

THE ROLE OF MONOSODIUM GLUTAMATE IN HEADACHE

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Abstract

Consumption of monosodium glutamate (MSG) can induce headache in young healthy individuals and migraine-like headache in migraineurs. Blood plasma levels of glutamate are also elevated in migraineurs, but it is unknown how elevated levels of glutamate contribute to headache. The current study was undertaken to investigate the hypothesis that monosodium glutamate induces headache through activation of peripheral glutamate receptors. To test the hypothesis, we combined *in vivo* electrophysiology, laser Doppler recordings of dural vasculature, and immunohistochemistry to investigate the effect of systemic administration of MSG on the trigeminovascular pathway. Immunohistochemical analysis of the dura, determined that the nerve fibres innervating dural blood vessels express NMDA, AMPA, kainate and mGlu5 receptors. The glutamate transporter EAAT2, but not EAAT1 or 3, is expressed by dural blood vessels. Systemic administration of 50mg/kg MSG induced a 24.5 and 20.6 percent increase in dural blood flow in male and female rats, respectively, as measured by laser Doppler flowmetry. Dural blood flow returned to baseline values by a mean of 170 seconds. In *in vivo* extracellular recordings of spinal trigeminal subnucleus caudalis (SpVc) neurons with dural receptive fields, intravenously administered MSG evoked an increase in neuronal discharge in 5/6 neurons in both male and female rats. MSG also induced mechanical sensitization in both sexes. When the NMDA receptor selective antagonist APV (5, 50mg/kg) was co-administered with MSG, it attenuated both the MSG evoked activation and mechanical sensitization of SpVc neurons. My

results suggest that MSG induced headache is mediated, in part, through activation of the peripheral NMDA receptor.

Preface

The experiments conducted in this thesis were conducted by me with assistance from Dr. Dong and Dr. Cairns. The experiments were conceived and supervised by Dr. Cairns and me. All animal procedures were reviewed and approved by the University of British Columbia Animal Care Committee (Animal Care Protocol Number: A13-0165).

I created Figures 1.5, 2.1, 2.2, and 2.10.

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List of Abbreviations

5-HT 5-hydroxytryptamine

APV DL-2-Amino-5-phosphonovaleric acid

AMPA α -amino-3-hydroxy-5-methyl-5-isoxazolepropionate

BoNT/A Botulinum neurotoxin A

CGRP Calcitonin gene related peptide

CM Chronic migraine

CNS Central nervous system

CSD Cortical spreading depression

CSF Cerebrospinal fluid

EAA Excitatory amino acid

EAAT Excitatory amino acid transporter

EM Episodic migraine

EPSCs Excitatory post-synaptic currents

GABA Gamma-Aminobutyric acid

iGluR Ionotropic glutamate receptor

IHS International headache society

KA Kainate

LT Low threshold

LTD Long term depression

LTP Long term potentiation

MA Migraine with aura

mAbs monoclonal antibodies

MCA Middle cerebral artery

mGluR Metabotropic glutamate receptor

MMA Middle meningeal artery

MO Migraine without aura

MOH Medication over-use headache

MSG Monosodium glutamate

MT Mechanical threshold

NK1 Neurokinin A receptor

NMDA N-methyl –D-aspartate

NO Nitric oxide

NS Nociceptive Specific

NSAID Non-steroidal anti-inflammatory drug

PAG Periaqueductal gray

PG Prostaglandin

PLC Phospholipase C

PNS Peripheral nervous system

PPE Plasma Protein Extravasation

RAMP Receptor activity-modifying protein

RCP Receptor component protein

SP Substance P

SpVc Spinal trigeminal subnucleus caudalis

SSS Superior sagittal sinus

TCA Tricyclic antidepressant

VPM Ventral posteromedial nucleus

WDR Wide dynamic range

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Chapter 1: Introduction

1.1. Project Overview

Migraine is a common form of headache; unfortunately, current drug treatments are not effective for all sufferers. An incomplete understanding of the pathophysiology of migraine drives continued research into the possible pathogenesis and congruently, potential drug targets. While the origins of migraine remain to be elucidated, the trigeminovascular system, particularly the afferent fibres which densely innervate the dura mater, have been implicated to play a key role (Cosentino et al. 2014). There are several pieces of evidence which suggest, glutamate, the primary excitatory neurotransmitter in the central nervous system (CNS), can be linked to migraine.

Glutamate is well known to be involved in the sensitization of afferent fibres (Cairns et al. 2007; Gazerani, Dong, et al. 2010; Laursen et al. 2014) as well as the transduction of nociceptive signaling (Klafke et al. 2012; Chan and MaassenVanDenBrink 2014). Blood plasma glutamate levels have also been found to be elevated in migraineurs long after a migraine attack (Martinez et al. 1993; Cananzi et al. 1995; Eufemia et al. 1997). And a genetic predisposition to migraine has been identified, a variant which down regulates glutamate transporters involved in maintaining glutamate homeostasis (Schürks 2012). How elevated levels of glutamate contribute to headache are unknown. My research hypothesis is that “monosodium glutamate (MSG) induces headache through activation of peripheral glutamate receptors”. Understanding the role that glutamate may play in proposed mechanisms of headache could identify potential targets for drugs in the treatment of migraine pain.

1.2. Migraine Clinical Significance

Migraine is a debilitating neurovascular disorder which affects up to 15% of people worldwide (Akerman et al. 2013), a large portion of this demographic are women who are two to three times more likely to suffer from migraine headache than men (Linde 2006). In the 2010 Global Burden of Disease Survey, migraine was listed as the third most prevalent disease and the seventh highest cause of disability (Society 2013). While current prophylactic and abortive treatments are effective in some individuals, there are still many who do not respond to pharmacological treatment (Giamberardino and Martelletti 2014). In women, the prevalence of migraine is highest during their fertile years (20-45 years of age) and may be triggered by menstruation, however the occurrence decreases post-menopause(Cairns and Gazerani 2009). Suffering from a debilitating disease during the most productive working years is also associated with high economic costs (Giamberardino and Martelletti 2014). In a recent study, migraine sufferers have been found to have more psycho-social difficulties, than those with other neurological conditions including stroke, multiple sclerosis or Parkinson's disease (Leonardi 2014). Population-base studies have determined that migraineurs are 2.2-4 times more likely to suffer from depression (Pompili et al. 2009). Discovering new treatments for migraine pain, is needed to combat the impaired quality of life of many migraine sufferers, who do not respond to current pharmacological treatment or cannot tolerate the adverse effects of these treatments (Bera et al. 2014).

1.2.1. Migraine Definition and Age of Onset

The International Headache Society (IHS) defines migraine (without aura, MO) as a:

Recurrent headache disorder manifesting in attacks lasting 4-72 hours. Typical characteristics of the headache are unilateral location, pulsating quality, moderate or severe intensity, aggravation by routine physical activity and association with nausea and/or photophobia and phonophobia.(Society 2013)

Migraine with aura (MA) has the same headache characteristics but is preceded by recurrent attacks of unilateral fully reversible visual, sensory or other central nervous system disturbances(Society 2013). Approximately 17% of migraine sufferers experience aura (Launer et al. 1999). Migraines are further characterized based upon frequency; migraines which occur ≥ 15 times/month for three consecutive months are designated as chronic migraine (CM), while less than 15 attacks per month are designated episodic migraine (EM)(Society 2013).

The onset of migraine can occur at any age but approximately 90% of those who suffer from migraines will have their first attack before age 40 (Russell et al. 1996; Davidoff 2002). Migraine attacks are most prevalent between the ages of 20-50 in both men and women but appear to peak later in life in women (O'Brien et al. 1994; Fernández-de-Las-Peñas et al. 2010; Schurks et al. 2011; Borsook et al. 2014). As previously mentioned women are more likely to suffer from migraine headache than men (Linde 2006), but they also report a higher frequency of attacks as well as more debilitating attacks than men who suffer from migraine headaches (Stewart et al. 1992; Davidoff 2002). Figure

1.1 depicts the relationship between migraine prevalence and age for both men and women.

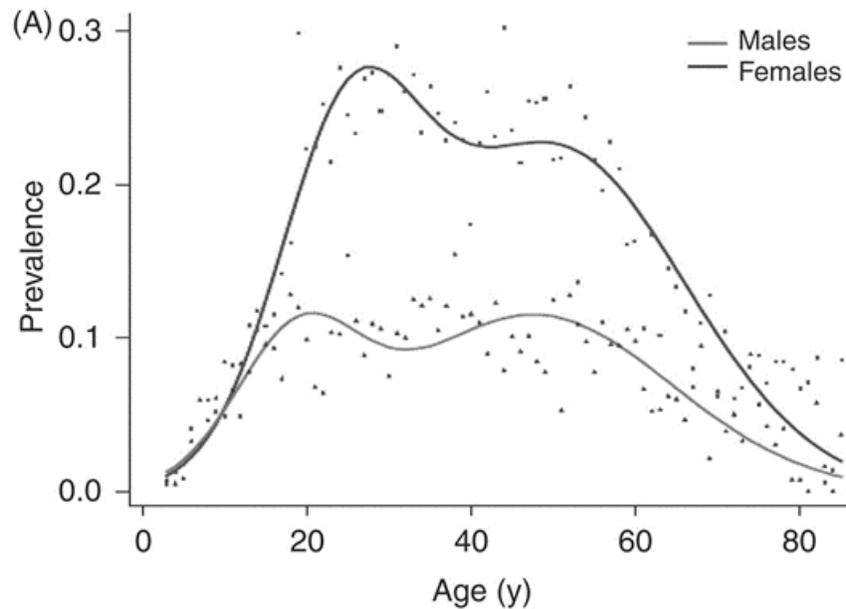


Figure 1.1: Age-specific prevalence of migraine in men and women. One-year period prevalence (ratio of prevalence) of self-reported, physician-diagnosed migraine obtained from the 2003 National Health Interview Survey (NHIS) conducted in the United States. In women, the highest prevalence occurs during the reproductive years and almost reaches a 30% prevalence in this population. Adapted with permission from (Victor et al. 2010).

1.2.2. Migraine Triggers

Triggers, also called precipitating factors, of migraine vary based on sex, classification of migraine (MA or MO), and frequency of attacks (chronic vs episodic) (Kelman 2007).

Triggers precede an attack by less than 48 hours in those who are susceptible, they are not universal and are not experienced by all migraineurs (Zagami and Bahra 2005).

Approximately 64-90% of migraineurs have known triggers; however they do not precipitate an attack each time they're encountered (Van den Bergh et al. 1987;

Rasmussen and Olesen 1992; Rasmussen 1993; Russell et al. 1996; Zagami and Bahra

2005; Kelman 2007). Common precipitating factors include stress, hormonal variations often associated with menstruation, sleep disturbances, alcohol, food, changes in weather, and environmental factors such as odors, lights or smoke (Selby and Ance 1960; Van den Bergh et al. 1987; Rasmussen and Olesen 1992; Rasmussen 1993; Robbins 1994; Zagami and Bahra 2005; Kelman 2007; Schurks et al. 2011). Stress or mental tension is the most prevalent (Selby and Ance 1960; Rasmussen and Olesen 1992; Rasmussen 1993; Russell et al. 1996; Kelman 2007). The frequency of common migraine triggers is found in Table 1.1.

Hormonal variations play a role in the onset of migraine in some women (Selby and Ance 1960; Rasmussen and Olesen 1992; Rasmussen 1993; Robbins 1994; Kelman 2007; Schurks et al. 2011). The IHS recognizes two separate classifications of MO which are related to menstruating; pure menstrual migraine and menstrually-related migraine, which differ based on the time of the attack with respect to menses (Society 2013). Changes in estrogen and progesterone levels have been implicated as the precipitating factor for these headaches (Massiou and MacGregor 2005; Schurks et al. 2011).

Hormonal contraception can also trigger an attack in women (Massiou and MacGregor 2005; Schurks et al. 2011).

Several types of food can trigger a migraine, they include chocolate, cheese, citrus fruits and mono-sodium glutamate (MSG)(Peatfield et al. 1984; Zagami and Bahra 2005). The consumption of MSG, a naturally occurring form of glutamic acid and common food-additive, has been commonly associated with adverse reactions in some individuals, including headache (Baad-Hansen et al. 2010; Shimada et al. 2013). The IHS lists MSG as

a trigger for headache. The MSG-related headache is classified as mild to moderate in non-migraineurs, but classified as episodic migraine in those who suffer from migraine (Society 2013). In a recent study of female migraineurs, 24.7% listed MSG as either a trigger or factor that worsened their migraine (Schurks et al. 2011). A study investigating the effect of MSG consumption in healthy subjects over a span of 5 days resulted in the occurrence of headache or dizziness in 57% of subjects and decreased pain thresholds in the masseter muscle of all subjects (Shimada et al. 2013).

| Triggers | Selby and Lance (Selby and Ance 1960) n=500 | Peatfield et al. (Peatfield et al. 1984) n=490 | Van den Berg et al. (Van den Bergh et al. 1987) n=217 | Rasmussen and Olesen (Rasmussen and Olesen 1992) n=58 | Rasmussen (Rasmussen 1993) n=119 | Peatfield (Peatfield 1984) n=490 | Robbins (Robbins 1994) n=494 | Russell et al. (Russell et al. 1996) n=222 | Kelman (Kelman 2007) N=1207 |
|----------------------|---|--|---|---|--|--|--|--|---|
| Stress | 67 | – | 49 | 43 | 44.5 | – | 62 | 38 | 80 |
| Food | 25 | 19 | 45 | 12 | 10 | 17 | 30 | 34 | 27 |
| Alcohol | – | 29 | 52 | 28 | 20 | 30 | – | 11 | 38 |
| Weather | – | – | 7 | 16 | 11 | – | 43 | – | 53 |
| Menstruation | 62 | – | 48 | 29 | 12 | – | 50 | – | 65 |
| Sensory Stimuli | 47 | – | 12 | – | – | – | 38 | 12 | – |
| Fatigue | – | – | 16 | – | – | – | – | 19 | 50 |
| One or more Triggers | – | – | 85 | 90 | 64 | – | – | 79 | 76 |

Table 1.1: The percentage of respondents to various triggers across multiple studies.

Table adapted with permission from (Zagami and Bahra 2005)

1.2.3. Acute Migraine Treatment

Non-steroidal anti-inflammatory drugs (NSAIDs) and triptans are the mainstay of acute migraine pharmacotherapy (Becker 2015). Several factors need to be considered when prescribing a treatment to abort migraine; the intensity, average duration, and the chronicity of attacks, and also whether the side-effect profile of therapy is acceptable for a given patient (Becker 2015). While NSAIDs are commonly prescribed for mild to moderate intensity attacks, triptans or a triptan/NSAID combination are often recommended for more severe attacks or for those who don't receive adequate relief from NSAIDs alone (Becker 2015).

NSAIDs act by inhibiting local synthesis of prostaglandins (PG) by modulating cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2)(Silberstein and Stirpe 2014). Both peripheral and central actions are thought to contribute to its efficacy in treating migraine headache (Kröger and May 2014; Silberstein and Stirpe 2014). Prostaglandins are found in the smooth muscle of dural blood vessels and when administered systemically, induce vasodilation in rats and humans –in part due to the release of calcitonin gene related peptide (CGRP)(Ebersberger et al. 1999; Jenkins et al. 2001; Wienecke et al. 2009; Myren et al. 2010). In migraineurs, PG induces a migraine-like headache (Antonova et al. 2013). Experimentally, NSAIDs have been found to decrease the release of CGRP at the level of the dura, and inhibit the firing of second order trigeminovascular neurons in the CNS (Sokolov et al. 2010; Silberstein and Stirpe 2014).

NSAIDs are not without side effects, they increase the risk of gastrointestinal complications (Silberstein and Stirpe 2014).

Triptans became available in the early 1990s and have largely replaced ergotamines as the drug of choice for the acute treatment of moderate to severe episodic migraine (Becker 2015). Triptans are agonists for the 5-hydroxytryptamine 1b and 1d (5-HT_{1b/1d}) receptors, however, their mechanism of action when used to treat migraine headache is not fully understood (Goadsby and Knight 1997; Levy et al. 2004; Saxena and Tfelt-Hansen 2005; Tfelt-Hansen 2010). It is thought that triptans act on 5-HT_{1b/1d} receptors on meningeal blood vessels and nerve terminals of the trigeminal system. It is proposed that activation of 5-HT_{1b} receptors constricts the intracranial vessels, activation of 5-HT_{1d} receptors decreases neurotransmitter and neuropeptide release from peri-vascular afferent fibers, while activation of 5-HT_{1b,d} and f receptor subtypes decreases trigeminal neuronal excitability centrally (Humphrey and Feniuk 1991; Humphrey and Goadsby 1994; Goadsby and Knight 1997; Razzaque et al. 1999; Saxena and Tfelt-Hansen 2005). Ongoing research into other potential actions of triptans within the CNS are inconclusive, but side effects consistent with central nervous system modulation are reported for some of the drugs (Bartsch et al. 2004; Tfelt-Hansen 2010; Asghar et al. 2012; Dussor 2015).

Seven triptans are currently available in North America: almotriptan, eletriptan, frovatriptan, naratriptan, rizatriptan, sumatriptan, and zolmitriptan. Although triptans are a major contribution to acute migraine treatment, only about 50-60% of patients

consistently respond to this type of medication (Ferrari et al. 2001; Tronvik et al. 2003). While within the class of triptans the drugs share a relatively similar molecular structure, they can differ greatly in pharmacokinetic and side effect profiles (Becker 2015). Individual patients respond in profoundly different ways to a given triptan, both in terms of effectiveness for pain relief and tolerance of side effects (Becker 2015). A recent meta-analysis which determined the relative efficacy of all triptans, concluded that eletriptan and rizatriptan had the highest rates of pain-free responses 2 hours after onset of an attack (Thorlund et al. 2014).

The overuse of triptans, use on 10 days/month or more, places the patient at a higher risk of medication over-use headache (MOH), thus the instance of refractory migraine and chronicity of attacks must be considered for those prescribed triptans (Society 2013; Becker 2015). Both eletriptan and frovatriptan have relatively low rates of headache recurrence (Thorlund et al. 2014; Becker 2015). Triptans are contraindicated in patients with cardiovascular disease or uncontrolled hypertension (Saxena and Tfelt-Hansen 2005; Becker 2015). Potential side effects of triptan therapy include tingling, numbness, pressure or tightness in the chest or neck, dizziness, and sedation (Martin and Goldstein 2005).

1.2.4. Prophylactic Migraine Treatment

Prophylactic treatments aim to reduce the frequency or severity of migraine attacks and are often recommended for those who suffer from three or more severe migraine

attacks per month or are at risk for MOH (Pringsheim et al. 2012; Tfelt-Hansen and Olesen 2012; Jackson et al. 2015). Prophylaxis can also be useful for episodic migraineurs who have an impaired quality of life despite appropriate use of acute therapies (Tfelt-Hansen and Olesen 2012). Beta-blockers, tricyclic antidepressants (TCAs), calcium channel blockers, anti-epileptics, and botulinum neurotoxin A (BoNT/A) are all used as prophylactic agents (Jackson et al. 2015; Luvisetto et al. 2015).

The efficacy of β -adrenergic receptor blockers as a migraine prophylactic is thought to be partially mediated via their inhibitory actions on thalamic neurons in the ventral posteromedial nucleus (VPM), thus impeding the transmission of pain to the primary somatosensory cortex (Shields and Goadsby 2005; Vecsei et al. 2015). Several beta-blockers are indicated for migraine prophylaxis; propranolol, atenolol, metoprolol, and timolol are all superior to placebo for the treatment of EM, both propranolol and atenolol have also been studied in CM but were not found to be effective (Andersson et al. 1983; Tfelt-Hansen et al. 1984; Johannsson et al. 1987; Jackson et al. 2015).

