afferents are consistent with previous evidence of glutamate-evoked dose-dependent increases in jaw muscle activity, activation and sensitization of deep nociceptive afferents, and pain in human masticatory muscles (Cairns *et al.* 1998, 2001a,b, 2002a, 2003a,b, 2006, 2007; Svensson *et al.* 2003, 2005; Dong *et al.* 2006, 2007). However, this is the first study to document capsaicin-induced activation and sensitization in trigeminal nociceptive afferents and Vc/UCC nociceptive neurons with deep craniofacial RFs. In contrast to previous studies (Takeshita *et al.* 2001; Okamoto *et al.* 2003, 2005b; Bereiter *et al.* 2005a; Tashiro *et al.* 2007) that required extensive exposure and retraction of muscle and connective tissue to stimulate the TMJ, the less invasive small skin incision overlying the TMJ in the present study allowed us to bypass superficial (i.e. cutaneous) afferent inputs and directly assess deep (i.e. TMJ) RF sites. As such, the present findings suggest that both peripheral and central sensitization mechanisms may contribute to the enhanced responses to EAA and TRPV1 receptor agonists (e.g. increase in Rmag, Rdur or Pfreq and/or a decrease in Rlat) and TMJ MAT reduction. However, the observed cutaneous RF expansion and cutaneous MAT reduction in Vc/UCC nociceptive neurons induced by injection of glutamate and capsaicin cannot simply be explained by peripheral sensitization and likely reflect central sensitization since glutamate and capsaicin were applied to a *deep* tissue RF site (TMJ) remote from the *cutaneous* RF sites assessed.

Although there have been no previous studies demonstrating glutamate-induced central sensitization in trigeminal or spinal dorsal horn neurons, the present findings of glutamate-induced central sensitization in Vc/UCC nociceptive neurons are consistent with trigeminal (Cairns *et al.* 1998, 2001a, 2003a,b, 2006; Svensson *et al.* 2003, 2005) and spinal (Lawand *et al.* 1997; Wang *et al.* 1997;

Warncke *et al.* 1997) findings in animals and humans that suggest a role for central sensitization (e.g. secondary hyperalgesia) in mediating glutamate-evoked pain. However, glutamate did not induce a significant reduction in cutaneous MAT for the Vc/UCC neuronal population as a whole but did significantly reduce MAT in the deep craniofacial afferents, which suggests that peripheral sensitization may play a greater role in glutamate-evoked mechanical hypersensitivity.

In the case of capsaicin, the present findings of capsaicin-induced central sensitization in Vc/UCC nociceptive neurons are also consistent with both trigeminal (Carstens *et al.* 1998; Sohn *et al.* 2000, 2004; Wang *et al.* 2002; Gazerani and Arendt-Nielsen 2005; Gazerani *et al.* 2006; Zanotto *et al.* 2007) and spinal (LaMotte *et al.* 1991, 1992; Simone *et al.* 1991; Raja *et al.* 1999; Witting *et al.* 2000a,b; Sluka 2002) evidence in animals and humans that suggest a role for central sensitization in mediating capsaicin-evoked pain. Indeed the findings that capsaicin produced no significant reduction in deep craniofacial afferent MAT compared with its significant reduction in cutaneous MAT for the Vc/UCC neuronal population as a whole is consistent with previous studies suggesting that central sensitization may play a greater role in capsaicin-evoked mechanical hypersensitivity (Baumann *et al.* 1991; LaMotte *et al.* 1991, 1992; Simone *et al*. 1991). In addition, the significant reduction in MAT directly at the deep (TMJ) site of capsaicin injection for the Vc/UCC neuronal population as a whole contrasts with the desensitized RF site of superficial (intradermal) capsaicin injection in previous studies (Baumann *et al.* 1991; LaMotte *et al.* 1991, 1992; Simone *et al*. 1991) and suggests differences in central nociceptive processing of deep versus cutaneous tissues to peripherally applied capsaicin.

The finding that glutamate or capsaicin may induce MAT reduction without prior afferent or neuronal activation may be explained by glutamate and capsaicin acting on peripheral non-neuronal cells in addition to neuronal cells and causing them to release mediators via paracrine activation that sensitize the afferent inputs to the Vc/UCC neurons (Lam *et al.* 2005; Chapter 3). There has been recent compelling evidence for the expression and function of glutamate

and capsaicin in signaling processes in several types of non-neuronal cells (Meddings *et al.* 1991; Skerry and Genever 2001; Kato *et al.* 2003; Rizvi and Luqman 2003; Li *et al.* 2005; Xin *et al.* 2005). Thus it may be possible that the activation of glutamate and capsaicin receptors on non-neuronal cells may result in the release of various other sensitizers including bradykinin, amines, prostanoids, growth factors, chemokines, cytokines, protons and ATP (Sikand and Premkumar 2007; Woolf and Ma, 2007) that may sensitize the afferent inputs to the Vc/UCC neurons.

Glutamate and Capsaicin Receptor Interactions in Deep Craniofacial Tissues

By using the same experimental paradigm in peripheral and central studies of glutamate and capsaicin-evoked effects, this is the first study to provide direct comparisons of peripheral versus central nociceptive neuronal responses to peripherally applied glutamate and capsaicin. Capsaicin-evoked activation of trigeminal afferents and Vc/UCC neurons following glutamate injection were significantly enhanced compared to capsaicin alone, suggesting that glutamate may sensitize the nociceptive afferents and neurons and produce more immediate (e.g. decrease Rlat), larger (e.g. increased Rmag and Pfreq) and more prolonged (e.g. increased Rdur) responses to subsequent noxious stimuli (e.g. to capsaicin). These results are consistent with findings that pre-injection of NMDA receptor antagonists into the TMJ region attenuates jaw muscle activity evoked by capsaicin (Chapter 3) or mustard oil (Yu *et al*. 1996). The inflammatory irritant mustard oil activates the peripheral TRPA1 (ANKTM1, see Bautista *et al.* 2006; McMahon and Wood 2006; Tai *et al.* 2008) receptor, also a member of the TRP family. A protein that is likely to mediate the interactions between peripheral NMDA and TRPV1 receptors is the Ca^{2+}-calmodulin-dependent kinase II (CaMKII). This protein is persistently activated after NMDA receptor stimulation (see Yamakura and Shimoji 1999; Petrenko *et al.* 2003; Paoletti and Neyton 2007) and phosphorylation of TRPV1 by CaMKII is required

for its ligand binding (Jung *et al.* 2004; Suh and Oh 2005; Tominaga and Tominaga 2005). Although no studies to date have demonstrated the co-localization of peripheral NMDA and TRPV1 receptors on the same trigeminal primary afferent terminal, nociceptive responses could be enhanced if the same nociceptive afferent expresses both EAA and TRPV1 receptors. Taken together, these findings suggest that the activation and/or sensitization of peripheral EAA receptors may be important in the mechanisms whereby capsaicin or mustard oil via TRP receptors evoke nociceptive trigeminal responses.

In addition to possible interactions between ionotropic receptors, there is evidence of a major coupling between G-protein-coupled receptors and some TRP channels in the membrane such as TRPA1 and TRPV1 (Sikand and Premkumar 2007; Woolf and Ma 2007). For example, TRPA1 may function as a receptor-operated channel for bradykinin by allowing Ca^{2+} influx following activation of the B2 receptor (Bautista *et al.* 2006). Bradykinin can also significantly potentiate TRPV1 activity by activating the Ca^{2+}/phospholipid-dependent kinase (PKC) pathway (Sikand and Premkumar 2007). Activation of the PKC pathway has also been shown to lower the heat threshold of TRPV1 below body temperature and sensitize TRPV1 receptor responses to capsaicin (Premkumar and Ahern 2000; Crandall *et al.* 2002). The mechanism behind this effect is thought to involve direct phosphorylation resulting in PKC-dependent insertion of TRPV1 receptors into the neuronal membrane (for review, see Hucho and Levine 2007). This type of coupling may also exist between TRPV1 and other G-protein-coupled receptors (Sikand and Premkumar 2007; Woolf and Ma 2007), such as the metabotropic EAA receptors, and provide an additional means for interactions between peripheral EAA and TRPV1 receptors.

The interactions between peripheral glutamate and capsaicin receptors on deep craniofacial afferent inputs to Vc/UCC nociceptive neurons suggest a convergent nociceptive signaling pathway (see Fig. 7). That is, the nociceptive signaling pathways are not separate; the glutamate and capsaicin-activated deep craniofacial afferents converge on Vc/UCC neurons. In the glutamate followed by capsaicin subgroup, the activation of four possible combinations of agonist-

responsive afferent inputs converge centrally to result in the activation of only two agonist-responsive Vc/UCC neurons that are both capsaicin-sensitive following glutamate, whereas in the glutamate followed by capsaicin subgroup, the activation of four possible combinations of agonist-responsive afferent inputs converge centrally to result in the activation of only two agonist-responsive Vc/UCC neurons that are both glutamate-insensitive following capsaicin. Glutamate-evoked activation and cutaneous RF expansion in the Vc/UCC nociceptive neurons following capsaicin injection were abolished or significantly reduced. These findings suggest that capsaicin may desensitize Vc/UCC neurons to subsequent noxious stimuli (e.g. to glutamate) which is consistent with previous evidence showing that capsaicin may desensitize central nociceptive neurons to subsequent noxious stimuli applied to craniofacial and other tissues (Baumann *et al.* 1991; LaMotte *et al.* 1991, 1992; Simone *et al*. 1991; Carstens *et al.* 1998). Capsaicin-induced activation of the Ca^{2+}-dependent phosphatase calcineurin may not only mediate dephosphorylation and desensitization of peripheral TRPV1 (Docherty *et al.* 1996; Jung *et al.* 2004) but it may also desensitize central NMDA and non-NMDA receptors (Swope *et al.* 1999; Rycroft and Gibb 2004) to subsequent noxious stimulation. However, our findings in Vc/UCC neurons contrast with our afferent data where many of the afferents remained responsive to glutamate following capsaicin injection. This difference in peripheral and central nociceptive processing of glutamate-evoked responses following capsaicin injection is difficult to explain. However, since deep craniofacial afferents remain responsive to glutamate following capsaicin injection, the peripheral contribution to the mechanisms behind capsaicin's desensitizing effect on Vc/UCC neurons appears to be minimal.

Nociceptive afferents may use glutamate, neuropeptides and proteins as neurotransmitters or synaptic modulators (Woolf and Salter 2000; Dubner 2005; Woolf and Ma 2007) and it is possible that there may be differences in the types of presynaptic receptors, neurotransmitters or modulators released from the central terminals of capsaicin-sensitive versus glutamate-sensitive afferents that may account for the differential sensitizing or desensitizing effects on Vc/UCC

neurons. The variety of these neurotransmitters may result in considerable spatial and temporal summation capabilities that are subject to both excitatory and inhibitory influences. In the present study, capsaicin-evoked activation was lower than that evoked by glutamate alone in the afferents tested. In contrast, capsaicin-evoked activation was analogous to that evoked by glutamate in the Vc/UCC neurons. These findings suggest that a greater proportion of convergent capsaicin-sensitive afferent inputs on Vc/UCC neurons may contribute to the enhanced capsaicin-evoked responses as well as the desensitizing effects on subsequent glutamate-evoked responses in Vc/UCC neurons. Since the majority of capsaicin-sensitive afferents were very slowly conducting afferents projecting to the Vc/UCC region and the vast majority of Vc/UCC neurons were capsaicin-sensitive, the findings suggest that these very slowly conducting afferents may be mainly responsible for the activation of Vc/UCC neurons by peripherally applied capsaicin. These capsaicin-sensitive afferent inputs may activate segmental and/or descending inhibitory influences on Vc/UCC neurons (for review, see Dubner and Bennett 1983; Sessle *et al*. 1992; Fields and Basbaum 1994; Yaksh and Malmberg 1994; Dubner and Ren 2004) that in the case of the capsaicin-induced desensitizing effects, might involve increased inhibitory GABAergic synaptic transmission through alterations in cation-chloride cotransporters such as NKCC1 or KCC2 on nociceptive neurons (Galan and Cervero 2005; Karlsson *et al.* 2005; Price *et al.* 2005, 2006; Garcia-Nicas *et al.* 2006; Pitcher *et al.* 2007). The present findings are consistent with recent evidence of other peripheral TRP receptors participating in the activation of central inhibitory circuits, such as the attenuation of the increased excitability of spinal nociceptive neurons following activation of TRPA1-related nociceptive afferent inputs (Kosugi *et al.* 2007; Merrill *et al.* 2008). Thus it is possible that the capsaicin-induced desensitization in the present study may occur despite subsequent glutamate-evoked activity in afferent inputs on Vc/UCC neurons via the activation of similar central inhibitory circuits. Further studies are necessary to clarify the mechanisms involved.

2.5 TABLES:

Table 1. Mechanoreceptive field (RF) and baseline mean pooled antidromic and orthodromic conduction velocity (CV) and mechanical activation threshold (MAT) of deep craniofacial nociceptive afferents.

	Aδ-fiber afferent Mean±SE			**C-fiber afferent** Mean±SE		
RF location	**n**	**Mean CV (m/s)**	**Mean MAT (g)**	**n**	**Mean CV (m/s)**	**Mean MAT (g)**
TMJ	31	9.8±1.1	29.6±3.5	9	1.6±0.1	30.0±8.8
Masseter	19	8.8±1.2	27.6±5.3	4	1.7±0.3	21.1±17.1
Temporalis	4	16.9±1.9	24.7±9.9	1	1.7	4.9
Pooled	**54**	**9.8±0.8**	**28.5±2.8**	**14**	**1.6±0.1**	**24.9±7.2**

Table 2. Response properties of trigeminal nociceptive afferents to injection of glutamate (or vehicle) and capsaicin (or vehicle) into deep craniofacial tissues (***p<0.001, Glutamate vehicle vs. Glutamate alone or Capsaicin vehicle post-Glutamate vs. Capsaicin post-Glutamate or Glutamate vehicle post-Capsaicin vs. Glutamate post-Capsaicin; **p<0.01, Capsaicin vehicle vs. Capsaicin alone; †p<0.05, ††p<0.01, Glutamate alone vs. Capsaicin alone; ‡p<0.05, ‡‡p<0.01, Capsaicin alone vs. Capsaicin post-Glutamate; Mann-Whitney U test).

	Glutamate ⇨ Capsaicin Median [interquartile range]		**Capsaicin ⇨ Glutamate** Median [interquartile range]	
Response Property	**Glutamate alone (n=28)**	**Capsaicin post-Glutamate (n=28)**	**Capsaicin alone (n=25)**	**Glutamate post-Capsaicin (n=22)**
Rmag (spikes)	168 [377] ††	170 [642] ‡	14 [24]	431 [487]
Rlat (sec)	5.8 [8.2]	7.5 [6.7]	9.8 [20]	5.6 [5.3]
Rdur (sec)	144 [138] †	70 [272]	30 [50]	149 [236]
Pfreq (Hz)	19 [25] ††	24 [36] ‡‡	3.0 [3.0]	19 [22]
Response Property	**Glutamate vehicle (n=4)**	**Capsaicin post-Glutamate vehicle (n=4)**	**Capsaicin vehicle (n=4)**	**Glutamate post-Capsaicin vehicle (n=4)**
Rmag (spikes)	0***	14 [31]	0**	149 [27]
Rlat (sec)	0***	6.0 [20]	0**	9.4 [2.5]
Rdur (sec)	0***	28 [32]	0**	131 [139]
Pfreq (Hz)	0***	4.0 [4.2]	0**	18 [14]
Response Property	**Glutamate alone (n=3)**	**Capsaicin vehicle post-Glutamate (n=3)**	**Capsaicin alone (n=4)**	**Glutamate vehicle post-Capsaicin (n=4)**
Rmag (spikes)	168 [550]	0***	14 [31]	0***
Rlat (sec)	3.2 [3.2]	0***	6.0 [20]	0***
Rdur (sec)	144 [148]	0***	28 [32]	0***
Pfreq (Hz)	20 [27]	0***	4.0 [4.2]	0***

Table 3. Properties of responses of TMJ-responsive Vc/UCC nociceptive neurons to injection of glutamate (or vehicle) and capsaicin (or vehicle) into the TMJ (***p<0.001, Glutamate vehicle vs. Glutamate alone or Glutamate alone vs. Glutamate post-Capsaicin or Capsaicin vehicle post-Glutamate vs. Capsaicin post-Glutamate; †††p<0.001, Capsaicin vehicle vs. Capsaicin alone or Glutamate vehicle post-Capsaicin vs. Glutamate post-Capsaicin; ‡p<0.05, Capsaicin alone vs. Capsaicin post-Glutamate; Mann-Whitney U test).

	Glutamate ⇨ Capsaicin Median [interquartile range]		**Capsaicin ⇨ Glutamate** Median [interquartile range]	
Response Property	**Glutamate alone (n=14)**	**Capsaicin post-Glutamate (n=13)**	**Capsaicin alone (n=16)**	**Glutamate post-Capsaicin (n=10)**
Rmag (spikes)	833 [1186]	2915 [4079]‡	161 [942]	0***
Rlat (sec)	4.3 [2.6]	5.3 [2.5]‡	16.4 [10.5]	0***
Rdur (sec)	98.6 [49.1]	377 [283]‡	112 [201]	0***
Pfreq (Hz)	31.7 [39.2]	45.1 [43.7]‡	16.6 [35.6]	0***
Response Property	**Glutamate vehicle (n=5)**	**Capsaicin post-Glutamate vehicle (n=5)**	**Capsaicin vehicle (n=5)**	**Glutamate post-Capsaicin vehicle (n=5)**
Rmag (spikes)	0***	131 [718]	0†††	643 [1396]
Rlat (sec)	0***	15.1 [14.4]	0†††	4.3 [2.6]
Rdur (sec)	0***	111 [153]	0†††	108 [50.6]
Pfreq (Hz)	0***	13.3 [29.8]	0†††	33.5 [41]
Response Property	**Glutamate alone (n=5)**	**Capsaicin vehicle post-Glutamate (n=5)**	**Capsaicin alone (n=4)**	**Glutamate vehicle post-Capsaicin (n=4)**
Rmag (spikes)	472 [1253]	0***	131 [718]	0†††
Rlat (sec)	3.6 [4.7]	0***	15.1 [14.4]	0†††
Rdur (sec)	126 [103]	0***	111 [153]	0†††
Pfreq (Hz)	21.7 [46.9]	0***	13.3 [29.8]	0†††

Table 4. Comparison of response properties of WDR and NS TMJ-responsive Vc/UCC nociceptive neurons and of deep craniofacial nociceptive afferents to injection of glutamate and/or capsaicin into deep craniofacial tissues. In general, both glutamate and capsaicin-evoked responses were greater in Vc/UCC neurons compared to afferents (i.e. increased activation incidence, Rmag or Rdur).

	Glutamate ⇨ Capsaicin				Capsaicin ⇨ Glutamate			
Response Property	Glutamate alone		Capsaicin post-Glutamate		Capsaicin alone		Glutamate post-Capsaicin	
	Afferent	Vc/UCC	Afferent	Vc/UCC	Afferent	Vc/UCC	Afferent	Vc/UCC
Activation	+	++‡	++†	+++†‡	+	++‡	+	-‡‡
Deep MAT	+	+	+††	+††	+	+	+	-
Cutaneous MAT	N/A	+	N/A	+	N/A	+	N/A	+
Cutaneous RF	N/A	+	N/A	+	N/A	+	N/A	+††

+ : Sensitized- increased excitability (↑Rmag, ↑Pfreq, ↑Rdur, ↓Rlat or ↓MAT)

- : Desensitized- decreased excitability (↓Rmag, ↓Pfreq, ↓Rdur, ↑Rlat or ↑MAT)

N/A: Not applicable

† : increased excitability compared to capsaicin alone

††: decreased excitability compared to glutamate/capsaicin alone

‡: increased excitability compared to afferents tested

‡‡: glutamate-evoked activity abolished

2.6 FIGURES:

Fig. 1A. Example of typical mechanoreceptive field and response properties of deep craniofacial nociceptive afferent to injection of glutamate vehicle followed by capsaicin (CV=2.8m/s, MAT=38 g, Aδ-fiber TMJ afferent). (a) Deep mechanoreceptive field (white circle) of nociceptive afferent involving the TMJ region indicated by arrow, (b) Afferent mechanical activation threshold determined with von Frey device, (c) No response in this afferent was evoked by injection of glutamate vehicle into the TMJ, (d) Afferent response evoked by injection of capsaicin following glutamate vehicle into the TMJ.

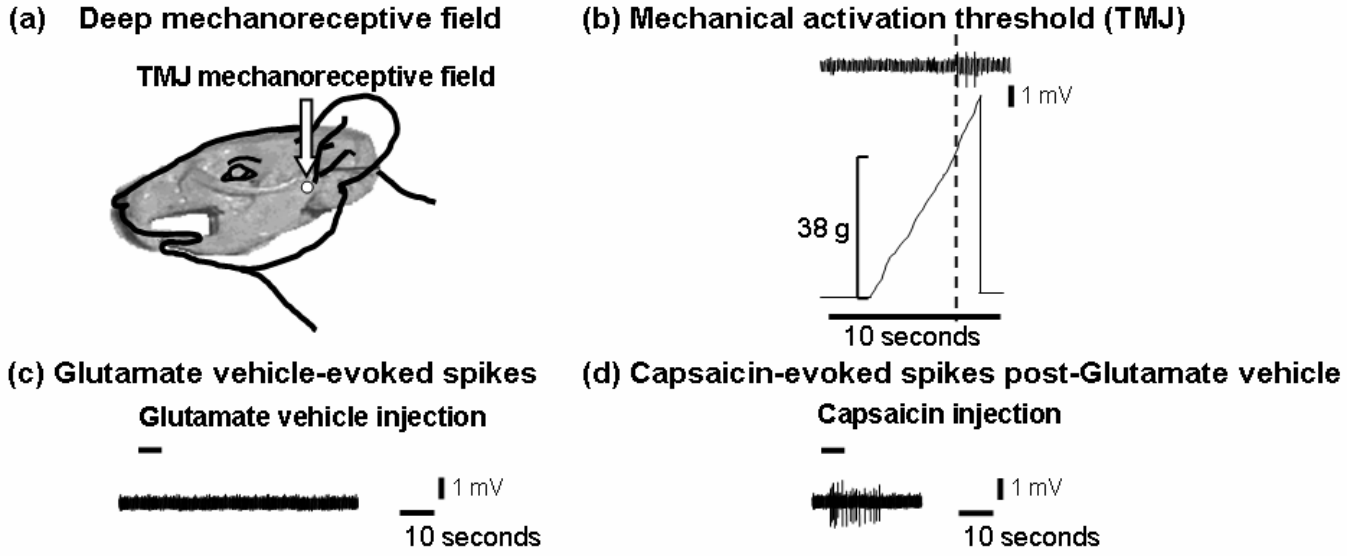


Fig. 1B. Example of typical mechanoreceptive field and response properties of deep craniofacial nociceptive afferent to injection of glutamate followed by capsaicin (CV=2.0m/s, MAT=12.7 g, C-fiber TMJ afferent). (a) Deep mechanoreceptive field (white circle) of nociceptive afferent involving the TMJ region indicated by arrow, (b) Afferent mechanical activation threshold determined with von Frey device, (c) Afferent response evoked by injection of glutamate into the TMJ, (d) Afferent response evoked by injection of capsaicin following glutamate into the TMJ, (e) *S*timulation of Vc/UCC (50 µs, 50 µA, 100 Hz) evoked an antidromic action potential (latency: 6.0 ms). By measuring the distance between the recording electrode and the stimulating electrode in Vc/UCC and dividing by the antidromic latency, the CV of this afferent was estimated to be 2.0 m/s, (f) Blunt mechanical stimulation of the TMJ tissue was used to evoke orthodromic spikes that served as a trigger for electrical stimulation of Vc/UCC (antidromic spike). Shortening the delay between the orthodromically evoked spike and the electrical stimulus applied to Vc/UCC resulted in a collision, as evidenced by the disappearance of the antidromic spike.

