AN ANIMAL MODEL OF BURNING MOUTH SYNDROME (BMS) FOR ASSESSMENT OF PERIPHERAL ɣ-AMINOBUTYRIC ACID A ($GABA_A$) RECEPTORS AS AN ANALGESIC TARGET

by

Sun Nee Tan

B.Sc. (Hon), University of London (King's College), 2007

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Abstract

Burning Mouth Syndrome (BMS) is a chronic pain syndrome characterised by burning pain in patients with clinically normal oral mucosa (Grushka et al., 2002). The prevalence of BMS increases with age and occurs far more commonly in women, with estimated female to male ratios ranging from 3:1 to 16:1 (Grushka, 1987b, van der Ploeg et al., 1987, Clark G.T, 2005). In women, there is a strong correlation between the development of BMS and the onset of menopause (Lipton et al., 1993, Bergdahl and Bergdahl, 1999). A proposed theory is that deprivation of oestrogen produces atrophic changes within the oral epithelium (Valimaa et al., 2004) leading to symptoms of BMS. Therefore, I employed immunohistochemistry and electrophysiology techniques to test the hypotheses that **ovariectomised rats will show reduced nerve fibre densities and lower proportions of peptidergic neuronal fibres (small-diameter fibres) in the tongues leading to reduced thermal and mechanical thresholds of afferent fibres.** A non-conventional treatment for BMS that seems effective (Gremeau-Richard et al., 2004) is sucking a tablet of the benzodiazepine (BZD), clonazepam, without swallowing. This treatment is speculated to decrease pain by activating peripheral γ-aminobutyric acid receptors A ($GABA_A$) receptors in the oral cavity. Expression of $GABA_A$ receptors in tongue afferent fibres has yet to be demonstrated. Also, evidence for a direct effect of BZDs on nociceptors in the oral mucosa is lacking. **My hypotheses are: $GABA_A$ receptors are present on both peptidergic and non-peptidergic nerve fibres. Activation of peripheral $GABA_A$ receptors will increase mechanical thresholds of tongue nerve fibres** which would explain the therapeutic efficacy of topical clonazepam in human BMS. This study found high proportions of $GABA_A\gamma_2$-containing, peptidergic (94%) and $GABA_A\gamma_2$-containing, non-peptidergic (93%) tongue

axonal fibres of intact female rats. In intact females, muscimol and γ-aminobutyric acid (GABA) solutions had no effect on relative mechanical thresholds of afferent fibres. After thermal stimulation, muscimol significantly increased relative mechanical thresholds of afferent fibres. Ovariectomised and sham-operated rats did not differ in any of the parameters measured. Topical applications of muscimol and bicuculline solutions did not have any significant effect on relative mechanical thresholds of nerve fibres in both rat groups.

Preface

- All procedures were performed in adherence with the principles of the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee (A10-0060).

- The control experiments on HEK-293 cells using Western blot analysis were conducted in collaboration with Dr. Ujendra Kumar's laboratory.

- Results of high expression levels of $GABA_A\gamma_2$ in peptidergic and non-peptidergic mucosal afferent fibres from intact female rats were published in an abstract in the official journal of the Canadian Pain Society, Pain Research & Management, May/June 2012, Vol 17 (3), 225.

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List of Abbreviations

ALA = Alpha Lipoic Acid

AP(s) = Action Potential(s)

BIC = Bicuculline

BMS = Burning Mouth Syndrome

BR = Blink Reflex

BZD = Benzodiazepine

CBT = Cognitive Behaviour Therapy

CNS = Central Nervous System

CV(s) = Conduction Velocity(ies)

DRG = Dorsal Root Ganglion

GABA = Gamma-Aminobutyric Acid

$GABA_A$ = Gamma-Aminobutyric Acid A receptor

$GABA_A\gamma_2$ = γ2 subunit of the Gamma-Aminobutyric Acid A receptor

HEK-293 = Human Embryonic Kidney Cell Line 293

HRT = Hormone Replacement Therapy

HA-γ_2GABA_A = Haemaglutinin-tagged cDNA for gamma2 subunit of $GABA_A$ receptor

MR = Mechanoreceptor

MT = Mechanical Threshold

MUS = Muscimol

NGF = Nerve Growth Factor

OVX = Ovariectomised/ Ovariectomy

PBS = Phosphate-Buffered Solution (pH=7.4)

PGP 9.5 = Polygene Protein 9.5

PM = Polymodal receptor

PNS = Peripheral Nervous System

P2X3 = Purinergic Receptor 3 (Ligand-Gated Ion Channel)

QST = Quantitative Sensory Testing

RT-PCR = Reverse Transcriptase Polymerase Chain Reaction

SHAM = operated but non-ovariectomised rats

SP	= Substance P
TMJ	= Temporomandibular Joint
THIP	= 4, 5, 6, 7-tetrahydroisoxazolo [5, 4-c] pyridin-3-ol
TRPV1	= Transient Receptor Potential Cation Channel Subfamily V Member 1
VAS	= Visual Analogue Scale
wt	= Wild Type/ Naïve

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Dedication

Especially dedicated to my much-loved parents,

supportive family members & dear friends.

You know who you are!

1 Chapter: Introduction

1.1 Anatomy and Histology of the Mammalian Tongue

1.1.1 Sensory Innervation of the Tongue

The anterior two-thirds of the dorsum of the human tongue, defined as the area in front of the circumvallate papillae, is innervated by the lingual branches of the mandibular nerve (V3) (Holland and Robinson, 1992, Zur et al., 2004). The mandibular nerve is the largest branch of the trigeminal nerves (V), whose cell bodies reside in the trigeminal ganglion at the base of the skull, outside the brain. The mucosa on the ventral surface of the tongue is also supplied by the lingual nerve. It enters the tongue from behind the sublingual salivary gland to the anterior of the circumvallate papillae (Holland and Robinson, 1992, Zur et al., 2004). It then bifurcates into medial and lateral branches, with each branch further subdividing into 2-5 terminals, to supply the middle and anterior two-thirds of the tongue mucosa, respectively. It was found that the sensory innervation of the tongue is concentrated near the lateral aspects of the tongue but is scarce around the central region of the anterior two-thirds of the tongue (Zur et al., 2004). The lingual nerve also carries sympathetic fibers (Matthews and Robinson, 1980) responsible for vasodilatation and probably proprioceptive fibers from the tongue muscles as well (Holland and Robinson, 1992).

The glossopharyngeal nerves (IX) supplies general and taste sensations to the posterior one-third of the tongue mucosa, which includes the circumvallate papillae (Zur et al., 2004). Some small areas on the posterior part of the tongue around the epiglottis are supplied by the superior laryngeal branches (internal branches) of the vagus nerve (X)

(Berkovitz, 2002). The chorda tympani branches of the facial nerves (VII) also innervate the anterior two thirds of the tongue and travel with the lingual nerve (Berkovitz, 2002). They carry both sensory (taste) and parasympathetic fibres (Zur et al., 2004). The anterior two-thirds of the dorsum of the tongue is supplied by the chorda tympani to discriminate the different taste modalities (Berkovitz, 2002).

In summary, the areas investigated in this particular project i.e. the mucosa covering the anterior two-thirds of the dorsum of the tongue and the ventral surface of the tongue are supplied by the lingual nerve (V3) which conveys mechanical (touch and pain) and thermal (heat and cold) sensations from the tongue to the central nervous system (CNS).

1.1.2 **Characteristics of Tongue Afferent Nerve Fibres**

Post-mortem examination of human tongues revealed that the average caliber (interior diameter) of the main trunk of the lingual nerve is about 3.5 mm (Zur et al., 2004). Subdivisions of the lingual nerve form axonal bundles measured at about 1 mm in diameter with the distal subdivisions approximating 0.5– 0.75 mm (Zur et al., 2004). The axonal terminals of sensory neuronal fibres of the oral mucosa measured approximately 2.5-3.0 micrometres in diameter (Watanabe, 2004). In short, the diameter spectrum of the lingual nerve extends from 1 to 15 μm (Blom, 1960, Biedenbach and Chan, 1971). In the oral mucosa, the afferent nerve fibres are myelinated but lose their myelin sheaths beneath the epithelial layer (Watanabe, 2004). Studies utilising transmission electron microscopy found that the lamina propria of tongue mucosa from aging rats contains both myelinated and

unmyelinated sensory nerve fibres. Interestingly, each axonal fibre, whether myelinated or not, has a thin lamina of Schwann cell sheet enveloping it (Grisolia, 2006). Tongue afferent fibres have a range of diameters and myelinated afferent fibres have varying degrees of thickness of the myelin sheets (Grisolia, 2006). The mean diameter of myelinated sensory fibres in the tongue mucosa is 4.5 μm, with the largest diameter recorded being 12 μm and smallest diameter is 1.4μm. Unmyelinated nerve fibres have an average diameter of 0.6 μm (maximum diameter= 3.17 μm; minimum diameter=0.2). It was reported that the conduction velocities (CVs) of lingual nerve fibres ranged from 11 to 53 m/sec with a mean of 30 m/sec and the measured average length of lingual nerve was 62 mm (Biedenbach and Chan, 1971). The tip of the tongue and the lateral border have the highest density of nociceptors innervated by the lingual nerve and chorda tympani, and the least density on the ventral surface (Biedenbach and Chan, 1971).

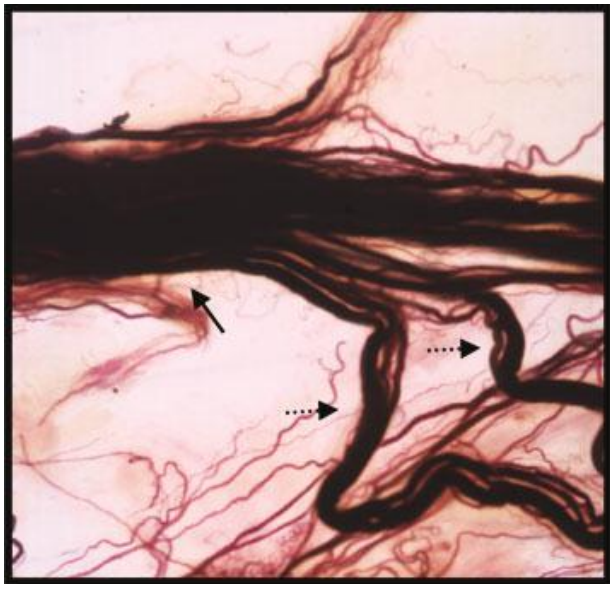


Figure 1: The main trunk of the lingual nerve (arrow) has an average diameter of 3.5 mm; each of the major subdivisions measures 1 mm in diameter and contains 3–5 bundles each (dashed arrows). Used with permission from (Zur et al., 2004).

It has been demonstrated that mucosal nociceptors are different from skin nociceptors in the frequency distribution of their types and certain physiological properties (Toda et al., 1997). For example, the oral mucosa is richly supplied with both Aδ- and C-innervated polymodal (PM) nociceptors (Toda et al., 1997) but Aδ-PM nociceptors are very scarce in the skin (Willis, 1985). All the C-fibres are solely the PM type whereas in the skin, there are also C-high threshold and C-mechanocold nociceptors in the skin (Willis, 1985). In one *in vitro* study, 64 out of 124 (54%) sensory lingual nerve fibres isolated from the medial side of the oral mucosa were classified as nociceptors (MT above 17.6mN/1.8g to the von Frey stimuli) (Toda et al., 1997). The investigators found 4 distinct types of nociceptors in rat oral mucosa namely Aδ-high threshold mechanoreceptors, Aδ-mechanoheat nociceptors, Aδ-polymodal nociceptors and C-polymodal nociceptors, with the majority of oral mucosal nociceptors being the polymodal (PM) type (Toda et al., 1997). They outlined 4 distinctive characteristics of oral mucosal nociceptors. First, the area of the receptive field of PM type was significantly larger than that of Aδ-high threshold mechanoreceptors or Aδ-mechanoheat nociceptors, and the C-polymodals had the largest receptive field in the oral mucosa (Toda et al., 1997). Second, these characteristics were very different from those found in skin nociceptors (Toda et al., 1997). Third, there was no significant difference in MTs among the different types of oral mucosal nociceptors (Toda et al., 1997). And last, the Aδ-polymodal showed significantly lower thermal threshold than other types of nociceptors and also had the lowest CVs among Aδ-innervated nociceptors (Toda et al., 1997).

1.2 Burning Mouth Syndrome (BMS)

1.2.1 Definition and Classification of BMS

The International Association for the Study of Pain (IASP) has identified primary BMS as a "distinctive nosological entity" characterised by the "unremitting oral burning or similar pain in the absence of detectable oral mucosa changes" (Merskey H, 1994, Scala et al., 2003). A widely-accepted definition of primary BMS is that it is "a chronic, idiopathic burning sensation or pain in clinically normal mucosa in which dental or medical causes have been excluded. The term syndrome implicates the simultaneous presence of several symptoms: most frequently a feeling of oral dryness (xerostomia) and altered taste (dysgeusia) in addition to a burning sensation in the oral mucosa." (Zakrzewska et al., 2001, Grushka et al., 2002, Pedersen et al., 2004). Other synonyms used interchangeably to describe BMS are stomatopyrosis, glossopyrosis, stomatodynia, glossodynia, sore mouth, sore tongue, and oral dysesthesia. In this thesis, only the term (primary) BMS is used and it is defined as above.

A second type is called burning mouth sensations (formerly known as secondary BMS) and is associated with known aetiologies such as local, systemic and psychogenic or psychiatric factors (see Table 1) (Maltsman-Tseikhin et al., 2007). Contrary to primary BMS, symptoms of burning mouth sensations will disappear when the underlying cause(s) is treated (Jaaskelainen, 2012).

Table 1: Possible Aetiological and Pathogenic Factors Associated with Secondary BMS. This table is modified from Table 3 published in (Pedersen et al., 2004).

Local Factors
Dental Treatment and Denture-related problems (Basker et al., 1978, Lamey and Lamb, 1988, Svensson and Kaaber, 1995)
Diseases of the oral mucosa e.g. oral lichen planus, oral candidiasis (Zegarelli, 1987, Samaranayake et al., 1989)
Xerostomia (sensation of dry mouth) and Hyposalivation (Grushka and Sessle 1991; Gorsky, Silverman et al. 1991; Gorsky, Silverman et al. 1987; Basker, Sturdee et al. 1978)
Temporomandibular Disorders (Paterson et al., 1995, Thorstensson and Hugoson, 1996)
Allergic Reactions (Kaaber et al., 1979, Haustein, 1988, Lamey and Lamb, 1988, Lamey et al., 1994)

Systemic Factors
Nutritional Deficiencies e.g. Vit B1, B2, B6 and zinc deficiencies (Lamey et al, 1986; Hugoson and Thorstensson, 1991; Vucicevic Boras et al, 2001)
Hormonal Disturbances e.g. Menopause and Estrogen Deficiency (Basker et al., 1978, Grushka, 1987b)
Immunological Disturbances e.g. Autoimmune diseases, HIV and AIDS (Grushka and Sessle 1991)

Psychogenic and Psychiatric Factors
Anxiety and Depression (Browning et al., 1987, Grushka, 1987b, Lamb et al., 1988, Bergdahl et al., 1995b, Jerlang, 1997)
Somatisation and Cancerophobia (Browning et al., 1987, Grushka et al., 1987a, Lamb et al., 1988, Bergdahl et al., 1995b, Jerlang, 1997)

Primary BMS has been further subdivided into three subtypes according to variations in daily pain intensity (Schoenberg et al., 1971, Lamey et al., 1994, Maltsman-Tseikhin et al., 2007, Pedersen et al., 2004). Type I BMS refers to patients who do not experience pain upon waking up but symptoms develop gradually as the day progresses, reaching its peak in the evenings (Pedersen et al., 2004, Maltsman-Tseikhin et al., 2007). These patients suffer from daily burning symptoms and have a relative frequency of occurrence at 35% (Maltsman-Tseikhin et al., 2007). It has been linked with non-psychiatric factors (Lamb et al., 1988, Lamey and Lamb, 1988). Patients with Type 2 BMS experience constant burning pain that is present daily, be it day or night. This type of BMS has the highest relative frequency of occurrence at 55% (Pedersen et al., 2004, Maltsman-Tseikhin et al., 2007) and is associated with psychiatric factors such as chronic anxiety (Lamb et al., 1988, Lamey and Lamb, 1988). Last, Type 3 BMS, with a relative frequency of 10%, is defined as intermittent pain and the painful sites also vary. This type of BMS usually affects unusual oral sites such as the buccal mucosa, the throat and the floor of the mouth (Pedersen et al., 2004, Maltsman-Tseikhin et al., 2007). Patients with Type 3 BMS are usually psychologically normal but it has been linked with intake of food additives or flavouring allergies (Lamb et al., 1988, Lamey and Lamb, 1988). Due to the heterogenous nature of manifestations of BMS symptoms, the diagnosis of BMS is highly challenging and it is very probable that, like many types of chronic pain, BMS has multiple aetiopathogenesis.

1.2.2 **Clinical Features of BMS**

1.2.2.1 Pain Characteristics

This condition is characterised by a spontaneous burning pain or in the oral mucosa, in the absence of clinically identifiable dental and medical pathology. Other descriptions of oral mucosal pain include scalding and tingling sensation as well as feeling numbness in the mouth (Scala et al., 2003). The pain intensity varies from mild to severe with average visual analogue scale (VAS) scores ranging from 5 to 7 but may reach 8 -10; in these studies VAS ranged from 0 indicating no pain to 10 indicating the worst imaginable pain (Danhauer et al., 2002, Gremeau-Richard et al., 2004, Petruzzi et al., 2004). One study reported that the average pain intensity in BMS patients is 8 (Lamey and Lamb, 1988) but a more recent one found a considerably lower score of 4.6 (Bergdahl and Bergdahl, 1999).

1.2.2.2 Location

The pain is usually located at the tip of the tongue and in the anterior two-thirds of the tongue (Grushka, 1987b, Lamey and Lamb, 1988, Gorsky et al., 1991, Bergdahl and Bergdahl, 1999, Scala et al., 2003, Maltsman-Tseikhin et al., 2007). Other sites that may be involved include the lateral right and left of the tongue, gingivae, upper and lower lip, hard palate, throat and mandibular-alveolar region (Grushka, 1987b, van der Ploeg et al., 1987, Dutree-Meulenberg et al., 1992, Svensson et al., 1993, Tammiala-Salonen et al., 1993, Eli et al., 1994, Grinspan et al., 1995, Svensson and Kaaber, 1995, Lamey et al., 1996, Scala et al., 2003, Maltsman-Tseikhin et al., 2007, Pedersen et al., 2004), 2007). The buccal mucosa and

the floor of the mouth are rarely involved (Waal, 1990, Scala et al., 2003). Pain is usually bilateral and may be confined to the tongue only or involve multiple sites in the oral mucosa (Grushka, 1987b, Minor and Epstein, 2011, Pedersen et al., 2004).

1.2.2.3 Temporal Pattern

A majority of BMS patients experience spontaneous onset of symptoms, without any obvious precipitating factor {Pedersen, 2004 #150}(Grushka, 1987a,Grushka and Sessle, 1991). The symptoms have a characteristic chronic, and unremitting pattern (Grushka, 1987b, Jerlang, 1997). Typically, BMS lasts for a duration of 2 to 3 years (Browning et al., 1987, Drage and Rogers, 1999, 2003, Maltsman-Tseikhin et al., 2007) and rarely persists for longer periods (from months to up to 18 years) (Maltsman-Tseikhin et al., 2007). However, it has been reported that only 3% of BMS patients may experience spontaneous remission (complete remission of oral burning sensations without any treatments) within 5 years after the onset of the syndrome (Sardella et al., 2006). Partial remission has been reported in about half to two-third of patients 6 to 7 years after onset. These patients experience semi-spontaneous relief of symptoms characterised by a change in pain pattern from constant to episodic burning pain (Grushka, 1987b,Ship et al., 1995, Bergdahl and Bergdahl, 1999). Individually, the pattern of daily symptoms is fairly stable with one-third of them experiencing symptoms day and night (Grushka, 1987b,van der Ploeg et al., 1987, Bergdahl and Bergdahl, 1999). The majority of patients report not experiencing obvious symptoms upon waking up. However, symptoms will gradually increase during the day to reach peak intensity in the evening (Grushka, 1987a). Sleep disturbances such as difficulty falling asleep

and being woken up during the night are experienced by one-third of patients (van der Ploeg et al., 1987, Grushka et al., 2002), possibly contributing to other problems, such as mood changes, irritability, anxiety and depression (Grushka et al., 1987a, Grinspan et al., 1995). The burning pain may be exacerbated by stress, fatigue, speaking and food ingestion (Grushka, 1987a).

1.2.2.4 Symptoms and Comorbidities

Some BMS patients also complain of dry mouth (xerostomia) and dysgeusia (Grushka et al., 1986, Gorsky et al., 1987, Grushka, 1987a, Bergdahl and Bergdahl, 1999). Dysgeusia can present in the form of altered taste perception, in terms of taste intensity or may appear as a persistent taste, predominantly bitter and/or metallic or a combination of both (Grushka et al., 1986, Scala et al., 2003, Pedersen et al., 2004). Together with the chief burning pain symptom, these three main complaints form the symptomatic triad of BMS (Scala et al., 2003). Full-blown syndrome i.e. presenting with all three aforementioned symptoms is commonly observed in a subgroup of patients especially elderly women who are going through peri-/post-menopause (Basker et al., 1978, Zachariasen, 1993, Ben Aryeh et al., 1996, Scala et al., 2003). BMS patients may be oligosymptomatic i.e. they either present with pain and dysgeusia or pain and xerostomia (Scala et al., 2003).

Particularly amongst postmenopausal women, there may be possible associations with anxiety, depression and personality disorders (Gorsky et al., 1991, Bergdahl and Bergdahl, 1999, Scala et al., 2003). However, it remains a controversy whether these psychogenic

factors have a causal relationship with BMS or they are simply secondary events that occur due to BMS patients experiencing persistent, chronic pain (Al Quran, 2004, Maina et al., 2005, Schiavone et al., 2012). Several studies have reported a high prevalence of psychiatric symptoms and/or other mental disorders amongst BMS patients (Rojo et al., 1994, Maina et al., 2005). For example, one study observed that 21% of BMS patients had personality profiles that suggested significant psychologic distress but as a whole, these patients did not show significant clinical depression, anxiety or somatisation (Carlson et al., 2000). The most recent study found that BMS patients have significantly higher scores of anxiety, depression and somatisation than the control group (Schiavone et al., 2012).

1.2.3 **Epidemiology**

There have been several studies conducted to assess the prevalence of BMS in various populations around the world. The prevalence of BMS increases with age (Bergdahl and Bergdahl, 1999, Minor and Epstein, 2011, Pedersen et al., 2004) and BMS typically affects only middle-aged and elderly individuals aged between 38 to 78 years (Basker et al., 1978, Lamey and Lamb, 1988, Tammiala-Salonen et al., 1993, Bergdahl and Bergdahl, 1999, Marbach, 1999, Clark G.T, 2005). In Italy, it is estimated that approximately 3.7% of the population, in their fifties to seventies, are affected by BMS (Scardina et al., 2008, Schiavone et al., 2012). BMS prevalence is estimated to be 0.7% in the United States (0.8% women and 0.6% men) (Lipton et al., 1993, Ship et al., 1995, Minor and Epstein, 2011). A study conducted amongst the Finnish population reported an estimate of 15% prevalence, although half of them have oral mucosal lesion or oral candidiasis (burning mouth sensations/ secondary BMS) (Tammiala-Salonen et al., 1993). The incidence of BMS is about 13% from

a study conducted in Naples (Femiano, 2002). A mail survey followed up by telephone interviews in Canada reported an estimate prevalence of 1.5%, of which 75% were women (Locker and Grushka, 1987b, a, 1988). Approximately 1.6% of men and 5.5% of women in Sweden were found to have BMS, with the highest prevalence reaching more than 12% among the oldest women (Bergdahl and Bergdahl, 1999). In this study, no men and women younger than 40 and 30 years old respectively were affected by BMS. In fact, BMS rarely occurs in people below the age of 30 (Waal, 1990, Danhauer et al., 2002, Barker and Savage, 2005, Clark G.T, 2005) and has not been reported in children (Maltsman-Tseikhin et al., 2007). Other studies and reviews have reported variable prevalence ranging from 0.7% to 15% (Basker et al., 1978, Grushka and Sessle, 1991, Bergdahl and Anneroth, 1993, Lipton et al., 1993, Hakeberg et al., 1997, Bergdahl and Bergdahl, 1999, Scala et al., 2003, Barker and Savage, 2005, Grushka et al., 2006, Pedersen et al., 2004), most likely due to the lack of standard diagnostic criteria for BMS (Scala et al., 2003, Barker and Savage, 2005, Maltsman-Tseikhin et al., 2007, Patton et al., 2007, Pedersen et al., 2004). Most importantly, such a wide range of reported prevalences from across the world reveal to us that BMS is a worldwide problem that is still poorly understood.