Numerous studies have indicated that propranolol can reduce headache frequency by up to 50%, and a Cochrane review determined that propranolol was as effective and safe as other prophylactic agents (Tfelt-Hansen et al. 1984; Ziegler et al. 1987; Diener et al. 1996; Diener et al. 2004; Linde and Rossnagel 2004). The most common adverse events following treatment with beta-blockers are: fatigue, sleep disturbances, nausea and weight gain. Less common adverse effects include depression and bradycardia (Tfelt-Hansen et al. 1984; Linde and Rossnagel 2004; Jackson et al. 2015; Vecsei et al. 2015).

Verapamil and flunarizine are calcium channel blockers currently available for migraine prophylaxis (Jackson et al. 2015). The side effects for verapamil, a selective L-type Ca^{2+} channel blocker, are serious and include heart palpitations and angina; studies show its more efficacious for cluster headache than migraine (Agnoli 1988; Jackson et al. 2015). Flunarizine, which has selectivity for T-type Ca^{2+} channels, is effective in decreasing the frequency of migraines, but its side effect profile includes weight gain, fatigue, somnolence and depression (Agnoli 1988; Jackson et al. 2015; Vecsei et al. 2015). Multiple prospective, open studies, comparing flunarizine and propranolol in migraine prophylaxis, found that the risk of depression is greater in patients taking flunarizine (Verspeelt et al. 1996; de Bock et al. 1997; Vecsei et al. 2015).

Two anti-epileptics drugs, valproate and topiramate are utilized for migraine prophylaxis (Jackson et al. 2015; Vecsei et al. 2015). Both drugs are thought to reduce the frequency of migraine attacks by reducing trigeminovascular activity of neurons both peripherally and centrally, but do so via different mechanisms (Vecsei et al. 2015). Valproate, by blocking Gamma-Aminobutyric acid (GABA) transaminase and also blocking sodium channels (Vecsei et al. 2015). Topiramate is associated with multiple cellular targets, including potentiating activity at GABA receptors and inhibiting activity at voltage gated calcium and sodium channels, AMPA, and kainate receptors (Vecsei et al. 2015). Several placebo-controlled trials of both drugs found a 50% decrease in the frequency of headaches (Klapper 1997; Freitag et al. 2002; Brandes et al. 2004; Diener et al. 2004; Silberstein et al. 2004). Side effects for valproate include dizziness, somnolence, tremor and gastrointestinal issues; it is not recommended for women who are in the

reproductive age range (Vecsei et al. 2015). The adverse events associated with topiramate are sedation, paresthesia, weight loss, fatigue, gastrointestinal issues, and cognitive deficits (Brandes et al. 2004; Vecsei et al. 2015).

Amitriptyline is a tricyclic antidepressant which, at doses lower than those used for treating depression, is prescribed for migraine prophylaxis (Jackson et al. 2015; Vecsei et al. 2015). Amitriptyline has several molecular targets which may contribute to the drug's analgesic effects; it acts as a norepinephrine and serotonin reuptake inhibitor, increases adenosine availability and activity at the adenosine A1 receptor, inhibits GABA transporters and increases activity at the GABA_B receptor (Sawynok et al. 2005; McCarson et al. 2006; G et al. 2008; Dharmshaktu et al. 2012). Several placebo-controlled trials have found that amitriptyline is superior to placebo in decreasing migraine headache frequency in patients with EM (Gomersall and Stuart 1973; Couch and Hassanein 1979; Couch 2011). Common side effects include dry mouth, fatigue, weight gain, and somnolence (Vecsei et al. 2015).

Botulinum neurotoxin A's analgesic effects are thought to be mediated peripherally via the inhibition of the release of neurotransmitters from sensory nerve fibers (Gazerani, Au, et al. 2010; Shao et al. 2013). Injection of BoNT/A into pericranial and neck muscles has been found to reduce the frequency of migraine headaches in several clinical trials in CM patients, and received regulatory approval for chronic migraine prophylaxis in 2010 (Freitag et al. 2008; Aurora et al. 2011; Lipton et al. 2011; Luvisetto et al. 2015). A recent study examining the extended use of BoNT/A found that 10% of patients whom had been receiving regular BoNT/A injections for a period of at least one year stopped

responding to the treatment (Cernuda-Morollon et al. 2015). Meanwhile it also determined that patients that respond to treatment after one year, reduced their use of abortive migraine medication by more than 50% after two years of regular treatment (Cernuda-Morollon et al. 2015). Side effects of treatment include injection pain and inflammation, neck pain, muscular weakness, and eye ptosis (Diener et al. 2014; Vecsei et al. 2015).

While there are more prophylactic agents available than acute abortive medications, a low percentage of patients who are candidates for their use, use them (Pringsheim et al. 2012; Vecsei et al. 2015). An epidemiological study conducted in the United States concluded that approximately one quarter of migraine sufferers were candidates for preventative therapy, while only 13% were currently taking a migraine prophylactic (Lipton et al. 2007). The underutilization of prophylactics was proposed to be due to lack of efficacy, intolerable side effects, and contraindications (Lipton et al. 2007; Pringsheim et al. 2012; Vecsei et al. 2015).

1.3. Theories of Migraine Pathogenesis

Following Ray and Wolff's seminal studies on pain-sensitive structures of the head, pain producing dural blood vessels came to the forefront as a clue into the pathophysiology of migraine headache, and for decades later migraine research focused on a vascular origin of the headache (Ray and Wolff 1940; Wolff et al. 1953). As migraine research evolved, the nerve fibres innervating these dural blood vessels, those of the trigeminovascular system, became implicated in the genesis of migraine. While it is difficult to determine the cause of migraine, partially because of the heterogeneity of

known triggers (Levy et al. 2009), there are two main theories that attempt to explain migraine pathophysiology. One theory proposes a central origin (The Central Neuronal Theory). This theory suggests that migraine is a result of altered brain excitability, which is capable of activating the trigeminovascular system that innervates the dura (Ho et al. 2010). A second theory proposes a peripheral origin (Neurogenic Inflammation). This theory postulates that sterile inflammation, occurring in the dura, activates and sensitizes dural afferent fibres, which leads to migraine headache pain (Ho et al. 2010; Nosedá and Burstein 2013). Despite decades of research, the source of migraine headaches remains unclear. In both theories, the pain which results is believed to be maintained by both peripheral and central sensitization, with the trigeminovascular system crucial to its pathogenesis (Nosedá and Burstein 2013).

1.3.1. The Central Neuronal Theory

Current views suggest that migraine is a complex neurological disorder which affects multiple brain and brainstem areas, in such that the brain of a migraineur is easily excitable (Burstein et al. 2015). While the mechanism of the headache pain once it has been initiated is thought to be understood, that is activation of the trigeminovascular system- what is not known is how dural afferents are in fact activated by a hyperexcitable brain (Nosedá and Burstein 2013; Burstein et al. 2015). A clue as to what may activate these neurons is found when examining the prodromes of a migraine attack. It should be noted that a migraine sufferers may not always experience prodrome symptoms but it provides important insight into the possible pathogenesis

(Burstein et al. 2015; Maniyar et al. 2015). Cortical, subcortical and brainstem regions have been implicated as key CNS regions because they regulate autonomic, affective, cognitive, and sensory functions which are often impaired during a migraine attack (Maniyar et al. 2014; Burstein et al. 2015; Maniyar et al. 2015).

A significant body of research points to the hypothalamus as a key component of this so called hyperexcitable brain. The hypothalamus can be linked to prodromes such as fatigue, irritability, food cravings, and yawning and also plays a key role in maintaining homeostasis as well as circadian rhythms, the sleep-wake cycle, and hormonal fluctuations. The role of the hypothalamus as it pertains to sleep in particular is interesting in that sleep can be both a trigger and a remedy for a migraine attack (Burstein et al. 2015; Maniyar et al. 2015).

There are multiple hypotheses as to how the hyperexcitable brain can activate the dural afferent fibres. The hypothalamus regulates the activity of parasympathetic and sympathetic neurons in the superior salivatory nucleus (SSN) and spinal intermediolateral nucleus, respectively (Burstein et al. 2015). The increased activity of SSN neurons can result in the release of acetylcholine, vasoactive intestinal peptide (VIP), and nitric oxide from dural fibres originating from the sphenopalatine ganglion (SPG). This can in turn activate dural nociceptive fibres (Burstein et al. 2015).

Modulation of SPG activity provides some pain relief in migraine sufferers and is currently being explored further (Khan et al. 2014).

Another potential scenario explores the role that the hypothalamus and brainstem play in modulating allostatic load in migraineurs. Its proposed that maladaptive responses to

stressors, either internal (e.g. changes in sleep patterns) or external (e.g. consumption of wine) induces a cascade of events which, via the hypothalamus and brainstem, can lower the threshold of relay thalamic neurons with projections to the cortex (Borsook et al. 2012; Burstein et al. 2015). That is, that the physiological response to stressors in migraineurs can be heightened and drive the activity of thalamic trigeminovascular neurons. This theory of thresholds proposes that the migraine could be initiated centrally or by peripheral input (Burstein et al. 2015).

Finally, cortical spreading depression (CSD) is another phenomenon which may be driven by a hyperexcitable brain. CSD is a slowly propagating wave (2-6mm/min) of depolarization and excitation of cortical neurons and glia, followed by a period of hyperpolarization and inhibition that lasts between 15-30 minutes (Nosedá and Burstein 2013; Burstein et al. 2015). CSD is attributed to the generation of aura which some migraine patients experience (Cutrer et al. 1998). The origin of CSD is unknown but may be linked to genetic factors (Eikermann-Haerter et al. 2009). The depolarization phase results in the local release of glutamate, ATP, K⁺ and H⁺ ions, CGRP and nitric oxide (Hansen and Zeuthen 1981; Schock et al. 2007; Nosedá and Burstein 2013). Through a variety of cellular mechanisms and the breakdown of the blood-brain barrier, these chemicals reach the trigeminal afferents of the dura, inducing neurogenic inflammation and activation of the trigeminovascular system (Bolay et al. 2002; Nosedá and Burstein 2013).

1.3.2. Neurogenic Inflammation

Sterile neurogenic inflammation was long thought to be critical to initiate a migraine. Neurogenic inflammation consists of plasma protein extravasation (PPE), vasodilation, and mast cell degradation; and is mediated by the release of neuropeptides by dural afferent fibres (Williamson and Hargreaves 2001). Preclinical testing of triptans, ergotamines, and NSAIDs all inhibited PPE, which suggested that vascular leaking may be important in migraine (Buzzi and Moskowitz 1990; Williamson and Hargreaves 2001). PPE is mediated by substance P, however the development of neurokinin 1 receptor antagonists were not effective in treating migraine pain (Connor et al. 1998; Diener 2003).

The dural vasodilation mediated by CGRP is still a hot topic in migraine research. CGRP is thought to further propagate nociceptive signaling indirectly by inducing the release of histamine, serotonin, bradykinin, nitric oxide and tumor necrosis factor- α , from immune cells, all of which can act as sensitizing inflammatory mediators (Burstein et al. 2011). This efflux of neurotransmitters and neuropeptides further sensitize the trigeminal afferent fibres, which project onto the trigeminal nucleus caudalis and subsequently converge onto higher order neurons resulting in the perception of pain (Nosedá and Burstein 2013).

1.3.3. Genetic Component of Migraine

Migraine is considered to have a genetic component as it often runs in families and several genetic variants have been identified which are linked to migraine (Burstein et

al. 2015). A monogenic subtype of migraine with aura familial hemiplegic migraine (FHM), has three known mutations which affect neurotransmission (Burstein et al. 2015; Tolner et al. 2015). The genes affected are *CACNA1A*-which encodes the pore-forming subunit of voltage-gated calcium channels, *ATP1A2*- which encodes the α -2 subunit of sodium-potassium ATPases, and *SCN1A*-which encodes voltage-gated sodium channels (Burstein et al. 2015). All of these mutations regulate glutamate availability via their roles in the generation of action potentials and neurotransmitter release (Burstein et al. 2015). Genome-wide studies in both MOA and MA have resulted in the discovery of more genetic variants which are linked to glutamate dysfunction (Schürks 2012; Burstein et al. 2015). Among these mutations include a minor allele in the gene rs1835740, which is involved in glutamate homeostasis, and increases the propensity to suffer from migraines by 18% (Schürks 2012). Researchers have also shown that this variant leads to down-regulation of *SLC1A2*, which codes for the excitatory amino acid transporter 2 (EAAT2) (Schürks 2012)-the major glutamate transporter in the CNS, responsible for >90% of glutamate uptake (Kim et al. 2011). This mutation may contribute to the maintained elevation of glutamate reported in migraine sufferers (Burstein et al. 2015). Another variant, in the gene rs11172113, alters the transcription of lipoprotein receptor-related protein 1 (LPR1), which is involved in synaptic transmission (Schürks 2012). LPR1 and NMDA receptors have been found to be co-expressed on neurons, and LPR1 has also been shown to modulate NMDA receptor function (Nakajima et al. 2013). Mutations which affect synaptic plasticity and development have also been identified, taken together with the above mentioned mutations they provide a strong argument

towards a hyperexcitable migraine brain, with glutamate being a potentially key regulator (Anttila et al. 2013; Burstein et al. 2015).

1.4. Neurotransmitters and Neuropeptides implicated in Migraine

The central and peripheral theories of migraine both implicate the same endogenous substances as key regulators in the activation of dural afferents, which causes the headache pain in migraine. Glutamate, calcitonin gene related peptide (CGRP) and substance P (SP) have been identified as potentially important mediators in the activation and subsequent sensitization of the peripheral trigeminal afferents (Messlinger et al. 2011; Gasparini and Griffiths 2013). It is thought that the activation of trigeminovascular system; via CSD, parasympathetic activation, a hyperexcitable CNS, or glutamate dysfunction, results in a large peripheral release of the excitatory neurotransmitter glutamate (Gasparini and Griffiths 2013). The activation of the peripheral ionotropic glutamate N-Methyl –D-Aspartate (NMDA) receptor mediates, via N- and L- type Ca²⁺ channels, the release of neuropeptides, SP and CGRP (McRoberts et al. 2001). Neurogenic inflammation is triggered by SP and CGRP which contribute to plasma protein extravasation and vasodilation at the level of the dura (Akerman et al. 2013).

1.5. Glutamate

Glutamate is the primary excitatory neurotransmitter in the CNS (Gasparini and Griffiths 2013). Formed from its precursor glutamine in the neuron, glutamate, once released from vesicles, binds to its receptors on neurons or astrocytes, or alternatively is taken

up by a glutamate transporter, of which there are several (Torgner and Kvamme 1990; Gasparini and Griffiths 2013). Glutamate stores are replenished in the neuron via the neuronal-astrocytic glutamine-glutamate cycle (Torgner and Kvamme 1990). This excitatory amino acid (EAA) has been linked to migraine for several reasons. Glutamate is well known to be involved in the sensitization of afferent fibres (Cairns et al. 2007; Gazerani, Dong, et al. 2010; Laursen et al. 2014), as well as the transduction of nociceptive signaling (Klafke et al. 2012; Chan and MaassenVanDenBrink 2014). Intravenously administered MSG, which resulted in an increased glutamate concentration in the masseter muscle of rats, caused a decrease of 25% in the threshold required to fire an action potential in masseter muscle afferent fibres in response to mechanical stimulus. The sensitization induced by administration of glutamate was attenuated when co-injected with ketamine, a NMDA-receptor antagonist, suggesting that its effects were mediated through NMDA receptors (Cairns et al. 2007). Because glutamate does not cross the blood-brain-barrier (Gasparini and Griffiths 2013), ingestion of MSG likely induces headache via a peripheral mechanism, which reinforces the interest in targeting peripheral glutamate receptors in migraine treatment.

As previously mentioned, the International Headache Society (IHS) lists MSG as a trigger for headache. MSG-related headache is classified as mild to moderate in non-migraineurs, but classified as episodic migraine in those who suffer from migraine (Society 2013). A study investigating the effect of MSG consumption in healthy subjects over a span of 5 days, found that headache or dizziness occurred in 57% of subjects and that decreased pain thresholds occurred in the masseter muscle of all subjects (Shimada

et al. 2013). Glutamate levels in blood plasma, platelets, and cerebrospinal fluid (CSF) have also been found to be elevated in migraineurs long after a migraine attack (Martinez et al. 1993; Cananzi et al. 1995; Eufemia et al. 1997), and several genetic variants affecting glutaminergic neurotransmission have been identified in migraine sufferers (Schürks 2012; Burstein et al. 2015).

1.5.1. Glutamate Receptors

Glutamate signalling is dependent upon transmembrane ionotropic and metabotropic receptors. The ligand-gated ionotropic receptors (iGluRs), are divided into two types, the NMDA receptor, and the non-NMDA receptors, which are named α -amino-3-hydroxy-5-methyl-5-isoxazolepropionate (AMPA) and kainate (KA) receptors as a result of the selective agonist that activates them (Kew and Kemp 2005). There are eight G-protein coupled metabotropic glutamate receptors, mGluR1-8, which are divided into three types, group I (mGluR 1&5), group II (mGluR 2-3), and group III (mGluR 4, 6-8)(Kew and Kemp 2005). An overview of glutamate receptor classes, subunits, and signal transduction is found in Figure 1.2.

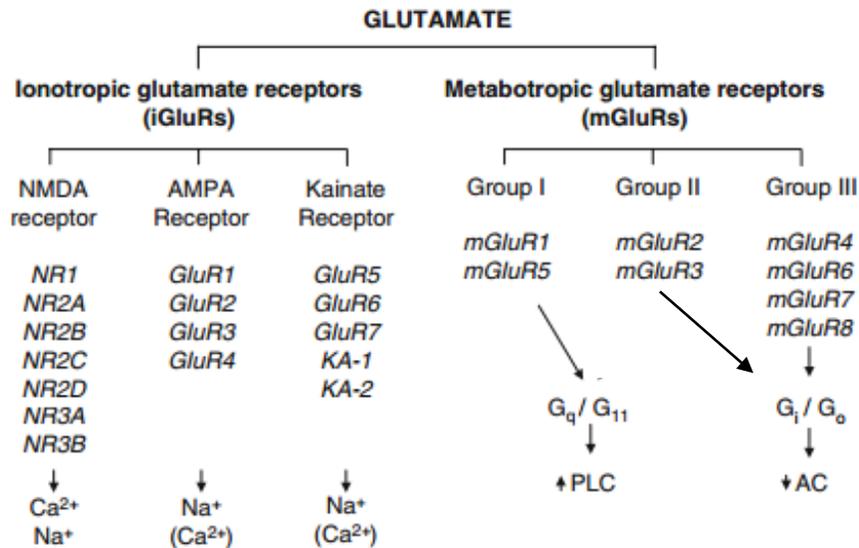


Figure 1.2: Overview of glutamate receptors, subunits and effects of activation. Ionotropic glutamate receptors, of which there are three; NMDA, AMPA, and kainate, allow the entry of sodium and calcium ions into the cell. Metabotropic glutamate receptors, which are divided into three groups (I-III), are coupled to various G-proteins which induce an increase in phospholipase C (PLC) or decrease in adenylate cyclase (AC). Preprinted in an adapted form with permission from (Kew and Kemp 2005).