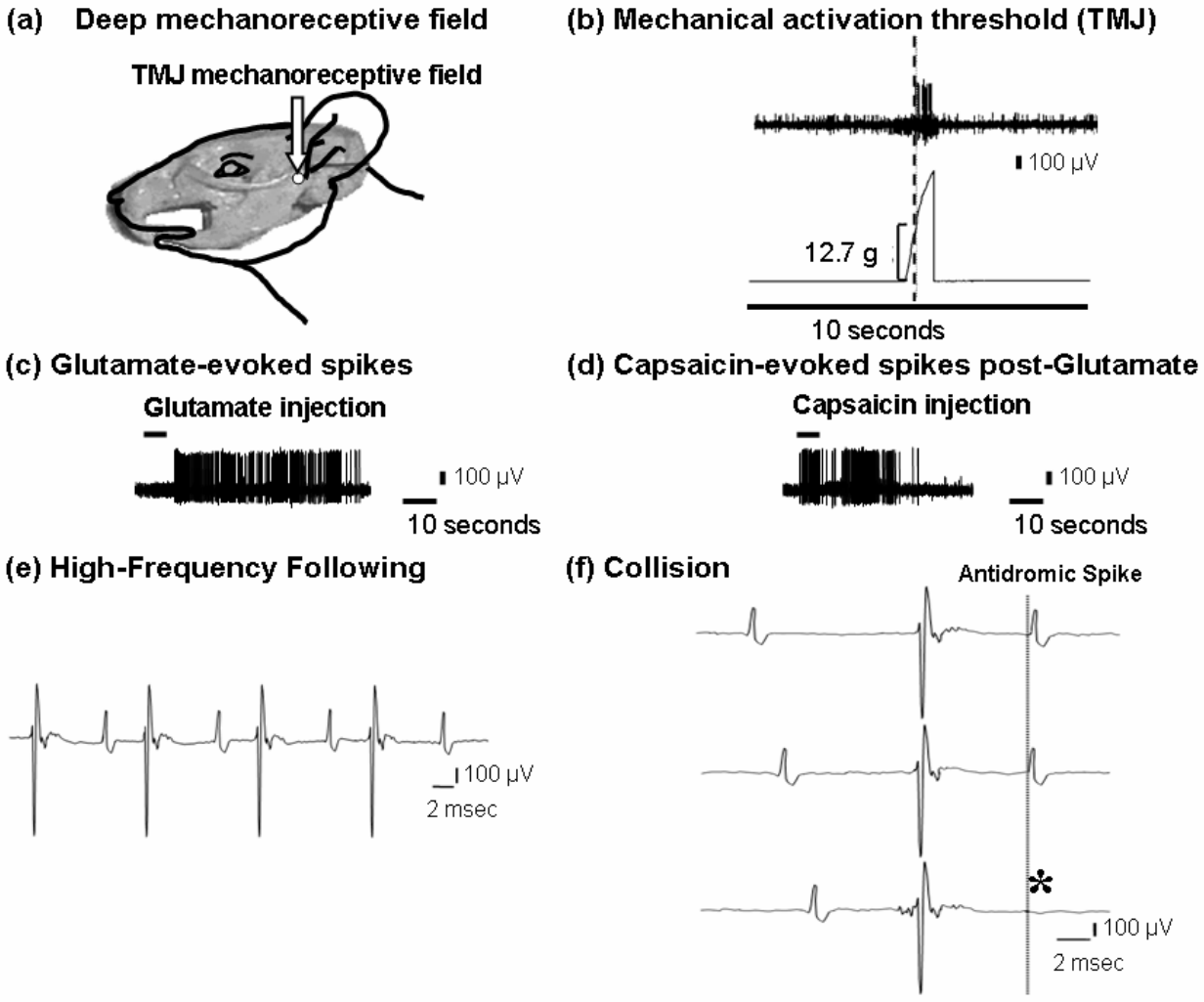


Fig. 2. The unit recording sites reconstructed from histology were plotted on diagrams of the brainstem (Paxinos and Watson 1997). Coronal sections at 3 different rostrocaudal levels of subnucleus caudalis/upper cervical spinal cord (Vc/UCC). Filled symbols represent wide dynamic range (WDR) neurons; opened symbols nociceptive-specific (NS) neurons. The dot and triangle symbols represent the glutamate injection followed by capsaicin injection and capsaicin injection followed by glutamate injection subgroups, respectively. The labels 3A, 3B, and 4 represent the NS neurons shown in Figs. 3 and 4.

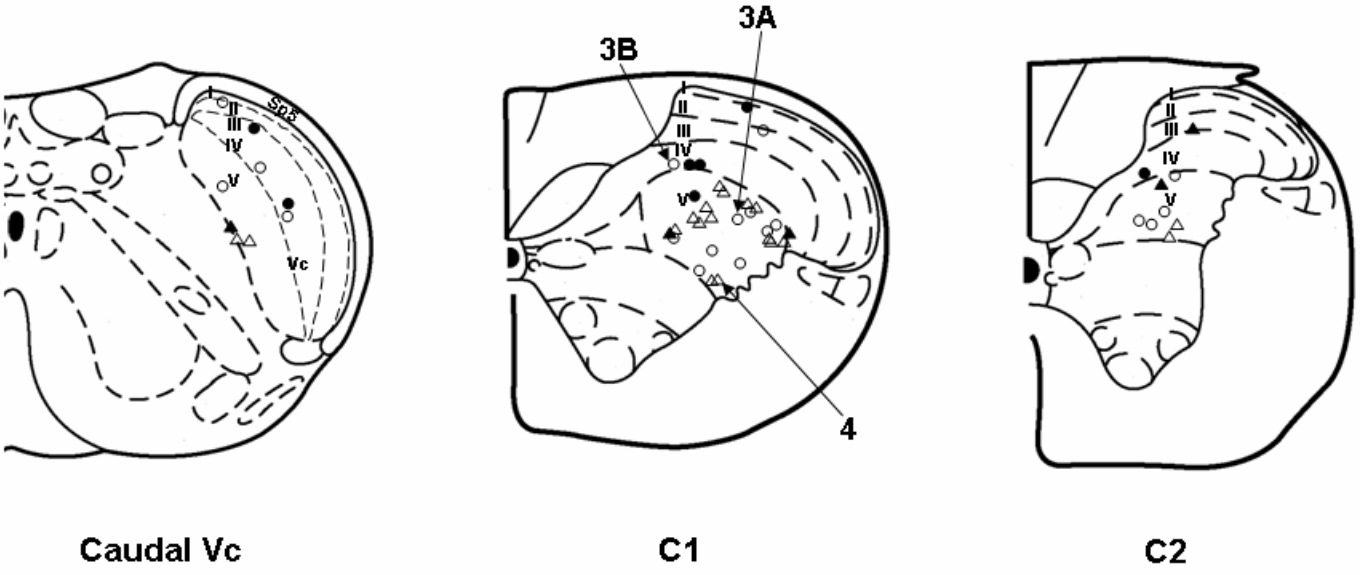


Fig. 3. Examples of typical cutaneous mechanoreceptive field (RF) and response properties of NS TMJ-responsive nociceptive neurons in the Vc/UCC region. **(a)** Cutaneous RF of a NS neuron involving the TMJ region and its lack of neuronal response evoked by injection of glutamate vehicle into the TMJ as well as its neuronal response evoked by injection of capsaicin following glutamate vehicle into the TMJ. Note: histologically confirmed lesioned NS neuronal recording site is shown in Fig. 2. **(b)** Cutaneous RF of a NS neuron involving the TMJ region and neuronal response evoked by injection of glutamate into the TMJ as well as its enhanced neuronal response evoked by injection of capsaicin following glutamate into the TMJ. Note: histologically confirmed lesioned NS neuronal recording site is shown in Fig. 2.

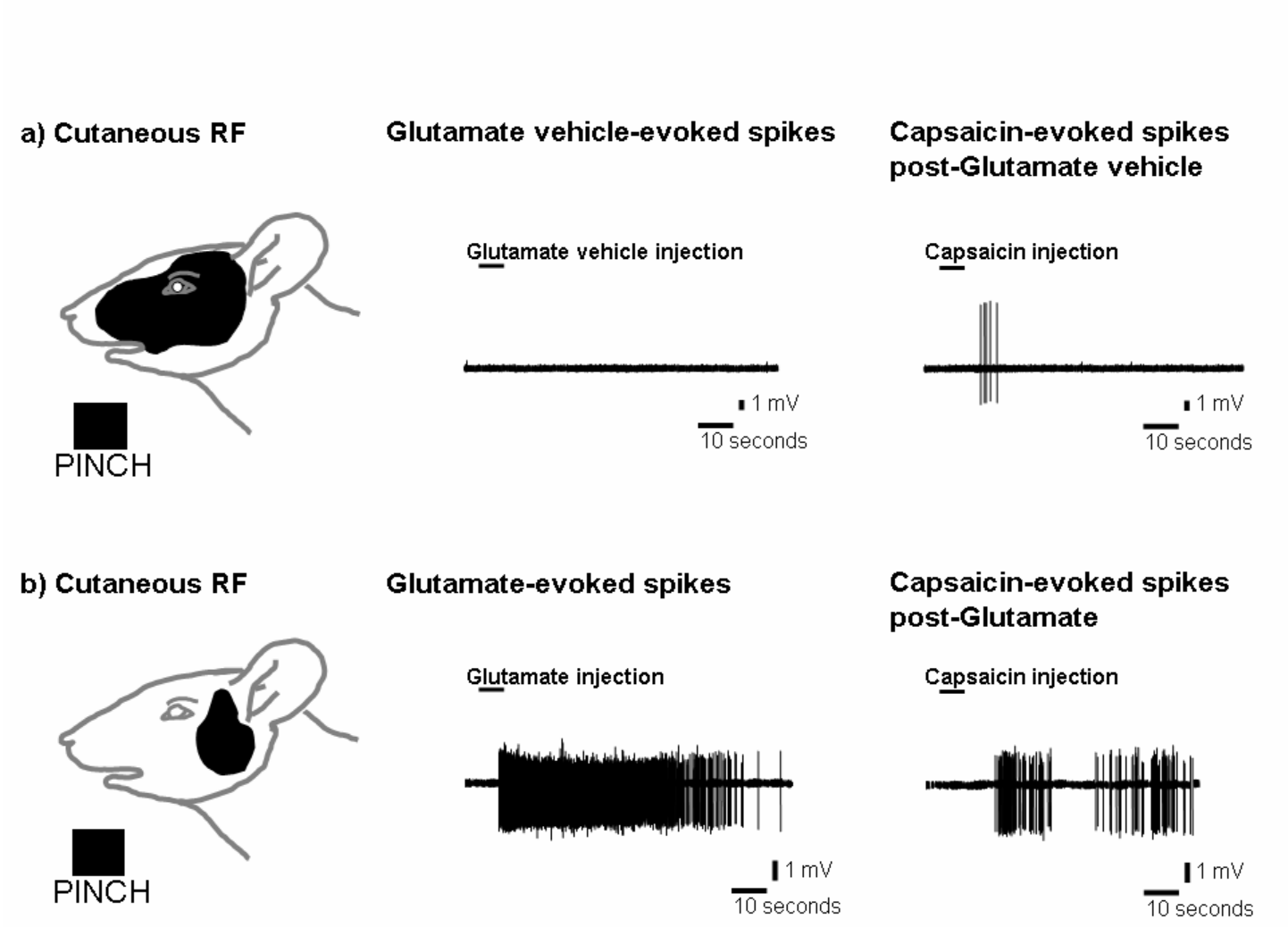


Fig. 4. Example of capsaicin and glutamate-induced central sensitization in a NS TMJ-responsive nociceptive neuron in the Vc/UCC region. **(a)** Neuronal activity at baseline and responses evoked by injection of capsaicin and glutamate into the TMJ, **(b)** Cutaneous mechanoreceptive field (RF) of NS neuron at baseline and following injection of capsaicin and glutamate into the TMJ, **(c)** Cutaneous mechanical activation threshold (MAT) of NS neuron at baseline and following injection of capsaicin and glutamate into the TMJ. Note: histologically confirmed lesioned NS neuronal recording site is shown in Fig. 2.

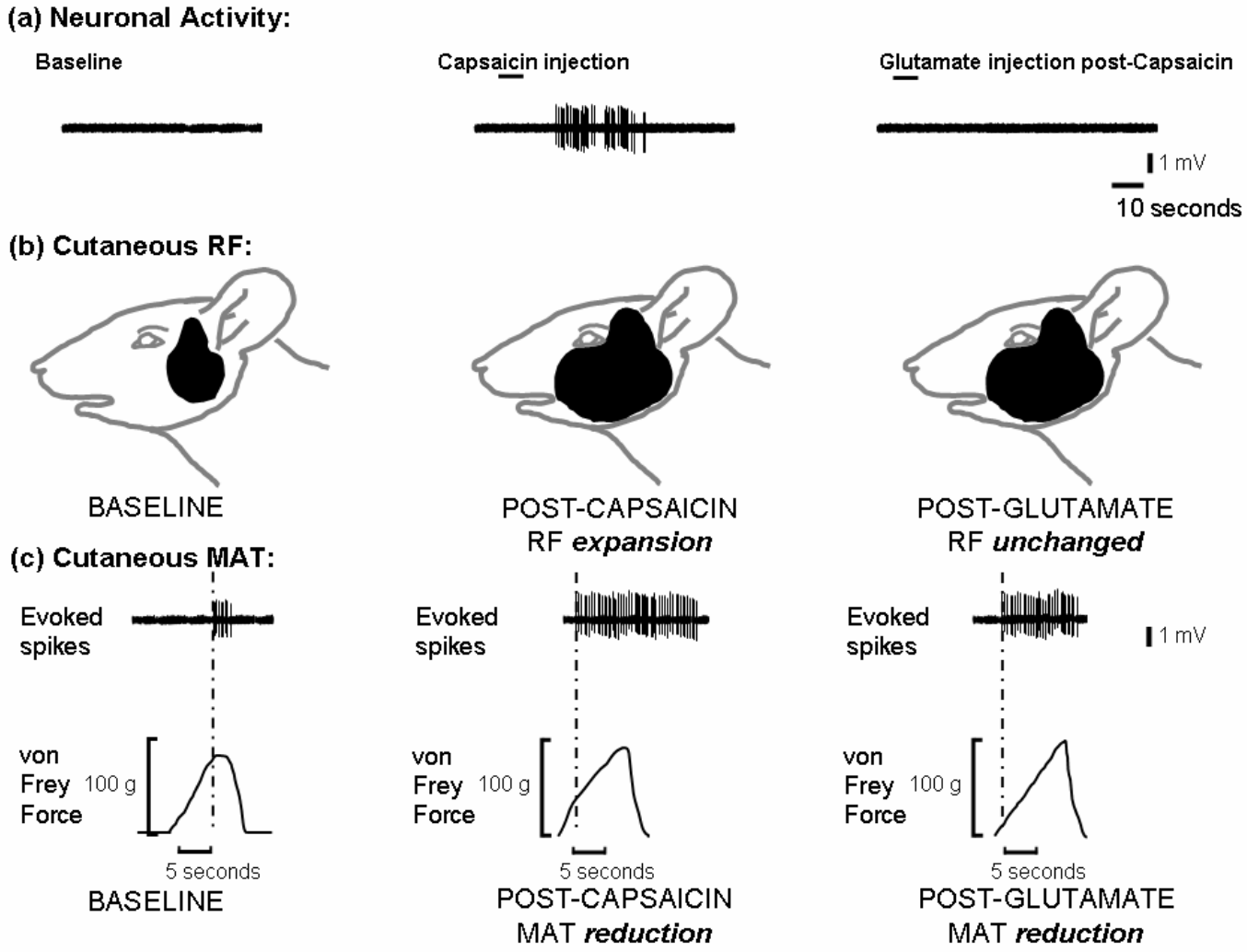
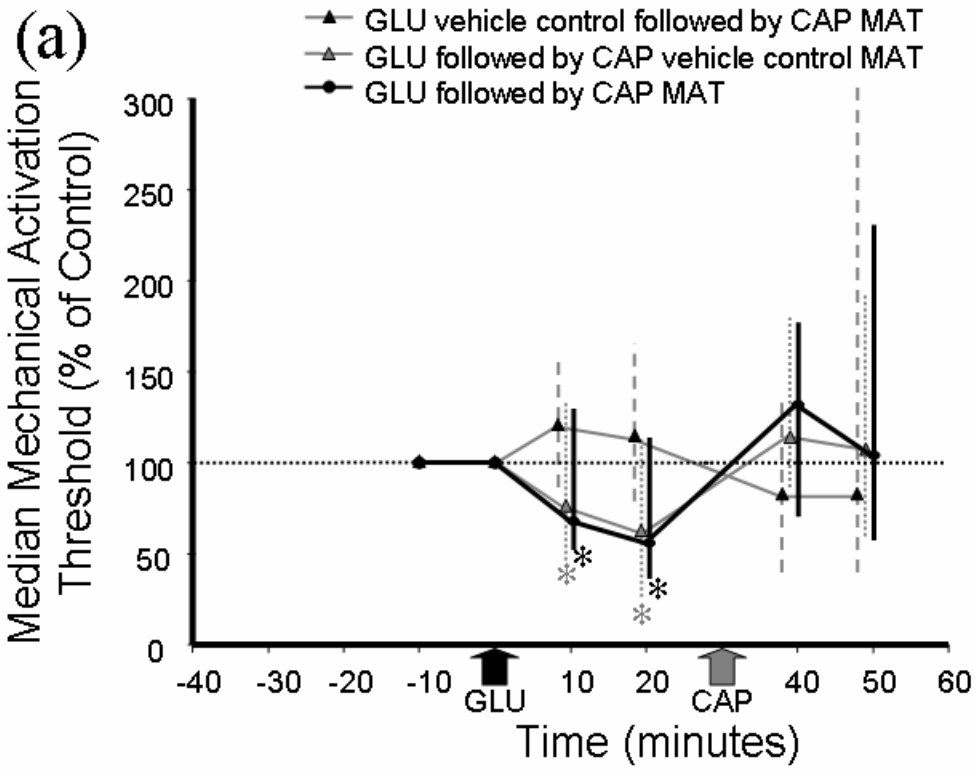


Fig. 5. The time courses of glutamate (GLU) and capsaicin (CAP)-induced mechanical activation threshold (MAT) in deep craniofacial afferents. Arrow indicates time point for injection of GLU (black) and CAP (grey) into deep craniofacial tissues. Circles indicate median normalized MAT following injection of GLU and CAP. Triangles indicate GLU or CAP-induced median normalized MAT before or after injection of vehicle controls for GLU or CAP. Raw MAT threshold values were normalized to the initial baseline pre-injection value of the first agonist. Lines: interquartile range. Note that injection of **(a)** GLU alone and **(b)** GLU following CAP into deep craniofacial tissues significantly reduced the MAT (*p<0.05, **p<0.01, ***p<0.001; RM ANOVA-on-ranks, Dunn's Method).