BMS occurs far more commonly in women than in men (Basker et al., 1978, Grushka, 1987b, Tammiala-Salonen et al., 1993, Bergdahl and Bergdahl, 1999, Maltsman-Tseikhin et al., 2007, Minor and Epstein, 2011) and the estimated ratio of BMS in women to men varies from 3:1 to 16:1 (Basker et al., 1978, Main and Basker, 1983, Grushka, 1987a, van der Ploeg et al., 1987, Lamey and Lamb, 1988, Gorsky et al., 1991, Ship et al., 1995, , Clark G.T, 2005, Pedersen et al., 2004). Perimenopausal and postmenopausal women have a

much higher incidence of BMS (Ferguson et al., 1981, Grushka, 1987a, Wardrop et al., 1989, Ship et al., 1995, Bergdahl and Bergdahl, 1999, Grushka et al., 2002, Barker and Savage, 2005, Clark G.T, 2005, Maltsman-Tseikhin et al., 2007, Minor and Epstein, 2011), up to about 13% (Ferguson et al., 1981, Bergdahl and Bergdahl, 1999, Pedersen et al., 2004). BMS develops amongst women who experienced menopause naturally, usually 3 years before to 12 years after menopause (Grushka, 1987a, b, Clark G.T, 2005, Pedersen et al., 2004), and occurs even in women who underwent hysterectomy (Ferguson et al., 1981, Ben Aryeh et al., 1996, Tarkkila et al., 2001). In actuality, about 1 in 5 young women who underwent ovariectomy developed symptoms of BMS (Tarkkila et al., 2001). **These data suggest that there is a correlation between BMS and hormonal alterations and that further research to understand the underlying pathogenesis of this disorder is warranted.**

1.2.4 **Aetiology of BMS**

Although there is still no confirmed cause of BMS and the pathogenic mechanisms underlying this syndrome are still uncertain (Waal, 1990, Zakrzewska, 1995, Zakrzewska et al., 2001, Scala et al., 2003, Minor and Epstein, 2011, Jaaskelainen, 2012, Pedersen et al., 2004), increasing evidence has led to the general consensus that BMS is a form of neuropathy leading to chronic, neuropathic pain (Ship et al., 1995, Minor and Epstein, 2011, Jaaskelainen, 2012, Pedersen et al., 2004).

Neuropathic pain has the characteristic smarting, burning sensation as a result of damage to the peripheral nervous system (PNS) and/or central nervous system (CNS) (Lund

et al., 2001, Pedersen et al., 2004). It is commonly associated with somatosensory disturbances such as feeling pain even with normally innocuous stimuli (allodynia) e.g. sunburn pain and/or having increased sensation of pain when stimulated by noxious materials (hyperalgesia) at the neuropathic pain site (Finnerup et al., 2007). Some classic examples of neuropathic pain conditions include diabetic polyneuropathies, postherpetic neuralgias and post spinal cord injury pain (Attal, 2012). Different levels in the nervous system are involved in pain modulation. Research on animal models of neuropathic pain has resulted in several postulated mechanisms involving the PNS and CNS. For example, it was discovered that peripheral afferent fibres and the dorsal root ganglion (DRG) neurons had heightened spontaneous tonic activity and increased excitability of non-nociceptive A-beta nerve fibres (Attal, 2012, McArthur, 2012, Stemkowski and Smith, 2012, Pedersen et al., 2004). In the CNS, the descending pathway originating from the mesencephalic periaqueductal gray matter (PAG) and raphe nuclei systems exert both facilitating and inhibitory effects upon dorsal horn neurons (Porreca et al., 2002, Suzuki et al., 2002). Normally, there is a balance between inhibition and facilitation at different synaptic relay systems in the nervous system but in chronic neuropathic pain patients, reduced descending inhibitory tone from the CNS can cause an imbalance between excitation and inhibition control leading to manifestation of chronic pain (Finnerup et al., 2007, McArthur, 2012, Stemkowski and Smith, 2012). Additionally, central maladaptive plasticity in the brain results in altered cortical processing of nociceptive inputs to ultimately lead to a shift in pain threshold and in response to suprathreshold stimuli (Finnerup et al., 2007, McArthur, 2012, Stemkowski and Smith, 2012). Since BMS appears to be a form of neuropathy, the same

peripheral and central mechanisms leading to neuropathic pain conditions may be responsible for BMS.

Recent neurophysiological, psychophysical, histopathological, neuropathological and brain imaging studies have led to the proposal of some neuropathic mechanisms that may play a key role in the development of primary BMS (Jaaskelainen, 2012). Results from these studies will be discussed in the following sections. It is still open to debate whether BMS is primarily a central neuropathic phenomenon or as a result of peripheral neuropathy. Nonetheless, it is noteworthy that a wide variety of other factors and associations remain open for investigations and cannot be neglected when researching the aetiopathogenesis of this syndrome.

1.2.4.1 Evidence of Neurogenic Dysfunction in BMS

The first systematic psychophysical study on BMS patients utilised the Quantitative Sensory Testing (QST) methods to investigate tactile and thermal sensory modalities within the orofacial region including tongue mucosa (Grushka, 1987a). The investigators found that BMS patients had lower tolerance to heat pain at the tip of the tongue compared to healthy controls (Grushka, 1987a, Jaaskelainen, 2012). Congruent with this, BMS patients also demonstrated a positive sign of higher intraoral mucosa vasoreactivity to dry ice stimulation when measured with laser Doppler flowmetry (Heckmann et al., 2001). In short, these BMS patients presented with altered sensory modalities in the form of decreased pain tolerance, as

often seen in neuropathic pain patients, hence supporting the notion that BMS is a form of neuropathy (Jaaskelainen, 2012).

In one study, BMS patients (n=40) were found to have significantly higher mean thermal pain threshold (thermal hypoalgesia) than healthy controls (Ito et al., 2001). Another negative sensory sign found in BMS patients is hypoesthesia whereby patients had significantly elevated thermal sensory thresholds (elevated detection thresholds to innocuous cooling and warming) (Granot and Nagler, 2005). Using QST with an argon laser stimulator, the study by Granot and Negler revealed the involvement of intraoral small-diameter afferent system in BMS because patients showed increased sensory and pain thresholds (negative signs of hypoesthesia and hypoalgesia) (Granot and Nagler, 2005). Furthermore, BMS patients also had lower pain to sensory threshold ratios on the tongue mucosa than healthy controls when stimulated with the argon laser (Svensson et al., 1993). Taken together, these findings indicate that BMS patients have somatosensory disturbances similar to other neuropathic conditions (Pedersen et al., 2004).

Neuroanatomical studies using specific immunohistochemical staining of the tongue mucosa have shown upregulation of nerve growth factor (NGF), TRPV1 ion channels and P2X3 receptors within the subepithelial nerve fibres of BMS patients (Yilmaz et al., 2007, Beneng et al., 2010, Jaaskelainen, 2012). It has been demonstrated in various animal models of neuropathic pain that these factors i.e. NGF, TRPV1 ion channels and P2X3 receptors are associated with the development of neuropathic pain symptoms (Yilmaz et al., 2007, Beneng

et al., 2010, Jaaskelainen, 2012), further supporting the notion that BMS is a form of neuropathic pain.

1.2.4.2 Proposed mechanisms: Evidence for BMS as a Peripheral Neuropathy

Lauria and colleagues provided evidence in favour of the idea that BMS is caused by a trigeminal small-fiber sensory neuropathy and proposed using superficial tongue biopsy to aid diagnosis of the condition (Lauria et al., 2005). In their study, 3-mm punch biopsies from the anterior two-thirds of tongues (lateral aspect) were collected from 12 BMS patients and 9 healthy controls. Using immunohistochemistry and confocal microscopy co-localization techniques, tongue samples were labelled with cytoplasmic, cytoskeletal, Schwann cell and myelin antibodies to identify any pathological changes in the tissues. Interestingly, BMS tongue samples had significantly lower epithelial (unmyelinated) nerve fibre density (ENFD) than controls. However, they did not find any correlation between density of fibres and severity of symptoms. Moreover, epithelial and subpapillary nerve fibres also showed diffuse morphological changes reflecting axonal regeneration, characterized by weaker and more fragmented immunoreactivity (IR) to PGP 9.5 and beta-tubulin antibodies (Lauria et al., 2005). Their findings resemble the histological picture of ‘burning feet syndrome’ which also present with loss of epidermal nerve fibres (Lauria et al., 2005). A significant correlation between reduction of epithelial nerve fibre density and lowered heat sensitivity was found in both chronic pain rat models (partial sciatic nerve lesion) on their glabrous skin of the hindpaw and healthy humans (on skin from forearm and inner thigh) (Khalili et al., 2001, Lindenlaub and Sommer, 2002, Malmberg et al., 2004, Gremeau-Richard et al., 2010).

An earlier study which was published in Italian also reported focal small-diameter nerve fibre system pathology in the tongue mucosa of BMS patients. Out of the 37 patients examined, a loss of function in small-diameter nerve fibres was observed in about 50% of BMS patients (Lauritano et al., 1998) and 70% of them had moderate atrophy in their tongue mucosa. However, this study is weakened by the lack of well-defined diagnostic criteria so secondary BMS patients or patients with major lingual or mandibular neuropathies might have been included (Jaaskelainen, 2012). Other studies have also repeatedly shown significant loss of epithelial small-diameter nerve fibres in the tongue mucosa of BMS patients (Yilmaz et al., 2007, Beneng et al., 2010, Puhakka et al., 2010). Despite consistent results from numerous studies, these data have to be interpreted with caution as most of these studies did not mention any control by blinding during the process of quantifying the epithetial nerve fibre density so investigator's bias cannot be ruled out. Moreover, these studies mostly involved a relatively small number of subjects (n=10 to 12) and the difference in mean age between the patients and controls did vary by a large margin, from 10 years (Lauria et al., 2005) to 22 years (Hwang and Yaksh, 1997, Yilmaz et al., 2007, Beneng et al., 2010). To date, there is only one convincing neuropathology study that demonstrated a significant reduction of epithelial nerve fibre density in tongue mucosa of BMS patients (Puhakka et al., 2010). The researchers involved in this project applied strict diagnostic criteria for primary BMS, had properly age-matched control group, meticulous neurophysiologic and QST examinations to selectively include only patients with small-diameter fibre dysfunction and excluded patients with other major neuropathies (Jaaskelainen, 2012).

Some studies have reported that BMS patients have a dysfunctional trigeminal nervous system (Jaaskelainen et al., 1997, Forssell et al., 2002). Most importantly, these findings strongly supported the hypothesis that neuropathic dysfunction is involved in the aetiopathogenesis of BMS. In their first study in 1997, Jaaskelainen and colleagues performed objective electrophysiologic examinations of the trigemino-facial system of 11 BMS patients and 10 healthy controls (Jaaskelainen et al., 1997). All subjects underwent evaluations of blink reflex and jaw reflex. Those with abnormal blink reflex further underwent needle electromyography to evaluate activities of their facial and masticatory muscles. Increased excitability of the trigeminal system is reflected in the form of deficient habituation of the R2 component of the blink reflex (Jaaskelainen et al., 1997, Forssell et al., 2002, Scala et al., 2003). Thin fibre dysfunction is indicated when patients had one or more sensory thresholds abnormalities (Forssell et al., 2002, Scala et al., 2003). Abnormal patterns of BR suggest existence of trigeminal neuropathy while brain stem pathology was confirmed through the use of MRI scan (Forssell et al., 2002). The BMS group did show significant abnormalities in their blink reflexes and these abnormalities appeared to be positively associated with disease duration (Jaaskelainen et al., 1997). As a follow-up study, a larger group of BMS patients was tested to evaluate the peripheral and central neural pathways of the trigeminal system using electrophysiological tests namely BR and QST. Of 52 BMS patients, 89% had abnormal recordings in one way or another (Forssell et al., 2002). To be more specific, 33 out of 46 patients tested with QST showed signs of hypoesthesia (increased sensory thresholds to warm and cold stimuli) and about one-fifth of the patients showed abnormal BR recordings (Forssell et al., 2002). About 21% of patients (11/52 cases) showed signs of increased excitability of the trigeminal system, 19% (10/52 cases) of patients showed

signs of brain stem pathology or peripheral trigeminal neuropathy, while 76% (35/46 cases) of them showed signs of pure thin-fibre dysfunction (also known as small-fibre neuropathy) (Forssell et al., 2002, Scala et al., 2003). Due to the heterogeneity of their findings, the authors concluded that BMS patients suffer from a generalised abnormality in the processing of somatosensory information, possibly at multiple levels, with the majority of them resulting from peripheral neurogenic mechanisms (Forssell et al., 2002, Scala et al., 2003).

1.2.4.3 Proposed mechanisms: Evidence for BMS as a Central Neuropathy

Involvement of the CNS and its interaction with the PNS has been subject to a lot of speculation in the debate over the aetiopathogenesis of BMS (Patton et al., 2007). It was proposed that damage to the gustatory system and disinhibition of central nociceptive regions lead to hyperactivity of both the sensory and motor components of the trigeminal nervous system to result in symptoms of BMS (Grushka et al., 2003, Bartoshuk et al., 2005, Eliav et al., 2007, Patton et al., 2007, Femiano et al., 2008). In a study using functional magnetic resonance imaging (fMRI), BMS patients showed less volumetric activation in the entire brain, and more specifically in the bilateral thalamus, when given painful thermal stimuli (Albuquerque et al., 2006), as also seen in neuropathic pain patients (Apkarian et al., 2005).

Since the blink reflex is also modulated by dopaminergic inhibitory control via connections between the basal ganglia to the facial motor nuclei (Evinger et al., 1993, Jaaskelainen et al., 2001), findings of abnormal blink reflexes in BMS patients have initiated a number of studies to investigate the role of brain dopamine system in pain (Jaaskelainen et

al., 2001, Hagelberg et al., 2003, Hagelberg et al., 2004, Jaaskelainen, 2012). By utilising modified positron emission tomography (PET), the nigrostriatal dopaminergic pathway was investigated in 10 BMS patients and 14 healthy controls (Jaaskelainen et al., 2001). BMS patients had significantly decreased uptake of fluoro-DOPA-tracer in the presynaptic terminals of the putamen on both sides. This indicates a lower level of dopamine in the nigrostriatal nerve terminals of BMS patients compared to healthy subjects, leading the authors to propose that BMS patients have decreased dopaminergic inhibition in the nigrostriatal pathway (Jaaskelainen et al., 2001, Minor and Epstein, 2011, Jaaskelainen, 2012). This hypothesis has yet to be substantiated with a sufficient body of experimental findings and validations.

1.2.4.4 Proposed mechanism: Dysregulation of Hormones and Neuroactive Steroids

There has been speculation that altered female sex hormone levels may predispose women to BMS. This is because BMS patients are predominantly peri-menopausal and/or postmenopausal women, reflecting a likely association with changes in gonadal sex hormones levels (e.g. depletion of oestrogen hormone). In fact, a positive correlation between BMS and marked menopausal symptoms has been reported (Grushka, 1987a, Woda et al., 2009). Furthermore, it appears that even young patients, from both genders, with imbalanced hormonal control could be at risk of developing BMS. For example, one publication reported that 18% of 145 ovariectomised (OVX) young women had burning sensation in the lips or tongue (Ferguson et al., 1981). Using a linear regression model, Tarkkila and colleagues found a correlation between the use of hormone replacement therapy (HRT) with an

increased risk for painful mouth with 13% of HRT users reported having painful mouth in their study (Tarkkila et al., 2001). There has been speculation that the lack of oestrogen during peri- and postmenopausal periods in women leads to atrophic changes in the oral epithelium which directly result in symptoms of BMS (Rojo et al., 1993). Ovariectomy also produced histological changes in the oral mucosa of female rats; OVX rats had reduced thickness of the keratinised epithelial oral mucosa, with the most significant effect at the tip of the tongue 6-months post-ovariectomy (Seko et al., 2005), which is also the most frequently affected site in BMS. HRT partially reverses these alterations in the oral mucosa. In a study using formalin tests, female rats had increased behavioural pain signs (licking and rubbing) in the perioral areas but not in the hindpaws, after OVX (Pajot et al., 2003). Male rats exhibited the opposite effects (Pajot et al., 2003).

The high prevalence of anxiety and depressive disorders among BMS patients indicate a possible association with malfunctioned hypothalamic-pituitary-adrenal (HPA) system. It has been shown that chronic stress, posttraumatic stress disorders, anxiety and major depression are capable of inducing dysregulation of the HPA axis and glucocorticoid homeostasis. Since, these psychiatric disorders and chronic and stressful, adverse life events are particularly associated with BMS (Hammaren and Hugoson, 1989, Woda et al., 2009), these patients are likely to suffer from HPA dysfunction as well. It was demonstrated that BMS patients had higher anxiety scores and salivary cortisol levels than control subjects (Amenabar et al., 2008). Similarly, patients under chronic stress and those with major depression also have exaggerated cortisol concentration (Tafet and Bernardini, 2003, Kahl et al., 2006), indicating hyperactivity of the HPA axis and impaired feedback control of the

HPA system (Parker et al., 2003, Swaab et al., 2005) which may ultimately lead to a hypothalamic hyperdrive or adrenal hypertrophy (Rubin and Phillips, 1993, Gervasoni et al., 2004). Taken together, these studies suggest that HPA dysfunction may underlie the pathophysiology of BMS (Woda et al., 2009).

Since BMS symptoms are strictly confined to the oral mucosa, it was also proposed that alterations in neuroactive steroid levels might be one of the many causative factors (Woda et al., 2009). This is because neuroactive steroids such as oestrogens and androgens can be synthesised by cutaneous cells, neurons, glials, and Schwann cells in the PNS and CNS (Rupprecht and Holsboer, 1999, Melcangi et al., 2005) and can act via paracrine, autocrine or intracrine methods (Gago et al., 2004, Belelli et al., 2006). Neuroactive steroids have the ability to exert limited steroid activity within the tissues or cell types that they are synthesised in, (Belelli et al., 2006) which coincides with the restricted location of intraoral burning pain as seen in BMS. It was speculated that the complex interactions and complementary activities of systemic and local neuroactive steroids may induce peripheral and/or central neuropathy as observed in BMS patients (Woda et al., 2009).

Based on this speculation that BMS may be caused by dysregulation of sex hormones, I attempted to develop an animal model of BMS using young, OVX female rats, in the hope to observe reduced density of small-diameter nerve fibre in the tongue mucosa of OVX rats, as had been observed in human BMS patients.

1.2.5 **Current Treatments and Clinical Trials**

Several reviews have assessed the effectiveness of currently employed therapies for management of BMS (Buchanan & Zakrzewska 2008; Zakrzewska & Forssell 2005; Moraes et al, 2012; Minguez Serra et al, 2007). Thus far, there is no sufficient evidence of a treatment that cures BMS. Some of the various therapeutic interventions for management of BMS that have been investigated in published trials are summarized in Table 2 below.

1.2.5.1 Alpha-Lipoic Acid (ALA)

ALA is a mitochondrial coenzyme with antioxidant properties (Minor and Epstein, 2011). It is able to increase the levels of intracellular glutathione to fight free radicals as well as regenerating other antioxidants such as Vitamins C and E (de Moraes et al., 2012). It has been shown that ALA has neuroprotective functions in previous studies (Ziegler et al., 1999, Femiano, 2002, Spanemberg et al., 2012) since ALA stimulates the production of neural growth factors (Femiano et al., 2000, Femiano et al., 2004, Carbone et al., 2009).

There had been 4 clinical trials which showed positive effects of treatment with ALA on BMS-related pain as measured by the VAS (Femiano et al., 2000, Femiano, 2002, Femiano and Scully, 2002, Femiano et al., 2004). Despite the positive results, these studies have not provided conclusive evidence to support the use of ALA to treat BMS for several reasons. First of all, these studies were all conducted by the same group of scientists in a single-centre institution within a similar timeframe. Only one of them was a randomized

controlled trial with double blind design (Femiano and Scully, 2002) but it had a relatively small group of subjects. The other 3 studies were either non-randomised or were not controlled by blinding. Furthermore, 3 other randomized, double-blind, placebo-controlled clinical trials of ALA for BMS conducted by other investigators and institutions (Carbone et al., 2009, Cavalcanti and da Silveira, 2009, Lopez-Jornet et al., 2009) found no significant benefit of using ALA compared to placebo which cast doubt on the efficacy of ALA to treat BMS (Minor and Epstein, 2011). In short, ALA's efficacy needs to be assessed in multi-institutional, double-blind, randomized-controlled trials to validate the use of this therapeutic.

1.2.5.2 Antidepressants

Clinicians have observed from anecdotal and clinical uncontrolled studies that low doses of tricyclic antidepressants can attenuate the burning pain reported by BMS patients (Grushka et al., 2002). A positive effect of BMS treatment with paroxetine was reported whereby 80% of patients (42/52) demonstrated significant pain reduction after only 12 weeks of treatment, with minor transient side effects (Yamazaki et al., 2009). This was an open-label, single-arm, dose-escalation pilot study but results suggest that paroxetine may be useful in treating BMS and warrant further research.

In Italy, a single centre, investigator-blinded, randomized-controlled trial with no placebo arm was conducted to compare paroxetine and sertraline, both of which are selective serotonin reuptake inhibitors with amisulpride, an atypical antipsychotic (a selective dopamine D2/D3 antagonist) (Maina et al., 2002). During the 8-week study, BMS patients

received 50mg sertraline, 20 mg paroxetine or 50 mg amisulpride daily. Although, 69.6% to 72.2% of patients from all 3 groups responded positively to these treatments, placebo effect cannot be ruled out to account for the positive results. A more recent open label study with no placebo control reported that BMS patients treated with amisulpride showed significant improvement in BMS symptoms after 24 weeks of daily intake of 50 mg amisulpride (Rodriguez-Cerdeira and Sanchez-Blanco, 2012). This early positive outcome convinces the investigators to push for future double-blinded, placebo-controlled trials to further assess the efficacy of amisulpride.

A single double-blinded, randomized-controlled trial was conducted in Finland to investigate the effectiveness of trazodone in 37 female BMS patients (Tammiala-Salonen and Forssell, 1999). Over the 8-week trial period, trazodone failed to have any positive effect on VAS pain scores in the treatment group when compared to placebo. A double-blind study investigated 253 patients treated with clomipramine and mianserin, both of which are tetracyclic antidepressants (Loldrup et al., 1989). Compared to the placebo group, there was no difference in magnitude of pain relief in both treatment groups.

Early evidence from these clinical trials suggest that some antidepressants may be effective in controlling symptoms of BMS but thus far, there is still insufficient evidence from well-designed and properly-controlled clinical trials to strongly support the use of any one of these antidepressants.

1.2.5.3 Gabapentin

The idea that BMS is a type of chronic neuropathy has generated interest in trying to use gabapentin (Neurontin) to treat BMS. An open, dose-escalation study conducted over 3 weeks found no significant improvement in pain scores in the 15 patients who took 300-2400 mg of gabapentin per day (Heckmann et al., 2006). A single case report was published (White et al., 2004) offering support for the use of gabapentin to manage BMS. However, at present, there is insufficient evidence to support the use of gabapentin. Large, well-designed clinical trials need to be conducted to confirm its efficacy for BMS (Minor and Epstein, 2011).

1.2.5.4 Hormone Replacement Therapy (HRT)

Despite a significant association between BMS and postmenopausal women, the effect of HRT in BMS is still controversial (Maltsman-Tseikhin et al., 2007). Three previously conducted clinical trials have found no significant beneficial effect of systemic or local oestrogen versus placebo treatments in BMS patients (Pisanty et al., 1975, Ferguson et al., 1981, Forabosco et al., 1992). Nonetheless, these studies were not randomized and were poorly-designed i.e. no clear diagnostic criteria, outcome measures and definition of BMS, method of randomization not specified, lack of blinding and a non-validated assessment scale for symptom improvement (Buchanan and Zakrzewska, 2008). Hence, results have to be interpreted with caution. Failure to distinguish between primary and secondary BMS patients is also one of the weaknesses of these studies (Danhauer et al., 2002, Santoro et al., 2005, Maltsman-Tseikhin et al., 2007). Further research to determine the efficacy of HRT in both primary and secondary BMS patients is required so that the right cohort of BMS patients

most likely to benefit from HRT treatment can be identified (Maltsman-Tseikhin et al., 2007).