1.5.1.1. NMDA Receptors

The NMDA receptor is a heterotetrameric receptor which requires both the NR1 and NR2X subunit to be functional (Kew and Kemp 2005). It's often a dimer of dimers, consisting of two NR1 subunits and two NR2X subunits, but in some cases has a NR3 subunit which alters cation permeability (Schorge and Colquhoun 2003; Kew and Kemp 2005). The receptor requires the binding of co-agonists glycine and glutamate to the NR1 and NR2X subunits, respectively, as well as the removal of the voltage-gated magnesium block, via depolarization of the membrane, for activation (Purves 2001; Kew and Kemp 2005). The requirement of membrane depolarization means that NMDA receptors only open after an initial excitatory input on the post-synaptic neuron (Liu and Salter 2010). NMDA receptors are mixed-cation channels and are permeable to Na⁺, K⁺

and Ca^{2+} . They differ from non-NMDA iGluRs in their relatively higher Ca^{2+} permeability (Purves 2001). The excitatory post-synaptic currents (EPSCs) elicited in neurons by NMDA and non-NMDA iGluRs are quite different and can be differentiated by speed and duration; the NMDA current is slower and lasts longer than the AMPA or KA EPSCs (Purves 2001; Liu and Salter 2010). The different isoforms created from the possible combinations of NR2 subunits result in a variety of post-synaptic responses. The NR2B subunit containing NMDA receptor is most associated with pain transmission (Liu and Salter 2010).

The NMDA receptor is expressed in peripheral, spinal, and supra spinal nociceptive pathways (Liu and Salter 2010). At the level of the spinal cord NMDA receptors are widely expressed; the NR1 and NR2A subunits are expressed throughout the gray matter, and NR2B subunits are mainly found in laminae I–II of the dorsal horn (Liu and Salter 2010). Peripherally, NMDA receptors are expressed on the cell bodies and axons of neurons found in the dorsal root ganglion (DRG) and trigeminal ganglion (Bleakman et al. 2006; Liu and Salter 2010; Wong et al. 2014). Following an inflammatory insult or nerve injury, the expression of NR2A and NR2B containing receptors are upregulated, both peripherally and centrally, which is thought to contribute to nociceptive behaviours in lab animals (Guanitz et al. 2002; Wilson et al. 2005; Bleakman et al. 2006; Iwata et al. 2007; Yang et al. 2009). The increase in NMDA receptor expression and altered channel kinetics (which enhance activity) following inflammation has been linked to the phosphorylation of the NR2B subunit (Salter and Kalia 2004; Liu and Salter 2010).

1.5.1.2.AMPA Receptors

The AMPA receptor is also heterotetrameric, consisting of various combinations of its four subunits (GluR1-4)(Kew and Kemp 2005; Liu and Salter 2010). It, like the NMDA receptor, is a mixed cation ion channel and is permeable to Na⁺, K⁺, and Ca²⁺ but to a smaller extent than NMDA receptors (Liu and Salter 2010). The composition of subunits of the AMPA receptor alters its functionality; the presence of the GluR2 subunit greatly reduces the calcium permeability of the receptor (Liu and Salter 2010). The receptor is associated with the fast ESPCs of a neuron following glutamate binding, and facilitates the activation of the NMDA receptor via membrane depolarization (Purves 2001; Liu and Salter 2010). The AMPA receptor is largely associated with its role in long-term potentiation (LTP) and long-term depression (LTD), due to the downstream effects of phosphorylating the GluR1 subunit (Kessels and Malinow 2009; Liu and Salter 2010). Similar to NMDA receptors, AMPA receptors are expressed in peripheral and central regions associated with nociception (Bleakman et al. 2006; Liu and Salter 2010). AMPA-R is found in the trigeminal and dorsal root ganglions, and their peripheral and central axons (Bleakman et al. 2006; Willcockson and Valtschanoff 2008; Liu and Salter 2010). It's also expressed in the dorsal horn and medullary dorsal horn (Bleakman et al. 2006; Polgar et al. 2008; Todd et al. 2009). The AMPA receptor has been implicated in the maladaptive pain associated with synaptic plasticity seen in chronic pain conditions, because of its role in LTP (Bleakman et al. 2006).

1.5.1.3. Kainate Receptors

The kainate receptors, like the other ionotropic glutamate receptors, are tetramers. Their subunits are assembled in various combinations of GluK1-3 and GluK4-5 (Liu and Salter 2010). GluK1-3 are low affinity subunits and GluK4-5 are high affinity subunits, the formation of a tetramer containing only subunits from GluK1-3 does not produce a functional receptor (Kew and Kemp 2005; Liu and Salter 2010). The KA receptor is also a mixed-cation ion channel (Kew and Kemp 2005). The potential role that kainate receptors may play in nociception is still largely unknown because of the difficulty of separating the pharmacology of non-NMDA receptors (Liu and Salter 2010). Emerging evidence suggests that the GluK1 subunit of KA receptors may be an important mediator of nociception (Liu and Salter 2010; Andreou et al. 2015).

Kainate receptors, while previously overlooked in the field of pain, are also expressed in relevant nociceptive pathways (Liu and Salter 2010). The receptor, in varying subunit combinations, has been found to be expressed in the trigeminal and dorsal root ganglion, and centrally in the dorsal horn and spinal trigeminal nucleus (Bleakman et al. 2006; Lucifora et al. 2006; Andreou et al. 2015).

1.5.1.4. Metabotropic Glutamate Receptors

The predicted mGluR structure includes a large N-terminal extracellular domain which forms a pocket containing the glutamate binding site, three extracellular and intracellular loops, and a cytoplasmic C-terminus, which is separated by seven transmembrane hydrophobic regions (Bleakman et al. 2006; Ferraguti and Shigemoto

2006; Liu and Salter 2010). The intracellular domains interact with G-proteins to initiate a signal transduction cascade (Ferraguti and Shigemoto 2006). The metabotropic glutamate receptors are separated into three groups based upon their sequence homology and are easily differentiated by their respective coupling to intracellular second messengers (Liu and Salter 2010). Group I (mGluR1 & mGluR5) receptors generally increase neuronal activity following glutamate binding via its coupling to phospholipase C (PLC)(Bleakman et al. 2006; Ferraguti and Shigemoto 2006). Both group II (mGluR2 & mGluR3) and group III (mGluR4,6-8) receptors are negatively coupled to adenylate cyclase (AC) and activation of these two groups of receptors primarily reduces neuronal excitability (Bleakman et al. 2006; Ferraguti and Shigemoto 2006).

MGLuRs are expressed both peripherally and centrally on neurons and glial cells, group I mGluRs are more commonly expressed postsynaptically, while group II and III mGluRs are more commonly expressed on axons and presynaptically (Kew and Kemp 2005). In the field of pain research, group I mGluRs have predominantly been the focus in the periphery due to their pain-producing behaviour following activation, even though they have the lowest percentage of expression in the DRG (Kolber 2015). In primary sensory afferents, mGluR1 & mGluR5 are functionally coupled with the transient receptor potential vanilloid (TRPV1) receptor, which has important implications in nociceptors (Jin et al. 2009; Liu and Salter 2010). Centrally, with the exception of mGluR6, all of the mGluRs are expressed, on a variety of cell types, which can lead to either pro- or anti-nociceptive effects upon activation(Liu and Salter 2010).

1.5.2. Glutamate Transport

In the central nervous system, extracellular glutamate concentrations, under normal conditions, are tightly regulated to prevent hyperexcitability neurons which could result in excitotoxicity and cell death (Danbolt 2001). Glutamate transporters located in neurons and glial cells are responsible for regulating the levels of glutamate (Gasparini and Griffiths 2013). There are two types of glutamate transport systems; vesicular glutamate transporters (VGLUTs) which allow glutamate to be taken up into synaptic vesicles, and excitatory amino acid transporters (EAATs) which take up glutamate from the extracellular space (Gasparini and Griffiths 2013).

There are five known types of EAATs, EAAT1-5, which are trans-membrane proteins that share 50-60% homology (Danbolt 2001). The transport of glutamate by EAATs is driven by the trans-membrane concentration gradients of sodium and potassium, regulated by the Na⁺/K⁺-ATPase pump (Gegelashvili and Bjerrum 2014). The transport is initiated by the simultaneous binding of glutamate, three sodium ions, and a proton to the extracellular face of the EAAT. This triggers a conformational change, resulting in the EAAT releasing its ligands into the cytoplasm of the cell. The counter-transport of a potassium ion returns the EAAT to its basal state where it can bind another glutamate molecule for transport (Danbolt et al. 1992; Levy et al. 1998; Kim et al. 2011).

With the exception of EAAT5 which is localized to the retina, the remaining transporters all appear to be expressed throughout the central nervous system (Danbolt 2001).

EAAT1 (also known as glutamate/aspartate transporter- GLAST) is solely expressed on glial cells and EAAT2 (also known as glutamate transporter 1-GLT1) is predominately

expressed in astrocytes but has also been detected in small amounts on hippocampal neurons (Danbolt 2001; Furness et al. 2009). The glutamate transport by EAAT2 is responsible for >90% of all glutamate uptake in the CNS (Kim et al. 2011).

Less is known about the regulation of glutamate levels in the peripheral nervous system. In the DRG, the satellite glial cells only express EAAT1, and EAAT2 is expressed in a small percentage of DRG neurons (Berger and Hediger 2000). In the TG, the satellite glial cells express both EAAT1 and 2 (Laursen et al. 2014). It is unknown what the tissue-specific distribution of EAATs are, and which transporters are responsible for the majority of glutamate uptake in the PNS.

It has been suggested that dysregulation or a decrease in expression of EAATs may contribute to pain states (Sung et al. 2003; Laursen et al. 2014). There is also of course the interest in the potential role that the down-regulation of EAAT2 (via a mutation in the SLC1A2 gene) plays in the pathogenesis of migraine (Schürks 2012; Gasparini and Griffiths 2013).

1.5.3. Glutamate Receptor Antagonists used Preclinically and Clinically for Migraine

Glutamate receptor antagonists have been evaluated in a number of animal models, which mirror a physiological component of migraine. Two common models, which are used in migraine research are (1) KCl or electrically induced cortical spreading depression, and (2) electrical or chemical stimulation of the dural blood vessels- and the subsequent measure of dural vasodilation or trigeminal activation.

Several NMDA receptor antagonists, including the non-competitive antagonists dizocilpine (MK-801) and memantine, and competitive antagonist selfotel (CGS 19755), have been tested in the CSD model and suppressed the number of CSD waves measured (Nellgard and Wieloch 1992; Peeters et al. 2007; Oláh et al. 2013). The AMPA antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) and non-selective iGluR antagonist kynurenic acid (KA) were also tested, but only KA decreased the number and amplitude of CSD waves, suggesting that the AMPA receptor may not play an important role in cortical spreading depression (Nellgard and Wieloch 1992; Oláh et al. 2013). Both kynurenic acid and dizocilpine were also found to decrease the permeability of the BBB during CSD (Oláh et al. 2013).

In contrast to the lack of efficacy of AMPA antagonists in the CSD model, antagonists of all iGluRs are able to suppress trigeminal activation in the dural stimulation model (Storer and Goadsby 1999; Chan et al. 2010; Andreou and Goadsby 2011; Andreou et al. 2015). Topiramate, an anti-convulsant which among other actions acts as an antagonist at AMPA and kainate receptors, inhibited the firing of SpVc and VPM neurons (Andreou and Goadsby 2011). The kainate receptor antagonist, LY466195 (a decahydroisoquinoline), and the non-competitive AMPA receptor antagonist, GYK 152466, both suppressed the firing of SpVc neurons (Storer and Goadsby 1999; Andreou et al. 2015). LY466195 was unable to inhibit dural vasodilation following electrical stimulation of the dura, while GYK 152466 on the other hand did inhibit CGRP induced dural vasodilation (Chan et al. 2010). The NMDA receptor antagonist dizocilpine inhibited the firing of trigeminovascular neurons in the SpVc and also attenuated dural

vasodilation evoked by electrical stimulation (Storer and Goadsby 1999; Chan et al. 2010).

Promising preclinical studies using glutamate antagonists led to clinical migraine trials. Memantine, tezampanel, and ADX 10059, which are antagonists to the glutamate receptors NMDA, AMPA, and mGluR5, respectively, have been found to be effective in treating headaches but off-target side-effects in the CNS has rendered them unsuitable (Chan and MaassenVanDenBrink 2014). The detrimental characteristic of these compounds is that they cross the blood-brain-barrier (Chan and MaassenVanDenBrink 2014), thus investigating whether peripheral glutamate receptor antagonists may also be effective in attenuating migraine pain, is a logical alternative.

1.6. Calcitonin Gene Related Peptide

CGRP is a 37–amino acid peptide, that along with calcitonin, amylin, and adrenomedullin make up the calcitonin family. There are two isoforms of CGRP; α -CGRP which is the product of alternative splicing of the calcitonin gene in neurons, and β -CGRP which is encoded by a different gene—thus far their biological activities have remained indistinguishable (Poyner 1992; Benarroch 2011). Peripherally, CGRP is expressed in a subset of sensory neurons (peptidergic neurons) and acts as both a neurotransmitter and potent vasodilator (Durham 2006; Benarroch 2011; Brain et al.). A δ and C nociceptive fibres originating from the dorsal root, trigeminal, and vagal ganglions express CGRP (Poyner 1992; Gu and Yu 2007; Lennerz et al. 2008; Benarroch 2011). Centrally, CGRP is found in the dorsal horn and spinal trigeminal nucleus, as well as in

neurons in the parabrachial nuclear complex (PBN) and the posterior intralaminar thalamic complex- which are key regions in the integration of sensory information (de Lacalle and Saper 2000; Dobolyi et al. 2005; Benarroch 2011; Unger and Lange). CGRP has been implicated as a key neuropeptide involved in the pathogenesis of migraine for several reasons. CGRP is released from peripheral nerve endings following depolarization, and is a key modulator of neurogenic inflammation which is thought to contribute to headache pain (Messlinger et al. 1995). The serum concentration of CGRP is transiently elevated during a migraine attack, and migraine treatments such as triptans and BoNTA suppress this elevation (Goadsby et al. 1990; Goadsby and Edvinsson 1993; Cernuda-Morollón et al. 2015). Similarly, to the actions of MSG, administration of α -CGRP can induce a migraine like headache in those who are susceptible to migraines (Hansen et al. 2010).

1.6.1. CGRP Receptor

A functional CGRP receptor consists of the coupling of a calcitonin receptor-like receptor (CLR) and a receptor activity-modifying protein 1 (RAMP1)(Benarroch 2011). CLR is a seven transmembrane domain that is G-protein coupled to $G_{\alpha s}$ which activates adenylyl cyclase (AC)(Tschopp et al. 1985; Benarroch 2011). RAMP1 is necessary to transport CLR to the cellular membrane and also mediates the receptor signaling (Walker et al. 2010; Benarroch 2011). A final protein, receptor component protein (RCP) is essential to associate CLR and RAMP1 intracellularly, and also couple the receptor to the G-protein (Walker et al. 2010; Benarroch 2011). Following the binding of CGRP, AC induces a

cAMP-PKA pathway that leads to several downstream actions, including modulation of K^+ and L-type Ca^{2+} channels, as well as the cAMP response element binding protein (Benarroch 2011). The result is vasodilation, neurotransmitter release, and increased neuronal excitability (Benarroch 2011). Figure 1.3 depicts the CGRP receptor and its transduction pathways.

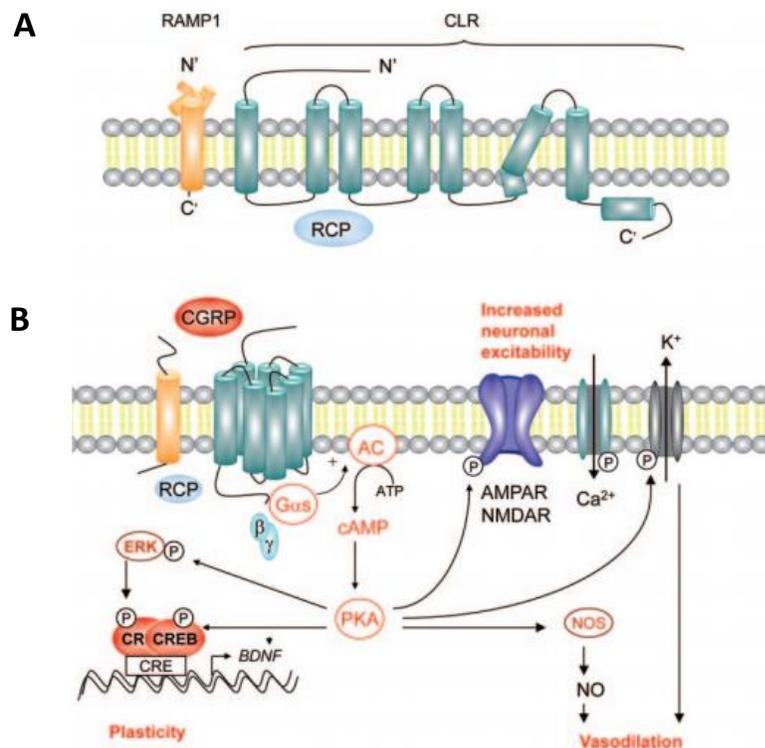


Figure 1.3: Components of the functional CGRP receptor and the downstream effects of its activation. A, a functional CGRP receptor consists of the transmembrane proteins RAMP1; receptor activity-modifying protein 1, and CLR; calcitonin receptor-like receptor, and the intracellular component RCP; receptor component protein. B, upon binding of CGRP, the G-protein coupled receptor activates adenylyl cyclase (AC), leading to the production of cyclic adenosine monophosphate (cAMP) and activation of protein kinase A (PKA). The effects of which are increased neuronal excitability, vasodilation and plasticity. (AMPA; AMPA receptor, BDNF; brain-derived neurotrophic factor, CRE; cyclic adenosine monophosphate response element, ERK; extracellular receptor activated kinase, NMDAR; NMDA receptor, NO; nitric oxide; NOS; nitric oxide synthase.) Preprinted with permission from (Benarroch 2011)

Histological analysis of the expression of the CGRP receptor, requires labelling of both the CLR and RAMP1 components. For the most part, the expression of the CGRP

receptor coincides with the expression of CGRP (Benarroch 2011). Peripherally, the CGRP receptor has been found in the TG and DRG, as well as extensively in blood vessels (Lennerz et al. 2008; Benarroch 2011; Eftekhari et al. 2013; Kestell et al. 2015). Centrally, in the dorsal horn, spinal trigeminal nucleus, PBN, thalamus, and the amygdala there exists a population of neurons expressing RAMP1 and CLR (Tschopp et al. 1985; Marvizón et al. 2007; Benarroch 2011). The localization of CGRP, CLR, and RAMP1 has been studied extensively in the trigeminovascular system because of its possible role in migraine. In the TG, neurons express either CLR and RAMP1 or CGRP, but not both CGRP and its receptor (Eftekhari et al. 2010). Satellite glial cells in the TG also contain the CGRP receptor (Eftekhari et al. 2010). Lennerz et al. investigated the expression of RAMP1 and CLR at the level of the dura as well as on the central projections of TG neurons (Lennerz et al. 2008). In the dura, the functional CGRP receptor was expressed in blood vessels, mononuclear cells, and Schwann cells, but not in the afferent fibres themselves (Lennerz et al. 2008). In the trigeminal spinal nucleus however, CLR and RAMP1 were expressed on the nerve fibres themselves, suggesting potentially different actions peripherally and centrally (Lennerz et al. 2008). A recent study examining expression in the dura, has discovered conflicting results in which unmyelinated fibres express CGRP, whereas CLR and RAMP1 were expressed in myelinated fibers identified with an A-fiber marker (Eftekhari et al. 2013).

1.6.2. CGRP Antagonists and Sequestering Antibodies tested Preclinically and Clinically for Migraine

A class of CGRP receptor antagonists, the gepants, were tested preclinically with very promising results. Olcegepant inhibited facial vasodilation in marmoset monkeys induced via trigeminal stimulation, suppressed nitric oxide induced activation of spinal trigeminal neurons in rats, and acted as a vasoconstrictor in isolated human cerebral arteries (Doods et al. 2000; Verheggen et al. 2002; Koulchitsky et al. 2009). Six drugs of the gepant family have undergone clinical trials, five of which had clinically relevant decreases in migraine frequency. The CGRP receptor antagonists have endured several setbacks as possible migraine analgesics however due to issues with formulation and toxicity. Olcegepant studies were discontinued due to poor bioavailability, and both telcagepant and MK-3207 have been discontinued due to hepatotoxicity (Bigal, Walter, et al. 2013; Karsan and Goadsby 2015).