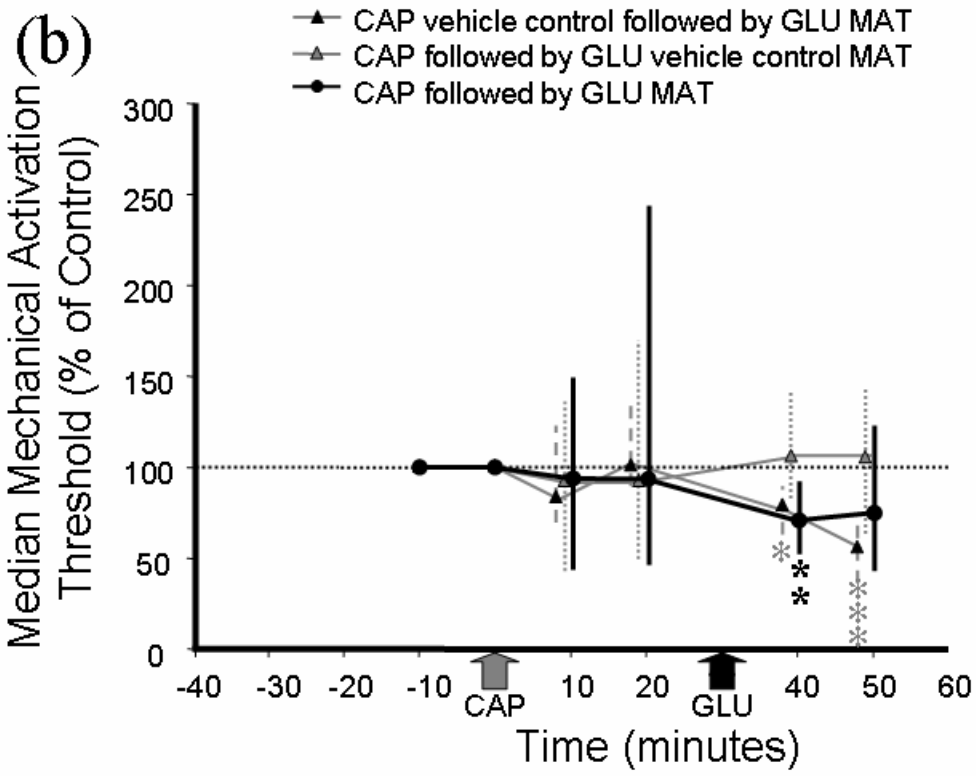
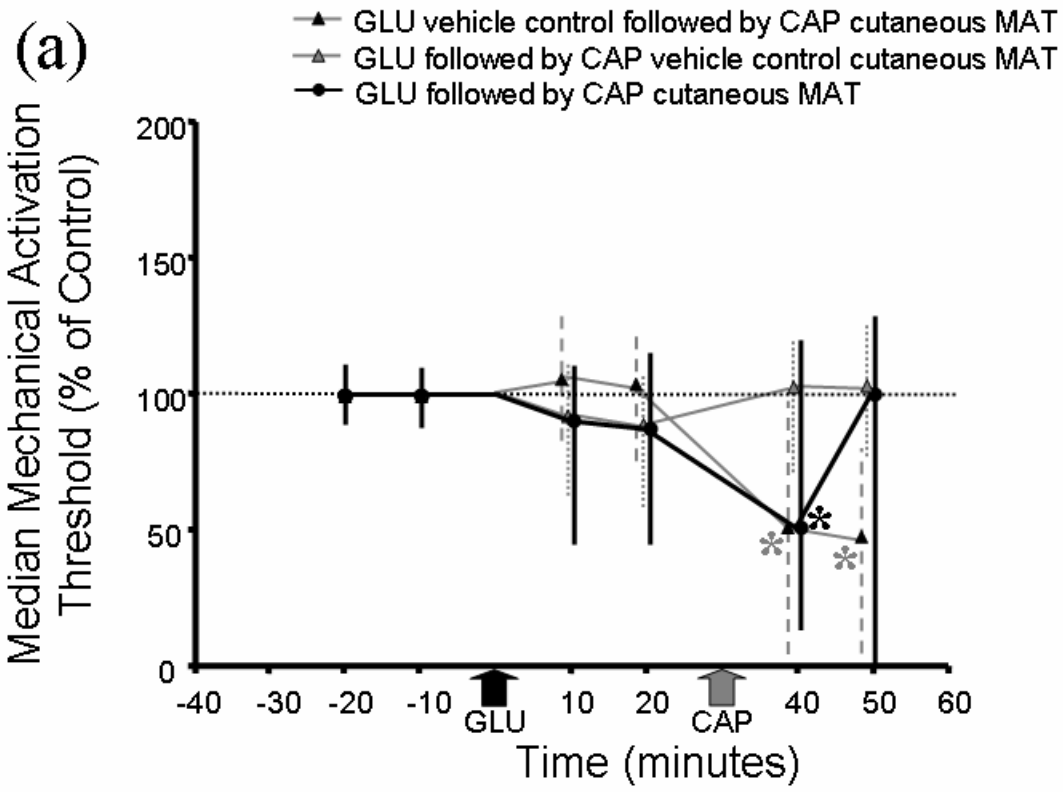
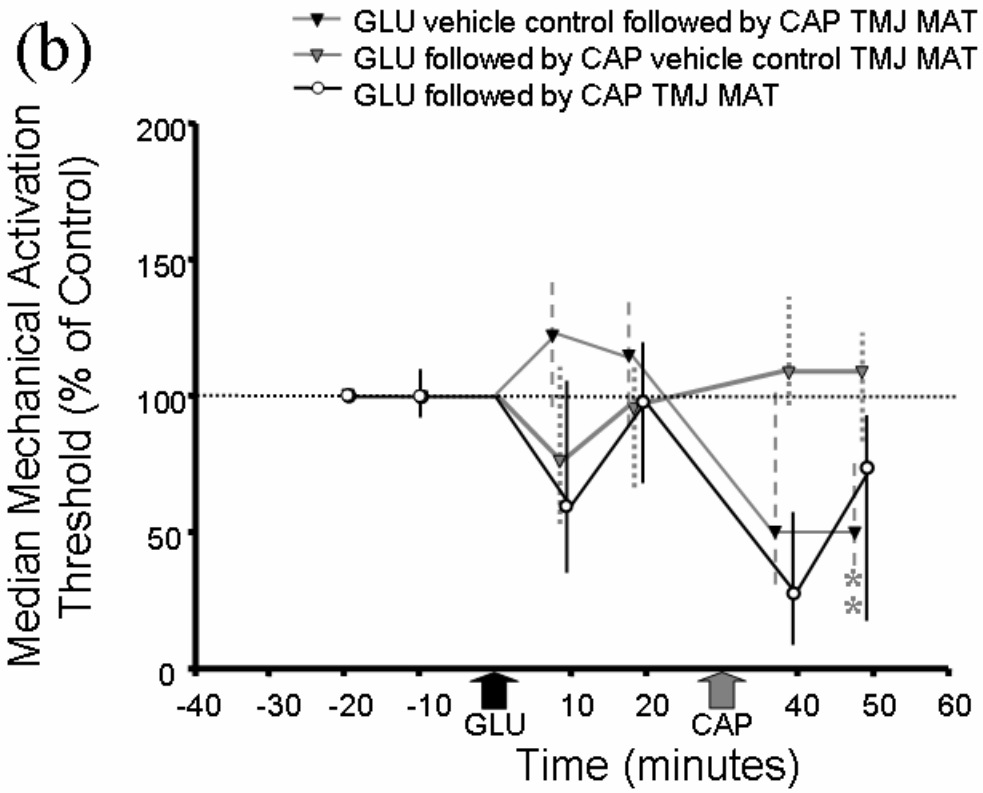
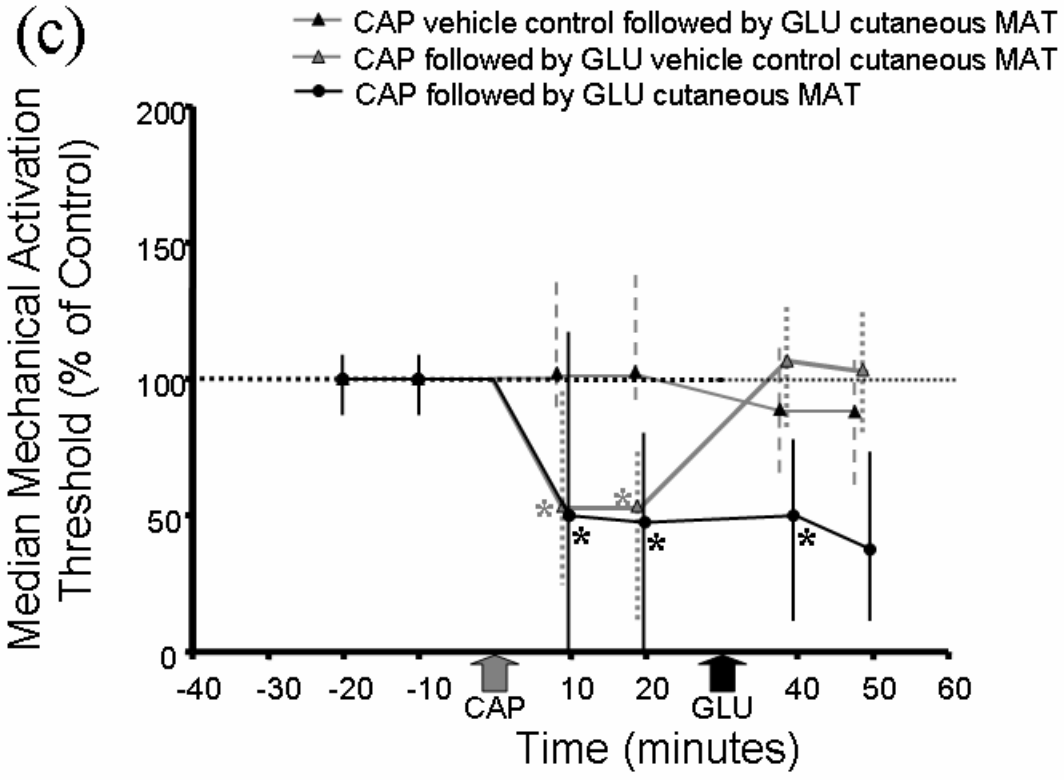

(b)
CAP vehicle control followed by GLU MAT
CAP followed by GLU vehicle control MAT
CAP followed by GLU MAT
Median Mechanical Activation Threshold (% of Control)
300
250
200
150
100
50
0
-40
-30
-20
-10
CAP
10
20
GLU
40
50
60
Time (minutes)

Fig. 6. The time courses of glutamate (GLU) and capsaicin (CAP)-induced cutaneous mechanical activation threshold (MAT) and TMJ MAT reduction in Vc/UCC nociceptive neurons. Arrow indicates time point for injection of GLU (black) and CAP (grey) into the TMJ. Circles indicate median normalized cutaneous MAT (black) and TMJ MAT (white) following injection of GLU and CAP. Triangles indicate GLU or CAP-induced median normalized cutaneous MAT and TMJ MAT before or after injection of vehicle controls for GLU or CAP. Raw MAT threshold values were normalized to the initial baseline pre-injection value of the first agonist. Lines: interquartile range. Note that injection of CAP following GLU vehicle control or CAP alone into the TMJ significantly reduced both the **(a and c)** cutaneous (*p<0.05) and **(b and d)** TMJ MAT (**p<0.01); whereas injection of **(a)** CAP following GLU, or **(c)** GLU following CAP significantly reduced the cutaneous MAT (*p<0.05; RM ANOVA-on-ranks, Dunn's method).




(b)
GLU vehicle control followed by CAP TMJ MAT
GLU followed by CAP vehicle control TMJ MAT
GLU followed by CAP TMJ MAT
Median Mechanical Activation Threshold (% of Control)
200
150
100
50
0
-40 -30 -20 -10 GLU 10 20 CAP 40 50 60
Time (minutes)


(c)
CAP vehicle control followed by GLU cutaneous MAT
CAP followed by GLU vehicle control cutaneous MAT
CAP followed by GLU cutaneous MAT
Median Mechanical Activation Threshold (% of Control)
200
150
100
50
0
-40 -30 -20 -10 CAP 10 20 GLU 40 50 60
Time (minutes)

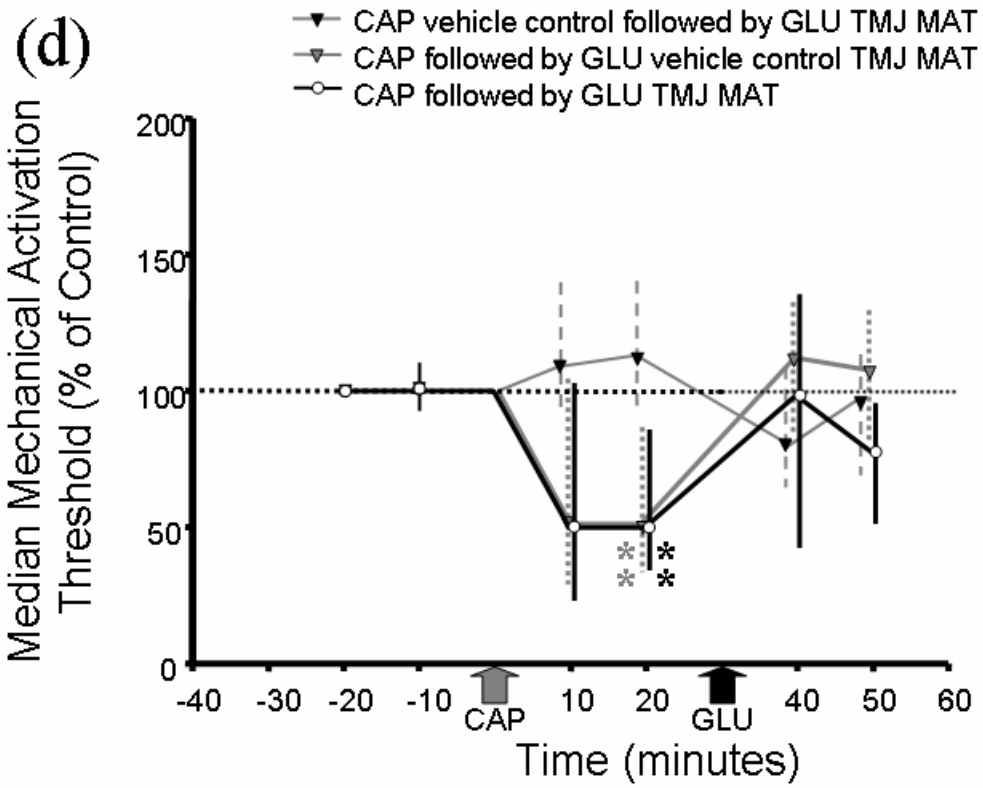
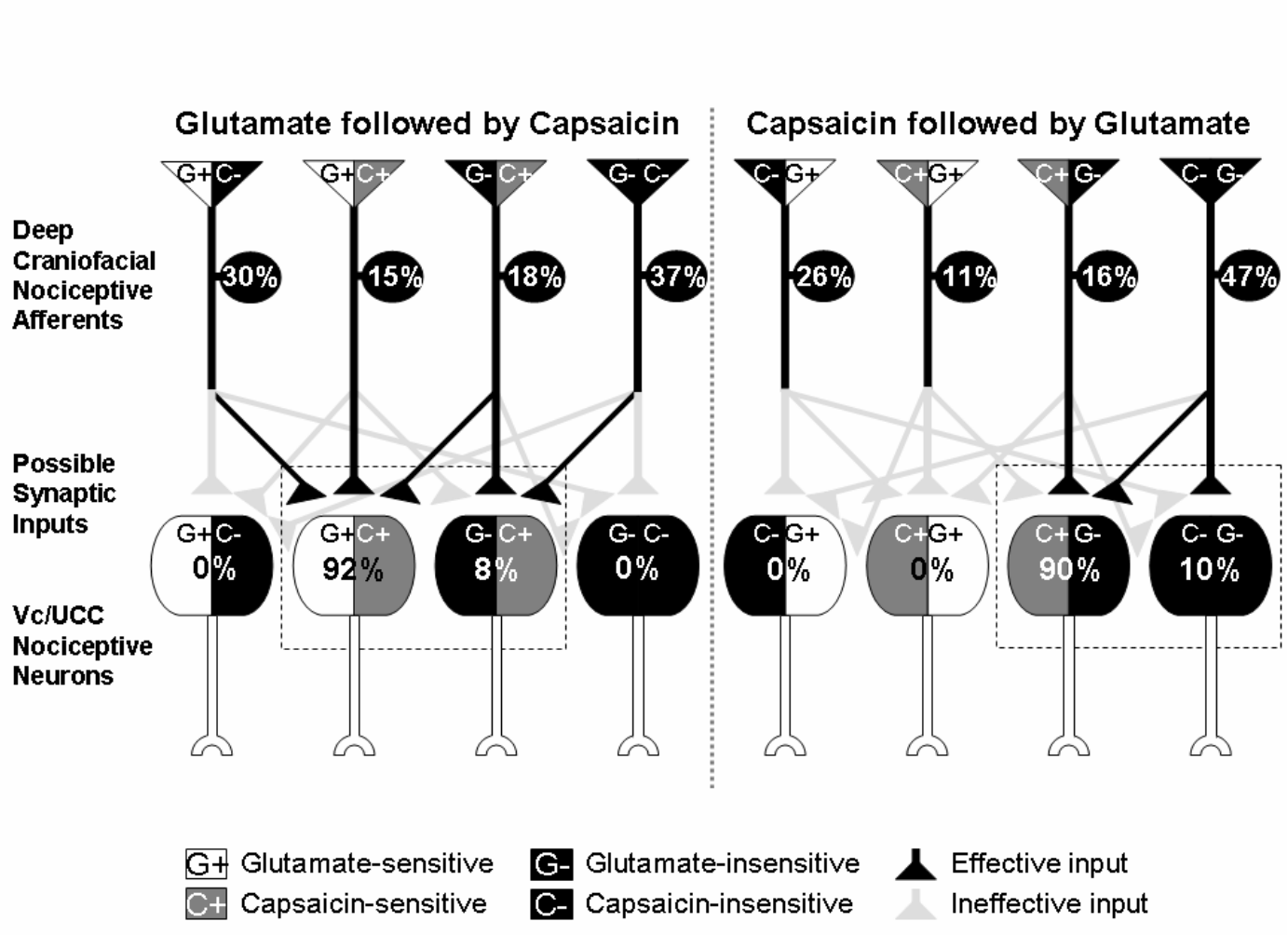

(d)
CAP vehicle control followed by GLU TMJ MAT
CAP followed by GLU vehicle control TMJ MAT
CAP followed by GLU TMJ MAT
Median Mechanical Activation Threshold (% of Control)
200
150
100
50
0
-40
-30
-20
-10
CAP
10
20
GLU
40
50
60
Time (minutes)

Fig. 7. Schematic diagram of glutamate and capsaicin-evoked activation responses and some of the possible convergent trigeminal nociceptive afferent inputs to Vc/UCC nociceptive neurons. Note that for both the glutamate followed by capsaicin subgroup and the capsaicin followed by glutamate subgroup, whereas there were four types of agonist-responsive afferents, there were only two types of agonist-responsive Vc/UCC neurons activated following injection of glutamate and capsaicin into deep craniofacial tissues. In addition, the vast majority of the Vc/UCC neurons were capsaicin-sensitive in both subgroups (100% of the glutamate followed by capsaicin subgroup and 90% of the Vc/UCC neurons were capsaicin followed by glutamate subgroup), whereas there were no glutamate-sensitive Vc/UCC neurons in the capsaicin followed by glutamate subgroup. Possible synaptic inputs depicted in black refer to inputs effective in activating the neurons and those in gray refer to relatively ineffective inputs. %: Percentages of each type of agonist-responsive deep craniofacial afferent or Vc/UCC nociceptive neuron found in the glutamate followed by capsaicin subgroup or capsaicin followed by glutamate subgroup.



Chapter 3

Peripheral NMDA receptor modulation of jaw muscle electromyographic activity induced by capsaicin injection into the temporomandibular joint of rats

3.0 ABSTRACT:

We have previously documented that peripheral N-methyl-D-aspartate (NMDA) receptor mechanisms are involved in nociceptive reflex increases in jaw muscle activity to injection of mustard oil or glutamate into the rat temporomandibular joint (TMJ). The aim of the present study was to determine whether peripheral NMDA receptor mechanisms are also involved in the nociceptive reflex responses in the jaw muscles evoked by injection of the inflammatory irritant and algesic chemical capsaicin into the TMJ. The effects of peripheral injection of NMDA receptor antagonists, MK-801 and APV, on the increases in electromyographic (EMG) activities of digastric and masseter muscles reflexly evoked by capsaicin injection into the TMJ were tested in halothane-anesthetized male rats. The capsaicin injection following pre-injection of vehicle evoked significant increases in EMG activity in both digastric and masseter muscles whereas pre-injection of MK-801 or APV into the TMJ resulted in a significant concentration-related reduction in the magnitude of capsaicin-evoked digastric and masseter EMG activity (ANOVA-on-ranks, $P<0.05$). This finding indicates that capsaicin-evoked digastric and masseter EMG activity can be attenuated by pre-injection into the TMJ of NMDA receptor antagonists, and that the activation of peripheral NMDA receptors may be important in the mechanisms whereby capsaicin evokes nociceptive trigeminal responses.

3.1 INTRODUCTION:

The vanilloid type 1 receptor, TRPV1, is activated by noxious heat, protons or the inflammatory irritant and small-fiber excitant capsaicin, and is found on small-diameter afferent nerve fibers and dorsal root ganglion neurons (Caterina *et al.* 1997; Tominaga *et al.* 1998; Jordt *et al.* 2003). TRPV1 receptors have also recently been described on trigeminal afferents and trigeminal ganglion neurons innervating the rat temporomandibular joint (TMJ) (Ichikawa *et al.* 2004). Furthermore, capsaicin injected into the rat TMJ reflexly evokes a dose-dependent increase in jaw muscle electromyographic (EMG) activity (Tang *et al.* 2004), produces an inflammatory response (Hu *et al.* 2005a), and induces activation and sensitization in brainstem nociceptive neurons (Chapter 2). Intramuscular injection of capsaicin in humans also results in intense pain and hyperalgesia (Witting *et al.* 2000b). Both trigeminal afferent (Chapter 2) and brainstem (Chapter 2) nociceptive neuronal responses to capsaicin injected into the TMJ can be significantly increased following glutamate injection into the TMJ. These findings raise the possibility that peripheral NMDA receptors may contribute to the mechanisms whereby capsaicin evokes nociceptive responses.

It has been previously shown that glutamate injection into the rat TMJ induces a concentration-related reflex increase in jaw muscle activity that can be significantly attenuated by co-injection of NMDA receptor antagonists (Cairns *et al.* 1998). Similarly, glutamate injection into the human masseter muscle causes pain that can be attenuated by co-injection of an NMDA receptor antagonist (Cairns *et al.* 2001a, 2003ab, Svensson *et al.* 2003). Increases in jaw muscle reflex activity as a result of mustard oil application to the TMJ are also attenuated by TMJ pre-injection of an NMDA receptor antagonist (Yu *et al.* 1996). In order to determine whether peripheral NMDA receptor mechanisms are involved in nociceptive reflex responses evoked by capsaicin application to the TMJ, the present study tested the possible effects of the peripheral (TMJ) application of NMDA receptor antagonists, MK-801 and APV, on the increases in jaw muscle EMG activity that could be reflexly evoked by capsaicin application to the TMJ.

A portion of this data has been previously presented in abstract form (Hu *et al.* 2003, Lam *et al.* 2003c).

3.2 MATERIALS AND METHODS:

ANIMAL PREPARATION. A total of 45 male Sprague Dawley rats (250–450 gm) were prepared for acute in vivo recording of jaw muscle EMG activity, as previously described (Hu *et al.* 1993; Yu *et al.* 1994, 1995, 1996). Briefly, under surgical anesthesia (O_2: 1 L/min; halothane: 1.5–2.5%), a tracheal cannula was inserted and artificial ventilation initiated. The rat's head was then placed in a stereotaxic frame to facilitate placement of EMG recording electrodes. Bipolar electrodes were fashioned out of 40 gauge Teflon-coated single-strand stainless steel wire and inserted into the left digastric and masseter muscles.

After completion of all surgical procedures, the halothane level was titrated (1–1.3%) until noxious pressure applied to the hindpaw could not induce a flexion reflex of the hindlimb to ensure that an adequate level of anesthesia was maintained for the duration of the experiment. Heart rate and body core temperature were continuously monitored throughout the experiment and kept within the physiological range of 330–430/min and 37–37.5°C, respectively. All methods and experimental approaches were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Canada).

DRUG SOLUTIONS. For the TMJ administration of drug solutions, two 27-gauge cannulae were first cemented side-by-side and connected to two Hamilton syringes with polyethylene tubes. One cannula was filled with either the non-competitive NMDA receptor antagonist (+)-MK-801 (0.001, 0.01 or 0.1M; 10 µL; n=6 per group; Research Biochemicals International, Natick, MA), competitive NMDA receptor antagonist (±)-D-2-amino-5-phosphonovalerate, APV (0.005 or 0.05M; 10 µL; n=6 per group Research Biochemicals International, Natick, MA),

or vehicle (sterile normal saline; 10 μL; n=6), and the other with 1% capsaicin (10% capsaicin in ethanol:Tween-80: sterile normal saline in a 1:1:8 ratio by volume; 10 μL; Calbiochem, La Jolla, CA). Both NMDA receptor antagonists were dissolved in isotonic saline (pH 7.2-7.4). The concentrations of the NMDA receptor antagonists and capsaicin were chosen on the basis of our previous findings (Yu *et al.* 1996; Cairns *et al.* 1998; Tang *et al.* 2004; Hu *et al.* 2005a). The two cannulae were then carefully inserted into the left TMJ, and capsaicin and NMDA receptor antagonists or vehicle were injected into the left TMJ via the needle and catheter. We have previously demonstrated that injection of capsaicin vehicle or isotonic saline into the TMJ region does not evoke any significant change in EMG activity in the digastric or masseter muscles (Yu *et al.* 1995, 1996; Tang *et al.* 2004).