1.2.5.5 Topical Capsaicin

Topical applications of low doses of capsaicin, 3 to 4 times a day, on painful intraoral area(s) seem effective in alleviating pain in 24 BMS subjects with 31.6% of patients achieving complete remission and another 31.6% had partial remission (Epstein and Marcoe, 1994, Lauritano et al., 1998, Scala et al., 2003). Over long treatment periods with topical capsaicin are thought to deplete Substance P (SP), and possibly other neurotransmitters, caused by degeneration and desensitization of C-fibre (Lynn, 1990, Simone and Ochoa, 1991, Maltsman-Tseikhin et al., 2007), with consequent loss of pharmacological effects (Scala et al., 2003). Therefore, the recommended management with topical capsaicin is a seven-day topical administration with periods of no treatment in between and capsaicin has to be removed after each application (Scala et al., 2003). However, management with topical capsaicin is often associated with poor patient compliance due to the bitter taste of the compound and the need for constant, repeated applications as it is quickly washed out from saliva flow and tongue movements (Petruzzi et al., 2004).

1.2.5.6 Systemic Capsaicin

A single-centre, doubled-blind, placebo-controlled pilot study concludes that systemic capsaicin can be therapeutically-effective for short-term treatment of BMS only due to major gastrointestinal side effects (Petruzzi et al., 2004). In this study, a total of 25 BMS patients were given 0.25% of capsaicin capsules, 3 times a day for 4 weeks and 22/25 of them

reported significantly lower pain scores (Petruzzi et al., 2004, Minguez Serra et al., 2007). In the placebo group, only one patient showed improvement (Petruzzi et al., 2004, Minguez Serra et al., 2007). Despite the apparent effectiveness of oral capcaisin, higher number of gastric toxicity cases was recorded compared to placebo group, limiting the use of systemic capsaicin to short-term treatment only.

1.2.5.7 Systemic Clonazepam

An open-label, dose-escalation pilot study found that systemic clonazepam may be helpful in the management of BMS because about 70% of subjects experienced pain reduction with effects at low doses (Grushka et al., 1998). Patients were prescribed a starting dose of 0.25mg daily, with an increase in dose of 0.25 mg on a weekly basis if symptoms continued. This led to a recent double-blind, placebo-controlled randomized clinical trial which found significant improvement in pain ratings in BMS patients (n=10) treated with systemic clonazepam (0.5mg/day) compared to placebo controls (n=10) (Heckmann et al., 2012). There were no significant differences in mood changes, depression scores, taste test and salivary flow rate between groups. The authors concluded that clonazepam appears to have a positive analgesic effect in BMS patients (Heckmann et al., 2012).

1.2.5.8 Topical Clonazepam

An open-label, pilot study pioneered by Alain Woda's group found that local application of clonazepam (0.5 or 1mg), two or three times daily had pain-relieving effect in a majority of BMS patients (Woda et al., 1998). Out of the 25 subjects tested, 10 of them

were totally cured, 9 patients with some improvements, and 6 patients reporting no benefit. Inspired by the success of this pilot study, a multi-centred, double-blinded, randomized-controlled trial was conducted in France on 84 BMS patients (40 women and 44 men) to study the potential therapeutic effect of topical clonazepam in treating BMS (Gremeau-Richard et al., 2004). Patients were instructed to suck a tablet (either 1mg clonazepam or placebo) three times a day, for 3 minutes each time. Before spitting out the medicine, they would swish the dissolved medicine around their painful sites without swallowing. The same swish and spit technique was used in the pilot study. Patients were on this regimen for 2 weeks. There was a significant reduction in pain scores – recorded using a standard numerical scale (0 to 10) – but no difference in adverse events between treatment group and placebo control. Blood levels of clonazepam were below therapeutic range in both studies, suggesting that clonazepam may have acted on peripheral $GABA_A$ receptors to exert its therapeutic effect. At present, the only effective pharmacological intervention demonstrated to relieve symptoms of BMS is topical administration of clonazepam because this protocol is the only one that has been validated by a randomized controlled trial of sufficient design quality to provide strong, validated conclusions (Gremeau-Richard et al., 2004, Zakrzewska et al., 2005, Amos et al., 2011, Klasser et al., 2011). However, no studies have demonstrated or quantified the expression of $GABA_A$ receptors by afferent fibres of tongue mucosa and this thesis addresses this gap of knowledge.

1.2.5.9 Combine Topical and Systemic Clonazepam

A pilot study which retrospectively evaluated clinical records audit of BMS patients diagnosed between January 2006 to June 2009 found that a large percentage (80%) of

patients had significant pain reduction (more than 50%) over the treatment period. The authors conclude that their preliminary results are indicative of the effectiveness of a novel protocol for management of BMS i.e. a combined topical and systemic administration of clonazepam (Amos et al., 2011). Despite the inherent limitations associated with retrospective studies, the authors were confident that the method of dissolving clonazepam tablets orally before swallowing is a promising management option for BMS and warrants future research (Amos et al., 2011).

1.2.5.10 Chlordiazepoxide

An interesting study conducted in 130 BMS patients tested multiple medications, one of which is chlordiazepoxide (Librium) in a large non-randomised, non-placebo-controlled trial. Out of the 78 patients placed on chlordiazepoxide (Librium), 14% experienced complete resolution, 35% had significant improvement, 15% with minor benefits, and 36% showed no change in pain scores (Gorsky et al., 1991). Unfortunately, the study design is of lower quality than studies of clonazepam. By comparison, clonazepam appears to have more positive outcomes (Grushka et al., 1998, Gremeau-Richard et al., 2004) and is used in the treatment of neuralgias and neuropathies whereas chlordiazepoxide is not (Epocrates, 2010, Minor and Epstein, 2011) although both drugs bind to benzodiazepine sites to positively modulate $GABA_A$ receptors.

1.2.5.11 Lafutidine

Lafutidine is an antagonist to histamine H2-receptors and is commonly used– outside of North America– to treat gastic ulcers by activating mucosal defensive mechanisms in the gastrointestinal tract by sensitising capsaicin-sensitive afferent neurons and augmenting the release of calcitonin gene-related peptide and nitric oxide in the stomach (Toida et al., 2009). Studies in animal models have suggested that lafutidine may relieve neuropathic pain by sensitising capsaicin-sensitive afferent neurons (Kan Y, 2005) and it was speculated to be a potential therapeutic for BMS (Minor and Epstein, 2011). This randomised-controlled trial was designed such that 34 BMS patients were blindly switched from their original H2-blocker to lafutidine while the other 30 subjects remained on their original H2-blocker (Toida et al., 2009). VAS scores were taken at three different time points i.e. week-4, 8 and 12 and results indicated that improvement rates in the lafutidine group were significantly higher than controls. Since there has been only one study investigating the effectiveness of lafutidine on BMS patients, this treatment option cannot be justified yet.

1.2.5.12 Other Medical Treatments

In an open non-controlled study of 33 patients, the use of topical anesthetic, dyclonine hydrochloride resulted in 12 subjects suffering from increased burning, 14 patients with no change and 7 with some improvements (Formaker et al., 1998). There was no significant effect of using benzydamine hydrochloride oral rinse, a topical anti-inflammatory to treat BMS (Sardella et al., 1999). To date, there is limited evidence to support the use of local anesthetic or analgesic agents to treat idiopathic BMS (Minor and Epstein, 2011). One

study that investigated the efficacy of sucralfate, a cytoprotective agent primarily used to treat duodenal ulcers, failed to show a convincing effect (Minor and Epstein, 2011). This small, open label study of 14 subjects reported improvements in 6 patients but worsening of symptoms in 4 subjects (Campisi et al., 1997). Hypericum perforatum (St. John's wort) failed to demonstrate any therapeutic effects in a placebo-controlled, double-blind, randomised control trial on 39 BMS patients (Sardella et al., 2008).

1.2.5.13 Cognitive Behavioural Therapy (CBT) and Group Psychotherapy

There has been only one randomised, placebo-controlled trial which investigated the effect of CBT to treat BMS. In this trial, CBT group received 12 to 15 one-hour sessions of CBT per week whereas placebo group received a similar number of sessions, but without the CBT techniques (Bergdahl et al., 1995a). A non-conventional VAS pain scale (scale of 1-7) which lacks validation was used in this study but CBT group did have a significant reduction in VAS scores compared to placebo group. This study is weakened by the lack of information on other baseline characteristics of all the BMS subjects (Minor and Epstein, 2011). Intervention using group psychotherapy found significant improvements in 71% of the 24 BMS patients who underwent active treatment compared to only 40% of the 20 subjects in the placebo group reported improvement (Miziara et al., 2009). This form of treatment can be explored further and may be a useful adjunct to pharmacological interventions since a majority of BMS patients also suffer from psychogenic/psychiatric disorders.

Table 2: Summary of interventions for management of burning mouth syndrome that have been investigated in published trials. Modified from (Klasser et al., 2011)

Behavioural therapy
• **Cognitive behavioural therapy**
• **Group psychotherapy**
• **Electroconvulsive therapy**

Topical medication
• **Benzodiazepine: clonazepam (swish and expectorate)**
• **Anesthetic: lidocaine (viscous gel)**
• **Atypical analgesic: capsaicin (cream)**
• **Antidepressant: doxepin (cream)**
• **Nonsteroidal anti-inflammatory: benzydamine (oral rinse)**
• **Antimicrobial: lactoperoxidase (oral rinse)**
• **Mucosal protectant: sucralfate (oral rinse)**

Systemic medication
• **Benzodiazepine (low dose): clonazepam, chlordiazepoxide**

- **Anticonvulsants: gabapentin, pregabalin, topiramate**
- **Atypical analgesic: capsaicin**
- **Antidepressants (low dose): amitriptyline, imipramine, nortriptyline, desipramine, trazodone**
- **Selective serotonin reuptake inhibitors: paroxetine, sertraline, trazodone**
- **Selective norepinepherine reuptake inhibitors: milnacipran, duloxetine**
- **Antioxidant: α-lipoic acid**
- **Antipsychotics: amisulpride, levosulpride**
- **Atypical antipsychotic: olanzipine**
- **Dopamine agonist: pramipexole**
- **Histamine$_2$ receptor antagonist: lafutidine**
- **Herbal supplement: *Hypericum perforatum* (St. John's wort)**
- **Salivary stimulants: pilocarpaine, sialor, cevimiline, bethanechol**

1.3 GABA$_A$ Receptors

1.3.1 Structure and Function of GABA$_A$ receptors

There are 2 types of GABA receptors i.e. GABA$_A$ and GABA$_B$ (Macdonald and Olsen, 1994, Olsen and Sieghart, 2008). Of these two types, GABA$_A$ receptors modulate the majority of GABAergic signalling and are considered the most important for maintaining inhibitory tone in the CNS (Olsen and Sieghart, 2008, Botzolakis, 2009, Olsen and Sieghart, 2009). Structurally, GABA$_A$ receptors are heteropentameric ion channels assembled from a

large family of homologous subunits (Macdonald and Olsen, 1994, Olsen and Sieghart, 2008, 2009). $GABA_A$ receptors are fast-activating chloride (Cl^-) channels from the Cys-loop superfamily of ligand-gated ion channels (Macdonald and Olsen, 1994, Olsen and Sieghart, 2008, 2009). Activation of $GABA_A$ receptors in adult's CNS causes membrane hyperpolarisation due to influx of Cl^- ions (Macdonald and Olsen, 1994, Olsen and Sieghart, 2008, Botzolakis, 2009, Olsen and Sieghart, 2009). On the contrary, $GABA_A$ receptors on immature CNS neurons and mature primary afferent neurons are depolarising as chloride ions flow out from the plasma membranes to result in either excitation or inhibition (Macdonald and Olsen, 1994, Olsen and Sieghart, 2008, Botzolakis, 2009). $GABA_A$ receptors have a rich pharmacology such that in addition to GABA, several GABA analogs such as muscimol (MUS) can directly activate the receptor whereas BZD can allosterically enhance $GABA_A$ receptors currents through different binding sites (Macdonald et al., 1989, Macdonald and Olsen, 1994, Olsen and Sieghart, 2008, 2009). BZD modulation requires the presence of the γ subunit because BZDs bind at the interface between the principal side of the α subunit and the complementary side of the γ subunit (Macdonald et al., 1989, Macdonald and Olsen, 1994, Olsen and Sieghart, 2008, 2009). In fact, it was found that 94% of BZD binding sites were absent in neonatal mouse brains following targeted disruption of the γ subunit gene, whereas the number of GABA sites were only slightly reduced (Gunther et al., 1995). BZDs increase the binding affinity of GABA for the receptor without altering channel mean open time, thus increasing channel opening frequency (Macdonald and Olsen, 1994, Olsen and Sieghart, 2008, Botzolakis, 2009, Olsen and Sieghart, 2009). Amongst the γ subunits, the **γ2 subtype is the most widely expressed subtype**, both in developing and adult brains (Herb et al., 1992, Botzolakis, 2009). Since BZDs such as clonazepam needs to

bind to $GABA_A$ receptors that contain the γ subunit and the γ_2 subunit is the most commonly expressed subtype, I decided to use an antibody against the $GABA_A\gamma_2$ to investigate the expression of this receptor subtype in rat tongue mucosa.

It is possible that clonazepam did bind to peripheral $GABA_A$ receptors in the oral cavity of BMS patients to produce antinociceptive effect through inhibition of sensory neurotransmission. The problem with this speculation is that although $GABA_A$ receptors are abundant in the CNS, their expression in the oral mucosal layer and specifically on the tongue has yet to be demonstrated. First, there is a need to demonstrate the expression of functional peripheral $GABA_A$ receptors in the tongue mucosa and these receptors have to have the BZD-binding surface i.e. at the interface between the α and γ subunits. Second, evidence for a direct antinociceptive effect from activation of peripheral $GABA_A$ receptors on nociceptors in the oral mucosa, specifically on the tongue, is lacking. Therefore, in the present study the expression of $GABA_A$ receptors in tongue nerve fibres was investigated using immunohistochemical methods and the effects of GABA and MUS, a selective agonist of $GABA_A$ receptors (Olsen and Sieghart, 2008) on the mechanical thresholds (MTs) of tongue afferent fibres were investigated by electrophysiological methods.

1.3.2 **Evidence for Peripheral $GABA_A$ receptors as Potential Analgesic Target**

The presence of $GABA_A$ receptors or their subunits (α, β or γ) has been reported in the DRG cell bodies and its associated central (Singer and Placheta, 1980, Persohn et al., 1991, Furuyama et al., 1992, Ma et al., 1993, Alvarez et al., 1996) and peripheral processes (Carlton et al., 1999), in the trigeminal ganglion (Hayasaki et al., 2006), the mandible, the

palate, the salivary glands (Watanabe et al., 2002) and in taste receptor cells in mouse tongue (Starostik et al., 2010, Dvoryanchikov et al., 2011) suggesting that these peripheral receptors are accessible for local pharmacologic manipulations. Approximately 10% of unmyelinated sensory axons in the glabrous skin of the cat paw are labeled for the β_2 and β_3 subunits and 14% are labeled for the α_1 subunit of the $GABA_A$ receptor (Carlton et al., 1999). Other lines of evidence indicating the presence of $GABA_A$ receptors on primary sensory neurons include *in situ* hybridization studies which identify the mRNA for several different subunits of the $GABA_A$ receptor in both large- and small-diameter DRG cells (Persohn et al., 1991, Furuyama et al., 1992, Ma et al., 1993) and immunohistochemical studies using an antibody directed against the β_2 and β_3 subunits of this receptor, which labelled virtually all DRG cells and preferentially stained the inner part of lamina II and lamina III where many primary afferent fibers terminate in the dorsal horn (Alvarez et al., 1996).

Intra-articular injection of GABA was found to suppress reflex jaw muscle activity evoked by noxious stimulation of the rat temporomandibular joint (TMJ) in a concentration related manner (Cairns et al., 1999, Cai et al., 2001). This GABA-mediated inhibition was reversed by co-application of the $GABA_A$ receptor antagonist, BIC but not by the $GABA_B$ receptor antagonist, phaclofen, which suggests that inhibition was mediated through activation of peripheral $GABA_A$ receptors in the TMJ. The finding that $GABA_A$ receptor activation appeared responsible for the inhibitory effect of GABA remains enigmatic, since activation of $GABA_A$ receptors on cultured dorsal root and trigeminal ganglion neurons is generally depolarizing; an effect which makes them more rather than less excitable (Puil and Spigelman, 1988, Ma et al., 2006). It has been speculated that activation of peripheral

$GABA_A$ receptors located on the afferent fiber endings in the TMJ results in a current shunt that interferes with generation of action potentials (Cairns et al., 1999).

Behavioural studies also indicate that local peripheral injection of a low concentration of the $GABA_A$ agonist, MUS (2.0 mM) attenuates formalin-induced nociceptive behaviors (Carlton et al., 1999). This effect was lessened as the concentration of MUS was increased and intraplantar injection of MUS alone at a high dose induced thermal hyperalgesia. When BIC was applied, it prevented these MUS-induced changes in behavior. It was proposed that local activation of $GABA_A$ receptors by low concentrations of MUS depolarizes peripheral primary afferent terminals also known as peripheral primary afferent depolarization (Carlton et al., 1999).

Studies have revealed that GABA receptor expression and function vary with the duration and intensity of a noxious stimulus (Hama and Borsook, 2005). Congruent with this, findings by Naik and colleagues (Naik et al., 2008) suggest that early intervention through pharmacological manipulations of peripheral $GABA_A$ receptors in the DRG neurons can prevent the development of neuropathic pain. The authors reported that topical applications of $GABA_A$ receptor agonists, MUS and THIP to the L5 DRG *in vivo at the time* of sciatic nerve crush, dose-dependently alleviated thermal hyperalgesia that developed in this neuropathic pain model. In fact, the highest doses of MUS (0.4mg/*ml*) and THIP (0.18 mg/*ml*) completely prevented the development of thermal hyperalgesia. Moreover, alleviation of thermal and/or mechanical hyperalgesia produced by topical application of MUS or THIP was completely reversed by BIC. On the other hand, applications of $GABA_A$

receptor antagonists, BIC and picrotoxin, caused an exacerbation of thermal hyperalgesia in this neuropathic pain model. Comparatively, modulations of spinal GABA-ergic system, although shown to be effective, provided only transient alleviation of neuropathic pain symptoms (Hwang and Yaksh, 1997). The authors concluded that manipulations of the peripheral GABA-ergic system via the DRG neurons appeared to be more effective in abolishing the development of neuropathic pain after peripheral nerve injury with particular emphasis on early intervention in the management of neuropathic pain.

These studies suggest that peripheral $GABA_A$ receptors have the potential to be targeted locally in the management of chronic, neuropathic pain conditions such as BMS.

1.4 Summary of Research Rationales

BMS has a greater prevalent in women than in men (Bergdahl and Bergdahl, 1999,Maltsman-Tseikhin et al., 2007, Minor and Epstein, 2011). Hence, female rats were used in this study. Several observations have led to the speculation that hormonal imbalance and low production or depletion of steroids during peri- or post- menopausal period may affect the nervous system causing nerve fibre degeneration and/or dysfunction (Woda et al., 2009). Thus, the present study examined the effects of ovariectomy (depletion of sex hormones) using OVX female rats. Human tongue biopsies also consistently demonstrated reduced density of epithelial nerve fibres in the tongue mucosa of BMS patients (Lauria et al., 2005,Yilmaz et al., 2007, Beneng et al., 2010) suggesting of small-diameter nerve fibre neuropathy. Therefore, this study quantified and compared axonal fibre densities on tongue mucosa between OVX and SHAM rats as well the proportions of peptidergic, small-diameter

nerve fibres between these rat groups. Clinical trials using topical clonazepam on BMS patients (Grushka et al., 1998, Woda et al., 1998) suggest that BZD modulation may be beneficial to relief painful symptoms of BMS. As such, expression of peripheral $GABA_{A\gamma 2}$ on tongue mucosal axonal fibres was investigated in this study and proportions of nerve fibres expressing $GABA_{A\gamma 2}$ were compared between OVX and SHAM female rats. *In vivo* extracellular electrophysiology experiments were performed on female rats with the recording electrode inserted into each animal's trigeminal ganglion in order to assess the effects of independent variables such as rat types (intact, SHAM vs. OVX) and drug types (GABA, MUS, or vehicle solutions) on the properties of afferent fibres innervating the tongue.

2 Chapter: Aims

2.1 General Aim

This study aims to investigate the feasibility of developing an animal model of BMS by using OVX female rats. Moreover, this study also aims to investigate the expression patterns, if any, of peripheral $GABA_A$ receptors on the tongue and the potential of using local pharmacotherapeutic applications to target these peripheral $GABA_A$ receptors to bring about analgesic effects.

$GABA_A$ receptors may be a novel peripheral receptor target for treatment of BMS and other neuropathic pain. Positive results from this study will lend support to the development of topical drugs such as benzodiazepine-containing lozenges and mouthwashes that target peripheral $GABA_A$ receptors to treat BMS-like pain. Furthermore, the use of topical agents to treat this condition will substantially reduce the negative central nervous side effects (e.g. dizziness, drowsiness) that come from the administration of systemic drugs.

2.2 Specific Aims

The specific aims of this study are outlined as below:

(1) To find out whether tongue afferent fibres express any peripheral $GABA_A$ receptors with the BZD binding site, by using an antibody against the γ_2 subunit.

(2) To investigate the differences in baseline properties (e.g. mean MT and CV, ratio of MRs to PMs, baseline MTs and TTs) of tongue afferent fibres between OVX and SHAM female rats.

(3) To compare nerve fibre densities, proportions of peptidergic (small-diameter) nerve fibres, proportions of $GABA_A\gamma_2$-containing, peptidergic and $GABA_A\gamma_2$-containing, non-peptidergic fibres between OVX and SHAM rats.

(4) To compare the effect(s), if any, of MUS and BIC on MTs of tongue afferent fibres in OVX and SHAM rats.

3 Chapter: Materials and Methods

3.1 Animal Models

Adult female Sprague-Dawley rats (270-395g; Charles River Inc., Wilmington, MA, USA) were used for all experiments. For the preliminary part of the study, only intact female rats (n=27) were used; 9 intact female rats were used primarily to assess the basic properties of tongue afferent fibres and the other 18 rats were divided into 3 groups to receive either GABA solution (51.5mg/ml; 0.5M), MUS solution (20µg/ml; 0.175mM) or vehicle (PBS). The second part of the study were performed on sham-operated (SHAM) (n=16) or ovariectomised (OVX) (n=19) female rats. SHAM and OVX rats were housed in the UBC Hospital for a total of 4 weeks, post-surgery to allow for total depletion of steroid hormones from their system, before they were selected for experimentation.

3.2 Methods

3.2.1 Immunohistochemistry

3.2.1.1 Immunohistochemistry Methods

Tongues from 6 intact female rats, 6 SHAM and 6 OVX female rats were removed and frozen with liquid nitrogen. The first 5 mm of frozen tongue tissue, measured from the tip of the tongue was cut coronally and embedded in O.C.T. compound. The tissue was then cut into 10µm-thick sections with a cryotome (Minotome PLUSTM). For the intact female rats, a total of 4 sections from each tongue were chosen for analysis, with each section separated from the previous section by a distance of at least 50µm. Since inter-animal and

intra-animal variabilities were relatively small, only 3 sections from each OVX and SHAM female rats, were chosen for analysis, with each section separated from the previous section by a distance of at least 50µm. The sections were mounted on glass slides and stored at -20°C. Sections were incubated in 0.2% Triton-X100 and 10% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 1 hour at room temperature. The slides were then washed with PBS three times and incubated overnight at 4°C with primary antibodies. Axonal fibres were identified using rabbit polyclonal antibody against rat, Protein Gene Product (PGP 9.5, 1:2000; Abcam Inc., Cambridge, MA, USA). PGP 9.5 is an axonal marker and serves to identify nerve fibers. $GABA_A$ receptors, specifically the γ_2 subunit were labelled with the use of goat polyclonal antibody against the $GABA_A\gamma_2$ (1:50, Santa Cruz Biotechnology, Inc., CA, USA). Additional experiments were conducted in collaboration with Dr. Ujendra Kumar's laboratory to determine the selectivity of this commercial antibody (see Appendix A). Peptidergic neuronal fibres were defined as those that contained SP and were identified using guinea pig polyclonal antibody (1:700; Abcam Inc., Cambridge, MA). Alexa Fluor 555 donkey anti-rabbit IgG antibody (1:700; Invitrogen, NY, USA) was used to visualise the PGP 9.5- IR; Alexa Fluor 488 donkey anti-goat IgG antibody (1:700; Invitrogen, NY, USA) was used to visualise $GABA_A\gamma_2$-IR and Alexa Fluor 633 goat anti-guinea pig IgG antibody (1:700; Invitrogen, NY, USA) was used to visualise the SP-IR. The following day, slides were washed with PBS three times and mounted with cover slips. Sections were scanned block by block to capture images of the entire mucosal area using a Leica TCS SPE confocal microscope.

As a control experiment, some tissue sections were incubated without primary antibodies to confirm that the fluorescence signals were due to the binding of the primary antibodies with the proteins of interest and not due to tissue binding to the secondary fluorescent antibodies (see Appendix B).