Interest in CGRP as a target for migraine treatment has instead moved towards CGRP sequestering antibodies, four of which are currently being tested for migraine pain (Bigal and Walter 2014). While CGRP antagonists provided clear evidence that modulating CGRP could be efficacious in mitigating migraine pain, monoclonal antibodies (mAbs) against CGRP or its receptor have several promising features that indicate they may be more useful clinically (Bigal and Walter 2014). CGRP mAbs have excellent target specificity, have long half-lives meaning they can be dosed less frequently, and finally mAbs are broken down into endogenous amino acids and therefore pose fewer safety concerns in terms of toxicity (Wu and Dall'Acqua 2005;

Bigal and Walter 2014; Karsan and Goadsby 2015). The four CGRP mAbs are currently at different stages of clinical trials, some of which the results have been released and suggest a statistically significant reduction of headache days compared to baseline measurements (Bigal, Escandon, et al. 2013; Bigal and Walter 2014; Dodick et al. 2014).

1.7. Substance P

Substance P (SP) is neuropeptide consisting of 11 amino acids, and is a member of the tachykinin family, also known as the neurokinin family. SP is expressed peripherally in sensory fibres and cell bodies in the TG and DRG, as well as in blood plasma and immune cells (Ma et al. 2001). Centrally, SP is expressed in neurons of the SpVc, dorsal horn, and PAG (Tajti et al. 2001; Smith et al. 2002; Uddman et al. 2002). Produced in the cell bodies of sensory neurons, SP is transported to peripheral and central axons where it acts a neurotransmitter, interestingly SP levels is 4-fold higher in the peripheral endings (Brimijoin et al. 1980). SP and CGRP are co-expressed in a population of A δ and C-nociceptive fibres and are thought to be cotransmitters in pain transmission, potentiating the effects of each other (Ambalavanar and Dessem 2009).

Substance P has been linked to the pathogenesis of migraine because of its ability to produce plasma protein extravasation (PPE), a component of neurogenic inflammation, and because SP levels are elevated in the saliva of migraineurs during an attack (Nicolodi and Del Bianco 1990). The ability of migraine therapies such as ergot amines and

triptans to block experimentally induced PPE further justified the interest in SP in migraine research (Markowitz et al. 1988; Buzzi and Moskowitz 1990).

1.7.1. Substance P Receptor

There are three neurokinin receptors, neurokinin receptor 1-3 (NK₁, NK₂, and NK₃); while tachykinins can bind to any of these receptors, SP preferentially binds to the NK₁ receptor (Ambalavanar and Dessem 2009). The NK₁ receptor is a seven transmembrane domain G-protein linked receptor which experimentally has been found to coupled to several types of G-proteins (Roush and Kwatra 1998; Harrison 2001). G_{q/11} coupled receptors activate phospholipase C (PLC), which in turn increases cytoplasmic IP₃ levels leading to a rise in intracellular Ca²⁺ (Harrison 2001). NK1 is expressed on cell bodies in the DRG and TG as well as their peripheral and central processes (Helme and Fletcher 1983; Ambalavanar and Dessem 2009). Autocrine activation of SP-containing neurons is possible because of the co-expression of NK₁ and SP in these cells (Zhang et al. 2008; Ambalavanar and Dessem 2009). NK1 is also expressed on vascular endothelium and mast cells which mediate SP's ability to induce vascular permeability and edema (Foreman and Jordan 1983; Markowitz et al. 1987; Brain and Williams 1989; Kruminis and Broomfield 1992; Ambalavanar and Dessem 2009).

1.7.2. Substance P Antagonists used Preclinically and Clinically for Migraine

Several NK1 antagonists were tested in animal models of trigeminal activation, and their ability to inhibit plasma protein extravasation at the dura, and suppress elevations in c-fos expression (a measure of neuronal activation) were analyzed. RPR 100893 and

GR265171 were able to block electrically induced PPE, as well as c-fos elevation in both the TG and SpVc (Cutrer et al. 1995; Shephard et al. 1995; Polley et al. 1997). Positive pre-clinical results from NK₁ antagonists and translation results of migraine treatments blocking PPE in animals, lead to enthusiastic trials of NK₁ antagonists in clinical trials. The same NK₁ antagonists, RPR 100893 and GR265171 were tested in double-blinded, placebo controlled trials, and were unable to mitigate headache pain (Connor et al. 1998; Diener 2003). After several failed trials, interest has moved away from substance P and the NK₁ receptor as targets in treating migraine.

1.8. Migraine categorized as a Vascular Headache?

Traditionally, migraine has been considered a vascular headache, largely based on the observation of dilation of retinal and temporal blood vessels during a migraine attack (Goltman 1936; Wolff et al. 1953). The efficacy of early migraine treatments such as ergotamine, dihydroergotamine, and the more recently some triptans, mitigating headache pain, while also acting as potent vasoconstrictors, supported this hypothesis (Aellig and Rosenthaler 1986; Williamson et al. 1997; Williamson and Hargreaves 2001). Asghar et al. measured an increase in blood flow in the middle meningeal artery (MMA) following administration of α -CGRP, which is known to induce migraine-like headaches (Asghar et al. 2010). Further evidence of a vascular mechanism comes from a study by Friberg et al., who measured blood flow in the middle cerebral artery (MCA) using a Doppler sonogram and estimated a 20% increase in the diameter of the MCA during a

migraine attack compared to 5 days post-attack (Friberg et al. 1991). In both studies, sumatriptan suppressed the vasodilation (Friberg et al. 1991; Asghar et al. 2010). Evidence contradicting the vascular component of migraine also exists; a migraine attack induced by sildenafil in ten out of twelve patients did not elicit any changes in MCA diameter, as measured by transcranial Doppler ultrasonography (Kruuse et al. 2003). Another study conducted by Ferrari et al., measured blood velocity of the MCA in migraineurs who suffer from unilateral attacks. Measurements taken by a transcranial doppler during and after attacks, did not provide evidence of vasodilation (Zwetsloot et al. 1993).

While there is conflicting evidence for or against a vascular theory of migraine, it cannot be ruled out. Two potential scenarios exist in which meningeal vasodilation can (1) activate dural afferent fibres, or (2) result from the activation of dural afferent fibres (Levy and Burstein 2011). In the first scenario, dilation of the highly innervated dural blood vessels can activate mechanoreceptors in the trigeminovascular afferent fibres, potentially leading to the sensitization of the trigeminovascular system (Levy and Burstein 2011). In the second scenario, dural afferent fibres which have already been activated may induce vasodilation via the release of CGRP and SP (Levy and Burstein 2011).

1.9. Trigeminal Anatomy

The trigeminal nerve, also known as cranial nerve V, is the largest of 12 cranial nerves.

The trigeminal ganglion (TG) located in Meckel's cave, contains the cell bodies of

unipolar trigeminal neurons, which project both peripherally and centrally. The peripheral axons exit the TG and divide into three divisions; V1 (the *ophthalmic* branch), V2 (the *maxillary* branch), and V3 (the *mandibular* branch) (Prasad and Galetta 2007). The three branches innervate deep and superficial structures within and beneath the highlighted dermatomes in figure 1.4. The ophthalmic and maxillary branches are comprised of sensory fibres while the mandibular branch contains both sensory and motor fibres necessary for mastication (Prasad and Galetta 2007). Peripheral branches of the trigeminal nerve also contain pre- or postganglionic parasympathetic fibres or post-ganglionic sympathetic fibres which supply blood vessels, and salivary, sweat, or other glands (Liu 2005).

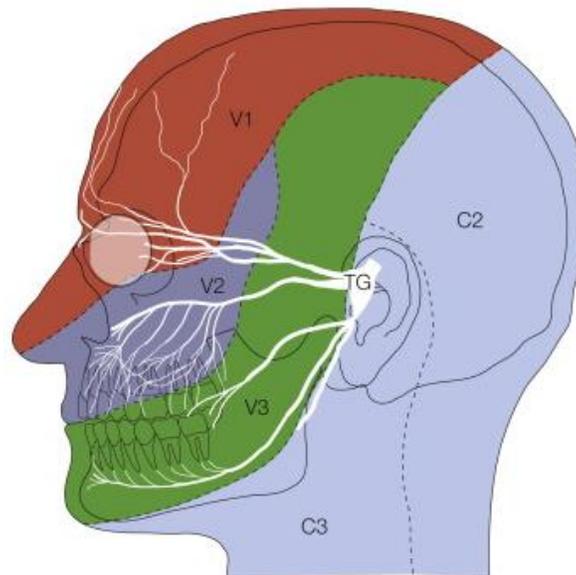


Figure 1.4: The face and head are innervated by the trigeminal nerve. The trigeminal nerve is the 5th cranial nerve and consists of three branches. The ophthalmic (V1), the maxillary (V2), and the mandibular (V3) branches innervate the highlighted dermatomes as well as the underlying vascular, muscular, and meningeal tissues. The head is also innervated by the upper cervical nerves, C1 and C2. TG; trigeminal ganglion. Preprinted with permission from Villanueva & Noseda 2013.

The central axons of the trigeminal nerve exit the TG in a single tract and enter the brainstem at the level of the pons upon which the axons diverge, spreading rostrally or caudally. The first order trigeminal neurons synapse in three sensory nuclei within the brainstem. These include the spinal trigeminal nucleus (Vsp), the principal sensory nucleus (Vp), and the mesencephalic nucleus (Vmes), each of which have a distinct trigeminal function (Prasad and Galetta 2007). The Vsp which receives nociceptive and cutaneous input is further divided into three subnuclei; the caudalis (Vc), interpolaris (Vi) and oralis (Vo) (Shigenaga and Yoshida 2007). The Vp receives input from fibres that convey tactile and pressure senses and the Vmes receives input from fibres involved in the kinesthetic sensations of the face (Prasad and Galetta 2007).

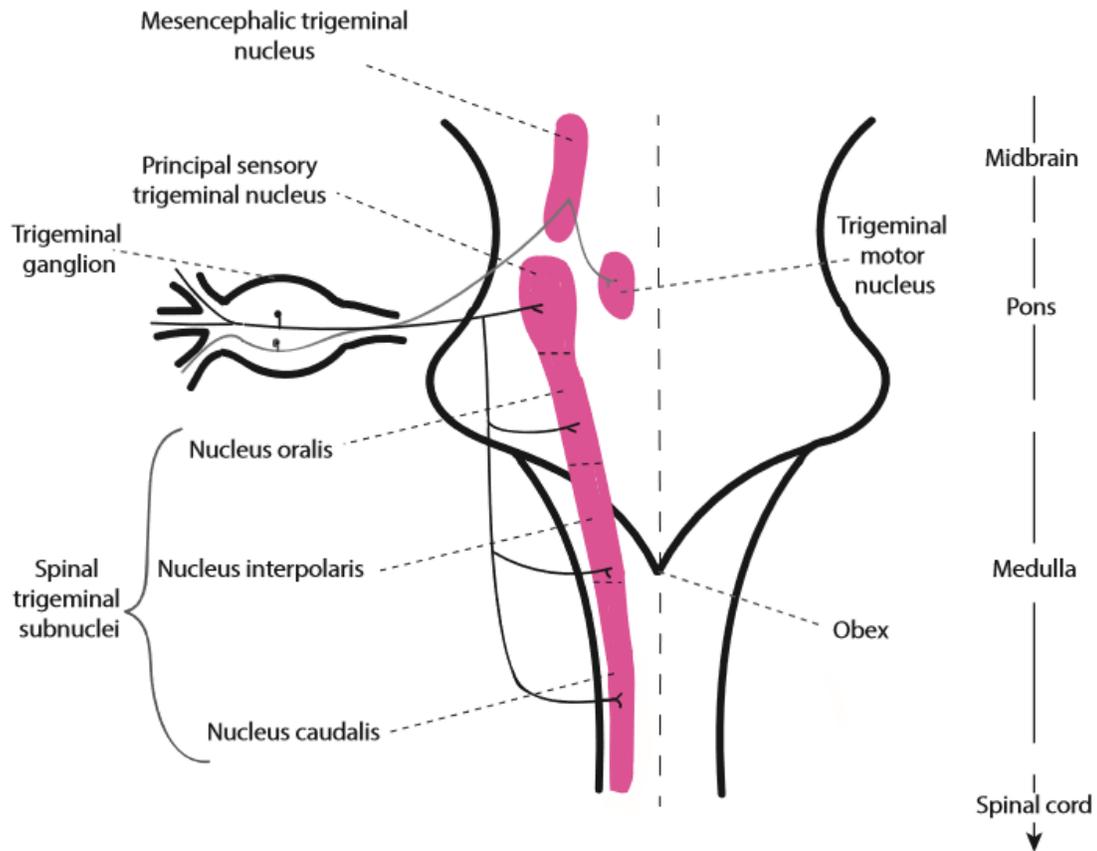


Figure 1.5: A simplified diagram of central trigeminal nuclei. Central projections of the trigeminal neurons with cell bodies in the trigeminal ganglion synapse at the mesencephalic, principal sensory, trigeminal motor, or spinal trigeminal nucleus. The spinal trigeminal nucleus is further divided into three subnuclei; the nucleus oralis, nucleus interpolar, and nucleus caudalis. Adapted from Saper 2000.

1.9.1. Innervation of the Dura

The cranial meninges that protect the brain are comprised of three fibrous layers; the dura, arachnoid, and pia maters. The meninges as a whole are divided into four anatomical areas; the falx cerebri, diaphragma sella, falx cerebelli, and tentorium cerebelli. The dura, which lies beneath the skull, and the pia, which adheres to the brain, are both vascular. The arachnoid mater lies between the dura and pia and surrounds cerebrospinal fluid (CSF) in the subarachnoid space above the pia. The dura contains

sinuses, which collect blood drained from the brain and cranial bones; the largest being the superior sagittal sinus (SSS), as well as blood vessels, the largest of which is the middle meningeal artery (MMA) which is the major supplier of blood to the dura. The pia contains smaller capillaries and also forms a layer around blood vessels entering the brain (Mancall 2011).

Fibres innervating the dura arise from all three branches of the trigeminal nerves, as well as the upper cervical nerves, sympathetic and parasympathetic nerves. Both A (myelinated) and C (unmyelinated) afferent fibres innervate the dura. They are found concentrated around blood vessels but non vascular regions are also innervated to a lesser extent (Messlinger et al. 2005). Blood vessels of the supratentorial dura mater are richly innervated by ipsilateral trigeminal afferent fibres originating from the ophthalmic branch and to a lesser extent the maxillary branch. The tentorial nerve and ethmoidal nerve are both meningeal branches of ophthalmic origin. The tentorial nerve innervates the tentorium cerebelli, the posterior portion of the falx cerebri, the dura mater of the parieto-occipital region, and the superior sagittal and transverse sinuses (RA Davidoff 2002; Kemp III et al. 2012). Ethmoidal branches innervate the middle meningeal artery, and follow rostrally along the sagittal sinus as far as the olfactory bulb (Andres et al. 1987; Kemp III et al. 2012). The middle meningeal nerve, a branch of maxillary origin, innervates the dura mater in the parietal region of the middle cranial fossa at the base of the skull and follows along the middle meningeal artery (Kemp III et al. 2012). The middle cranial fossa also receives some innervation from the nervus spinosus, which

contains sensory fibres from the mandibular branch and also postganglionic sympathetic fibres (Andres et al. 1987; Uddman et al. 1989a; Mancall 2011; Kemp III et al. 2012).

Meningeal branches of the upper three cervical spinal nerves (C1-C3) innervate the dura covering the lateral and posterior walls of the posterior cranial fossa (Kemp III et al. 2012). The superior cervical ganglion provides sympathetic innervation to the dura (Uddman et al. 1989a; Mancall 2011). Parasympathetic innervation is provided by the vagus nerve in addition to the postganglionic fibres arising from the sphenopalatine and otic ganglion within the trigeminal sensory branches (Uddman et al. 1989a; Liu 2005; Mancall 2011).

Histological studies on the meninges have determined that a large number of the fibres innervating it contain neuropeptides. In particular, substance P (SP), neurokinin A, and calcitonin-gene related peptide (CGRP) are expressed by many sensory nerve fibres (Edvinsson et al. 1988; Tsai et al. 1988; von Düring et al. 1990; Keller and Marfurt 1991). CGRP-immunopositive afferent fibres and trigeminal cell bodies are more abundant than those which are SP-immunopositive (Uddman et al. 1989b). Dural sympathetic fibres express neuropeptide Y, while parasympathetic fibres are immunopositive for vasoactive intestinal peptide (VIP) (Messlinger et al. 2005) which allows these fibres to be differentiated from sensory fibres.

Recently it has been proposed that nerve fibres innervating the dura may have dual projections to the periosteum above the skull and pericranial muscles in human and rat (Schueler et al. 2013; Schueler et al. 2014; Zhao and Levy 2014). Zhao and Levy theorize that the fibres reach the periosteum through the calvarial sutures, which become fused

during cranial development (Zhao and Levy 2014). They suggest V1 and V2 dural branches are capable of having these collateral branches, while Schueler et al., characterize V2 and V3 branches which innervate the cranial dura, periosteum, and pericranial muscles (Schueler et al. 2014; Zhao and Levy 2014). Further histological studies are needed to confirm this new development.

1.9.2. The Spinal Trigeminal Nucleus Caudalis

The trigeminal brainstem nuclear complex (TBNC), comprised of the Vp and Vsp, integrates innocuous and noxious input from the trigeminal afferent fibres and also receives projections from facial, glossopharyngeal, vagus nerves and upper cervical nerves (Shigenaga and Yoshida 2007). Clinical observations and animal models have implicated the Vsp, perhaps most importantly the subnucleus caudalis (SpVc), as the primary region of relaying facial pain information to higher level regions of the brain (Greenwood and Sessle 1976; Sessle and Greenwood 1976; Lisney 1983; Hayashi et al. 1984; Amano et al. 1986). The SpVc is similar to the dorsal horn of the spinal cord in both laminar structure and physiological properties, thus it is also known as the medullary dorsal horn (Gobel et al. 1981; Messlinger et al. 2005).

Within the medullary dorsal horn, along with the central projections of the trigeminal nerve are intrinsic interneurons and projection neurons, as well as axons from descending brainstem pathways (Dubner and Bennett 1983). Axonal tracing and histology have been used to characterize primary afferent projections to the SpVc and the subsequent projections of second and third order neurons in the spinothalamic

tract. Retrograde tracing of sensory innervation of dural vasculature has determined projections to the caudalis and interpolaris subnuclei of the Vsp (Arbab et al. 1986; Arbab et al. 1988; Uddman et al. 1989a; Liu et al. 2008). Some neurons in the SpVc have been found to project directly to the thalamus (Fukushima and Kerr 1979; Bruce et al. 1987; Kemplay and Webster 1989; Sessle 2000), with the ventral posteromedial thalamic nucleus (VPM) receiving the majority of this input (Peschanski 1984). SpVc neurons can also project to the thalamus via poly-synaptic paths utilizing relays in the reticular formation and adjacent brainstem areas (Sessle 2000). Ipsilateral projections to other regions within the TBNC from the SpVc have also been characterized and include the subnucleus oralis (Hockfield and Gobel 1982; Ikeda et al. 1984; Sessle 2000). Additionally projections to other areas of the brain from the SpVc include the superior colliculus (Huerta et al. 1983; Bruce et al. 1987), the parabrachial nucleus (PB) (Cechetto et al. 1985), the cerebellar cortex (Matsushita et al. 1982; Huerta et al. 1983; Magnusson et al. 1987), and the lateral periaqueductal grey (PAG) matter (Nosedá et al. 2008).