STIMULATION AND RECORDING TECHNIQUES. Bipolar recordings were made of the EMG activities of the left digastric (jaw-opening) and left masseter (jaw-closing) muscles (Hu *et al.* 1993; Yu *et al.* 1995) before and after injections of MK-801, APV, or vehicle control, and capsaicin. EMG activity was amplified (gain, 500x; bandwidth, 30–1000 Hz) and fed into a computer equipped with a CED 1401 Plus board and analysis software (Spike2; Cambridge Electronics; signal sampling rate was 2000 Hz). Recorded EMG activity was stored electronically and analyzed offline. The EMG activities with the jaw in resting position were first recorded for 20 min to establish a baseline, and either MK-801, APV or saline vehicle was administrated into the left TMJ. In another series of experiments, MK-801 (0.1M, n=6) or APV (0.05M, n=3) was injected into the right TMJ to control for possible systemic effects. Five minutes following the administration, capsaicin was injected into the left TMJ and the resulting changes in EMG activity were continuously recorded for another 30 min. All drug solutions were slowly injected into the TMJ over ~5 sec.

DATA ANALYSIS AND STATISTICS. Recorded EMG data were rectified off-line, and EMG area bins (microvolts per minute) were calculated. Baseline EMG

activity was calculated as a mean of EMG area bins recorded over the first 20 min before injection of agents into the TMJ. Relative EMG activity was calculated by normalizing EMG area bins to the baseline EMG activity and was used to illustrate the results of individual experiments. Agents applied to the TMJ were considered to have evoked jaw muscle activity if the value of the first EMG bin after TMJ application was 2 SD above the baseline (Yu *et al.* 1995). The value of the baseline plus 2 SD was chosen as a signal-to-noise limit because it represents an approximation of the 95% confidence interval for the mean baseline activity. The relative area under the EMG response curve (AUC) was calculated by summing the value of the first and all subsequent EMG area bins greater than 2 SDs above the mean baseline (20 min) EMG activity and defined as the overall response. ANOVA-on-ranks and post-hoc Dunnett's or Dunn's tests were used as appropriate ($p<0.05$ considered to reflect statistically significant differences).

3.3 RESULTS:

Consistent with our previous findings (Yu *et al.* 1994, 1995), none of the animals revealed any variation in baseline EMG activities greater than two standard deviations (e.g. Fig. 1) during the initial 20 min period prior to capsaicin injection into the TMJ. Compared with baseline EMG activity, administration of either the saline vehicle (n=6), MK-801 (0.001, 0.01 or 0.1M; n=6 per group), or APV (0.005 or 0.05M; n=6 per group) into the left TMJ did not evoke any significant change in EMG activity (e.g. Fig. 1). In contrast, compared with the baseline EMG activity, the capsaicin injection into the left TMJ following the pre-injection of saline vehicle evoked significant increases in EMG activity in both digastric and masseter muscles of all animals (6/6) tested that lasted up to 20 min (Figs. 1-3).

Effects of MK-801 on capsaicin-induced EMG responses

Compared with the EMG responses in the animals receiving pre-injection of saline vehicle, the pre-injection of MK-801 at all 3 concentrations tested (0.001, 0.01, and 0.1M) into the left TMJ significantly reduced the AUC of the capsaicin-induced EMG response in both digastric and masseter muscles; the reduction was greater in the masseter muscle than in the digastric muscle and was most pronounced with the highest concentration of MK-801 (Figs. 1 and 2). Thus, compared with pre-injection of saline, pre-injection of MK-801 into the left TMJ resulted in a significant concentration-related reduction in the magnitude of capsaicin-evoked digastric and masseter EMG responses (ANOVA-on-ranks, Dunnett's test, $p<0.05$) (Figs. 1 and 2).

Experiments to rule out systemic effects of MK-801 were conducted by determining if pre-injection of the highest concentration of MK-801 into the right TMJ can attenuate the EMG responses induced by capsaicin injected into the left TMJ via a systemic route. There was however no significant difference in capsaicin-evoked EMG activity following the pre-injection of vehicle into the left TMJ or 0.1M MK-801 into the right TMJ (ANOVA-on-ranks, Dunnett's test, $p>0.05$). In contrast, there was a significant difference in capsaicin-induced EMG activity following the pre-injection of 0.1M MK-801 into the left TMJ when compared to pre-injection of 0.1M MK-801 into the right TMJ (ANOVA-on-ranks, Dunnett's test, $p<0.05$) (Fig. 3).

Effects of APV on capsaicin-induced EMG responses

Compared with the EMG responses in the animals receiving pre-injection of saline vehicle, the pre-injection of APV at a concentration of 0.005M into the left TMJ did not significantly reduce the AUC of the capsaicin-induced EMG response in the digastric muscle although it did significantly reduce the capsaicin-induced EMG response in the masseter muscle. The pre-injection of 0.05M APV did significantly reduce the AUC of the capsaicin-induced EMG response in both digastric and masseter muscles; the reduction was greater in the masseter muscle than in the digastric muscle and was most pronounced with

the highest concentration of APV (Figs. 1 and 2). Thus, compared with pre-injection of saline, pre-injection of APV into the left TMJ resulted in a significant reduction in the magnitude of capsaicin-evoked digastric and masseter EMG responses (ANOVA-on-ranks, Dunnett's test, $p<0.05$) (Figs. 1 and 2).

There was no significant difference in capsaicin-evoked EMG activity following the pre-injection of vehicle into the left TMJ or 0.05M APV into the right TMJ (ANOVA-on-ranks, Dunn's test, $p>0.05$). However, there was a significant difference in capsaicin-induced EMG activity following the pre-injection of 0.05M APV into the left TMJ when compared to pre-injection of 0.05M APV into the right TMJ (ANOVA-on-ranks, Dunn's test, $p<0.05$) (Fig. 3).

3.4 DISCUSSION:

Local application of the small fiber-excitant and inflammatory irritant capsaicin to the left TMJ increased jaw muscle activity and these capsaicin-evoked increases in EMG activity were attenuated by pre-injection of NMDA receptor antagonists into the left TMJ. It may be argued that the attenuation in capsaicin-evoked increases in EMG activity may be due to mechanisms unrelated to NMDA receptors since although MK-801 non-competitively antagonizes excitation mediated by the NMDA receptor, at higher concentrations (~100 microM) MK-801 also blocks sodium channels (Rothman 1988; Halliwell *et al.* 1989). Another concern is that MK-801 at lower concentrations (0.3-30 microM) also blocks nicotinic acetylcholine channels (Halliwell *et al.* 1989; Galligan and North 1990; Ramoa *et al.* 1990; Amador and Dani 1991 Briggs and McKenna 1996). However, our finding that the competitive NMDA receptor antagonist APV also similarly attenuates capsaicin-evoked EMG activity confirms the involvement of NMDA receptors in the mechanism behind capsaicin-evoked nociceptive EMG responses. It is unlikely that the effect of MK-801 or APV injected into the left TMJ was produced by a systemic action since there was no significant effect of either MK-801 or APV injection into the right TMJ on capsaicin-evoked EMG

activity. We have interpreted these results to suggest that the activation of peripheral NMDA receptors contributes, in part, to the mechanism whereby capsaicin injection into the TMJ evokes nociceptive jaw muscle responses.

Peripheral neural mechanisms underlying the reflex activation of jaw muscles

We have previously reported that application of mustard oil or glutamate to the rat TMJ region results in a characteristic increase in the EMG activity of both the digastric and masseter muscles that involves a brainstem reflex circuit involving trigeminal brainstem subnucleus caudalis (Yu *et al.* 1994, 1995, 1996; Bakke *et al.* 1998; Cairns *et al.* 1998, 2001c; Hu *et al.* 1997; Tsai *et al.* 1999). It has also been shown that injection of glutamate into the masseter muscle of humans caused significantly higher levels of pain and pain spread than injection of isotonic saline (Cairns *et al.* 2001a, 2003b, Svensson *et al.* 2003). Peripheral NMDA receptors may play a role in these effects of glutamate since recent evidence indicates that ketamine, an NMDA receptor antagonist, applied in combination with glutamate, decreases glutamate-evoked muscle pain in humans (Cairns *et al.* 2003a). The results of the present study indicate that application of capsaicin to the rat TMJ evokes a similar co-activation of these jaw muscles, and thus it is possible that capsaicin may activate the same putative nociceptive reflex pathways as mustard oil, glutamate and other algesic chemicals. The receptor mechanisms underlying the mustard oil or capsaicin-evoked activation of TMJ afferents remain unclear. However, our present results along with our earlier data that local application to the TMJ region of the NMDA antagonist MK-801 blocks mustard oil-evoked jaw muscle activity (Yu *et al.* 1996) indicate that peripheral NMDA receptors appear to play a role in mediating mustard oil and capsaicin-evoked increases in jaw muscle activity.

Since TRPV1 receptors are activated by heat, protons, or capsaicin (Caterina *et al.* 1997; Tominaga *et al.* 1998; Benham *et al.* 2003; Jordt *et al.* 2003), the results of this study suggest there may be functional TRPV1 receptors located within the TMJ region. Our findings are supported by recent findings of TRPV1

receptors in TMJ tissues of the rat (Ichikawa *et al.* 2004). However, the use of specific receptor antagonists would be required to confirm the involvement of the TRPV1 receptor. One possible mechanism to account for our finding that antagonism of peripheral NMDA receptors contributes to capsaicin-evoked jaw muscle activity is via autocrine and/or paracrine activation of peripheral NMDA receptors (Carlton 2001; Hinoi *et al.* 2004). That is, activation of peripheral TRPV1 receptors via noxious heat, protons or capsaicin may result in the release of glutamate from neuronal terminals. The glutamate released could then further activate NMDA receptors on the same neuronal terminal or adjacent surrounding peripheral terminals. Activation of peripheral NMDA receptors could then lead to the release of more glutamate in the peripheral tissues and might alter TRPV1 receptor responsiveness to enhance nociceptive responses. Another possible mechanism, albeit a much slower process, is that blockade of calcium (Ca^{2+}) influx via peripheral NMDA receptors in nociceptive afferents may prevent the potentiation of NMDA and/or TRPV1 activity (Petrenko *et al.* 2003; Geppetti and Trevisani 2004). No studies to date have demonstrated the co-localization of peripheral NMDA and TRPV1 receptors on the same trigeminal primary afferent terminal but our recent evidence that peripherally applied glutamate and capsaicin may activate the same TMJ or craniofacial muscle fiber (Chapter 2) suggests that both receptors may be found on a single trigeminal primary afferent. Since mustard oil excites sensory nerve fibers through activation of the peripheral TRPA1 (ANKTM1) receptor (Jordt *et al.* 2004; Bandell *et al.* 2004), also a member of the TRP family, the mechanisms proposed above for capsaicin-evoked jaw muscle activity may also account for previous findings in our laboratory that antagonism of peripheral NMDA receptors can reduce mustard oil-evoked nociceptive responses (Yu *et al.* 1996). Thus, these findings suggest that peripheral NMDA receptors may play a role in mediating the nociceptive responses elicited by the activation of these TRP receptors.

It is possible that under conditions that are associated with deep tissue pain, glutamate may be released from afferent fiber terminals and act on peripheral EAA receptors to excite nociceptive fibers. Glutamate is present in and released

from the central terminals of small-diameter spinal cord and trigeminal afferents, including TMJ afferents (Salt and Hill 1983; Wanaka *et al.* 1987; Battaglia and Rustioni 1988; Kai-Kai 1989; Westlund *et al.* 1989; Clements and Beitz 1991; Clements *et al.* 1991; Kai-Kai and Howe 1991; Azerad *et al.* 1992; Boucher *et al.* 1993; Bereiter and Benetti 1996). It is not known whether glutamate is also released from the peripheral endings of trigeminal afferent fibers but capsaicin application to the sciatic nerve has been shown to result in the neurogenic release of glutamate from peripheral terminals (deGroot *et al.* 2000). In addition to the cytosolic release of glutamate from affected neuronal terminals, non-neuronal cells such as macrophages (Piani *et al.* 1991), blood serum (McAdoo *et al.* 1997) or Schwann cells (Parpura *et al.* 1995) may also contribute to the increase in peripheral levels of glutamate following activation of TRPV1 receptors. It has been demonstrated that application of inflammatory irritants such as mustard oil and capsaicin to the TMJ region results in significant plasma extravasation (Haas *et al.* 1992; Yu *et al.* 1995, 1996; Hu *et al.* 2005a). Plasma extravasation can occur within a few seconds after a noxious stimulus is applied (Raab 1992). The concentration of glutamate in plasma is ~300 μM which is greater than the reported ED_{50} for activation of peripheral glutamate receptors (Erdo 1991; Ault and Hildebrand 1993a,b). Therefore, plasma extravasation into the TMJ region could rapidly elevate glutamate concentrations to a level that could activate EAA receptors located within the TMJ region. Thus, changes in peripheral glutamate levels through cytosolic release from tissue damage or inflammation or through neurogenic release as a result of nociceptive activation may all play a role in modulating the sensitivity of deep craniofacial tissues through autocrine and/or paracrine regulation of ionotropic glutamate receptor mechanisms.

Clinical relevance

Peripheral glutamate receptor mechanisms may have an important role in craniofacial pain since peripheral glutamate levels are elevated during cutaneous or deep tissue inflammation (Omote *et al.* 1998; Lawand *et al.* 2000; McNearny

et al. 2000). We have demonstrated that injection of glutamate into the rat masseter muscle or TMJ can activate and induce peripheral sensitization in masseter muscle and TMJ afferent fibers (Cairns *et al.* 2001a,b, 2002a; Chapter 2), activate and induce central sensitization in brainstem nociceptive neurons (Chapter 2), and reflexly increase jaw EMG activity (Cairns *et al.* 1998, 2001c), through the activation of peripheral glutamate receptors. It has also been shown that injection of glutamate into the masseter muscle induces pain in humans (Cairns *et al*. 2001a, 2003b, Svensson *et al.* 2003). These findings suggest that activation of peripheral glutamate receptors, NMDA receptors in particular, may excite nociceptors that contribute to pain responses. The glutamate-evoked peripheral sensitization may contribute to the primary hyperalgesia or allodynic states characteristic of craniofacial pain conditions such as temporomandibular disorders (TMD) (see Carlsson and LeResche 1995, Stohler 1995,1999). The glutamate-evoked central sensitization as reflected in receptive field expansion, mechanical activation threshold reduction, and increases in responses to suprathreshold stimuli and neuronal spontaneous activity may contribute to pain spread and referral, allodynia, hyperalgesia, and pain at rest in TMD (see Carlsson and LeResche 1995, Stohler 1995,1999). The finding in the present study that increases in capsaicin-evoked jaw muscle reflex activity can be attenuated by NMDA receptor antagonists indicates that peripheral NMDA receptors are involved in the mechanisms whereby capsaicin evokes nociceptive reflex responses.

The demonstration of a role for peripheral NMDA receptor mechanisms in modulating nociceptive trigeminal responses evoked by the activation of various peripheral nociceptive receptors is important in the targeting of treatment for craniofacial pain disorders of peripheral origin. Our findings that nociceptive responses evoked by the peripheral application of mustard oil, glutamate or capsaicin may be attenuated by the peripheral application of NMDA receptor antagonists suggest that it might prove more efficacious to target peripheral NMDA receptors rather than each of those activated by the above receptor agonists in the treatment of craniofacial disorders. The formulation of peripheral

NMDA receptor antagonists that do not cross the blood brain barrier may be of potential benefit by reducing peripheral nociceptive excitability while avoiding many harmful side effects that may be found with antagonism of central NMDA receptors. Although we have demonstrated a novel antinociceptive role for peripheral NMDA receptor antagonists in the animal and human craniofacial pain studies discussed above, there has been a paucity of reports on the effects of locally administered NMDA receptor antagonists in other human experimental pain models and data from the few existing studies are not consistent. For example, Warncke and colleagues (1997) showed, in a forearm burn injury model, that the development of mechanical hyperalgesia is inhibited with peripheral ketamine pretreatment and Pederson and colleagues (1998) showed, using a similar burn injury model, that peripheral ketamine pretreatment reduced spontaneous pain during burn injury induction and increased the heat pain threshold. In contrast to the results of the burn injury models, Gottrup and colleagues (2000, 2004), using a capsaicin model, failed to find an effect on pain and hyperalgesia after peripheral ketamine pretreatment. Differences in pain models, tissues (i.e. skin vs. deep craniofacial), dosage and timing of NMDA receptor antagonists and injury type and extent may explain the discrepancy in experimental results. Further studies are required to achieve a better understanding of the role of peripheral NMDA receptors in the pathobiological mechanisms underlying craniofacial pain conditions of peripheral origin.

3.5 FIGURES:

Fig. 1. Time course of left digastric and masseter EMG responses to NMDA receptor antagonist pretreatment followed by 1% capsaicin injected in the left TMJ. Compared with pre-injection of saline, pre-injection of APV or MK-801 into the left TMJ resulted in a significant concentration-related reduction in the magnitude of capsaicin-evoked left digastric (A and B) and masseter (C and D) EMG responses. Horizontal dotted line represents the baseline plus 2SD for the saline control.

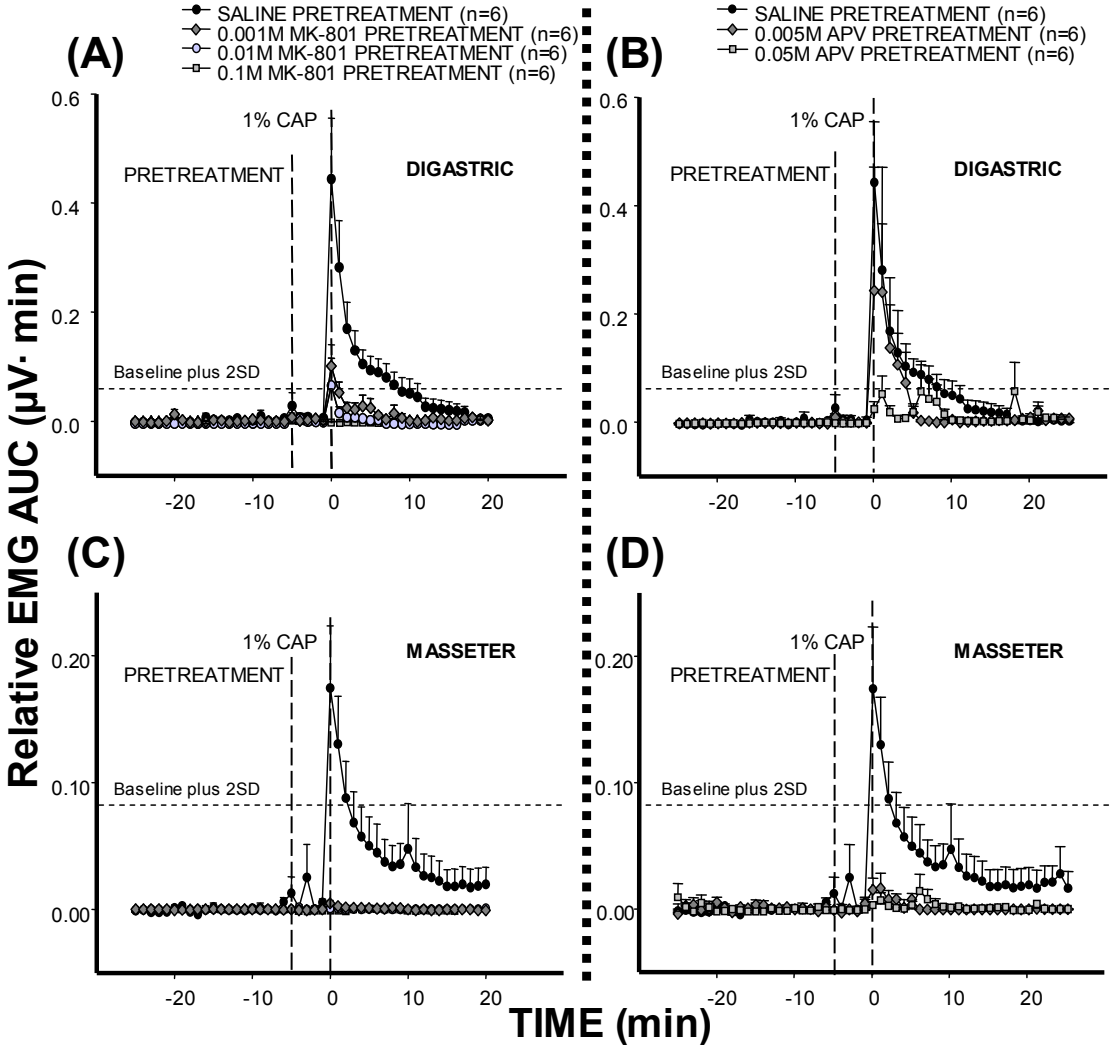


Fig. 2. Median EMG responses to NMDA receptor antagonist pretreatment followed by 1% capsaicin injected in the left TMJ. Compared with pre-injection of saline, pre-injection of APV or MK-801 into the left TMJ resulted in a significant reduction in the magnitude of capsaicin-evoked left digastric (A) and masseter (B) EMG responses (ANOVA-on-ranks, *p<0.05, **p<0.01, ***p<0.001). Median: transverse line within box; 75th percentile: top half box; 25th percentile: bottom half box.