3.2.1.2 Analysis and Quantification of Immunohistochemistry Data

All images were examined by scanning block by block to cover all areas of the mucosae. For this study, a nerve fibre was defined as any fibril-shaped fluorescence of at least 4.0 μm in length which stained positive for PGP9.5 antibody. Nerve fibres were considered positively-stained for any of the antibodies used when the intensity of the fluorescence signals were more than 2 standard deviations (SD) above the mean background intensity. The number of neuronal fibres on the mucosae, with or without colocalisation with SP and $GABA_A\gamma_2$ were counted using Image J software program (National Institutes of Health Image). **Throughout the entire tissue preparation and quantification processes of nerve fibres in SHAM and OVX female rats, I was <u>blinded</u> to the rat type.** The proportions of peptidergic fibres were calculated by dividing the total numbers of SP-positive, peptidergic fibres by the total numbers of PGP 9.5-positive neuronal fibres. The proportions of $GABA_A\gamma_2$ -containing, peptidergic fibres were counted by dividing the total numbers of SP-positive fibres that also showed $GABA_A\gamma_2$ -IR by the total numbers of SP-positive neuronal fibres. The proportions of $GABA_A\gamma_2$ -containing, non-peptidergic fibres were calculated by dividing the total numbers of SP-negative neuronal fibres which did not colocalise with $GABA_A\gamma_2$-IR by the total numbers of SP-negative neuronal fibres. The

density of nerve fibres from each section was counted by dividing the total number of PGP 9.5-positive fibres in the mucosal layer by the area of the entire tissue section. Images of the entire tissue sections were captured using a digital camera (Olympus) and areas of the tissue sections were calculated using Image J software program (National Institutes of Health Image) (see Figure 2).

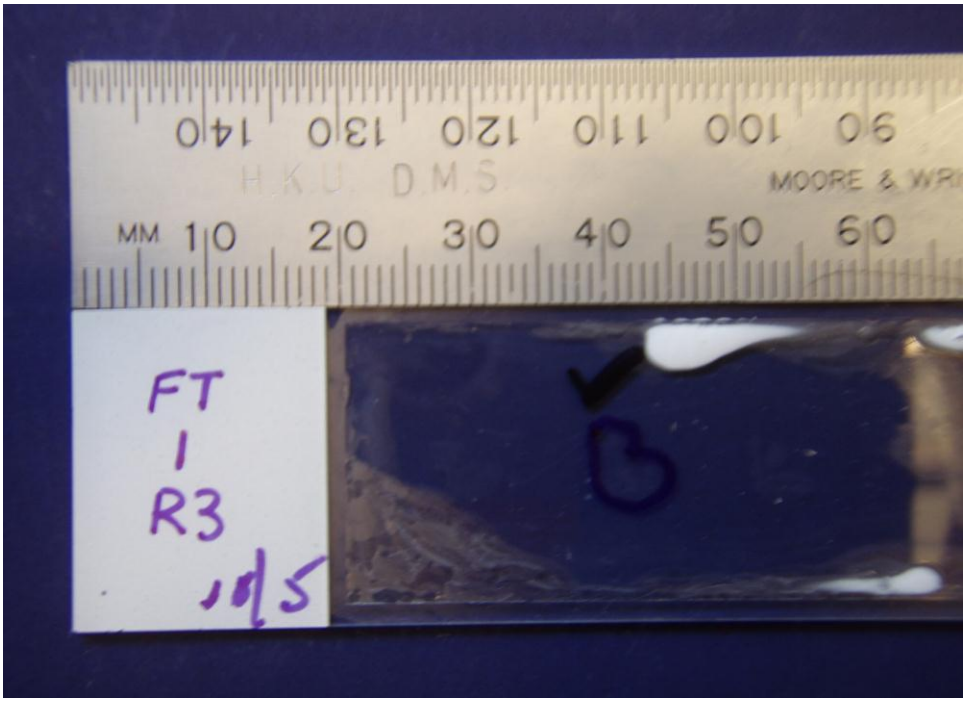


Figure 2: An example of a coronal tongue tissue section circumscribed with a marker. The area of the entire coronal tongue section, which included the mucosal and muscle layers of the tongue, was calculated using the ImageJ software.

3.2.2 *In vivo* electrophysiology

3.2.2.1 Sample Size Estimation

The preliminary data from intact rats indicated that MUS increased relative MT by about 50% compared to vehicle, although this difference did not reach statistical significance. Therefore, for the study of OVX and SHAM rats, a t-test sample size estimate was performed. The t-test sample size estimate indicated that to detect a difference in the relative MT between the MUS and vehicle groups of 50% or greater with alpha=0.5, beta= 0.2, and a coefficient of variance= 60%, 24 afferent fibres per group would be needed. On average, preliminary data collected in the intact rats indicated that I could find on average 1.5 tongue afferent fibres per rat, thus it was estimated that 16 OVX and 16 SHAM female rats would be required.

3.2.2.2 Surgical Preparation

Adult female Sprague-Dawley rats (270-395g; Charles River Inc., Wilmington, MA, USA) were used for acute, single-unit *in vivo* recording of extracellular action potentials (APs) of trigeminal ganglion neurons. Rats were prepared under surgical anaesthesia (O_2: 0.3- 0.4 l/min; isofluorane 2.5-3.0%) (Cairns et al., 2001). A rectal thermometer was used to monitor core body temperature while EEG leads were placed to measure heart rate. A vaginal lavage was performed from each rat to determine individual's oestrus stage through microscopic examination of epidermal cells (Cairns et al., 2001). A tracheal cannula was inserted to initiate artificial ventilation and to maintain anaesthesia with isofluorane. The carotid artery was catheterised to measure blood pressure and to allow administration of intravenous fluids. The rat's head was then placed in a stereotaxic frame, the skin over the

dorsal surface of the skull was reflected and a trephination was made on the right side of the skull. A parylene-coated tungsten-recording electrode was lowered into the trigeminal ganglion via the trephination to record APs from single trigeminal ganglion neurons (Cairns et al., 2002). A suture was placed in the middle of the tongue to stretch and hold it in place (see Figure 3). A stimulating electrode was used to electrically induce orthodromic APs from afferent fibres in the tongue (10 mA, 0.5 msec duration at 1Hz frequency) (see Figure 3). The conduction velocity (CV) of each recorded afferent fibre was estimated by measuring the estimated conduction distance (see section 3.2.2.5). A blunt probe was used to mechanically stimulate the tongue to identify receptive fields of afferent fibres in the tongue that project to the trigeminal ganglion neurons (Cairns et al., 2001, Cairns et al., 2002). Throughout the entire experiment, heart rate, mean blood pressure and core body temperature were continuously monitored and kept within physiological ranges of 300-400 beats/min, 60-80mm Hg and 36.8-37.1°C, respectively. Upon completion of all surgical procedures, the isofluorane level was reduced to 1.5-2.5% which maintained sufficient level of analgesia as determined by a continued absence of toe pinch reflex. All procedures were performed in adherence with the principles of the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee. All efforts were made to minimise the number of animals used and their suffering.

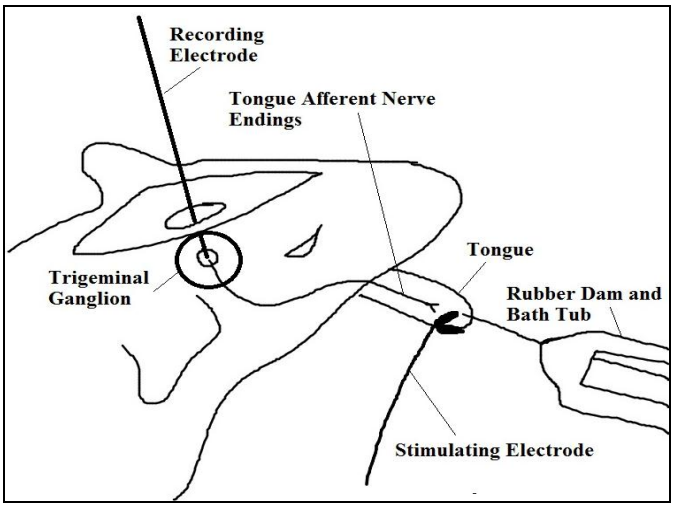


Figure 3: A diagram illustrating the electrophysiology experimental setup. A stimulating electrode was used to electrically stimulate afferent fibres on the tongue and a recording electrode was placed in the trigeminal ganglion to record the corresponding action potentials.

3.2.2.3 Electrophysiology Experiment

These experiments were carried out to study the effects on MTs of tongue MRs and PMs through pharmacological manipulation of $GABA_A$ receptors on or near the surface of tongue. One or more lingual nerve fibres were recorded from each rat. In the following text, *n* number refers to the number of afferent fibres and not the number of rats. When the recorded action potentials (APs) from each rat had different mechanical receptive fields, MTs, AP shapes and orthodromic latencies hence different CVs, they were treated as separate and independent afferent fibres. In the preliminary study on intact female rats, a total of 18 rats were randomly assigned to receive a 10-minute topical bath application of either PBS as control solution (2.0-4.0 ml, 1% PBS; n=12), aqueous GABA solution (2.0-4.0 ml, 0.5M; n=8) or MUS solution (2.0-4.0 ml, 0.175mM/ 20µg/ml; n=10). I was blinded to the type of drug solution used. In the second part of this study, a total of 16 SHAM female rats

and 19 OVX female rats were used. These rats were randomly assigned to receive PBS/PBS, PBS/BIC, MUS/PBS or MUS/BIC combinations. In this part of the study, again, I was blinded to the types of rat being operated on and the types of drug solutions being applied.

An electronic von Frey hair (model 160IC, IITC) was used to determine the MTs of the afferent fibres on the tongue at one minute intervals for a total of 10 minutes (see Figure 4). The baseline MT of each MR and PM was determined by averaging the thresholds measured from 10 consecutive mechanical stimuli before topical drug application. After 10 minutes of recording of spontaneous activity, the randomly assigned drug (approximately 2.0-4.0ml) was injected into a small tub and the tongue immersed in the bath solution for 10 minutes (see Figure 4). Then, the solution was removed and MT recorded every minute for the next 10 (for OVX and SHAM rats) or 30 minutes (for intact females) (see Section 3.3; Figures 8 and 9).

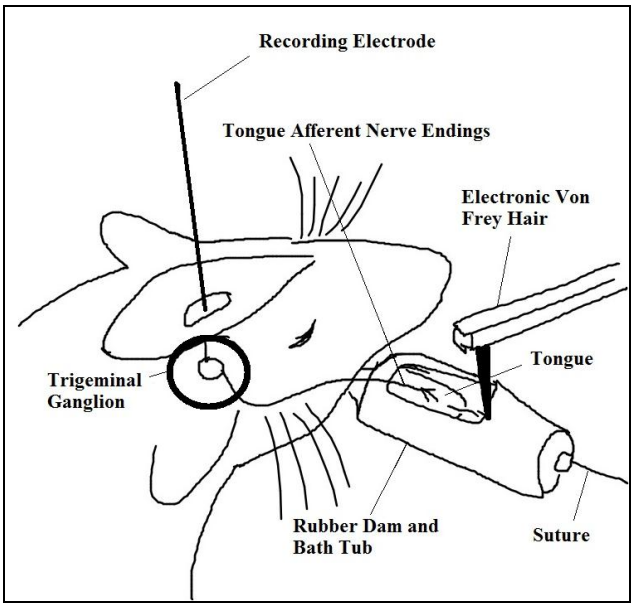


Figure 4: An electronic Von Frey hair was used to determine the MTs of afferent fibres before and after topical drug applications. A rubber dam sealed the bath tub to prevent

leaking of drug solutions so that tongue could be immersed in the bath solution for 10 minutes each time. A suture was used to fix tongue in a tensed position.

After that, a thermal probe was inserted into the tongue to measure changes in tongue temperatures and hot water (60°C) was injected into the bath tub to identify polymodal receptors (PMs) (see Section 3.2.2.4.3). The hot water was removed once temperature of the tongue had reached a plateau or the maximum to prevent irreversible damage to the tongue. The tongue was then cooled down with room-temperature saline until the tongue temperature had reached its baseline value. Then, MT was recorded every minute for 10 (for OVX and SHAM rats) or 30 minutes (for intact female rats) (see Section 3.3 Figures 8 and 9).

3.2.2.4 Analysis of Electrophysiology Data

Spike2 software was utilised to create templates of APs based on their shapes and amplitudes. This method enabled objective assortment and accurate identification of APs fired from multiple afferent fibres in response to mechanical and thermal stimuli.

3.2.2.4.1 Frequency of Spontaneous Baseline Firing

The number of spontaneous firing of afferent fibres before and during the administration of drug was recorded for 10-minute durations.

The frequency of baseline spontaneous firing (Hz) was calculated as the sum of spontaneous APs within 10 minutes divided by 600 seconds. This formula was used to calculate the frequency of baseline spontaneous afferent activity from each sensory fibre collected from OVX and SHAM rat groups and the results are reported in Section 4.2.2 (see Tables 5, 6 and 7).

Frequency(Hz) = total number of spontaneous APs within 10 min/ 600 seconds

Afferent fibres reacted differently when bathed in different drug solutions. Some showed an increase in the number of spontaneous firing activities while others had decreased numbers of spontaneous firings when different types of drugs were administered. Neuronal fibres that were non-spontaneous and did not show any change in spontaneous activities when bathed in drug solutions were not taken into account (see Section 4.2.2 and 4.2.3). Since the action of injecting the drug solutions into the bath tub and sucking them out might have mechanically stimulated some fibres causing them to fire, APs evoked within the first and last minutes of recordings were eliminated as these could be due to mechanical stimulations rather than spontaneous activities. The sum of spontaneous discharge during drug administration was therefore calculated as the total number of APs from minute-1 to minute-9 during drug administrations. This was compared with the baseline spontaneous activity level which was readjusted to the total number of spontaneous APs within the first 8 minutes of recording before any drug administration. The difference between the frequency of spontaneous discharge during drug administration with the frequency of baseline spontaneous discharge determined whether there was an increase or decrease of spontaneous discharge with drug applications (Section 4.2.3; Tables 8 and 9).

3.2.2.4.2 Mechanical Thresholds (MTs)

An electronic Von Frey hair was used to apply mechanical pressure onto the receptive field on the tongue until the first AP was fired from the afferent fibre. Spike2 software was used to create a template of all APs fired by each afferent fibre based on shape and amplitude of the APs. The lowest magnitude of mechanical pressure required to evoke an AP from the nerve fibre was taken as its MT (see Figure 5).

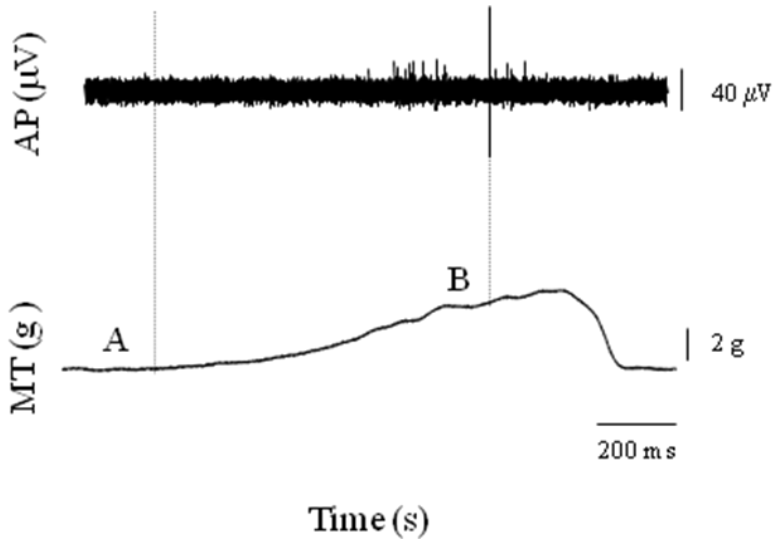


Figure 5: Method to record and calculate mechanical threshold (MT) from a tongue afferent fibre. At one minute intervals throughout the recording, mechanical pressure was applied using the electronic Von Frey instrument to the afferent receptive field until an action potential (AP) (top trace, near B) was fired. The lowest magnitude of mechanical pressure required generate an AP from this sensory fibre was taken as its MT (from bottom bar; value B minus value A).

For all MT recordings, 10 consecutive recordings were made, one at every minute and those values were averaged to obtain the mean MTs values for baseline MTs, post-drug application MTs and post-thermal stimulation MTs. Relative MTs were obtained by normalizing the post-drug MTs with the baseline MTs and those relative MTs values were averaged to obtain a mean value for each rat group or afferent fibre type (either MRs or PMs).

3.2.2.4.3 Identification of a PM and Determination of Thermal Thresholds (TTs)

When an afferent fibre with no baseline spontaneous activity fired APs during the addition of hot water, it was categorised as a polymodal (PM) (see Figure 6).

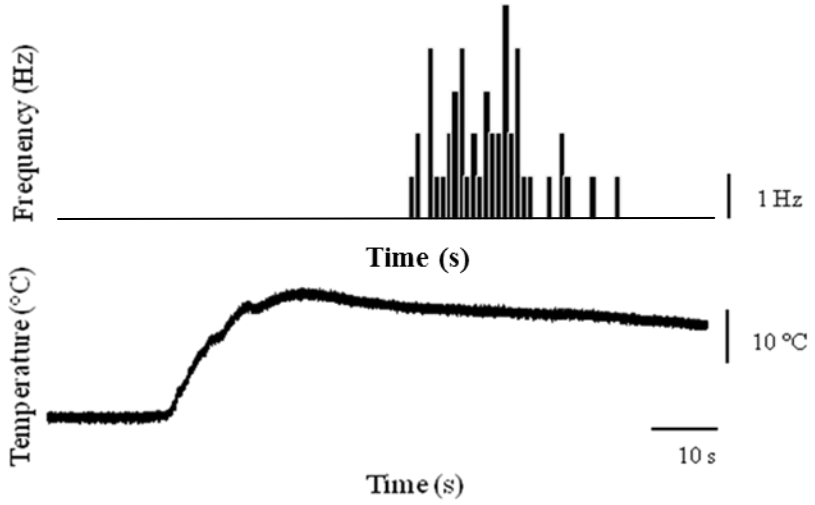


Figure 6: An example of an afferent fibre with no spontaneous discharge. A template was created by the Spike2 software to identify all action potentials (APs) fired by this particular afferent fibre (upper bar). The baseline tongue temperature for this sensory fibre was 23.3°C. As temperature gradually rose, the lowest temperature when this fibre started firing APs was taken as its thermal threshold i.e. 42.0°C.

For afferent fibres that had spontaneous firing, the baseline frequency of spontaneous firing was calculated and compared with the frequency of evoked APs. The baseline frequency of spontaneous firing was calculated from the total number of spontaneous APs recorded within 60 seconds prior to application of the hot water, divided by 60 seconds. The frequency of evoked APs by addition of hot water was calculated by dividing the sum of APs fired within 60 seconds after addition of hot water divided by 60 seconds. If the frequency of APs evoked with addition of hot water exceeded the highest frequency of baseline spontaneous firing, it was considered as a positive response and that particular afferent fibre was categorised as a PM (see Figure 7). The lowest temperature at which the frequency of evoked APs exceeded the maximum frequency of baseline spontaneous discharge was taken as the thermal threshold for that particular afferent fibre (see Figure 7).

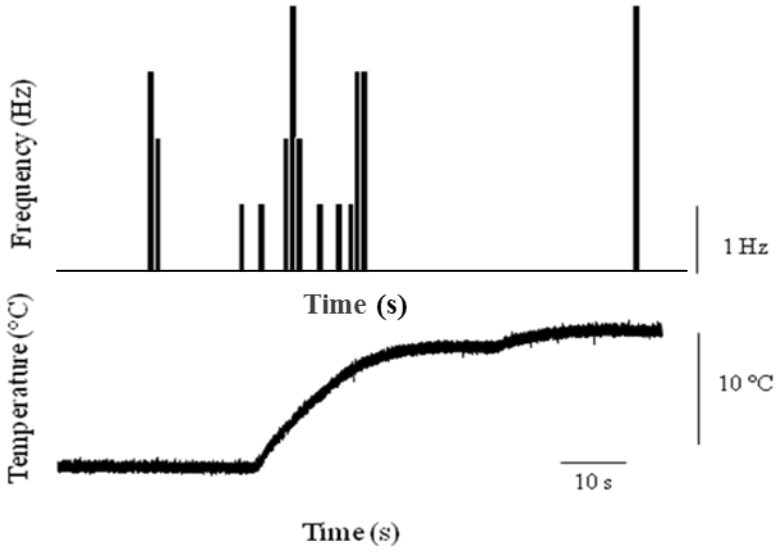


Figure 7: An example of an afferent fibre with baseline spontaneous discharge. A template was created by the Spike2 software to identify all action potentials (APs) fired by this particular afferent fibre (upper bar). The highest frequency of baseline spontaneous discharge

was 2.3 Hz and the baseline tongue temperature for this sensory fibre was 26.0°C. As temperature gradually rose, if the frequency of AP discharged exceeded frequency of baseline spontaneous discharge, in this case 2.3 Hz, it was considered as a positive response to thermal stimulation. Hence, this fibre was categorised as a PM receptor with thermal threshold of 33.5°C.

3.2.2.5 Terminal Procedures

At the end of each electrophysiology experiment, a blood sample was collected before rats were terminated with an overdose of pentobarbital (100 mg/kg i.v.; Euthanyl, Bimeda-MTC Animal Health Inc., Cambridge, ON). The conduction distance of lingual nerve was approximated to the nearest millimetre by using a suture to trace the anatomical distribution of the nerve from the external meatus to the respective receptive fields on the tongue. The tongue was removed and fast-frozen in liquid nitrogen for immunohistochemical analysis.

3.2.3 Drug Preparation

Stock solutions of GABA, MUS and BIC were prepared and frozen before commencement of the project. At the start of each experiment, a vial containing the assigned solution was thawed by immersing in room-temperature water. GABA powder was dissolved in 1% PBS solution to a concentration of 51.5mg/ml (0.5M) while MUS was first dissolved in 0.05M hydrochloric acid followed by 1% PBS to a concentration of 20µg/ml (0.175mM). Bicuculline methiodide powder was dissolved in 1% PBS solution to a concentration of 2.5 mg/ml (0.05M) of BIC solution. A few drops of yellow food colouring were added to the

PBS solution to mimic the yellow colour of the BIC solution so that blinding could be maintained.

The concentrations of GABA (51.5mg/ml or 0.5M) and BIC (2.5 mg/ml or 0.05M) were selected based on previously reported studies which found that such concentrations were successful at pharmacologically manipulating peripheral $GABA_A$ receptors in muscles of the TMJ (Cairns et al., 1999, Cai et al., 2001). The concentration of MUS (20µg/ml or 0.175mM) was chosen based on studies investigating topical applications of MUS on the PNS (Gwak et al., 2006, Anseloni and Gold, 2008, Carr et al., 2010, Lee et al., 2010). Upon obtaining positive results from preliminary studies on intact female rats, the same concentration of MUS was used on OVX and SHAM female rats.

3.3 Experimental Protocols

In the first part of this project, a pilot series of experiments were performed on intact female rats (n=18). The rats were randomised to receive either bath applications of PBS, GABA or MUS solutions. Randomisation was performed by Dr. Xudong Dong, a research scientist in Dr Cairns' laboratory. Baseline MTs were recorded every minute for a total of 10 minutes before the start of all experiments. Then, spontaneous firing activities of afferent fibres were recorded for 10 minutes, followed with 10 minutes of bath application of the designated solution. After that, post-drug MTs were recorded every minute for a total of 30 minutes. In order to identify whether the afferent fibre was a pure MR or a PM nociceptor, hot water at 60 °C was placed into the bath tub and the gradual temperature increase was

monitored using a thermal probe. Afferent fibres were cooled down to baseline tongue temperature before the final 30-minute period of MT recording (See Figure 8).