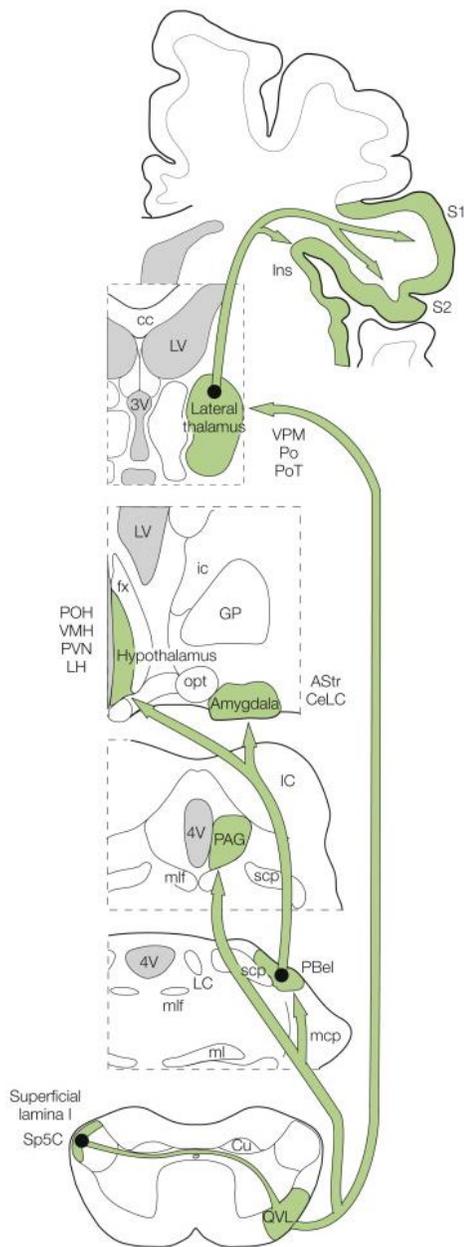
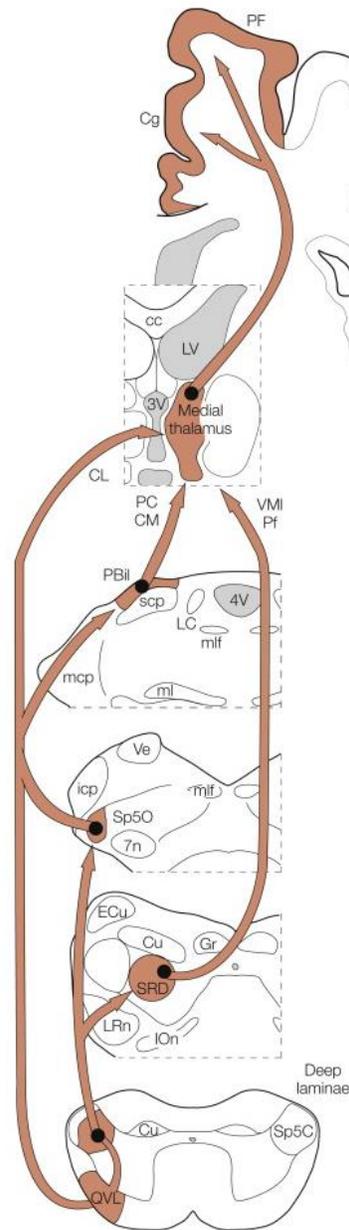
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Figure 1.6: The main ascending projections from superficial (A) and deep (B) SpVc neurons.

Superficial lamina I and deep lamina V-VI SpVc neurons send input to several regions implicated in somatosensory, motor, arousal, and attentional processing of nociceptive input. A, Lamina I SpVc neurons project to the pBel; graylateral parabrachial nucleus, PAG; periaqueductal gray, amygdala, and subnuclei of the hypothalamus and thalamus. The thalamic neurons which receive input from superficial SpVc neurons project to the Ins; insular cortex, and S1 and S2; the primary and secondary somatosensory cortices. B, Lamina V-VI (deep) SpVc neurons project to the SRD; subnucleus reticularis dorsalis, SpVo; spinal trigeminal subnucleus oralis, pBil; lateral parabrachial nucleus, and the thalamus. The thalamic neurons which receive input from deep SpVc neurons project to the PF; prefrontal cortex, and the Cg; cingulate cortex. The diagram is not exhaustive of all SpVc projections. Preprinted with permission from Villanueva & Nosedá 2013.

(Astr, amygdalostriatal transition area; cc, corpus callosum; CeLC, central amygdaloid nucleus, lateral capsular part; Cg, cingulate cortex; CL, centrolateral thalamic nucleus; CM, central medial thalamic nucleus; ECu, external cuneate nucleus; fx, fornix; GP, globus pallidus; Gr, gracile nucleus; ic, internal capsule; icp, inferior cerebellar peduncle; Ins, insular cortex; IOn, inferior olive nucleus; LC, locus coeruleus; LH, lateral hypothalamic nucleus; LRn, lateral reticular nucleus; LV, lateral ventricle; mcp, middle cerebellar peduncle; ml, medial lemniscus; mlf, medial longitudinal fasciculus; 7n, facial nucleus; opt, optic tract; PAG, periaqueductal gray; PBel, lateral parabrachial nucleus, external part; PBil, lateral parabrachial nucleus, internal part; PC, paracentral thalamic nucleus; pf, parafascicular thalamic nucleus; PF, prefrontal cortex; Po, posterior thalamic nuclear group; POH, preoptic hypothalamic region; Pot, posterior thalamic triangular nucleus; PVN, paraventricular hypothalamic nucleus; QVL, ventrolateral quadrant; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; scp, superior cerebellar peduncle; SRD, subnucleus reticularis dorsalis; 3V, third ventricle; 4V, fourth ventricle; Ve, vestibular nucleus; VMH, ventromedial hypothalamic nucleus; VMI, ventromedial thalamic nucleus, lateral part; VPM, ventral posteromedial thalamic nucleus.)

1.9.3. Functional Characteristics of Spinal Trigeminal Nucleus Caudalis Neurons with

Meningeal input

As aforementioned, anatomical mapping of trigeminal dural afferents has revealed the SpVc as an important region in dural vascular fibre input, and electrophysiological data also supports this theory. Electrophysiological responses of caudalis neurons have been recorded in cats (Davis and Dostrovsky 1986; Strassman et al. 1986; Davis and Dostrovsky 1988a; Davis and Dostrovsky 1988b) and rats (Burstein et al. 1998; Ellrich et al. 1999; Schepelmann et al. 1999) following electrical, mechanical and chemical stimulation of the dura. The pioneering electrophysiological studies by Davis (Davis and Dostrovsky 1986) and Strassman (Strassman et al. 1986) in cats concluded that these neurons had convergent input from the skin of the face, primarily with receptive fields in the ophthalmic dermatome, and to a lesser extent the maxillary dermatome. Additional studies completed in rats provided similar results. Rat SpVc neurons with meningeal input also had receptive fields mapped to the skin, predominantly in the V1

region (Burstein et al. 1998; Ellrich et al. 1999; Schepelmann et al. 1999). These SpVc neurons were further characterized by their response to chemical or thermal stimuli, after being located via an electrical search stimulus or by mechanically probing the dural receptive field (Burstein et al. 1998; Schepelmann et al. 1999). Responses to chemical stimulation of the dura, which included activation or sensitization, were recorded in 55-78% of neurons depending on the chemical agent applied (Burstein et al. 1998; Schepelmann et al. 1999). The mechanical sensitivity of SpVc neurons was also assessed with innocuous (light brushing) and noxious (pinching) stimuli applied to the facial receptive field. Low-threshold mechanoreceptive neurons (LT) (responding only to innocuous stimulus), wide-dynamic range neurons (WDR) (responding to both innocuous and noxious stimulus), and high-threshold mechanoreceptive or nociceptive-specific neurons (NS) (responding to only noxious stimulus) were all identified; WDR being the most common (Burstein et al. 1998; Ellrich et al. 1999; Schepelmann et al. 1999). These studies suggest that most SpVc neurons which receive input from the dura, respond to multiple stimuli and often do not respond exclusively to the dura (Burstein et al. 1998; Ellrich et al. 1999; Schepelmann et al. 1999; Messlinger and Ellrich 2001).

1.9.4. Functional Characteristics of Meningeal Primary Afferents

Electrophysiological studies have characterized the properties of primary afferent fibres innervating the dura via recording cells within the trigeminal ganglion of rats. Recording from the TG allowed calculation of conduction velocities of neurons and thus further classification; both myelinated A δ and unmyelinated C-fibre populations have been

identified (Dostrovsky et al. 1991; Strassman et al. 1996; Messlinger and Ellrich 2001; Levy and Strassman 2002). Similar to electrophysiological studies conducted in the SpVc, afferent fibres recorded in the TG responded to chemical, mechanical, and thermal stimuli. Dural afferent fibres responded similarly to nociceptors in other tissues by demonstrating chemosensitivity and sensitization to mechanical stimuli (Dostrovsky et al. 1991; Strassman et al. 1996; Levy and Strassman 2002; Strassman and Levy 2006).

1.9.5. The Vasculature of the Meninges

The dura mater is richly supplied with arteries and veins. The dural arterial supply consists of branches of the internal carotid, maxillary, ascending pharyngeal, occipital, and vertebral arteries. The largest supplier of blood is the middle meningeal artery (MMA). The largest vein is the middle meningeal vein, which drains into the pterygoid venous plexus or sphenoparietal sinus. The superior sagittal sinus also extends along the sagittal suture within the dura mater. The pia mater is also vascular and contains the middle cerebral artery (MCA) and smaller capillaries. Superior cerebral veins which lie beneath the meninges penetrate them to drain into the superior sagittal sinus (Messlinger et al. 2005; Mancall 2011).

1.9.6. Modulation of Dural Blood Flow

The blood vessels of the dura are highly innervated and induce headache like pain when stimulated in humans (Ray and Wolff 1940). These blood vessels are innervated by sensory, sympathetic and parasympathetic nerve fibre that modulate vasodilation and constriction. Bolay et al. (Bolay et al. 2002) showed that an animal model of CSD induced

a 37% increase in MMA blood flow as measured by speckle- imaging. Transecting the nasociliary branch of the trigeminal nerve or parasympathetic innervation to the meninges abolished the increase in dural blood flow. CSD following a rhizotomy of the trigeminal root also did not elicit an effect on dural blood flow. The selective NMDA receptor antagonist, MK-801, when applied to the dura was also able to suppress CSD induced MMA vasodilation, further implicating glutamate in neurogenic inflammation (Bolay et al. 2002).

Glutamate can induce vasodilation by several different mechanisms. The excitatory neurotransmitter can activate sensory fibres, which results in the release of the potent vasodilator CGRP (Messlinger et al. 1995). The activation of glutamate receptors, particularly the NMDA receptor, can also increase intracellular Ca^{2+} levels, which may in turn increase the production of nitric oxide (NO) by activating the Ca^{2+} /calmodulin-dependent NOS pathway (Meng et al. 1995). NO released by sensory neurons subsequently binds to and activates guanylyl cyclase in the endothelium of blood vessels; the downstream action of this causes smooth muscle relaxation and hence vasodilation (Garthwaite 1991; Meng et al. 1995). Finally, glutamate, through a trigeminal-autonomic reflex may also induce dural vasodilation mediated by innervation of vasculature by parasympathetic fibres. Polysynaptic connections between the SpVc and the superior salivatory nucleus (SSN) may activate parasympathetic fibres leading to dilation (Bolay et al. 2002).

1.10. Experimental Hypothesis

Based upon the above mentioned animal and human experimental evidence, I hypothesize that, **Monosodium glutamate induces headache through activation of peripheral glutamate receptors.** I've employed several research aims to examine this hypothesis:

***Aim 1:** To determine the level of expression of peripheral glutamate receptor subtypes and excitatory amino acid transporters in dural afferent fibres.*

***Aim 2:** To determine whether MSG induces changes in dural blood flow.*

***Aim 3:** To determine whether MSG induced dural vasodilation activates central trigeminovascular neurons, which have dural input, and whether a peripherally restricted glutamate receptor antagonist can attenuate any potential effects of MSG.*

Within this aim there are two experimental questions being proposed:

1. Is MSG induced dural vasodilation capable of activating and/or sensitizing mechanoreceptors on dural afferent endings, as recorded from second order neurons in the trigeminal spinal subnucleus caudalis (SpVc)?
2. Can a peripherally restricted glutamate receptor antagonist attenuate potential MSG induced dural afferent activation or sensitization?

Chapter 2: MSG induces Dural Vasodilation and excites Spinal Trigeminovascular Neurons with Dural Receptive Fields

2.1. Introduction

Due to the uncertainty in the cause of migraine, it is unknown if drugs targeting peripheral glutamate receptors will be efficacious in treating migraine headache, but there is a growing accumulation of evidence that supports that glutamate regulation could act in a prophylactic manner (Ferrari et al. 2009; Huang et al. 2014). Because glutamate receptors have been identified in areas associated with both the peripheral and central mechanisms of migraine (Nosedá et al. 2014) it is possible that blocking peripheral glutamate receptors could decrease excitatory transmission and subsequent sensitization, in both peripheral and central systems, and thus reduce the pain and allodynia associated with migraine (Gomez-Mancilla et al. 2014). It is unknown which glutamate receptors or transporters are expressed at the level of the dura.

While migraine is categorized as a neurovascular headache, current research is questioning how large of a role extracranial vasodilation plays in migraine attacks (Levy and Burstein 2011). The dural vasodilation model, whether induced by electrical or chemical stimulation, however, has been widely used and has identified receptors that are capable of modulating nociceptive stimuli, which have been subsequently shown to be clinically relevant (Akerman et al. 2013). As previously mentioned MSG has the ability to sensitize trigeminal afferent fibers (Cairns et al. 2007) as well induce a headache in nonmigraineurs (Shimada et al. 2013). MSG was used in this experiment to determine if it is able to induce dural vasodilation as well as activate or sensitize SpVc neurons with

dural input. In *in vivo* electrophysiological experiments, the peripherally restricted NMDA receptor antagonist, APV, was coadministered with MSG to determine if it is capable of attenuating any potential effect of MSG.

2.2. Methods

2.2.1. Animals

Male (305–480 g, n=30) and female (245-330 g, n=28) Sprague-Dawley rats were used for these experiments. Animals were housed in groups of two or three and were subjected to a 12-h light/dark cycle. Food and water were available ad libitum. Experiments were conducted in the light cycle, between 8am-5pm. All animal procedures were reviewed and approved by the University of British Columbia Animal Care Committee.

2.2.2. Immunohistochemistry

Male (n=4) and female (n=4) Sprague-Dawley rats were anesthetized (AErrane, Baxter Corporation, Mississauga, ON, Canada; 2-2.5% in oxygen 97-98%) and perfused with cold saline followed by paraformaldehyde (4%). The head of the rat was incubated in 20% sucrose followed by 40% sucrose for 48 hours each to dehydrate the tissue. The dura over the right and left sagittal sinus was carefully removed from the brain, and separated into four regions to analyze four glutamate receptors (NMDAr, AMPAr, KAR, mGluR5) in each rat. Sections were mounted on glass slides and treated with 0.3% Triton-X 100 for 25 minutes and subsequently washed 3 times with phosphate-buffered

saline (PBS). Slides were treated with 10% normal goat serum (NGS) in PBS for 1 h followed by overnight incubation with primary antibodies in 1% normal goat serum. All tissue samples were stained with antibodies against protein gene product 9.5 (rabbit polyclonal 1:1500, Abcam) and von Willebrand Factor (sheep polyclonal 1:750, Cedarlane), to identify nerve fibres and blood vessels, respectively. All tissues were also incubated with antibodies against a glutamate receptor, either the NR2B subunit of the NMDAR (mouse monoclonal 1:400, Abcam), the Glur1 subunit of the AMPAR (mouse monoclonal 1:1000, Stressmarq), the GluR 5+6+7 subunits of the KAR (mouse monoclonal 1:1000, Abcam) or mGluR5 (mouse monoclonal 1:500, Abcam). The expression of CGRP (sheep polyclonal 1:500, Abcam) and EAAT1-3 (sheep polyclonal 1:250, Santa Cruz) was also assessed. 24 hours later, sections were washed several times with phosphate-buffered saline and then incubated for 1 h at room temperature in the dark in the presence of appropriate fluorescence-conjugated secondary antibodies (Invitrogen). After several washes in buffer, tissue sections were mounted on slides with coverslips and visualized with Leica TCS SPE high resolution spectral confocal microscope. Images were taken in the x-y plane and z-stacking was used to view the tissue within 3-dimensions.

Fibres were identified if they meet the following criteria: minimum of 4 μm in length and intensity of the signal exceeded the two standard deviations of the mean background intensity (an estimate of the 95% confidence interval). All images were analyzed for positive immunoreactivity using the same criteria for signal intensity using ImageJ software.

The specificity of the antibodies was also tested. Omission of the primary antibody from the assay, and preabsorption with the appropriate antigen (for EAAT1-3 only) acted as negative controls. Treatment of tissues with known expression of the epitope of interest acted as a positive control. Slices from the trigeminal ganglion (TG) and brainstem of perfused animals were used to confirm the expression of EAAT1-3, CGRP and GluRs.

2.2.3. Surgical procedure for Doppler and Electrophysiological Recordings

Male and female Sprague-Dawley rats were anesthetized with isoflurane (AErrane, Baxter Corporation, Mississauga, ON, Canada; 2-2.5% in oxygen 97-98%) and artificially ventilated via a trachea tube. Temperature was continually measured with a rectal thermometer and maintained at $37\pm 0.5^{\circ}\text{C}$ using a heating pad (Fine Science Tools, 21060-01). Blood pressure was measured via carotid artery cannulation and maintained within a range of 60-90mmHg. Heart rate was also continuously measured throughout the procedure as a measure of depth of anaesthesia and maintained above 300 beats per minute. The femoral vein was cannulated to administer drugs. In cases of unsuccessful vein cannulation, drugs were administered via the carotid artery. Animals were placed in a stereotaxic frame, and an incision was made in the skin over the dorsal region of the skull to expose the bone.

The closed window method was used to measure dural flux with a Doppler flowmeter. Briefly, the parietal bone was thinned over the middle meningeal artery using a small drill, creating a “closed window”. The Doppler probe was held in place over the window by a cotton-tipped applicator lowered in position by a micro manipulator. Mineral oil was used to keep the cotton-tipped applicator and exposed bone moist.

For electrophysiology experiments, the right parietal and the frontal bone was removed from the lamboid suture to anterior of the orbit, as depicted in figure 2.3. An incision was also made in the skin and muscle over the neck to expose the brainstem, the dura was removed and a C1 laminectomy was performed. The exposed brainstem was bathed in mineral oil.

2.2.3.1. Doppler Flux Recording

Five male and five female Sprague-Dawley rats were used to measure potential changes in dural blood flow following MSG administration. Dural blood flux (measured in perfusion units, PU) was sampled at a rate of 1Hz. A 30-minute recovery period was allocated following the completion of surgery, before beginning the dural flux recording. The Doppler probe was calibrated in a polystyrene calibration solution prior to each use and mineral oil was applied once the probe was held in position over the closed window to mitigate noise artifacts. After the recovery period, a five-minute baseline measurement of dural blood flux was recorded, followed by injection of 50mg/kg MSG into the femoral vein or carotid artery. MSG was flushed with 0.5ml of normal saline. The response to MSG was recorded. Animals were terminated via an overdose of pentobarbital.