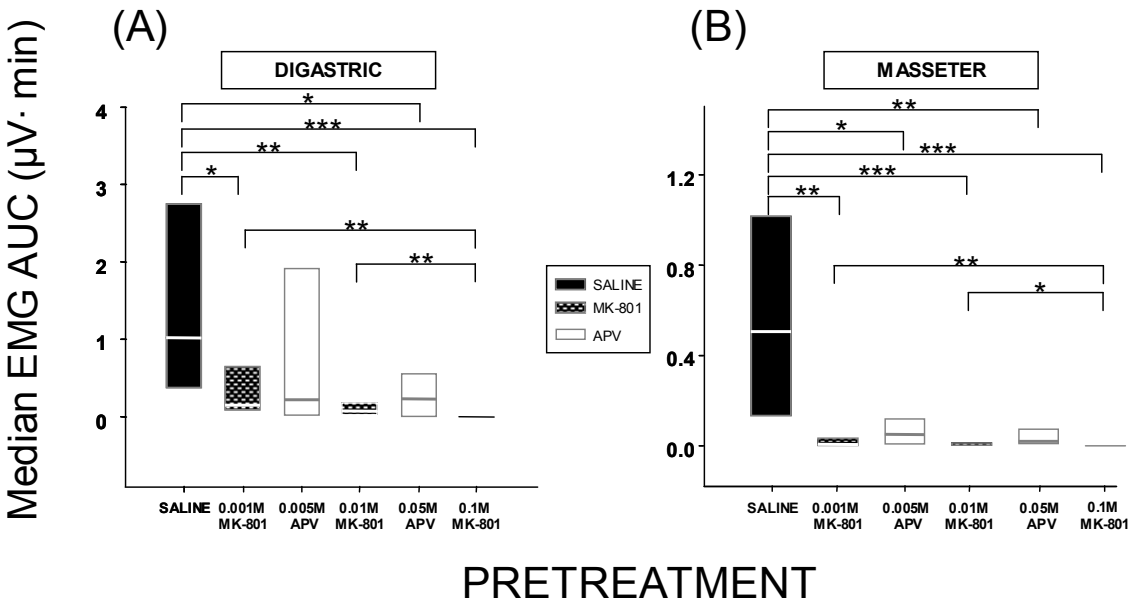
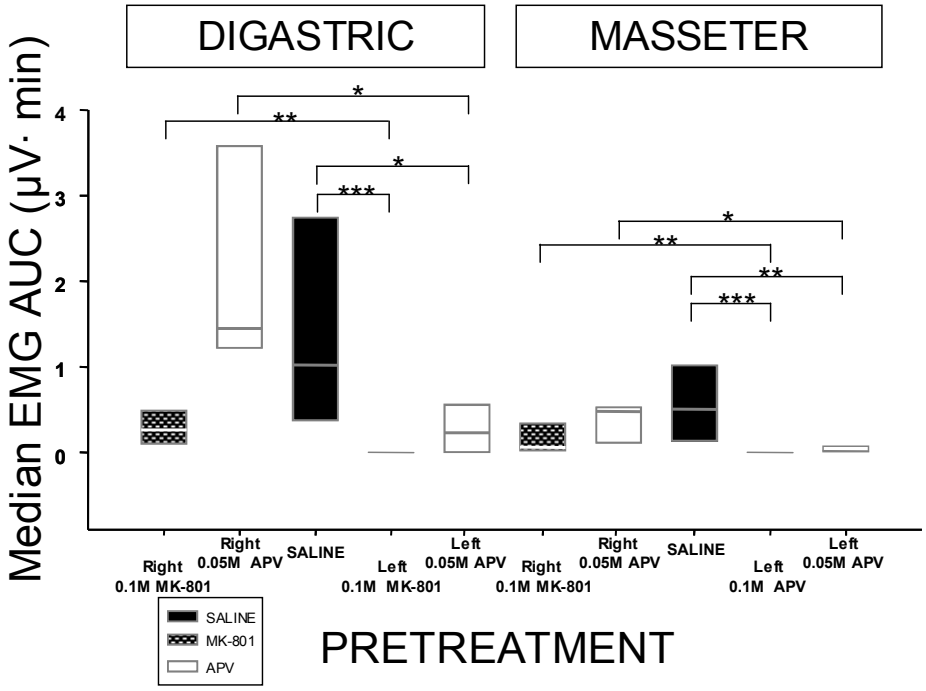


Fig. 3. Comparison of left versus right NMDA receptor antagonist pretreatment effects on median digastric and masseter EMG responses to 1% capsaicin injected in the left TMJ. There was a significant difference in capsaicin-induced EMG activity following the pre-injection of APV or MK-801 into the left TMJ when compared to pre-injection of APV or MK-801 into the right TMJ (ANOVA-on-ranks, *p<0.05, **p<0.01, ***p<0.001). Median: transverse line within box; 75th percentile: top half box; 25th percentile: bottom half box.



Chapter 4

Surgical incision can alter capsaicin-induced central sensitization in rat brainstem nociceptive neurons

4.0 ABSTRACT:

Surgical trauma can affect spinal neuronal excitability, but there have been no studies of the effects of surgical cutaneous injury on central nociceptive processing of deep afferent inputs evoked by noxious stimuli such as capsaicin. Thus our aim was to test the effect of surgical cutaneous incision in influencing central sensitization induced by capsaicin injection into the temporomandibular joint (TMJ). The activity of single nociceptive neurons activated by noxious mechanical stimulation of the TMJ was recorded in the trigeminal subnucleus caudalis/upper cervical cord of halothane-anaesthetized rats. The cutaneous mechanoreceptive field (RF), cutaneous mechanical activation threshold (MAT) and TMJ MAT of neurons before and after both surgical cutaneous incision alone and capsaicin injection were compared with results of incision and lidocaine pretreatment of the facial skin overlying the TMJ and capsaicin injection into the TMJ. Incision itself induced a barrage of neuronal spikes and excitability increases reflecting central sensitization (cutaneous RF expansion, cutaneous MAT reduction) in most neurons tested whereas lidocaine pretreatment significantly attenuated the barrage and central sensitization. Capsaicin injection into the TMJ induced cutaneous RF expansion, cutaneous MAT reduction and TMJ MAT reduction following lidocaine pretreatment of the cutaneous incision site whereas most neurons tested with capsaicin following incision alone showed no capsaicin-induced excitability increase. These findings suggest that central sensitization induced by capsaicin alone or by cutaneous incision alone can readily occur in TMJ-responsive nociceptive neurons and that following incision-induced excitability increases, nociceptive neurons may be temporarily refractory to any further capsaicin-induced changes reflecting central sensitization.

4.1 INTRODUCTION:

The trigeminal subnucleus caudalis/upper cervical spinal cord region (Vc/UCC) is an important relay site of nociceptive information from the temporomandibular joint (TMJ) and TMJ nociceptive afferent inputs can induce central sensitization in Vc/UCC (Takeshita *et al.* 2001; Bereiter *et al.* 2005; Hu *et al.* 2005b). A peripheral receptor that may be involved in craniofacial nociceptive mechanisms is the vanilloid TRPV1 receptor which is activated by the inflammatory irritant capsaicin, protons or noxious heat (Caterina *et al.* 1997; Dray 2005). We have documented that capsaicin injected into the rat TMJ activates nociceptive afferents and Vc/UCC neurons (Chapter 2), reflexly evokes increases in jaw muscle electromyographic activity (Tang *et al.* 2004; Chapter 3) and induces inflammation within these tissues that can be blocked by TRPV1 receptor antagonists (Hu *et al.* 2005a).

The intense, injury-induced afferent barrage caused by surgical trauma may in itself produce central sensitization and enhance neuronal excitability. Indeed, rat hindlimb (Brennan *et al.* 1996; Vandermeulen and Brennan,2000; Pogatzki *et al.* 2002a; Kawamata *et al.* 2005a) and human forearm (Kawamata *et al.* 2002) postoperative pain models have demonstrated central sensitization following surgical incision. Evidence from the rat incision models suggests that incision-induced central sensitization effects may vary depending on the injured skin type (hairy or glabrous) (Kawamata *et al.* 2005a). In addition, previous studies suggest that activation of cutaneous afferents is much less effective than deep tissue afferents in inducing central sensitization in spinal (Woolf and Wall 1986; Witting *et al.* 2000b; Sluka 2002) and trigeminal systems (Yu *et al.* 1993). However, the effect of cutaneous incision on central nociceptive neurons with deep afferent inputs has not been investigated in the above studies and furthermore, the effect of incision of facial cutaneous tissues on Vc/UCC nociceptive neurons has not been tested. Therefore, the aims of the present study were to determine: (1) if a surgical incision of the facial skin and fascia overlying the TMJ can induce central sensitization in nociceptive neurons receiving TMJ afferent input in the Vc/UCC region, (2) if injection of capsaicin into the TMJ can induce central sensitization in these neurons, and (3) if the surgical cutaneous incision can influence the central sensitization induced by the

subsequent injection of capsaicin into the TMJ in these neurons. A portion of this data has been previously presented in abstract form (Lam *et al.,* 2004c).

4.2 MATERIALS AND METHODS:

ANIMAL PREPARATION. The methods used for animal preparation and neuronal recording and classification were similar to those described previously in detail (Hu 1990; Hu *et al.* 2005b) and so will only be briefly outlined. Ninety adult male (250-400 g) Sprague-Dawley rats were prepared for acute recording of TMJ-responsive nociceptive neurons in the Vc/UCC under surgical anaesthesia (O_2: 1 L/min; halothane: 1.5–2.5%; Cairns *et al.* 2001). A cannula was inserted into the trachea and artificial ventilation initiated. The head of the rat was then stabilized in a stereotaxic frame, and surgery was performed to allow placement of a recording microelectrode in contact with the left Vc/UCC. Upon completion of surgery, the halothane level was slowly reduced to a level (1–1.3%) that was just sufficient to produce reflex suppression of the hindlimb to noxious pressure applied to the hindpaw to ensure that an adequate level of anaesthesia was maintained for the duration of the experiment (Cairns *et al.* 2001). Heart rate and body core temperature were continuously monitored and kept within the physiological range of 330–430/min and 37–37.5°C, respectively. All methods and experimental approaches were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Canada).

RECORDING OF NEURONAL ACTIVITY. One hour after completion of surgical procedures, a round dental burnisher (1-mm diameter) was applied as a noxious mechanical search stimulus (~100 g) to the skin overlying the TMJ while an epoxy-resin-coated tungsten microelectrode was lowered to identify a TMJ-responsive nociceptive neuron. A nociceptive neuron was considered *TMJ-responsive* when the neuron was found to respond to direct, blunt noxious mechanical stimulation of the TMJ region. Baseline spontaneous neuronal activity (spikes/second) was observed for a 10-minute period prior to mechanical and chemical stimulation. To classify each TMJ-responsive

nociceptive neuron as a wide dynamic range (WDR) or nociceptive-specific (NS) neuron, mechanical stimuli (brush, pressure, and pinch) were applied to the cutaneous tissues (Hu 1990). The cutaneous mechanoreceptive field (RF) and mechanical activation threshold (MAT) of the nociceptive neurons were assessed at the time intervals specified under the experimental paradigm (see below). A brush, blunt probe, and a pair of nonserrated forceps were used to determine the extent of the neuronal cutaneous RF which was outlined on a life-size drawing of the rat's head (Hu 1990). The neuronal MAT was determined with an electronic von Frey device (SENSELab Model 735, 1.0-mm diameter probe tip, Somedic Sales AB, Sweden) applied to the center of the cutaneous RF or the TMJ region. The MAT of the neuron was defined as the force (g) required to evoke the first spike, or a firing rate of greater than 2 standard deviations above baseline when the neuron was spontaneously active, measured at the neuronal RF site with a ramp of gradually increasing force (see example in Fig. 2).

DRUG ADMINISTRATION. The local anaesthetic Lidocaine (2% in 10 μL; AstraZeneca Canada Inc., Mississauga, ON) was infiltrated subcutaneously in one experimental group of rats to anaesthetize the skin overlying the left TMJ prior to incision of the skin. One percent capsaicin (10% capsaicin in ethanol:Tween-80: sterile normal saline in a 1:1:8 ratio by volume; 10 μL; Calbiochem, La Jolla, CA) or vehicle control (ethanol:Tween-80: sterile normal saline in a 1:1:8 ratio by volume; 10 μL) was then injected into the left TMJ in both experimental groups of rats. The concentration of capsaicin was chosen on the basis of its effectiveness in evoking inflammation and jaw muscle electromyographic activity and we have previously documented that its injected volume is localized to the TMJ region (Tang *et al.* 2004; Hu *et al.* 2005a).

EXPERIMENTAL PARADIGM. In each animal, only one TMJ-responsive nociceptive neuron was tested with capsaicin after the surgical cutaneous incision with or without lidocaine pretreatment. Experimental animals were divided into two groups according to the presence (n=45) or absence (n=45) of lidocaine pretreatment prior to incision: neuronal properties of rats with cutaneous incision alone and injection of capsaicin (or

vehicle) were compared with properties of neurons in the other group of rats with cutaneous incision and lidocaine pretreatment and injection of capsaicin (or vehicle).

A similar experimental paradigm was applied to both groups: 10 minutes after a TMJ-responsive nociceptive neuron was identified as a WDR or NS neuron, the cutaneous RF and MAT were determined and served as baseline values. A short-acting local anaesthetic (2% Lidocaine in 10 μL) was infiltrated subcutaneously 15 minutes prior to incision of the facial skin overlying the left TMJ for one experimental group of rats (Pogatzki *et al.* 2002a; Sun *et al.* 2004). The skin overlying the TMJ was then raised with tissue forceps to avoid damage to the TMJ and deep tissues during the surgical incision (<2 mm long, 30 seconds duration) made with a 16-gauge needle through skin and fascia overlying the left TMJ. The cutaneous incision was made to confirm a deep tissue afferent input by allowing direct mechanical stimulation of the TMJ, allow for the later injection of capsaicin into the TMJ and allow also direct assessment of the MAT of the TMJ before and after capsaicin injection.

A s*urgical incision-induced barrage* in TMJ-responsive nociceptive neurons was defined as the total number of evoked spikes, greater than 2 standard deviations above baseline spontaneous neuronal activity, from completion of incision up to 10-minutes post-incision or a 1-minute quiescent period. If no baseline activity was present before incision, the total number of evoked spikes following incision was considered as the incision-induced barrage. Changes in cutaneous RF, cutaneous MAT and TMJ MAT were assessed 10 and 20-minutes post-incision. This 30-minute observation period for incision-induced neuronal excitability central sensitization effects was limited to the acute phase of injury and was based on previous studies demonstrating that incision-induced neuronal excitability was significantly increased in spinal dorsal horn neurons for up to 30 minutes after the incision and returned to baseline thereafter (Kawamata *et al.* 2005b).

Thirty minutes after incision, the tip of a catheter (a 27-gauge needle connected by polyethylene tubing to a Hamilton syringe, 100 μL) was inserted into the left TMJ through the incision site. Baseline neuronal activity was recorded for 10 minutes and then capsaicin or vehicle control was slowly injected into the left TMJ (over a 5-second period), and any resulting neuronal responses were monitored for 10 minutes after the

injection. Response properties of TMJ-responsive nociceptive neurons to injection of capsaicin into the TMJ were assessed as: (1) *Response magnitude*: the total number of evoked spikes, or a firing rate of greater than 2 standard deviations above baseline neuronal activity when the neuron was spontaneously active, following capsaicin or vehicle injection, (2) *Response latency*: the total time (seconds) from capsaicin or vehicle injection to the first spike following capsaicin injection, (3) *Response duration*: the total time (seconds) from the first spike following capsaicin or vehicle injection to the last spike, and (4) *Peak frequency:* the highest firing rate in a one second period (Hz) during the Rdur. The above response properties were measured over the 10-minute period post-injection of capsaicin. Changes in cutaneous RF, cutaneous MAT and TMJ MAT were also assessed 10 and 20 minutes post-injection of capsaicin or vehicle control. This 30-minute observation period for capsaicin-induced neuronal excitability and central sensitization effects was limited to the acute phase of injury and was based on our previous studies demonstrating that mustard oil injected into rat craniofacial tissues induced central sensitization, as reflected in RF and MAT changes, that was readily observed in Vc/UCC nociceptive neurons for up to 30 minutes post-injection but dissipated by 40 minutes (Hu *et al.* 1992; Yu *et al.* 1993; Chiang *et al.* 1998, 2002) and that capsaicin injection into the rat TMJ reflexly evokes increases in jaw muscle electromyographic activity for up to 30 minutes (Tang *et al.* 2004; Chapter 3).

The cutaneous RF, cutaneous MAT and TMJ MAT of nociceptive neurons before and after both cutaneous incision alone and injection of capsaicin were compared with results with incision and lidocaine pretreatment and injection of capsaicin. *Cutaneous RF expansion* was defined as expansion in RF size to include a predetermined point lying 5 mm outside the perimeter of the baseline cutaneous RF. This methodology for assessment of RF reduces the possibility of iatrogenically induced sensitization by avoiding multiple repeated sites of noxious stimulation. In a small number of neurons, the border of the cutaneous RF was mapped in detail for illustration purposes as in the examples shown in Figs. 2 and 3. *Cutaneous MAT reduction* was defined as ≥ 50% threshold reduction from baseline MAT score measured at the center of the cutaneous RF site. *TMJ MAT reduction* was defined as ≥ 50% threshold reduction from baseline MAT score measured at the TMJ RF site (note: this property could only be assessed

following surgical incision of skin overlying the TMJ in order to directly expose the TMJ). Raw MAT threshold values measured were normalized to the initial baseline pre-incision value to illustrate population responses.

In order to further assess the time course of the surgical incision-induced refractory period to further capsaicin-induced central sensitization effects, capsaicin was injected into the left TMJ with the above procedure at 60-90 minutes post-incision without lidocaine pretreatment rather than the usual 30 minutes post-incision in a small number of neurons (n=3),

TERMINAL PROCEDURES. At the completion of each experiment, electrolytic lesions were made in the Vc/UCC recording site of rats by applying a monophasic current pulse (10 μA, 10 seconds) for subsequent histological verification of the recording site. Rats were then euthanized with the agent T61 (Hoechst, Canada). The unit recording sites confirmed histologically were subsequently reconstructed and plotted on standardized diagrams of the brainstem (Paxinos and Watson 1997). Postmortem dissection of the TMJ region also confirmed the correct placement of the catheter tip (see above) in the TMJ region, in accordance with our previous studies (Tang *et al.* 2004; Hu *et al.* 2005a).

DATA ANALYSIS. The activity of identified TMJ-responsive nociceptive neuronal activity was stored electronically and analyzed off-line (Hu 1990). Experiments were designed to record from only a single neuron in each animal and all neurons were isolated and characterized for baseline properties. However, for some neurons, every test stimulus could not be applied to each neuron; the number of neurons completing each part of the experimental paradigm are reported. Population data are reported as mean±SE or, if not normally distributed, as median values with interquartile ranges indicated in square brackets; median [IQR]. Mann-Whitney U test, Fisher exact test and RM ANOVA-on-ranks were used as appropriate ($p<0.05$ considered to reflect statistical significance).

4.3 RESULTS:

The properties of 90 nociceptive neurons (59 NS, 31 WDR) that responded to noxious mechanical stimulation of the TMJ region and that were recorded in the deep laminae (III-V) of Vc/UCC were studied (Figs. 1-3). Less than 6% (5/90) of these neurons displayed baseline spontaneous activity (0.29±0.14 spikes/sec) prior to mechanical and chemical stimulation. All 59 NS neurons and 31 WDR neurons had at baseline (i.e. before surgical cutaneous incision) an ipsilateral cutaneous RF that involved the facial skin overlying the TMJ and included both the maxillary and mandibular divisions. There were no differences in baseline spontaneous activity, laminae location (i.e. superficial vs. deep), or response to surgical cutaneous incision and capsaicin-induced activation and sensitization (i.e. Rmag, Rdur, Rlat and Pfreq; cutaneous RF expansion and cutanteous/TMJ MAT reduction) between the WDR and NS TMJ-responsive neurons ($p>0.05$, Mann-Whitney U test; $p>0.05$, Fisher exact test) and as a result, the data from WDR and NS neurons were pooled together for analysis of incision and capsaicin-induced activation and sensitization. Table 1 provides a summary of incision and capsaicin-evoked effects in Vc/UCC nociceptive neurons. Examples of incision and capsaicin-evoked effects in neurons with or without lidocaine pretreatment of the cutaneous incision site are shown in Figs. 2 and 3.

Surgical incision effects:

NEURONAL ACTIVATION. The cutaneous RF of neurons tested in both groups [i.e. with (n=34) or without (n=31) lidocaine pretreatment] included the facial skin overlying the TMJ (i.e. responsive to mechanical stimulation at the cutaneous incision site). Following lidocaine pretreatment of the incision site and immediately prior to the incision of the skin overlying the TMJ, neurons tested (n=34) were still responsive to mechanical stimulation of the underlying deep tissues (TMJ) but were not responsive to mechanical stimulation of the skin overlying the TMJ. In the absence of lidocaine pretreatment, incision of the skin overlying the TMJ produced a barrage of spikes in all neurons tested (105 [571] spikes; n=31; e.g. Fig. 2). In contrast, following lidocaine pretreatment (15 minutes prior to incision), the incision-induced barrage was almost completely abolished

(0 [2] spikes; n=34; p<0.001, Mann-Whitney U test; e.g. Fig. 3). When neuronal responses were assessed 10 minutes post-incision (25 minutes post-lidocaine pretreatment), mechanical stimulation of the cutaneous (skin overlying the TMJ) RF site as well as the deep (TMJ) RF site activated all neurons tested in both groups.

CUTANEOUS RF EXPANSION. In the absence of lidocaine pretreatment of the incision site, incision of the facial skin overlying the TMJ induced cutaneous RF expansion in 50% (8/16; 2/8 NS, 6/8 WDR) of neurons tested (Fig. 2) whereas following lidocaine pretreatment, the incision induced cutaneous RF expansion in only 7% (2/29; 1/20 NS, 1/9 WDR) of neurons (Fig. 3) tested (p<0.01, Fisher exact test).

CUTANEOUS MAT REDUCTION. There was no difference in baseline cutaneous MAT (i.e. prior to local anaesthetic infiltration) between neurons tested in the absence or presence of lidocaine pretreatment (p>0.05, Mann Whitney U test). The incidence of cutaneous MAT reduction (≥ 50% threshold reduction from baseline MAT score) was 68% (19/28; 8/14 NS, 11/14 WDR) of neurons tested in the absence of lidocaine pretreatment (Fig. 2) whereas following lidocaine pretreatment, the incidence of incision-induced cutaneous MAT reduction was only 12% (3/26; 3/17 NS, 0/9 WDR) (Fig. 3) (p<0.001, Fisher exact test). Similarly, in the absence of lidocaine pretreatment of the incision site, incision of the facial skin overlying the TMJ induced a significant reduction in median cutaneous MAT at 10 and 20 minutes post-incision relative to the pre-incision baseline of 65.7 [46.3] g (p<0.01, RM ANOVA-on-ranks, Dunn's method), whereas in the presence of lidocaine pretreatment, incision did not induce a significant median cutaneous MAT reduction from a baseline of 64.2 [70.6] g (p>0.05, RM ANOVA-on-ranks) (Fig. 4).

Capsaicin effects:

NEURONAL ACTIVATION. The capsaicin-induced response properties were generally similar between both groups: in the presence of lidocaine pretreatment of the incision site prior to surgical cutaneous incision, capsaicin injected into the TMJ activated 88%

(14/16) (Response magnitude: 161 [942] spikes; Response latency: 16.4 [10.5] seconds; Response duration: 112 [201] seconds; Peak frequency: 16.6 [35.6] Hz) of the neurons tested (e.g. Fig. 3) and in the absence of lidocaine pretreatment prior to incision, capsaicin activated 67% (8/12) (Response magnitude: 411 [531] spikes; Response latency: 8.4 [12.9] seconds; Response duration: 17.9 [18.0] seconds; Peak frequency: 70.4 [29.4] Hz) of these neurons ($p>0.05$, Mann-Whitney U test; e.g. Fig. 2). Vehicle injected into the TMJ in the presence (n=6) or absence (n=3) of lidocaine pretreatment prior to incision did not activate any of the 9 neurons tested ($p>0.05$, Fisher exact test).

CUTANEOUS RF EXPANSION. In the presence of lidocaine pretreatment prior to cutaneous incision, capsaicin injected into the TMJ induced cutaneous RF expansion in 60% (9/15; 7/11 NS, 2/4 WDR) of neurons tested (Fig. 3) whereas in the absence of lidocaine pretreatment prior to incision, capsaicin induced cutaneous RF expansion in 0% (0/12; 0/7 NS, 0/5 WDR) of neurons tested (Fig. 2) ($p<0.001$, Fisher exact test). Vehicle injected into the TMJ in the presence (n=6) or absence (n=3) of lidocaine pretreatment prior to incision induced no significant RF expansion in the 9 neurons tested ($p>0.05$, Fisher exact test).

CUTANEOUS MAT REDUCTION. In the presence of lidocaine pretreatment prior to incision, the incidence of capsaicin-induced cutaneous MAT reduction (≥ 50% threshold reduction from baseline MAT score) was 53% (8/15; 7/12 NS, 1/3 WDR) of the neurons tested (Fig. 3), whereas in the absence of lidocaine pretreatment prior to incision, capsaicin induced cutaneous MAT reduction in only 17% (2/12; 1/7 NS, 1/5 WDR; $p>0.05$, Fisher exact test) of neurons tested (Fig. 2). Similarly, in the presence of lidocaine pretreatment prior to incision, capsaicin injected into the TMJ induced a significant reduction in median cutaneous MAT at 10 and 20 minutes post-injection relative to the baseline of 64.2 [70.6] g ($p<0.05$, RM ANOVA-on-ranks, Dunn's method), whereas in the absence of lidocaine pretreatment prior to incision, capsaicin induced a significant increase in median cutaneous MAT at 10 and 20 minutes post-injection relative to the baseline of 65.7 [46.3] g ($p<0.01$, RM ANOVA-on-ranks, Dunn's method) (Fig. 4). Vehicle injected into the TMJ in the presence (n=6) or absence (n=3) of

lidocaine pretreatment prior to incision induced no significant cutaneous MAT reduction in the 9 neurons tested ($p>0.05$, Fisher exact test).

TMJ MAT REDUCTION. There was no significant difference in baseline TMJ MAT (i.e. after cutaneous incision to expose the TMJ for direct assessment) between neurons tested in the absence or presence of lidocaine pretreatment ($p>0.05$, Mann Whitney U test) (Fig. 4). In the presence of lidocaine pretreatment prior to incision, the incidence of capsaicin-induced TMJ MAT reduction (≥ 50% threshold reduction from baseline MAT score) was 64% (9/14; 8/11 NS, 1/3 WDR) of the neurons tested (Fig. 3), whereas in the absence of lidocaine pretreatment prior to incision, capsaicin injection into the TMJ induced TMJ MAT reduction in only 17% (2/12; 2/7 NS, 0/5 WDR; $p<0.05$, Fisher exact test) of neurons tested (Fig. 2). Similarly, in the presence of lidocaine pretreatment prior to incision, capsaicin injected into the TMJ induced a significant reduction in median TMJ MAT at 20 minutes post-injection relative to the pre-injection baseline of 80.5 [63.1] g ($p<0.01$, RM ANOVA-on-ranks, Dunn's method), whereas in the absence of lidocaine pretreatment prior to incision, capsaicin induced a significant increase in median TMJ MAT at 10 minutes post-injection relative to the pre-injection baseline of 47.5 [49.3] g ($p<0.05$, RM ANOVA-on-ranks, Dunn's method) (Fig. 4). Vehicle injected into the TMJ in the presence (n=6) or absence (n=3) of lidocaine pretreatment prior to incision induced no significant TMJ MAT reduction in the 9 neurons tested (Fisher exact test, $p>0.05$).

The above findings of capsaicin-induced activation and central sensitization were documented in experiments wherein capsaicin was injected into the TMJ 40 minutes after the cutaneous incision. Such effects were also seen in 3 neurons tested with capsaicin injected into the TMJ 60 min (n=2) or 90 min (n=1) post-incision; in the absence of lidocaine pretreatment prior to incision, capsaicin induced activation and cutaneous RF expansion, cutaneous MAT reduction, and TMJ MAT reduction in all 3 neurons tested.

4.4 DISCUSSION:

This is the first study to examine the influence of cutaneous incision injury on central nociceptive processing of deep afferent inputs evoked by noxious stimuli such as capsaicin. In addition, our findings have provided the first demonstration in the trigeminal system that surgical incision of facial skin can readily activate and evoke central sensitization, a process reflected in cutaneous RF expansion and MAT reduction, in TMJ-responsive nociceptive neurons in the Vc/UCC and can render most of these neurons temporarily refractory to sensitization induced by capsaicin injected into the TMJ. However, local anaesthetic application to the skin prior to incision enables capsaicin injected into the TMJ to induce central sensitization despite skin incision. Both peripheral and/or central sensitization mechanisms may contribute to the TMJ MAT reduction in this study. However, the observed cutaneous RF expansion and cutaneous MAT reduction cannot be explained by peripheral sensitization and likely reflect central sensitization since the *cutaneous* central sensitization is induced by capsaicin applied to a *deep* tissue RF site (TMJ) remote from the *cutaneous* RF sites assessed. These findings suggest that cutaneous injury may modify central nociceptive processing of deep tissue afferent inputs.

Surgical incision-induced central sensitization

This is the first study to show that, in the absence of local anaesthetic pretreatment, surgical incision of facial skin may induce a barrage and subsequent central sensitization in trigeminal nociceptive neurons receiving deep afferent input. Incision-induced cutaneous RF expansion and cutaneous MAT reduction may contribute to the pain spread and referral and to the development of allodynia that have been documented in postoperative pain states (see Melzack *et al.* 2001; Kawamata *et al.* 2002). Differences in surgical preparation, anaesthetic type and concentration, experimental paradigm, time course and quantification of central sensitization effects make it difficult to directly compare amongst findings in previous studies as well as with

those in the present study. Nonetheless, the present finding that surgical incision of facial skin may induce central sensitization in trigeminal nociceptive neurons receiving deep afferent input are in general agreement with previous findings in other rat cutaneous incision models (Brennan *et al.* 1996; Zahn and Brennan 1999; Vandermeulen and Brennan 2000; Pogatzki *et al.* 2002a; Sun *et al.* 2004; Kawamata *et al.* 2005a) where central sensitization was induced in spinal dorsal horn neurons following surgical incision, although none of these previous studies tested for the presence of deep nociceptive inputs in the spinal dorsal horn neurons under study.

Local anaesthetic pretreatment attenuates incision-induced effects

Our findings that local anaesthetic pretreatment attenuates the incision-induced barrage and subsequent central sensitization in TMJ-responsive nociceptive neurons are in agreement with many past animal and human surgical pain models (see Dahl and Kehlet 1993, Katz 2001, Melzack *et al.* 2001; Kawamata *et al.* 2002). Our results are also consistent with rat hindpaw incision models in which local anaesthetic pretreatment prevented spinal *c-fos* expression (Sun *et al.* 2004) and rat pain behaviors for several hours after incision (Pogatzki *et al.* 2002a; Sun *et al.* 2004). However, in these previous rat hindpaw incision models, the increased *c-fos* expression or pain behavior was reported to last only as long as the duration of the local anaesthetic used; this suggests that continuing afferent input or ongoing spontaneous afferent activity from the incision injury site after the local anaesthesia has dissipated is critical to sensitization of spinal dorsal horn neurons in the plantar incision model. In contrast, in our rat facial skin injury model, the local anaesthetic effect had dissipated when neuronal response properties were assessed 10 minutes following surgical incision, and yet no central sensitization of the Vc/UCC neurons was apparent. This finding suggests that the short-acting local anaesthetic pretreatment may attenuate incision-induced activation and central sensitization in TMJ-responsive nociceptive neurons by blocking the initial intense incision-induced barrage of afferent inputs to these nociceptive neurons, and that any subsequent afferent input from the facial cutaneous incision site is not sufficient to

induce Vc/UCC central sensitization. Although our observation period for incision-induced Vc/UCC central sensitization was restricted to the acute phase of injury, our results provide support for the use of preemptive regional analgesia in addition to general anaesthesia prior to surgery in order to attenuate or prevent incision-induced activation and central sensitization in nociceptive pathways both in experimental models of pain as well as in clinical surgical cases.

Capsaicin-induced neuronal activation and central sensitization

Capsaicin activated and induced central sensitization in a large proportion of TMJ-responsive nociceptive neurons and the effects were documented in neurons tested with capsaicin injected into the TMJ at 40, 60 and 90 minutes following cutaneous incision. The capsaicin-induced effects were especially apparent in the presence of local anaesthetic pretreatment prior to surgical incision, and are in agreement with those shown previously in the spinal and trigeminal systems. Capsaicin applied to skin has been shown to activate and sensitize spinal afferents and dorsal horn neurons (Simone *et al.* 1991; LaMotte *et al.* 1992) and evoke intense pain and secondary hyperalgesia in humans (LaMotte *et al.* 1991, 1992; Witting *et al.* 2000a; Magerl and Treede 2004; Poyhia and Vainio 2006). TRPV1-immunoreactive sensory neurons have been demonstrated in both the rat (Helliwell *et al.* 1998; Guo *et al.* 1999; Ichikawa and Sugimoto 2001, 2004) and human (Hou *et al.* 2002; Renton *et al.* 2003; Morgan *et al.* 2005) trigeminal ganglion. TRPV1 receptors have also been demonstrated on trigeminal afferents innervating the rat TMJ (Ichikawa *et al.* 2004). Capsaicin has also been shown to activate and sensitize both trigeminal afferents (Liu and Simon 1994, 1996, 2003; Strassman *et al.,* 1996; Chapter 2) and Vc nociceptive neurons (Carstens *et al.* 1998; Zanotto *et al.* 2007; Chapter 2) and induce secondary hyperalgesia, allodynia and jaw muscle pain in humans (Sohn *et al.* 2000, 2004; Wang *et al.* 2002; Gazerani and Arendt-Nielsen 2005; Gazerani *et al.* 2006). Injection of capsaicin into the TMJ was also shown to evoke an increase in jaw muscle electromyographic activity (Tang *et al.* 2004; Chapter 3) with a time course comparable with the Vc/UCC central sensitization changes revealed in this study. More importantly, the time course of capsaicin-induced activation

and central sensitization effects in the present study correlate with findings in human spinal and trigeminal studies where pain and mechanical sensitization effects were almost immediate and reached their maximal levels over 15-30 minutes following capsaicin injection (LaMotte *et al.* 1991; Simone *et al.* 1991; Wang *et al.* 2002; Sohn *et al.* 2004; Gazerani and Arendt-Nielsen 2005; Gazerani *et al.* 2006). Taken together, these findings suggest a role for peripheral TRPV1 receptor mechanisms in enhanced pain responses such as allodynia, as reflected in MAT reduction, as well as in pain spread in deep craniofacial tissues, as reflected in cutaneous RF expansion. However, the use of specific TRPV1 receptor antagonists should be used to confirm the role of TRPV1 in trigeminal nociceptive processing.

Incision effects on capsaicin-induced neuronal activation and central sensitization

Our findings suggest that surgical cutaneous incision-induced excitability increases in TMJ-responsive nociceptive neurons may render most of these neurons temporarily refractory to sensitization induced by capsaicin injected into the TMJ. Although the mechanisms underlying noxious mechanical transduction are not fully understood, several candidate high-threshold mechanotransducers such as TRPs, acid-sensing ion channels (ASICs), and K^+ channels (Hu *et al.* 2006; Woolf and Ma 2007) may be activated following surgical cutaneous incision. Peripheral TRP receptors that may be involved in incision-induced nociceptive mechanisms are the TRPV4 receptor which is activated by mechanical stimuli and shear stress (Caterina *et al.* 1997; Nilius *et al.* 2004; Woolf and Ma 2007) and the TRPA1 receptor which is activated by chemical (e.g., mustard oil), thermal or mechanical noxious stimuli (Kwan *et al.* 2006; Woolf and Ma 2007). Some of these TRP receptors are coupled to G protein-coupled receptors (GPCRs) (Bautista *et al*. 2006; Woolf and Ma 2007) and surgical incision itself could therefore conceivably activate afferent inputs to the Vc/UCC directly by activating these GPCR-coupled receptors or indirectly by injury-induced release of inflammatory mediators that activate GPCR and second messenger-mediated phosphorylation and sensitization of central EAA receptors (Kress and Zeilhofer 1999; Wallace 2006). The possible involvement of one or more of these TRP receptor mechanisms in incision-

induced nociceptive processing may modulate the effects of subsequent TRPV1 activation on TMJ-responsive nociceptive neurons.

However, a combination of incision-induced excitatory and inhibitory effects most likely accounts for the TMJ-responsive nociceptive neurons being temporarily refractory to further central sensitization induced by capsaicin. Surgical incision without local anaesthetic pretreatment may increase brainstem EAAs and/or activate descending facilitatory mechanisms (Dubner and Ren 2004) and impose a ceiling effect on subsequent sensitizing effects of capsaicin by limiting any further capsaicin-evoked brainstem increases in EAAs. That is, the neurons may have reached their maximal increases in cutaneous RF expansion and MAT reduction following incision without local anaesthetic pretreatment such that they could not be sensitized further by capsaicin 40 minutes post-incision. It has also been proposed that the large increase in EAAs in the spinal cord as a result of noxious stimulation is followed (Sorkin *et al.* 1992) or accompanied (Sluka and Westland 1992) by increased concentrations of inhibitory amino acids (IAAs), which may reflect activation of endogenous inhibitory responses, consistent with findings in the trigeminal system (Yu *et al.* 1994; Hu *et al.* 1997). Increased concentrations of the IAA serine have been shown to parallel increased concentrations of the EAAs glutamate and aspartate in the spinal cord for 10-30 minutes after plantar incision (Zahn *et al.* 2002). We also have recent evidence that central inhibitory effects may be activated following a capsaicin-induced afferent barrage and that these inhibitory effects may contribute to the marked attenuation of glutamate-evoked activation and central sensitization effects in Vc/UCC nociceptive neurons following capsaicin injection 40 minutes prior to the injection of glutamate into the TMJ (Chapter 2). Central sensitization in spinal dorsal horn nociceptive neurons with afferent input from the cat knee is attenuated by a progressive enhancement of descending inhibition during the development of inflammation induced by the intra-articular injection of kaolin and carrageenan (Schaible *et al.* 1991b). Accordingly, the incision-induced barrage without local anaesthetic pretreatment may also conceivably activate or enhance descending inhibitory modulation of Vc/UCC nociceptive neurons from the brainstem and suprabulbar areas (Chiang *et al.* 1991, 1994; Sessle *et al.* 1992; Fields and Basbaum 1994; Dubner and Ren 2004) although in the case of incision-induced

central sensitization, peripheral or segmental inhibitory mechanisms may play a greater role since it has been reported that incision-induced central sensitization effects on spinal dorsal horn neurons are not modulated by descending influences from the brain (Pogatzki *et al.* 2002b; Kawamata *et al.* 2005a).

Whether incision-induced central facilitatory or inhibitory actions predominate may also depend on the magnitude and local distribution of intracellular Ca^{2+} (Kress and Zeilhofer 1999; Swope *et al.* 1999; Rycroft and Gibb 2004). The importance of increased Ca^{2+} influx in mediating spinal sensitization after incision has been demonstrated since a spinally administered antagonist for Ca^{2+}-permeable non-NMDA receptors reduced central sensitization after incision (Pogatzki *et al.* 2003; see Pogatzki-Zahn 2006). Increased intracellular Ca^{2+} may also activate the phosphatase calcineurin, which can dephosphorylate and desensitize central EAA receptors (Swope *et al.* 1999; Rycroft and Gibb 2004). Indeed, the finding that, in the absence of lidocaine pretreatment prior to incision, capsaicin induced significant increases in both cutaneous MAT and TMJ MAT provides further evidence for enhanced central inhibitory mechanisms. This finding wherein cutaneous injury may modify central nociceptive processing of deep tissue afferent inputs is consistent with the recent finding that cutaneous injury evoked by mustard oil application to the rat hindpaw attenuated the subsequent electrically-evoked increased excitability of spinal nociceptive neurons (Merrill *et al.* 2008).

Since incision-induced increases in EAAs and IAAs have been shown to return to baseline 60 minutes post-incision (Zahn et al. 2002; see Pogatzki-Zahn 2006), it provides an explanation for our present finding that capsaicin can readily induce central sensitization when injected into the TMJ 60-90 minutes after an incision-induced barrage without lidocaine pretreatment. Also, we have previously documented that mustard oil injected into deep craniofacial tissues evokes not only Vc central sensitization and jaw muscle electromyographic activity but also a subsequent opioid-related inhibitory effect that persists 50-60 minutes following mustard oil injection into the TMJ (Yu *et al.* 1993, 1994; Hu *et al.* 1992, 1997). As a result, the 40-minute period post-incision, coupled with our previous finding of a 50-60 minute opioid depressive period (Yu *et al.* 1994; Hu *et al.* 1997), suggests that the surgical incision-induced refractory period in the present study may follow a time course on the order of 40-60 minutes post-incision.

4.5 TABLE:

Table 1. Summary of statistical comparisons for incision and capsaicin-evoked effects in TMJ-responsive nociceptive neurons in the subnucleus caudalis/upper cervical spinal cord (Vc/UCC) region. Although capsaicin-evoked neuronal activation was equivalent between both groups with and without lidocaine pretreatment of the cutaneous incision site, capsaicin failed to evoke significant central sensitization in TMJ-responsive nociceptive neurons following incision without lidocaine pretreatment.

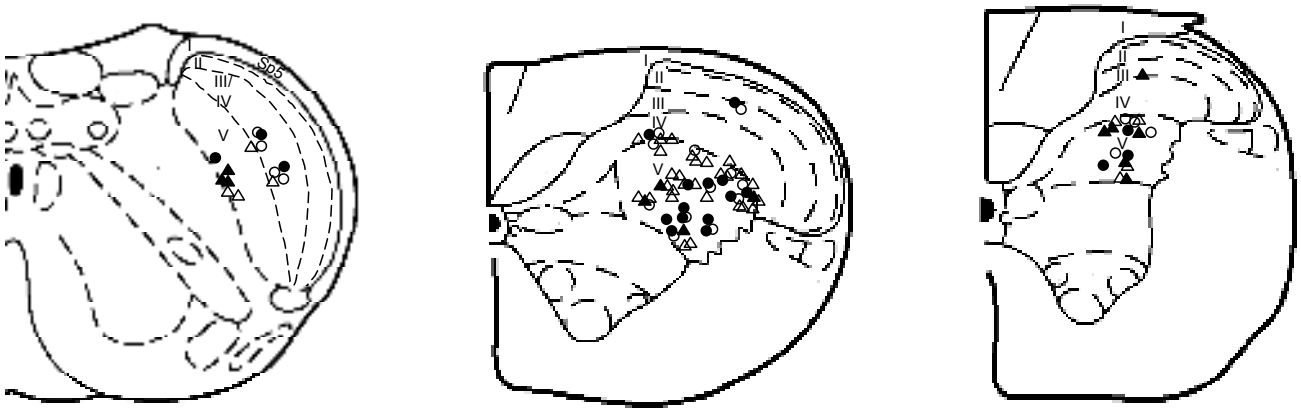
	SURGICAL INCISION		CAPSAICIN	
PRETREATMENT OF INCISION SITE	**Activation**	**Central Sensitization**	**Activation**	**Central Sensitization**
No Lidocaine	+***	+**	+	-
Lidocaine	-	-	+	+*

\+ = effect induced by incision or capsaicin, - = no or reduced effect induced by incision or capsaicin

* = p<0.05, ** = p<0.01, *** = p<0.001, Mann-Whitney U-test or Fisher's exact test as appropriate to compare Lidocaine vs. No Lidocaine pretreatment groups

4.6 FIGURES:

Fig. 1. The unit recording sites in subnucleus caudalis/upper cervical spinal cord (Vc/UCC). The sites were reconstructed from histological sections and were plotted on diagrams of the brainstem (Paxinos and Watson 1997). Coronal sections are shown at 3 different rostrocaudal levels of Vc/UCC. Filled symbols represent wide dynamic range (WDR) neurons; opened symbols nociceptive-specific (NS) neurons. The dot and triangle symbols represent, respectively, neurons in animals receiving the injection of capsaicin following incision without lidocaine pretreatment and neurons in animals receiving injection of capsaicin following incision with lidocaine pretreatment.



Caudal Vc **C1** **C2**

Fig. 2. Example of incision and capsaicin-evoked effects in a TMJ-responsive nociceptive neuron in the Vc/UCC region without lidocaine pretreatment of the cutaneous incision site. The asterisk in (a) represents the lesioned neuronal recording site confirmed histologically; tissue stained by conventional hematoxylin and eosin techniques. Note that without lidocaine pretreatment, surgical cutaneous incision of the facial skin overlying the TMJ resulted in a large incision-induced neuronal activation (b), cutaneous mechanoreceptive field (RF) expansion (d) and cutaneous mechanical activation threshold (MAT) reduction (e.g. 100-g von Frey force was ineffective pre-incision but effective post-incision in activating the neuron) (e). In contrast, subsequent neuronal activation induced by capsaicin injection into the TMJ without lidocaine pretreatment (c) did not result in cutaneous RF expansion (d) or cutaneous MAT reduction (e).

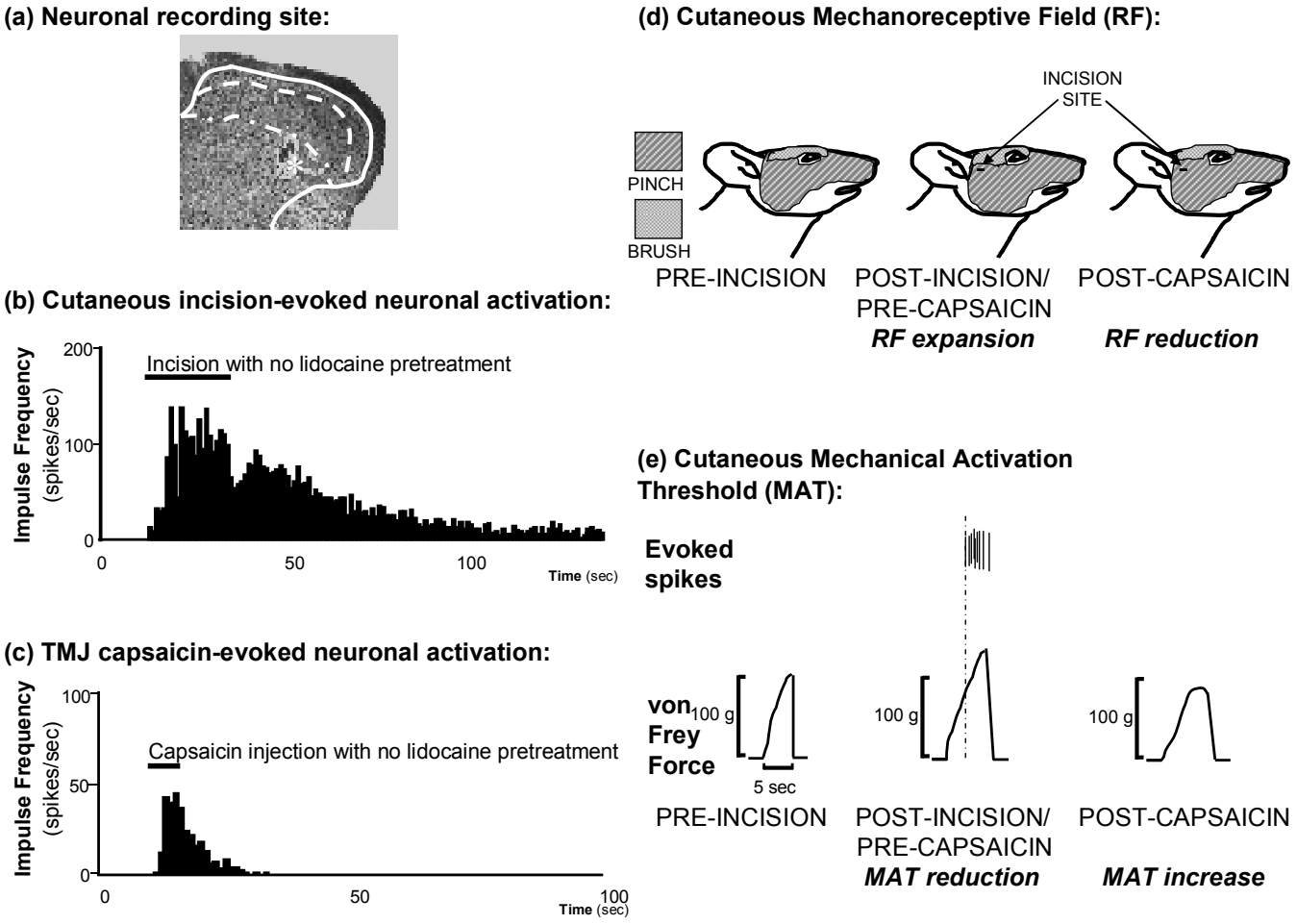


Fig. 3. Example of incision and capsaicin-evoked effects in a TMJ-responsive nociceptive neuron in the Vc/UCC region with lidocaine pretreatment of the cutaneous incision site. The asterisk in (a) represents the lesioned neuronal recording site confirmed histologically; tissue stained by conventional hematoxylin and eosin techniques. Note that with lidocaine pretreatment, surgical cutaneous incision of the facial skin overlying the TMJ resulted in minimal incision-induced neuronal activation (b), no cutaneous mechanoreceptive field (RF) expansion (d) or cutaneous mechanical activation threshold (MAT) reduction (e). In contrast, subsequent neuronal activation induced by capsaicin injection into the TMJ with lidocaine pretreatment (c) resulted in both cutaneous RF expansion (d) and cutaneous MAT reduction (e).

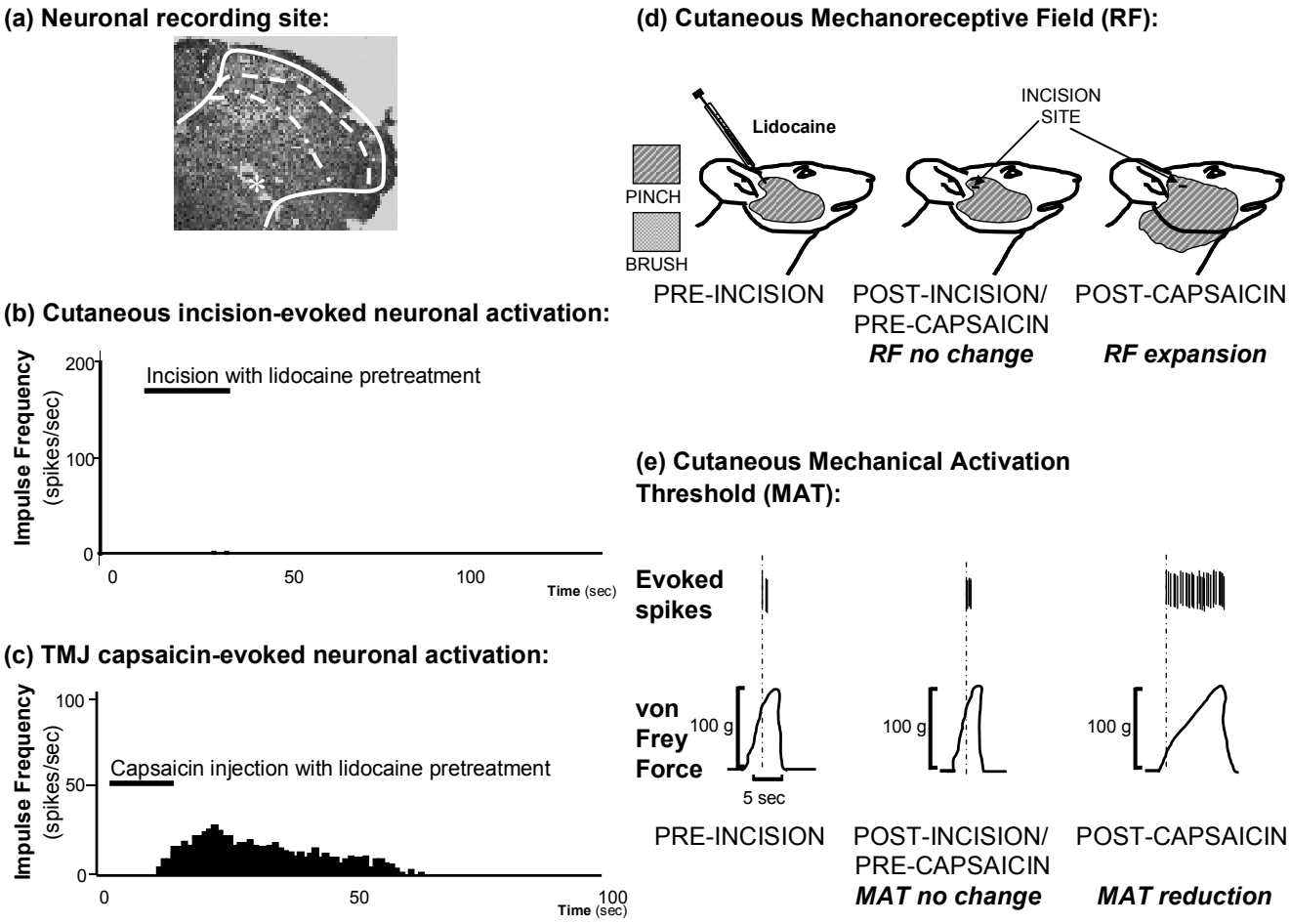
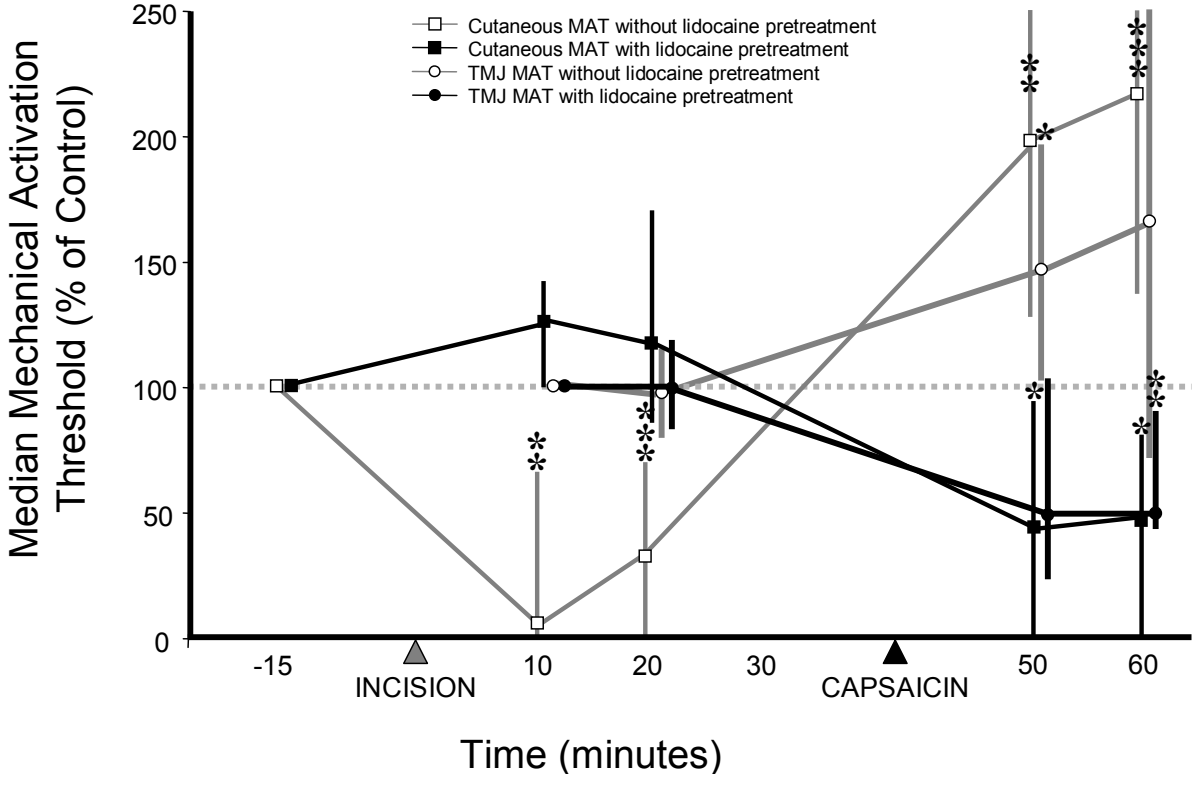


Fig. 4. The time course of surgical cutaneous incision and capsaicin-induced cutaneous mechanical activation threshold (MAT) and TMJ MAT reduction in TMJ-responsive nociceptive neurons with (black) or without (white) lidocaine pretreatment. Triangle indicates time point for incision (grey) and injection of capsaicin into the TMJ (black). Squares indicate median normalized cutaneous MAT and circles indicate median normalized TMJ MAT. Raw MAT threshold values were normalized to the initial baseline pre-incision value. Lines: interquartile range. Note that incision alone without lidocaine pretreatment significantly reduced the cutaneous MAT; injection of capsaicin following incision with lidocaine pretreatment significantly reduced both the cutaneous and TMJ MAT, whereas capsaicin injection following incision without lidocaine pretreatment significantly increased the cutaneous and TMJ MAT (*p<0.05, **p<0.01, ***p<0.001, RM ANOVA-on-ranks, Dunn's method).



Chapter 5

General Discussion

5.0 GENERAL DISCUSSION:

The present doctoral study is the first to provide evidence in support of the following hypotheses:

Hypothesis 1: ***Peripheral glutamatergic and capsaicin-sensitive mechanisms modulate the properties of primary afferents and brainstem neurons processing deep craniofacial nociceptive information.***

Hypothesis 2: ***Surgical cutaneous incision modulates the properties of brainstem neurons processing deep craniofacial nociceptive information.***

Peripheral glutamatergic and capsaicin-sensitive mechanisms do indeed influence both peripheral and central nociceptive processing in deep craniofacial tissues and there are differences in peripheral compared to central nociceptive processing of these peripheral receptor mechanisms. By using the same experimental paradigm in both peripheral and central processing studies on glutamate and capsaicin-evoked responses in nociceptive afferents supplying deep craniofacial tissues, direct comparisons of peripheral versus central nociceptive neural responses to peripherally applied glutamate and capsaicin were possible. Both peripheral and/or central sensitization mechanisms may contribute to enhanced responses to glutamate and capsaicin (e.g., increase in Rmag, Rdur or Pfreq and/or a decrease in Rlat) and TMJ MAT reduction. However, cutaneous RF expansion and cutaneous MAT reduction induced by injection of glutamate and capsaicin likely reflect central sensitization since the cutaneous central sensitization in TMJ-responsive nociceptive neurons in the

Vc/UCC region was induced by glutamate and capsaicin applied to a *deep* tissue RF site remote from the *cutaneous* RF sites assessed. We have also shown for the first time that the increased EMG activity in both jaw-opening and jaw-closing muscles as a result of capsaicin injection into the TMJ can be attenuated in a dose-dependent manner by TMJ pre-injection of an NMDA receptor antagonist. Taken together, these novel findings support Hypothesis 1 and suggest that both peripheral glutamate and capsaicin receptor mechanisms may be involved in the nociceptive processing of deep craniofacial afferent inputs and may interact to modulate both activation as well as peripheral and central sensitization evoked from these tissues.

In addition, this is the first study to detail the effect of surgical cutaneous incision on V nociceptive neuroplasticity. The present findings support Hypothesis 2 and suggest that TMJ-responsive nociceptive neurons in the Vc/UCC region can be activated by surgical incision of the skin overlying the TMJ and this incision-induced afferent barrage can cause nociceptive neurons to be refractory to further central sensitization effects mediated by capsaicin.

5.1 SPECIFIC HYPOTHESES AND STUDY AIMS:

5.1.1 Glutamate and capsaicin activate and induce peripheral sensitization in nociceptive afferents supplying TMJ or associated musculature and projecting to the caudal Vc/UCC

Studies addressing AIMS 1-1 to 1-3 have demonstrated that TMJ and muscle nociceptive afferents projecting to the caudal Vc/UCC are activated and modulated by peripheral glutamatergic and capsaicin-sensitive mechanisms. The present study appears to be the first to document the capsaicin-induced activation and sensitization of V nociceptive afferents with deep craniofacial RFs. These findings are consistent with previous data that capsaicin evokes dose-dependent increases in jaw muscle EMG activity when injected into the rat TMJ (Tang *et al.* 2004) and induces an inflammatory process within these tissues (Hu

et al. 2005a). The present findings are also consistent with studies in humans that have shown capsaicin-induced craniofacial pain (e.g. Sohn *et al.* 2000, 2004; Wang *et al.* 2002; Gazerani and Arendt-Nielsen 2005; Gazerani *et al.* 2006), and with findings that capsaicin can activate and sensitize V afferents (Liu and Simon 1994, 1996, 2003; Strassman *et al.* 1996) and spinal afferents (Baumann *et al.* 1991; Peterson and LaMotte 1991; LaMotte *et al.* 1992; Marchettini 1996; Simone *et al.* 1997; Dray 2005; Gold 2005).

Although glutamate-evoked activation and sensitization have not been demonstrated in spinal primary afferents, the findings in the present study in deep craniofacial afferents (masseter and temporalis muscle; TMJ) are consistent with previous evidence of glutamate-evoked activation and sensitization of masseter nociceptive afferents, jaw muscle EMG activity and pain in human masseter and trapezius muscles (Cairns *et al.* 1998, 2001ab, 2002a, 2003ab, 2006; Svensson *et al.* 2003; Ge *et al.* 2005).

TMJ and muscle nociceptive afferents projecting to the caudal Vc/UCC are not only activated and modulated by peripheral glutamatergic and capsaicin-sensitive mechanisms but there are also interactions between these peripheral mechanisms that influence the properties of these nociceptive afferents. This is the first study to document that a large proportion of V nociceptive afferents innervating deep craniofacial tissues may be activated and sensitized by the peripheral application of glutamate, capsaicin, or both. These changes in response properties reflect peripheral sensitization as shown in enhanced activation to agonist injection (*e.g.*, increase in Rmag and Pfreq) and/or a MAT reduction in V nociceptive afferents. In addition, glutamate sensitized afferent responses to subsequent noxious stimulation of the deep craniofacial tissues by capsaicin, whereas capsaicin neither sensitized nor desensitized afferent responses to subsequent noxious stimulation by glutamate. These findings suggest that both peripheral glutamatergic and capsaicin-sensitive mechanisms may be involved in the activation and sensitization of deep craniofacial nociceptive afferents and may interact to modulate the activation and peripheral sensitization in some nociceptive afferents supplying deep craniofacial tissues.

5.1.2 Glutamate and capsaicin activate and induce central sensitization in TMJ-responsive nociceptive neurons in the caudal Vc/UCC

Studies addressing AIMS 1-4 to 1-6 have demonstrated that TMJ-responsive nociceptive neurons in the caudal Vc/UCC are not only activated and modulated by peripheral glutamatergic and capsaicin-sensitive mechanisms but there are also interactions between these peripheral receptor mechanisms that influence the properties of nociceptive neurons in the CNS. This is the first study to document that a large proportion of TMJ-responsive nociceptive neurons in the caudal Vc/UCC can be activated and sensitized by the peripheral application to the TMJ of glutamate, capsaicin or both, and that peripheral glutamate and capsaicin receptor mechanisms interact to modulate activation and central sensitization in deep craniofacial tissues. Glutamate applied to the TMJ may sensitize TMJ-responsive nociceptive neurons, and thereby produce more immediate, larger and more prolonged responses to noxious stimulation of the TMJ by capsaicin. In contrast, capsaicin applied to the TMJ may desensitize these neurons to subsequent noxious stimulation of the TMJ by glutamate.

The present findings of a role for peripheral glutamatergic and capsaicin-sensitive mechanisms in central processing of nociceptive afferent inputs to Vc/UCC from deep craniofacial tissues are in agreement with previous findings of an important role for peripheral EAA and TRPV1 receptors in central sensitization, and are consistent with both V (Carstens *et al.* 1998; Sohn *et al.* 2000, 2004; Wang *et al.* 2002; Gazerani and Arendt-Nielsen 2005; Gazerani *et al.* 2006; Zanotto *et al.* 2006) and spinal (LaMotte *et al.* 1991,1992; Simone *et al.* 1991; Raja *et al*. 1999; Gottrup *et al.* 2000, 2004; Witting *et al.* 2000ab; Sluka 2002; Gazerani *et al.* 2006; Poyhia and Vainio 2006) evidence in animals and humans that suggest a role for central sensitization (e.g., secondary hyperalgesia) in mediating capsaicin-evoked pain in peripheral tissues. Similarly, this project's findings for Vc/UCC nociceptive neurons are consistent with previous evidence of glutamate-evoked jaw muscle EMG activity that could be attenuated by NMDA receptor antagonist application to Vc and of pain evoked by

glutamate in human masseter and trapezius muscles (Cairns *et al.* 1998, 2001a, 2003ab, 2006; Svensson *et al.* 2003; Ge *et al.* 2005). The present findings are also consistent with previous spinal evidence of thermal and mechanical hyperalgesia induced by subcutaneous or intra-articular (knee) glutamate injection (for review, see Carlton 2001; Carlton *et al.* 2003). In addition, peripheral application of a selective EAA receptor antagonist has been shown to attenuate irritant chemical-induced hyperalgesia and dorsal horn *c-fos* expression in animals (Jackson *et al.* 1995; Davidson *et al.* 1997; Lawand *et al.* 1997; Wang *et al.* 1997; Davidson and Carlton 1998; Carlton 2001; Carlton *et al.* 2003) and inhibit the development of secondary hyperalgesia in humans (Warncke *et al.* 1997).

5.1.3 Differences in peripheral and central nociceptive processes evoked by the peripheral application of glutamate and capsaicin to deep craniofacial tissues

The same experimental paradigm was employed in the studies of V primary nociceptive afferents (AIMS 1-1 to 1-3) and caudal Vc/UCC neurons (AIMS 1-4 to 1-6) to test for possible modulating effects of glutamate and capsaicin in order to allow direct comparisons between peripheral and central nociceptive responses to the peripheral application of glutamate and capsaicin. Our findings that the glutamate-evoked responses were similar to those evoked by capsaicin in TMJ-responsive nociceptive neurons contrast with our V nociceptive primary afferent results where glutamate-evoked responses in the nociceptive afferents were significantly greater than capsaicin-evoked responses. These findings suggest the involvement of convergent nociceptive signaling pathways may contribute to the markedly enhanced incidence of activation and response properties evoked by glutamate and capsaicin in Vc/UCC neurons.

The finding that both V nociceptive afferent and brainstem nociceptive neuronal responses to capsaicin injection were significantly increased following glutamate injection suggests that the activation of peripheral glutamate receptors by glutamate may sensitize both primary afferents and Vc/UCC nociceptive

neurons supplying deep craniofacial tissues to subsequent stimuli. Peripheral NMDA receptors, in particular, may play a role in mediating the glutamate-induced peripheral and/or central sensitization as evidenced by our finding that jaw muscle activities reflexly evoked by capsaicin injection into the rat TMJ can be attenuated by preinjection into the TMJ of NMDA receptor antagonists. These results suggest that NMDA and possibly capsaicin receptors are located within the TMJ and the activation of NMDA receptors contributes, in part, to the mechanism whereby capsaicin evokes nociceptive responses. One possible mechanism involving autocrine and/or paracrine regulation of nociceptive excitability via ionotropic glutamate receptors may contribute to the mechanisms whereby capsaicin evokes nociceptive jaw muscle activity. Similar peripheral NMDA receptor mechanisms may also play an important role in the nociceptive responses evoked by mustard oil. Jaw muscle activity evoked by mustard oil injected into the TMJ (Yu *et al.* 1996) as well as nocifensive behaviour (Ro 2003), oedema formation (Ro 2003) and *c-fos* expression in the V brainstem nuclei (Ro *et al.* 2004) evoked by mustard oil injected into the masseter are also similarly attenuated by local TMJ or muscle MK-801 pretreatment. In addition to possible interactions between ionotropic receptors, there is evidence of a major coupling between G-protein-coupled receptors and some TRP channels in the membrane such as TRPA1 and TRPV1 (Sikand and Premkumar 2007; Woolf and Ma 2007). This type of coupling may also exist between TRPV1 and other G-protein-coupled receptors (Woolf and Ma 2007), such as the metabotropic EAA receptors, and provides a means for potential interactions between peripheral EAA and TRPV1 receptors.

The interactions between peripheral glutamate and capsaicin receptors on deep craniofacial afferent inputs to Vc/UCC nociceptive neurons suggest a convergent nociceptive signaling pathway (see Fig. 1). That is, the nociceptive signaling pathways are not separate; the glutamate and capsaicin-activated deep craniofacial afferents converge on Vc/UCC neurons. In contrast to the glutamate-induced sensitizing effects on deep craniofacial afferent responses to subsequent noxious stimulation by capsaicin, there was a lack of capsaicin-

induced modulation of glutamate-evoked afferent excitability. Indeed many of the afferents remained responsive to glutamate following capsaicin injection. However, the glutamate-evoked activation responses and incidence of cutaneous RF expansion in the TMJ-responsive nociceptive Vc/UCC neurons following capsaicin injection were abolished or significantly reduced. These findings suggest that capsaicin may desensitize Vc/UCC neurons to subsequent noxious stimuli (e.g. to glutamate) which is consistent with previous evidence showing that capsaicin may desensitize Vc neurons to subsequent activation by various irritant chemicals applied to craniofacial tissues (Carstens *et al.* 1998). It is possible that there may be differences in the types of convergent presynaptic receptors or the neurotransmitters released from the central terminals of capsaicin-sensitive and glutamate-sensitive afferents that may account for the differential sensitizing or desensitizing effects on Vc/UCC neurons (Woolf and Salter 2000; Dubner 2005; Woolf and Ma 2007). The variety of these neurotransmitters may result in considerable spatial and temporal summation capabilities that are subject to both excitatory and inhibitory influences. The capsaicin-induced desensitizing effects on Vc/UCC neurons may involve increased inhibitory GABAergic synaptic transmission and alterations in cation-chloride cotransporters such as NKCC1 or KCC2 (Galan and Cervero 2005; Karlsson *et al.* 2005; Price *et al.* 2005, 2006; Garcia-Nicas *et al.* 2006; Pitcher *et al.* 2007). The present findings are also consistent with recent evidence of other peripheral TRP receptors such as TRPA1 participating in the activation of central inhibitory circuits (Kosugi *et al.* 2007; Merrill *et al.* 2008). Thus it is possible that the capsaicin-induced desensitization in Vc/UCC neurons in the present study may occur via the activation of similar central inhibitory circuits.

5.1.4 Targeting peripheral glutamate and capsaicin receptor mechanisms

The glutamate-evoked sensitization of primary afferent fibers in the present study may contribute to the primary hyperalgesia or allodynic states found in craniofacial pain conditions such as TMD (for review, see Carlsson and LeResche 1995, Stohler 1995,1999; Sessle 1999, 2005; Svensson and Sessle

2004; Lam *et al.* 2005). Glutamate injection into the TMJ also evoked activity and central sensitization in Vc/UCC nociceptive neurons. This glutamate-evoked central sensitization, reflected as RF expansion, MAT reduction, and increase in neuronal responses to subsequent noxious stimuli, may be central mechanisms contributing to pain spread and referral, allodynia, pain at rest, and hyperalgesia in craniofacial pain conditions. However, further studies are required to achieve a better understanding of the role of peripheral glutamate receptors in the pathobiological mechanisms underlying craniofacial pain conditions. These should involve the use in female as well as male subjects of specific ionotropic and metabotropic EAA receptor agonists, antagonists and gene knockouts in both acute and chronic craniofacial pain models studying peripheral (primary afferent) and central (Vc, thalamic, somatosensory cortex) nociceptive processing. Moreover, since the present study suggests that peripheral glutamate receptors may modulate the activity of other peripheral nociceptors (i.e., TRPV1 receptor), future studies could examine the modulatory role of peripheral EAA receptors on other peripheral nociceptors (e.g., P2X receptors). The demonstration of a relationship between peripheral glutamate receptor mechanisms and craniofacial pain may lead to the development of novel diagnostic and therapeutic approaches for TMD and other craniofacial pain conditions of peripheral origin. Thus, peripheral glutamate receptors may be potential novel targets for the treatment of craniofacial pain conditions and may provide a non-opioid rationale for pain therapy. The formulation of specific peripheral ionotropic glutamate receptor antagonists that do not cross the blood brain barrier may be of potential benefit by reducing peripheral nociceptive excitability while avoiding any harmful central side effects associated with central glutamate receptor antagonism.

Currently, TRPV1 receptors have been targeted successfully in the treatment of some neuropathic pain and chronic pain syndromes such as postherpetic neuralgia, postmastectomy neuroma, reflex sympathetic dystrophy syndrome, diabetic neuropathy, and rheumatoid arthritis (Rumsfield and West 1991; Sawynok 2005). However, further studies are needed to elucidate the role of

TRPV1 receptors in deep craniofacial pain. These include some approaches analogous to those suggested above to elucidate glutamate receptor mechanisms (e.g., sex differences), plus the use of specific TRPV1 receptor agonists, antagonists and gene knockouts in both acute and chronic craniofacial pain models studying peripheral (primary afferent) and central (Vc, thalamic, somatosensory cortex) nociceptive processing. Moreover, the role of other members of the TRP family (e.g., TRPA1, see Jordt *et al*. 2004) and any sex differences in these receptor properties in craniofacial pain should also be investigated with receptor-specific agonists and antagonists. The demonstration of a relationship between peripheral capsaicin receptor mechanisms and craniofacial pain may lead to the further development of diagnostic and therapeutic approaches for TMD and other craniofacial pain conditions of peripheral origin. However, since the present study suggests that peripheral glutamate receptors, NMDA in particular, may modulate TRPV1 receptor activity, the targeting of peripheral glutamate receptors may be a novel approach to indirectly target TRPV1 receptors.

5.1.5 Surgical incision-induced excitability

Studies addressing AIMS 2-1 to 2-2 are the first to demonstrate that surgical cutaneous incision can readily activate and evoke central sensitization in TMJ-responsive nociceptive neurons in the Vc/UCC and can render most of these neurons temporarily refractory to sensitization induced by capsaicin injected into the TMJ. Our findings that local anaesthetic pretreatment attenuates the incision-induced barrage and subsequent central sensitization in TMJ-responsive nociceptive neurons are in general agreement with many past animal and human surgical pain models (for review, see Dahl and Kehlet 1993, Abram 1996, Katz 2001, Melzack *et al.* 2001; Kawamata *et al.* 2002; Pogatzki *et al.* 2002a; Sun *et al.* 2004). However, there are some interesting differences demonstrated between incision-induced effects on V compared to spinal nociceptive processing. Previous studies have shown minimal to no changes in cutaneous and deep (muscle) tissue incision-induced cutaneous MAT reduction

(Vandermeulen and Brennan 2000) or cutaneous RF size (Kawamata *et al.* 2005a), whereas cutaneous incision-induced cutaneous MAT reduction and cutaneous RF expansion were markedly robust in Vc/UCC nociceptive neurons. This difference suggests that the V system may be more readily sensitized to peripheral injury than the spinal system. However, the effect of cutaneous incision on spinal nociceptive neurons with deep afferent inputs has not been investigated since the above studies did not test for the presence of a deep afferent RF in the spinal dorsal horn neurons tested. Incision-induced cutaneous RF expansion and cutaneous MAT reduction may contribute to the pain spread and referral and to the development of allodynia that have been documented in postoperative pain states (for review, see Melzack *et al.* 2001; Kawamata *et al.* 2002).

Particularly interesting is that surgical incision can induce central sensitization in TMJ-responsive nociceptive neurons and can render most of these neurons temporarily refractory to sensitization induced by capsaicin injected into the TMJ. This finding suggests that cutaneous injury may modify central nociceptive processing of deep tissue afferent inputs. Possible explanations for the observed effects may be due to incision-induced activation of TRP channels coupled to G protein-coupled receptors, competing peak excitatory and inhibitory responses in the brainstem, or descending inhibitory modulation.

The present results provide support for the use of preemptive regional analgesia in addition to general anaesthesia prior to surgery in order to attenuate or prevent incision-induced activation and central sensitization in nociceptive pathways both in experimental models of pain as well as in clinical surgical cases. Furthermore, given that this is the first study to suggest that there may be differences in sensitivity to surgical incision between V and spinal systems, further studies of incision-induced injury on V processing are warranted. These include some approaches analogous to those suggested above to elucidate glutamate and capsaicin receptor mechanisms, plus studies on the possible incision-induced central excitatory and inhibitory mechanisms that may contribute to the subsequent refractory period in VC/UCC nociceptive neurons. Future

studies may also investigate potential differences between superficial and deep tissue injury as well as acute and chronic injury on peripheral (primary afferent) and central (Vc, thalamic, somatosensory cortex) craniofacial nociceptive processing. Moreover, in addition to studies with local anaesthetics, further investigations of the role of peripheral receptor antagonists (e.g., EAA and TRPV1 receptor antagonists) in incision-induced craniofacial nociceptive processing may provide novel targets for the treatment and management of traumatic tissue injuries and surgical patients.

5.2 STUDY LIMITATIONS:

5.2.1 Identification of nociceptive neurons supplying deep craniofacial tissues

The accurate identification of deep RFs in these studies is complicated by two factors. First, when pressure, chemical or electrical stimuli are applied to deep structures, they can activate afferent fibers remote from the stimulus source. Second, extensive dissection is required with a risk of damaging the afferent terminal/receptor itself (Bove and Light 1995). In spite of these limitations, we have employed a novel methodology to study identified afferent fibers that project from the TMJ or masticatory muscles to the caudal Vc/UCC (see Cairns *et al.* 2001ab, 2002a) as well as TMJ-responsive nociceptive neurons in the caudal Vc/UCC. We avoided damaging the tissue and associated afferent terminals while studying the afferent responses to glutamate and capsaicin by delaying the verification of the afferent's RF until the end of the experiment where skin and connective tissue overlying the RF was removed and mechanical and electrical stimuli reapplied. Similarly, in the study of TMJ-responsive Vc/UCC neurons, our conservative surgical preparation allowed the craniofacial tissues within the neuronal RF being studied to remain intact throughout the experiment with the exception of the small (<2mm long) incision in the experiments involving local anaesthetic pretreatment of skin overlying the TMJ. We have demonstrated that the local anaesthetic pretreatment of skin overlying the TMJ serves to attenuate

the surgical incision-induced activation and central sensitization in TMJ-responsive nociceptive neurons. As a result, the more conservative surgical preparation in the present study has allowed us to study nociceptive neurons closer to their resting state with minimal prior excitability.

5.2.2 Assessment of neuronal sensitization

The use of inhalational anaesthesia may potentially confound the assessment of neuronal excitability since halothane may modulate thermal responses of cutaneous nociceptive afferents (Campbell *et al.* 1984) and the MAT of spinal nociceptive neurons (Herrero and Headley 1995; Yamamori *et al.* 1995). However, the concentrations employed in these studies were different than that utilized in the present study and the effect of halothane on the response of deep afferents to chemical stimuli has yet to be evaluated. Other studies have not shown a difference in sensitization of rat dorsal horn neurons between 1% halothane anaesthesia, pentobarbital anaesthesia or decerebrate preparations (Laird and Cervero 1989). Thus, although unlikely, the anaesthetic regimen could have lead to iatrogenically enhanced primary afferent or central neuronal responses.

A further limitation of the study of nociceptive processing is that despite the more conservative surgical measures instituted, the surgery required to expose the V ganglion and/or the caudal Vc/UCC may excite and/or sensitize the afferent or neuron under study. Moreover, the determination of sensitization relies on repeated mechanical stimuli - which can, in itself, result in sensitization. In order to prevent or limit this iatrogenic sensitization, we instituted a novel protocol that limited the stimulation magnitude, duration, frequency and extent required to determine sensitization. The cutaneous RF expansion in the present study was defined as expansion in cutaneous RF size to include a predetermined point lying 5 mm outside the perimeter of the baseline RF. However, this approach does not measure the full extent of RF expansion and the RF may not expand in the direction of the predetermined point; as a result, the true incidence of RF expansion may actually be underrepresented with this methodology. In addition,

most of these Vc/UCC neurons tested for central sensitization were located in the deep laminae of the Vc/UCC. Thus further investigations of the effects of glutamate, capsaicin and surgical incision on neurons in the superficial laminae are warranted in future studies, given the importance of this region in central nociceptive processing.

5.2.3 Evidence for peripheral EAA and TRPV1 receptor mechanisms in deep craniofacial nociception

Studies from this thesis project have demonstrated that peripheral glutamatergic and capsaicin-sensitive mechanisms do indeed influence deep craniofacial nociceptive processing. However, with the exception of a role for peripheral NMDA receptors in capsaicin-evoked EMG activity (see Chapter 2), there is a lack of direct evidence for NMDA, non-NMDA and TRPV1 receptor mechanisms in V nociception in the present studies. A role for both NMDA and non-NMDA receptors in deep craniofacial nociceptive processing has been previously demonstrated in animal (Cairns *et al.* 1998; Dong *et al.* 2006, 2007) and human craniofacial pain models (Cairns *et al.* 2001a, 2003ab, 2006; Svensson *et al.* 2003, 2005). Thus, the results of this thesis project may be consistent with the involvement of functional NMDA and non-NMDA receptor mechanisms in deep craniofacial nociception shown previously.

In the craniofacial region, TRPV1 receptors have also been demonstrated on V afferents innervating the rat TMJ (Ichikawa *et al.* 2004; Ioi *et al.* 2006). Capsaicin injection into the rat TMJ reflexly evokes a dose-dependent increase in jaw muscle EMG activity (Tang *et al.* 2004) and produces an inflammatory response within these tissues that can be blocked by TRPV1 receptor antagonists (Hu *et al.* 2005a). Since TRPV1 receptors are activated by capsaicin (Caterina *et al.* 1997; Tominaga *et al.* 1998; Benham *et al.* 2003; Jordt *et al.* 2003), the results of this study suggest there may be functional TRPV1 receptors located within the TMJ region. However, the use of specific receptor antagonists would be required to confirm the involvement of the TRPV1 receptor.

5.2.4 Sex differences in deep craniofacial nociception

It was beyond the scope of the present study to investigate sex-related differences in V nociceptive processing. However, the prevalence of TMD-related pain is higher among women of reproductive age compared to postmenopausal women or to men (Carlsson and LeResche 1995, LeResche *et al*. 1997; for review, see Fillingim and Ness 2000). Estrogen receptors are found in muscle (Dalberg 1982; Meyer and Raap 1985), TMJ (Aufdemorte *et al.* 1986; Milam *et al.* 1987), and dorsal root ganglion neurons (Sohrabji *et al.* 1994), indicating that deep tissues as well as peripheral ganglia are potential targets for sex steroids to modulate sensory functions. Also, in view of the finding of Vc estrogen receptors (e.g., Amandusson *et al.* 1996; Bereiter *et al.* 2005b), central actions of estrogen on nociception cannot be ruled out. The present study used only male rats and consequently could not investigate sex-related differences in V nociceptive processing of glutamatergic and capsaicin-sensitive mechanisms. Previous studies in animal (Cairns *et al.* 2001ab, 2002b; Dong *et al.* 2007) and human (Cairns *et al.* 2001a, 2003a,b, 2006; Svensson *et al.* 2003; Castrillon *et al.* 2007) craniofacial pain models support the view that females may exhibit an enhanced neural responsiveness to glutamate injected into the deep craniofacial tissues, which may be due, in part, to a sex-related increase in the excitability of V primary afferents as well as central nociceptive neurons. However, it is not known whether there are any sex differences with respect to peripheral TRPV1 receptor mechanisms nor if there are any sex-related interactions between peripheral EAA and TRPV1 receptor mechanisms. As noted above, this would be a fruitful future direction.

5.2.5 Translation of experimental animal models to human pain states

The findings in the present rat TMJ injury model suggest that peripheral glutamatergic and capsaicin-sensitive mechanisms may contribute to craniofacial pain states in humans. However, one may question the applicability of translating the results of experimental animal models to human pain states due to genetic and species differences. In this context, there is a strong genetic influence on rat

responses to stress and susceptibility to inflammatory conditions (Cizza and Sternberg 1994). Similarly, genetics influence morphine effects on mice (Mogil *et al.* 1996; Belknap *et al.* 1998; Elmer *et al.* 1998) as well as mice responses to various nociceptive tests (Mogil 1999, see 2005). There is also remarkable variability among humans in terms of susceptibility to chronic orofacial pain and its characteristics, which suggests that these syndromes are complex heritable traits controlled by alleles of certain polymorphic genes that interact with the environment (Seltzer *et al.* 2004).

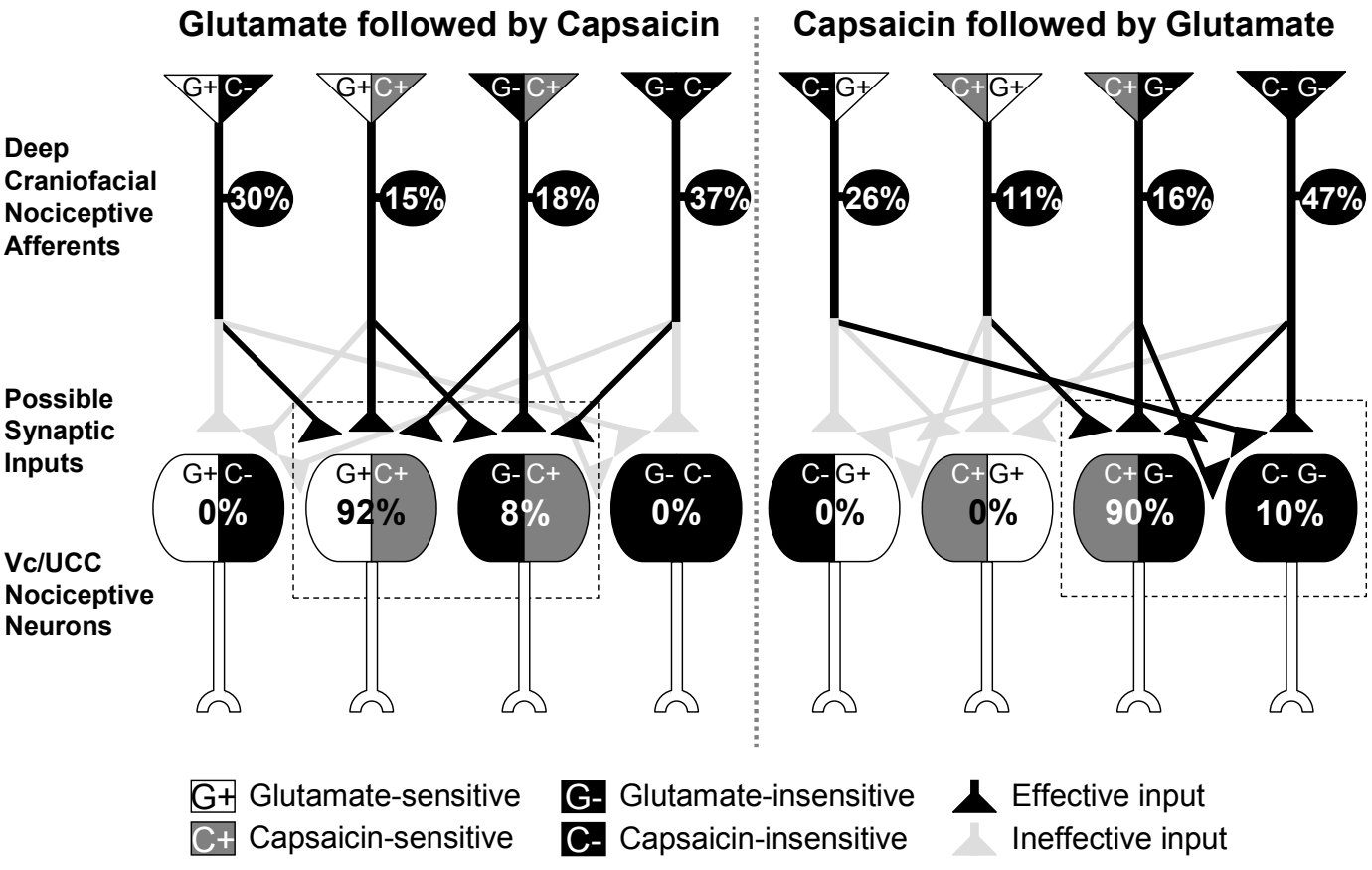
In many respects, the organization of the somatosensory system of the rat is comparable to that of other animals including humans (Kunc 1970; Afshar and Dykes 1984; Dallel *et al.* 1989; Tracey and Waite 1995; Brown 1997; Usunoff *et al.* 1997; Pajot *et al.* 2000; Jawahar *et al.* 2001; Paxinos 2004). Taken together, data from rats, cats and monkey suggest similar organizational and functional features of primary afferents and spinal and medullary dorsal horns (Grant 1995; Paxinos 2004). Although there are some differences with higher-order structures, e.g., unlike carnivores and primates, no interneurons or GABAergic cells are present in the VPM of rats (Barbaresi *et al.* 1986; Williams and Faull 1987, Waite and Tracey 1995; Amadeo *et al.* 2001), and the representation in the CNS of the face and vibrissae in the rat occupies a disproportionately larger area in the primary somatosensory cortex than in many higher mammals (Welker 1971; Waite and Tracey 1995; Brett-Green *et al.* 2001; Frostig 2006). In addition, despite the mandibular condyle extending anterior-posteriorly in rats as opposed to the lateromedial direction in humans, the rat TMJ articular apparatus is very similar to that of humans in that they both possess a reciprocally fitting condylomeniscal joint and meniscotemporal joint (Bermejo *et al.* 1993; Fletcher *et al.* 2004). More importantly, the demonstration of a novel nociceptive role for peripheral glutamatergic and capsaicin-sensitive mechanisms in our animal models agrees with findings in humans (for review, see Chapter 1).

Although the same limitations as above apply to the incision study, our findings that local anaesthetic pretreatment attenuates the surgical cutaneous incision-induced barrage and subsequent central sensitization in TMJ-responsive

nociceptive neurons are in agreement with past animal and human surgical pain models (see Dahl and Kehlet, 1993, Abram, 1996, Katz, 2001, Melzack *et al.,* 2001; Kawamata *et al.,* 2002). Moreover, although our observation period for incision-induced central sensitization was only during the acute phase of injury, our findings provide support for the use of preemptive regional analgesia in order to attenuate or prevent incision-induced activation and central sensitization in nociceptive pathways in clinical surgical cases.

5.3 FIGURE:

Fig. 1. Schematic diagram of glutamate and capsaicin-evoked activation responses and the possible convergent trigeminal nociceptive afferent inputs to Vc/UCC nociceptive neurons. Note that for both the glutamate followed by capsaicin subgroup and the capsaicin followed by glutamate subgroup, whereas there were four types of agonist-responsive afferents, there were only two types of agonist-responsive Vc/UCC neurons activated following injection of glutamate and capsaicin into deep craniofacial tissues. In addition, the vast majority of the Vc/UCC neurons were capsaicin-sensitive in both subgroups (100% of the glutamate followed by capsaicin subgroup and 90% of the Vc/UCC neurons were capsaicin followed by glutamate subgroup), whereas there were no glutamate-sensitive Vc/UCC neurons in the capsaicin followed by glutamate subgroup. Possible synaptic inputs depicted in black refer to inputs effective in activating the neurons and those in gray refer to relatively ineffective inputs. %: Percentages of each type of agonist-responsive deep craniofacial afferent or Vc/UCC nociceptive neuron found in the glutamate followed by capsaicin subgroup or capsaicin followed by glutamate subgroup.



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