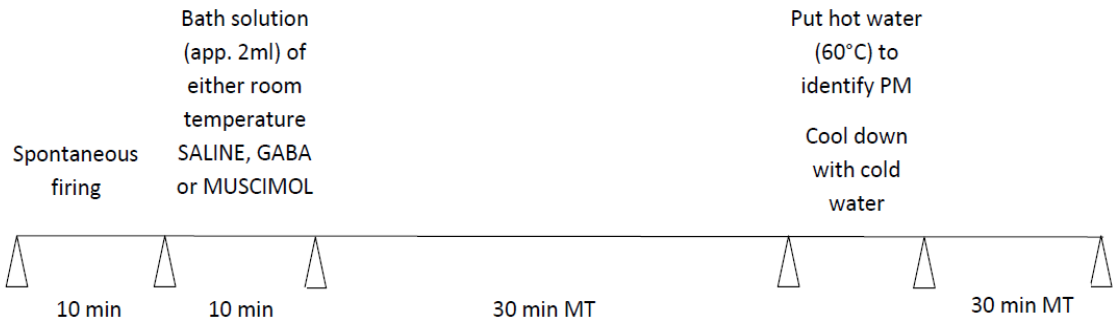


Figure 8: The standard operating procedure for *in vivo* electrophysiology on intact female rats

In the second part of this project, in vivo electrophysiology experiments were performed on either SHAM or OVX female rats. Following consultation with my supervisory committee, a revised operating procedure (see Figure 9), was designed and approved. Baseline MTs (B_{MT}) and spontaneous firing activities were still recorded every minute for 10 minutes as in previous design. Randomisation was conducted by Dr. Dong, and I was blinded to the type of rat and drug being used. Drug A was injected into the bath tub and tongue was immersed in this drug for a total of 10 minutes. Drug A could be either MUS or PBS solution. This was followed by 10-minute MT recording (MT_A), with MT measurements taken at every minute. The drug was then removed and replaced with saline solution to wash out drug A. After 30 minutes of washout period, a second baseline MT (B_{2MT}) recording was performed before bath application of drug B for. Both sessions lasted 10 minutes. Drug B could be either BIC or PBS solution. MTs after the administration of drug B (MT_B) was taken every minute for a total of 10 minutes. The washout period is again, 30 minutes using

saline solution. After that, a third baseline MT (B_{3MT}) recording was taken before the addition of hot water at 60 °C. Baseline tongue temperature and the gradual increase in tongue temperature were recorded using a thermal probe inserted into the mucosal layer of the tongue. This step enabled me to identify whether the afferent fibre(s) was a pure MR or a PM receptor. Lastly, post-thermal MTs (MT_C) were recorded, every minute for a total of 10 minutes.

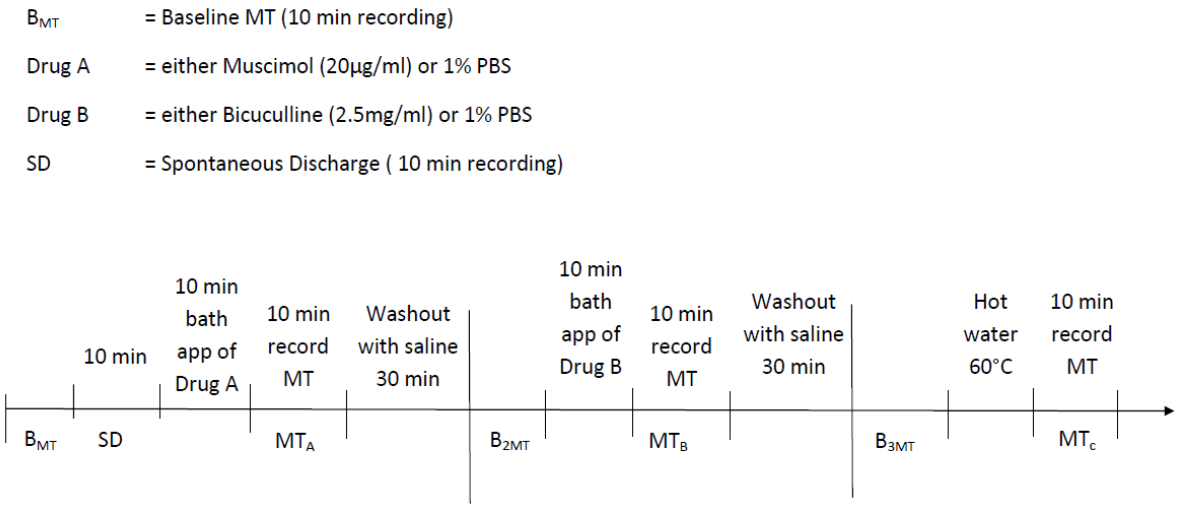


Figure 9: Experimental Protocol for *in vivo* electrophysiology experiments on SHAM and OVX female rats.

3.4 Data Analysis

Where data were not normally distributed, they were reported as median values with interquartile ranges indicated in square brackets. Mean values were indicated with SEM in parentheses. Comparisons between two groups were made with a Mann Whitney rank sum test for non-parametric data or a paired t-test if the data had a normal distribution.

Comparisons among more than two groups were made with a Kruskal- Wallis One-Way Analysis of Variance (ANOVA) on ranks, ANOVA or Two-Way Repeated Measures ANOVA One Factor Repetition, as deemed appropriate. A Spearman rank order correlation was used to investigate the relationships between different parameters. In all tests, the level of significance was set at $P< 0.05$.

4 Chapter: Results

4.1 Results from Intact Female Rats

4.1.1 Basic Properties of Afferent Fibres

A total of 60 mechanically-responsive afferent fibres were collected from 27 intact female Spraque-Dawley rats in this study. In the earliest part of the study, the initial 9 female rats were used primarily to assess baseline properties of afferent fibres whereas the following 18 female rats were divided into 3 groups to receive either bath application of PBS, GABA or MUS solutions. Afferent fibres were recorded from all 27 of these intact female rats. Forty two (70% of the population) of the afferent fibres were pure MRs, whereas the other 18 (30% of the population) were PM receptors. Afferent fibres were categorised as PM if they responded to both mechanical and thermal stimulations (see Section 3.2.2.4.3). The estimated CVs of the collected fibres ranged from 0.99 – 53.13 m/s which indicated that Aβ (myelinated) fibres, Aδ (thinly-myelinated) fibres, and C- fibres (unmyelinated) were examined. A total of 41 Aβ fibres (> 12 m/s), 16 Aδ fibres (2- 12 m/s) and 3 C-fibres (< 2 m/s) were collected.

Unlike masseter muscle afferent fibres from male rats which showed significant inverse relationship between baseline MT and CV (Mann et al., 2006), there was no significant relationship between CV and MT of female tongue afferent fibres (P> 0.05, Spearman Rank Order Correlation) (see Figure 10), which is consistent with the results found in masseter muscle afferent fibres of female rats (Mann et al., 2006). Comparisons of the basic properties between MRs and PMs showed no significant differences between the two

types of sensory receptors. The CVs (Mean ± SEM) between MRs (19.48 m/s ± 1.76) and PMs (18.16 m/s ± 2.30) were not significantly different (P> 0.05, Student's t test) (see Figure 11A). Similarly, the baseline MTs (median [interquartile range]) between MRs (2.13g [1.41-3.57g]) and PMs (2.11g [1.19-3.03g]) were also not significantly different (P>0.05, Mann-Whitney rank sum test) (see Figure 11B). As shown in Figure 12, the differences in median values of the baseline MTs among Aβ (2.19g [1.23-3.30g]), Aδ (2.05g [1.57- 4.38g]) and C-fibres (1.98g [1.39-4.42g]) were also not significantly different (P> 0.05, Kruskal-Wallis ANOVA on ranks).

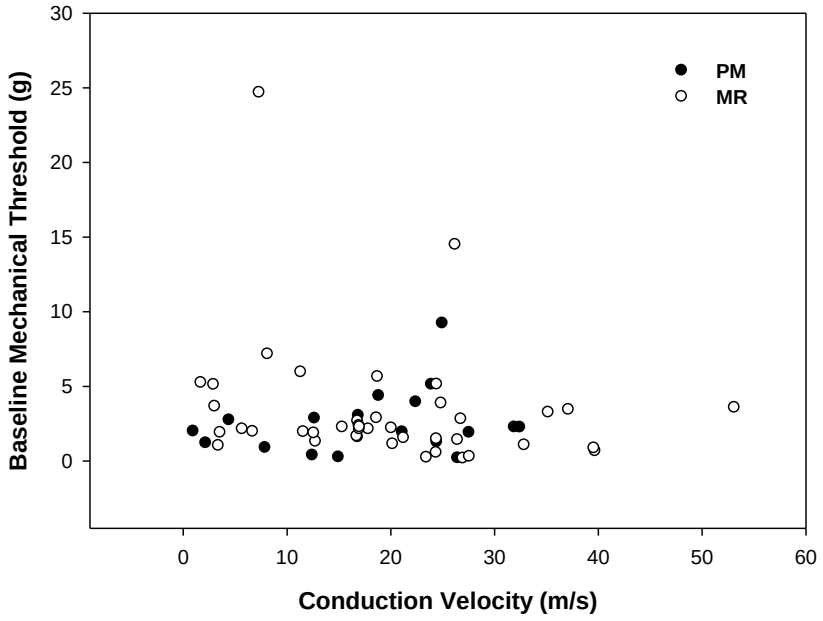


Figure 10: There was no significant relationship between baseline MT and CV of tongue afferent fibres. MRs (n=42), PMs (n=18) (P> 0.05, Spearman Rank Order Correlation).

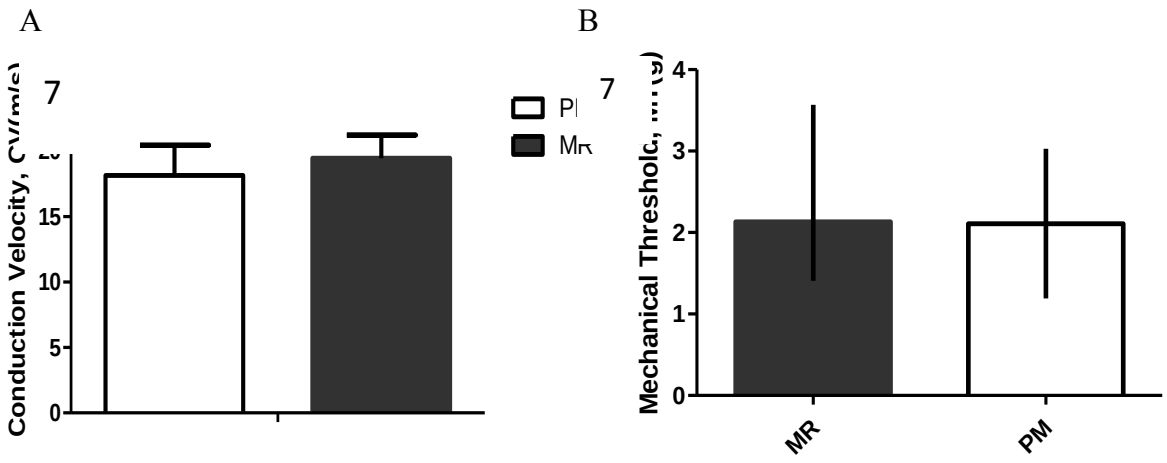


Figure 11: Comparisons of mean CVs and median MTs between MRs and PMs. A) There was no significant differences in the mean values of CVs between PMs (n=18) and MRs (n=42) (P> 0.05, Student's t test). B) There was no significant differences in the median values of baseline MTs between PMs (n=18) and MRs (n=42) (P> 0.05, Kruskal-Wallis ANOVA on ranks).

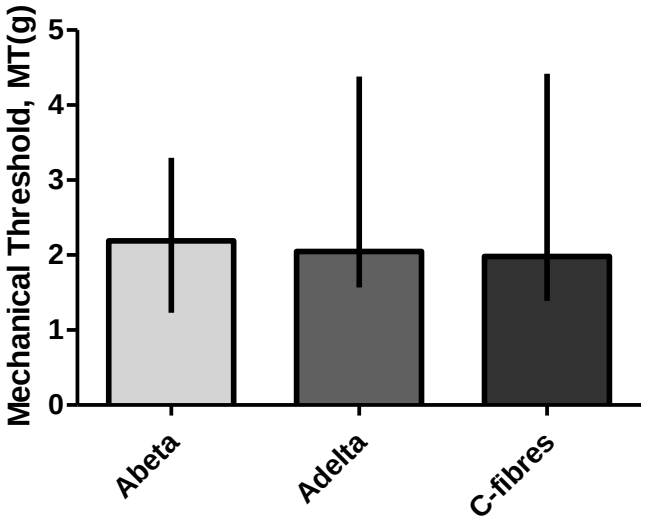


Figure 12: There were no significant differences in the median values of the baseline MTs among Aβ (n= 41) (2.19g [1.23-3.30g]), Aδ (n=16) (2.05g [1.57- 4.38g]) and C-fibres (n=3) (1.98g [1.39-4.42g]) (P> 0.05, Kruskal-Wallis ANOVA on ranks).

4.1.2 **Results from Immunohistochemistry**

Two independent assessors (A and B) were assigned to quantify and classify all the neuronal fibres (see Figure 13 and 14). They had no knowledge of the other person's results until the end of the quantification process. Values from both assessors were averaged.

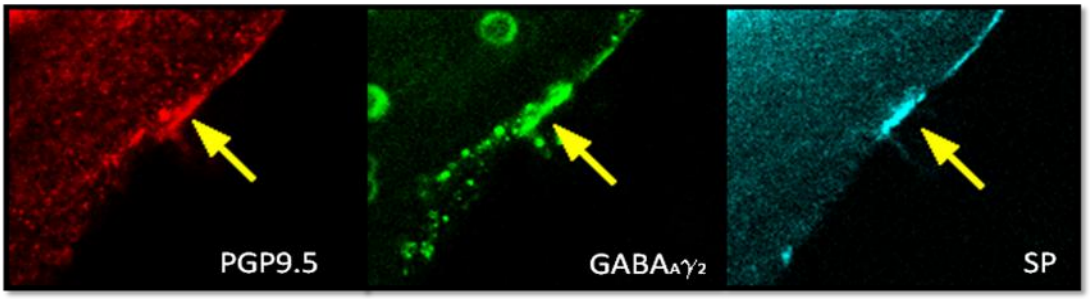


Figure 13: An example of a peptidergic, $GABA_A\gamma_2$-containing axonal fibre. The yellow arrows were pointing at a PGP 9.5-positive, axonal fibre *(most left)*, which also expressed the neuropeptide, Substance P (SP) *(most right)* as well as the γ_2 subunit of the $GABA_A$ receptor *(middle)*.

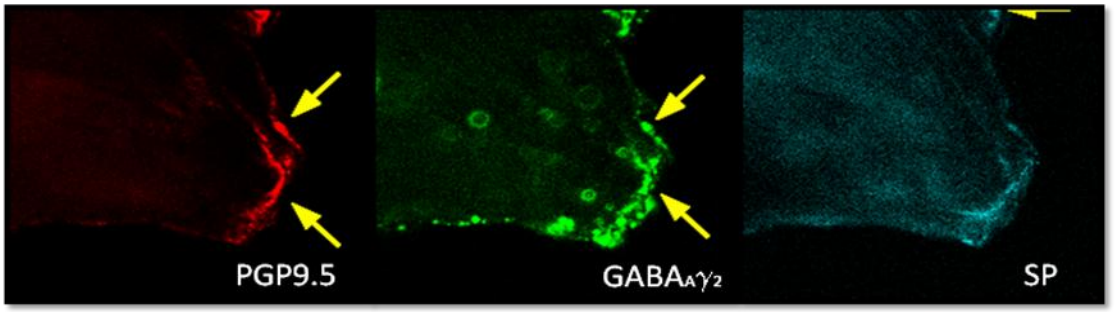


Figure 14: An example of two non-peptidergic, $GABA_A\gamma_2$-containing axonal fibres. The yellow arrows were pointing at two PGP 9.5-positive axonal fibres *(most left)*, both of which

did express the γ_2 subunit of the $GABA_A$ receptor *(middle)*. However, these axonal fibres did not express any immunoreactivity for the antibody against the neuropeptide, Substance P (SP) *(most right)*.

The mean±SD neuronal density value was 4.0±0.3 nerve fibres/ mm^2 calculated from 4 tongue sections/rat and a total of 6 intact female rat tongues. Only PGP9.5-positive nerve fibres found on the mucosa of tongue sections were quantified. It was found that 25% ± 4% of axonal fibres in the tongue mucosa were positive for SP antibody and therefore were considered as peptidergic neuronal fibres. An average of 94% ± 4% of these peptidergic fibres co-expressed the $GABA_A\gamma_2$. In addition, 93% ± 0% of non-peptidergic fibres also expressed the $GABA_A\gamma_2$. These results are summarized in Table 3 below. There was no significant difference between the median values [interquartile ranges] of proportions of $GABA_A\gamma_2$-containing peptidergic fibres (94% [89- 100%]) and $GABA_A\gamma_2$-containing non-peptidergic fibres (93% [92- 96%]) ($P > 0.05$, Mann-Whitney Rank Sum Test) (see Figure 15).

	Assessor A	Assessor B	Mean	SD
Nerve Fibre Density (fibres/mm^2)	4.3	4.7	4.5	0.3
Proportion of Peptidergic Fibre (%)	22.0	28.0	25.0	4.0
Proportion of GABA$_A\gamma_2$+Peptidergic Fibre (%)	91.0	96.0	94.0	4.0
Proportion of GABA$_A\gamma_2$+ Non-peptidergic (%)	93.0	93.0	93.0	0.2

Table 3: This table summarises the immunohistochemistry results from a total of 6 intact female rats with 4 tongue sections taken from each rat.

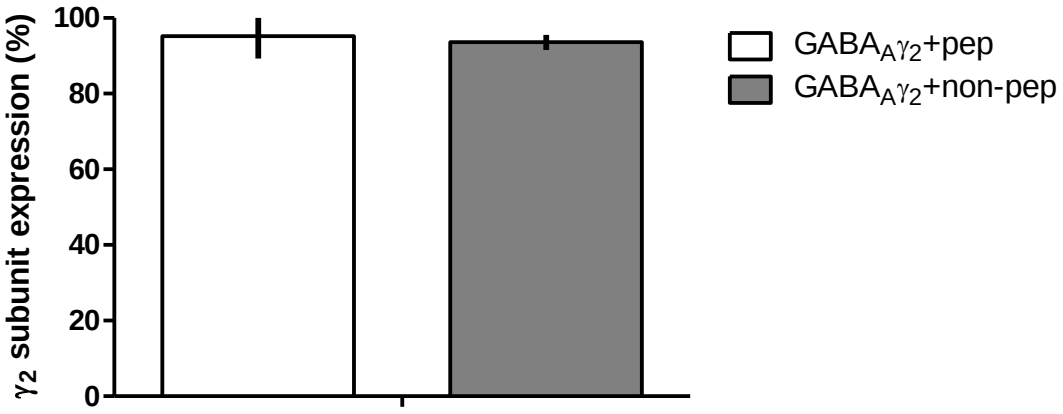


Figure 15: There was no significant difference between the proportions of peptidergic fibres expressing the GABA$_A\gamma_2$ (n=24) (94% [89- 100%]) and non-peptidergic fibres that expressed GABA$_A\gamma_2$ (n=24) (93% [92-96%]) (P> 0.05, Mann-Whitney Rank Sum Test).

4.1.3 **Results from Electrophysiology Recordings**

4.1.3.1 Effects of Treatments and Time on Mechanical Thresholds of Afferent Fibres

A total of 8, 10 and 12 fibres (n=6 female rats per group) were subjected to treatments with GABA, MUS and vehicle (0.01M PBS) solutions respectively. Two way repeated measures ANOVA indicated a significant effect of time, but not treatment or the interaction between treatment and time on the MTs of afferent fibres examined (see Figure 16).

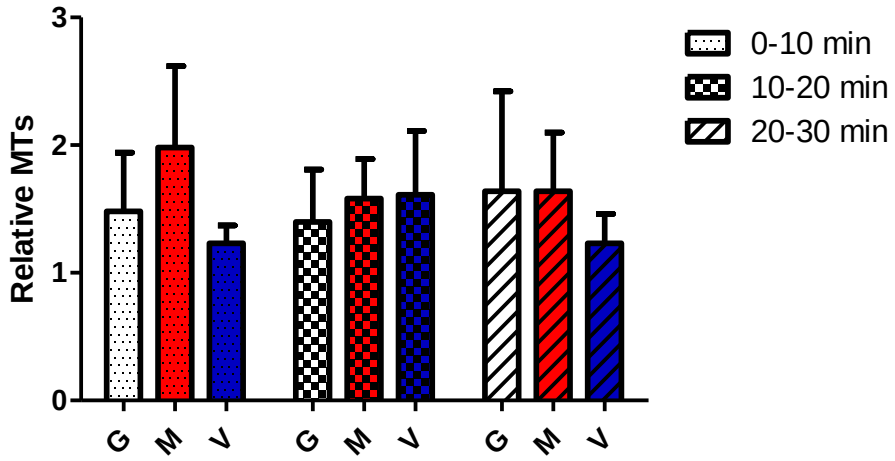


Figure 16: Relative mechanical thresholds (MTs) from afferent fibres bathed in topical solutions of GABA (G) (n=8), MUS (M) (n=10) and vehicle (V) (n=12). There was no significant effect of treatments, or the interaction between treatment and time on the MTs of afferent fibres. However, there was a significant effect of time ($p<0.05$, Two Way Repeated ANOVA).

4.1.3.2 Effect of Treatments on Mechanical Thresholds of Thermally-Stimulated Afferent Fibres

MTs were taken after thermal stimulations from 5, 6 and 8 fibres treated with GABA, MUS and vehicle (0.01M PBS) solutions respectively. There was no significant effect of treatment with GABA on the MTs of all fibres after noxious thermal stimulation (P> 0.05, One Way ANOVA). Interestingly, after thermal stimulation (hot water at 60°C), the relative MT of muscimol-treated fibres (at 0-10 min after thermal stimulation) was significantly greater than vehicle-treated fibres (*: P< 0.05, One Way ANOVA) (see Figure 17).

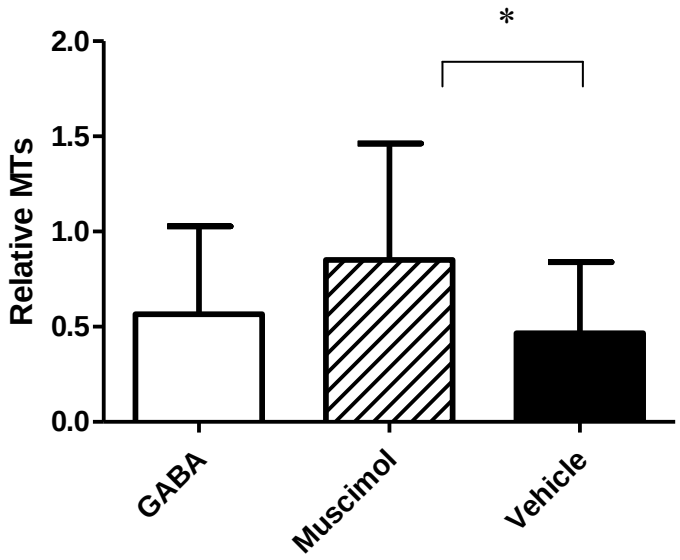


Figure 17: After thermal stimulation (hot water at 60°C), the relative MT of muscimol-treated fibres was significantly greater than vehicle-treated fibres (*: P< 0.05, One Way ANOVA). GABA (n=6), MUS (n=5), vehicle (n=8)

4.2 Results from OVX and SHAM female rats

4.2.1 Baseline Properties of Afferent Fibres

For assessment of baseline afferent fibre properties, fibres were collected from a total of 19 OVX and 16 SHAM female Spraque-Dawley rats. In the OVX group, 30 fibres were MR, 10 PM nociceptors, and 2 undefined fibre type (but mechanically-responsive). In the SHAM group, 15 fibres were MRs, 6 PMs, and 3 undefined fibre type (but mechanically-responsive). Fibres were categorised as undefined (UD) when they stopped responding to mechanical stimuli before thermal stimulation (hot water at 60°C) could be applied. Therefore, it could not be ascertained whether these fibres would be pure MRs or PMs. An afferent fibre was categorised as a PM receptor if it responded to both mechanical and thermal stimulations (see Section 3.2.2.4.3).

A total of 42 and 24 mechanically-responsive afferent fibres were collected from OVX and SHAM rats respectively. In the OVX group, 30 (63% of the population) of them were pure MRs whereas the other 11 (37% of the population) were PMs. In the SHAM group, 15 (71% of the population) of them were pure MRs whereas the other 6 (29% of the population) were PMs, which is consistent with the ratio of MR: PM obtained from intact female rats. UD fibres were not included in this calculation. Comparison of the proportions of MRs to PMs amongst the three rat groups (see Table 4) revealed no significant differences in these proportions ($p>0.05$; Chi-Square Test).