2.2.3.2. In-vivo Electrophysiological Recording

Extracellular action potentials of SpVc neurons with dural receptive fields were recorded with a parylene-coated tungsten microelectrode (0.10", 2M Ω , A-M Systems Inc., Carlsborg, WA, USA) in male (n=21) and female (n=19) Sprague-Dawley rats. In some

animals, multiple neurons, which could be differentiated by their action potential characteristics (amplitude, waveform, duration), shared a dural receptive field; in those cases, the acquired data from both neurons were used and spike sorting was applied with the program Spike 2. The recording electrode was lowered into the exposed brainstem in the area of the nucleus caudalis (SpVc), 1-2mm lateral and 1-2mm caudal to the obex and at depth ranging from 50-2800 μm at a 33° angle. The V1 and V2 dermatomes of the skin were brushed while the electrode was lowered, to identify potential areas of the SpVc which may have dural input. Mechanoreceptors innervating the dura were identified by their response to mechanical stimulation applied to the dura with the tip of an electronic von-Frey hair. The neurons were characterized by their receptive fields, their response to innocuous (brush) and noxious (pinch) stimuli, and also the latency to fire following electrical stimulation of the dura. To differentiate receptive fields in the temporalis muscle and the overlying skin, the skin was gently pulled away from the muscle and both the skin and muscle were brushed and pinched. Following the identification of a SpVc neuron with a dural receptive field, a baseline mechanical threshold (MT) was recorded. Briefly, the electronic von-Frey hair (model 1601C, Life Science, Woodland Hills, CA, USA) was pressed against the dural receptive field with increasing force until it elicited an action potential, the smallest force which elicited an action potential was recorded as the MT. The baseline consisted of five evoked responses separated by one minute intervals, from which the mean baseline MT was calculated. Following a five-minute baseline to assess spontaneous activity of the neuron, 50mg MSG was administered alone or in combination with APV (5mg/kg or

50mg/kg), and flushed with normal saline. Another five-minute period was recorded to measure spontaneous activity following administration of drug(s). The MT was again assessed for a period of 10 minutes. Mean MT values were calculated for two 5-minute increments. In a separate group of animals (n=3 male and female), saline was administered in a volume which matched that of the MSG treated animals, to assess for a potential volume effect. After completion of the experimental protocol a blood sample was taken and the animals terminated with an overdose of pentobarbital.

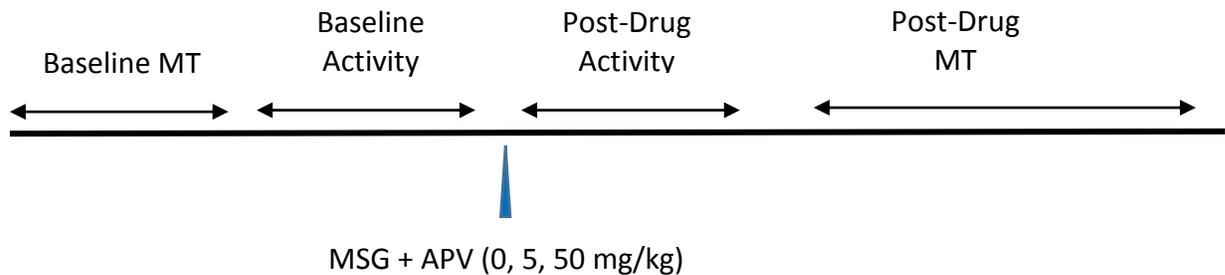


Figure 2.1: The experimental protocol to assess the effect of MSG (50mg/kg) administered alone or with APV (5, 50mg/kg) on the spontaneous activity and mechanical threshold (MT) of a SpVc neuron with dural input. The baseline MT consisted of evaluating the MT once a minute for five minutes using an electronic von-Frey hair. Baseline and post-drug activity consisted of five minutes of neuronal recording in the absence of mechanical stimulation. Post-drug MT consisted of accessing the MT for a period of ten minutes.

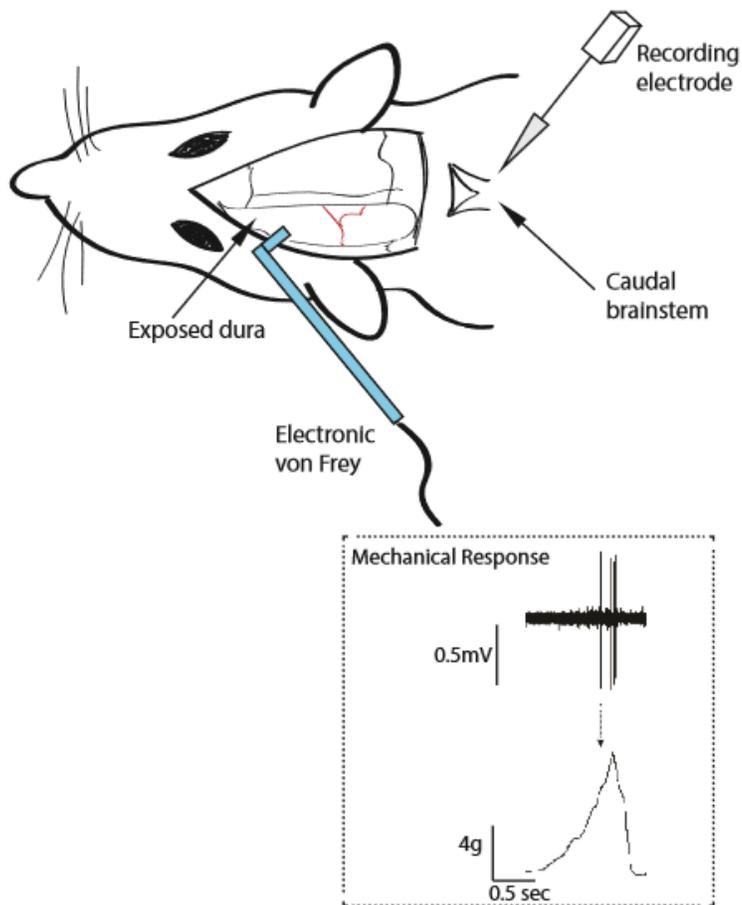


Figure 2.2: The experimental set up of a rat for electrophysiological recordings. An example of a mechanical threshold (MT) measurement is shown. An electronic von Frey hair was used to apply increasing force to the exposed dura (lower trace) until an action potential was generated in the SpVc (upper trace). The arrow indicates the MT for the SpVc neuron, which in this experiment was 8.7g.

2.2.3.3. Drugs

L-glutamic acid sodium salt hydrate (Sigma Aldrich) was dissolved in water to a final concentration of 169.1mg/ml and administered at doses of 50mg/kg. Fresh solutions of MSG were prepared weekly. DL-2-Amino-5-phosphonovaleric Acid (APV; Santa Cruz Biotechnology) was dissolved in 0.2M NaOH and phosphate buffered saline (PBS; Sigma Aldrich). Two concentrations of APV were prepared, 20mg/ml and 2mg/ml which were

administered at doses of 50mg/kg and 5mg/kg. The experimenter was blinded to the dose of APV being administered.

2.2.3.4. Plasma Estrogen Concentration

Collected blood samples were centrifuged for 10 min at 3000 RPM to separate plasma and blood cells. Plasma was extracted, labelled and stored at - 20°C until analysis. The concentration of 17 β -estradiol (pg/ml) was measured with an enzyme linked immunosorbent assay (ELISA) kit (40-056-205004, Genway). Samples were assayed in duplicate.

2.2.4. Data Analysis

The relative blood flux following MSG administration was calculated by the following equation: relative blood flux = median MSG flux / median baseline flux. The 'median MSG flux' was calculated in 30s bins up to a maximum of 5 minutes, and the 'median baseline flux' was calculated for the 5-minute baseline period. The mean of the maximal value of relative blood flux for each animal was calculated and denoted the mean relative flux. The duration of the flux response following MSG was estimated from the raw experimental traces.

The mean of the 5 mechanical thresholds (MTs) taken during the baseline period was calculated for each animal. Two mean MTs were calculated from the MTs taken over a ten-minute period following drug delivery, one from 5-10 minutes and the other from 10-15 minutes post injection. The relative MT was then calculated by the following equation: relative MT = (post-drug MT/baseline MT) x 100. The mean of the relative MT

was calculated for the two post drug time points for each treatment group. Three-way repeated measures ANOVA was performed on the relative MT at the two different time points with sex and treatment as factors. The Fisher's least significant difference (LSD) test was used post-hoc. The ongoing discharge of neurons was also measured before and after drug delivery, the change in discharge was calculated as: total discharge post-drug - total discharge pre-drug. The proportion of neurons in a treatment group that responded with an increase in ongoing discharge was calculated. The median change in neuronal discharge in the MSG group and MSG+APV (5 or 50mg/kg) groups were compared using a 2-way ANOVA on ranks with sex and treatment as factors. The correlation between serum estrogen concentration and relative MT was assessed with a Pearson correlation test. A probability level of less than 0.05 was considered significant for all tests.

2.3. Results

2.3.1. Glutamate Receptor and Transporter expression in the Dura

Histological analysis revealed that the NMDA, AMPA, Kainate and mGlu5 glutamate receptors are all expressed by nerve fibers innervating dural blood vessels. Blood vessels were identified with anti-von Willebrand factor. Differentiation of blood vessels and nerve fibres are lacking in other immunohistochemical studies in the dura (Edvinsson et al. 1988; Harriott and Gold 2008; Lennerz et al. 2008; Eftekhari et al. 2010). While networks of nerve fibers, as identified by expression of the axonal marker PGP 9.5,

innervate the dura, the expression of glutamate receptors was localized to the nerve fibre endings at blood vessels (Figures 2.3 and 2.4).

The expression of EAAT1-3 was also assessed in the dura, EAAT2 (GLT1) was the only transporter identified, and was expressed by dural blood vessels (Figure 2.5). As a control, brainstem and TG slices were stained with EAAT1-3, and all three transporters were identified.

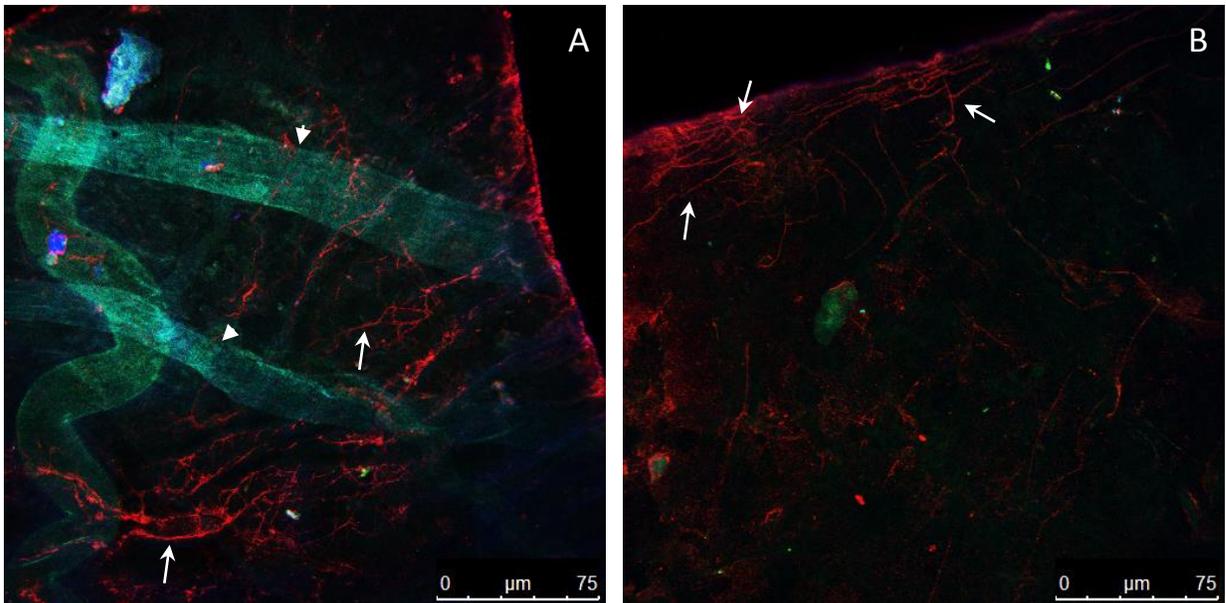


Figure 2.3: Photomicrographs show dural afferent fiber innervation of the dura. Nerve fibers innervate in the proximity of blood vessels (A) as well as in non-vascular areas of the dura (B). Nerve fibres, indicated by arrows, show immunopostivity for the pan-axonal marker PGP 9.5. Blood vessels, indicated by the arrowheads, show immunopostivity for the endothelial marker vWF. Images taken at 40X magnification.

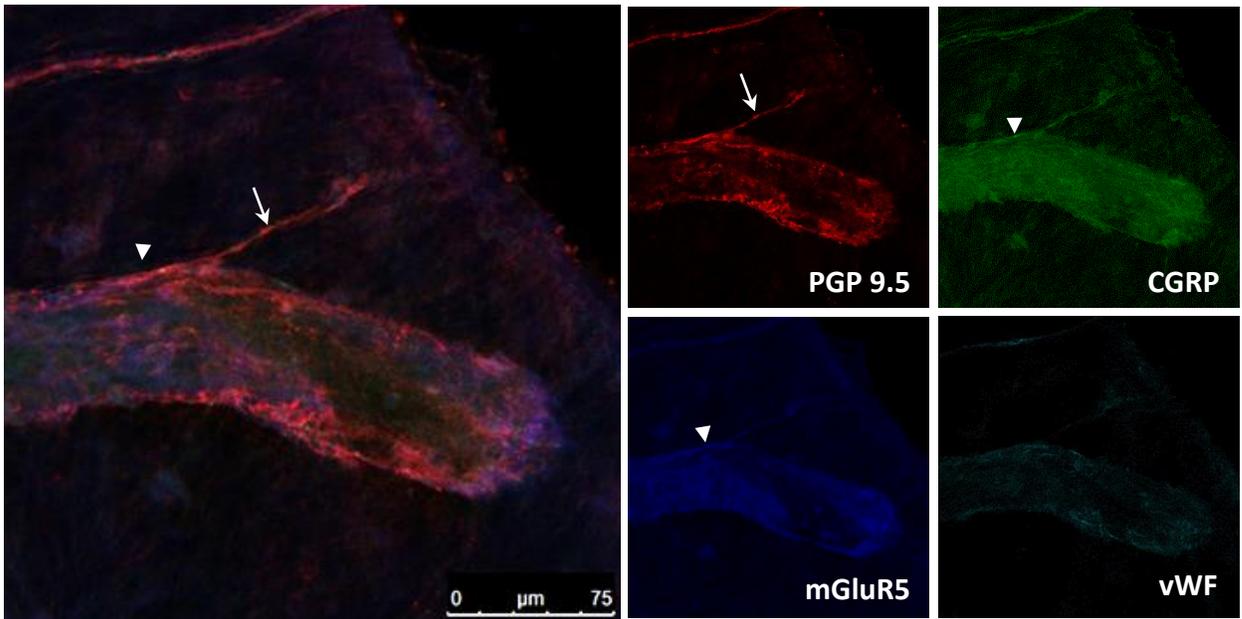


Figure 2.4: Photomicrographs show dural afferent fiber innervation of a blood vessel. The nerve fiber, indicated by an arrow, and blood vessel were identified by PGP 9.5 and vWF, respectively. Afferent labelling for calcitonin gene related peptide (CGRP) and mGluR5 is indicated by the arrowheads. Images taken at 40X magnification.

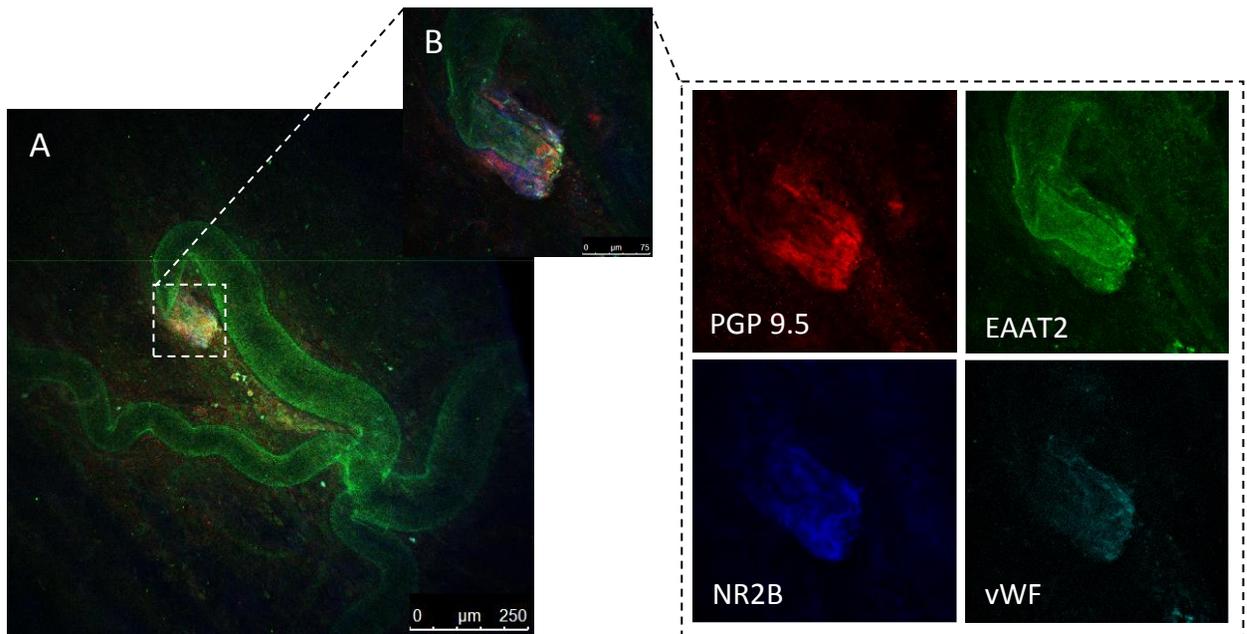


Figure 2.5: Photomicrographs show expression of EAAT2 and NMDA receptor on a dural blood vessel. A; composite image at 10X magnification, highlighted box shown at 40X magnification in B. Magnification in B, reveals the blood vessel, identified with the endothelial marker von Willebrand factor (vWF), is also being innervated by nerve fibres, as identified with immunopositivity for protein gene peptide 9.5 (PGP 9.5), which express the NR2B subunit of the NMDA receptor.

2.3.2. MSG induces Dural Vasodilation

MSG (50mg/kg) administered intravenously or intra-arterially induced a mean maximal increase of $24.5 \pm 4.4 \%$ and $20.6 \pm 2.8 \%$ in the dural flux, relative to baseline, as measured in male and female rats, respectively (Figure 2.6). The average duration of the MSG evoked surge in flux was approximately 170s across both sexes. Systemic blood pressure, also increased with a few seconds delay behind the rising dural flux.

Experimental traces of dural blood flux and systemic blood pressure are depicted in Figure 2.7. A control experiment, in which saline was administered rather than MSG did not elicit an increase in dural flux or heart rate (Figure 2.8). The laser Doppler response to pentobarbital, which was used to terminate the animals at the end of the

experiment, is shown in Figure 2.9. This was measured as a control to ensure proper functioning of the Doppler flowmeter, readings of dural flux should reach a 'biological zero' value as the animal dies.

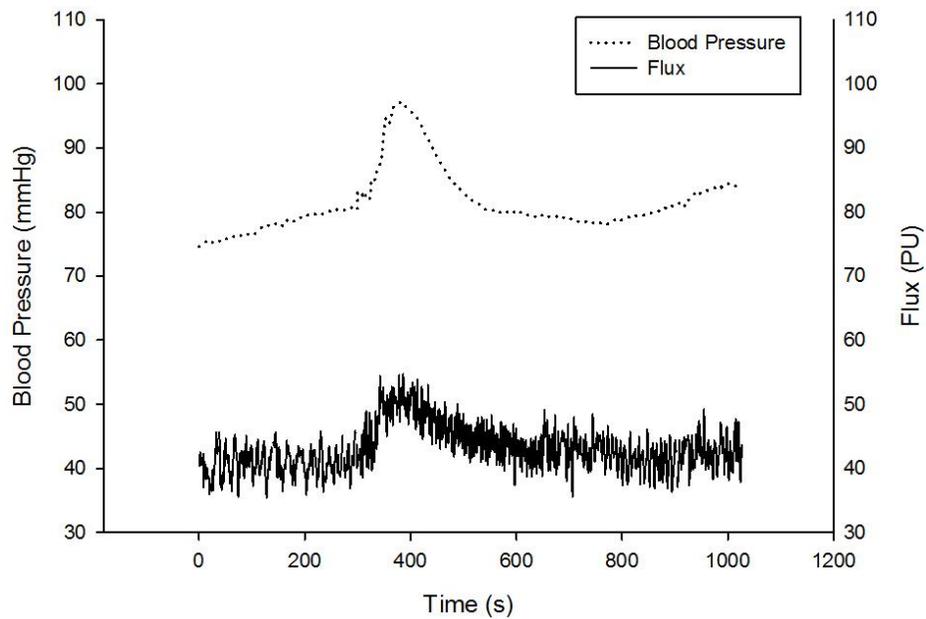


Figure 2.6: An example of an experimental trace showing the response to 50mg/kg MSG administered intravenously in a male rat. Flux, measured in perfusion units (PU), was recorded by a laser Doppler probe placed above the middle meningeal artery and blood pressure, measured in mmHg, was measured via carotid artery cannulation. Both flux and blood pressure increase transiently following MSG (50mg/kg) administration at 300s.

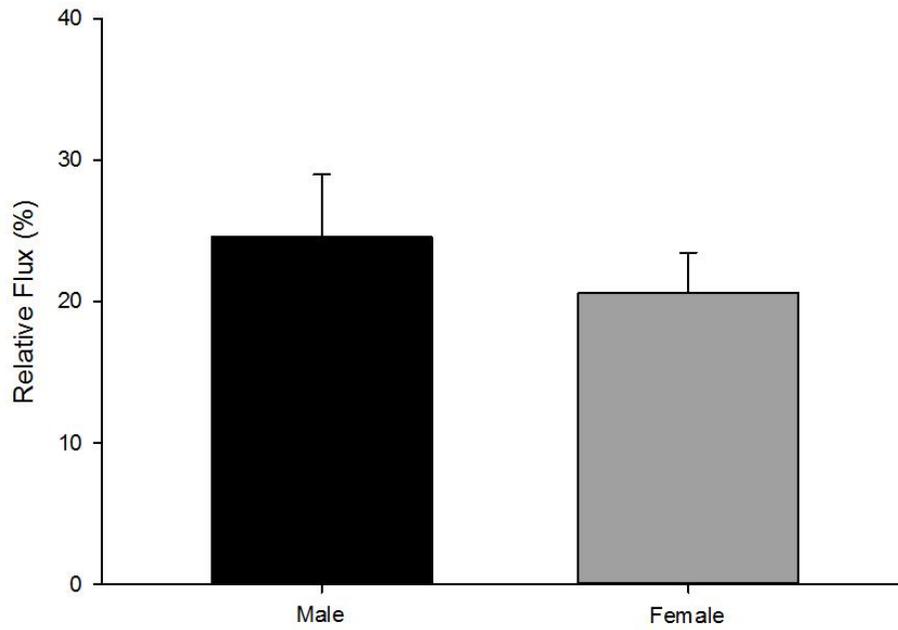


Figure 2.7: Mean maximal dural flux relative to baseline, following systemic administration of 50mg/kg MSG in male and female rats (n=5, error bars=SEM). In male rats, MSG induced a 24.5 ± 4.4 % and in female rats a 20.6 ± 2.8 % increase in dural flux relative to baseline measurements.

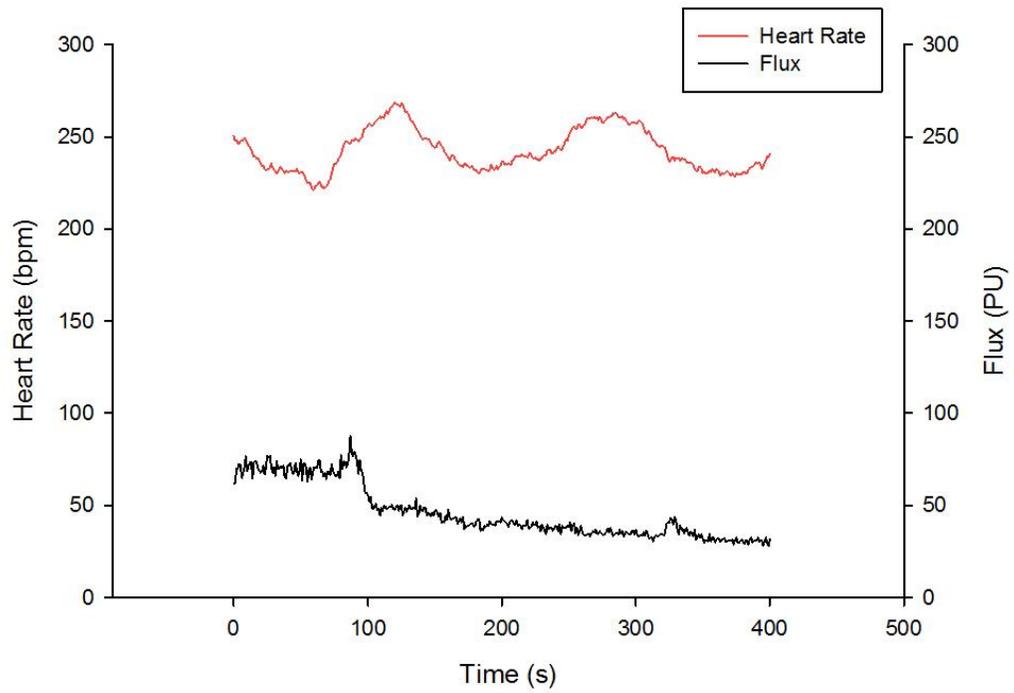


Figure 2.8: An example of an experimental trace showing the response to saline administered intravenously in a female rat. Flux, measured in perfusion units (PU), decreased following saline administration at 100s, while the heart rate, measured in beats per minute (bpm), fluctuated normally.

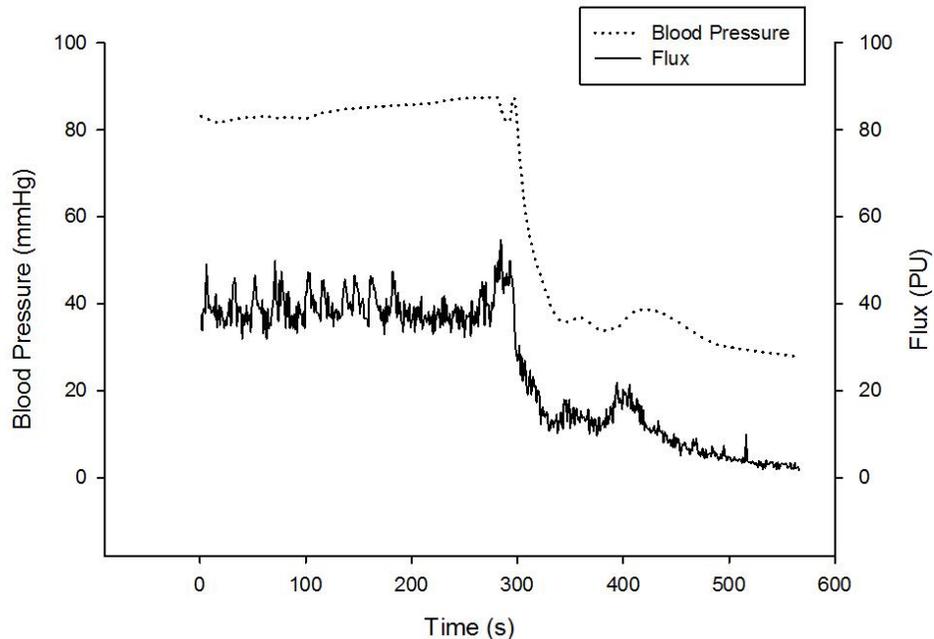


Figure 2.9: An example of an experimental trace showing the response to pentobarbital administered intravenously in a male rat. Dural flux, measured in perfusion units (PU), decreased drastically towards biological zero, while system blood pressure (mmHg) followed with a slight delay following administration of pentobarbital.

2.3.3. Characteristics of SpVc Neurons

To determine the potential effect of MSG and APV (0, 5, 50mg/kg) on the spontaneous and evoked activity of SpVc neurons with dural receptive fields, electrophysiological recordings from 36 neurons were undertaken. All neurons had receptive fields on the dura and most (34, 94%) also had additional receptive fields on the face. Receptive fields were predominantly located in the V1 (ophthalmic) dermatome, most commonly around the eye, but also could include both V1 and V2 (maxillary) dermatomes. The majority of neurons innervated both the skin and the temporalis muscle. Two neurons only responded to mechanical stimulation of the dura. Ninety-two percent of neurons were classified as wide-dynamic range (WDR), responding to both innocuous (brush)

and noxious (pinch) stimuli, the remaining were classified as either nociceptive specific (NS, 5%) or low threshold (LT, 3%). Response to electrical stimulation was assessed in 25 neurons, 10 (40%) responded with a mean latency of 11.8 ± 2.2 ms. In the remaining neurons either deteriorating conditions or inaccessibility to the receptive field due to bone or bleeding inhibited the ability to electrically stimulate the dura.

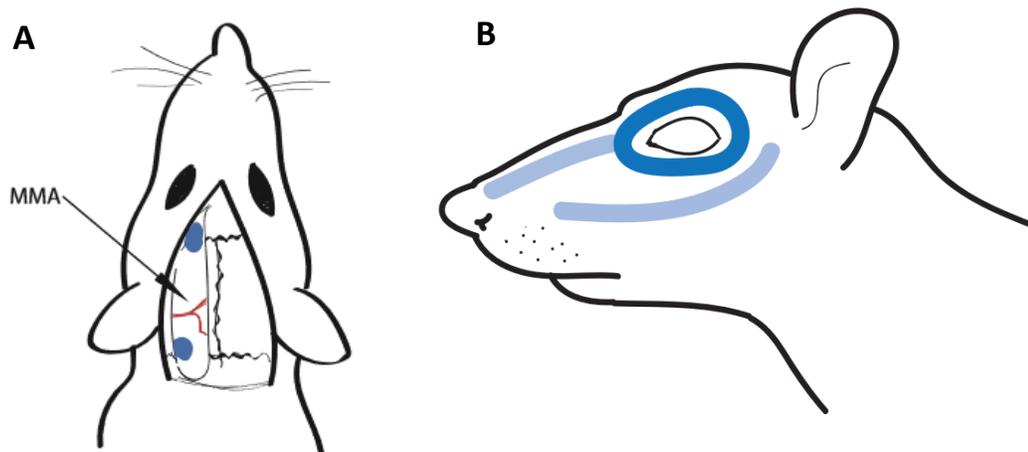


Figure 2.10: Dural and facial receptive fields of the SpVc neurons recorded. All caudalis neurons recorded had dural input and 94% received additional input from the face. A, blue spots indicate common dural receptive fields as determined via mechanical stimulation of the dura. B, blue regions indicate common facial receptive fields, darker blue represents the most common facial receptive field which includes the temporalis muscle and periorbital skin. Facial receptive fields were identified via brushing and pinching of the skin and muscle.

2.3.4. MSG Activates SpVc Neurons with Dural Input

The intravenous administration of 50mg/kg MSG induced an increase in the activity of SpVc neurons as measured by the number of action potentials fired in 5-minute periods before and after MSG was given. In 5/6 male and 5/6 female rats, MSG evoked an increase in neuronal discharge when compared with the baseline spontaneous activity. An example of the responses from SpVc neurons to MSG is shown in Figure 2.11. The

median increase in the number of action potentials fired following MSG administration was 11 and 25 for male and female rats respectively. Co-administration of 5mg/kg, but not 50mg/kg APV, with MSG resulted in a statistically significant decrease in the median change in discharge following drug administration ($p= 0.015$, 2-way ANOVA on RANKS, post-hoc Holm-Sidak). There was no statistically significant difference between the change in discharge in each treatment group between male and female rats (Figure 2.12).

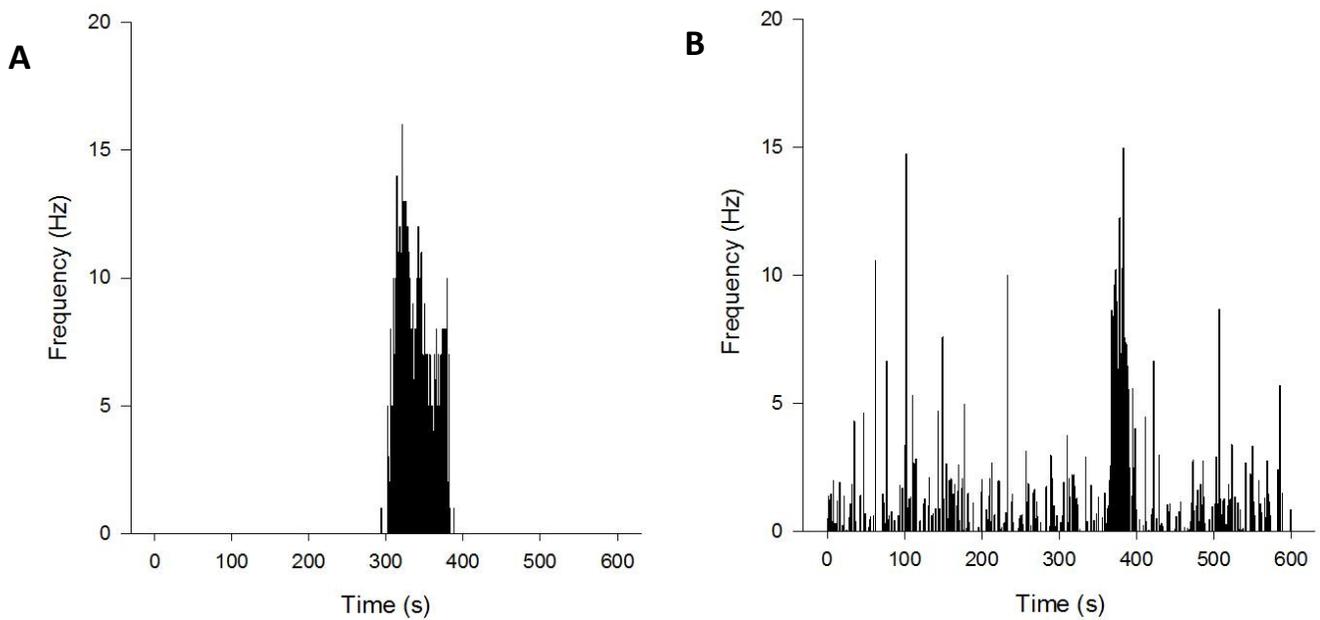


Figure 2.11: The peri-stimulus histograms depict the increase in frequency of action potentials fired as recorded from the SpVc following administration of 50mg/kg MSG at 300s. A and B show caudalis neurons that are quiet and have on-going activity prior to the administration of MSG.

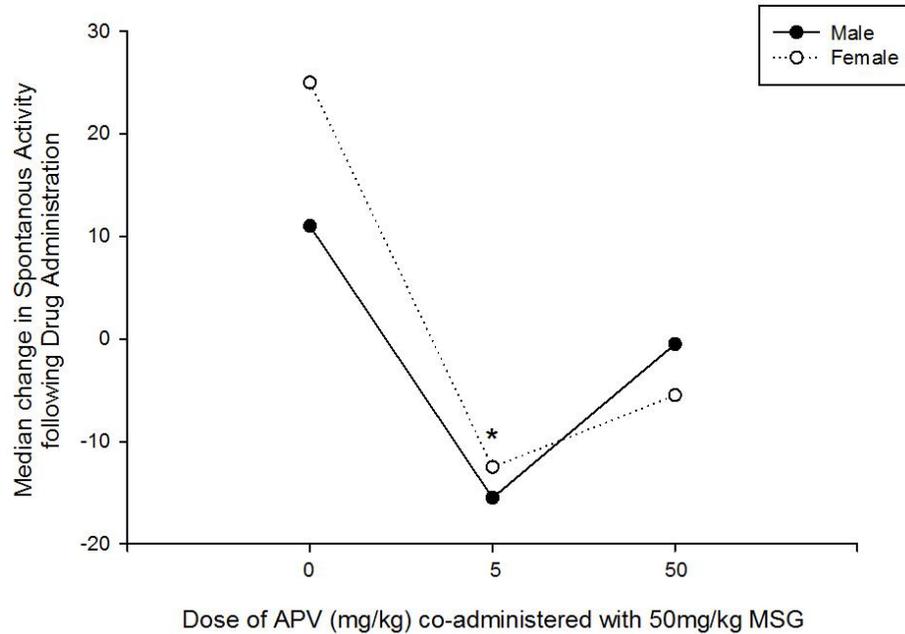


Figure 2.12: The median difference in action potentials fired following administration of MSG + APV (0, 5, 50mg/kg, n=6). Difference in action potentials determined by subtracting pre-drug discharge from post-drug discharge. The asterisk indicates a statistically significant difference between the 5mg/kg APV treated groups and 0mg/kg APV treated groups ($P < 0.05$, post-hoc Holm-Sidak multiple comparison test). There is not a statistically significant difference based on sex ($P = 0.142$). Raw data for each treatment group is found in Appendix B.

2.3.5. Effect of APV on MSG Evoked Sensitization of SpVc Neurons

Mechanical sensitization of SpVc neurons was assessed by measuring mechanical thresholds prior to and after intravenous administration of 50mg/kg MSG. Two relative MTs were calculated, at 5-10 minutes and 10-15 minutes post MSG administration. In male rats, MSG induced an $11.1 \pm 5.7\%$ decrease in MT, while in female rats the effect was smaller with a $0.4 \pm 12.5\%$ decrease in MT, 5-10 minutes after receiving MSG. APV co-administration dose-dependently increased the mechanical threshold in both male and female rats. The combination of 50mg/kg APV with MSG induced a $21.2 \pm 15.0\%$ and $37.6 \pm 15.4\%$ increase in MT in male and female rats, respectively at 5-10 minutes

post injection. At 10-15 minutes after the drug administration, the relative MT in the 50mg/kg APV + MSG treated animals had increased by $43.0 \pm 30.6\%$ and $67.8 \pm 12.1\%$ in male and female rats, respectively. The effect of time and treatment were both statistically significant. The relative mechanical thresholds from 15 minutes post drug administration are larger than those measured at 10 minutes post drug administration ($p=0.014$, 3-way rm ANOVA). The relative MTs of animals treated with MSG and 50mg/kg APV are statistically different from those who received MSG alone ($p=0.010$, 3-way rm ANOVA, post-hoc Fisher's least significant difference test). There was not a statistically significant difference in MTs between male and female rats. The effects of MSG administered alone or combined with APV on mechanical threshold are seen in Figure 2.13.

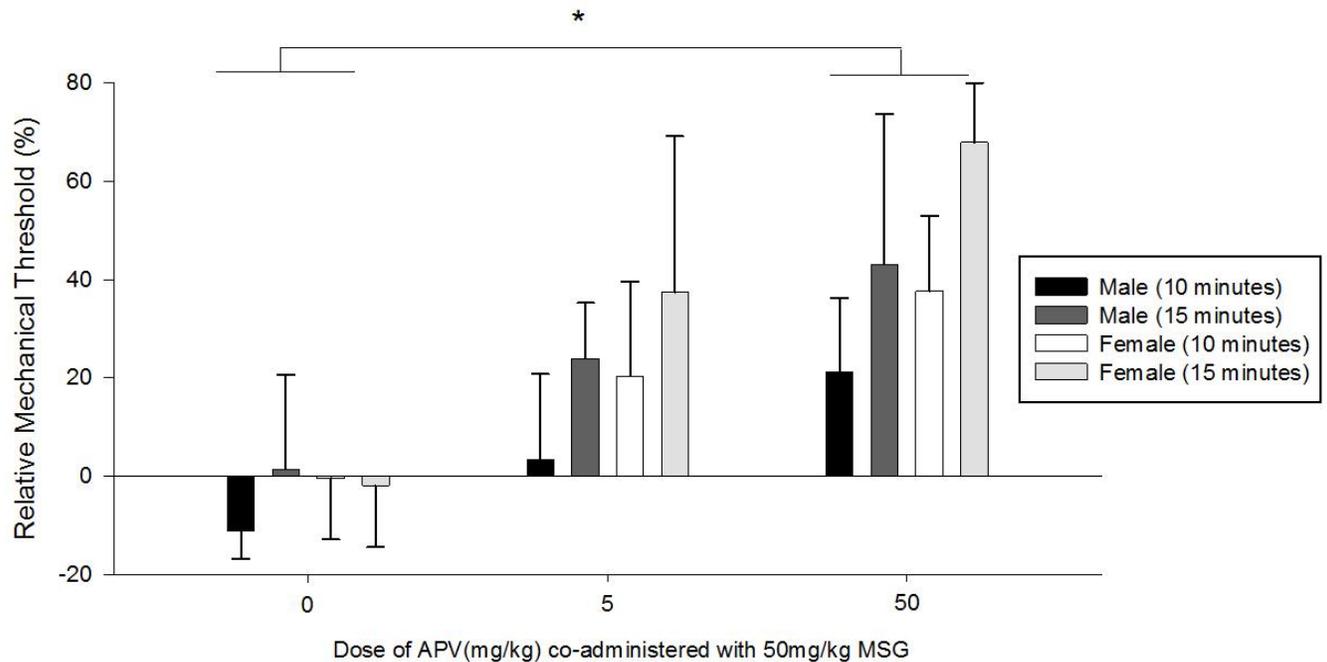


Figure 2.13: Co-administration of APV attenuates the MSG induced mechanical sensitization of SpVc neurons at 10 and 15 minutes post drug administration (n=6). The relative mechanical threshold was calculated by comparing the two post-drug MTs to the baseline MT. For each treatment group the relative MT is plotted for both male and female animals at two time points post MSG (10 and 15 minutes). Asterisks indicate thresholds significantly different between 50mg/kg APV + MSG and MSG treatment groups ($p < 0.05$, post-hoc Fisher's LSD test). There was no statistical difference based on sex ($p > 0.05$).

2.3.6. Effect of Saline on SpVc Neurons with Dural Input

The response to saline was measured in 7 SpVc neurons in 6 animals, 3 male and 3 female. Saline administered at equal volume to that of MSG, did not induce an increase in ongoing discharge in SpVc neurons. There was no median change in the number of action potentials fired following saline administration. Saline did however induced mechanical sensitization in SpVc neurons, the mechanical threshold decreased by $5.7 \pm 3.2\%$ and $1.8 \pm 8.0\%$ at 10 and 15 minutes post injection.

2.3.7. Plasma Estrogen Levels do not correlate with Baseline MT in SpVc Neurons

The levels of plasma estradiol were determined in animals used for electrophysiology experiments because it has been determined that estrogen levels correlate with the level of expression of the NMDA receptor in trigeminal ganglion neurons (Dong et al. 2007). In male rats the plasma estradiol concentration was lower and more tightly clustered than in female rats. No significant correlation was observed between the baseline MT and plasma estradiol concentration in female ($r = -0.196$) or male rats ($r = 0.199$), as determined by the Pearson Correlation Test (Figure 2.14).

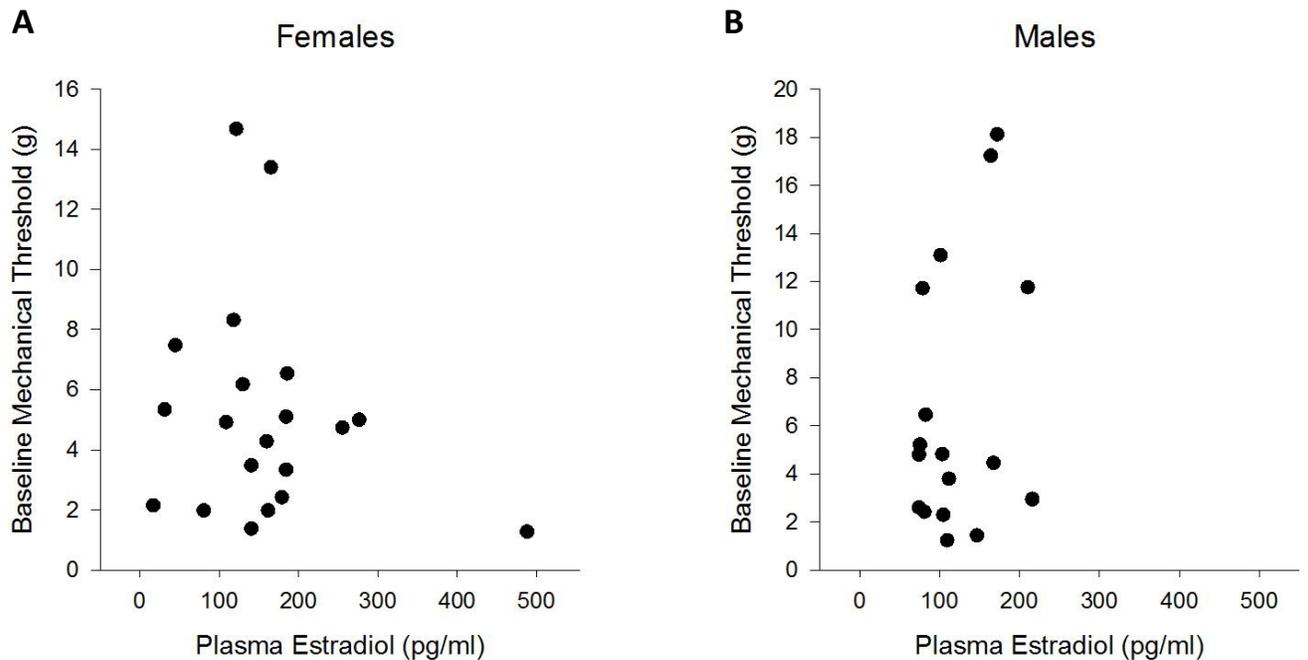


Figure 2.14: The scatter plots show the relationship between baseline mechanical threshold and plasma estradiol concentration in female (A) and male (B) rats. Plasma estradiol concentration was quantified using an enzyme linked immunosorbent assay. In both female ($r = -0.196$) and male ($r = 0.199$) no significant correlations were determined (Pearson Correlation Test, $p > 0.05$).

2.4. Discussion

2.4.1. MSG and Headache

The consumption of MSG has long been associated with unpleasant reactions such as heart palpitations, muscle pain and weakness, and headache (Geha et al. 2000). These unpleasant sensations were initially termed “Chinese Restaurant Syndrome”, which was later renamed “MSG symptom complex” once it was determined to result from ingesting high levels of MSG (Geha et al. 2000). MSG is commonly added in high levels to processed foods but also occurs naturally in protein rich food such as meat, tomatoes, cheese and milk (Nelson et al. 2000). The daily intake is estimated to be between 50 and 200mg/kg/day in industrialized countries, but varies based on dietary preferences (Geha et al. 2000; Nelson et al. 2000; Shimada et al. 2013). A recent study found that a single oral dose of 150mg/kg taken consecutively for five days resulted in headache and muscle tenderness when given to healthy young men (Shimada et al. 2013). Older work concluded that MSG consumption did not induce symptoms of pain or sensitivity but many of these studies have been scrutinized for their poor methodology (Tarasoff and Kelly 1993). Without definitive proof that MSG is harmful, it has been cleared in the United States and Canada as safe for human consumption and can be added to foods without regulation from Health Canada.

Consumption of MSG results in accumulation of glutamate in tissues, such as skeletal muscle and salivary glands. Orally ingested MSG undergoes first-pass metabolism and thus relatively high doses (100+ mg/kg) are used to induce large increases in systemic

glutamate levels in humans (Battezzati et al. 1995; Graham et al. 2000). Graham et al., provided single 150mg/kg oral doses of MSG to 9 healthy volunteers (8 males, 1 female), to assess the plasma and muscle concentrations of glutamate following ingestion (Graham et al. 2000). All volunteers reported transient headaches. Venous plasma glutamate levels increased by approximately 700-800%, thirty minutes after ingestion. Muscle biopsies from the vastus lateralis muscle in the thigh were taken at various time points to assess changes in glutamate concentrations. The concentrations of glutamate rose significantly by 45-75 minutes post ingestion, the mean increase was 3.56mmol/kg dry weight. This increase represents approximately 40% of the MSG administered, indicating that muscles are a major sink for orally ingested glutamate. By 105 minutes, the plasma and muscle glutamate levels had declined to baseline measurements (Graham et al. 2000). More recently, healthy male subjects given daily 150 mg/kg doses were found to have elevated salivary glutamate levels and transiently elevated blood pressure that were associated with decreased pain thresholds in response to mechanical stimulation of the masseter muscle (Shimada et al. 2013). More than 50% of subjects also reported headaches. These findings suggest that elevated tissue levels of glutamate from consumption of high dose MSG leads to increased pain sensitivity in otherwise healthy subjects.

The ingestion of MSG could induce a headache by multiple mechanisms. Because glutamate does not cross the blood-brain-barrier, ingestion of MSG likely induces headache via a peripheral mechanism (Gasparini and Griffiths 2013). Proposed mechanisms include vasodilation of dural blood vessels, and sensitization of dural

sensory and pericranial muscle afferent fibers (Baad-Hansen et al. 2010). In human studies, ingestion of MSG has induced increased blood pressure (Graham et al. 2000; Baad-Hansen et al. 2010; Shimada et al. 2013). In healthy individuals increases in arterial blood pressure do not commonly induce pain, in fact, higher resting blood pressure is associated with an increased pain threshold (Bruehl and Chung 2004). In chronic pain patients however, increases in blood pressure can alter acute pain sensitivity by lowering the individuals pain threshold (Bragdon et al. 2002; Bruehl and Chung 2004). This may play a role in MSG inducing migraine-like headache in migraine patients. In rats, intravenous or intramuscular administration of MSG or NMDA results in an increase in blood pressure which last for 5-15 minutes (Dong et al. 2006; Dong et al. 2007). Glutamate has also been found to regulate cerebral blood flow in animals (Meng et al. 1995; Yang et al. 1998). Dural vasodilation in rats induced by harmaline, a CNS stimulant, is attenuated by the AMPA receptor antagonist, NBQX (Yang et al. 1998). Topical administration of glutamate onto the exposed dura in pigs induced vasodilation which was attenuated by the NMDA receptor antagonist, MK-801 when co-applied topically or administered systemically (Meng et al. 1995). Experiments in this thesis demonstrated that 50mg/kg MSG induced a 24.5 ± 4.4 % and 20.6 ± 2.8 % increase in dural flux in male and female rats, respectively. Flux is proportional to the product of the concentration of blood cells and the velocity at which they are moving past the laser emitted from the laser Doppler flowmeter (Tonnesen et al. 2005). Flux is thus not an absolute measure of blood perfusion but allows for the measurement of changes in blood perfusion (Tonnesen et al. 2005). The MSG induced increase in dural flux correlate

with vasodilation of the MMA, which was beneath the laser Doppler probe. The arterial blood pressure also increased following MSG administration. Taken together, these findings support the idea that ingestion of MSG induces headaches by vasodilating dural blood vessels.

MSG appears to induce dural vasodilation indirectly. Glutamate-induced dural vasodilation in pigs was blocked by L-NNA (*N*^G-nitro-L-arginine), a potent nitric oxide synthetase inhibitor (Meng et al. 1995). This suggests that the glutamate induced dural vasodilation is mediated by NMDA-dependant increase of nitric oxide (NO), which is a potent vasodilator (Garthwaite 1991; Meng et al. 1995). NO induces headache in healthy individuals and a migraine-like headache in migraineurs (Thomsen et al. 1994; Van Gelderen and Saxena 1996). It is also commonly used as a model of migraine in animal studies (Akerman et al. 2013). NO's effects however may mirror the downstream effects of MSG administration and subsequent glutamate receptor activation. Nitric oxide is not the only vasodilatory substance mediated by glutamate receptor activation; glutamate can also activate sensory fibres causing the release of CGRP which when released by dural afferent fibers induces vasodilation (Messlinger et al. 1995; Gazerani, Dong, et al. 2010). Thus, increased blood glutamate concentrations promote cerebral vasodilation through the release of several vasodilators, which include NO and CGRP.

Another potential source of MSG induced headache is that elevated blood glutamate concentration may also directly modify the response properties of peripheral afferent

fibres which innervate pericranial muscles and the dura. Skeletal muscles, which take up more than a third of orally ingested MSG in people, are often tender following MSG ingestion (Graham et al. 2000; Shimada et al. 2013). The mechanical pain thresholds of the masseter muscle also decrease following chronic (5 day) MSG intake (Shimada et al. 2013). Systemic administration of 50mg/kg MSG in rats resulted in a 2- to 3- fold increase in interstitial glutamate concentration in the masseter muscle (Cairns et al. 2007). It also induced neuronal discharge and an approximately 25% decrease in the mechanical threshold of masseter muscle afferent fibres. Pre-treatment with NMDA receptor antagonist, ketamine, prevented MSG's ability to induce masseter afferent sensitization (Cairns et al. 2007). In the present study, MSG induced an increase in the discharge of SpVc neurons in 5/6 male and 5/6 female rats. It also induced a decrease in the mechanical threshold of SpVc neurons with dural receptive fields. The neurons recorded also often had receptive fields in the temporalis muscle and skin and thus, the peripheral input was not solely originating from the dura. These findings suggest that MSG acting at sites other than the dura could enhance the discharge recorded from central SpVc neurons. At the level of the dura, activation of trigeminal afferent fibres could result from the surge in circulating glutamate following MSG ingestion. Conversely, mechanoreceptors which innervate dural blood vessels could become activated following distention of the blood vessels as MSG induces dural vasodilation (Levy and Burstein 2011). Trigeminal afferent innervation of pericranial muscles and the dura converge at the spinal trigeminal nucleus (Davis and Dostrovsky 1986; Ellrich et al. 1999; Schepelmann et al. 1999). The convergence provides an explanation for the

muscle tenderness and facial allodynia that often accompanies migraine headache (Burstein et al. 2015).

The effects of systemic MSG on dural vasodilation, systemic blood pressure, and neuronal discharge in these experiments were relatively brief compared to the effects of MSG on healthy subjects who ingested MSG. In my study, dural flux and systemic blood pressure had returned to baseline by a mean of 170s. The neuronal discharge evoked by MSG was substantial and discrete in some cells while tonic in others. The experimental design, which included mechanical threshold determinations, precluded its assessment beyond a 5-minute post injection recording period. In my experiments MSG was administered intravenously, however, because orally administered MSG is slowly absorbed from the gastrointestinal tract into the circulation, there is a more prolonged elevation in glutamate blood concentration. In healthy human subjects, glutamate concentration in plasma and muscle remains elevated for more than 90 minutes (Graham et al. 2000). The longer exposure to elevated concentrations of glutamate may explain the increased reports of craniofacial pain and sensitivity, which include headache and masticatory muscle tenderness.

Prolonged elevations of blood and tissue glutamate concentrations may explain the association between MSG and migraine headache. Extracellular glutamate concentrations are tightly regulated in the CNS predominantly by EAAT2 (Danbolt 2001; Zhou and Danbolt 2013), but in the periphery, however, less is known about glutamate

uptake. In my study, EAAT2 expression was found in the dural blood vessel walls, which suggests that this EAAT subtype is critically important for the clearance of glutamate from the blood. Plasma levels of glutamate vary in individuals but are elevated in migraineurs (Cananzi et al. 1995). A proportion of migraine sufferers have a mutation which results in the downregulation of the gene that encodes EAAT2, which could contribute to inhibited clearance of glutamate (Schürks 2012; Gasparini and Griffiths 2013). Thus, it is possible that the sensitivity of certain migraineurs to MSG results from their inability to clear glutamate from the blood, although future research is required to establish whether this is indeed the reason for elevated blood glutamate concentrations in migraine headache patients.

In the current study, the effects of MSG on SpVc neurons were found to be mediated through peripheral NMDA receptors. APV; a competitive NMDA receptor antagonist, inhibited MSG's ability to activate and sensitize SpVc neurons with dural input. Moreover, it dose-dependently increased the mechanical threshold at the dural receptive field over the baseline threshold. APV was chosen because it poorly crosses the blood brain barrier and thus its effects can be attributed solely to peripheral actions (Lodge et al. 1988; Whitten et al. 1990). The results implicate peripheral NMDA receptors as a major mediator of MSG induced excitation of SpVc neurons with dural receptive fields. Glutamate receptor antagonists have been tested in clinical migraine trials as acute and prophylactic agents and provided promising results (Chan and MaassenVanDenBrink 2014). The AMPA/Kainate receptor antagonist tezampanel was

superior to placebo in reducing headache intensity at 2 hours and also producing a pain-free state at the same time point (Sang et al. 2004; Chan and MaassenVanDenBrink 2014). A negative allosteric modulator of mGlu5, ADX10059, was also superior to placebo at producing a pain-free state 2 hours after treatment (Goadsby and Keywood 2009; Chan and MaassenVanDenBrink 2014). Both drugs had adverse CNS side effects which would hinder their acceptability as abortive treatments for migraine (Chan and MaassenVanDenBrink 2014). Memantine, a NMDA receptor antagonist has also been tested as an prophylactic migraine treatment (Bigal et al. 2008). It significantly reduced the number of headache days during a 3-month period and the disability assessment scores of study participants (Bigal et al. 2008). Adverse CNS side effects have limited the use of all of glutamate receptor antagonists as prophylactics against migraine (Bigal et al. 2008; Huang et al. 2014). The limitation of using these compounds is that they cross the blood-brain-barrier, thus investigating whether peripheral glutamate receptor antagonists may also be effective in attenuating migraine pain, is a logical alternative.

2.4.2. Immunohistochemistry of the Dura

Immunohistochemical analysis of the dura revealed the expression of the NMDA, AMPA, kainate, and mGlu5 receptors on densely innervated dural blood vessels. The expression of CGRP, substance P and their receptors as well as 5HT1b and 1d receptors in the dura have been assessed previously (Edvinsson et al. 1988; Harriott and Gold 2008; Lennerz et al. 2008; Eftekhari et al. 2010). In these studies, neither a dye to trace dural fibres nor a marker of blood vessels have been used to differentiate nerve fibres from small blood vessels (Edvinsson et al. 1988; Harriott and Gold 2008; Lennerz et al.

2008; Eftekhari et al. 2010). In contrast, blood vessels in my study were identified in dural tissues using an antibody against von Willebrand factor (vWF). CGRP co-expressed with each of the glutamate receptors on dural blood vessels. The findings also revealed that EAAT2 but not EAAT1 or 3 were expressed in the dura. In the CNS, EAAT2 is expressed in astrocytes (Danbolt 2001; