# EFFECT OF LOCAL ANAESTHESIA

# ON

# CAPSAICIN-INDUCED NEUROGENIC INFLAMMATION

by

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**Effect of Local Anaesthesia on Capsaicin-Induced Neurogenic Inflammation.**

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## ABSTRACT

Neurogenic inflammation has been associated with the development of temporomandibular disorders and noxious stimulation of the temporomandibular joint (TMJ) region has led to reflex excitation of masticatory muscles. The aim of this study was to evaluate the neurogenic component in acute TMJ inflammation by investigating the effect of local anesthetic blockade of afferent innervation on the development of capsaicin-induced edema in the rat TMJ region and on reflex jaw muscle activity. The application of capsaicin into the saline pre-treated TMJ region led to edema development and reflex jaw muscle activity. However, pre-treatment with bupivacaine failed to inhibit capsaicin-induced edema; whereas, reflex jaw muscle activity was abolished. Therefore, edema of the rat TMJ region developed independent of axonal conduction, suggesting inflammatory mediators such as neuropeptides may be directly released from primary afferent nerve terminals regardless of functional nerve conduction. Moreover, capsaicin-induced reflex jaw muscle activity appears to be centrally mediated.

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**Abbreviations**

| | |
|---|---|
| ANOVA | analysis of variance |
| AUC | area under the curve |
| BK | bradykinin |
| $B_2$ | bradykinin receptor subtype 2 |
| CGRP | calcitonin gene-related peptide |
| CNQX | 6-cyano-7-nitroquinoxaline-2,3-dione |
| COLD | thermoreceptive specific |
| DRG | dorsal root ganglion |
| DRRs | dorsal root reflexes |
| EMG | electromyographic |
| $GABA_A$ | gamma-amino-butyric acid receptor subtype A |
| HRP | horseradish peroxidase |
| IL-1 | interleukin-1 |
| -LI | like immunoreactivity |
| LTM | low threshold mechanoreceptors |
| NGF | nerve growth factor |
| NK-1 | neurokinin receptor subtype 1 |
| NKA | neurokinin A |
| NMDA | N-methyl-D-aspartate |
| non-NMDA | non N-methyl-D-aspartate |
| NPY | neuropeptide Y |
| NS | nociceptive specific |
| PAD | primary afferent depolarization |
| PE | plasma extravasation |
| PG | prostaglandins |
| RF | receptive field |
| SP | substance P |
| SPGN | sympathetic post-ganglionic neuron |
| TMDs | temporomandibular disorders |
| TMJ | temporomandibular joint |
| TRP | transient release potential |
| V | trigeminal nerve |
| V1 | ophthalmic nerve |
| V2 | maxillary nerve |
| V3 | mandibular nerve |
| VBSNC | trigeminal brainstem nuclear complex |
| Vc | subnucleus caudalis |
| Vi | subnucleus interpolaris |
| VIP | vasoactive intestinal polypeptide |
| Vo | subnucleus oralis |
| VR1 | vanniloid receptor subtype 1 |
| WDR | wide dynamic range |
| 5-HT | 5-hydroxytryptamine (serotonin) |
| 6-OHDA | 6-hydroxydopamine |

# CHAPTER 1

# INTRODUCTION & REVIEW OF LITERATURE

## 1.1 RATIONALE

Temporomandibular Disorders (TMDs) encompass an array of problems involving the temporomandibular joint (TMJ) and/or the masticatory musculature. Epidemiologic studies have concluded that the most common signs and symptoms of TMDs consist of pain and tenderness, sounds in the TMJ, limitation or other disturbances of mandibular movement (Carlsson, 1999). Specifically, 10% of the adult population complains of pain associated with the temporomandibular region (Carlsson, 1999) and the signs and symptoms of TMDs are more prevalent in females (Carlsson, 1999; Milam & Schmitz, 1995).

Although the signs and symptoms may assist in the identification of TMDs, however, the objective criteria for the diagnosis and the indications for treatment of TMDs remain controversial (Goldstein, 1999; Milam & Schmitz, 1995). This is solely due to the poor understanding of the biologic processes underlying TMJ adaptation to mechanical stress and the pathophysiology of TMDs (Milam & Schmitz, 1995). In addition, the limited number of studies investigating the underlying nociceptive mechanisms of the TMJ and associated masticatory muscles (Sessle, 1999) contributes to the lack of definitive diagnosis and treatment of TMDs.

Despite the lack of information, preliminary animal and clinical studies of the TMJ and other body joints have suggested three mechanisms of injury implicated in the development of TMDs (see Figure 1) (Milan & Schmitz, 1995). These are a) direct mechanical injury, b) hypoxia-reperfusion injury, and c) neurogenic inflammation. The first

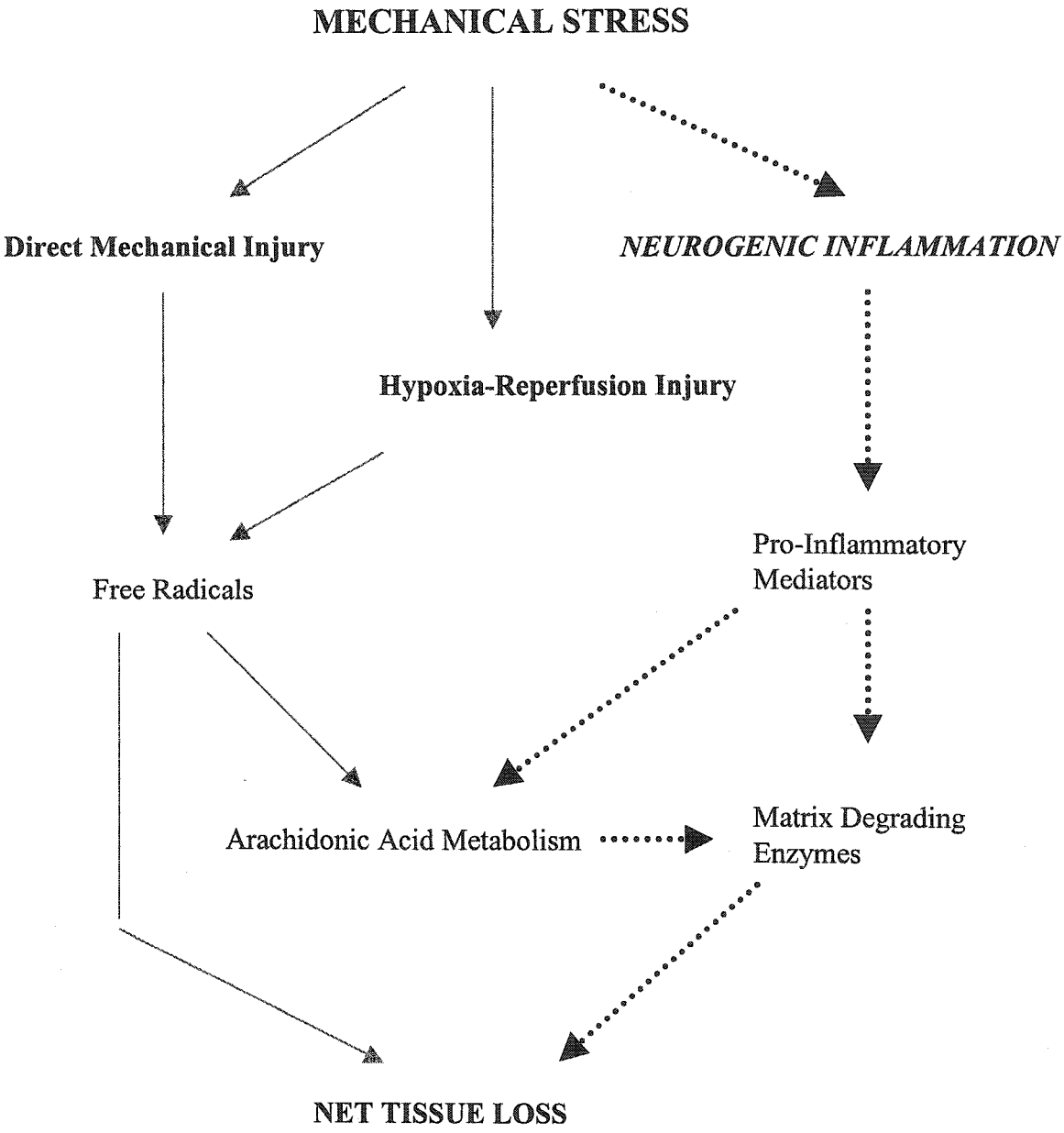


**Figure 1** **Common Pathways for Three Proposed Mechanisms of TMDs**
*Adapted from Milam & Schmitz, 1995.*

two proposed mechanisms involve the production of free radicals that subsequently lead to an increase in arachidonic acid metabolism and a net loss of tissue with associated pain. On the other hand, the latter mechanism involves the release of neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) from peripheral endings of nociceptive primary afferents to evoke pro-inflammatory effects in peripheral tissues (Holzer *et al.*, 1995; Lembeck & Holzer, 1979; Maggi, 1997). However, the evidence supporting this pro-inflammatory neurogenic mechanism implicated in the development of TMDs is minimal.

Nevertheless, in 1999, Yoshida *et al.* (1999) compared the TMJ aspirates from patients with internal derangement of the TMJ with control specimens and demonstrated more intense SP-like immunoreactivity expression and pain ratings from affected patients. Similarly, Alstergren *et al.* (1995) revealed the presence of SP in both the plasma and TMJ fluid aspirates of TMJ arthritic patients. In addition to human studies, there have been investigations on experimentally induced arthritic TMJ rats attempting to validate neurogenic inflammation as one of the possible mechanisms underlying TMDs (Carleson *et al.*, 1997a, b; Spears *et al.*, 1998). Thus, these studies jointly demonstrated a possible pro-inflammatory neurogenic role in the development of TMDs.

In addition to assessing neuropeptide content, a pharmacological tool used in the investigation of neurogenic inflammation is capsaicin, the pungent extract of hot chili peppers. This chemical is a small fiber excitant and inflammatory irritant known to evoke acute neurogenic inflammation when applied to peripheral tissues (Jancso *et al.*, 1967, 1968; Inoue *et al.*, 1995). It has been well established that capsaicin acts on specific membrane vanniloid receptors termed VR1 receptors, (Caterina *et al.*, 1997) located along the entire length of primary sensory neurons (Guo *et al.*, 1999). The resulting depolarization and

discharge of action potentials in the peripheral nerve endings (Holzer, 1988, 1991) cause the release of inflammatory peptides such as SP and CGRP (Holzer, 1988, 1991; Caterina *et al.*, 1997), evoking burning pain, local edema and plasma extravasation (PE) (Holzer, 1988, 1991). Therefore, capsaicin is an essential functional marker for a subset of neurons specialized to detect noxious stimuli and an indispensable agent for the evaluation of neurogenic inflammation.

Previously, in our laboratory, the application of capsaicin to the rat TMJ region has been shown to be markedly inflammatory, resulting in plasma protein extravasation and edema (Fiorentino *et al.*, 2000). In addition, the reduction of edema following the pre-treatment of the TMJ tissues with ruthenium red (non-competitive VR1 receptor antagonist) or capsazepine (competitive VR1 receptor antagonist) prior to the application of capsaicin confirmed the presence of VR1 receptors in the TMJ region (Fiorentino *et al.*, 2000). Therefore, these findings suggest that capsaicin can be applied to the rat TMJ region to theoretically evoke a neurogenic inflammatory response; however, the presence or absence of a capsaicin-induced neurogenic component in the inflammatory reaction in the TMJ tissues has not been specifically investigated. Hence, with the aid of the pharmacological tool capsaicin, this present study can evaluate the neurogenic mechanism of injury implicated in the development of TMDs by assessing the effects of a complete conduction blockade provided by local anaesthetic pre-treatment of the TMJ region.

Furthermore, investigations assessing the effect of local anesthetic blockade of afferent innervation on the actions of capsaicin applied to the rat TMJ have not been performed. Theoretically, the application of local anaesthetic will block the nerve conduction and consequently inhibit the release of neuropeptides elicited by the binding of

capsaicin to VR1 receptors. Hence, the pre-treatment of TMJ tissues with local anaesthetic may inhibit neurogenic inflammation and subsequently prevent the development of TMDs.

Interestingly, in our laboratory, Wong *et al.* (2001) demonstrated the ineffectiveness of the pre-treatment of rat TMJ tissues with 5% lidocaine or 0.5% bupivacaine in blocking the mustard oil-induced neurogenic inflammation even though the abolishment of mustard oil-induced electromyographic (EMG) activity of the jaw muscles confirmed the efficacy of the local anaesthetic. Therefore, the authors hypothesized that either mustard oil acted non-neurogenically to produce edema or mustard oil released mediators of neurogenic inflammation by direct action on nociceptive terminals independent of axonal depolarization (Wong *et al.*, 2001). However, since the collected EMG data were comprised of only one rat per experimental group, the validity of the confirmation of a complete conduction blockade is questionable; thus, the hypotheses made may be erroneous. In addition, the precise molecular and cellular actions of mustard oil remain unknown and specific 'mustard oil' receptors have not been identified; hence, the assumption that this algesic agent only activates nociceptive terminals is poorly supported. Consequently, an agent that specifically stimulates nociceptors is better suited for the evaluation of the neurogenic inflammatory process in TMJ tissues. Therefore, since capsaicin binds only to VR1 receptors located on primary sensory afferents, it is the ideal agent for the present study.

Moreover, the application of algesic agents such as mustard oil (Yu *et al.*, 1995) or non-inflammatory substances such as glutamate (Cairns *et al.*, 1998) to the rat TMJ region evokes a reflex jaw muscle activity that mimics the protective reflex activation of jaw musculature as a result of injury to the TMJ region. Recently, an investigation demonstrated that the application of capsaicin to the TMJ region elicits a dose-dependent sustained and

reversible increase in EMG activity of the ipsilateral masseter and digastric muscles (Hu *et al.*, 2001). This discovery is beneficial in the present investigation for evaluating the efficacy of the local anaesthetic pre-treatment of the rat TMJ tissues with the successful blockade of afferent innervation theoretically corresponding to the lack of capsaicin-induced reflex activity of the jaw muscles.

In summary, the goal of this present study is to test the hypothesis that neurogenic inflammation plays an important role in the development of TMDs. A better appreciation for the peripheral nociceptive transmission of TMJ noxious stimuli will provide insights on the pathophysiological mechanism underlying TMDs and a rational basis for future pharmacological research targeting analgesics that act peripherally, selectively interfering with nociceptor activation.

## 1.2 NEUROGENIC INFLAMMATION

### *1.2.1 Definition and Current Understanding of Neurogenic Inflammation*

Neurogenic inflammation is defined as an increase in vascular permeability and PE triggered by antidromic stimulation of sensory nerve fibers (Chahl, 1988; Lynn, 1996). This is attributed to an effector function of primary afferent terminals initiated by the release of vasoactive peptides, such as SP and CGRP (Jancso *et al.*, 1967, 1968; Lewis 1927) and is often associated with local axon reflexes (Lewis, 1927). However, it can also be initiated centrally, in the spinal cord, by primary afferent depolarization (PAD) large enough to trigger dorsal root reflexes, (Eccles *et al.*, 1961, 1962; Koketsu, 1956; Willis, 1999). Furthermore, the current accepted model of neurogenic inflammation involves a stimulating event to occur at the neuronal terminal, followed by a resultant antidromic depolarization from the central

nervous system (CNS) and release of neurotransmitters such as SP, neurokinin A (NKA) and CGRP (Maggio & Hunter, 1984; Gamse & Saria, 1985). SP and NKA subsequently act on mast cells to release histamine (Jorizzo *et al.*, 1983), whereas CGRP acts to vasodilate and facilitate the actions of histamine, SP and NKA (Gamse & Saria, 1985).

### *1.2.2 Historical Perspective*

This positive feedback phenomenon neurogenic inflammation, stemmed from a related concept known as antidromic vasodilatation, which has been under investigation for over a century. In 1876, Stricker (1876) stimulated peripheral ends of cut dorsal roots, resulting in an increase in skin temperature due to cutaneous vasodilatation. He concluded this resultant vasodilatation was elicited by efferent fibers originating from the dorsal roots. This phenomenon contradicted the Bell-Magendie Law of separation which stated dorsal roots have sensory functions and ventral roots have motor functions (cf. Lynn, 1996). In 1901, Bayliss (1901) verified these results and illustrated that the disruption of the dorsal root between the ganglia and the spinal cord did not eliminate the vasodilatation. However, the removal of afferent nerves from the dorsal root resulted in the degeneration of the peripheral nerves and the abolishment of the vasodilatatory response. Therefore, Bayliss concluded that vasodilatation was initiated by the efferent function of afferent fibers and termed this type of conduction as 'antidromic'. A few years later, Bruce (1913) induced vasodilatation with the application of mustard oil to the conjunctiva of the cat and observed that the response depended on intact peripheral nerves; however, a disruption in the connection of the afferent nerves with the spinal cord did not abolish the vasodilatatory response. Thus, he suggested it was the result of a concept called 'axon reflex' in which one single nerve is responsible for

the vasodilatation. Moreover, in 1921, the physiological role of antidromic vasodilation became inherent when Langley (1921) proposed that the flare component of inflammatory reactions and antidromic vasodilation involved the same nervous components.

In 1927, Lewis (1927) made a significant contribution by describing the 'triple response' of an inflammatory reaction that included redness, flare and wheal. He believed the flare and wheal component were independent events and his assumption was confirmed when an investigation demonstrated that PE is due to endothelial cell contraction (Majno *et al.,* 1969). Furthermore, Lewis suggested that the flare component involved a local axon reflex and the area affected was dependent on the network of axon collaterals and vessels associated with the activated nerve. He proposed the release of neurotransmitters from nerve terminals followed by their action on the vasculature explained the delay between the time of nerve stimulation and the onset of vasodilatation. Initially, histamine was suggested as a possible neurotransmitter, however, Lewis later rationalized that one chemical could not account for the various inflammatory reactions. Also, itching, a histamine-induced response did not always occur with vasodilatation. In 1953, Lembeck (1953) proposed that substance P (SP) may be the main mediator of antidromic vasodilatation. By the 1980s, many studies agreed that SP appeared to be the most appropriate candidate (cf. Chahl, 1988); however, it was not the sole mediator (Holzer *et al.,* 1991).

Subsequently in the field of research embracing antidromic vasodilatation, the identification of nerves responsible for this phenomenon became important. Hinsey and Gasser (1930) revealed that dorsal root stimulation of sufficient intensity produced antidromic vasodilatation and a 'C' wave on the neurogram. Then, in 1953, Celander and

Folkow (1953) became the first to prove that sensory fibers responsible for nociception were involved in antidromic vasodilation.

In Hungary, during the 1940s, Jancso and his co-workers began experimenting with capsaicin, the pungent extract of hot chili peppers, to investigate the flare and wheal response on skin (cf. Chahl, 1988). In 1968, Jancso *et al.* (1968) observed that the flare response was dependent on intact nerve conduction since local anaesthesia abolished it. Interestingly, local anaesthesia did not abolish the wheal which is defined as a circumscribed plaque of edema of the skin (Dorland, 1994). In denervated skin, there was an absence of a flare and wheal response to the topical application of capsaicin. Therefore, although the wheal response required the presence of functional nerves, it did not rely on neuronal conduction. Furthermore, of great importance is the resultant desensitization of the entire animal achieved by parental neonatal pretreatment with capsaicin, suggesting that this agent selectively targeted sensory neurons (Jancso *et al.,* 1967). In addition, the capsaicin-induced desensitization abolished the edema response normally elicited by the antidromic stimulation of the rat saphenous nerve. Thus, this provided direct evidence that antidromic stimulation of sensory nerves evoked increased vascular permeability, a concept termed as neurogenic inflammation. In addition to the animal models employed by Jancso and his colleagues, the investigation of neurogenic inflammation was later extended to human subjects by other researchers (Lamotte *et al.,* 1991).

Further studies concluded that neurogenic inflammation depended on stimulation intensities sufficient to excite C fibers (Chahl & Ladd, 1976; Szolcsanyi, 1977) and particularly depended on stimulation of nociceptive fibers (Kenins, 1981). In addition, investigation of the pharmacology of neurogenic inflammation (Lembeck & Holzer, 1979;

Chahl & Ladd, 1976) revealed a mediator that mimicked SP, which had been associated with antidromic vasodilatation. Therefore, it became apparent that the mechanisms and mediators of both antidromic vasodilatation and neurogenic inflammation are similar.

### *1.2.3 Concepts of Neurogenic Inflammation from Past to Present*

Over the past century, the concept of neurogenic inflammation has evolved and encompassed various essential aspects (see Figure 2). Lewis (1927) proposed that the classical model of axon reflex was part of a host defense mechanism for noxious stimuli, which he termed the 'nocifensor' system. This defense system consisted of branching axons with sensory receptors receiving stimuli on one end and an effector terminal innervating vessels on the other end; such that the excitation of the receptor ending activated the neuroeffector collateral ending at the vessels via an uni-directional axon reflex. Lewis' initial concept of a 'nocifensor' system was not strongly supported until other investigators demonstrated that substance P was released by the stimulation of cutaneous nerves and led to vasodilatation, PE and subsequent recruitment of secondary mediators of inflammation (Chahl, 1988). This recognition of the role of neurally derived mediators in neurogenic inflammation was later joined by evidence citing the involvement of mast cells (Lewis, 1927; Kiernan, 1971; Kiernan, 1972). However, the theory of mast cells eliciting a direct action on afferent terminals was soon changed to an action on the vasculature (Arvier *et al.,* 1977). Moreover, with the knowledge that SP appeared to be the primary mediator of neurogenic inflammation (Lembeck *et al.,* 1977; Lembeck & Holzer, 1979), the concept of an axon cascade was established (Lembeck & Gamse, 1982). Mediators initiated this axon cascade that was propagated centrally as nociceptive information and peripherally to release SP from

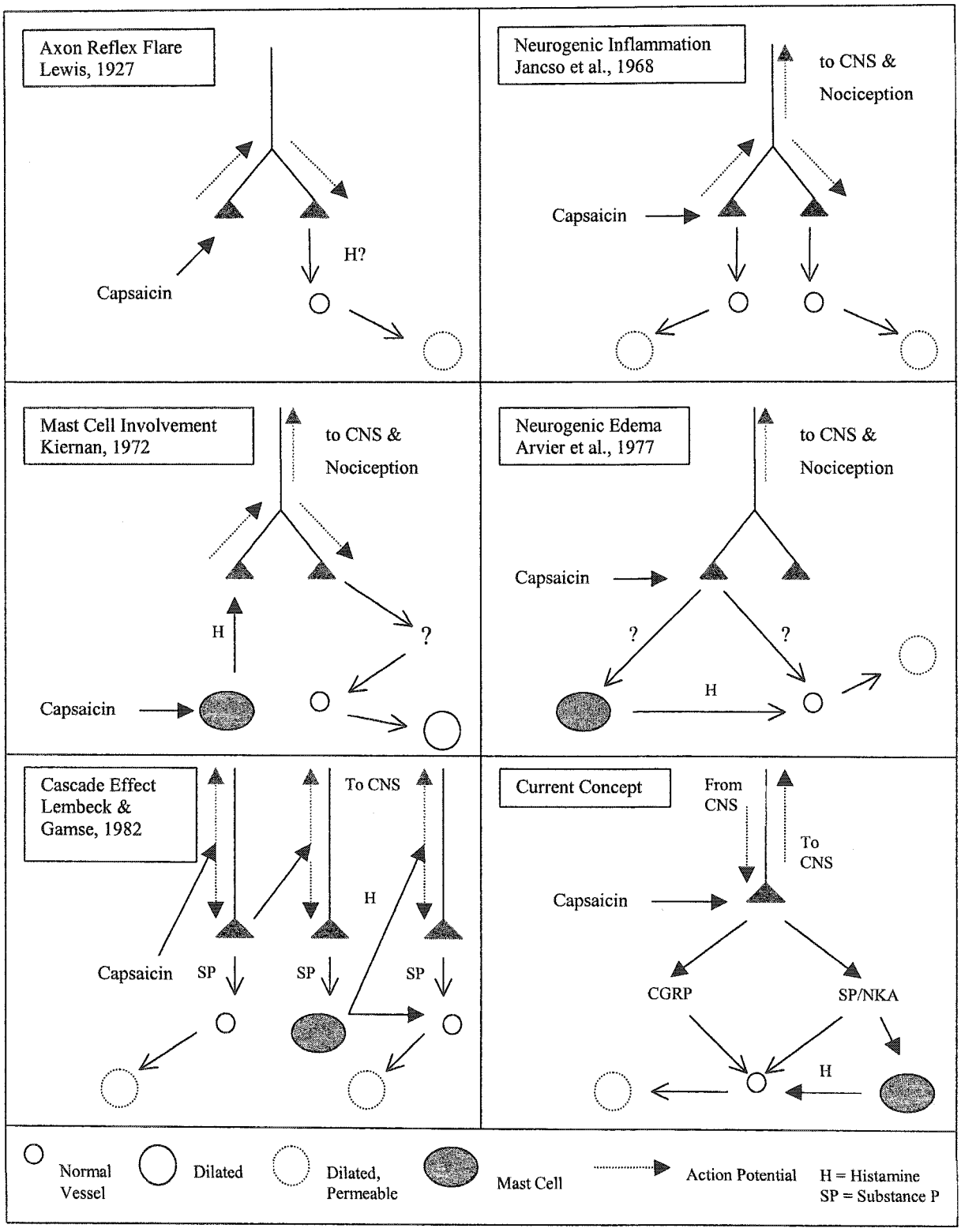


**Figure 2** **Concepts of Antidromic Vasodilatation and Neurogenic Inflammation**
Adapted from *Chahl, 1988.*

neuronal terminals. However, this latter theory has been replaced. The current accepted model of neurogenic inflammation involves a stimulating event to occur at the neuronal terminal, followed by a resultant antidromic depolarization from the CNS and release of neurotransmitters such as SP, neurokinin A (NKA) and CGRP (Maggio & Hunter, 1984; Gamse & Saria, 1985). SP and NKA subsequently act on mast cells to release histamine (Jorizzo *et al.,* 1983), whereas CGRP acts to vasodilate and facilitate the action of histamine, SP and NKA (Gamse & Saria, 1985).

### *1.2.4 Neurogenic Mediators of Neurogenic Inflammation*

The release of neuropeptides from primary sensory afferent endings as a result of antidromic stimulation is an essential component for the resultant increased vascular permeability and vasodilatation in the process of neurogenic inflammation. The principal neuropeptide implicated in neurogenic inflammatory process is substance P (SP) (Chahl, 1988); however, calcitonin gene-related peptide (CGRP) has also been demonstrated to play an important vasodilatatory role (Holzer, 1998). In addition to SP and CGRP, other neuropeptides such as vasoactive intestinal polypeptide (VIP), neurokinin A (NKA), and neuropeptide Y (NPY) contribute to these vascular effects.

#### 1.2.4.1 Substance P (SP)

SP, a tachykinin, was first associated with antidromic vasodilatation when its injection into tissue led to hyperemia (von Euler & Gaddum, 1931). Later, several researchers suggested that SP was a neurotransmitter released from sensory afferent endings as well as a mediator of antidromic vasodilatation (Hellauer & Umrath, 1947; Lembeck,

1953). Further investigations found that SP was synthesized in cell bodies and transported to peripheral nerve endings (Andrews & Holten, 1958) in addition to its central terminals (Takahashi & Otsuka, 1975). SP has been synthesized and its peptide sequence determined (Powell *et al.,* 1973). The demonstration of its anatomical distribution is signified by SP-like immunoreactivity (Hokfelt *et al.,* 1975a, b; Nilsson *et al.*, 1975). Since SP actions mimicked the effects of antidromic stimulation of nerves, SP was named the primary neurotransmitter responsible for mediating neurogenic inflammation (Chahl, 1988).

Foreman *et al.* (1983) first hypothesized that SP elicits PE via the specific interaction of its C-terminus and vascular receptors. Subsequently, these vascular receptors were identified as $NK_1$ receptors on venular endothelium (Brain, 1996; Holzer, 1992). Studies also revealed that the binding of SP to $NK_1$ receptors coupled to phosphoinositide signaling pathway leads to vasodilatation (Maggi, 1995a) and plasma protein leakage due to increased vascular permeability (Brain, 1996; Holzer, 1992). Furthermore, the introduction of $NK_1$ receptor antagonists inhibited the neurogenic exudative responses to a variety of stimuli (Brain, 1996; Holzer, 1992; Walsh *et al.,* 1995). These data provide more evidence supporting SP as the main neuropeptide in neurogenic inflammation.

### 1.2.4.2 Calcitonin Gene-Related Peptide

In the 1980s, the neuropeptide CGRP was discovered (Amara *et al.,* 1982; Rosenfeld *et al.,* 1983) and its potent vasodilatory effect was demonstrated (Brain *et al.,* 1985). Subsequent studies found CGRP to be co-localized with SP in capsaicin-sensitive spinal and trigeminal primary sensory neurones of the rat (Skofitsch & Jacobowitz, 1985; Lee *et al.,* 1985). In addition to vasodilatation, CGRP was shown to potentiate tachykinin-induced

plasma protein extravasation (Gamse & Saria, 1985) after its release from afferent endings in response to nerve stimulation (Holzer, 1992). Therefore, CGRP is considered as one of the many neuropeptides involved in the process of neurogenic inflammation.

The potent vasodilatatory effect of CGRP is mediated by $CGRP_1$ receptors coupled to the adenylate cyclase system and a direct action of CGRP on vascular smooth musculature (Brain, 1996). Further confirmation of the role of CGRP in neurogenic inflammation stems from pharmacological antagonism of this endogenously released neuropeptide. The application of $CGRP_1$ receptor antagonist, $CGRP_{8-37}$, inhibits the vasodilatation of cutaneous arterioles evoked by nerve stimulation (Brain, 1996).

Moreover, CGRP does not directly cause protein leakage; however, it inhibits the degradation of SP (Brain, 1996; LeGreves *et al.*, 1985; Holzer, 1992). In other words, CGRP indirectly enhances the increased vascular permeability due to nerve stimulation by preventing the removal of SP. Thus, CGRP is primarily a vasodilator in the process of neurogenic inflammation.

### *1.2.5 The Sympathetic Nervous System and Neurogenic Inflammation*

The involvement of the sympathetic nervous system in the modulation of neurogenic inflammation has been extensively studied in many different research models. However, depending on the agent utilized for the experimental induction of PE, the sympathetic efferents may or may not play a role in the process of neurogenic inflammation. Nevertheless, the variation in protocols may account for such controversy.

Bradykinin (BK) is a potent pain-producing agent that vasodilates and increases vascular permeability (Kadar, 1989); thus, renders it to be an ideal compound for artificially

inducing neurogenic inflammation. The resultant PE is the measure used to evaluate the involvement of sympathetic post-ganglionic neuron (SPGN) terminals in neurogenic inflammation. Chemically induced sympathectomy can markedly attenuate the PE induced by BK (Lee *et al.,* 1991); therefore suggesting SPGNs contribute to the neurogenic inflammatory process. The role of SPGNs in the regulation of PE was further investigated with the co-perfusion of various SPGN-derived mediators with BK into the rat knee joint (Green *et al.*, 1993a). The co-administration of norepinephrine (NE) or neuropeptide Y (NPY) with BK reduced PE whereas the infusion of prostaglandin $E_2$ (a pro-inflammatory mediator) increased PE. Thus, SPGN-derived mediators may either inhibit or potentiate BK-induced PE depending on which ones are released. Moreover, Green *et al.* (1993b) revealed that BK induced PE is diminished in sympathectomized rats and BK itself is capable of releasing NE in the rat knee joint. Similarly, a recent study by Lo *et al.* (1999) demonstrated the dependence of BK- induced PE on SPGN terminals. In addition, the evaluation of BK-induced neurogenic migration of neutrophils led to the suggestion that BK acts on sympathetic nerve terminals in the rat knee joint, causing the attraction of neutrophils, which enhances PE (Lo *et al.,* 1999). Therefore, the above studies support the role of the SPGN terminal involvement in inflammatory PE induced by BK.

Another agent used to induce PE is mustard oil, an algesic agent known to induce neurogenic inflammation. Komoroski *et al.* (1996) studied the effect of a guanethidine sympathectomy on mustard oil-evoked PE in dental pulps of molars in response to mustard oil. The authors found no difference between the amount of PE in sympathectomized and non-sympathectomized rats. Therefore, it was concluded that sympathetic efferents have no modulatory effect on mustard oil-induced PE. Likewise, a previous study evaluating the

effect of topical mustard oil application after a chemical sympathectomy also demonstrated no change in the resultant PE (Donnerer *et al.,* 1991). Therefore, the sympathetic nervous system does not appear to be involved in mustard oil- induced neurogenic inflammation.

The lack of a sympathetic influence on mustard oil-induced PE could be due to an incomplete sympathectomy (Donnerer *et al.,* 1991). Neonatal guanethidine treatment can reduce 86% of the NE levels with a consequent reduction of PE in response to an antidromic stimulation. However, 6-hydroxydopamine (6-OHDA) used for the sympathectomy only led to a 66% reduction in NE levels and an unchanged amount of antidromic stimulated PE. Hence, inadequate sympathectomy may lead to inaccurate findings.

SP has also been utilized as a compound to induce neurogenic inflammation; however, the involvement of sympathetic efferents in SP- induced PE is controversial. Rats pre-treated with 6-OHDA reduced SP- induced PE (Khalil & Helme, 1989). However, guanethidine sympathectomy increased PE induced by SP (Mathison & Davison, 1994). Therefore, these studies reveal both an excitatory and an inhibitory effect of sympathetic efferents on SP- induced PE, respectively.

The above inhibitory effect of sympathetic efferents on PE is also demonstrated in afferent nerve- induced PE (Kerezoudis *et al.,* 1993). Electrical stimulation of afferent nerves in chronically sympathectomized rats increased afferent nerve- induced PE (Kerezoudis *et al.,* 1993). This suggests that sympathetic nerves inhibit the PE in response to afferent nerve stimulation. On the contrary, since stimulation of afferent nerves by capsaicin in sympathectomized rats did not diminish or enhance PE (Sulakvelidze *et al.,* 1994); sympathetic nerves may not be essential for capsaicin- induced PE.

### *1.2.6 The Parasympathetic Nervous System and Neurogenic Inflammation*

In contrast to the sympathetic nervous system, the evaluation of the parasympathetic efferents in association with the process of neurogenic inflammation is limited and ill defined. Delepine and Aubineau (1997) assessed the contribution of the parasympathetic nervous system on the enhanced PE induced by the electrical stimulation of the sphenopalatine ganglion in rats. An atropine infusion inhibited the release of acetylcholine from post-ganglionic parasympathetic fibers and completely eliminated the PE response, suggesting the involvement of parasympathetic efferents in neurogenic inflammation.

### *1.2.7 The Central Nervous System and Neurogenic Inflammation*

The effector function of primary afferent fibers in neurogenic inflammation is often associated with the axon reflex (Lewis, 1927) concentrated in the peripheral nervous system; however, the CNS has also been shown to contribute to the development of neurogenic inflammation via dorsal root reflexes (DRRs) (see Figure 3) (Eccles *et al.,* 1961, 1962; Koketsu, 1956; Willis, 1999).

Chahl and Ladd (1976) first demonstrated that the antidromic stimulation of the saphenous nerve innervating one limb resulted in an edema response in the contralateral limb. Similarly, Levine *et al.* (1985) found that a mild injury to one hindpaw produced swelling and hyperalgesia in the contralateral paw. This contralateral response was attenuated in acute and chronic peripherally denervated, sympathectomized and capsaicin treated rats. Therefore, the contralateral response appears to be mediated by a spinal circuit connection across the spinal cord.

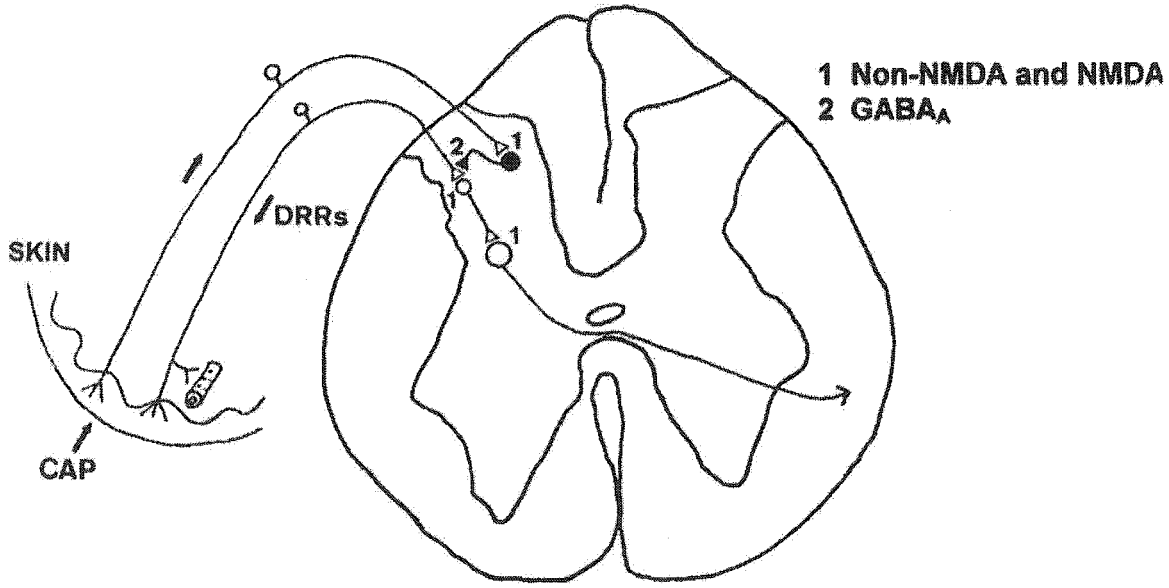


**Figure 3** **Proposed Model of Dorsal Root Reflexes in Neurogenic Inflammation**
Adapted from *Lin et al., 1999.*

Sluka *et al.* (1994) investigated the involvement of central processes controlling acute inflammation induced by intra-articular injection of kaolin and carrageenan into the rat knee joint cavity. A dorsal rhizotomy resulted in a significant reduction of the inflammatory response; however, a spinal cord transection was ineffective in altering joint inflammation. Therefore, the data indicate a local spinal circuit as opposed to descending modulatory influences is responsible for the resultant inflammatory response.

Subsequently, Rees *et al.* (1994, 1995, 1996) extensively studied the generation of a local spinal circuit in response to an articular inflammatory reaction. Mechanical stimulation of the hindlimb following the induction of acute arthritis elicited efferent activity in knee joint afferents (Rees *et al.,* 1994). The abolishment of this activity was achieved in dorsal rhizotomized rats, thus the efferent activity was concluded to represent DRRs. Likewise, the recording of activity in the proximal stump of the medial articular nerve following the induction of acute arthritis demonstrated the production of antidromic action potentials (Rees *et al.,* 1995), which were abolished by a dorsal rhizotomy procedure. In addition, the central administration of $GABA_A$ receptor antagonist, bicuculline or the non-NMDA receptor antagonist, CNQX, also eliminated the DRRs. Therefore, both studies suggest that articular inflammation leads to an increase in activity of articular efferents and dorsal horn neurons, resulting in the activation of central terminals of primary afferents by enhancing PAD. Consequently, these DRRs could lead to the release of mediators that further contribute to the inflammatory process.

The above studies concentrated on joint inflammation, but recently Lin *et al.* (1999) assessed the presence of DRRs and cutaneous neurogenic inflammation in response to the intradermal injection of capsaicin by evaluating the resultant flare and edema. The acute

transection of the ipsilateral sciatic and femoral nerves or the dorsal rhizotomies almost abolished the flare reaction, significantly reduced the edema and diminished the enhanced DRR activity in response to capsaicin. In addition, the intrathecal application of bicuculline, CNQX, or a NMDA receptor antagonist (AP7) led to similar results. Therefore, all these data taken together suggests that capsaicin- induced peripheral cutaneous inflammation involves the CNS.

Moreover, Sluka *et al.* (1995) and Rees *et al.* (1996) demonstrated that DRRs are found in all fibers types (Aβ-, Aδ- and C- fibers) innervating the joint; however, Rees *et al.* (1996) revealed that DRRs recorded from the contralateral afferents of a unilateral arthritic rat knee joint were only dependent on activity in fine afferent fibers such as C- fibers. Furthermore, Lin *et al.* (2000) conclude that the Aδ- and C- fibers are responsible for conveying DRRs following the intradermal injection of capsaicin in rats.

The evaluation of the levels of the mediators implicated in neurogenic inflammation is an alternative method for assessing the involvement of the central processes in the inflammatory response. The acute phase of the unilateral rat knee joint inflammation produced a bilateral increase in both SP and CGRP levels in the dorsal horn and dorsal root ganglion (DRG); however, the chronic phase revealed unchanged levels of SP and CGRP in the contralateral side and reduced amounts in the ipsilateral spinal cord and DRG (Mapp *et al.*, 1993). Therefore, this is suggestive of a neurochemical change in the CNS that is associated with an inflammatory reaction.

### *1.2.8 The Cellular Immune System and Neurogenic Inflammation*

Inflammation is a complex reaction of tissue and its microcirculation to a pathogenic insult, which is characterized by the generation of inflammatory mediators and the movement of fluid and leukocytes from the vasculature into the extravascular tissues (Rubin & Farber, 1994). Therefore, the neurogenic release of inflammatory mediators is only one component of this highly integrated non-specific immune defense system. In other words, neurogenic mediators may interact with non-neurogenic inflammatory factors released in the process of inflammation. These latter mediators include factors derived from immune cells, membrane fatty acids, vascular- and tissue-derived precursors such as cytokines, eicosanoids, kinins, histamine and serotonin. However, the relationships between non-neurogenic and neurogenic mediators in the process of neurogenic inflammation remain unclear.

Cytokines refer to a group of low molecular weight proteins that are secreted by host defense cells involved in the process of inflammation (Rubin & Farber, 1988). Interleukin-1 (IL-1) is a principal cytokine that is mainly derived from macrophages when stimulated by noxious stimuli, immune complexes and neuropeptides (Kimball *et al.,* 1988; Oppenheim *et al.,* 1986; Scott *et al.,* 1994). The role of IL-1 in the inflammatory process includes activating lymphocytes, stimulating the production of prostaglandins (potent pro-inflammatory mediators) and the breakdown of cartilage proteoglycans (Kopp, 2001). The specific interaction of IL-1 with neurogenic inflammation lies in its ability to stimulate afferent nerve fibers or augment their excitability via the release of prostaglandins (Dray, 1995). Moreover, IL-1 has been demonstrated to enhance capsaicin-induced neurogenic vasodilatation in the rat skin through the possible facilitation of neuropeptide release from afferent nerve endings (Herbert & Holzer, 1994).

The eicosanoids are derived from the membrane phospholipids in response to injury and comprise the prostaglandins (PG), thromboxanes and leukotrienes (LT), which are potent inflammatory mediators. For example, prostaglandins $D_2$ ($PGD_2$), $E_2$ ($PGE_2$), and $F_2$ ($PGF_2$) have vasodilatory effects and potentiate the development of edema. PG are associated with the process of neurogenic inflammation by their direct action on sensory fibers leading to a state of inflammatory hyperalgesia (Dray, 1995). Rats pre-treated with a neurotoxic dose of capsaicin inhibited the PG-induced PE (Holzer, 1998); thus, providing evidence to support the role of PG in the modulation of neurogenic inflammation. Moreover, LT may be potent chemotactic agents, promoting the aggregation of neutrophils ($LTB_4$) or enhancing vascular permeability ($LTC_4$, $D_4$, $E_4$, $F_4$) (Pace-Asciak, 1989); thus, contributing to the inflammatory process.

The complement system consists of a group of 20 plasma proteins that induce the secretion of histamine by mast cells and basophils (C3a, C5a), promote vascular permeability (C4, C2) or are chemotactic for immune cells. These complement components interact to elicit a variety of pro-inflammatory effects. Another system involved in inflammation is the kinin system, which represents vasoactive peptides of low molecular weight (Rubin & Farber, 1994). An example of a vasoactive kinin is bradykinin (BK), which is generated in response to tissue injury and acidification (Dray, 1995). It is a potent pain-producing agent that vasodilates and enhances vascular permeability, leading to edema formation (Kadar, 1989). In addition, BK has been shown to directly activate nociceptive afferents with BK $B_2$ receptors (Holzer, 1998) and subsequently lead to the release of neuropeptides from the sensory afferent nerve terminal (Green *et al.,* 1993c), regulating the process of neurogenic inflammation.

Furthermore, histamine and serotonin are two biogenic amines found in mast cells and platelets (Dray *et al.,* 1997; Scott *et al.,* 1994) that are able to sensitize or activate afferent neurons by stimulating $H_1$ and 5-$HT_3$ receptors, respectively (Dray, 1995; Holzer, 1992; Maggi, 1995b). In addition, there is evidence showing the release of SP from afferent endings leads to increased vascular permeability and PE via the degranulation of masts cells (Lam & Ferrell, 1990). Therefore, histamine and serotonin mediate inflammation by a direct action on the vasculature and by evoking neurogenic release of neuropeptides from nerve terminals.

### *1.2.9 The Temporomandibular Joint and Neurogenic Inflammation*

The possibility of the nervous system playing a crucial role in the development and severity of inflammation involving the TMJ is principally supported by the presence of neuropeptides both in nerve endings innervating the articular tissues, in the synovial fluid of the TMJ and in the trigeminal ganglia. By assessing these mediators, both animal and human studies have provided some insight into the involvement of sensory afferents and sympathetic efferents in the pathophysiology of inflammatory joint disease.

In several animal studies, adjuvant-induced rat TMJ arthritis has led to increased levels of neuropeptides associated with inflammation (Carleson *et al.,* 1996, 1997a, b; Hutchins *et al.,* 2000). Carleson *et al.* (1996) examined the cerebrospinal fluid, plasma and TMJ perfusates in rats inoculated at the base of the tail (polyarthritic rats) or in one TMJ (monoarthritic rats) for SP-, NKA-, CGRP- and NPY- like immunoreactivity (-LI). Results revealed significant increases in all four neuropeptide-LI in perfusates of both TMJs at 1 hour and 12 hours post-adjuvant injection, which were more pronounced for monoarthritic rats.

Therefore, it appears that the increased neuropeptide levels were related to the induced inflammation and a centrally mediated reflex could be responsible for its bilateral appearance.

Carleson *et al.* (1997a) also evaluated the levels of SP-, CGRP- and NPY- LI in the TMJ perfusates and trigeminal ganglion 28 days after bilateral TMJ injection of adjuvant. These results were consistent with the previous study, whereby significantly increased levels of all three neuropeptide-LI were present in the arthritic TMJ fluid; however, only a significant increase in CGRP-LI was demonstrated in the arthritic trigeminal ganglion. Thus, in this investigation, peripheral SP-, CGRP- and NPY- LI were implicated in the inflammatory process of arthritis whereas only central CGRP-LI was associated with TMJ arthritis. One limitation of this investigation was the single time point of study, namely 28 days after the induction of inflammation. To address this issue, Hutchins *et al.* (2000) assessed the immunoreactivity of SP and CGRP in the trigeminal ganglia and the brainstem subnucleus caudalis at multiple time intervals. The levels of adjuvant-related neuropeptides were compared with those of the contralateral vehicle-related tissues and non-injected controls at 6, 24 and 48 hours, in addition to 10 days after the induction of arthritis. In the trigeminal ganglion, CGRP-LI was significantly increased relative to the control at 6 hours, then decreased at each of the following time intervals but remained significantly elevated. Conversely, SP-LI in the trigeminal ganglion was significantly increased at all four time points. In the brainstem subnucleus caudalis, CGRP-LI was significantly elevated at all the observed time intervals. However, SP-LI in the subnucleus caudalis was significantly increased for the first three time points (6, 24 and 48 hours), but its levels declined to that of the control by day 10. Therefore, there were differences between SP- and CGRP- LI levels

following the induction of arthritis in addition to the variation in pattern of change between the trigeminal ganglion and the brainstem. In light of these findings, the researchers hypothesize that SP and CGRP may have different roles in the inflammatory process along with different mechanisms at the brainstem and trigeminal ganglion that may modulate the course of inflammation.

Adjuvant-induced arthritis has been criticized for its non-specific action on nociceptive neurons. It has been suggested that mycobacterial proteins in lymphoid structures activate peptidergic neurons and this activation leads to polyarthritis (Freund, 1951). Therefore, in order to directly study the neural influence on joint peptide levels, either the TMJ region was pre-treated with capsaicin or surgical denervation of the trigeminal nerve was performed.

Carleson *et al.* (1997b) evaluated the effects of capsaicin applied into the TMJ or surgical denervation of the mandibular branch of the trigeminal nerve in adjuvant-induced rat TMJ arthritis on neuropeptide levels in the trigeminal ganglia and TMJ perfusates after 29 days. As expected, the application of adjuvant to the rat TMJ led to increased levels of SP- and CGRP- LI in both the trigeminal ganglion and TMJ perfusates as compared with the controls. However, capsaicin pretreatment of the TMJ tissues in arthritic rats revealed decreased levels of SP- and CGRP- LI in both the trigeminal ganglion and TMJ perfusates. Similarly, surgical denervation prevented the increase of the two neuropeptide levels in response to the adjuvant injection. These data suggest that intact afferent innervation is essential for the increase in the intra-articular levels of pro-inflammatory mediators such as SP and CGRP.

In contrast, Spears *et al.* (1998) demonstrated the temporal effect of an intra-articular injection of capsaicin to the TMJ on CGRP- LI levels in the trigeminal ganglion of the rat. The authors showed that the levels of CGRP- LI fluctuated above and below that of the controls throughout the 21 days of inflammatory response to capsaicin. Thus, this suggested that the reduction of CGRP- LI does not necessarily reflect the absence of a neurogenic component in the inflammatory process of TMDs.

In addition to animal investigations, neuropeptide- LI levels have been measured in humans. TMJ synovial fluid in patients with TMDs contains SP-, NKA-, CGRP-, NPY- and vasoactive intestinal polypeptide- (VIP-) LI in subjects with TMDs (Holmlund *et al.,* 1991). Interestingly, there is no correlation between the levels of neuropeptide- LI and the clinical signs and symptoms or arthroscopic findings. Likewise, Alstergren *et al.* (1995) assessed the levels of the same neuropeptide- LI with the exception of VIP- LI in both the TMJ joint fluid and the plasma of patients that were categorized into two diagnostic groups: inflammatory or degenerative/non-specific joint disease. The findings revealed elevated levels of all neuropeptide- LI with no significant difference between the two groups; however, the levels of all the examined pro-inflammatory mediators in subjects diagnosed with inflammatory TMD were highly correlated. These studies suggest an association between the presence of neuropeptides in the joint fluid and inflammatory TMDs. However, it is critical to confirm this conclusion with the comparison of these levels with healthy individuals.

Yoshida *et al.* (1999) measured SP in the human TMJ in patients with internal derangement of the TMJ and healthy controls. The results demonstrated a more intense SP expression in the diseased individuals in contrast to the diffuse presentation seen in the

healthy controls. These data provide an indirect link between elevated SP- LI levels and the presence of a neurogenic inflammatory component in TMDs.

In contrast, Mapp *et al.* (1990) revealed a reduction in the numbers of CGRP- and SP-immunoreactive nerves innervating the superficial synovium in rheumatic arthritis patients versus those without. Therefore, the presence of neuropeptide expression may not be a definitive measure of joint disease.

Nevertheless, the presence of a neurogenic inflammatory component in TMDs is supported by the high concentrations of CGRP- and NPY- LI in the TMJ fluid associated with pain, impairment of mandibular mobility and occlusal signs of TMJ destruction in patients with rheumatoid arthritis (Appelgren *et al.,* 1995). However, the same study revealed that SP- LI was not associated with TMJ pain or restricted mandibular function. Moreover, SP was negatively correlated to the TMJ pain perceived by patients with inflammatory disorders (Appelgren *et al.,* 1995). Therefore, this suggests that CGRP and NPY are involved as mediators or modifiers of TMJ pain and dysfunction, while SP is associated with an anti-nociceptive effect in TMDs (Kopp, 2001).

## 1.3 ANIMAL MODELS FOR STUDYING NEUROGENIC INFLAMMATION

### *1.3.1 General*

With the availability of an assortment of animal models, the mechanism and mediators involved in neurogenic inflammation as well as its contribution to various inflammatory diseases such as TMDs can be evaluated. As a general rule, each model observes a change in a certain component of neurogenic inflammation, such as PE or tissue expansion, under the influence of different agonists and antagonists.

Traditionally, PE is evaluated by quantifying dye-labeled extravascular leakage of proteins in tissues subjected to an acute inflammatory reaction. The original procedure used Typhan blue dye (Ramsdell, 1928). The methodology was modified and improved upon with the use of Evan's blue dye. In 1971, Harada *et al.* (1971) devised a protocol for dye extraction and Saria and Lundberg (1983) quantified PE with spectrophotometry. Radiolabeled compounds have also been used to evaluate the amount of dye present (Newbold & Brain, 1985).

Since the above dyes bind to albumin (plasma protein), protein binding dyes are used to quantify PE. During inflammation, the increase in vascular permeability leads to the leakage of plasma proteins into surrounding tissues. Therefore, the regions that are involved in this inflammatory process will be stained and can be visualized with the injected dye.

To assess the effects of various compounds on PE, the agent of interest is first injected into the tissues. If the area of attention is the joint, the contralateral joint serves as the control to account for plasma extravastion induced by the introduction of the catheter used to inject agents and the mechanical distention of fluid. After the course of the experiment, Evan's blue dye is delivered intravenously, the animal is sacrificed at a pre-determined time, and the tissue of interest is dissected and analyzed for Evan's blue content.

This methodology has been used to study neurogenic inflammation in many tissues including the knee joint (Lam & Ferrell, 1991), TMJ (Haas *et al.,* 1992; Yu *et al.,* 1996; Wong *et al.,* 2001), skin (Louis *et al.,* 1989; Dux *et al.,* 1996), tracheal mucosa (Lundberg & Saria, 1983), esophagus, bladder, ureter, conjunctiva and duodenum (Saria *et al.,* 1983).

A different protocol has also been used to study the knee joint was designed (Green *et al.,* 1993a). The joint capsule is exposed and the Evan's blue dye is delivered intravenously.

A catheter connected to a syringe pump is placed into the joint space, perfusion is initiated and concomitantly, the injected perfusate is allowed to flow out of a second catheter positioned into the joint space. The agents of interest are added to the perfusate delivered by the first catheter and the fluid is collected with the second catheter at pre-set intervals and analysis for Evan's blue content is performed spectrophotometrically. The advantage of this modified methodology is the capability of quantifying plasma extravasation over a continuous period of time.

Another advance in the quantification of PE involves the use of digital imagery. The PD induced by a burn injury to the rat abdomen induced PE has been quantified over a continuous period of time by use of the digital image colour analysis (Jonsson *et al.,* 1998).

Another measure of neurogenic inflammation is the tissue expansion due to increased vascular permeability. Unlike the evaluation of PE, the physical measurement of expanded tissue is confounded with varying degrees of accuracy and precision.

A direct circumferential measurement of a swollen rat knee joint can be taken with a measuring tape (Houghton *et al.,* 1998; Lawand *et al.,* 1999; Rees *et al.,* 1995), and fine micrometer calipers can be used to measure the mouse ear thickness (Inoue *et al.,* 1995) and rat paw thickness (Levine *et al.,* 1985; Lin *et al.,* 1999) induced by neurogenic inflammation. In addition, volumetric displacement of fluid has been used to measure changes in tissue volume as a direct measurement of the resultant edema (Lippe *et al.,* 1993).

### *1.3.2 Models Involving the Temporomandibular Joint*

The evaluation of a neurogenic inflammation in the TMJ region is a challenge. Unlike the knee joint, the TMJ capsule is located deep to both muscle and fascia; therefore,

direct access to the TMJ is hindered and precludes a direct measure of the circumferential expansion distance with a measuring tape or with a pair of micrometer calipers. The rat TMJ is quite small. This renders it very difficult to introduce two catheters into the joint space to accurately evaluate PE. It is also a challenge to position both catheters into the same joint space on the first attempt and even if successful, the passing of two catheters may obliterate the capsule and afferent supply.

Traditionally, the protocol for assessing edema in the rat TMJ area has involved the quantification of PE with spectrophotometry of Evan's blue content (Haas *et al.,* 1992; Yu *et al.,* 1995) based on Saria and Lundberg (1983). However, recently a simple method of evaluating edema development in the rat TMJ region was developed (Fiorentino *et al.,* 1999); allowing the continuous measurement of tissue expansion over a prolonged period of time (see Section 3.1.5). Therefore, this methodology is employed in the present investigation.

## 1.4 CAPSAICIN

### *1.4.1 Properties*

The pungent extract of hot chili peppers, capsaicin (8-methyl-N-vannillyl-6-nonenamide), is a lipophilic vanilloid compound, small fiber excitant and inflammatory irritant known to evoke acute neurogenic inflammation when applied to peripheral tissues (Jancso *et al.,* 1967, 1968).

### *1.4.2 Historical Perspective to Current Research*

Hot chili peppers (genus *Capsicum*) are known to be pungent, poorly absorbed, affect thermoregulation and activate autonomic reflexes (Szallasi & Blumberg, 1999). Over 7000

years ago, the cultivation of hot peppers began in South America and since the 16th century, the rest of the world has followed suit (Caterina & Julius, 2001). In the mid-19th century, Thresh (1846) isolated the active ingredient in hot peppers, naming it capsaicin and predicted the chemical structure of this principal pungent component of hot peppers and vanillin to be closely related. A few decades later, Hogyes suggested that *Capsicum* extracts promote the sensation of pain and trigger heat loss through sweating by selectively acting on sensory neurons (cf. Caterina & Julius, 2001). By 1919, Nelson (1919) determined the exact chemical structure of capsaicin as being an acylamide derivative of homovanillic acid, 8-methyl-N-vannillyl-6-noneamide (see Figure 4). A decade later, in 1930, Spath and Darling completed the synthesis of capsaicin (cf. Szallasi & Blumberg, 1999). However, it was not until the 1950s and 1960s before the physiological properties of capsaicin were revealed. Jancso and his colleagues (1967, 1968) demonstrated that capsaicin activates sensory neurons and the parental neonatal pretreatment of animals with this agent led them to be resistant to painful stimuli. Moreover, these studies investigating the activation of primary afferent nociceptors by capsaicin revealed the ability of some nociceptors to act in an efferent fashion to stimulate inflammation, later termed as neurogenic inflammation. Subsequently, these findings rendered capsaicin sensitivity to be extensively used as an essential functional marker for a subset of neurons specialized to detect noxious stimuli (Caterina & Julius, 2001) and an important pharmacological tool for the exploration of the neurogenic component of inflammation.

In 1988, further advances were made in the field of vanniloid research when Wood *et al.*, (1988) identified a non-specific cation-channel blocker ruthenium red which had the capacity to non-competitively antagonize the effects of capsaicin. Immediately following, in

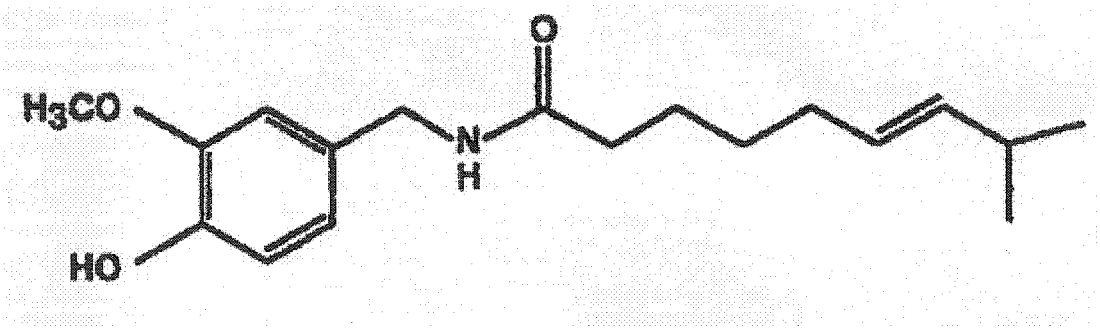


**Figure 4** **Chemical Structure of Capsaicin**
Adapted from *Caterina & Julius, 2001*

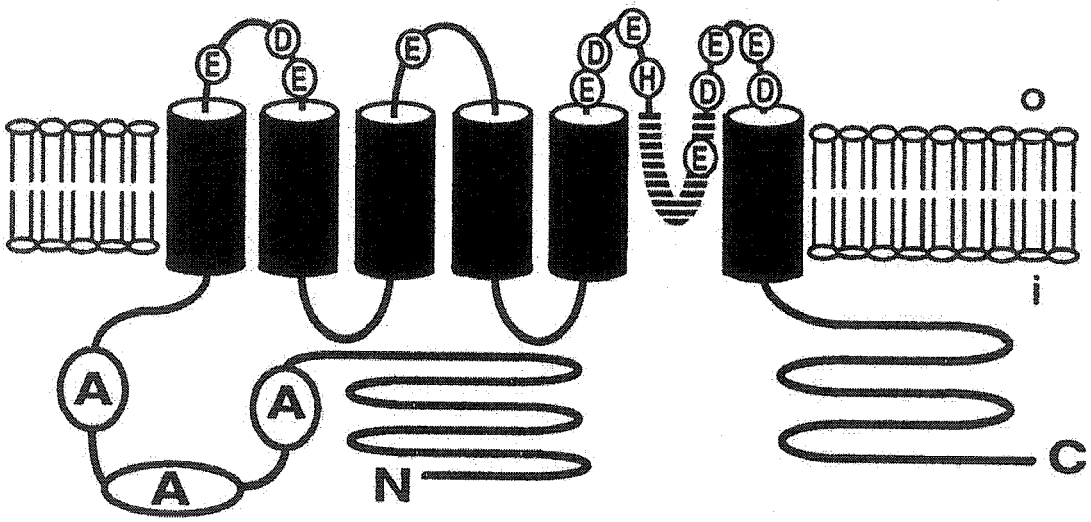


**Figure 5** **Predicted Membrane Topology and Domain Structure of the Vanniloid Receptor Subtype 1**
Adapted from *Jordt et al., 2000*

1992, Bevan *et al.* (1992) developed the first competitive capsaicin antagonist, capsazepine. In addition to antagonists, an extremely potent capsaicin analogue was discovered. This compound, resiniferatoxin was derived from Euphorbia plants and mimicked the cellular actions of capsaicin (de Vries & Blumberg, 1989; Szallasi & Blumberg, 1989). Consequently in 1997, all three previously mentioned agents along with capsaicin assisted Caterina *et al.* (1997) in the cloning and identification of a specific capsaicin receptor, termed the vanniloid receptor subtype 1 (VR1). Further investigations revealed this ionotropic receptor to be activated not only by capsaicin but also by noxious heat (>48°C) and low pH (Tominaga *et al.*, 1998).

Presently, researchers have generated a VR1 knockout mouse, in which the VR1 gene is disrupted, for the genetic analysis of the VR1 receptor (Caterina *et al.*, 2000). In addition, the search for endogenous ligands has begun and a recent study suggested that lipids with structural similarities to capsaicin might act as such. Specifically, investigations have suggested that endocannabinoid anandamide may possibly function as an endogenous agonist for VR1 receptors (Szallasi & Di Marzo, 2000). Researchers have demonstrated that at low concentrations, anandamide activates nociception via ionotropic VR1 receptors; however at high concentrations, nociception is suppressed via metabotropic cannabinoid receptors through the inhibition of neuropeptide release (cf. Szallasi & Di Marzo, 2000). Therefore, more conclusive evidence is required before any compound is designated as the endogenous ligand for the VR1 receptor.

### *1.4.3 Effects of Capsaicin on Sensory Neurons*

Capsaicin excites a subset of primary sensory neurons with their somata in dorsal root ganglion (DRG) or trigeminal ganglion (Holzer, 1988) or nodose ganglion (Acs *et al.,* 1994; Szallasi *et al.,* 1995). In general, these capsaicin-sensitive neurons are peptidergic, small diameter neurons giving rise to thin, unmyelinated C-fibers that are predominantly polymodal nociceptors which release the pro-inflammatory neuropeptides from their peripheral terminals (Buck & Burks, 1996; Holzer, 1991). However, at least 5% of small diameter DRG neurons do not respond to capsaicin (Lawson & Nickels, 1980) and 40% of medium diameter neurons, predominantly Aδ-type fibers are activated by this vanniloid agent (Nagy *et al.*, 1983). These vanilloid-sensitive Aδ-fibers mainly function as mechano-heat sensitive nociceptors. Therefore, capsaicin-sensitive neurons are morphologically, neurochemically and functionally heterogeneous, including several subclasses of DRG neurons (cf. Szallasi & Blumberg, 1999).

The binding of capsaicin to VR1 elicits depolarization via an influx of calcium and discharge of action potentials in the peripheral nerve endings (Holzer, 1988, 1991). This will lead to the antidromic stimulation of adjoining afferents (Jancso *et al.,* 1967, 1968) and release of neuropeptides from the peripheral neuronal terminals (Caterina *et al.,* 1997; Holzer, 1988, 1991; Maggi & Meli, 1988). Subsequently, these neuropeptides elicit a sensation of burning pain and initiate the process of neurogenic inflammation (Chahl, 1988; Holzer, 1991; Caterina *et al.,* 1997). Moreover, the excitation of sensory neurons by capsaicin leads to a period of enhanced sensitivity to painful thermal and mechanical stimuli (Caterina & Julius, 2001). Interestingly, the initial stimulation evoked by capsaicin is followed by a lasting refractory state traditionally known as desensitization (Handwerker *et*

*al.,* 1984; Lynn *et al.,* 1984; cf. Szallasi & Blumberg, 1999). In addition, a recent investigation studied one of the afferent actions of capsaicin-sensitive neurons, namely capsaicin-evoked jaw muscle activity (Hu *et al.,* 2001).

### 1.4.3.1 Capsaicin-Evoked Excitation

Early *in vivo* and *in vitro* studies revealed that capsaicin depolarizes sensory neurons by promoting an influx of sodium and calcium ions (Baccaglini & Hogan, 1983; Heyman & Rang, 1985; Marsh *et al.,* 1987; Williams & Zieglgansberger, 1982) followed by a discharge of action potentials in the peripheral nerve endings. *In vitro* whole-cell voltage clamp experiments on cultured sensory neurons (Baccaglini & Hogan, 1983; Liu & Simon, 1994), along with biochemical (Wood *et al.,* 1988) and microscopic studies (Winter, 1987) demonstrated that the resulting cationic current response exhibited a preference for divalent cations. Furthermore, electrophysiological investigations (Forbes & Bevan, 1988; Oh *et al.,* 1996) showed that capsaicin triggered the membrane-delimited opening of discrete ion channels and displayed an outwardly rectifying current-voltage relationship. Although the activation of VR1 receptors is known, the transmission of information from central terminals of capsaicin-sensitive neurons to second order neurons of dorsal horn has yet to be identified (Szallasi & Blumberg, 1999).

### 1.4.3.2 Efferent Functions of Capsaicin Sensitive Neurons

The peripheral activation of capsaicin-sensitive afferent neurons triggers the release of neuropeptides from the central terminals in the spinal cord dorsal horn, resulting in an acute pain response (Gamse *et al.,* 1979; Theriault *et al.,* 1979). In addition, the activation of

these neurons by capsaicin also elicits the release of neuropeptides from their peripheral terminals (Holzer, 1988, 1991; Maggi & Meli, 1988), which initiate the cascade of neurogenic inflammation characterized by increased vascular permeability and PE (Chahl, 1988). Moreover, the physiological importance of the efferent actions of capsaicin-sensitive neurons includes the recruitment of serum factors and inflammatory cells, as well as eliciting non-inflammatory responses and contributing to maladaptive inflammation associated with many conditions under pathological circumstances (Caterina & Julius, 2001).

The efferent effects of capsaicin on cutaneous peripheral sensory afferents have been extensively studied in humans and animals (cf. Buck & Burks, 1986; cf. Holzer, 1991). In addition, capsaicin-sensitive neurons innervating the heart, tooth pulp, respiratory tract, urogenital tract and the gastrointestinal tract have been investigated (cf. Buck & Burks, 1986; cf. Holzer, 1991). However, a minimal amount of information exists on the effect of capsaicin on deep peripheral sensory afferents innervating joints, particularly the TMJ.

An intradermal injection of capsaicin in humans elicits a flare response and an area of primary hyperalgesia to heat and mechanical stimuli at the site of injection (Simone *et al.,* 1987, 1989; LaMotte *et al.,* 1991). This reaction is followed by the development of a secondary mechanical hyperalgesia and allodynia in the area surrounding the primary hyperalgesia site (Simone *et al.,* 1991; Torebjork *et al.,* 1992).

Similarly, the topical application of capsaicin to rat skin results in antidromic vasodilatation as measured by the increase in blood flow (Lin *et al.,* 1999; Lynn *et al.,* 1992; Szolcsanyi, 1988) and this spread of vasodilatation is consistent with the flare response seen in humans (LaMotte *et al.,* 1991; Lin *et al.,* 1999). In addition, the use of albumin-binding Evan's blue dye quantified the resultant increased vascular permeability (Jancso *et al.,* 1967).

Although limited, there is evidence suggesting that sensory nerve mechanisms also contribute to development of inflammatory processes in joints (Holzer, 1988). The antidromic stimulation of the posterior articular nerve innervating the cat knee joint resulted in PE into the synovial joint cavity (Ferrell & Russell, 1986). In addition, the application of capsaicin to the rat TMJ region has been shown to be markedly inflammatory, resulting in plasma protein extravasation and edema (Fiorentino *et al.,* 2000). Moreover, the reduction of edema following the pre-treatment of the TMJ tissues with ruthenium red (non-competitive antagonist) or capsazepine (competitive receptor antagonist) prior to the application of capsaicin verified the presence of VR1 receptors in the TMJ region (Fiorentino *et al.,* 2000).

Recently, Caterina and Julius (2001) suggested several mechanisms for the peripheral release of neuropeptides: a) activation of a collateral terminal of the same nociceptor, b) retrograde activation of capsaicin-sensitive afferent neuron by an antidromic electrical stimulus, and c) direct activation of the peripheral capsaicin-sensitive terminal.

#### *1.4.3.2.1 Activation of a Collateral Terminal of the Same Nociceptor*

This is closely related to the axon reflex described by Lewis (1927), whereby the event takes place entirely within a single neuron that bifurcates in the periphery. One branch receives the irritant stimulus, such as capsaicin and the other supplies a blood vessel. The antidromic stimulation from the former branch causes the release of neuropeptides from the latter neuronal terminal, resulting in the 'triple response' of inflammation: reddening, wheal and flare.

This axon reflex is supported morphologically by the extensive arborizations of sensory nerve processes and the proximity of some branches to blood vessels (Chapman &

Goodell, 1964; Helme & McKernan, 1985). In addition, the transection of peripheral processes of afferents abolishes the flare component (Bayliss, 1901), but the antidromic stimulation is unaffected by a disruption in the connection of the afferent nerves with the spinal cord (Bruce, 1913). Moreover, the spread of flare is prevented by the application of local anaesthetic (Dux *et al.,* 1996; Jancso *et al.,* 1968; Lewis, 1927) and the flare reflex is dependent on intact sensory innervation. However, some studies demonstrated the area of flare does not coincide with the receptive field (RF) of the stimulated afferent (Helme & McKernan, 1985; Van Hees & Gybels, 1981). Furthermore, a recent study exhibited the capsaicin-induced release of sensory neuropeptides to occur even in the presence of lignocaine or tetrodotoxin (Szolcsanyi *et al.,* 1998); thus, contradicting the presence of a local axon reflex. Therefore, evidence from various studies supports the existence of an axon reflex, however other investigations suggest the possibility of other factors playing a role in the release of neuropeptides following the capsaicin-induced activation of peripheral neuronal terminals.

#### *1.4.3.2.2 Retrograde Activation of Capsaicin-Sensitive Afferents*

In contrast to a peripherally-mediated mechanism, this explanation suggests the prospect of a centrally-mediated reflex release of neuropeptides associated with capsaicin-activated sensory afferents whereby the reflex is initiated in the spinal cord by PAD large enough to trigger dorsal root reflexes (DRRs) (Eccles *et al.,* 1961, 1962; Koketsu, 1956; Willis, 1999).

A recent study by Lin *et al.* (1999) concluded that peripheral cutaneous inflammation induced by intradermal injection of capsaicin involves the CNS. They found that the area of

flare was much larger than that explained by the branching of sensory afferents. In addition, the sectioning of peripheral nerves and dorsal rhizotomies resulted in a reduction of the flare response to capsaicin. Therefore, according to this investigation, the decreased flare response is the result of the blockade of DRRs that elicit the release of neuropeptides from the peripheral neuronal terminals.

Furthermore, the surgical removal of sympathetic postganglionic neurons innervating the rat paw skin or the pre-treatment with non-selective α or selective α-1 adrenoreceptor antagonists, phentolamine or prazosin respectively prevented the development of secondary hyperalgesia induced by capsaicin (Kinnman & Levine, 1995). Similarly, in humans, phentolamine inhibited the hyperalgesia that develops after intradermal injection of capsaicin (Kinnman *et al.,* 1997). Therefore, these studies imply a role for the autonomic system in the efferent function of peripheral nerves stimulated by capsaicin.

#### *1.4.3.2.3 Direct Activation of the Peripheral Capsaicin-Sensitive Terminal*

In addition to nerve conduction mediated mechanisms, some researchers have suggested that the influx of calcium produced by VR1 receptor activation is sufficient for the release of neuropeptides (Caterina & Julius, 2001). However, the number of studies supporting this direct activation of capsaicin-sensitive peripheral terminals is limited. Nevertheless, some studies have demonstrated that neither local anaesthetics nor tetrodotoxin can inhibit the neurogenic inflammation induced by capsaicin applied to the cutaneous tissues (Jancso *et al.,* 1968; Szolcsanyi, 1984, Szolcsanyi *et al.,* 1998). Hence, the release of neuropeptides from sensory endings can be independent of axonal depolarization (Saria *et al.,* 1983; Szolcsanyi, 1984).

Moreover, other algesic chemicals known to cause neurogenic inflammation such as mustard oil have been implicated in this possible efferent function of peripheral afferents. Wong *et al.* (2001) demonstrated that mustard oil-induced neurogenic inflammation of the TMJ could not be suppressed by the prior application of local anaesthetics; supporting the theory that neuronal terminals may be directly acted upon to cause the release of pro-inflammatory neuropeptides without axonal depolarization.

#### 1.4.3.3 Capsaicin-Evoked Desensitization

The initial excitation and sensitization of primary sensory afferents exposed to capsaicin is followed by a lasting refractory state which is characterized by the relative resistance to specific painful stimuli or the unresponsiveness of sensory neurons to subsequent capsaicin challenge (Jancso, 1992; Jancso *et al.,* 1967). The timeframe of desensitization involving functional and morphological changes varies depending on capsaicin dose, duration of treatment, route of administration, subject's age and species involved (Caterina & Julius, 2001; Szallasi & Blumberg, 1999).

Experiments have shown that neonatal rats or mice systemically treated with high dosages of capsaicin (50 mg/kg) display a selective degeneration of C fiber and some A fiber axons with an irreversible loss of > 80% of small-diameter sensory neuron cell bodies (Jancso, 1984; Jancso *et al.,* 1977; Lawson and Nickels, 1980; Nagy *et al.,* 1983). In addition, Kwan *et al.,* (1996) demonstrated a 70% reduction in low-threshold mechanoreceptive (LTM) neurons in the neonatal capsaicin-treated rat trigeminal (V) subnucleus oralis and nucleus principalis. However, a similar procedure performed on adult rats produces less pronounced effects with either no (Joo *et al.,* 1969) or partial (Jancso,

1981; Jancso *et al.,* 1985; Palermo *et al.,* 1981) degeneration of C fiber axons. Clinically, despite minimal effects on peripheral innervation, systemic capsaicin treatment resulted in unresponsiveness to noxious stimuli and the abolition of neurogenic inflammation in adult rats.

Furthermore, after the initial burning sensation and hyperalgesia to heat and mechanical stimuli evoked by capsaicin, there is a period of decreased sensitivity to noxious chemical, mechanical or thermal stimulants (Simone *et al.,* 1987, 1989; Simone & Ochoa, 1991). In contrast to animal studies, histological examination of the injection site reveals degeneration and subsequent reinnervation of cutaneous nerve endings (Simone *et al.,* 1998).

In addition, *in vitro* investigations on the desensitization component of capsaicin-evoked effects on sensory neurons have been carried out. Studies have demonstrated that the removal of extracellular calcium reduced desensitization to capsaicin (Santicioli *et al.,* 1987; Liu & Simon, 1996). Therefore, it was speculated that not only does the rise in intracellular calcium serve to activate VR1 receptors, but it also leads to VR1 desensitization (Szallasi & Blumberg, 1999).

#### 1.4.3.4 Afferent Functions of Capsaicin Sensitive Neurons

Although the efferent function of capsaicin-sensitive sensory neurons is of greatest interest in this particular investigation, the classical afferent role of these fibers should not be overlooked. Nonetheless, several studies have evaluated the afferent effects of applying capsaicin through various routes of administration (Hu *et al.*, 2001; LaMotte *et al.*, 1991; Witting *et al.*, 2000).

The resultant afferent effects of the intradermal application of capsaicin to the human skin have been extensively studied. LaMotte *et al.* (1991) demonstrated the injection of capsaicin into the human volar forearm led to an experience of intense burning pain along with the formation of hyperalgesic areas surrounding the injection site. In addition, the investigation revealed the spread of mechanical hyperalgesia to be peripherally mediated by cutaneous nerve fibers; however, the maintenance of this area became dependent on central influences. Therefore, the sensitization of interneurons in the dorsal horn by capsaicin-sensitive peripheral nerve fibers was termed 'neurogenic hyperalgesia' (LaMotte *et al.*, 1990).

Furthermore, Witting *et al.* (2000) compared the afferent effects of the intradermal and intramuscular injection of capsaicin into humans. Interestingly, the intradermal application produced a more intense local pain that was later counteracted by the experience of referred pain. On the other hand, the perception of local pain was significantly less via the administration of capsaicin into the brachioradial muscle; however referred pain occurred more frequently. Therefore, the experience of local and referred pain following noxious stimulation of different tissues may be the result of the variation in neurophysiological mechanisms (Witting *et al.*, 2000).

Recently, researchers demonstrated the application of capsaicin to the rat TMJ region evoked a dose-dependent sustained and reversible increase in EMG activity of the ipsilateral masseter and digastric muscles (Hu *et al.*, 2001). This discovery is beneficial in the present investigation for evaluating the efficacy of the local anaesthetic pre-treatment of the rat TMJ tissues with the successful blockade of afferent innervation theoretically corresponding to the lack of capsaicin-induced reflex activity of the jaw muscles.

### *1.4.4 Vanniloid Receptor Subtype 1 (VR1)*

Due to a vanilloid moiety that constitutes an essential chemical component of both capsaicin and its ultra potent analogue, resiniferatoxin, the cloned capsaicin receptor was named vanilloid receptor subtype 1 (VR1) (Caterina *et al.,* 1997). It consists of six transmembrane domains and a short, pore-forming hydrophobic stretch between the fifth and sixth membrane spanning regions (see Figure 5). In addition, there are three ankyrin repeat domains at its amino terminal (Caterina *et al.,* 1997) and three potential protein kinase A phosphorylation sites hypothesized to play a role in desensitization (Szallasi & Blumberg, 1999). Moreover, VR1 receptor is a non-selective cation channel structurally related to members of the transient release potential (TRP) family of store-operated calcium channels (cf. Szallasi & Blumberg, 1999; Tominaga & Julius, 2000).

Not only is VR1 receptor activated by capsaicin, but noxious heat (>43°C) and low pH can also activate this non-selective cation channel (Tominaga *et al.,* 1998). Interestingly, only heat has the capability of opening the channel pore of VR1, whereas capsaicin and protons serve to lower the heat threshold at which the receptor can be activated. As a result, even room temperature is able to gate the VR1 receptor in the presence of mildly acidic environments and/or capsaicin (Tominaga *et al.,* 1998). Therefore, VR1 receptors are known as polymodal detectors of noxious physical and chemical stimuli.

#### 1.4.4.1 Localization of VR1 Receptor

As previously mentioned, capsaicin sensitivity is most likely the best functional marker of C-fiber nociceptors (Szolcsanyi *et al.,* 1988). The majority of unmyelinated peptidergic and non-peptidergic neurons with their somata within dorsal root, trigeminal and

nodose sensory ganglia express this VR1 receptor (Tominaga *et al.,* 1998; Guo *et al.,* 1999; Michael & Priestly, 1999).

Although the expression of VR1 is dominantly located along the entire length of primary sensory neurons (Guo *et al.,* 1999), VR1 receptor expression is not restricted to sensory neurons. Several independent investigations have revealed VRs in various brain nuclei (Acs *et al.,* 1996), and non-neuronal tissues such as mast cells (Biro *et al.,* 1998b) and glial cells (Biro *et al.,* 1998a).

Furthermore, immunohistochemical studies reveal VR1-like immunoreactivity in the spinal dorsal horn (Guo *et al.,* 1999). The most prominent staining is located in lamina I, however, abundant labeling is also present in the inner layer of lamina II. This distribution of VR1-like immunoreactivity is consistent with the known central projection patterns of most C fibers (Caterina & Julius, 2001).

#### 1.4.4.2 VR1 Receptor Knockout Mice

Recently, the genetic analysis of the VR1 receptor functions was initiated with the generation of the VR1 knockout mice. Despite the absence of VR1 receptors, the sensory ganglion development was unaltered; however, the disruption of the VR1 receptor gene led to specific nociception deficits (Caterina *et al.,* 2000).

The electrophysiological responses and neurogenic inflammation elicited by capsaicin or resiniferatoxin along with the aversion to capsaicin-containing water were characteristics absent in the knockout mice (Caterina *et al.,* 2000). In addition, the knockout mice demonstrated a reduction in the percentage of neurons activated by both heat and protons when compared with wild type mice. Therefore, this demonstrated that VR1 receptors are

essential for transducing nociceptive and inflammatory effects of vanilloid compounds, and VR1-independent mechanisms exist for the detection of noxious heat and presence of protons (Caterina & Julius, 2001).

### *1.4.5 Clinical Application of Capsaicin*

Apart from its culinary usage and a pharmacological tool for scientific investigations, capsaicin has been employed for combat/self-defense and as a form of analgesic. This latter mode of clinical application stems from the knowledge that capsaicin desensitizes neurons and thus, this has provided a rational basis for the use of this agent and related compounds in the treatment of many disorders, ranging from the control of the micturition reflex to neuropathic pain conditions (Szallasi & Blumberg, 1999).

The recognition for the therapeutic potential of capsaicin as an analgesic began as early as 1850 when alcoholic hot pepper extract was used for the instant relief of a toothache (Turnbull, 1850). Nowadays, capsaicin is used to treat an overreactive bladder by decreasing the sensitivity of C-fibers responsible for the micturition reflex (Cruz, 1998). In addition, this vanniloid compound has been found to be effective in improving vasomotor rhinitis (Blom *et al.,* 1997) and relieving cluster headaches (Marks *et al.,* 1993).

Topical capsaicin has been employed as a supplemental analgesic for a variety of neuropathic pain conditions such as postherpetic neuralgia (Watson *et al.,* 1988), painful diabetic neuropathy (Low *et al.,* 1995) and rheumatoid arthritis (Matucci-Cerinic *et al.,* 1995). However, the true therapeutic value of capsaicin is difficult to assess due to its initial characteristic burning sensation and high placebo response rate as reported by many clinical trials (Szallasi & Blumberg, 1999). Moreover, the effectiveness of topical capsaicin to

desensitize nerve endings in human skin is compromised by its combined low potency and poor bioavailability (Kasting *et al.,* 1997; Munn *et al.,* 1997). Therefore, a drug similar to capsaicin but with improved potency, bioavailability and desensitization to irritation ratio may be of greater clinical value.

## 1.5 LOCAL ANAESTHETICS

### *1.5.1 Properties*

Local anaesthetics are drugs that inhibit the generation and propagation of nerve impulses, resulting in the loss of pain sensation. This block is reversible and the restoration of normal nerve function is achieved with the removal of the local anaesthetic via the bloodstream (Yagiela, 1998).

### *1.5.2 Review of the Physiology of Neuronal Depolarization and Conduction*

The function of a nerve is to transport messages from one location to another and these messages are passed along the axon in the form of electrical impulses called action potentials. Moreover, these action potentials are transient membrane depolarizations that result from a brief increase in permeability of the nerve membrane to sodium and a subsequent delayed increase in permeability to potassium (Heavner, 1991). This increase in permeability to sodium and potassium is in response to an excitation of a nerve segment by either a chemical, thermal, mechanical or electrical stimuli. Membrane excitation disrupts its resting equilibrium and results in an initial phase of slow depolarization. Subsequently, if the firing threshold is reached, an extremely rapid phase of depolarization arises leading to the initiation of an action potential (Malamed, 1997). This impulse is moved along the axon by

way of a self-perpetuating mechanism of impulse propagation. The depolarized segment of the nerve produces local currents in adjacent resting membrane areas and this current flow disrupts the resting equilibrium towards its firing threshold. Once the firing threshold is met, complete depolarization occurs and the entire process is repeated. Therefore, the impulse is conducted through the entire length of the axon.

### *1.5.3 Mechanism of Action of Local Anaesthetics*

The primary action of local anaesthetics is to prevent nerve conduction by inhibiting the influence of stimulation on sodium conductance as opposed to altering the normal resting potential of the nerve membrane (Yagiela, 1998). In the past, the membrane expansion theory was proposed to explain the pharmacological action of local anaesthetic. Specifically, this concept involved the diffusion of local anaesthetic molecules into the hydrophobic regions of the nerve membranes to physically prevent sodium channel opening by disrupting the organization of the membrane lipids (Trudell, 1977). However, there is no direct evidence that proves this membrane expansion theory accounts for the entire nerve conduction blockade nor does this concept explain the differential blocking potencies of stereoisomers of local anaesthetics (Yagiela, 1998).

Recently, research has provided substantial evidence to support a specific receptor theory whereby local anaesthetics interact directly with the sodium channel to inhibit nerve conduction. Thus, the physical blockade of the channel, an allosterically mediated change in channel conformation or a distortion of the local electrical field may be actions that could contribute to the subsequent block of nerve conduction (Yagiela, 1998).

### *1.5.4 Rationale for Use of Bupivacaine*

Since the application of local anaesthetics block the generation and propagation of nerve impulses, then theoretically, the resultant antidromic stimulation in the neurogenic inflammatory cascade would be inhibited and consequently, the neuropeptides would not be released from peripheral afferent endings. Therefore, with the pre-treatment of the TMJ region with local anaesthetic, the stimulation of primary afferents with capsaicin would not result in both PE and vasodilatation.

The use of bupivacaine stems from a study performed by Wong *et al.* (2001). The complete duration of local anaesthetic blockade provided by lidocaine and bupivacaine was determined to be at least 10 and 30 minutes, respectively. Since lidocaine was shown to be only effective for such a short period of time, the duration of afferent innervation blockade may be inadequate for the inhibition of neurogenic inflammation. Therefore, in this investigation, only bupivacaine, the local anaesthetic with the longer duration of action, was used to assess the efficacy of blocking capsaicin-induced neurogenic inflammation.

## 1.6 TRIGEMINAL SYSTEM

### *1.6.1 Gross Anatomy*

The craniofacial tissues are principally innervated by the fifth cranial nerve known as the trigeminal (V) nerve; however, branches from the upper cervical spinal nerves or other cranial nerves have been demonstrated to supply various areas of the head. The V nerve consists of three peripheral branches along with its peripheral receptors, a ganglion containing the majority of sensory neuron somata and a central root that enters the pontine brainstem. This is a mixed nerve, with both sensory and motor components. The three

subdivisions of the V nerve arise from the Gasserian (trigeminal) ganglion: the ophthalmic (V1), maxillary (V2), and mandibular (V3) nerve. Each branch is associated with various peripheral receptors distributed among the orofacial tissues. The V1 nerve supplies sensory branches to the forehead, nasal cavity and skin, cornea eyeball and dural tissue. The V2 nerve provides purely somatosensory information from the zygomatic area, upper lip and nasal structures to the CNS. In addition, the V2 nerve also innervates a portion of the nasal and oral cavity such as maxillary teeth and its associated periodontium. In contrast to the other two branches of the V nerve, the third subdivision of the V nerve supplies both sensory and motor fibers. The V3 nerve supplies sensory afferents to the remaining extra- and intra-oral structures, specifically the mandibular teeth and periodontium, the skin covering the mandible, the chin and the TMJ, and provides voluntary motor efferents that mainly innervate to the muscles of mastication.

The majority of V primary afferents cell bodies reside in the Gasserian ganglion and through this structure, sensory information is conducted to the brainstem. This V ganglion is organized in a somatotopic fashion (Dubner *et al.,* 1978; Jacquin *et al.,* 1986a) such that the cell bodies of V1 are located anteriomedially, V3 are situated posterolaterally and V2 are inbetween the other two subdivisions. The cell bodies of some periodontal afferents and muscle spindle afferents are the exception to the rule and reside in the V mesencephalic nucleus located within the CNS (Shigenaga *et al.,* 1990).

The V nerve projects to the trigeminal brainstem nuclear complex (VBSNC), a bilateral, multinucleated structure in the dorsolateral part of the brainstem to form synaptic connections. 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 reflex function (for review, see Dubner *et al.,* 1978; Lund, 1991; Capra & Dessem, 1992; Hannam & Sessle, 1994).

### *1.6.2 Primary Afferent Anatomy and Physiology*

The V primary afferent fibers consist of the large diameter, fast-conducting, myelinated Aβ- fibers, the medium diameter, moderate-conducting, myelinated Aδ- fibers, and the small diameter, slow-conducting, unmyelinated C-fibers. All these fibers innervate a wide range of peripheral receptors located throughout the craniofacial region (for review, see Darian-Smith, 1966; Dubner *et al.,* 1978).

Many V primary afferent fibers terminate in specialized receptor organs such as the Pacinian or Meissner corpuscles, Merkel's disks and hair follicles. The large diameter Aβ-fibers are associated with these sense organs, 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 (LTM). These specialized receptors either respond in a rapidly-adapting (Pacinian/Meissner corpuscles) or slowly-adapting manner (Merkel's disk); however, some slowly adapting mechanoreceptors have been shown to respond to innocuous cooling (Brown *et al.*, 1981). On the other hand, receptors responsive to proprioceptive stimuli such as movement of hair in a certain direction are known as proprioceptors. Apart from the ones in the superficial tissues, there are some proprioceptors located in deep tissues (joint and muscles) termed as muscle spindles or Golgi tendon organs. Therefore, through the activation of these sense organs, the sensory information regarding the location, intensity,

modality and duration of light tactile and proprioceptive stimuli can be transmitted to the CNS.

Other V primary afferents terminate as free nerve endings responding to noxious or intense mechanical stimuli and are known as nociceptors. The type of fibers that convey the sensory information of noxious stimuli are the Aδ- and C- fibers. Currently, there are three known classes of V nociceptive primary afferents supplying the cutaneous and mucosal craniofacial tissues (Cooper *et al.,* 1993, Dubner *et al.,* 1978; Dubner & Bennett, 1983; Hu & Sessle, 1988). The first class are the high-threshold mechanoreceptive afferents. These are Aδ- fibers which only respond to intense mechanical stimulation and their activation commonly signifies a well-localized acute mechnical tissue injury. The mechanothermal nociceptive afferents are the second class of V nociceptive primary afferents. Similar to the former category, these are Aδ- fibers which are activated by noxious mechanical and noxious heat stimuli (>45°C). An example would be the heat-induced pricking pain perceived after a heat-induced injury. Alternatively, these Aδ- nociceptive afferents can be classified functionally as type I and type II Aδ nociceptors (Leem *et al.,* 1993; Meyer & Campbell, 1981; Treede *et al.,* 1995). The first set of afferents termed as type I are activated by intense mechanical stimuli or by noxious heat at temperatures higher than 52°C. The type II afferents are activated by both mechanical and heat stimuli but at a lower thermal threshold of 43°C.

The final class are the polymodal nociceptive fibers. In contrast to the other two types of nociceptive primary afferents, these are C- fibers that are activated by intense mechanical, thermal and chemical stimuli. Due to its slow conducting velocity, these afferents have been associated with the signaling of 'second' pain that is poorly localized,

dull and aching in nature (Price *et al.,* 1977). In addition, these C- fibers can be subdivided anatomically as being peptidergic or non-peptidergic (Snider & McMahon, 1998). The former group encompasses the afferents that contain pro-inflammatory neuropeptides such as SP and CGRP, and their growth is regulated by the nerve growth factor (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-derived neurotrophic factor is essential. Nevertheless, this classification of IB4 positive and IB4 negative is not absolute (Patruska *et al.,* 2000).

Studies have demonstrated that the TMJ is innervated by free nerve endings (for review, see Dubner *et al.,* 1978; Sessle, 2000) associated with Aδ- and C- fiber primary sensory afferents. Therefore, one peripheral structure in the craniofacial region that transmits noxious information to the CNS is the TMJ (Casatti *et al.,* 1999; Kido *et al.,* 1995; Widenfalk & Wiberg, 1990).

In summary, the V system consists of Aβ- fibers that are responsible for conveying sensory information regarding light tactile and proprioceptive stimuli to the CNS, whereas the Aδ- and C- fibers detect noxious stimuli.

### 1.6.2.1 Innervation of the Temporomandibular Joint

The innervation of TMJ, in terms of the properties of nociceptors and vascular interactions, are important determinants in the pathogenesis of TMDs (Sessle & Hu, 1991), particularly for the possible role of a neurogenic inflammatory component in TMDs.

The human, Macaque monkey and rat TMJ is innervated by the branches of the third division of the trigeminal nerve (Schmid, 1969; Bernick, 1962). The auriculotemporal nerve innervates the posterior and lateral aspects of the TMJ capsule, the masseteric and deep temporal nerve supply the anterior capsule and the medial aspect is innervated by both the auriculotemporal and masseteric nerve.

In the 1960s, the light microscope demonstrated that the mouse and monkey TMJs consisted of neurons terminating in the synovial lining layer of the TMJ (Frommer & Monroe, 1966; Keller & Moffett, 1968). These neurons were identified as being sensory (Ichikawa *et al.,* 1989; Johansson *et al.,* 1986; Kido *et al.,* 1993) and CGRP and SP -LI was demonstrated in and around the synovial lining of rat and monkey TMJs. Subsequently, Heym *et al.* (1993) revealed that CGRP and SP like immunoreactivity was not restricted to sensory primary afferent neurons and included small diameter sympathetic fibers. This finding was problematic since autonomic efferents were also identified in the rat TMJ (Widenfalk & Wiberg, 1990). Therefore, the specific type of nerves innervating the TMJ could not be distinguished based on immunohistochemical studies as previously assumed. Nevertheless, other anatomical tracing techniques could be used for this purpose. For instance, neuronal labeling with neurotracer horseradish peroxidase (HRP) can transport HRP in an anterograde direction from the trigeminal ganglion to the neural terminal. This protocol can be used to visualize the fine anatomical distribution of sensory afferents in the TMJ (Kido *et al.*, 1995). The nerve bundles entered the joint anteriorly, laterally and posteriorly, where branches are mainly distributed in the peripheral portion of the disc with the lateral aspect being the most densely innervated. No fibers were observed in the central region of the disc and only a few fibers were located in the periosteum of the mandibular

condyle and temporal bone. Nerve fibers 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 fiber 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. 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 (Kido *et al.,* 1995).

Most anatomical studies demonstrate that the TMJ is supplied by nerve fibers originating mainly from the trigeminal ganglion among other sensory and sympathetic ganglia. The involvement of fibers from the trigeminal mesencephalic nucleus and parasympathetic ganglia are unclear. Recently, Uddman *et al.* (1998) and Casatti *et al.* (1999) performed retrograde immunocytochemical and retrograde axonal tracing studies respectively to identify the complete range of ganglia that supply nerve fibers to the TMJ. The retrograde immunocytochemical study (Uddman *et al.,* 1998) found that the majority of fibers originate from the trigeminal ganglion. However, the otic ganglion, sphenopalatine ganglion, superior cervical ganglion, stellate ganglion, nodose ganglion and dorsal root ganglia at levels C2 – C5 also contribute to the nerve fibers found in the TMJ. Among the autonomic ganglia, the otic and sphenopalatine ganglia provided the parasympathetic fibers, and the superior cervical and stellate ganglia supplied the sympathetic ones. Likewise, the retrograde axonal trading study (Casatti *et al.,* 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 trigeminal 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 trigeminal mesencephalic nucleus nor large diameter perikarya observed, indicating the lack of large diameter afferents such as the α- and β-fibers. Therefore, there is a roughly equal distribution of nerve fibers of sensory and autonomic origin (predominantly sympathetic) innervating the rat TMJ.

### *1.6.3 Central Organization of the Trigeminal System*

As mentioned above, nearly all cell bodies of V primary afferents are located in the Gasserian (trigeminal) ganglion with the exception of some periodontal and muscle spindle afferents. The somata of the V ganglion send their axons to the CNS via the V nerve root and proceed to ascend or descend the V spinal tract. The central axons of these afferent fibers terminate in the trigeminal brainstem nuclear complex (VBSNC) and synapse with second order neurons within or adjacent to this complex. The organization of VBSNC is comparable to that of the spinal system whereby primary afferents of the latter system with cell bodies in the DRG 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 process 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 VBSNC from the spinal cord (Sessle, 2000). Moreover, other than activating second order neurons in the VBSNC, some V afferents may also project to 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; Sessle, 1996).

The morphology of central terminals of V primary afferents has been extensively studied with anatomical tracing techniques such as neuronal labeling with neurotracer HRP and localization of neurochemicals with immunohistochemistry (Jacquin *et al.,* 1986b, 1993; Johnson *et al.,* 1991; Marfurt & Turner, 1983, 1984; Takemura *et al.,* 1991). The development of such sophisticated methodology enabled the localization of V primary central projections in the V spinal tract, the detection of terminal regions in different V brainstem nuclei and the central arborization of functionally identified V primary afferents.

The VBSNC is subdivided into the main (principal) sensory nucleus and the spinal tract nucleus. The latter nucleus consists of three subnuclei: subnucleus oralis (Vo), subnucleus interpolaris (Vi) and subnucleus caudalis (Vc). The main sensory nucleus and the two rostral subnuclei (Vo, Vi) are made up of relatively uniform heterogeneous populations of second order neurons of various sizes and projection targets. For example, some are short-range projection neurons which serve as connections to other brainstem subnuclei, whereas, neurons projecting to the thalamus or cerebellum are long-range (Falls & Alban, 1986; Jacquin *et al.,* 1986a; Jacquin & Rhoades, 1990). In contrast, the caudal portion of the V spinal tract nucleus is analogous to the dorsal horn of the spinal cord in which the neurons are organized in a laminated structure. Therefore, the Vc is also known as the medullary dorsal horn (Dubner & Bennett, 1983; Gobel *et al.,* 1981; Sessle, 2000).

The neurons comprising the VBSNC are arranged in a somatotopic organization described as an inverted medially directed face (Kruger & Michel, 1962; Nord, 1968; for review, see also Dubner *et al.,* 1978; Sessle, 2000). The V3 primary afferents send terminal

central projections to the dorsal part of each of its nuclei or subnuclei. The V1 primary afferents synapse with second order neurons located ventrally and V2 primary afferents terminate in the central portion of VBSNC. In addition, neurons with an oral receptive field (RF) (defined as the peripheral region which, when stimulated, actives the neuron) are located medially and those with a facial RF are situated laterally. However, in the Vc, the above somatotopic organization may be slightly altered. In other words, the oral and peri-oral sensory inputs are sent to the rostral compartment of Vc whereas the caudal portion represents the more lateral regions of the face. Moreover, the somatotopic organization of neurons in Vc has been referred to as the 'onion skin' arrangement since it is comprised of different layers known as lamina.

There are five major types of V brainstem neurons that are classified based on function and cutaneous RF properties (cf. Sessle, 2000). The first type is the LTM neuron that receives sensory information from the Aβ- fibers and therefore, responds to innocuous mechanical stimuli. The second category is the wide dynamic range (WDR) neuron responding to both innocuous and noxious stimuli in a graded manner and receives extensive convergent input from Aβ-, Aδ- and C- fiber afferents. The third class termed nociceptive specific (NS) neurons respond only to noxious mechanical, thermal or chemical stimuli conveyed by Aδ- and/or C- fiber afferents. The fourth group is polymodal nociceptive neurons that convey noxious heat, pinch or cold stimuli from C-fiber afferents. The final type is thermoreceptive specific (COLD) neurons that are excited by innocuous cutaneous cooling and inhibited by cutaneous warming.

These second order neurons in the VBSNC contribute to the delivery of somatosensory information to higher brain centers such as the thalamus (cf. Sessle, 2000).

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 centers, whereas a few may bypass the thalamus and project to the cerebellum, superior colliculus, pontine parabrachial nucleus, periaqueductal gray and spinal cord. Moreover, there are intrinsic connections between subnuclei within the VBSNC, which are termed the deep bundle system (for review, see Darian-Smith, 1966; Dubner *et al.*, 1978).

#### 1.6.3.1 VBSNC Involvement in Craniofacial Nociceptive Transmission

The VBSNC is the major brainstem relay for various types of somatosensory inputs from the craniofacial region, including the TMJ and its surrounding tissues. Many studies using morphological, clinical, behavioural, and electrophysiological approaches have verified the involvement of VBSNC in the transmission of craniofacial nociceptive information.

The morphology of the caudal components of VBSNC resembles that of the spinal dorsal horn, which is the fundamental component of spinal nociceptive processing. This similarity applies especially to the subnucleus caudalis due its laminated structure and various neuronal types that are analogous to those in the spinal dorsal horn (Gobel *et al.*, 1981; Renehan *et al.*, 1986). Moreover, a common feature shared with the spinal dorsal horn is the presence of the substantia gelatinosa layer (lamina II) where Aδ- and C- fiber primary afferents terminate. In addition to lamina II, Vc neurons receive small diameter (Aδ- and C-fiber) afferent convergence in lamina I, V and VI (for review, see Sessle, 2000). Specifically, an abundance of Aδ- and C- fiber primary afferents innervate the TMJ and its surrounding tissues, and these afferents have been shown to primarily project directly to Vc

and caudal parts of Vi (Broton *et al.,* 1988; Hayashi *et al.*, 1984; Jacquin *et al.,* 1986a; Sessle & Hu, 1991). Thus, the relay of nociceptive input from the craniofacial region, including the TMJ and its surrounding tissues to higher brain centers essentially involves the subnucleus caudalis of the VBSNC.

Recently, the development of immunocytochemical approaches allowed the examination for proto-oncogene (such as c-fos) expression as a marker for the identification of neurons responding to peripheral noxious stimulation (Sessle, 2000). An increased expression of c-fos in the Vc region was demonstrated when noxious stimuli were applied to different types of craniofacial tissues (Hathaway *et al.,* 1995; Lu & Bereiter, 1995; Martinez & Belmonte, 1996; Nozaki *et al.,* 1992). 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 in comparison with that induced by innocuous stimuli (Strassman & Vos, 1993). Therefore, immunocytochemical and various anatomical studies indicate a strong role played by Vc in craniofacial nociceptive processing.

Clinical and behavioral investigations have found that by disrupting the components of VBSNC, facial pain and nociceptive behavior are altered in both humans and animals. One such neurosurgical procedure is termed V tractotomy whereby the V spinal tract is commonly transected at the rostral pole of the Vc. The result of this surgery is the patient's reduced ability to perceive noxious stimuli while tactile sensation is unaffected (Sjoqvist, 1938; for review see Dubner *et al.,* 1978; Fromm & Sessle, 1991). Similarly, animal studies demonstrate that lesions or chemical injections to Vc interfere with the perception of noxious stimuli applied to craniofacial region and induce a change in nociceptive behavior (Broton *et al.,* 1988; Duale *et al.,* 1996; Dubner *et al.,* 1978). In addition, noxious craniofacial stimuli

have been associated with reflex effects in several craniofacial muscles, including the reflex action of jaw opening. The inactivation of Vc attenuates jaw muscle activity reflexly evoked by injection of mustard oil or glutamate into the TMJ capsule (Cairns *et al.,* 1998, 2001b; Hu *et al.,* 1997; Tsai *et al.,* 1999). Therefore, both clinical and behavioral studies support the involvement of Vc in nociceptive transmission and nociceptive induced reflex muscle activity.

Electrophysiological recordings in the superficial and deep laminae of Vc have demonstrated many neurons can be activated by cutaneous noxious stimuli, specifically the NS and WDR neurons (for review, see Sessle, 2000). Other types of peripheral afferent inputs, such as those supplying the TMJ, can converge onto a majority of these neurons and activate them. One study revealed that most neurons activated by electrical stimulation of TMJ afferents have deep and cutaneous RF (Sessle & Hu, 1991). This convergence of input was commonly used to explain the poor localization of deep pain and the phenomenon of referred pain; however, recently the concept of 'central sensitization' of these second-order neurons in the VBSNC has been implicated. This latter explanation allows the "unmasking" or "strengthening" of convergent afferent inputs (Hu *et al.,* 1997) and leads to the diffuse nature of the perceived pain along with the referral of pain to distant areas.

In summary, the identification of second order nociceptive neurons along with small caliber afferents terminating in Vc, plus clinical and behavioural evidence suggest the possibility of the subnucleus caudalis playing a major role in the processing of nociceptive inputs from the craniofacial region (Dubner & Bennett, 1983; Dubner *et al.,* 1978; Sessle, 2000; Yokota, 1989).

### *1.6.4 Development of Animal Model for TMJ Nociceptive Reflex Jaw Muscle Activity*

An insult to the TMJ region has been associated with a protective reflex activation of jaw musculature (cf. Sessle, 2000). Therefore, the animal model used to investigate the effects of acute TMJ injury on jaw muscle activity should be analogous to this excitatory reflex of jaw opening muscles and inhibitory reflex of jaw closing muscles.

Small diameter afferents innervating the TMJ have been implicated in pain and nociceptive reflexes associated with trauma, disease or dysfunction (Klineberg, 1971; Dubner *et al.*, 1978); however, it was not until recently in which studies revealed the reflex activation of the masticatory musculature in response to noxious stimulation of the TMJ. The application of algesic chemicals such as potassium chloride, histamine, 7% sodium chloride and mustard oil, to animal TMJ regions reflexly evoked activity in the jaw musculature as measured by increased EMG activity (Bakke *et al.*, 1998; Broton *et al.*, 1988; Broton & Sessle, 1988; Yu *et al.*, 1995, 1996). In addition, a recent study demonstrated that the nociceptive stimulation of rat TMJ afferents with capsaicin evoked reflex jaw activity in both the digastric and masseter muscles (Hu *et al.*, 2001).

Furthermore, experimentally induced lesions in Vc have resulted in a disruption of this reflex jaw opening activity evoked by noxious stimulation of the TMJ region (Cairns *et al.*, 1998, 2001b; Hu *et al.*, 1997; Tsai *et al.*, 1999). Therefore, theoretically the complete blockade of conduction from the primary afferents supplying the TMJ should eliminate the evoked reflex jaw muscle activity. Indeed, studies have demonstrated the abolishment of reflex EMG activity in digastric and masseter muscle when the TMJ region was pre-treated with 2% and 5% lidocaine 5 minutes prior to the application of the algesic chemical (Yu *et al.*, 1995; Wong *et al.*, 2001).

Therefore, the above acute inflammatory rat TMJ model induced by capsaicin can be used to study the peripheral mechanism of nociceptive reflex jaw muscle activity and to evaluate the efficacy of the conduction blockade of primary afferent fibers supplying the rat TMJ region.

# CHAPTER 2

# AIM AND HYPOTHESIS

## 2.1 AIM OF THIS STUDY

In an acute inflammation rat model, the process of acute neurogenic inflammation of the temporomandibular joint (TMJ) area will be investigated by evaluating the effect of local anaesthetic blockade of the afferent innervation on the development of capsaicin-induced edema in the rat TMJ and capsaicin-induced reflex jaw muscle activity.

## 2.2 HYPOTHESES

### *2.2.1 Hypothesis #1*

*The application of local anaesthetic to the rat TMJ region prior to the administration of capsaicin will suppress the capsaicin-induced neurogenic inflammation.*

The application of capsaicin to the TMJ region has been shown to be markedly inflammatory, resulting in plasma protein extravasation and edema in the TMJ region (Fiorentino *et. al*, 2000). In addition, the reduction of edema following the pre-treatment of the TMJ tissues with ruthenium red (non-competitive antagonist) or capsazepine (competitive receptor antagonist) prior to the application of capsaicin confirmed the presence of VR1 receptors in the TMJ region (Fiorentino *et. al*, 2000). Thus, capsaicin can be applied to the rat TMJ region to induce neurogenic inflammation; however, the presence or absence of a capsaicin-induced neurogenic component in the inflammatory reaction in the TMJ tissues has not been specifically investigated. Therefore, to evaluate the neurogenic role in inflamed TMJ tissues, local anaesthetic can be employed.

Local anaesthetics function by binding onto specific receptors located on sodium channels at the neural membrane surface. This union results in the inhibition of sodium ion influx, which in turn prevents axonal depolarization and subsequent nerve conduction. Therefore, theoretically, the prior application of local anaesthetic to the TMJ region prior to the administration of capsaicin will inhibit nerve conduction and consequently prevent the release of neuropeptides when capsaicin binds to VR1 receptors. Hence, the neurogenic inflammatory response of the TMJ tissues to capsaicin will be abolished by the pre-treatment of TMJ tissues with local anaesthetic and will be indicated by the absence or reduction of resultant tissue expansion.

### *2.2.2 Hypothesis #2*

*The application of local anaesthetic to the rat TMJ region prior to the administration of capsaicin will suppress the capsaicin-induced reflex jaw muscle activity.*

Capsaicin applied to the TMJ region evokes a sustained and reversible increase in EMG activity of the digastric and masseter muscles (Hu *et. al*, 2001). Similar to above, local anaesthetic blockade of afferent innervation in the TMJ region will block the nerve conduction and consequently prevent the capsaicin-induced reflex jaw muscle activity. Previous studies have shown the abolishment of mustard oil-induced reflew jaw muscle activity with the pre-treatment of TMJ tissues with local anaesthetic (Wong *et al.*, 2001; Yu *et al.*, 1995). Therefore, in parallel to earlier investigations, TMJ tissues pre-treated with local anaesthetic will not elicit an increase in EMG activity when capsaicin is injected.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 EXPERIMENTAL MODEL

### *3.1.1 General Methodology*

Male Sprague-Dawley rats weighing 225-400 g were prepared for the continuous measurement of one-dimensional tissue expansion as previously described by Fiorentino *et al.* (1999) and Wong *et al.* (2001) and for the acute recording of jaw muscle electromyographic (EMG) activity as previously described by Cairns *et al.* (1998, 1999). At a constant room temperature of 21 ± 1°C with relative humidity between 40-60% in a 12 hour light/dark cycle, rats were housed two per cage with access to food pellets and water *ad libitum*.

Prior to the surgery, the rats were weighed and the weight was recorded. Under surgical inhalational anaesthesia ($O_2$, 0.3-0.4 l/min; $N_2O$, 0.6 l/min; halothane, 1.5-2%), an electronic temperature sensor probe was placed into the rectum. A heating pad was activated and deactivated by the sensor to maintain a core temperature between 37-37.5°C. Using a 21-gauge needle, two pieces of 30-gauge wire were placed subcutaneously over the right and left chest wall to monitor the heart rate. Leads were attached to the wires and the signal was fed into an oscilloscope. The heart rate readings were between 300-400 beats per minute. In addition, a foot pad connected to a pulse-oximeter was placed on the right hindpaw to monitor the oxygen level. Oxygen saturation level readings were between 90-100%.

A tracheotomy was performed for the insertion of an artificial tracheal cannula and the rat was artificially ventilated for the duration of the experiment. Bipolar electrodes were

fabricated out of 36-gauge Teflon-coated single stranded stainless steel wires and were inserted unilaterally into the left digastric and masseter muscles.

The rat's head was mounted on a stereotaxic frame and an incision was made to expose the dorsal surface of the skull. Two screws were inserted into the right and left parietal bones anterior to the parieto-occipital suture and lateral to the inter-parietal sutures. A screw on the end of a vertical support bar connecting to the stereotaxic frame was ligated to the screws in the skull with a 30-gauge stainless steel wire. The three screws were coated with dental plaster and allowed to set. The ear bars were removed and the right side was further stabilized. Dental plaster was attached to a horizontal bar connected to the stereotaxic frame and covered the right side of the head. Therefore, the head was stabilized at all three axes: the vertical, incisal and horizontal bars. A diagrammatic illustration of the set up is presented in Figure 6.

The hair overlying the left TMJ area was trimmed. Antero-posteriorly, this area extended from the outer canthus of the left eye to just anterior to the left ear and supero-inferiorly, from just above the zygomatic arch to the midpoint of the mandibular ramus.

The left zygomatic arch was palpated, and simultaneously the lower jaw was retruded and advanced to locate the posterior infero-lateral aspect of the zygomatic arch. A double barrel cannula consisting of two 27 gauge dental needles connected by polyethylene tubing to two 25 µl Hamilton syringes was inserted at this landmark. The catheter was placed to a depth of 2 mm where it contacted bone and was further advanced 0.5 mm anteriorly. The catheter and the tubing were immobilized to the frame with masking tape. A 50 mm sharp tungsten needle was bent 90° 3 mm from its distal tip and its proximal end was stabilized with a drop of cyanoacrylate adhesive over the left TMJ region. The distal tip was allowed to

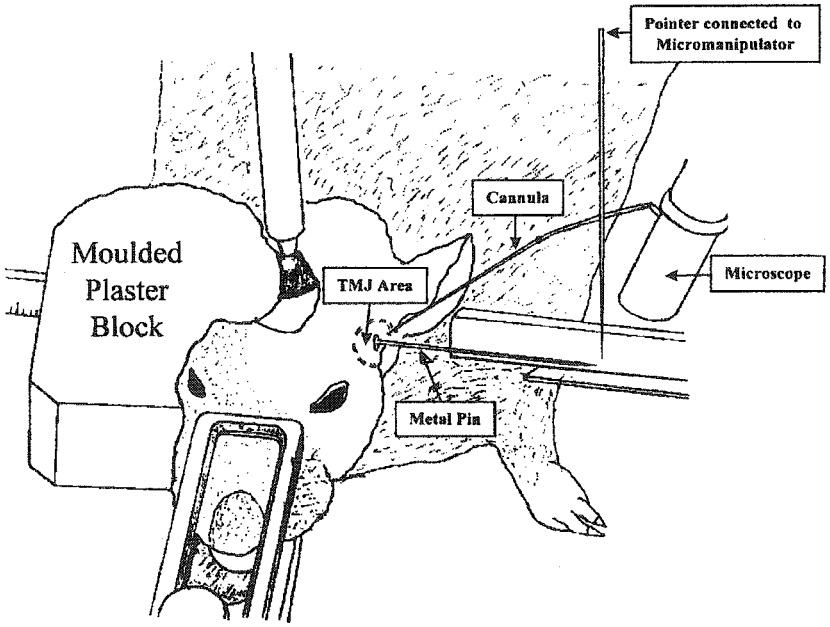


**Figure 6** **Set Up of Tissue Expansion Model of Neurogenic Inflammation**
Adapted from *Fiorentino et al., 1999.*

rest passively on a paper support track fixed lateral to the left TMJ area. This needle served as a physical marker of lateral tissue expansion. A second needle was fixed to a micromanipulator and the tip of the first was matched with the tip of the second with the aid of a 1.6X dissecting microscope.

On completion of the surgery, the halothane level was gradually reduced (0.9 -1.3%) until noxious pressure applied to the hindpaw toes produced a slight hindlimb withdrawal reflex, ensuring an adequate level of anaesthesia was maintained for the duration of the experiment (Cairns *et al.*, 1998, 1999).

### *3.1.2 Drug Itemization and Preparation*

All drugs masses were measured with a Mettler PM2000 electronic scale.

#### 3.1.2.1 Capsaicin

Capsaicin powder (8-methyl-N-vannillyl-6-nonenamide, Sigma Chemical Co., St. Louis, MO, USA) 50 mg was measured and placed into a test tube. 500 µl of 100% ethanol was added to the powder to make a 10% stock solution. Prior to each experiment, an aliquot of the desired concentration (1.0% or 0.1%) was prepared by diluting a volume of the 10% capsaicin stock solution with the appropriate volume of Tween-80 (Polyoxyethylenesorbitan Monooleate, Sigma Chemical Co., St. Louis, MO, USA) and sterile normal saline (0.9% sodium chloride injection USP, Baxter, Toronto, Ontario), in a 1:1:8 ratio, respectively. The mixture was sonicated for 60 seconds with the Branson 3200 ultrasonic cleaner (Branson Cleaning Equipment Co., Shelton, CT, USA), immediately after dilution and prior to the loading of the solution into the delivery catheter.

### 3.1.2.2 Bupivacaine HCl

Bupivacaine hydrochloride powder (1-butyl-n-(2,6-dimethylphenyl)-2-piperidinecarboxamide, Sigma Chemical Co., St. Louis, MO, USA) 5 mg was measured and placed in 1 ml vial. 1 ml of sterile normal saline was added to the powder to make a 0.5% solution. The mixture was then agitated by hand for 2 minutes.

### 3.1.2.3 Halothane, Nitrous Oxide, Oxygen

Inhalational anaesthesia was provided by Halothane® (2-bromo-2-chloro-1,1,1-trifluoroethane, Halocarbon Laboratories, River Edge, NJ, USA), Nitrous Oxide (BOC Gases, Mississauga, Ontario, Canada) and Oxygen (BOC Gases, Mississauga, Ontario, Canada) with the aid of a 7025 Rodent Ventilator (UGO Basile, Comerio-Varese, Italy) at a rate of 93 strokes per minute, delivering a total of 4 ml of gases.

### 3.1.2.4 Evan's Blue Dye

Evan's Blue Dye powder (Sigma Chemical Co., St. Louis, MO, USA) 100 mg was measured and placed into a test tube. 10 ml of sterile water (Abbott Laboratories Ltd., Saint-Laurent, Quebec, Canada) was added to the powder to make a 10 mg/ml solution. The mixture was agitated with a Maxi Mix Plus® (Bernstead/Thermolyne, Dubuque, Iowa) mixer for 2 minutes.

#### 3.1.2.5 Proprietary Heparin, Normal Saline, and T-61

Hepalean® (Heparin Sodium U.S.P., Organon Teknika, Toronto, Ontario, Canada) 1000 U/ml was used to aid in draining blood from the rat during the saline perfusion. This allowed the visualization of tissue with Evan's Blue markings of plasma extravasation.

Normal sterile saline for injection (0.9% sodium chloride solution, Baxter Corporation, Toronto, Ontario, Canada) was used for the saline pre-load agent and for the transcardial perfusion.

T-61® (Hoechst, Regina, Saskatchewan, Canada), an euthanasia agent, was used to sacrifice the rat at the end of the experiment.

### *3.1.3 Rationale for Specific Concentrations*

#### 3.1.3.1 Capsaicin

The rationale for the selection of 1.0% and 0.1% solutions for the evaluation of capsaicin-induced neurogenic inflammation in the TMJ region were based on Fiorentino *et al.* (2000) and a pilot study illustrated in Appendix A. Fiorentino *et al.* (2000) demonstrated that the tissue expansion induced by concentrations of capsaicin higher than 1.0% were equivalent to that elicited by 1.0% capsaicin solutions. Furthermore, a pilot study (see Appendix A) assessing the effect of 0.01% capsaicin on tissue expansion demonstrated the resultant edema was similar to that induced by the vehicle solution. Therefore, solutions above 1.0% concentration and below 0.1% were not evaluated in this study.

In addition, the amount of capsaicin injected into the TMJ region was modified from previous investigations (Fiorentino *et al.*, 2000; Hu *et al.*, 2001) based on a pilot study (see Appendix A). Fiorentino *et al.* (2000) and Hu *et al.* (2001) utilized 20 μl of capsaicin;

however, a pilot study (see Appendix A) demonstrated that the pre-treatment of tissues with 10 μl of 0.5% bupivacaine was ineffective in blocking the reflex jaw muscle EMG activity evoked by 20 μl of capsaicin regardless of the latency between the injection of the local anaesthetic and capsaicin (i.e., administration of capsaicin 5, 10 or 20 minutes post bupivacaine injection). Therefore, to eliminate the possibility that the 20 μl of capsaicin may have distributed to a larger area than that anaesthesized by the 10 μl of bupivacaine, the volume of capsaicin was reduced to 10 μl.

#### 3.1.3.2 Bupivacaine HCl

As mentioned in Section 1.5.4, the rationale for the utilization of 0.5% bupivacaine stems from Wong *et al.* (2001). Bupivacaine has been reported to have an extended duration of action when compared with the prototype local anaesthetic lidocaine (Malamed, 1997). Likewise, Wong *et al.* (2001) revealed the duration of complete conduction blockade in the TMJ region to be longer for 0.5% bupivacaine than 5 % lidocaine. Therefore, bupivacaine appeared to be the more ideal agent for the investigation of the efficacy of a local anaesthetic in preventing capsaicin-induced neurogenic inflammation. In addition, a solution of 0.5% was chosen due to its clinical relevance and a volume of 10 μl was selected based on Wong *et al.* (2001).

### *3.1.4 Set Up and Loading of Double Barrel Catheter*

The double barrel catheter consists of two 27-gauge dental needles connected by polyethylene tubing to two 25 μl Hamilton syringes. First, mineral oil was injected into the polyethylene tubing prior to attachment of the Hamilton syringes. Then, 10 μl of the pre-

load agent (saline/bupivacaine) and capsaicin/vehicle was drawn up into their respective catheter. The introduction of mineral oil ensured that the complete volume of the aqueous solution was delivered and no amount was refluxed back into the tubing. In addition, its non-polar nature prevented any contamination between the two phases.

### *3.1.5 Experimental Groups*

The experimental groups are outlined in Table 1. The left TMJ region of rats in group 1, 2 and 3 were pre-treated with saline (0.9%; 10μl), followed with the application of 1.0% capsaicin (10μl), 0.1% capsaicin (10μl) or vehicle control (10μl), respectively. The left TMJ region of rats in group 4, 5 and 6 were pre-treated with bupivacaine (0.5%; 10μl), followed with the application of 1.0% capsaicin (10μl), 0.1% capsaicin (10μl) or vehicle control (10μl), respectively. For the measurement of expansion distance (see Section 3.1.6), each experimental group consisted of eight (8) rats each. For the EMG recording of jaw muscle activity (see Section 3.1.7), groups 1 and 2 consisted of six (6) rats, group 3 consisted of ten (10) rats and groups 4-6 consisted of eight (8) rats each.

**Table 1:**

**Experimental Groups**

| *Group* | *Descriptor* |
|---|---|
| 1 | 10 μl Saline + 10 μl Vehicle |
| 2 | 10 μl Saline + 10 μl 0.1% Capsaicin |
| 3 | 10 μl Saline + 10 μl 1.0% Capsaicin |
| 4 | 10 μl 0.5% Bupivacaine + 10 μl Vehicle |
| 5 | 10 μl 0.5% Bupivacaine + 10 μl 0.1% Capsaicin |
| 6 | 10 μl 0.5% Bupivacaine + 10 μl 1.0% Capsaicin |

### *3.1.6 Measurement of Expansion Distance*

The measurement of tissue expansion in the TMJ region is based on Fiorentino *et al.* (1999) and Wong *et al.* (2001). As the periarticular tissues expanded, the needle fixed to the left TMJ region was displaced laterally. The realignment of the two needle tips using the micromanipulator allowed the quantification of tissue expansion. Baseline measurements were taken every 5 minutes for 15 minutes prior to the injection of the pre-load agent and were denoted as $time_{-15}$, $time_{-10}$, $time_{-5}$ (see Figure 7). Subsequently, the pre-load agent (0.9% saline/0.5% bupivacaine; 10μl) was injected at $time_0$ and measurements were taken in one-minute intervals until $time_5$; at which time, capsaicin (1.0/0.1%; 10μl) was delivered. Again, the amount of expansion was recorded in one-minute intervals from $time_6$ to $time_{10}$, inclusive. From $time_{12}$ to $time_{70}$, the recording interval was increased to two minutes and from $time_{75}$ to $time_{180}$, measurements were taken every 5 minutes. Therefore, the expansion distance was recorded over a continuous period of 180 minutes.

### *3.1.7 EMG Recording of Jaw Muscle Activity*

Implanted bipolar electrodes were used to record EMG activity from the ipsilateral digastric and masseter muscles (Cairns *et al.*, 1998, 1999). The EMG recording of jaw muscle activity parallels that of the expansion distance measurements. Thus, baseline EMG activity was observed for 15 minutes before the administration of the pre-load agent and continuous EMG activity was recorded following the injection of the pre-load agent (0.9% saline/0.5% bupivacaine; 10μl) and capsaicin (1.0 or 0.1%; 10μl). The recording of EMG activity was terminated 30 minutes after the reflex activity induced by capsaicin returns to baseline levels. EMG activity was amplified (gain, 500X; bandwidth, 30-1000 Hz),

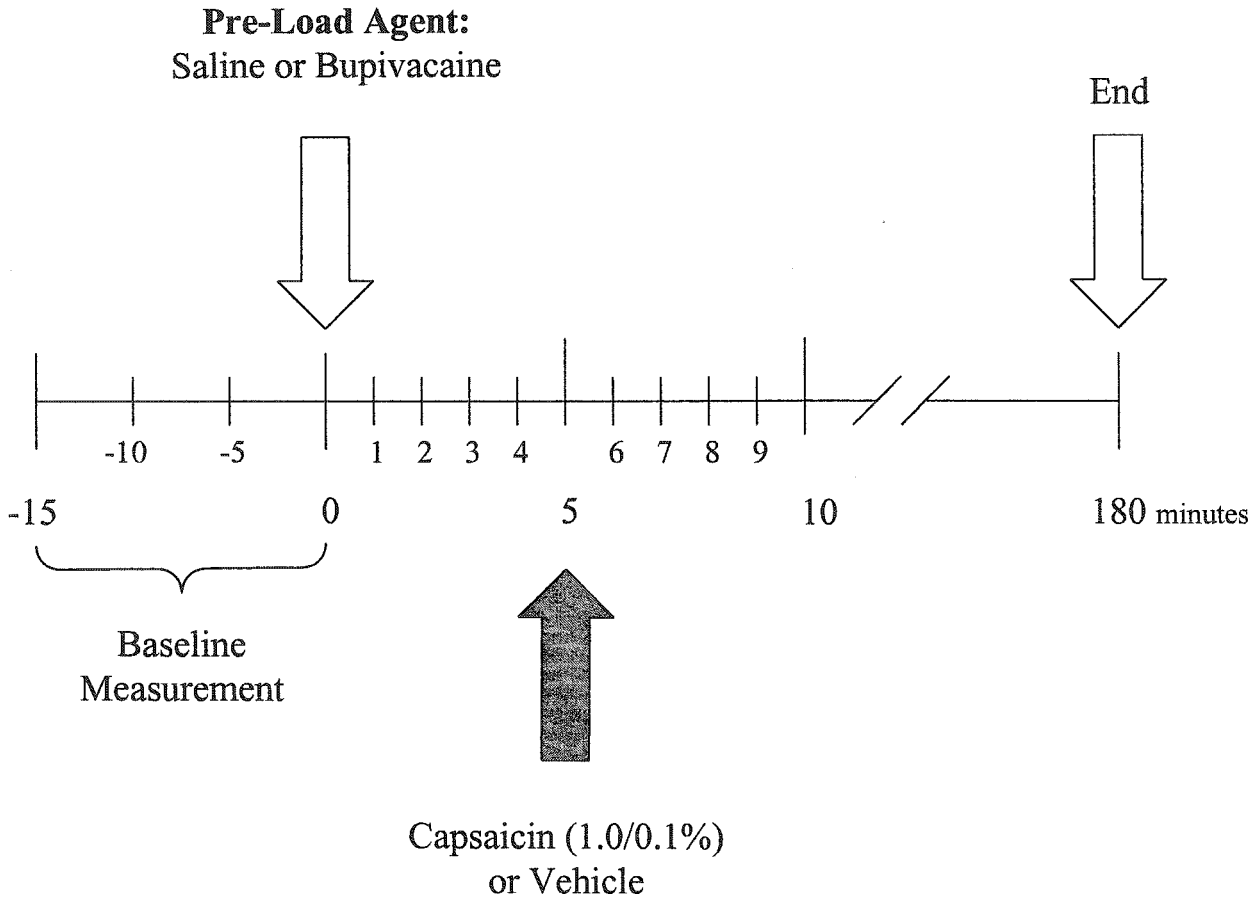


**Figure 7** **Timing of Injection of Pre-Load Agent and Capsaicin/Vehicle**
(Tick marks indicate times of measurement of tissue expansion.)

displayed on an oscilloscope and fed into a computer equipped with a CED 1401 board and analysis software (Spike2). The recorded EMG data was stored electronically and analyzed offline.

### *3.1.8 Confirmation of Complete Conduction Blockade*

To confirm the complete local anaesthetic conduction blockade at the time of the injection of capsaicin, 10 μl of capsaicin (1.0% or 0.1%) was injected 5 minutes following the injection of 10 μl of 0.5% bupivacaine and the resultant EMG activity relative to baseline was evaluated. Since the application of capsaicin to the rat TMJ region elicits reflex jaw muscle activity (Hu *et al.*, 2001), the absence of increased EMG activity in the digastric and masseter muscle above baseline levels following the injection of capsaicin indicated a complete conduction blockade.

### *3.1.9 Confirmation of Catheter Position & Termination Procedures*

After tissue expansion measurements and EMG recordings are collected, an intravenous cannula was placed into the right femoral vein and Evan's blue dye (10mg/ml, 20mg/kg) was slowly injected into the vein. After 15 minutes, an anticoagulant Heparin® (0.2 ml) was injected into the vein to aid the transcardial perfusion. After 5 minutes, the rat was sacrificed with T61 (0.2 ml, Hoechst) and transcardial perfusion was performed with 0.9% saline solution. A post-mortem dissection consisted of the retraction of the skin and superficial muscles (masseter and temporalis) to expose both the ipsilateral and contralateral TMJ. Both joints along with the surrounding tissues were visually examined and the presence of obvious blue staining of the disk and capsule signified the correct placement of

the catheter and provided the indication of plasma protein extravasation into the TMJ region (Haas *et al.*, 1992; Yu *et al.*, 1995).

## 3.2 STATISTICAL METHODS

### *3.2.1 Data Analysis for Tissue Expansion Measurements*

The analysis of the capsaicin-induced neurogenic inflammation was based on Wong *et al.* (2001). The mean expansion distance recorded over the first 15 minutes before injection of the pre-load agent (saline/bupivacaine) into the TMJ region determined the mean baseline tissue expansion. Residual tissue expansion distances were calculated by subtracting the mean baseline tissue expansion from each recorded expansion measurement. If the value of the first reading after TMJ application of the pre-load agent was greater than 2 SD above the baseline, the agent was considered to cause tissue expansion. The baseline plus 2 SD was determined since it represents an approximation of the 95% confidence interval for the mean tissue expansion. The overall response was defined as the cumulative area under the tissue expansion curve (AUC) calculated as the sum of values of the first and all subsequent relative tissue expansion distances corresponding to the raw tissue expansion measurements greater than 2SD above the mean baseline tissue expansion. The significant differences between cumulative AUC for the various experimental groups from $time_{-15}$ to $time_{150}$ were determined with two-way ANOVA to evaluate the presence or absence of an interaction between the different pre-load agents and the varying concentrations of capsaicin. In addition, the significant differences for the mean expansion distances at each recorded time point (from $time_{-15}$ to $time_{180}$) between the different experiment groups were also determined with ANOVA or the paired t-test, and post-hoc Tukey tests were performed when

appropriate with the aid of SigmaStat 2.03 Software (SPSS Inc., Chicago, IL, USA). Moreover, if the variances of the different experimental groups were not equal, the non-parametric Kruskal-Wallis ANOVA test was employed. A p-value less than 0.05 was determined as statistically significant.

### *3.2.2 Data Analysis of EMG Activity*

Recorded EMG data were rectified off-line and EMG area bins (microvolts per minute) were calculated (Cairns *et al.*, 1998, 1999). The mean of EMG area bins recorded over the first 20 minutes before injection of the pre-load agent into the TMJ region determined the mean baseline EMG activity. Residual EMG activity bins were calculated by subtracting the mean baseline EMG activity from each EMG area bins. If the value of the first EMG bin after TMJ application of either agent was greater than two standard deviations (2 SD) above the baseline, the agent was considered to evoke reflex jaw muscle activity. The baseline plus 2 SD was determined as a signal to noise limit since it presents an approximation of the 95% confidence interval for the mean baseline activity. The overall response was defined as the cumulative area under the EMG response curve (AUC) calculated as the sum of values of the first and all subsequent residual EMG area bins corresponding to the raw EMG area bins greater than 2 SD above baseline EMG activity. Significant differences in the cumulative AUC for the various experimental groups were determined with two-way ANOVA to evaluate the presence or absence of an interaction between the different pre-load agents and the varying concentrations of capsaicin, and post-hoc Tukey tests were performed when appropriate with the aid of SigmaStat 2.03 Software (SPSS Inc., Chicago, IL, USA). In addition, the significant differences for each residual

EMG area bins between the different experiment groups were also determined with ANOVA, and post-hoc Tukey tests were performed when appropriate. Moreover, if the variances of the different experimental groups were not equal, the non-parametric Kruskal-Wallis ANOVA test was employed. A p-value less than 0.05 was determined as statistically significant.

# CHAPTER 4

# RESULTS

## 4.1 CAPSAICIN-INDUCED NEUROGENIC INFLAMMATION

### *4.1.1 Dose Response Curve for Capsaicin-Induced Tissue Expansion*

The experimental groups are outlined in Section 3.1.5 and the dose-dependent time course of capsaicin-induced tissue expansion for Groups 1 through 3 is displayed in Figure 8. Visual analysis revealed that both concentrations of capsaicin solution (Groups 2 and 3) stimulated a greater amount of tissue expansion in the rat TMJ region than the vehicle solution (Group 1) (see Figure 8). From $time_{-20}$ to $time_0$, which corresponds to the time of catheter insertion to the time of saline pre-load injection respectively, there was no significant difference in the resultant tissue expansion between all three groups (ANOVA, $p<0.05$; Table 2). Thus, the mean baseline expansion distances caused by the trauma induced with the insertion of the catheter were similar among all groups. Likewise, all groups produced similar expansion measurements in response to the injection of capsaicin or vehicle at $time_6$ (ANOVA, $p>0.05$; Table 2). Following the administration of capsaicin, there was a reduction in expansion among all groups from $time_6$ to $time_{32}$. At $time_{32}$, the tissue expansion in Group 3 steadily increased for the remainder of the 180 minute time course. On the contrary, both Groups 1 and 2 exhibited a gradual decrease in tissue expansion, reaching a plateau at approximately $time_{155}$ and $time_{125}$, respectively.

Statistical analysis revealed only 1% capsaicin- (Group 3) evoked tissue expansion to be significantly different from the vehicle group (Group 1) at $time_{28}$ and onwards (ANOVA, $p<0.05$). In addition, the comparison of tissue expansion induced by 1% with 0.1% capsaicin

(Group 3 and 2, respectively) indicated statistical significance from $time_{48}$ until the end of the recording period (ANOVA, $p<0.05$). Therefore, in addition to the demonstration of a dose-dependent capsaicin-induced tissue expansion in the rat TMJ region, a difference in time point at which edema development is statistically significant between various concentrations of capsaicin was revealed.

The capsaicin-induced edema development was also evaluated by the cumulative amount of tissue expansion (Figure 13, solid circles ●). A dose-dependent edema response to the increasing capsaicin concentration was demonstrated and the tissue expansion evoked by the 1% solution significantly differed from that produced by the 0.1% concentration of capsaicin and vehicle control.

**Table 2:**

**Mean Expansion Distance (Mean) ± Standard Error (SE) at time 0, 6, 60, 120, 180 ($t_0$, $t_6$, $t_{60}$, $t_{120}$, $t_{180}$)**

| Group | Mean ($t_0$) ± $SE_0$ | Mean ($t_6$) ± $SE_6$ | Mean ($t_{60}$) ± $SE_{60}$ | Mean ($t_{120}$) ± $SE_{120}$ | Mean ($t_{180}$) ± $SE_{180}$ |
|---|---|---|---|---|---|
| 1 | 13 ± 4 μm | 190 ± 31 μm | 75 ± 12 μm | 45 ± 14 μm | 30 ± 11 μm |
| 2 | 16 ± 4 μm | 232 ± 18 μm | 128 ± 20 μm | 90 ± 18 μm | 86 ± 21 μm |
| 3 | 23 ± 6 μm | 227 ± 16 μm | 210 ± 25 μm | 295 ± 44 μm | 339 ± 46 μm |
| 4 | 12 ± 4 μm | 205 ± 19 μm | 45 ± 12 μm | 42 ± 13 μm | 50 ± 12 μm |
| 5 | 13 ± 4 μm | 183 ± 22 μm | 76 ± 26 μm | 60 ± 23 μm | 90 ± 24 μm |
| 6 | 25 ± 11 μm | 272 ± 34 μm | 247 ± 46 μm | 283 ± 37 μm | 392 ± 55 μm |

### *4.1.2 Effect of Local Anaesthetic Block on Inflammation*

The mean baseline expansion and the expansion induced after the injection of the pre-load (saline/bupivacaine) agent did not significantly differ among all 6 experimental groups (two-way ANOVA, $p>0.05$). The effect of 0.5% bupivacaine pre-treatment of the TMJ region on capsaicin-evoked edema was evaluated by comparing the saline pre-load with their respective bupivacaine pre-load groups. Specifically, comparisons were completed between Groups 3 and 6 (Figure 9), Group 2 and 5 (Figure 10), and Group 1 and 4 (Figure 11), revealing statistically insignificant differences in the resultant tissue expansion (*a priori* paired t-test, $p>0.05$). In other words, 0.5% bupivacaine was ineffective in reducing the capsaicin-induced tissue expansion in the TMJ region. A summary plot of all 6 experimental groups is given in Figure 12.

Alternatively, the cumulative tissue expansion for both the saline pre-treated and bupivacaine pre-treated groups were plotted in Figure 13. This graph (Figure 13) illustrates the similarity between the edema induced by capsaicin regardless of the type of pre-load agent utilized. In agreement with section 4.1.1, a two-way ANOVA demonstrated a dose-response effect with respect to the different concentrations of capsaicin on tissue expansion ($F_{2,42} = 40.76$, $p<0.001$) and the *post-hoc* Tukey test revealed statistical significance between the tissue expansion induced by 1.0% capsaicin and that evoked by 0.1% concentration and the vehicle control (Tukey test, $p<0.001$). However, the type of pre-load agent applied did not affect the resultant edema development ($F_{1,42} = 0.186$. $p>0.05$); thus, there was no significant difference between the saline pre-treated and bupivacaine pre-treated groups. Moreover, the two-way ANOVA did not demonstrated an interaction between the concentration of capsaicin and type of pre-load agent used ($F_{2,42} = 0.403$, $p>0.05$).

## 4.2 CAPSAICIN-INDUCED REFLEX JAW MUSCLE ACTIVITY

### *4.2.1 Capsaicin-Induced Raw EMG Activity Data*

Examples of the recorded raw EMG activity data for the saline pre-treated groups (Group 1, 2, and 3) are displayed in Figures 14, 15 and 16, respectively. The injection of saline at $time_{1200}$ does not evoke an increase in EMG activity, whereas the injection of capsaicin or vehicle at $time_{1500}$ elicits a sustained and reversible reflex ipsilateral masseter and digastric EMG response. Twelve rats responded to the injection of saline and EMG activity did not return to baseline levels prior to the injection of capsaicin/vehicle; thus, the resultant EMG data were not included in the analysis.

In addition, an example of the recorded raw EMG activity data for Group 6, the bupivacaine pre-treated 1% capsaicin group is illustrated in Figure 17. The injection of bupivacaine at $time_{1200}$ as well as capsaicin at $time_{1500}$ does not evoke an increase in reflex ipsilateral masseter and digastric EMG activity. Therefore, the nerve conduction blockade by the pre-treatment of TMJ tissues with bupivacaine was confirmed by the absence of reflex EMG activity in response to capsaicin. Two rats responded to the injection of 1% capsaicin despite the pre-treatment of the TMJ tissues with bupivicaine; therefore, the resultant tissue expansion data was discarded.

### *4.2.2 Dose Response Curve for Capsaicin-Induced EMG Activity*

The dose-dependent time course of relative mean masseter and digastric EMG activities for Groups 1 through 3 were expressed as the area under the curve (AUC) (Figures 18 and 19, respectively). In addition, the dose response curve of the capsaicin-induced AUC

relative mean EMG activity in the masseter and digastric muscles for Groups 1 through 6 are presented in Figures 20 and 21, respectively.

Figure 18 illustrates the dose-dependent time course of masseter EMG activity elicited by the injection of different concentrations of capsaicin and the vehicle into the rat TMJ region. Statistical analysis revealed a significant difference in EMG activity only between 1.0% capsaicin (Group 3) and the vehicle (Group 1) at $time_{1500}$ (Kruskal-Wallis ANOVA, $p<0.05$). On the other hand, the digastric EMG activity evoked by 1.0% capsaicin (Group 3) was statistically significant from that elicited by the vehicle (Group 1) at $time_{1500}$, $time_{1560}$ and $time_{1620}$ (Kruskal-Wallis ANOVA, $p<0.05$) (Figure 19). Although both masseter and digastric muscles responded to 0.1% capsaicin, the increased EMG activity at all time points did not significantly differ from that elicited by the vehicle (Kruskal-Wallis ANOVA, $p<0.05$).

Similar results were derived from the evaluation of the AUC cumulative mean EMG activity for both masseter and digastric muscles in Groups 1 through 3 (Figure 20 and 21). A two-way ANOVA revealed the concentration of capsaicin and the type of pre-load agent administered affected the increased EMG reflex jaw muscle activity and an interaction between those two factors was demonstrated. Specifically, the AUC EMG activity for masseter and digastric muscles among the different concentrations were statistically significant ($F_{2,37} = 3.69$, $p<0.05$; $F_{2,37} = 9.401$, $p<0.001$, respectively) and increased in a dose-dependent manner. In addition, the *post-hoc* Tukey test revealed the increased EMG activity evoked by 1% capsaicin (Group 3) to be only statistically significant from that elicited by the vehicle control (Group 1) (Tukey test, $p<0.05$). Moreover, the AUC EMG activity for Groups 4 through 6 plotted in Figures 20 and 21 indicates the lack of reflex

activity upon the pre-treatment of TMJ tissues with 0.5% bupivacaine prior to the injection of various concentrations of capsaicin or the vehicle. Statistical analysis demonstrated significance among the increased EMG activity in both masseter and digastric muscles evoked by capsaicin in saline pre-treated and bupivacaine pre-treated rats ($F_{1,37} = 6.604$, $p<0.05$; $F_{1,37} = 20.568$, $p<0.001$, respectively). Furthermore, the two-way ANOVA revealed an interaction between the concentration of capsaicin and the type of pre-load agent administered on the resultant reflex EMG activity in masseter and digastric muscles ($F_{2,37} = 3.69$, $p<0.05$; $F_{2,37} = 9.401$, $p<0.001$, respectively).

## 4.3 POST-MORTEM DISSECTION OF THE TMJ REGION

Qualitative analysis of the post-mortem dissection by the naked eye revealed a graded Evan's blue dye response in the ipsilateral temporomandibular articular and periarticular tissues in all experimental groups. A digital photograph of an example of a dissection is provided in Figure 22. The 1% capsaicin demonstrated a more intense staining compared with that produced by the 0.1% solution or vehicle control. Two rats with the absence of staining in the ipsilateral TMJ region were excluded from the analysis. In addition, examination of the contralateral rat TMJ revealed an absence of Evan's blue dye staining of the temporomandibular articular or periarticular tissues.

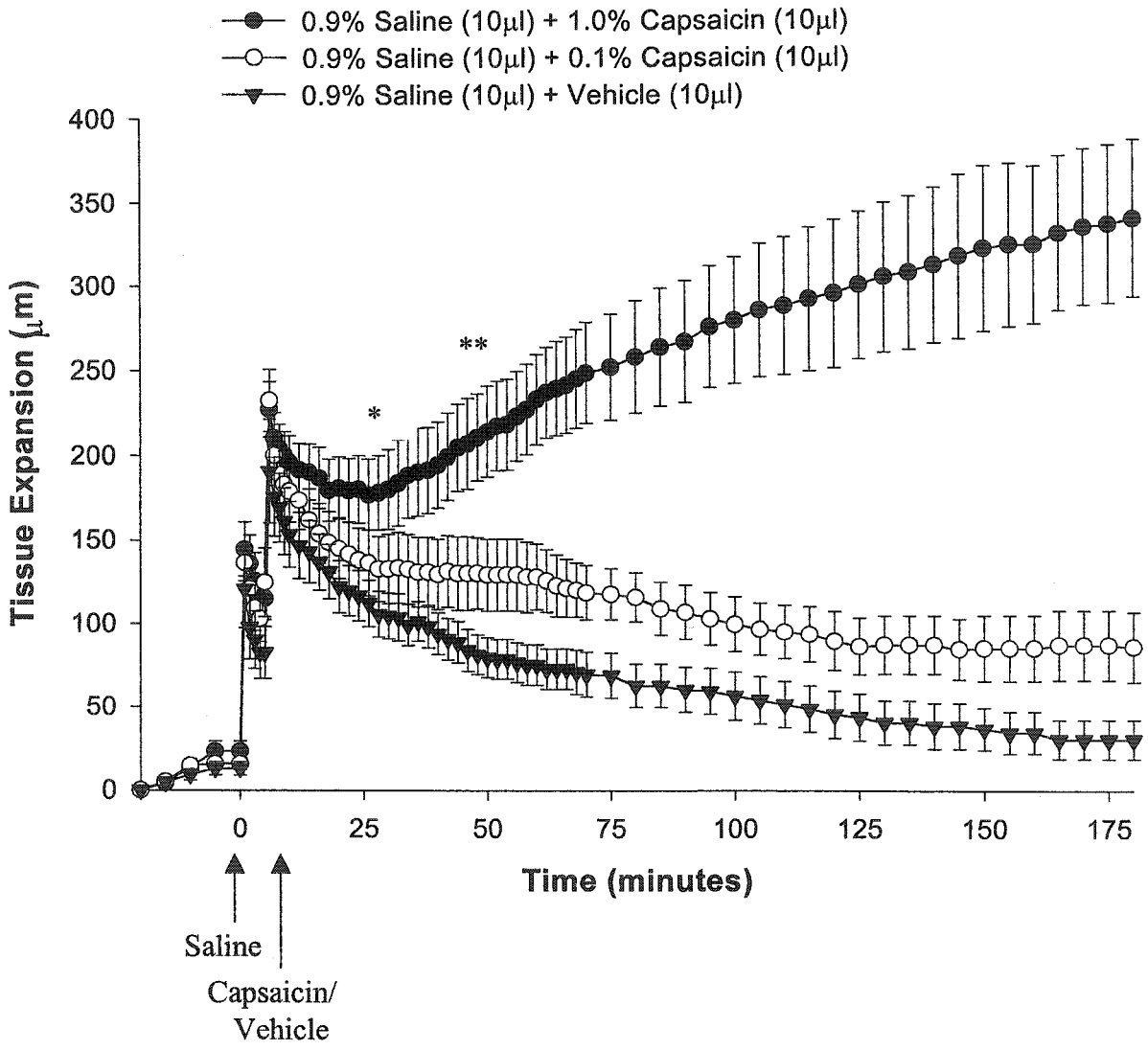


**Figure 8** ***Dose Response Curve: Tissue Expansion versus Increasing Capsaicin Concentration:*** The mean changes in tissue expansion evoked by the injection of various concentrations of capsaicin or vehicle control into the left TMJ region. Saline and capsaicin/vehicle were injected at $time_0$ and $time_5$, respectively. Each data point represents the mean tissue expansion ± SE for a certain time point over the 180 minute time course in eight rats. '*' and '**' indicates that the tissue expansion was significantly higher in comparison with that evoked by the vehicle control at $time_{28}$ and onwards, and that evoked by the 0.1% solution at $time_{48}$ and onwards (ANOVA, $p<0.05$).

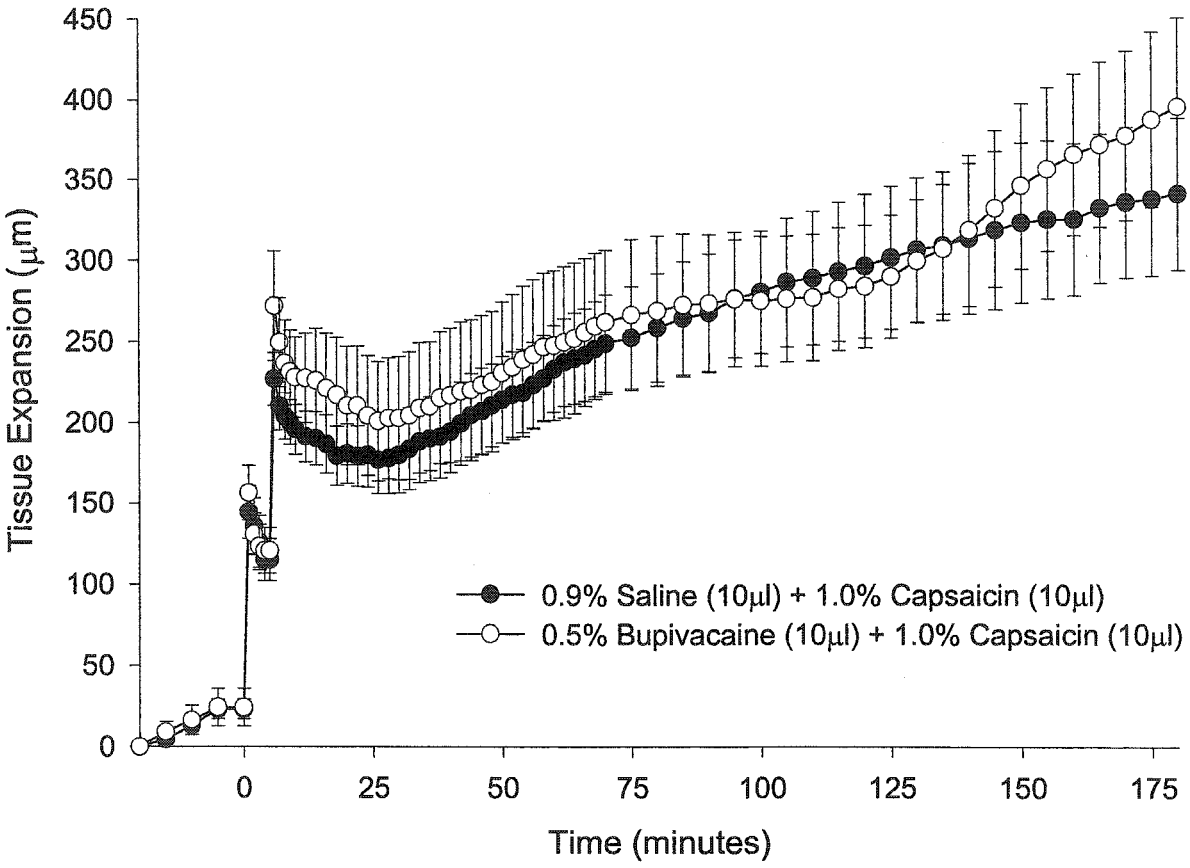


**Figure 9** ***Effect of 0.5% Bupivacaine on 1.0% Capsaicin-Induced Tissue Expansion:*** The mean changes in tissue expansion evoked by the injection of 1.0% capsaicin into the left TMJ region pre-treated with either a saline pre-load or a bupivacaine pre-load. Bupivacaine and capsaicin/vehicle were injected at $time_0$ and $time_5$, respectively. Each data point represents the mean tissue expansion ± SE for a certain time point over the 180 minute time course in eight rats. No statistical difference was demonstrated between the tissue expansion induced by 0.1% capsaicin in the saline pre-treated and bupivacaine pre-treated experimental groups.

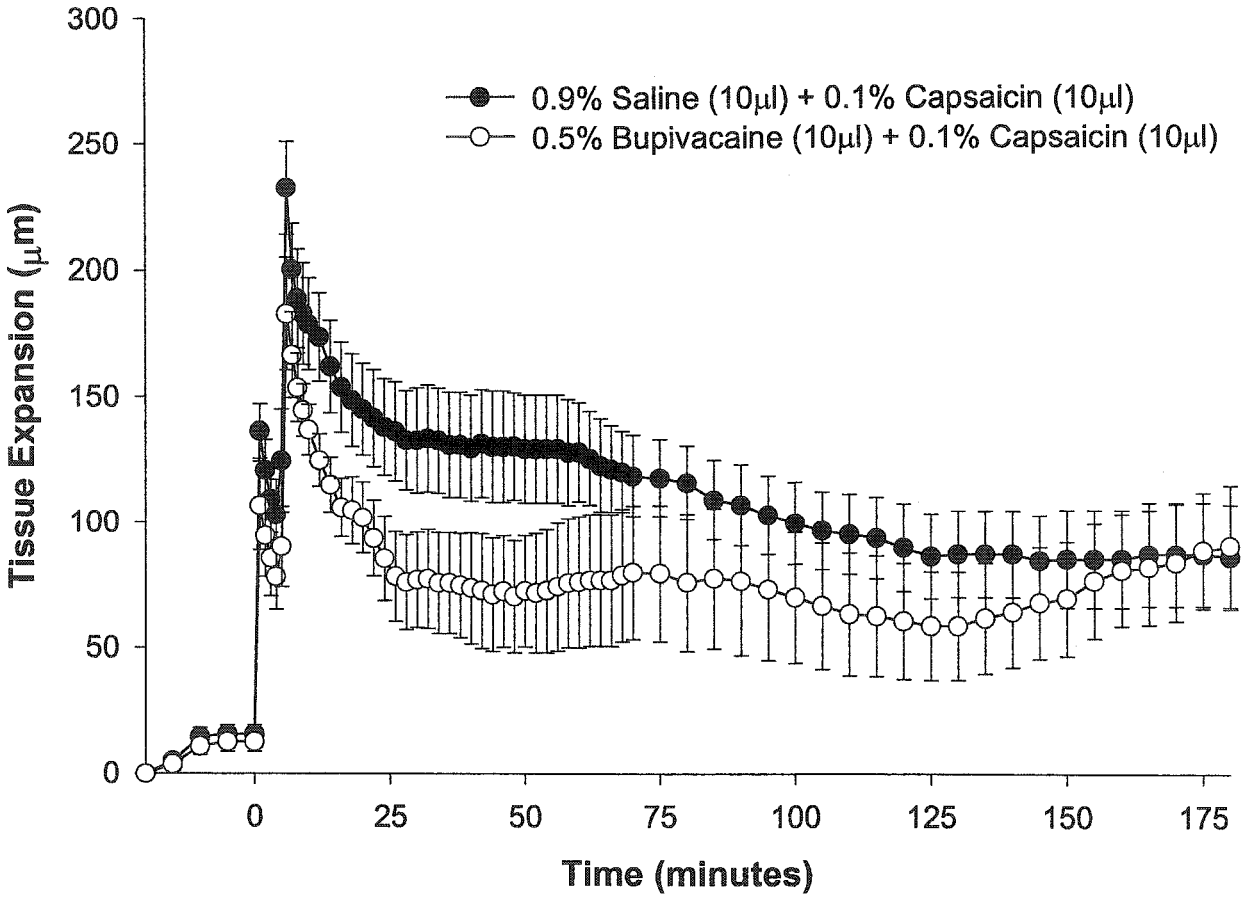


**Figure 10** ***Effect of 0.5% Bupivacaine on 0.1% Capsaicin-Induced Tissue Expansion:*** The mean changes in tissue expansion evoked by the injection of 0.1% capsaicin into the left TMJ region pre-treated with either a saline pre-load or a bupivacaine pre-load. Bupivacaine and capsaicin/vehicle were injected at $time_0$ and $time_5$, respectively. Each data point represents the mean tissue expansion ± SE for a certain time point over the 180 minute time course in eight rats. No statistical difference was demonstrated between the tissue expansion induced by 1.0% capsaicin in the saline pre-treated and bupivacaine pre-treated experimental groups.

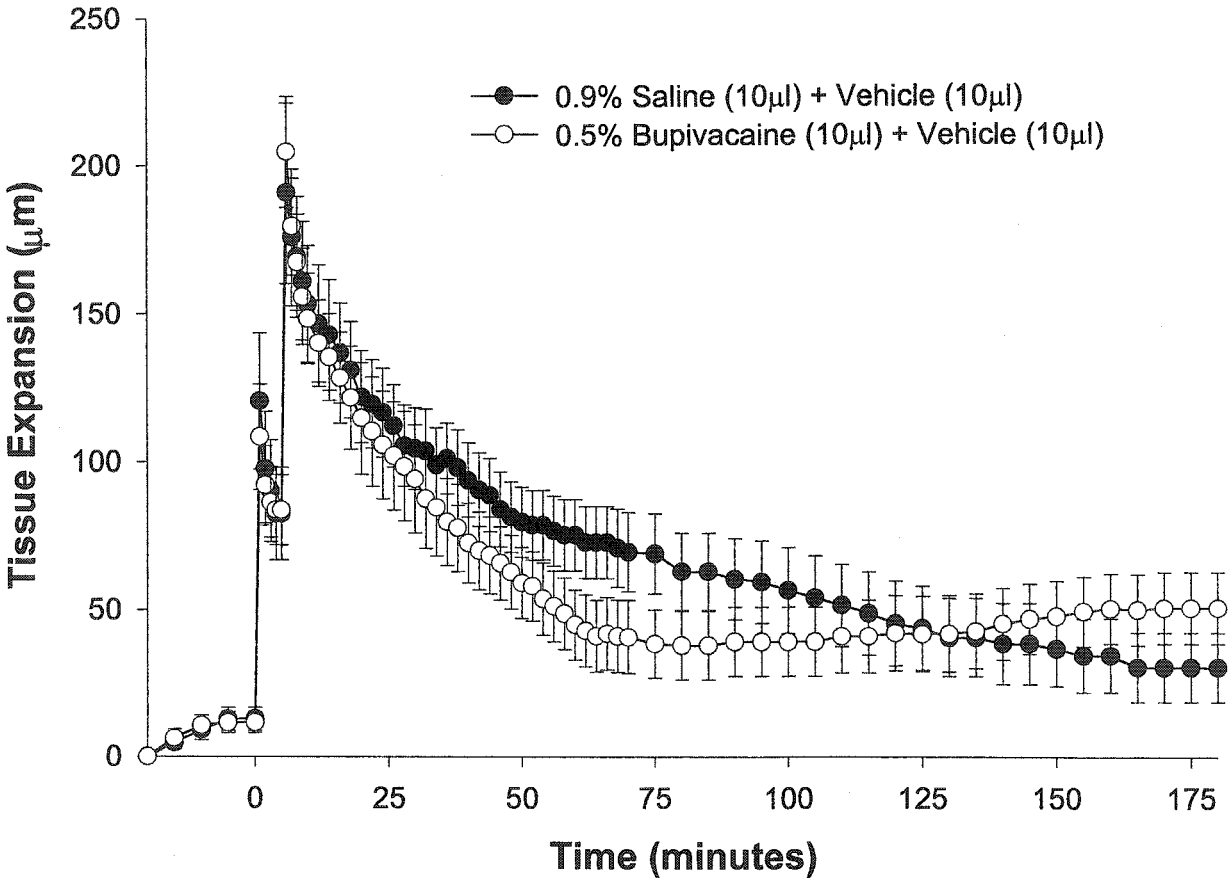


**Figure 11** ***Effect of 0.5% Bupivacaine on Vehicle-Induced Tissue Expansion:*** The mean changes in tissue expansion evoked by the injection of vehicle control into the left TMJ region pre-treated with either a saline pre-load or a bupivacaine pre-load. Bupivacaine and capsaicin/vehicle were injected at $time_0$ and $time_5$, respectively. Each data point represents the mean tissue expansion ± SE for a certain time point over the 180 minute time course in eight rats. No statistical difference was demonstrated between the tissue expansion induced by the vehicle control in the saline pre-treated and bupivacaine pre-treated experimental groups.

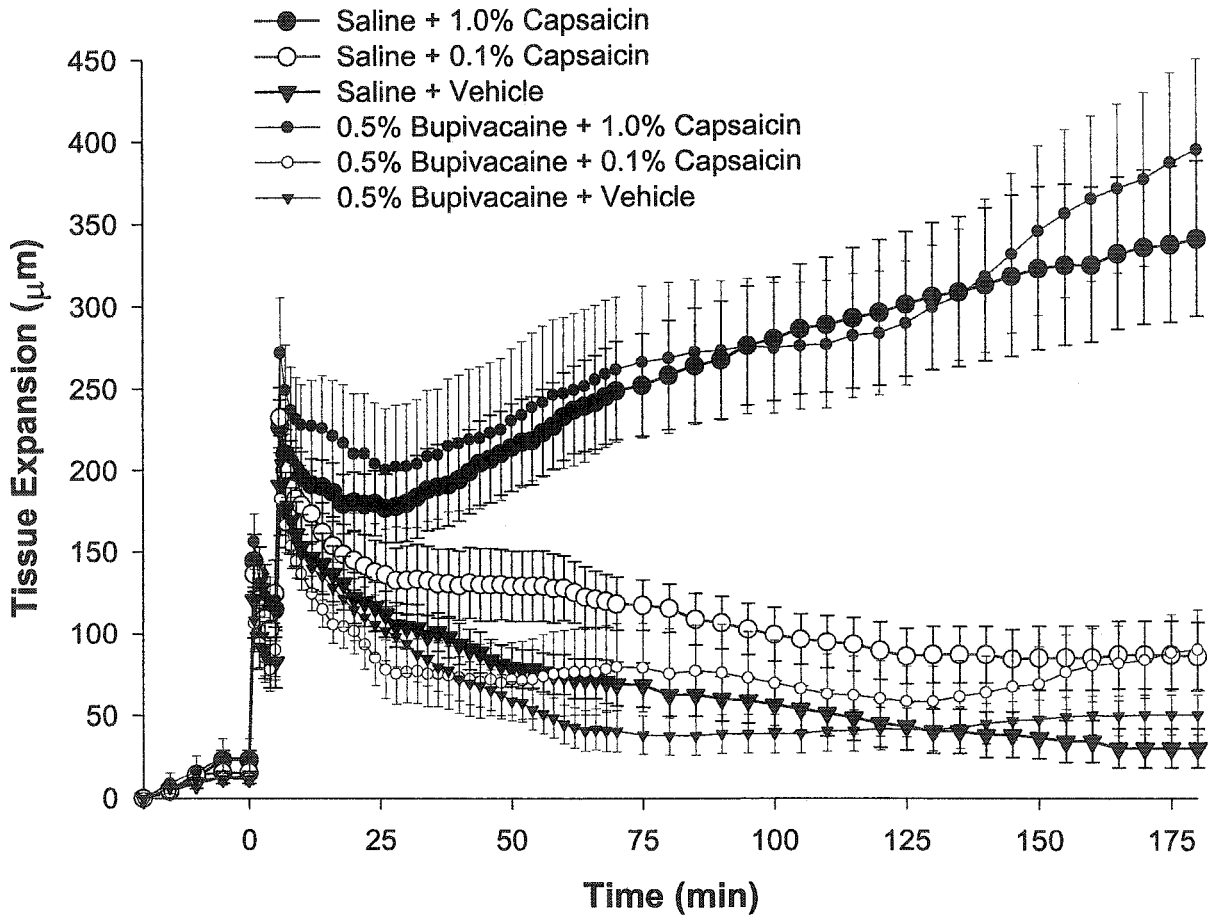


**Figure 12** ***Summary Plot of Various Concentrations of Capsaicin-Induced Tissue Expansion in Saline or Bupivacaine Pre-Treated TMJ in Comparison with a Vehicle Control:*** The mean changes in tissue expansion evoked by the injection of various concentrations of capsaicin or vehicle control into the left TMJ region pre-treated with either a saline pre-load or a bupivacaine pre-load. Saline/bupivacaine and capsaicin/vehicle were injected at $time_0$ and $time_5$, respectively. Each data point represents the mean tissue expansion ± SE for a certain time point over the 180 minute time course in eight rats.

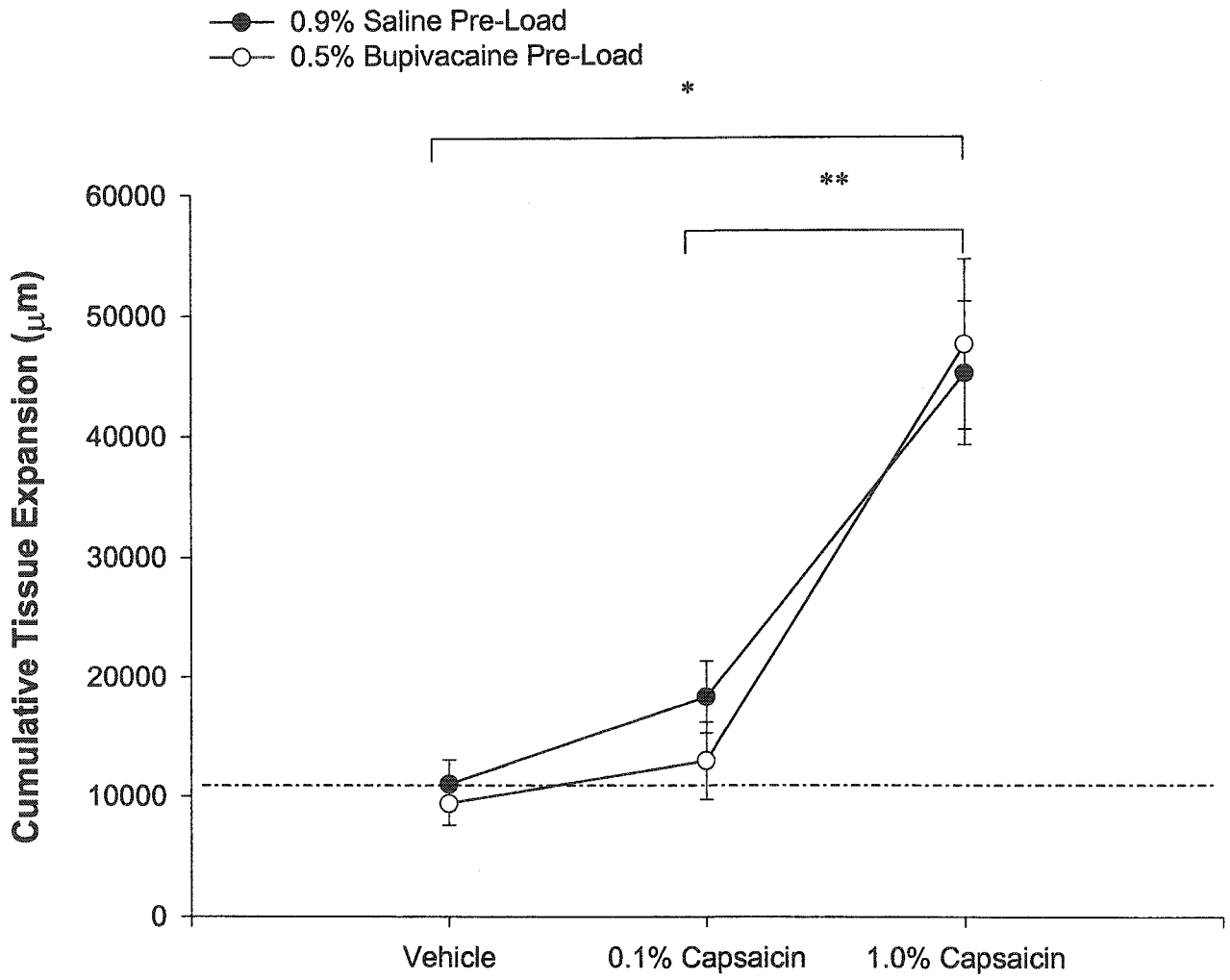


**Figure 13** ***Capsaicin-Induced Cumulative Tissue Expansion:*** The mean cumulative tissue expansion evoked by the injection of various concentrations of capsaicin or vehicle control into the left TMJ region pre-treated with either a saline pre-load or a bupivacaine pre-load. Each data point represents the mean ± SE of the normalized values relative to the baseline tissue expansion in eight rats. The horizontal dotted line indicates the mean baseline cumulative tissue expansion distance. '*' indicates that the cumulative tissue expansion induced by 1% capsaicin was significantly higher in comparison with that evoked by the vehicle control (Tukey test, $p<0.05$). '**' indicates that the tissue expansion of that data point was significantly higher in comparison with that induced by the 0.1% capsaicin solution (Tukey test, $p<0.05$). No statistical difference was demonstrated between the cumulative tissue expansion induced by the various concentrations of capsaicin or vehicle control in the saline pre-treated and bupivacaine pre-treated experimental groups.

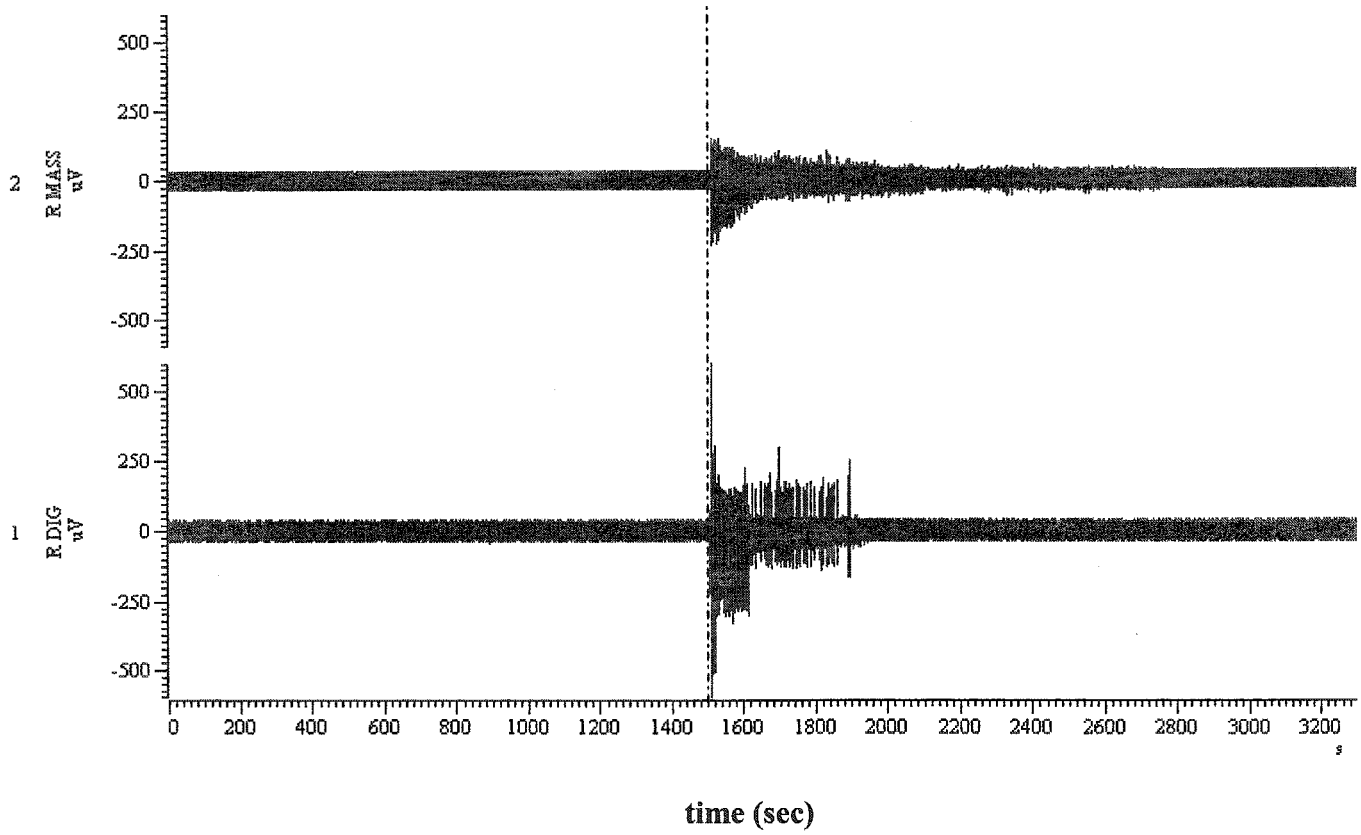


**Figure 14** ***Raw EMG Activity Data: 1.0% Capsaicin with Saline Pre-Load:*** An example of the increased EMG activity in both left masseter and digastric muscle evoked by the application of 1.0% capsaicin into the saline pre-treated left TMJ region. The horizontal dotted line indicates the injection of capsaicin time point.

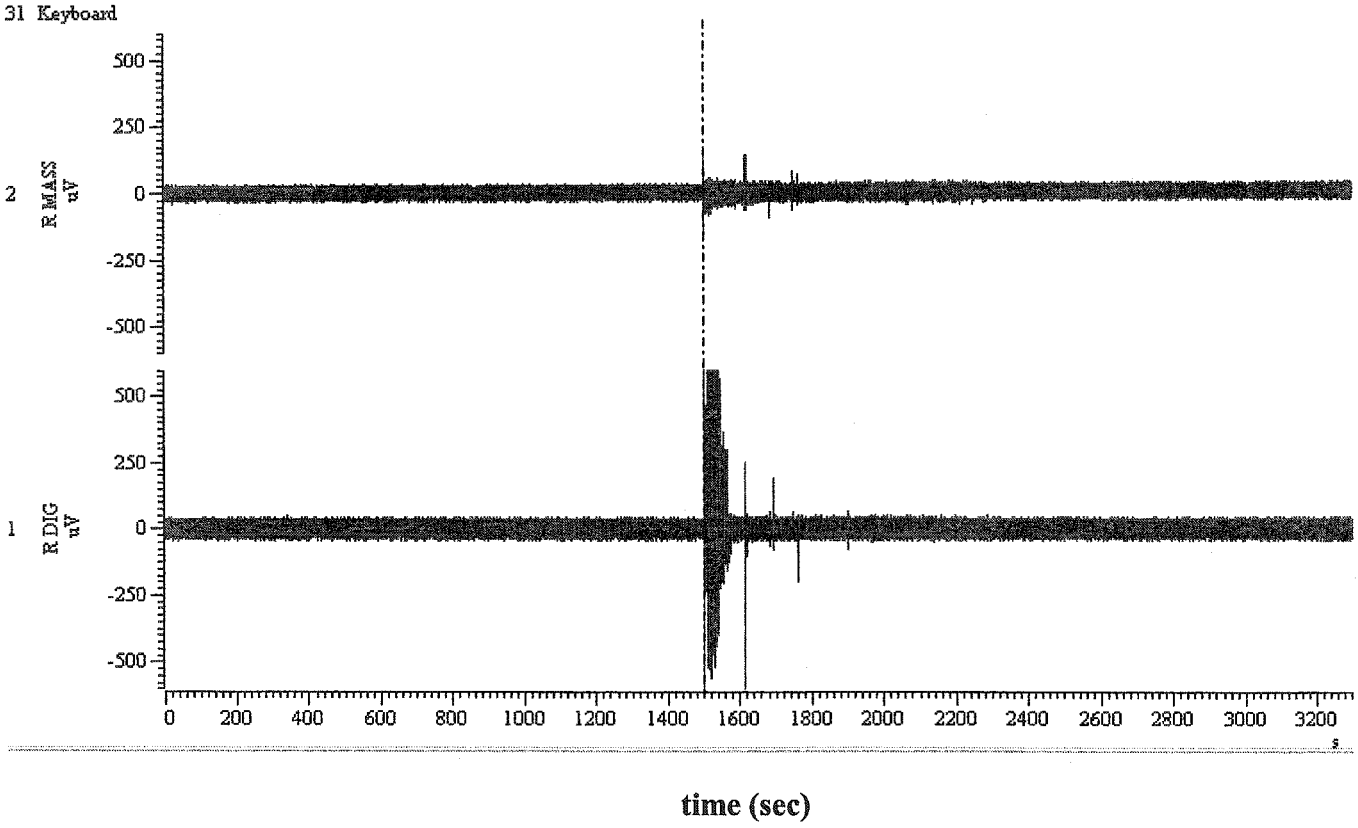


**Figure 15** ***Raw EMG Activity Data: 0.1% Capsaicin with Saline Pre-Load:*** An example of the increased EMG activity in both left masseter and digastric muscle evoked by the application of 0.1% capsaicin into the saline pre-treated left TMJ region. The horizontal dotted line indicates the injection of capsaicin time point.

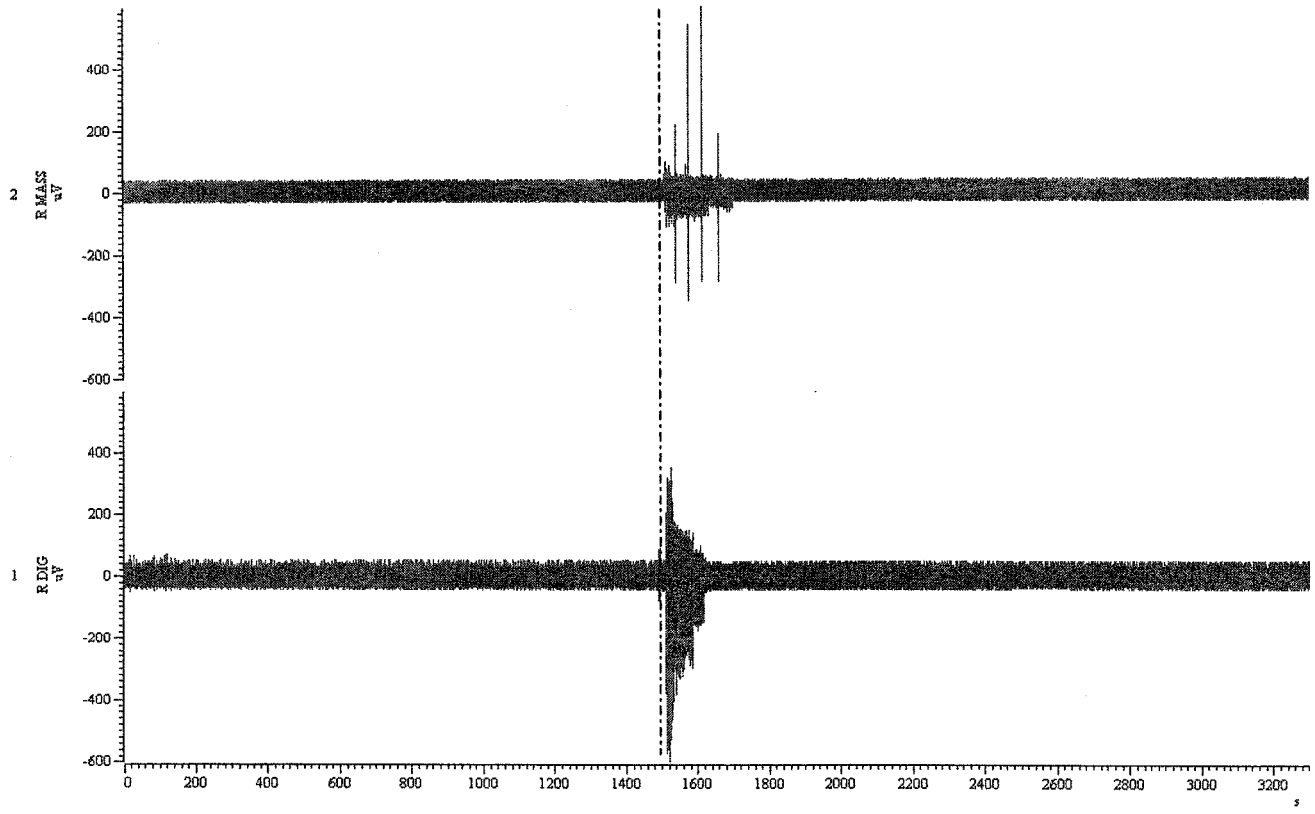


**Figure 16** ***Raw EMG Activity Data: Vehicle with Saline Pre-Load:*** An example of the increased EMG activity in both left masseter and digastric muscle evoked by the application of the vehicle control into the saline pre-treated left TMJ region. The horizontal dotted line indicates the injection of capsaicin time point.

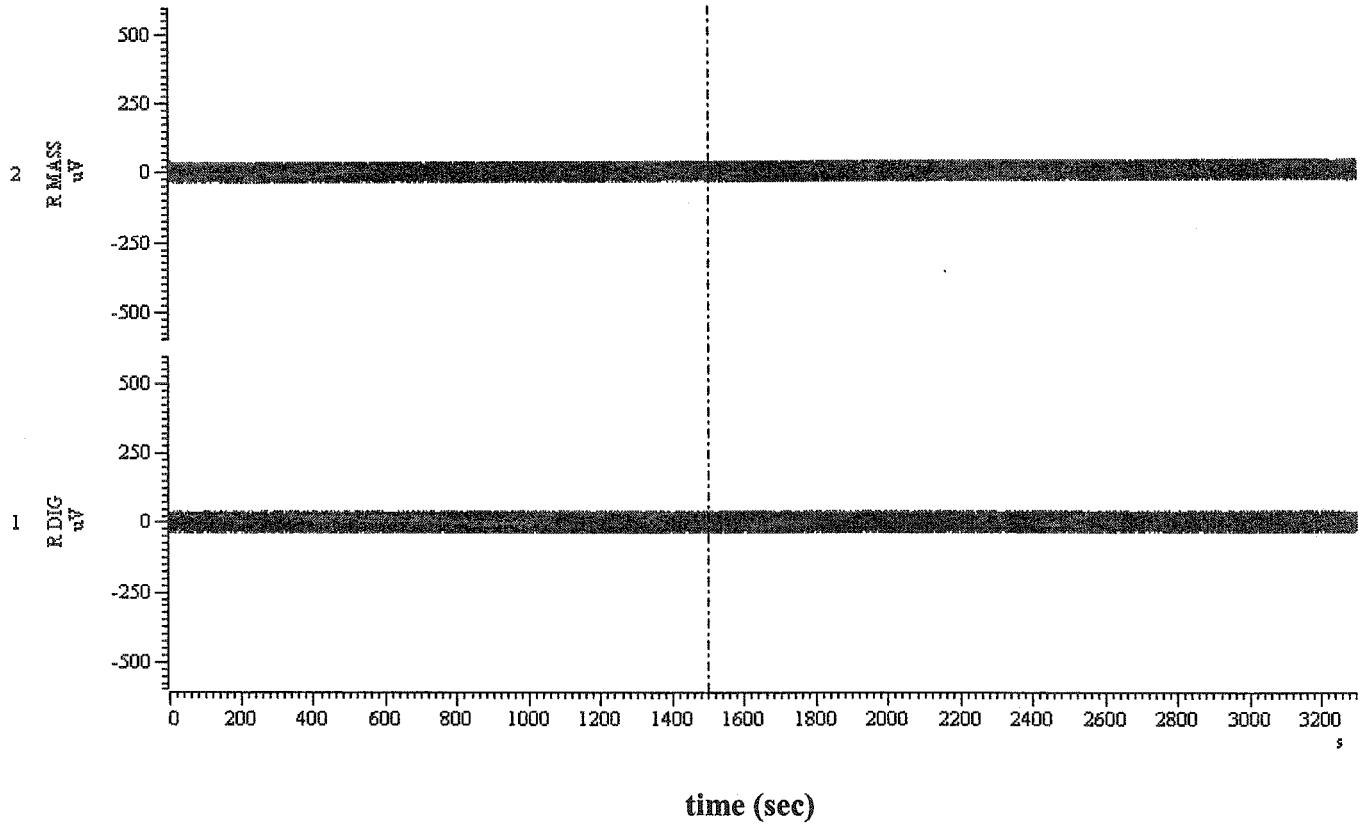


**Figure 17** ***Raw EMG Activity Data: 1.0% Capsaicin with 0.5% Bupivacaine Pre-Load:*** An example of the increased EMG activity in both left masseter and digastric muscle evoked by the application of 1.0% capsaicin into the bupivacaine pre-treated left TMJ region. The horizontal dotted line indicates the injection of capsaicin time point.

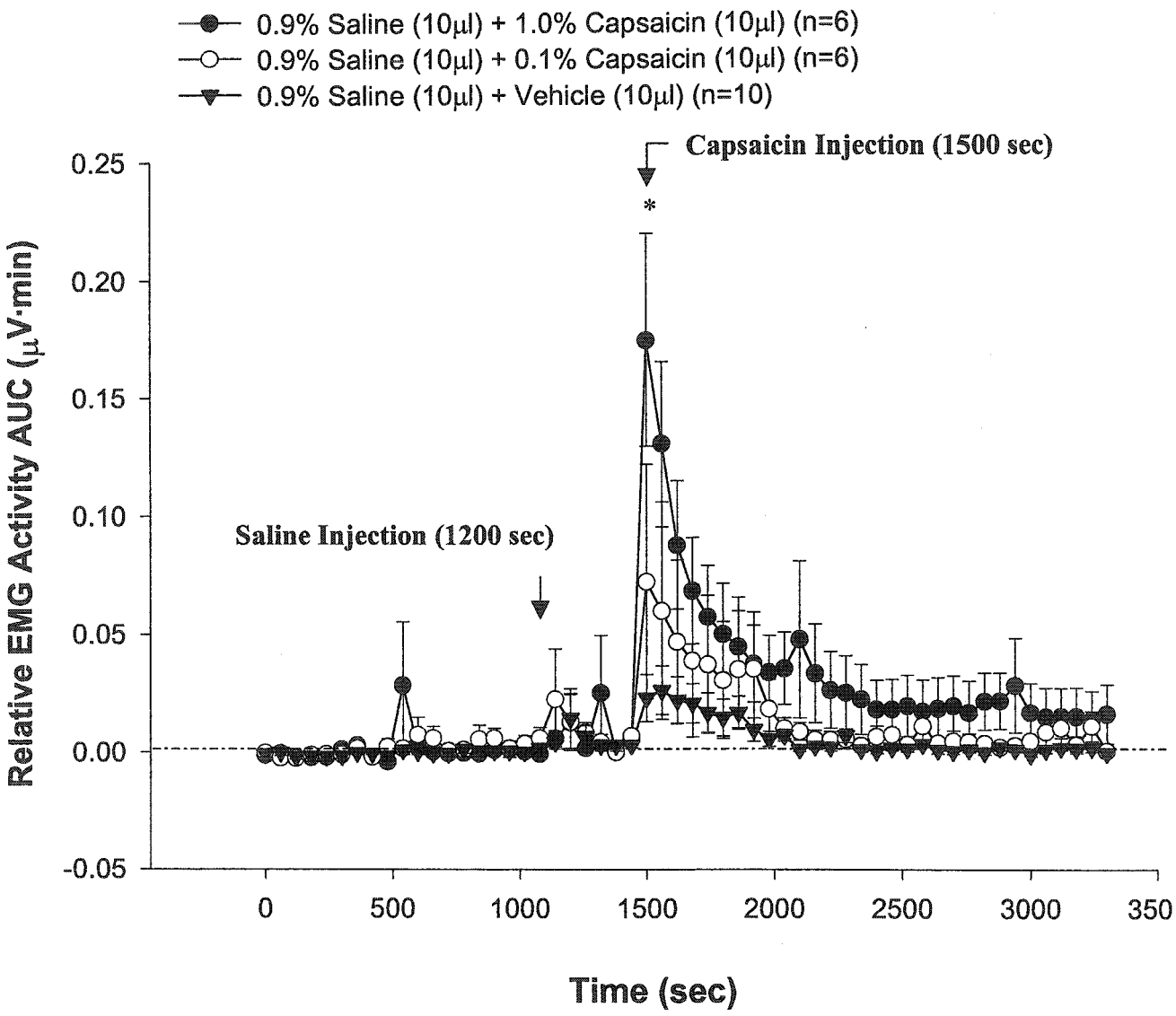


**Figure 18** ***Time Course of Capsaicin-Evoked Reflex Masseter EMG Activity:*** The mean changes in EMG area in the left masseter muscle evoked by the injection of various concentrations of capsaicin or vehicle control into the ipsilateral TMJ region. Each data point represents the mean ± SE of the normalized values relative to the baseline EMG activity in each rat and the horizontal dotted line indicates the mean baseline activity. '*' indicates the amplitude of the EMG activity of that data point was significantly higher than that evoked by the vehicle control (Tukey test, $p<0.05$).

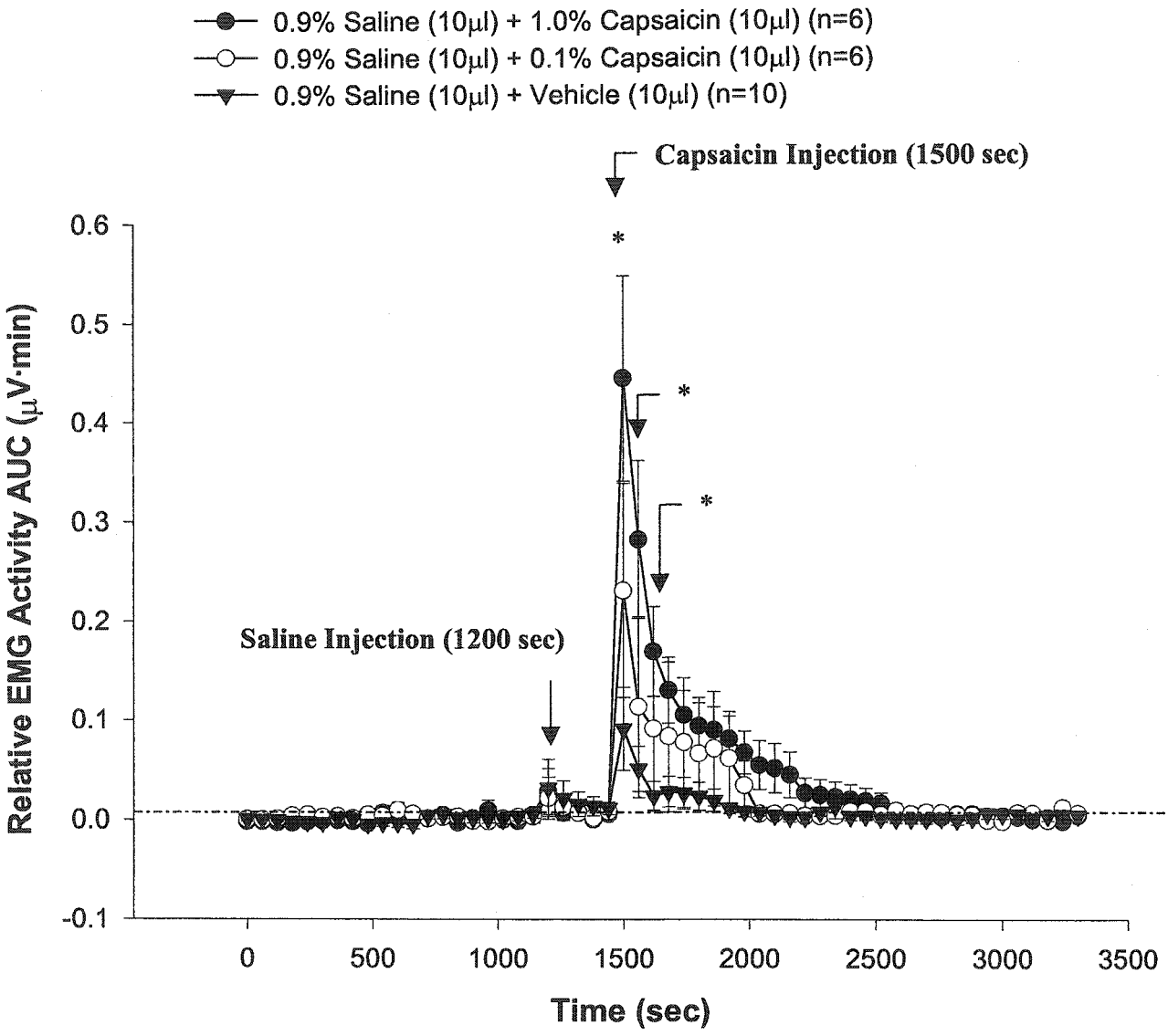


**Figure 19** ***Time Course of Capsaicin-Evoked Reflex Digastric EMG Activity:*** The mean changes in EMG area in the left digastric muscle evoked by the injection of various concentrations of capsaicin or vehicle control into the ipsilateral TMJ region. Each data point represents the mean ± SE of the normalized values relative to the baseline EMG activity in each rat and the horizontal dotted line indicates the mean baseline activity. '*' indicates the amplitude of the EMG activity of that data point was significantly higher than that evoked by the vehicle control (Tukey test, $p<0.05$).

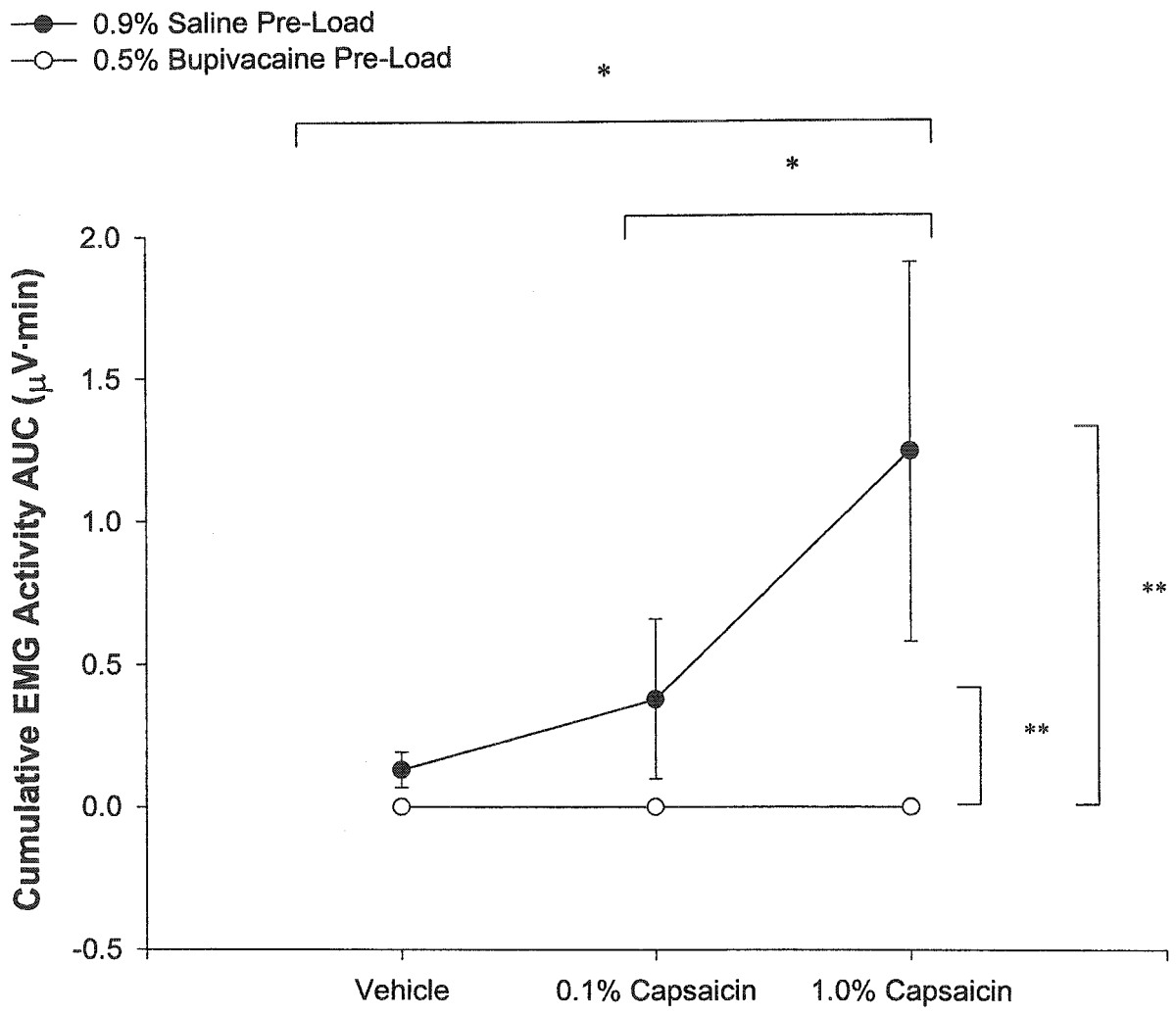


**Figure 20** ***Capsaicin-Induced Cumulative Masseter EMG Activity:*** The cumulative mean changes in EMG area in the left masseter muscle evoked by the injection of various concentrations of capsaicin or vehicle control into the ipsilateral TMJ region. Each data point represents the mean ± SE of the normalized values relative to the baseline EMG activity in each rat and the horizontal dotted line indicates the mean baseline activity. '*' indicates the cumulative EMG activity induced by 1% capsaicin was significantly higher in comparison with that evoked by both the 0.1% capsaicin solution and the vehicle control (Tukey test, $p<0.05$). '**' indicates statistical significance among the increased EMG activity in the masseter muscle evoked by capsaicin of various concentrations in saline pre-treated and bupivacaine pre-treated rats (Tukey test, $p<0.001$). In addition, the interaction between the concentration of capsaicin and the type of pre-load agent administered on the resultant reflex EMG activity in the masseter muscle was statistically significant.

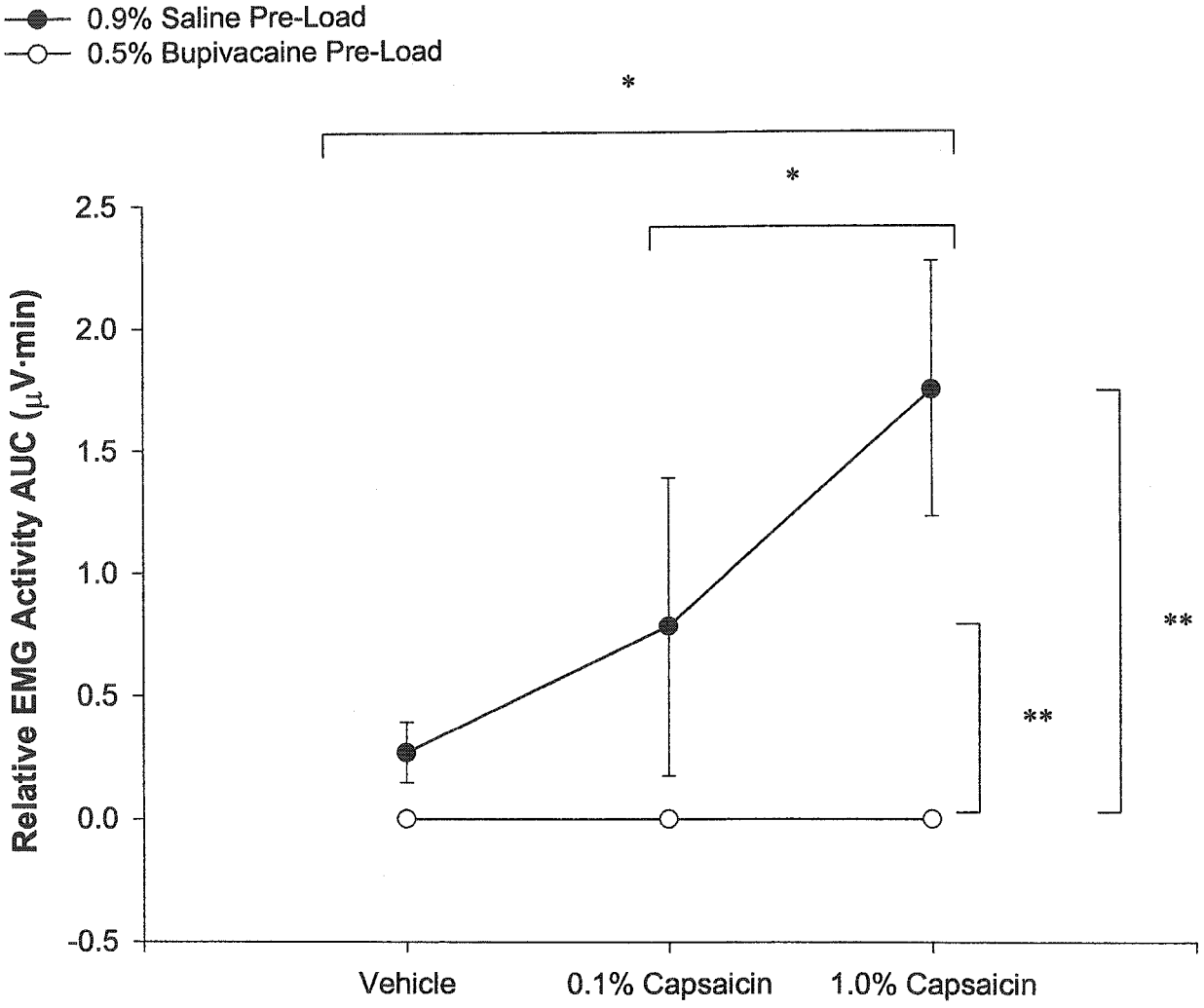


**Figure 21** ***Capsaicin-Induced Cumulative Digastric EMG Activity:*** The cumulative mean changes in EMG area in the left digastric muscle evoked by the injection of various concentrations of capsaicin or vehicle control into the ipsilateral TMJ region. Each data point represents the mean ± SE of the normalized values relative to the baseline EMG activity in each rat and the horizontal dotted line indicates the mean baseline activity. '*' indicates the cumulative EMG activity induced by 1% capsaicin was significantly higher in comparison with that evoked by both the 0.1% capsaicin solution and the vehicle control (Tukey test, $p<0.05$). '**' indicates statistical significance among the increased EMG activity in the digastric muscle evoked by capsaicin of various concentrations in saline pre-treated and bupivacaine pre-treated rats (Tukey test, $p<0.001$). In addition, the interaction between the concentration of capsaicin and the type of pre-load agent administered on the resultant reflex EMG activity in the digastric muscle was statistically significant.

**Figure 22** ***Example of a Post-Mortem Dissection***: Blue staining (outlined by a dotted black line) detected in the left TMJ region indicates plasma extravasation and correct placement of the catheter. (The black line indicates the zygomatic arch and the black circle indicates the external auditory meatus.)

# CHAPTER 5

# DISCUSSION

## 5.1 EFFECT OF CAPSAICIN APPLIED TO THE RAT TMJ REGION

### *5.1.1 Capsaicin-Induced Inflammation*

The application of capsaicin to the rat TMJ region resulted in an inflammatory reaction illustrated by the expansion of the periarticular tissue and plasma extravasation as demonstrated by Evan's blue dye staining in this area of interest. Since capsaicin binds to VR1 receptors located on primary sensory afferents innervating the rat TMJ region (Fiorentino *et al.,* 2000) to produce edema and plasma extravasation, the findings in this study suggest that acute inflammation of the rat TMJ region encompasses a neurogenic component.

The edema induced by capsaicin occurred in a dose-dependent manner whereby the resultant tissue expansion produced by the 1% capsaicin solution was significantly greater than that elicited by the 0.1% concentration and the vehicle solution. Contrary to a previous study (Fiorentino *et al.,* 2000), no significant difference in tissue expansion was demonstrated between the 0.1% capsaicin solution and the control. A possible explanation for this discrepancy may be the slight modification in the methodology for the application of capsaicin to the rat TMJ region. In this present investigation, 10 μl of capsaicin was applied after the administration of a 10 μl saline pre-load, whereas Fiorentino *et al.* (2000) employed a volume of 20 μl of capsaicin in the absence of a pre-load agent. The minute amount of 0.1% capsaicin solution administered may have been further diluted to a lower concentration as a result of the prior injection of a 10 μl saline pre-load in the rat TMJ region; thus, the

potential reduction in concentration may account for the lack of statistical significance in the amount of tissue expansion when compared with the vehicle group. Nevertheless, the findings in this present investigation are to some extent consistent with those by Fiorentino *et al.* (2000); therefore, suggest that the application of capsaicin to the rat TMJ region elicits an inflammatory response as indicated by tissue expansion.

Although the resultant plasma extravasation evoked by capsaicin injection into the TMJ region was not quantitatively analyzed with spectrophotometry, the observation of a graded response to the increasing concentration of capsaicin could be made with the naked eye. In addition, Fiorentino *et al.* (1999) demonstrated that edema development parallels plasma extravasation as measured by Evan's blue dye markings. Therefore, the capsaicin-induced plasma extravasation suggests that the acute inflammatory response in the TMJ region potentially acts through a neurogenic mechanism.

### *5.1.2 Capsaicin-Induced Reflex Jaw Muscle Activity*

A dose-dependent sustained and reversible increase in ipsilateral masseter and digastric muscle activities were evoked with the deposition of capsaicin into the rat TMJ region. Similar to Section 5.1.1, the 1% capsaicin solution evoked a significant increase in reflex EMG activity when compared with the 0.1% concentration and the control. Moreover, the 0.1% capsaicin solution did not elicit an increase in reflex EMG activity statistically different from that evoked by the vehicle solution. Again, this insignificance may be due to the smaller volume administered and the dilution of the 0.1% concentration by the prior injection of a saline pre-load; thus, potentially accounting for the discrepancy found between this present investigation and the study by Hu *et al.* (2001). Nevertheless, these results

support the hypothesis that the binding of capsaicin to VR1 receptors in the TMJ region elicits the activation of nociceptors and leads to a reflex jaw muscle response.

## 5.2 EFFECT OF LOCAL ANAESTHETIC PRE-TREATMENT ON CAPSAICIN APPLIED TO THE RAT TMJ REGION

### *5.2.1 Local Anaesthetic Effects on Capsaicin-Induced Inflammation*

To understand the characteristics of this potential neurogenic mechanism in the development of acute inflammation in the TMJ region evoked by capsaicin, the evaluation of the consequence of nerve conduction inhibition was performed. Although a complete blockade of nerve conduction was confirmed with the lack of EMG response to capsaicin, the amount of edema and plasma extravasation generated by the application of different concentrations of capsaicin did not significantly differ among the saline pre-treated and bupivacaine pre-treated rats. The comparison of the resultant tissue expansion elicited by 0.1% capsaicin depicted in Figure 10 revealed a trend towards the initial reduction in edema development in the bupivacaine pre-treated rats. However, this trend was not duplicated in the 1.0% capsaicin-induced tissue expansion and biological variance among the rats may explain for this difference. Thus, this present study demonstrated that the administration of local anaesthetic prior to the injection of capsaicin failed to significantly reduce the resultant tissue expansion in the TMJ region

### *5.2.2 Local Anaesthetic Effects on Capsaicin-Induced Reflex Jaw Muscle Activity*

Based on previous studies whereby the pre-treatment of the TMJ region with local anaesthetic inhibited the increased jaw muscle EMG response to mustard oil (Wong *et al.*,

2001; Yu *et al.*, 1995), it was hypothesized that the same results would be demonstrated when capsaicin was applied in lieu of mustard oil. Consistent with this assumption, the present study revealed that the pre-administration of local anaesthetic to the TMJ region prior to the application of capsaicin abolished the reflex ipsilateral masseter and digastric muscle activities. Regardless of the concentration of capsaicin injected, the local anaesthetic application blocked the resultant increased EMG activity. However, two out of twenty-six rats pre-treated with local anaesthetic elicited a reflex jaw muscle response to capsaicin. The possible reasons for failure to inhibit the increased activity may be due to the distribution of capsaicin to areas not anaesthesized, the ineffectiveness of the local anaesthetic block due to an inadequate mixture or the variation in onset of action of the local anaesthetic between different rats due to biological variance. Therefore, the hypothesis stating the application of local anaesthetic to the rat TMJ region prior to the administration of capsaicin will suppress the capsaicin-induced reflex jaw muscle activity is supported by the results in this present investigation.

## 5.3 INTERPRETATION OF RESULTS

### *5.3.1 Capsaicin-Induced Inflammation*

Based on the definition of neurogenic inflammation given in Section 1.2.1, the resultant plasma extravasation induced by capsaicin administration in the rat TMJ region suggests a neurogenic component in its inflammatory process; however, the lack of a significant reduction in capsaicin-induced edema in the local anaesthetic pre-treated TMJ region questions the true mechanism underlying neurogenic inflammation and the pure neurogenic nature of the inflammatory irritant, capsaicin. The findings from this

investigation can be interpreted in two ways. Firstly, capsaicin evokes the direct release of pro-inflammatory mediators from neuronal terminals without the conduction of action potentials along the axon. Alternatively, capsaicin predominantly acts in a non-neurogenic fashion when applied to the TMJ region due to its inherent inflammatory nature or the activation of VR1 receptors located on other cellular components.

#### 5.3.1.1 Capsaicin-Induced Direct Release of Pro-Inflammatory Mediators from Neuronal Terminals Without Axonal Conduction

In the event that capsaicin is indeed purely acting in a neurogenic fashion, the lack of the necessity for nerve conduction disproves both the classical and current concepts of neurogenic inflammation. Past studies have argued that the axon reflex (Lewis, 1927) and dorsal root reflex (Eccles *et al.,* 1961, 1962; Koketsu, 1956; Willis, 1999) are mechanisms underlying the neurogenic inflammatory process. In addition, both the CNS and autonomic nervous system have been implicated in the development of neurogenic inflammation. However, the results in the present investigation suggest otherwise. In the absence of central and autonomic descending influences, spinal reflex loops and antidromic conduction from adjacent terminal nerve branches, the resultant capsaicin-induced neurogenic inflammatory reaction was equivalent to that produced in the presence of functional nerve conduction mechanisms. Therefore, this finding leads to the hypothesis that pro-inflammatory mediators can be directly released from afferent nerve terminals without the conduction of nerve impulses.

Unfortunately, the body of evidence supporting the above potential mechanism in the development of neurogenic inflammation is limited and the findings from previous studies

evaluating the effect of local anaesthetic on the neurogenic inflammatory process have been inconsistent.

Earlier studies by Jancso *et al.* (1968) examined the effects of local anaesthetic on PE in the rat trigeminal system. The pain reaction induced by the application of capsaicin to the eye was prevented by pre-treatment with local anaesthetic; however, no difference was detected in the level of plasma extravasation when compared with controls. Consequently Jancso and his colleagues extended the evaluation of the effect of local anaesthetic on capsaicin-induced neurogenic inflammation to humans. Initially, capsaicin was applied intradermally to patients with an unilateral desensitized arm due to previous sensory nerve injury and the reaction was compared with the one produced on the contralateral arm. The capsaicin only induced a minor inflammatory reaction on the insensitive arm; however, the contralateral arm elicited an edema and hyperemic response. Subsequently, local anaesthetic was applied to healthy volunteers prior to the application of capsaicin and results revealed the abolishment of the flare response, but edema and redness still persisted. Therefore, Jancso *et al.* (1968) suggested that discrepancy between the reaction in subjects with sensory nerve damage and subjects with intact afferents may be explained by the depletion of mediators in damaged afferents. Thus, these findings demonstrated that axonal conduction is not necessary for the capsaicin-induced plasma extravasation and edema development since prior local anaesthetic treatment does not abolish the reaction; however, intact afferent innervation is a crucial element in the neurogenic inflammatory process.

Similarly, Szolcsanyi *et al.* (1998) demonstrated the release of sensory neuropeptides such as SP and CGRP from the peripheral endings of sensory nerves innervating the rat trachea in the presence of local anaesthesia, suggesting a direct mechanism for peptide

release not mediated by an axon reflex or central descending influences. Therefore, this finding further supports the hypothesis that capsaicin-induced neurogenic inflammation may lead to the direct release of pro-inflammatory mediators without the need for the conduction of nerve impulses.

Recently, Wong *et al.* (2001) provided further evidence in agreement with the direct activation of neuronal terminals in the development of neurogenic inflammation. Similar to the protocol utilized in the present study, Wong *et al.* (2001) revealed the ineffectiveness of local anaesthetic pre-treatment of the TMJ region on mustard-oil induced neurogenic inflammation and thus, concluded the direct release of inflammatory mediators from neuronal terminals to be the potential mechanism underlying the neurogenic component of inflammation in the TMJ.

Contrary to the above findings, Lundberg and Saria (1983) demonstrated the effectiveness of lidocaine in preventing neurogenic PE in the airway mucosa and argued against the direct release of pro-inflammatory mediators from afferent nerve terminals. Subsequently, Dux *et al.* (1996) re-examined the effect of local anaesthetic on the neurogenic inflammatory response of the rat skin. The pre-treatment of the skin with local anaesthetic followed by the application of mustard oil led to the inhibition of mustard oil-induced neurogenic inflammation. Therefore, in contrast to the findings in this present investigation, Dux *et al.* (1996) revealed that axonal conduction blockade inhibited PE in a dose-dependent manner. Moreover, these findings suggest that functional nerve conduction is necessary for the development of neurogenic inflammation; thus, contradicting the present findings. Nonetheless, the discrepancies between various investigations may be due to tissue specificity and specie differences.

### 5.3.1.2 Capsaicin-Induced Inflammation by Non-Neurogenic Mechanisms

The assumption that capsaicin may induce inflammation predominantly in a non-neurogenic fashion is paradoxical since it is known as an important pharmacological tool used to distinguish a subset of nociceptive sensory neurons as depicted in Figure 22. However, since this agent is an inflammatory irritant, it is conceivable that a general inflammatory response may be evoked by the innate immune defense system due to its recognition of capsaicin as a foreign substance. Therefore, an inflammatory response is activated regardless of functional nerve conduction.

As mentioned in Section 1.2.8, the neurogenic release of pro-inflammatory mediators is only one component of a highly integrated non-specific immune defense system. Along with neuropeptides released from neuronal terminals, non-neurogenic inflammatory factors may be released by capsaicin. For example, an *in vitro* investigation has localized VR1 receptors on mast cells (Biro *et al.,* 1998b); however, the application of capsaicin was unsuccessful at degranulating the mast cells. Nonetheless, the assumption that similar events may occur *in vivo* cannot be made and further investigation is required to support this hypothesis. In the event that mast cells degranulate with the binding of capsaicin to its VR1 receptors, there is the possibility that the release of histamine may mediate inflammation by its direct action on the vasculature. Thus, hypothetically, if capsaicin acts purely in a non-neurogenic manner and activates the degranulation of mast cells in the TMJ region, the secretion of histamine may be sufficient for the initiation of an inflammatory response. Furthermore, the non-neurogenic behaviour of capsaicin may be due in part to the characteristics of the primary sensory neurons innervating the rat TMJ regions. A study has revealed that the ability of afferents to produce neurogenic inflammation is dependent on the

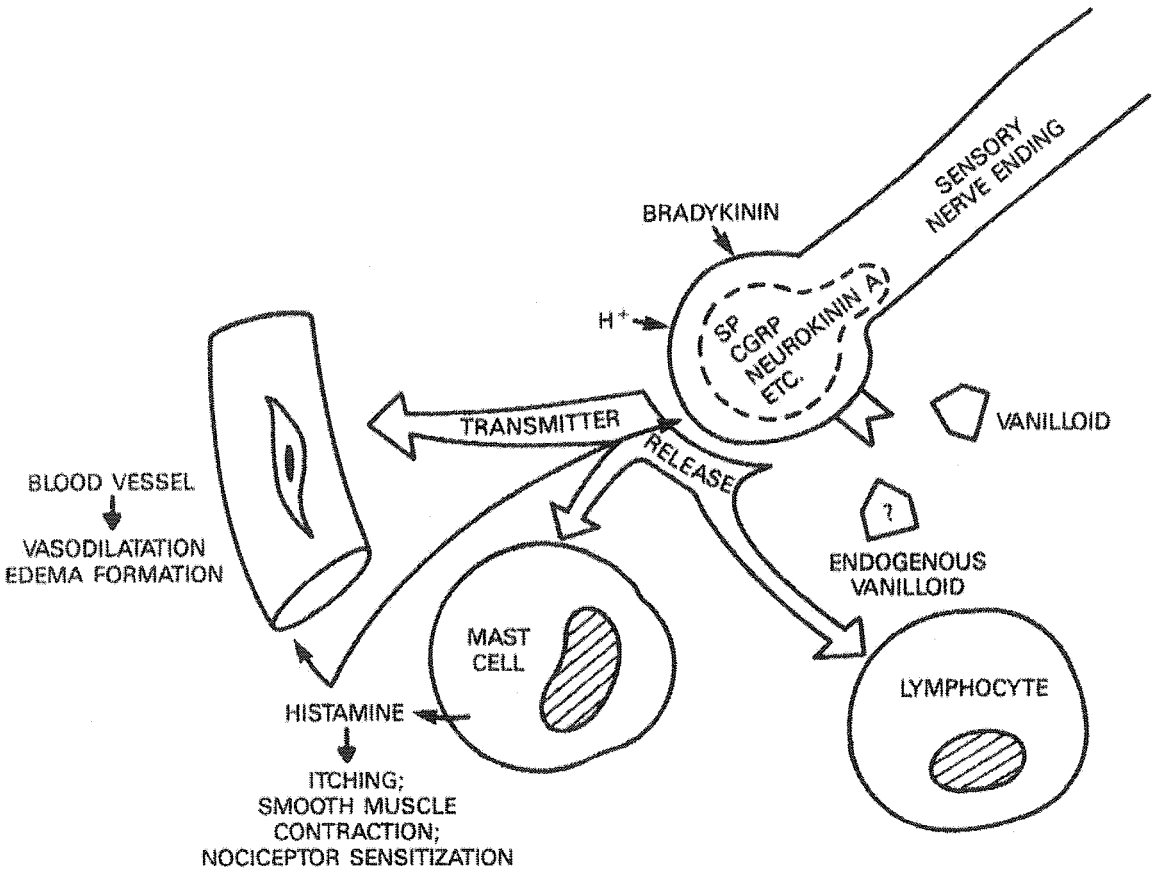


**Figure 23** **Schematic Illustration of the Role of Peripheral Capsaicin Sensitive Nerve Endings in Evoking Neurogenic Inflammation.**
Adapted from *Szallasi & Blumberg, 1999.*

particular tissue it innervates (McMahon *et al.*, 1989). Cutaneous afferents re-innervating muscle and muscle afferents re-innervating skin led to a decrease and increase in neurogenic edema, respectively. Therefore, it was suggested that there may be some tissue-specific trophic influences on the development of neurogenic inflammation. Although this investigation did not evaluate afferents innervating joint spaces, there is a possibility that the afferents innervating the TMJ region cannot elicit neurogenic inflammation and instead the surrounding tissues respond to capsaicin by eliciting a generalized inflammatory response initiated by the host defense system.

Moreover, the sensory innervation of the TMJ region may not have the physiological ability to generate neurogenic inflammation. Uddman *et al.* (1998) demonstrated that the primary afferents supplying the rat TMJ consist of only a few SP containing nerves. Therefore, due to the lack of the main neuropeptide implicated in neurogenic inflammation, the administration of capsaicin to this periarticular temporomandibular region could potentially lead to the release of other inflammatory factors by non-neurogenic mechanisms. However, the study by Kido *et al.* (1993) contradicts the aforementioned investigation. A moderate amount of SP-LI nerve fibers were located in the joint capsule, articular disc, periosteum and synovial membrane. Thus, the ability or inability of sensory nerves innervating the rat TMJ to elicit neurogenic inflammation is unclear.

### *5.3.2 Capsaicin-Induced Reflex Jaw Muscle Activity*

The application of capsaicin to the rat TMJ region evoked a reflex EMG response in the ipsilateral masseter and digastric muscles, and the prior application of local anaesthetic to the TMJ region abolished the capsaicin-induced reflex jaw muscle activity. Therefore, these

findings suggest that capsaicin-induced activation of primary afferent nociceptors generates action potentials conducted to the CNS and subsequently, efferent nerves innervating the ipsilateral masseter and digastric muscles are stimulated to elicit a reflex response. Moreover, the inhibition of reflex jaw muscle activity by the pre-treatment of the TMJ region with local anaesthetic provides evidence for the existence of a central neural pathway that associates capsaicin-induced activation of afferents innervating the TMJ region and the reflex jaw muscle activity.

Unfortunately, the methodology for this investigation cannot confirm the exact central neural pathway underlying the capsaicin-evoked reflex EMG response in the jaw muscles. However, based on previous investigations on reflex jaw muscle activity in response to inflammatory (Yu *et al.*, 1995, 1996) and non-inflammatory irritants (Cairns *et al.*, 1998, 1999, 2001a, b, c) administered to the rat TMJ region, an hypothesis on the potential central-mediated reflex can be drawn. Although Vo and Vi have been implicated in the orofacial nociceptive mechanisms (Dallel *et al.*, 1988, 1990), Vc has been demonstrated to be the essential relay site for nociceptive information from superficial and deep craniofacial tissues (Cairns *et al.*, 2001b; Tsai *et al.*, 1999). Hence, the extrapolation of the findings from other studies on the reflex jaw response leads to the reasonable assumption that capsaicin sensitive primary afferents innervating the TMJ also project to the Vc for conduction of nociceptive information to higher brain centers and the subsequent activation of efferent nerves innervating the jaw muscles (see Figure 23).

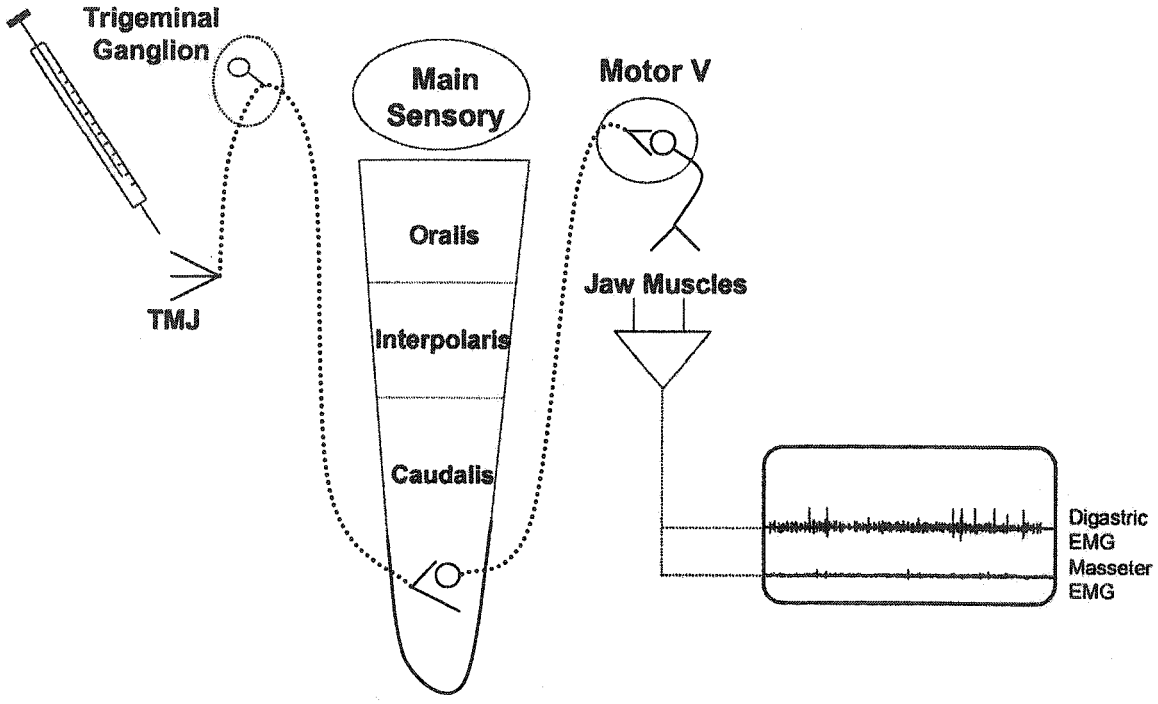


**Figure 24** **Proposed Neural Pathway for the Application of Capsaicin into Rat TMJ Region to Evoke Ipsilateral Jaw Muscle Reflex Activity**
Adapted from *Cairns et al., 2001a.*

### *5.3.3 Paradoxical Action of Local Anaesthetic*

In light of the tissue expansion results, the hypothesis of the release of neuropeptides from sensory afferent nerve terminals may be explained by the recent study illustrating the paradoxical action of local anaesthetics. Vlachova *et al.* (1999) demonstrated the application of procaine and bupivacaine led to an excitation of capsaicin-sensitive DRG neurons; thus, the administration of local anaesthetic may result in the paradoxical activation of sensory afferents innervating the rat TMJ region. However, the lack of capsaicin-induced reflex jaw muscle response to the locally anaesthetized periarticular temporomandibular region is not consistent with the above finding. In addition, the aforementioned investigation is an *in vitro* study and may not be applicable in an *in vivo* situation. Nevertheless, the excitation of afferent nerves may only occur peripherally and nerve impulses may not be conducted to the CNS.

## 5.4 STRENGTHS AND LIMITATIONS OF METHODOLOGY

Modifications to this rat model of acute inflammation in the TMJ region have rendered it a great advantage over earlier studies. Previous investigations utilized the Evan's blue dye staining technique and sacrificed the rat at a single time point to retrieve inflammatory tissue representing that particular time point for spectrophotometric analysis (Haas *et al.*, 1992; Midroni, 1996). However, the adoption of the modified technique by Fiorentino *et al.* (1999) has allowed the development of the inflammatory process to be followed over a continuous time course. Therefore, one rat can provide many data points as opposed to a single rat per time point. In addition, the Evan's blue dye technique (for example, Midroni, 1996) required the dye to be distributed through the vascular system for at least 10 minutes before the rat can be sacrificed. Thus, if the initial 5 minutes of the

inflammatory reaction were being assessed and the tissue cannot be retrieved until 10 minutes later; thus, the resultant plasma extravasation being evaluated is theoretically the 15 minute mark. Therefore, technically, the clear indication of an inhibition in edema development at 5 minute mark cannot be evaluated. Thus, the evaluation of tissue expansion over an entire time course provides an improved means of assessing the inflammatory reaction.

Although improvements in the protocol have been made, there are a few limitations associated with this particular animal model. First, the stabilization of the rat's head requires a block of plaster to be placed on the contralateral side to the injection site. This results in the loss of the contralateral side as the control and additional animals are required to serve as appropriate controls. In addition, the stabilization led to the inability to record EMG readings from the contralateral masseter and digastric muscles; thus, the presence of spinal reflex loops associated with capsaicin-induced jaw muscle reflex could not be evaluated.

Secondly, the rat TMJ is very small and the placement of the catheter is mainly performed by tactile sensation; thus, the confirmation of the correct location of the catheter can only be associated with Evan's blue dye markings in and around the TMJ. However, this link may be inaccurate since the catheter may not be in the TMJ capsule and merely in the TMJ region only. Therefore, conclusions from this investigation must carefully elucidate the application of capsaicin into the TMJ region as opposed to into the joint *per se*. In addition, the introduction of the catheter into the TMJ capsule may have potentially damaged some of the nociceptive afferents, leading to inaccurate EMG recordings and resultant tissue expansion. However, an electromyographic study has demonstrated that a substantial

amount of afferents is left functionally intact to provoke jaw reflexes despite the physical trauma from catherization (Yu *et al.,* 1995).

Thirdly, the use of anaesthesia may potentially confound the EMG recordings since there is evidence demonstrating the ability of halothane to sensitize cutaneous nociceptive afferents to noxious thermal but not mechanical stimulation (Campbell *et al.,* 1984). However, the concentrations employed were at levels greater than that utilized in this protocol and the effect of halothane on the response of deep afferents to chemical stimuli has yet to be evaluated. Thus, the anaesthetic regimen could have lead to erroneously enhanced EMG reflexes and neurogenic tissue expansion.

Lastly, the feasibility of the extrapolation of results from an animal study to humans is questionable. Recent evidence has revealed dissimilarities between rats and humans with respect to biochemical mechanisms of neurogenic inflammation and renders researchers less inclined to apply animal findings to humans. With the use of microdialysis techniques, Petersen *et al.* (1997) evaluated *in vivo* levels of SP and histamine following capsaicin application to the human volar forearm. In contrast to rat skin, the intradermal or topical capsaicin application to human skin did not lead to the release of histamine or SP. On the other hand, the injection of SP resulted in a similar inflammatory reaction and evoked the release of histamine. Thus, these investigators concluded that in human skin, SP is not the mediator of neurogenic inflammation. Likewise, a study by Schmelz *et al.* (1997) revealed equivalent findings. Moreover, Sauerstein *et al.* (2000) evoked a neurogenic inflammatory response in both rat and human skin by transcutaneous electrical stimulation via microdialysis technique. While this stimulus provoked neuropeptide release and vasodilation in both rat and human skin, only neurogenic protein extravasation was evoked in rat skin.

Therefore, the above studies demonstrate the existence of species differences in the mechanism of neurogenic inflammation and extrapolations from animal studies to humans must be made with caution.

## 5.5 FUTURE DIRECTIONS

The results of this investigation leads one to question whether or not capsaicin can indeed elicit neurogenic inflammation without axonal conduction. The suggestion that pro-inflammatory mediators are released from afferent nerve terminals despite conduction blockade leads to the hypothesis that the depletion of mediators within afferent neuronal terminals will eliminate the PE response evoked by capsaicin. This theoretical concept assumes that capsaicin functions purely in a neurogenic manner; however, in the event that capsaicin has both non-neurogenic and neurogenic potential to elicit plasma extravasation, the proportion of inflammation due to neurogenic influence will be evident.

Two possible methods for depleting the mediators would be either by capsaicin-induced desensitization or the surgical denervation of the nerve of interest. Desensitization of primary afferent nerve fibers with the use of capsaicin has been shown to selectively deplete primary afferent nociceptive neurons of mediators such as SP by causing an initial violent release from terminals (Jancso *et al.,* 1980). Therefore, one prospective study could render the primary afferents desensitized with the pre-treatment of tissues with capsaicin and the response to subsequent administration of capsaicin can be evaluated. The second option is the surgical denervation of the trigeminal nerve by cauterization, leading to the degeneration of afferent supply to the TMJ (Jancso *et al.,* 1967) and the subsequent depletion of mediators. Thus, if the neurogenic mechanism underlying the inflammatory response in

the TMJ involves the direct activation of neuronal terminals without axonal conduction, the lack of mediators in nociceptive terminals would significantly reduce the edema response evoked by capsaicin.

Furthermore, an indirect protocol for evaluating the hypothesis that mediators are released despite axonal conduction blockade would be to evaluate the effect of the local anaesthetic with and without the administration of a SP antagonist, specifically a $NK_1$ receptor antagonist, prior to the injection of capsaicin. Since SP is the main mediator involved in neurogenic inflammation, by preventing its action through the blockage of $NK_1$ receptors, the resultant edema response should be significantly diminished. In addition, antagonists for other pro-inflammatory mediators can be used to pre-treat the tissue, such as CGRP receptor antagonists.

Other assumptions drawn from the results include the lack of a necessity for a central and sympathetic involvement in capsaicin-induced edema in the TMJ region. To specifically assess the potential involvement of the central nervous system, antagonists (such as $GABA_A$ receptor antagonists, bicuculline or the non-NMDA receptor antagonist, CNQX) can be deposited into the dorsal horn of the spinal cord to validate the role of a dorsal root reflex in capsaicin-induced edema or alternatively, lesions in the trigeminal brainstem subnucleus caudalis can be made to evaluate the involvement of higher brain centers in the development of neurogenic inflammation. In addition, the existence of a sympathetic component in capsaicin-induced edema can be evaluated with the surgical procedure sympathectomy through several chemical means such as guanethidine and 6-OHDA.

In addition to the potential involvement of central and autonomic nervous systems, the cellular and biochemical mechanisms involved in capsaicin-induced inflammation also

require investigation since the possibility that capsaicin may act non-neurogenically in the TMJ region must not be disregarded. The specific roles played by histamine and mast cells are of greatest concern due in part to the existence of VR1 receptors on mast cells (Biro *et al.*, 1998b) and the activation of mast cells by SP to release histamine. The application of $H_1$ antagonists prior to the injection of capsaicin should reveal whether inflammation in the TMJ is heavily dependent on the release of mast cells. Alternatively, genetically manipulated mast cell deficient rats can be utilized to evaluate the proportion of the acute inflammatory response in the TMJ which relies on the binding of capsaicin to VR1 receptors on mast cells.

Moreover, the EMG data have demonstrated that the application of capsaicin into the TMJ region evokes reflex jaw muscle activity through the activation of VR1 receptors located on afferent nerve terminals and the finding that local anaesthetic is capable of abolishing this reflex indicates the requirement for functional nerve conduction pathways for the increased muscle activity to occur. However, the exact central neural pathways underlying the capsaicin-evoked reflex EMG response in the jaw muscles are presently unknown. Therefore, a future investigation should entail the surgical placement of lesions in the various subnuclei (Vo, Vi, Vc) of the trigeminal brainstem spinal tract to determine which subnuclei is involved in the relay of the noxious stimuli leading to capsaicin-induced reflex jaw muscle activity. In addition, the contralateral masseter and digastric muscles should also be evaluated to determine if there are spinal reflex loops leading to bilateral reflex EMG responses.

Therefore, the results from this present investigation provide the stepping stone for future studies involving the mechanism of neurogenic inflammation in the TMJ region and other tissues, along with preliminary information on the central neural pathway involved in

capsaicin-induced reflex jaw muscle activity. The demonstration of the potential existence of the release of pro-inflammatory mediators in the absence of central and autonomic descending influences, and axon reflexes question the true mechanisms underlying neurogenic inflammation. Thus, more investigations on capsaicin-induced neurogenic inflammation in various tissues are warranted.

# CHAPTER 6

# CONCLUSION

## 6.1 SUMMARY

The application of capsaicin to the rat TMJ region evoked a dose-dependent increase in tissue expansion and reflex EMG activity in the ipsilateral masseter and digastric muscles. However, only the 1% capsaicin solution generated a significant edema and jaw reflex response.

Regardless of the concentration of capsaicin administered, the pre-treatment of the rat TMJ region with local anaesthetic failed to inhibit the capsaicin-induced tissue expansion. Thus, the edema development occurred independent of axonal conduction, suggesting the direct release of pro-inflammatory mediators from primary afferent nerve terminals. Hence, contrary to traditional belief, this hypothesis leads to the assumption that central and autonomic descending influences, spinal reflex loops along with antidromic stimulation from adjacent nerve terminals are not required for the development of neurogenic inflammation.

Alternatively, the lack of local anaesthetic effect on the resultant inflammatory response may be due to the potential non-neurogenic nature of capsaicin in the TMJ region or the inability of primary afferents innervating the TMJ region to produce neurogenic inflammation.

On the other hand, the capsaicin-induced reflex EMG activity in the jaw muscles was abolished by the prior injection of local anaesthetic to the rat TMJ region. Thus, this finding suggests the jaw muscle response to capsaicin applied to the rat TMJ region is centrally mediated; however, the central neural pathway remains unknown.

## 6.2 CLINICAL IMPLICATION OF CONCLUSIONS

The findings in this present investigation suggest the activation of VR1 receptors on afferent nerves innervating the TMJ region evokes edema development and plasma extravasation; thus, indicating the possibility of the presence of a neurogenic component in the inflammatory process involving the TMJ region. Unlike other tissues, the TMJ region may produce inflammation independent of axon reflexes, dorsal root reflexes, and central and autonomic descending influences; thus, afferents innervating this region may release pro-inflammatory mediators in the absence of axonal conduction. Therefore, to prevent the effects of neurogenic inflammation in the TMJ, anti-inflammatory agents should either deplete the mediators in neuronal terminals or directly inhibit the action of pro-inflammatory mediators.

# CHAPTER 7

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# APPENDIX A

# PILOT STUDY

***Purpose:***

To determine the dosages of capsaicin effective in producing a neurogenic inflammatory response in the rat TMJ region pre-treated with saline and to determine the timing and the amount of capsaicin to be injected after locally anaesthesizing the rat TMJ region.

***Hypotheses:***

1. *The following dosages will be effective in producing a neurogenic inflammatory response: 1.0%, 0.1% and 0.01% Capsaicin.*

   The rationale underlying this hypothesis is based on Fiorentino *et al.* (2001), whereby the authors demonstrated the administration of the above concentrations produced significant amounts of tissue expansion with respect to each of the different concentrations and the control. Therefore, the addition of a saline pre-load should not alter the resultant inflammatory response to capsaicin.

2. *A volume of 20 μl of capsaicin injected 20 minutes after the pre-treatment of the rat TMJ region with 0.5% bupivacaine will not evoke a reflex EMG activity.*

   The rationale underlying this hypothesis is based on Wong *et al.* (2001) and Hu *et al.* (2001). Wong *et al.* (2001) demonstrated the duration of action for 0.5% bupivacaine to be no less than 30 minutes and prior trial experiments revealed an

increase in EMG activity in response to the saline pre-load; thus, the 20 minute interval allowed sufficient time for the muscle activity to return back to baseline levels and the conduction blockade in the TMJ region will remain present. In addition, Hu *et al.* (2001) illustrated the reflex EMG response of the masseter and digastric muscles to the application of capsaicin to the rat TMJ region. Therefore, theoretically, the pre-treatment of the TMJ region with local anaesthetic should prevent the conduction of nerve impulses and subsequent reflex EMG activity will be abolished.

***Methodology:***

The general set-up and specific description of the Tissue Expansion/Nociceptive Reflex Jaw Muscle Activity model are presented in Chapter III; however, there are differences in the amount and concentrations of capsaicin administered, the timing of the injection of capsaicin, and the time intervals at which tissue expansion measurements were taken.

A volume of 20 µl of 1.0% or 0.01% capsaicin was injected at $time_{20}$ (i.e., 20 minutes after the injection of the pre-load agent) or $time_{10}$ or $time_5$, and tissue expansion measurements were taken every 5 minutes over a continuous period of 150 minutes. Subsequently, the volume of capsaicin was reduced to 10 µl and injected at $time_5$ (i.e., 5 minutes after the injection of the pre-load agent).

***Results:***

The application of capsaicin to the TMJ region led to the development of edema and plasma extravasation. The resultant tissue expansion occurred in a dose-dependent manner as illustrated in Figure A. The 1.0% capsaicin solution produced a significant amount of edema as compared with the 0.01% concentration and the control; however, the 0.01% concentration did not elicit an edema response different from that of the control.

Regardless of the time interval between the pre-treatment of the TMJ region with local anaesthetic and the injection of 20 μl capsaicin, a 2-3 second delayed increase in EMG activity was evoked by the administration of capsaicin. However, the application of 10 μl of capsaicin after the injection of local anaesthetic did not elicit a reflex jaw muscle response.

***Discussion & Conclusion:***

The dose response curve in Figure A clearly depicts the inefficacy of the 0.01% capsaicin concentration in evoking a significant edema response; thus, concentrations equal to or less than 0.01% would not provide valuable information on the acute inflammatory response in the rat TMJ region. Therefore, only 1.0% and 0.1% capsaicin solutions are utilized in the present investigation.

This pilot study illustrated that the volume of capsaicin administered was a crucial factor. The initial methodology incorporated an amount of 20 μl of capsaicin as based on Fiorentino *et al.* (2000), however, regardless of the time interval between the injection of the local anaesthetic and capsaicin (5, 10 or 20 minutes), a reflex EMG response would be elicited. Therefore, the volume of capsaicin was reduced to 10 μl on the assumption that the original volume of capsaicin distributed to a greater area than the local anaesthetic.

Moreover, there was a delayed increase in EMG activity and this latency may be due to the time required to spread to areas not anaesthesized. Previously, Midroni (1996) had addressed an equivalent dilemma by doubling the amount of local anaesthetic with respect to the volume of mustard oil administered and found no difference in the resultant tissue expansion. Similarly, Wong *et al.* (2001) ruled out the possibility of volumetric explanations by arguing the fact that mustard oil is a viscous agent, while local anaesthetic is an aqueous solution; thus, the area of distribution of mustard oil most likely does not spread a substantial distance due to its viscous nature. However, the reduction of the volume of capsaicin to 10 μl along with the pre-treatment of the TMJ region with local anaesthetic proved to eliminate the capsaicin-induced reflex increase in the ipsilateral masseter and digastric muscle activities. Therefore, there is sufficient evidence to support the utilization of 10 μl of capsaicin in the present study.

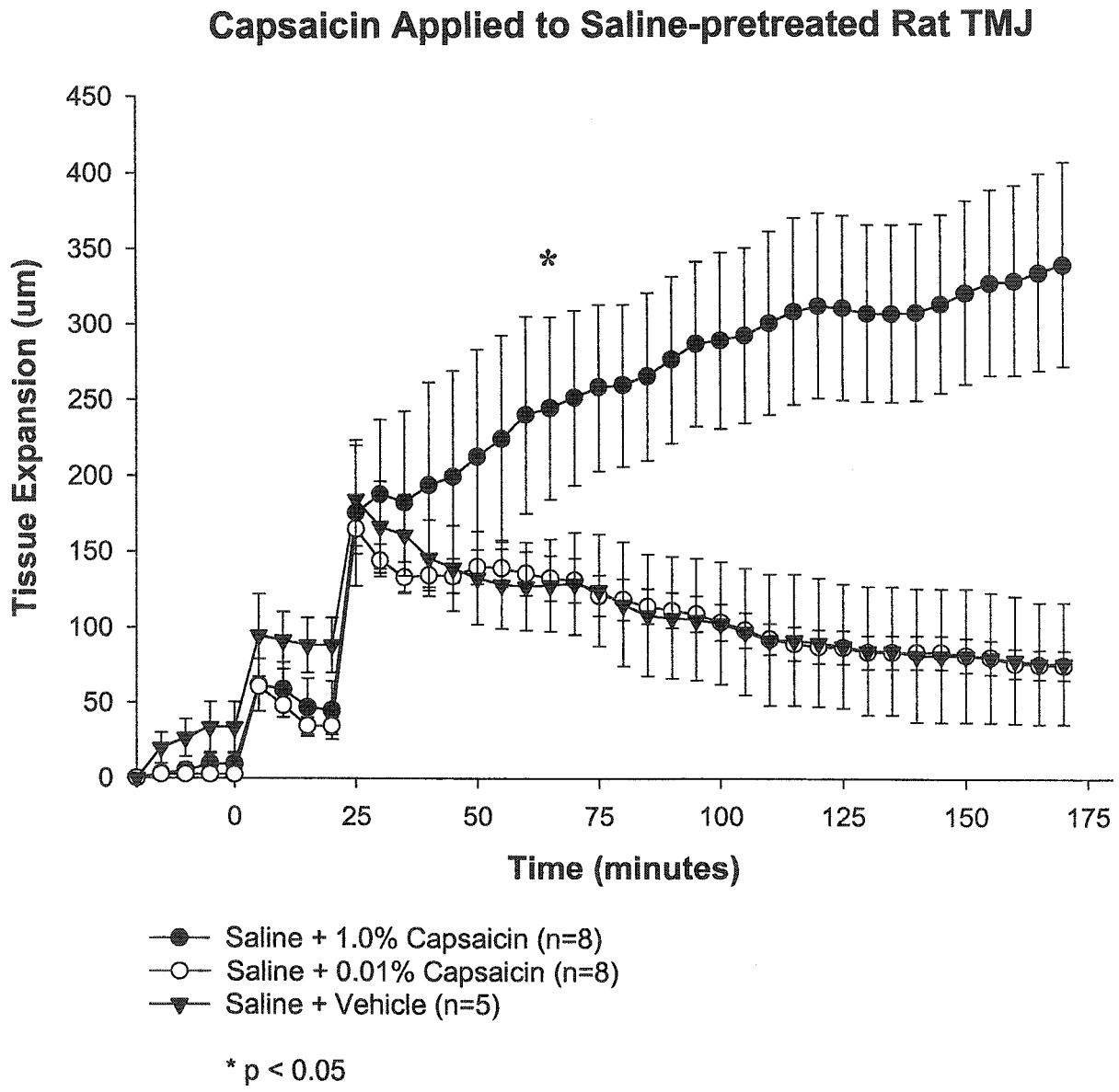


**Figure A** ***Dose Response Curve: Tissue Expansion versus Increasing Capsaicin Concentration:*** The mean changes in tissue expansion evoked by the injection of various concentrations of capsaicin or vehicle control into the left TMJ region. Each data point represents the mean tissue expansion ± SE for a certain time point over the 180 minute time course in eight rats. '*' indicates that the tissue expansion was significantly higher in comparison with that evoked by the 0.1% solution and vehicle control from $time_{65}$ and onwards (ANOVA, $p<0.05$).