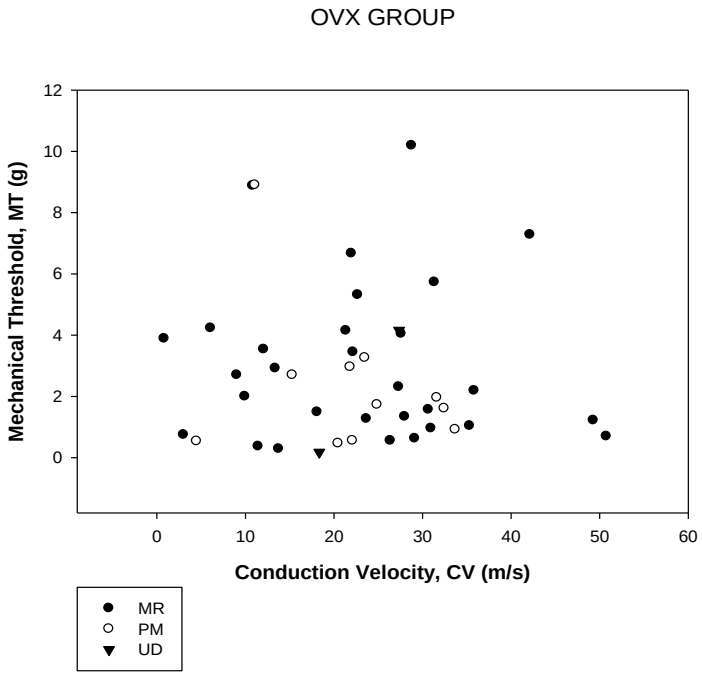
Rat Group	Number of MRs	Number of PMs
OVX	30	10
SHAM	15	6
Intact	42	18

Table 4: There was no significant difference in the proportions of mechanoreceptors (MRs) to polymodal receptors (PMs) in OVX, SHAM and intact female ($p>0.05$; Chi-Square Test).

In the OVX group, the estimated CVs of the collected fibres ranged from 0.8 – 50.8 m/s which indicated that Aβ (myelinated) fibres, Aδ (thinly-myelinated) fibres, and C- fibres (unmyelinated) were collected and examined; 33 Aβ fibres (> 12 m/s), 8 Aδ fibres (2- 12 m/s) and 1 C-fibres (< 2 m/s) were collected. In the SHAM group, the range of estimated CVs collected from tongue fibres ranged from 8.3- 35.8 m/s, out of which 22 were Aβ fibres (> 12 m/s) and 2 Aδ fibres (2- 12 m/s). No C fibres were found from the 16 female SHAM rats.

Similar to results obtained from masseter muscle afferent fibres of female rats (Mann et al., 2006) and tongue afferent fibres of intact female rats (see Section 4.1.1), there was no significant relationship between CV and MT of tongue afferent fibre in both groups of female rats ($P> 0.05$, Spearman Rank Order Correlation) (Figure 18A and B).

A



B

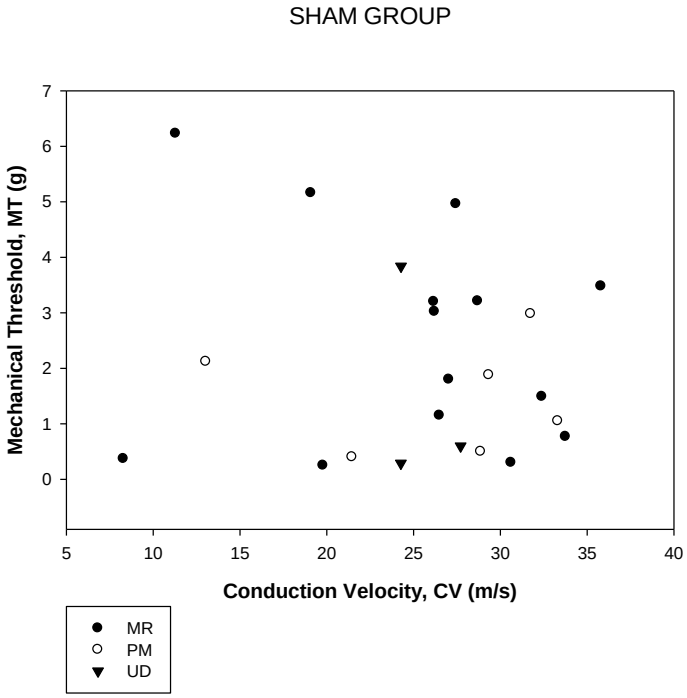


Figure 18: The scatter plots (A and B) illustrate the relationship between baseline MT and CV of tongue afferent fibres in OVX and SHAM female rats, respectively. (A) There was no significant relationship between baseline MT and CV of tongue afferent fibres (P> 0.05, Spearman Rank Order Correlation) collected from OVX rats (n=42). (B) There was no

significant relationship between baseline MT and CV of tongue afferent fibres (P> 0.05, Spearman Rank Order Correlation) collected from SHAM rats (n=24).

In both groups of rats, comparisons of the basic properties between MRs and PMs showed no significant differences between the two types of receptors (see Figure 19A and 19B). For OVX group, the CVs (median [interquartile range]) between MRs (23.2 m/s [12.1-30.7]) and PMs (22.1 m/s [16.6-30.0]) were not significantly different (P>0.05, Mann-Whitney rank sum test). The CVs (median [interquartile range]) between MRs (26.8 m/s [19.8-30.6]) and PMs (29.1 m/s [21.5-31.8]) from the SHAM group were also not significantly different (P>0.05, Mann-Whitney rank sum test).

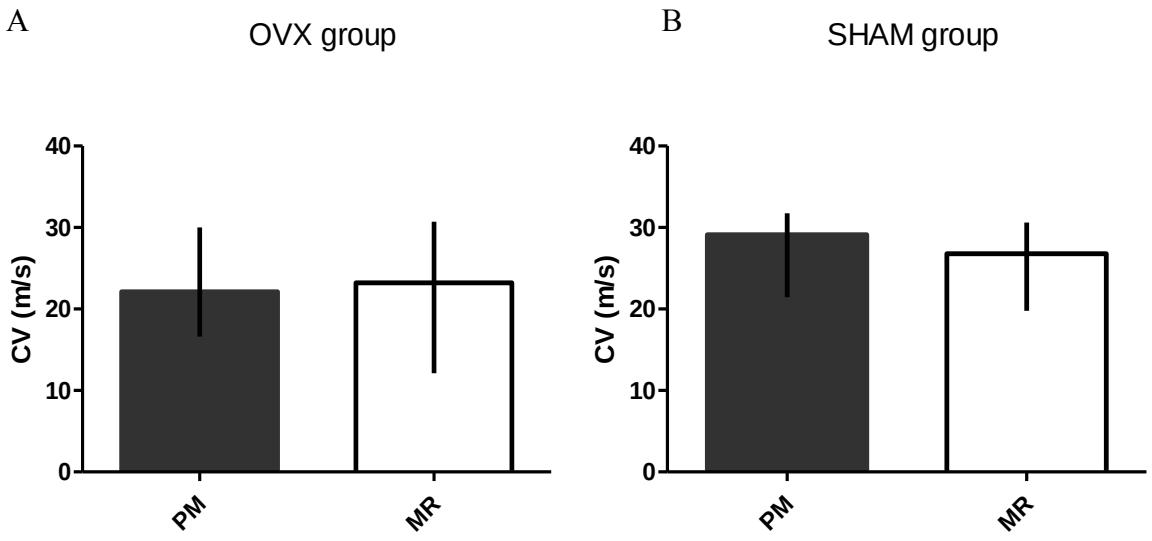


Figure 19: (A) There was no significant difference in the median values of CVs between PMs (n=11) and MRs (n= 30) collected from OVX rats (P> 0.05, Mann-Whitney rank sum test). (B) In the SHAM group, there was no significant difference in the median values of CVs

between PMs (n= 6) and MRs (n= 15) (P> 0.05, Mann-Whitney rank sum test). Open-ended bars represent median [interquartile range] values.

Similarly, the baseline MTs (median [interquartile range]) between MRs and PMs were also not significantly different in both rat groups (see Figure 20A and 20B). In the OVX group, the baseline MTs (median [interquartile range]) between MRs (2.3g [1.0-4.2]) and PMs (1.7g [0.7-2.9]) were not significantly different (P>0.05, Mann-Whitney rank sum test). Likewise, the baseline MTs (median [interquartile range]) between MRs (2.4g [0.5-2.1]) and PMs (1.5g [0.5-2.1]) from the SHAM group were also not significantly different (P>0.05, Mann-Whitney rank sum test).

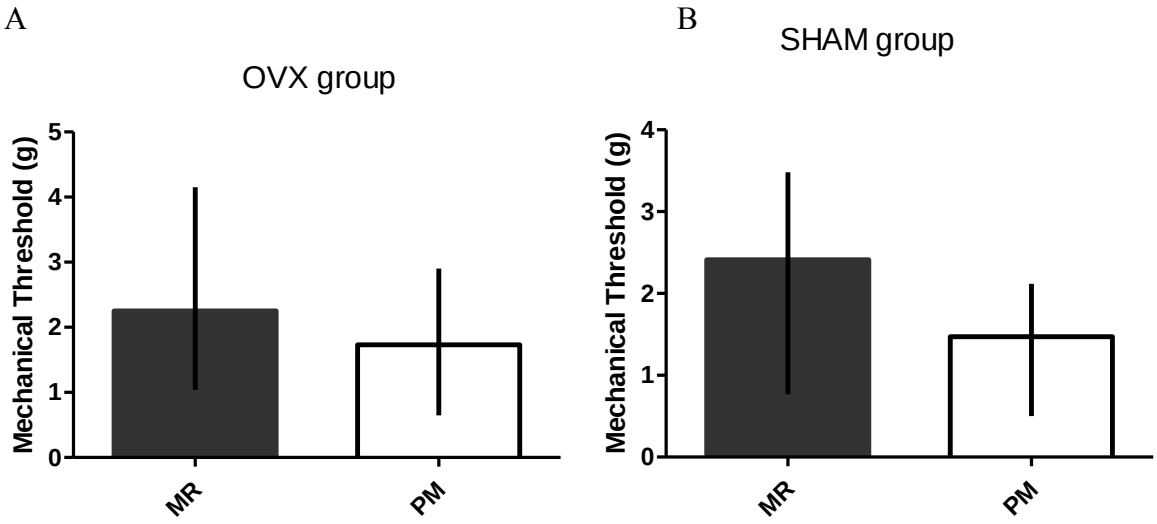


Figure 20: (A) There was no significant difference in the median values of baseline MTs in the OVX group between PMs (n= 11) and MRs (n=30) (P> 0.05, Mann-Whitney rank sum test). (B) There was no significant difference in the median values of baseline MTs between PMs (n= 6) and MRs (n=15) in the SHAM rats (P> 0.05, Mann-Whitney rank sum test). Open-ended bars represent median [interquartile range] values. Overall, the two groups of

rats showed similar baseline properties in terms of CVs and baseline MTs of both MRs and PMs (see Figure 21A and 21B).

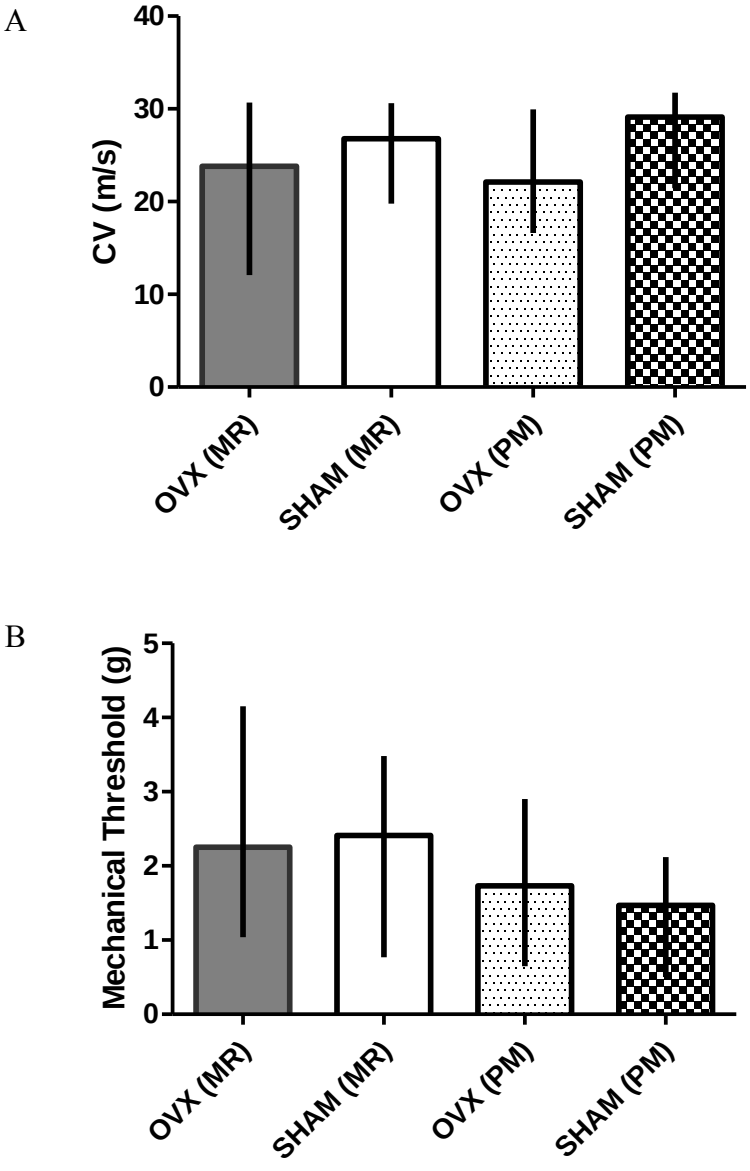


Figure 21: (A) There were no significant differences in the median values of CVs for both MRs and PMs collected from OVX and SHAM groups ($p>0.05$, Mann-Whitney rank sum test). (B) There were no significant differences in the median values of baseline MTs for MRs and PMs across the two rat groups ($p>0.05$, Mann-Whitney rank sum test). OVX: MR

(n=30), PM (n=11). SHAM: MR (n=15), PM (n=6). Open-ended bars represent median [interquartile range] values.

4.2.2 **Spontaneous Discharge of Afferent Fibres**

The ranges of frequencies of spontaneous afferent activity from all sensory fibres collected from both rat groups are summarised in Table 5 below.

		Range of frequency (Hz)
OVX	MR	0.002-0.137
	PM	0.003-0.138
	UD	0.043
SHAM	MR	0.002-0.027
	PM	0.002-0.043
	UD	0.002-1.367

Table 5: The ranges of frequencies of spontaneous firing of afferent fibres. MR= mechanoreceptor, PM= polymodal receptor, UD= undefined receptor, OVX=ovariectomised rats, SHAM= operated but non-ovariectomised rats.

Some neuronal fibres had spontaneous firing activities even before any drug administration whereas others did not. These afferent fibres were categorised into spontaneous (S) versus non-spontaneous (NS) groups and the frequency of these occurrences were recorded in Table 6 below.

	OVX			SHAM		
	MR	PM	UD	MR	PM	UD
S	11	5	1	6	5	2
NS	19	6	0	9	1	2

Table 6: There was no significant difference in the frequency of occurrence of afferent fibres with spontaneous (S) discharge and those that lacked spontaneous discharge (not-spontaneous; NS) for the various treatments MR= mechanoreceptor, PM= polymodal receptor, UD= undefined receptor, OVX=ovariectomised rats, SHAM= operated but non-ovariectomised rats.

As discussed in section 4.2.1, there was no significant difference in the baseline properties between MRs and PMs in both groups of female rats. Therefore, the above data from MRs and PMs in both rat groups were tabulated together to determine whether the populations of afferent fibres between OVX and SHAM rats displayed any differences in the frequencies of spontaneous and non-spontaneous firing activities (see Table 7).

	OVX	SHAM	Total
S	17	13	30
NS	25	12	37
Total	42	25	67

Table 7: There was no significant difference in the frequency of occurrence of afferent fibres with spontaneous (S) discharge and those that lacked spontaneous discharge (not-spontaneous; NS) ($p=0.448$; Fisher Exact Test).

4.2.3 **Effect of Muscimol (MUS) and Bicuculline (BIC) on Spontaneous Firing of Afferent Fibres**

The number of afferent fibres that showed increase and decrease in the levels of spontaneous activities were tabulated in the tables below (see Tables 8 and 9).

	PBS	MUS	Total
INCREASE	10	13	23
DECREASE	7	4	11
Total	17	17	34

Table 8: There was no significant difference in the frequency of occurrence of afferent fibres that exhibited increased spontaneous discharge or decreased spontaneous discharge when bathed in MUS as compared with vehicle (i.e. PBS) (P = 0.465; Fisher Exact Test).

	PBS	BIC	Total
INCREASE	2	3	5
DECREASE	10	3	13
Total	12	6	18

Table 9: There were no significant differences in the number of afferent fibres with increased and decreased spontaneous discharges when bathed in BIC as compared with PBS (P = 0.465; Fisher Exact Test).

4.2.4 **Immunohistochemistry Results**

In OVX and SHAM groups, the mean neuronal density values were 8 nerve fibres/ mm^2 (SEM: ± 0.47) and 8 nerve fibres/ mm^2 (SEM: ± 0.65), respectively (see Table 10A and 10B). These values were calculated from averages from 3 tongue sections/rat and a total of 6 female rat tongues per group. Only PGP9.5-positive nerve fibres found on the mucosa of tongue sections were quantified.

A

OVX Group	MEAN	SEM
Neuronal Fibre Density(fibres/mm^2)	8.00	0.47
Proportion of Peptidergic Fibres	24.17%	0.03
Proportion of $GABA_A\gamma_2$-positive, peptidergic fibre	97.25%	0.01
Proportion of $GABA_A\gamma_2$-positive, non-peptidergic fibre	94.35%	0.00

B

SHAM Group	MEAN	SEM
Neuronal Fibre Density (fibres/mm^2)	8.00	0.65
Proportion of Peptidergic Fibres	23.84%	0.03
Proportion of $GABA_A\gamma_2$-positive, peptidergic fibre	97.49%	0.01
Proportion of $GABA_A\gamma_2$-positive, non-peptidergic fibre	93.97%	0.01

Table 10: (A) This table provides mean and standard error of the mean values for all data collected from OVX rat tongues (n=18). (B) This table provides mean values and standard error of the mean values for all data collected from SHAM rat tongues (n=18).

It was found that the mean±SEM proportion of SP-IR axonal fibres in the tongue mucosa of both rat groups was 24% ± 3%. These SP-positive fibres were categorised as peptidergic neuronal fibres. Again, in both groups, an average±SEM of 97% ± 1% of these

peptidergic fibres co-expressed the $GABA_A\gamma_2$ while 94% (± 1% for SHAM group only) of non-peptidergic fibres in both rat groups also expressed the $GABA_A\gamma_2$. In all four parameters quantified and compared, there were no significant differences between the two rat groups ($p>0.05$, Student's t-test) (see Table 10A and 10B).

4.2.5 **Electrophysiology Results**

In order to ensure that the 30-minute washout periods were sufficient to restore the baseline values of MTs, mean values of B_{MT}, B_{2MT} and B_{3MT} from the different treatment paradigms (see Section 3.3 and Figure 9) were compared and they were found to be not significantly different ($p>0.05$; Two Way Repeated Measures ANOVA (One Factor Repetition)). This result also indicated that MTs of tongue afferent fibres did not change significantly over the course of the experiments.

4.2.5.1 Effect of Muscimol (MUS) on Mechanical Threshold (MT)

The mean MTs of afferent fibres after 10 minutes of topical bath with drug solution (either PBS or MUS) were normalised to the mean baseline MTs values before any drug application. In both OVX and SHAM groups, treatment with MUS did not have a statistically significant effect on the relative MTs of afferent fibres post drug administration ($p>0.05$; Two Way ANOVA) (see Figure 22). In the OVX group, mean±SEM relative MTs of afferent fibres bathed in PBS (n=22) and MUS (n=20) solutions were 1.3±0.2 and 1.2±0.1. Similarly,

mean±SEM relative MTs of afferent fibres bathed in PBS (n=16) and MUS (n=8) solutions were 1.3±0.1 and 1.2±0.1 in the SHAM group.

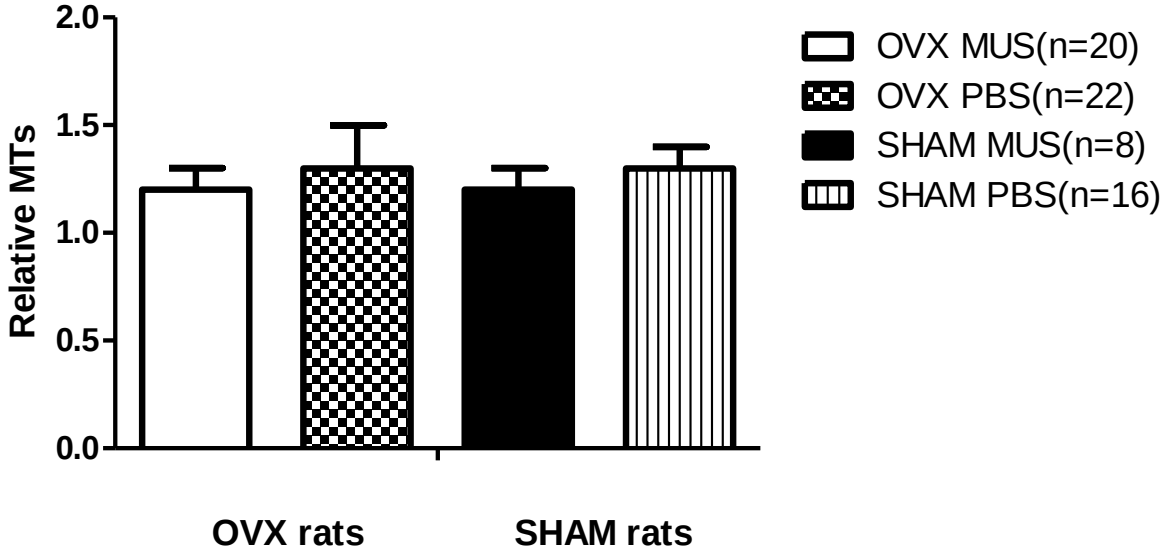


Figure 22: There was no significant difference in the mean values of relative MTs between muscimol (MUS) and vehicle (PBS) treatments. There was also no significant difference in the mean values of relative MTs between OVX and SHAM groups. There was no significant interaction between treatment and rat groups ($p > 0.05$, Two Way Analysis of Variance). Error bars indicate standard error of the mean (SEM).

4.2.5.2 Effect of Bicuculline (BIC) on Mechanical Threshold (MT)

The mean MTs of afferent fibres after 10 minutes of topical bath with drug solution (from treatment paradigm of either PBS/PBS or PBS/BIC) were normalised to the mean baseline MTs values before any drug application. In both OVX and SHAM groups, treatment

with BIC did not have a statistically significant effect on the mean MTs of afferent fibres post drug administration ($p>0.05$; Two Way Analysis of Variance) (see Figure 23). In the OVX group, mean±SEM relative MTs of afferent fibres bathed in PBS (n=9) and BIC (n=9) solutions were 1.1±0.1 and 0.9±0.1. Similarly, mean±SEM relative MTs of afferent fibres bathed in PBS (n=6) and BIC (n=8) solutions were 0.90±0.02 and 1.10±0.10 in the SHAM group.

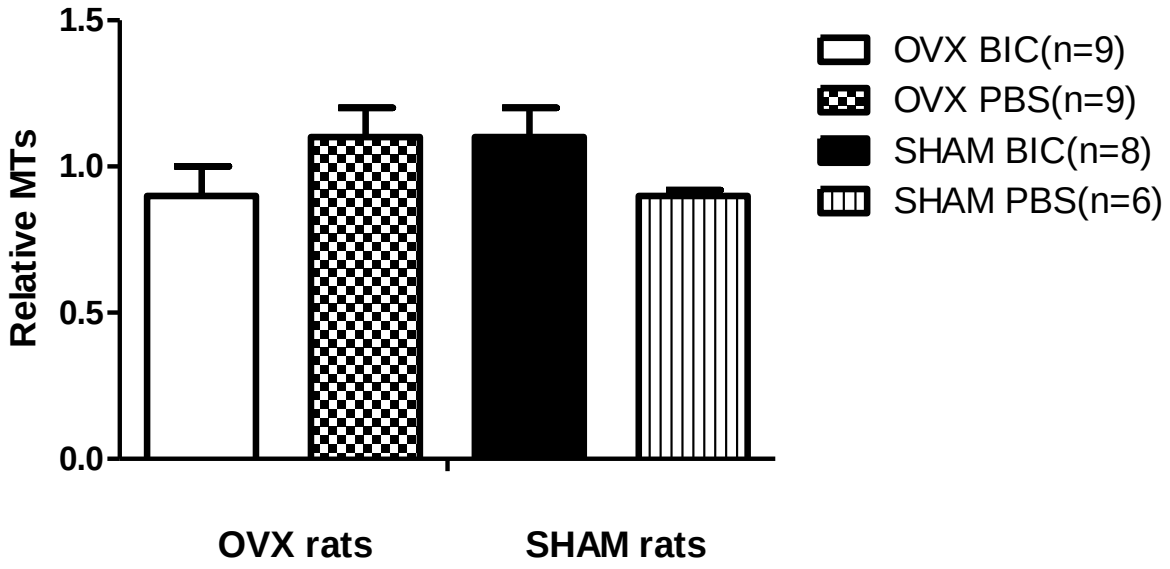


Figure 23: There was no significant difference in the mean values of relative MTs between bicuculline (BIC) and vehicle (PBS) treatments. There was no significant difference in mean values of relative MTs between OVX and SHAM groups. There was no significant interaction between treatment and rat groups ($p> 0.05$, Two Way Analysis of Variance). Error bars indicate standard error of the mean (SEM).

4.2.5.3 Comparison of Thermal Thresholds of Polymodal Receptors (PMs)

Baseline tongue temperatures were recorded before hot water was added to the bath. The lowest temperature at which an afferent fibre fired in response to thermal stimulation (addition of hot water at 60 °C) was taken as the thermal threshold for that particular neuronal fibre. An afferent fibre was defined as responsive to thermal stimuli when the evoked frequency of APs was higher than the frequency of baseline spontaneous discharge (if any) (see Section 3.2.2.4.3). The median values [interquartile range] of thermal thresholds (°C) from the OVX and SHAM group were 31.4 [25.2-34.7] and 33.7 [32.1-36.0] respectively. These values were not significantly different between the groups ($p > 0.05$, Mann-Whitney Rank Sum test) (see Figure 24). The temperature difference between baseline tongue temperature and thermal threshold for each afferent fibre was calculated and median values were compared between the two rat groups. There was no statistical significance between the median values [interquartile range] of temperature differences in the OVX (14.4 [4.6-15.2]) and SHAM (13.7 [12.7-17.7]) groups ($p > 0.05$, Mann-Whitney Rank Sum test) (see Figure 25).

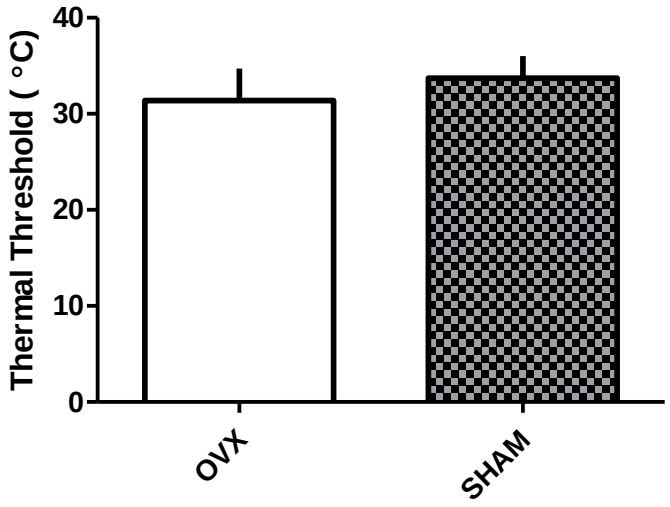


Figure 24: There was no significant difference in the median values of thermal thresholds between PMs collected from OVX (n= 11) and SHAM groups (n=6) (p> 0.05, Mann-Whitney Rank Sum test). Open-ended bars indicate median [interquartile range] values.

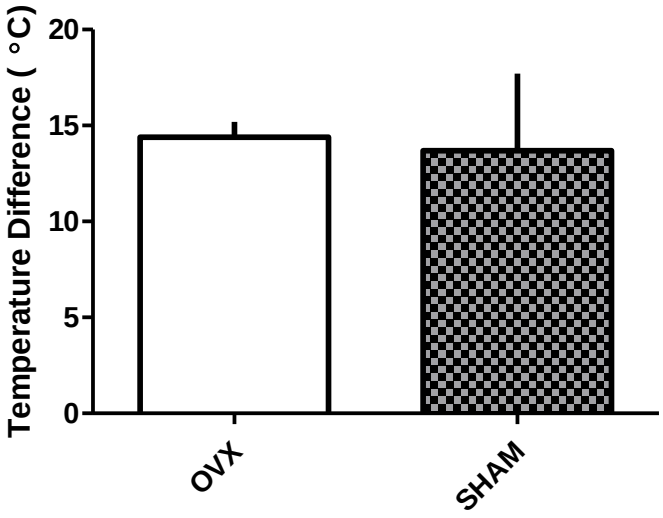


Figure 25: There was no significant difference in the median values of temperature differences between baseline tongue temperatures and thermal thresholds for sensory fibres between OVX (n=11) and SHAM (n=6) groups (p> 0.05, Mann-Whitney Rank Sum test). Open-ended bars indicate median [interquartile range] values.

4.2.5.4 Effect of Hot Water on MTs

Baseline MTs before afferent fibres were treated with hot water (B_{3MT}) were compared with post-thermal MTs in both groups of rats. Median values [interquartile range] of B_{3MT} and post-thermal MTs in the OVX group were 1.81 [0.8-3.3] and 1.52 [0.7-2.5], as well, in the SHAM group, the values were 0.85 [0.5-2.7] and 0.72 [0.5- 3.2] respectively. The pre-thermal MTs (B_{3MT}) were not significantly different from the post-thermal MTs ($p > 0.05$; Wilcoxon Rank Sum Test) in both groups of rats (see Figure 26). In short, hot water did not significantly affect the MTs of sensory fibres in both groups of rats.

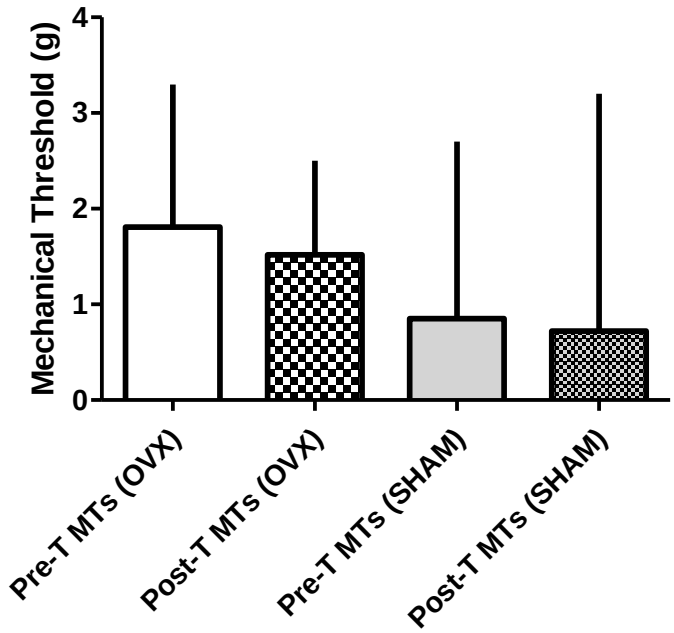


Figure 26: In the OVX (n=20) and SHAM (n=15) groups, there was no significant difference between the pre-thermal (Pre-T) MTs from the post-thermal (Post-T) MTs ($p > 0.05$; Wilcoxon Rank Sum Test). Open-ended bars indicate median [interquartile range] values.

5 Chapter: Discussion and Conclusion

The principal finding from this study is that nerve fibres on tongue mucosa express an abundance of $GABA_A$ receptors containing the γ_2 subunit ($GABA_A\gamma_2$). Immunohistochemistry analysis revealed that high proportions of axonal fibres in tongue mucosa, both peptidergic and non-peptidergic ones, expressed the $GABA_A$ receptor, suggesting that these receptors are likely to serve important function(s) in the oral cavity. From the functional aspect, thermally-stimulated tongue afferent fibres from intact female rats demonstrated significantly higher MTs after being topically-bathed in MUS solutions. Together with the high expression levels of $GABA_A\gamma_2$ in tongue axonal fibres, activation of peripheral $GABA_A$ receptors on the tongue is likely to modulate sensory (pain and touch) neurotransmission to the trigeminal ganglion.

My investigation of the effect(s) of loss of female sex hormones (e.g. menopause) on the innervation patterns of the tongue did not yield any detectable differences between OVX and SHAM rats in terms of the nerve densities values, proportions of peptidergic, small-diameter nerve fibres, proportions of $GABA_A\gamma_2$- positive, peptidergic fibres and proportions of $GABA_A\gamma_2$- positive, non-peptidergic fibres. Similarly, electrophysiology recordings from sample populations of tongue MRs and PMs in OVX and SHAM rats did not detect any significant differences in any of the parameters examined i.e. proportions of MRs: PMs, mean baseline MT, thermal threshold and CV. Based on observations from this study, , I conclude that depletion of sex hormones via 28 day-post ovariectomy was ineffective to induce any detectable changes in innervation patterns on female rat tongue mucosa.

5.1 Expression and Role of Peripheral $GABA_A$ receptors

I discovered in this study that high proportions of axonal fibres in the tongue mucosa did express $GABA_A\gamma_2$. In this study, axonal fibres in tongue mucosal layer of OVX, SHAM and intact female rats were found to have high proportions of $GABA_A\gamma_2$; 94% and 93% of peptidergic and non-peptidergic axonal fibres, were labelled positive for the $GABA_A\gamma_2$-IR in all three groups of rats. A study reported that 100% of large-sized (out of 142 cells) and small-sized (out of 416 cells) neuronal cell bodies in the trigeminal ganglion were found to express the $GABA_A\gamma_1$, $GABA_A\gamma_2$ or $GABA_A\gamma_3$ (Hayasaki et al., 2006). Reverse transcription polymerase chain reaction (RT-PCR) on cleanly isolated taste buds of mice also detected the presence of RNAs encoding all known subunits of $GABA_A$ receptor and $GABA_B$ receptor (Dvoryanchikov et al., 2011). Interestingly, the RNA coding for the $GABA_A\gamma_2$ was only detected from the vallate papilla tissue samples, which contained muscle tissues, connective tissues and importantly, neuronal tissues, but none was found in taste buds samples or in lingual epithelium tissues of mice (Dvoryanchikov et al., 2011). Another study which employed the RT-PCR and immunohistochemical methods also detected the presence of the $GABA_A\alpha_1$, $GABA_A\alpha_2$, $GABA_A\alpha_3$, $GABA_A\alpha_4$, and $GABA_A\alpha_6$ RNA in the circumvallate taste buds of mice (Starostik et al., 2010). In fact, $GABA_A$-IR was detected in 100% of taste buds (237 taste buds) that were labelled with anti-$GABA_A$ antibodies (Starostik et al., 2010). The authors also observed that $GABA_A\alpha_1$ expression was not restricted to taste buds as there was positive-labelling in the lingual epithelium (Starostik et al., 2010). Similar results were reported in another study which found that about two-third (202 out of 301) of the taste buds in either the foliate or circumvallate papilla were positively-labelled for the $GABA_A\alpha_1$ (Cao

et al., 2009). Taken together, such consistent reports of high levels of expression of the different subunits of the $GABA_A$ receptor from different types of cells in the tongue and neuronal cell bodies in the trigeminal ganglion are congruent with the results obtained from this study. It is worth noting that in my study, only axonal fibres on the most superficial aspect of the mucosa i.e. nerve fibres in the epithelial cells around and in between tongue papilla were quantified. Moreover, $GABA_A$ receptors had also been discovered in the mandible, the palate and the salivary glands (Watanabe et al., 2002). Taken together, such an abundance of peripheral $GABA_A$ receptors in the oral cavity indicates that these receptors are likely to have important functional roles and are likely to be accessible for local pharmacologic manipulation.

In fact, several studies have found that peripheral $GABA_A$ receptors could be activated to inhibit sensory information. For example, intra-articular injection of GABA was found to suppress reflex jaw muscle activity, evoked by noxious stimulation of the rat temporomandibular joint (TMJ) in a concentration related manner (Cairns et al., 1999, Cai et al., 2001). This GABA-mediated inhibition was reversed by co-application of the $GABA_A$ receptor antagonist, BIC but not by the $GABA_B$ receptor antagonist, phaclofen. These observations suggest that the inhibition was mediated through activation of peripheral $GABA_A$ receptors in the TMJ. Behavioural studies also indicated that local peripheral injection of a low concentration of the $GABA_A$ agonist, MUS (2.0 μM) attenuated formalin-induced nociceptive behaviors (Carlton et al., 1999). This effect was lessened as the concentration of MUS was increased and intraplantar injection of MUS alone at a high dose induced thermal hyperalgesia. When BIC was applied, it prevented these MUS-induced

changes in behavior. From these observations, I speculate that activation of peripheral $GABA_A$ receptors with low concentration of MUS results in a current shunt that interferes with the conduction of APs (Cairns et al., 1999), whereas activation of these receptors with high concentration of MUS leads to depolarisation of primary sensory endings.

In the first part of this study, MUS was found to significantly increase MTs of afferent fibres, but only after they were briefly heated with hot water (60°C). There was no significant effect of MUS on MTs of sensory fibres before these fibres were thermally-stimulated. Similarly, experiments conducted on OVX and SHAM rats also detected no significant effect of MUS on MTs of afferent fibres. It is probable that the addition of hot water increased blood flow to the tongue resulting in better permeability of MUS into tongue mucosal layer. In retrospect, based on this speculation, had I injected MUS into the receptive field, I might have been able to detect significant changes to the MTs.

It is worth reiterating that taste alterations and/or taste damage (also known as dysgeusia) are very common complaints in BMS patients (Scala et al., 2003, Maltsman-Tseikhin et al., 2007). A study observed that when taste neurotransmission was blocked through anaesthesia of the chorda tympani nerve, there was an intensified burning sensation in the contralateral anterior tongue when capsaicin was applied (Tie et al., 1999), indicating that under normal circumstances, taste input has an inhibitory effect on trigeminal sensory input (Bartoshuk et al., 2005). Grushka and colleagues postulated that BMS might result from damage to the taste buds leading to a loss of inhibition normally exerted on trigeminal sensory neurons encoding pain information from the oral cavity (Bartoshuk et al., 2005).

Hence, application of a $GABA_A$ receptor modulator such as clonazepam might be able to counteract this effect by inhibiting sensory afferent fibres that convey mechanical and thermal nociceptive information.

5.2 Epithelial Nerve Fibre Density (ENFD)

Several studies had investigated and compared tongue epithelial nerve fibre densities (ENFD) in human BMS patients and healthy controls using immunohistochemical techniques. Lauria and colleagues reported mean ±SD of the epithelial fibres/papilla of 2.00 (±2.17) in BMS patients (n=11) and 4.13(±1.85) in healthy controls (n=8) (Lauria et al., 2005). Based on results from this study, there was more than a one-fold reduction of epithelial nerve fibre density in BMS patients compared to healthy controls (Lauria et al., 2005). Following this report, another study also found that BMS patients had significantly lower epithelial nerve fibre density (Yilmaz et al., 2007). The mean±SEM of the epithelial fibres/papilla were 0.92±0.19 in healthy controls and 0.27±0.04 in BMS patients (Yilmaz et al., 2007). Both studies also employed 3-mm punch biopsies of the tongue and tissues were obtained from relatively similar site in the tongue i.e. the anterolateral aspect of the tongue close to the tip. However, only the first study controlled for operators' bias during the quantification process through blinding (Lauria et al., 2005) but there was no mention of such control in the second study (Yilmaz et al., 2007). In addition, both studies utilised different primary antibodies to identify epithelial nerve fibres with the former using the antibody against PGP 9.5 (Lauria et al., 2005) and the latter adopting several antibodies against peripherin and neurofilament proteins (Yilmaz et al., 2007) to label epithelial nerve fibre. It is worth noting that Lauria and colleagues did not use age-matched controls with BMS group

having a mean age of 14 years more than the control group (Lauria et al., 2005). Despite some of the aforementioned weaknesses in the design of these two studies, others have also repeatedly shown significant reduction of epithelial small-diameter nerve fibres in the tongue mucosa of BMS patients (Yilmaz et al., 2007,Beneng et al., 2010, Puhakka et al., 2010). The most recent investigation (Puhakka et al., 2010) has by far the most convincing demonstration of a significant reduction of epithelial nerve fibre density in tongue mucosa of BMS patients because strict diagnostic criteria for primary BMS were applied with properly age-matched control group. Furthermore, meticulous neurophysiologic and QST examinations were performed to selectively include only patients with small-diameter fibre dysfunction and excluded patients with other major neuropathies (Jaaskelainen, 2012).

In my study, I adopted slightly different approaches from the above studies, to quantify and compare nerve densities of rat tongue mucosal fibres. In an attempt to develop an animal model of BMS, one of the aims of this study was to investigate whether depletion of female sex hormones achieved through ovariectomy could induce alterations to tongue innervation densities, specifically a significant reduction of small-diameter nerve fibres in OVX female rats, as had been observed in human BMS patients. Unfortunately, in both OVX and SHAM rats, immunohistochemical analyses found no significant differences in nerve densities with both groups having an average of 8 nerve fibres/mm^2 of coronal tongue tissue (10µm thick). Similar to the other studies, tongue specimens were dissected coronally from the most anterior part of the tongue i.e. 0.5 mm from the tip of the tongue. However, only axonal fibres running parallel to the plane of section were quantified as these fibres were distinctly recognisable with a fibril-like morphology. These were intra-epithelial nerve fibre

on the most superficial aspect of the mucosa. By comparison, in the study by Lauria and colleagues, epithelial nerve fibre arising from the sub-papillary nerve bundles were quantifed whereas in the study by Yilmaz and colleagues, only intra-epithelial nerve fibre densities were different between BMS patients and controls with no significant differences of sub-epithelial nerve fibre densities. Due to the tortuous course of nerve fibres, some nerve fibres running perpendicular or oblique to the planes of section could not be confidently identified, hence were not counted. Instead of quantifying the number of nerve fibres per papilla, I decided to quantify the total number of epithelial nerve fibre running parallel to the most superficial aspect of the mucosa of each tissue section (PGP 9.5-positive labelling) before dividing by the total area size of that particular tissue section. From my initial observations, there were many superficial axonal fibres running parallel to the mucosal surface in between each papilla (inter-papillary epithelial nerve fibres). With this estimation method, these fibres were taken into account. As for measurement of area sizes, the most accurate and efficient technique known to me, was to measure the entire coronal section of each tongue tissue from digital images using the ImageJ software. I would like to point out that because of this chosen technique, the measured area sizes encompassed not only the mucosal layers, but also the muscle and connective tissue layers of the tongue, whereas only axonal fibres in the mucosal layer were quantified. Still, the same techniques were consistently employed in both rat groups, so that fair comparisons could be made. Retrospectively, by analysing several confocal images (x10) of these tissue sections, I found that the mucosal layer made up about 10% of the entire coronal tissue section. Therefore, as a rough approximation, both rat groups had an average of 80 axonal nerve fibres/mm^2 of mucosal tissue.

Perplexingly, the mean nerve density found in intact female rats was around 4 nerve fibres/mm^2 tongue tissue or 40 nerve fibres/mm^2 of tongue mucosal tissue. Most importantly, immunohistochemical analysis of intact female rats was performed by two independent assessors, both unaware of each other's results. The variabilities between results obtained by Assessor A and B were very small (see Table 3) which convinced me that this quantification and estimation technique allows for relatively reliable repeatability and reproducibility. This number is onefold lower than the values obtained from OVX and SHAM rats. The two variables not controlled for in intact females were: 1) Intact female rats were at least 4 weeks younger than OVX and SHAM female rats and had lower mean body weight, 2) Intact female rats also had smaller mean±SD (19.14±5.51 mm^2) area size of the coronal tongue tissue sections than those from OVX (23.54±5.32 mm^2) and SHAM (27.83±4.46 mm^2) rats. In spite of these differences, there was no obvious explanation for the differences in ENFD between the intact females and the OVX/SHAM female rats.

5.3 Nerve Fibres expressing Substance P (SP)

In this study, immunohistochemical analyses of tongue mucosal layers from all three groups of rats namely intact females, SHAM and OVX female rats revealed that approximately a quarter of all labelled axonal nerve fibres (positive PGP9.5-IR) of rat tongue expressed the neuropeptide, SP. A large body of immunohistochemical investigations of the DRG (Hokfelt et al., 1975a, b, Chan-Palay and Palay, 1977b, a) and trigeminal ganglion (Hokfelt et al., 1975b, Cuello et al., 1978, Del Fiacco and Cuello, 1980, Tervo et al., 1981) have discovered that SP-IR was selectively confined to a distinct, small population of small-sized ganglion cells giving rise to small-diameter, thin afferent fibres. Electrophysiological

(Henry, 1976, Andersen et al., 1978, Henry et al., 1980), immunocytochemical (Ma et al., 1996, Ribeiro-da-Silva and Hokfelt, 2000) as well as behavioural (Hayes and Tyers, 1979, Piercey et al., 1981) evidence has strongly indicated that SP-IR is not random and is contained within afferent fibres that predominantly convey pain sensory modality. Taken together, this means the 25% of the SP-IR axons identified from rat tongue mucosa in this study were small-diameter nerve fibres that could have conveyed nociceptive information.

It was found in Sprague-Dawley albino rats (n=30) and guinea pigs (n=20) that an average of 10-30% of trigeminal ganglion neurons demonstrated SP-IR (Lehtosalo et al., 1984). These SP-containing neurons were distributed throughout the trigeminal ganglion with no somatotopic segregation (Lehtosalo et al., 1984). They were either round or elongated cells small-sized neurons with average diameter of 15 to 50 µm (Lehtosalo et al., 1984). Smaller-sized neurones and those larger than 50 micron did not display any SP-IR and it was observed that in many cases these SP-IR ganglion cells gave rise to SP-containing, thin nerve fibers, seen throughout the trigeminal ganglion (Lehtosalo et al., 1984). Qualitatively-noted by the authors, the two species investigated were found to have similar proportions of SP-containing trigeminal neurons (Lehtosalo et al., 1984). Further confirming this finding, a second study utilising the axonal tracer, horseradish peroxidise, also found an approximate of 30% of SP-IR neurones in trigeminal ganglion cells, specifically from those supplying the cornea (the ophthalmic division, V1 of the trigeminal cranial nerve, Vth CN) (Lehtosalo, 1984). Once again, these cells giving rise to V1 nerve fibres, were small-sized neurones (15-50 µm), distributed throughout the anteromedial part of the ganglion, with no somatotopic organisation (Lehtosalo, 1984). In both studies, only unmyelinated nerve fibres in the

trigeminal ganglion displayed SP-IR as the investigators were not able to find any myelinated nerve fibres in the trigeminal ganglion which showed SP-IR. These findings of 30% SP-IR in the trigeminal ganglion neurons are consistent with the results from my study (25% SP-IR of tongue afferent fibres). These SP-positive fibres are considered to be small-diameter afferent fibres because electrical stimulation of myelinated, large-diameter fibres (Aα and Aβ fibers) did not result in a release of SP, whereas depolarization of relatively small-diameter fibres (Aδ and C-fibers) did cause the release of SP (Yaksh et al., 1980).

Despite no quantification being performed, numerous other studies had also located SP-containing nerve fibers in the epithelial layer of the palate of *Rana pipiens* frog (Hernandez et al., 2003) and in the fungiform and filiform papillae of the tongue of the bullfrog, *Rana catesbeiana* (Kusakabe et al., 1996) as well as in vallate papilla (Crescimanno et al., 2004), circumvallate and fungiform papilla (Nagy et al., 1982) of rat tongue. Moderate to few SP-IR was detected in epithelial nerve fibre from dog tongue (Hino et al., 1993) and from vallate papilla of guinea pigs (Huang and Lu, 1996). Skin biopsy from humans also revealed an abundance of SP-IR nerve fibres in the epidermis and dermis of cutaneous facial skin (Nolano et al., 2012).

The following discussion will focus on quantification results from a few studies investigating and comparing the density of neuropeptide-containing nerve fibres of healthy and diabetic rats. This is because diabetes-induced oral complications share some common complaints such as xerostomia and taste alterations with BMS symptoms (Batbayar et al., 2004). Also, both conditions are associated with peripheral neuropathy. Immunohisto- and

immunocytochemical analyses of tissues taken from the root of the tongue of healthy male Wistar rats found numerous SP-containing nerve fibres with approximate density of 20-49 SP-IR nerve fibres/mm^2 in the epithelium, moderate density (5-19 SP-IR nerve fibres/mm^2) in the subepithelial tissues and blood vessels, but few SP-IR nerve fibres (1-4 SP-IR nerve fibres/mm^2) in glandular cells in the tongue (Batbayar et al., 2004). On the contrary, no SP-IR was detected in the epithelial tissues taken from the alimentary tracts (pylorus, duodenum and colon) of healthy male Wistar rats (Feher et al., 2006). The authors reported an average of 5-19 SP-IR nerve fibres/mm^2 of sub-epithelial alimentary tract tissues, 1-4 SP-IR nerve fibres/mm^2 tissues from the glands and blood vessels in the alimentary tracts (Feher et al., 2006). Both studies reported an initial decrease in the total number of neuropeptide-containing nerve fibres followed by a dramatic increase four weeks-post streptozotocin treatment, suggesting an increased synthesis and/or regeneration of these nerve fibers to restore a diabetes-associated depletion of these neuropeptides (Batbayar et al., 2004, Feher et al., 2006).Particularly being observed was higher numbers of SP-containing nerve processes in epithelial, sub-epithelial tissues, and around the blood vessels, as well as marked increase in diameter of these SP-IR nerve fibres (Feher et al., 2006). Furthermore, following induction of diabetes, a large number of the immunocompetent cells (i.e. lymphocytes, plasma cells, and mast cells) showed IR for SP, with 12.3% of all immunocompetent cells in the tongue containing SP (Feher et al., 2006). Given observations of significant reduction of small-diameter epithelial nerve fibre from tongue biopsies of BMS patients and plasticity of neuropeptides, particularly expression levels of SP in diabetic rat models, suffice to say, ovariectomy alone (at 28 days-post surgery) was unable to induce similar marked alterations believed to be associated with classic observations of peripheral neuropathy. In the present

study, an animal model of BMS could not be developed by using OVX female rats to produce small-fibre neuropathy hence more research is warranted to understand this enigmatic condition.

5.4 Justification of Timeline and Effects of Ovariectomy (OVX)

This study compared tongue nerve fibres in female rats, at least 28 days after they were ovariectomised with those from SHAM female rats. I chose to wait 28 days after the surgeries before examining the rats after considering results from several published studies indicating that this period of time was sufficient to significantly deplete the systemic circulation of oestrogen levels as well as other practical considerations. One study that investigated the role of oestrogen on pain sensitivity and neuropeptide expression showed that long term (28 days) OVX of adult rats induced a profound thermal and mechanical hyperalgesia of the hindpaw and tail (Sarajari and Oblinger, 2010). In their study, SP was found to be almost exclusive to small-sized DRG neurons (<600 mm^2), with OVX rats expressing significantly higher number of SP-containing DRG neurons than oestrogen-treated OVX rats (Sarajari and Oblinger, 2010). Serum oestradiol levels, measured at all 5 different time points namely 24 hour-, 1 week-, 2 week-, 4 week- and 8 week- post-surgery, were significantly different between OVX and SHAM rats (Qu et al., 2012). Moreover, it was reported that mRNA levels for oestrogen receptors in rat uterus decreased 3- to 6-fold post-OVX (21-28 days) (Shupnik et al., 1989). These studies provided reasonable evidence and support to justify the 28-day post-surgery timeline used in this project. However, it is worth noting that the only study which reported observing alterations in rat tongue mucosa i.e. thinning of mucosal layer, irregular keratinised surface and disappearance of the lingual

papilla was seen in female rats at 2 months-, 4 months- and 6 months- post-OVX (Seko et al., 2005).

Since results from this study suggest no major differences between the two rat groups when measured more than 28 days post-surgery, it remains open to investigation whether ovariectomy can induce changes to nerve fibre innervations of tongue mucosa to mimic those seen in human patients. It may be possible that a longer duration is required post-OVX to produce visible and measurable changes to the densities of afferent fibres in the tongue mucosa. Other than that, it is also possible that the hypothesised small-diameter neuropathy in tongue mucosa may only be induced in older, chronically-stressed OVX female rats, since most BMS patients are older than 30 years old (Bergdahl and Bergdahl, 1999, Minor and Epstein, 2011) with the majority of them reported having suffered from chronic anxiety, depression or personal trauma (Grushka et al., 1987b, Carlson et al., 2000).

5.5 Methodology Considerations

There were several technical difficulties during the electrophysiology recording sessions which might have affected the results of this study. Firstly, the soft nature of the tongue made it very difficult to precisely apply standard pressure of mechanical stimulations to the receptive field at every minute interval. Despite steps being taken to keep the tongue tensed and fixed by using a suture, some receptive fields in the ventral aspect of the tongue and on the lateral border of the tongue were more challenging to apply standard mechanical stimuli each minute. Secondly, due to the wet nature of the tongue, a marker could not be placed on the receptive field in order to ensure accurate positioning of the von Frey tip with

each mechanical stimulation. Since superficial units of afferent nerve fibres have very small mucosal receptive field, found to be ranging from 1- 19.6 mm^2 (geometric mean= 2.4 mm^2) (Trulsson and Essick, 1997), any slight deviations from the central receptive field might have affected the resultant recorded MTs. Despite these challenges, careful considerations and measures were undertaken to minimise variations of recorded MTs. For example, I was the only person handling the electronic Von Frey tool to record the MTs, the same von Frey instrument was used in the entire study, thresholds were recorded at every minute for 10 minutes to obtain the mean values. Moreover, I was trained and practised for several months to consciously applying similar mechanical pressures from the same direction prior to commencement of this study.

In the immunohistochemistry aspect of this project, it is fair to state that to a small extent, subjective judgement was required in determining the background fluorescence from the images. In order to prevent false positives, I always chose the highest magnitude of background fluorescence to be deducted before quantifying the nerve fibres with positive immunoreactivities. Secondly, due to the tortuous nature of nerve fibres running in the tongue, I am aware that the values quantified were not likely to be absolute values. Nerve fibres running perpendicular to the cutting plane might have appeared just as dots of fluorescence in the images scanned. Again, I would like to emphasise that this study is primarily intended to compare several qualities of tongue nerve fibres between OVX and SHAM rats, therefore, as long as consistent methods were employed while quantifying these fibres in both rats groups, a meaningful comparison can be made. During the planning phase of this project, I had outlined very strict, standard criteria to identify nerve fibres and

immunoreactivities (IR) in the tongues of both rat groups. The labelled structure had to have a fibril-like shape with a minimum length of 4.0 μm to be considered as an axonal fibre in this study. The most fluorescent background areas were always used as the background values to be deducted. Only fluorescence values which were more than 2 standard deviations above the mean background fluorescence values were considered as a positive IR. Moreover, in the preliminary study, another independent assessor was involved in quantifying nerve fibres from intact female rats. Values obtained between the independent assessor and me were very similar so I am confident that these criteria were sufficient to allow for a relatively objective quantification process in both rat groups.

The main strength of this study is that both assessors (Assessor A and B) involved in the immunohistochemical quantification process were blinded and worked independently from each other. Furthermore, I was blinded to the type of rat and drugs applied during the electrophysiology experiments. Previous studies reporting positive results obtained from comparing epithelial nerve fibre density from human tongue biopsies of BMS patients and healthy controls (Yilmaz et al., 2007, Beneng et al., 2010) did not mention any blinding being performed.

5.6 Properties of Tongue Afferent Fibres

It was found in this study that female tongue afferent fibres did not have any significant correlation between the CV and MT. This result is consistent with what had been found in facial cutaneous nerve fibres of female rats (Gazerani et al, 2010) and masseter muscles of female rats (Mann et al., 2006). On the contrary, afferent fibres in masseter

muscle of male rats showed significant inverse correlation between CV and MT (Mann et al., 2006). Given these observed sex differences, one has to be careful not to generalise that nociceptors in females also have certain ranges of CVs.

In this study, the ratio of MR to PM was 3:1 in intact and SHAM female rats. On the contrary, *in vitro* single-unit recordings of the rat lingual nerve by Toda and colleagues found that 73% (n=49) of their recorded fibres were PMs which led them to conclude that the majority of oral mucosal nociceptors consisted of the polymodal type (Toda et al., 1997). This discrepancy in findings could be due to the different techniques used in our studies. For example, theirs was an *in vitro* recording whereas this study employed *in vivo* electrophysiological recordings. They used a thermode with a tip diameter of 3mm to supply the thermal stimulations (up to 50°C) but in my study, hot water (at 60 °C) was utilised as the thermal stimuli and the entire tongue was bathed in the hot water to gradually raise the tongue temperature. Furthermore, a PM nociceptor in their study was defined as a sensory nerve fibre which responded to all mechanical, thermal and chemical (bradykinin solution) stimuli whereas in my study, all afferent fibres which responded positively to both mechanical and thermal stimuli were categorised as PMs. Such differences in the techniques employed may be the reason behind our contradictory findings. Furthermore, the mean thermal threshold of PMs collected in this study were 30.9°C, 33.8°C and 33.9°C from OVX, SHAM and intact female rats whereas the Aδ-MHs found in the other research had mean thermal threshold of 47.6°C (Toda et al., 1997) indicating that different populations of thermal receptors were investigated in our studies.

5.7 Future Considerations

Future attempts to develop an animal model of BMS may utilise a combination of electrophysiology, immunohistochemistry and behavioural techniques to investigate older female rats, preferably chronically-stressed as well as having been ovariectomised for a longer duration than 28 days.

5.8 Summary

This study found that high proportions of axonal tongue fibres (peptidergic and non-peptidergic axonal fibres) express the $GABA_A\gamma_2$, suggesting that there is an abundance of peripheral $GABA_A$ receptors in the tongue capable of binding with BZDs, such as clonazepam. This is because the BZD-binding site in the $GABA_A$ receptor is in between the α and γ subunits of this receptor. This discovery lends support to the hypothesis that clonazepam brought about pain relief in a subpopulation of BMS patients via modulation of peripheral $GABA_A$ receptors in the oral cavity. Although tongue biopsies from human BMS patients found significantly lower epithelial nerve fibre density than healthy subjects (Lauria et al., 2005, Yilmaz et al., 2007, Beneng et al., 2010, Puhakka et al., 2010) and a study on rat tongue showed other histological alterations such as thinner keratinised epithelial layer (Seko et al., 2005), in my study, OVX female rats (after 28 days post-surgery) did not manifest any significant changes in terms of nerve density values, proportions of small-diameter (peptidergic fibres), proportions of $GABA_A\gamma_2$-containing nerve fibres, and baseline MTs than SHAM female rats. Therefore, I can only conclude that ovariectomy (after 28 days) alone could not induce any detectable histological alterations to model BMS.

MUS did have a significant effect of increasing relative MTs but only after a brief thermal stimulation was given to tongue afferent fibres from intact females. Interestingly, this positive effect was absent when no thermal stimuli were given to tongue afferent fibres from intact, OVX and SHAM groups which led us to speculate that the addition of hot water may have increased blood flow to the tongue resulting in better permeability of MUS into tongue mucosal layer. Moreover, given the high proportions of $GABA_A\gamma_2$ in the axonal fibres on rat tongue, these results most likely reflect the inability of topical drug delivery to the site of action, rather than $GABA_A$ receptors having no effects on MTs of afferent fibres.

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Appendices

Appendix A

Method: $GABA_A$ Receptor Constructs and Cell Lines

Rat cDNA for the $GABA_A\gamma_2$ receptor tagged with hemagglutinin (HA-$GABA_A\gamma_2$) was purchased from Origene (Rockville,MD, USA). The stable transfection of HA-$GABA_A\gamma_2$ into HEK-293 cell cultures was prepared using the Lipofectamine transfection reagent (Biored Inc.) according to the protocol provided by the manufacturing company (Somvanshi et al., 2009). HEK293 cells stably expressing HA-$GABA_A\gamma_2$ were selected and maintained in Dulbecco's MEM supplemented with 10% fetal bovine serum (FBS) and 700 μg/ml neomycin.

Method: Western Blot Analysis

In order to determine that the commercial polyclonal antibody for the $GABA_A\gamma_2$ used in the immunocytochemistry study of this project is selective for the γ_2 subunit, homogenate of transfected and *wt* cells was treated with a combination of antibodies. Membrane fractions prepared from *wt* and transfected HEK-293 cells were lysed using RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0). 15μg of the membrane preparation was fractionated by electrophoresis on 10% SDS-polyacrylamide gel which was transferred to nitrocellulose Hy-Bond ECL membrane (Amersham Ltd. Oakdale, ON, Canada). Membranes were processed with HA-tagged monoclonal antibody (Sigma) and polyclonal antibodies for the alpha and beta chains of the

$GABA_A$ receptors and compared it with the $GABA_A\gamma_2$ polyclonal antibody (Sigma). Membranes were developed by using chemiluminescence method and images were captured using Alpha Innotech FluorChem 8800 gel box imager (Alpha Innotech Co., San Leandro, CA).

Results from Western Blot Analysis

Western blot analysis shows that HEK-293 cells transfected with the rat cDNA of HA-$GABA_A\gamma_2$ was successful. Many ligand-gated and G-protein coupled receptors or protein subunits have been reported to be endogenous to *wt* HEK-293 cells such as glycine receptors, $GABA_A$ and $GABA_B$ receptors (Thomas and Smart, 2005). As illustrated in Figure A1, transfected HEK-293 cells had significantly higher protein expression compared to *wt* HEK-293 cells as detected by the commercial $GABA_A\gamma_2$ antibody that is being used in this project. Most importantly, this antibody detected only one band of protein (MW= 65 kDa) in the *wt* HEK-293 cells which had the same molecular weight (MW) as the protein detected from transfected HEK-293 cells. This shows that the $GABA_A\gamma_2$ antibody used was selective to the γ_2 subunit of the $GABA_A$ receptor. To further confirm that this commercial antibody was able to label the $GABA_A\gamma_2$, we exploited the HA- tag present in the transfected cDNA or the expressed $GABA_A\gamma_2$ protein in transfected HEK-293 cells. As illustrated in Figure A2, when treated with a monoclonal antibody against HA, a strong band of protein at similar molecular weight (65 kDa) was detected from transfected HEK-293 cells which were expressing higher level of HA-$GABA_A\gamma_2$, but no protein was detected in *wt* HEK-293 cells because HA is not endogenous to *wt* HEK-293 cells. Importantly, all the antibodies detected the protein

expression at the similar molecular weight (65 kDa) in transfected HEK-293 cells whereas weak or no expression of $GABA_A\gamma_2$ was detected in *wt* cells. This series of experimental results conclusively shows that this commercial polyclonal antibody $GABA_A\gamma_2$ was indeed selective in binding to the $GABA_A\gamma_2$.

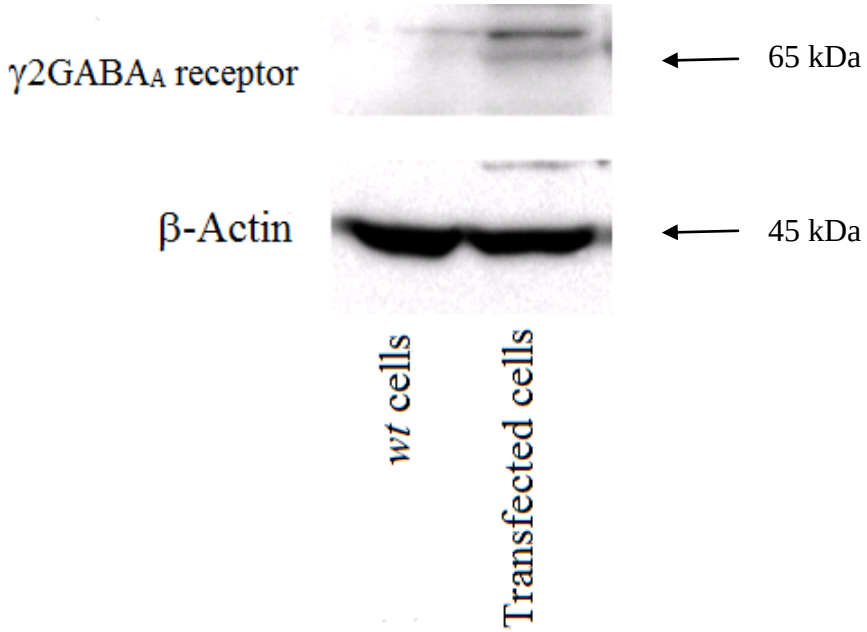


Figure A1: Transfected HEK-293 cells had significantly higher amount of proteins than *wt* cells as labelled by the commercial $GABA_A\gamma_2$ antibody.

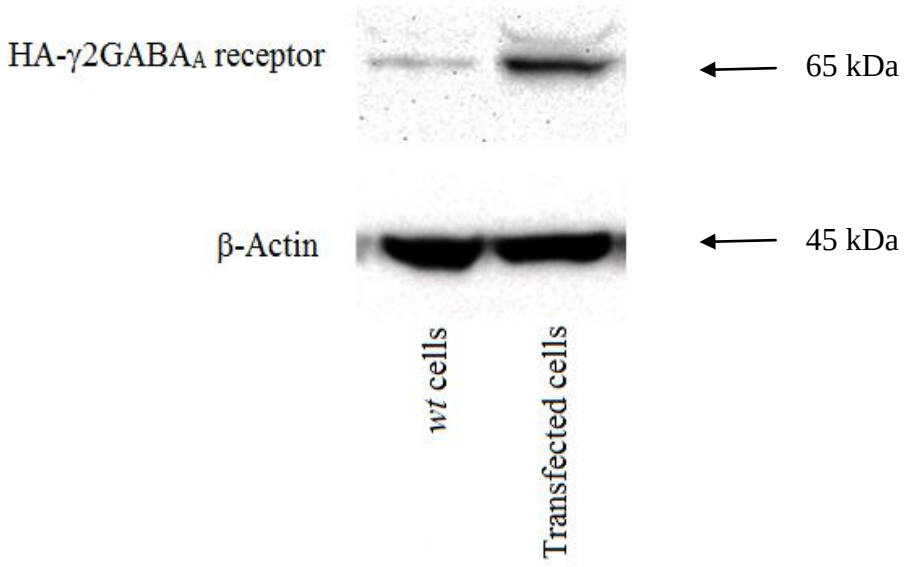
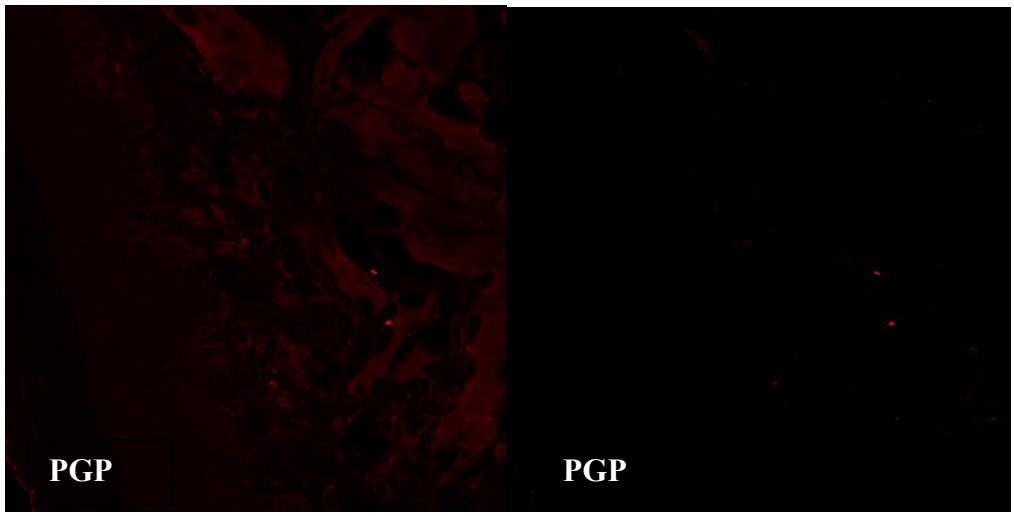
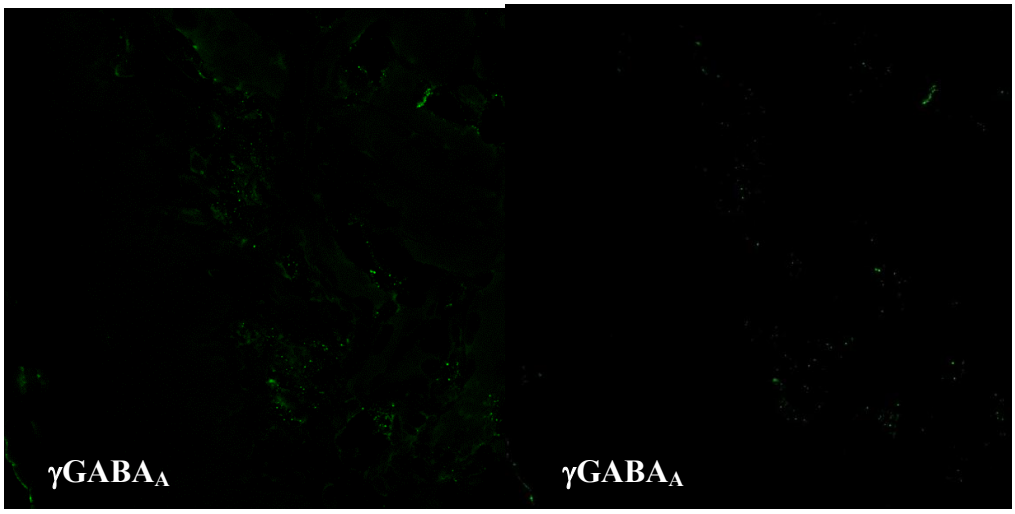


Figure A2: Qualitatively, transfected HEK-293 cells express significantly higher amount of HA-GABA$_A\gamma_2$ in comparison to wild type *(wt)* HEK-293 cells, indicating that the cell transfection process was successful.

Appendix B

Immunohistochemistry Controls





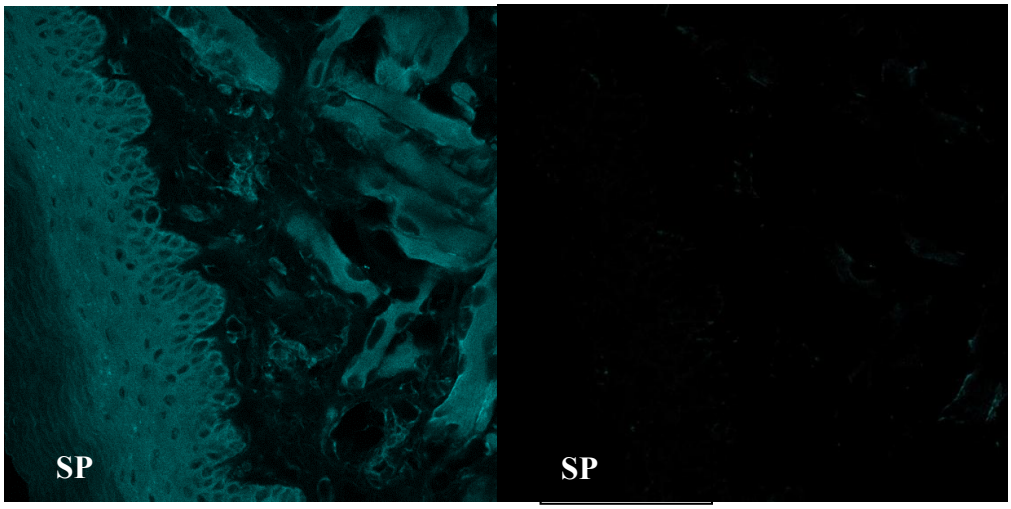


Figure B1: These confocal microscopy images were obtained from tissue sections labelled with only secondary, fluorescent antibodies without any primary antibodies. After deducting for background fluorescence (right side), these images qualitatively show that the secondary antibodies did not bind to other proteins not bound with any primary antibodies.

Appendix C

CELL #		1	2	3	4	5	6	7	8	MEAN	SD	SEM
RELATIVE MT	GABA (n=6)	1.5	1.1	0.8	0.9	1.0	0.9	NA	NA	1.0	0.3	0.1
	MUS (n=5)	1.3	1.1	1.7	2.3	1.0	NA	NA	NA	1.5	0.5	0.2
	VEHICLE(n=8)	0.9	0.3	0.9	1.0	1.0	1.0	0.6	1.1	0.8	0.3	0.1

Table C1: Raw data corresponding to results displayed in Figure 18. One Way Analysis of Variance (multiple comparisons versus control group (Holm-Sidak method)) found that differences in the mean values among the treatment groups were greater than would be expected by chance (P=0.021).

Distance (mm)	48.5	65.0	65.0	75.0	43.0	51.0
	63.0	63.0	62.7	76.3	59.3	52.0
	49.3	60.3	60.3	60.3	50.7	58.0
	41.0	62.0	51.0	50.0	52.0	64.0
	46.0	51.7	58.7	42.3	61.2	72.0
	71.8	71.8	71.8	73.0	53.8	66.7
	64.3	71.7	71.7	49.2	61.3	63.5
	51.0	58.0	57.0	44.0	45.0	56.0
	42.0	62.7	65.5	65.5	63.0	63.0
	73.3	62.7	62.7	62.7	68.8	68.8
	68.0	62.0	69.0	52.7	64.2	58.7
	61.2	56.3	58.3	70.7	61.7	60.8
	66.7	55.0	66.0	48.0	58.3	50.7
	46.7	64.5	50.5	50.5	50.5	50.5
Median	**61.0**					
Mean	**59.3**					

Table C2: The distribution of all lingual nerve lengths collected in this study. The mean and median lingual nerve lengths were 59.0 mm and 61.0 mm respectively.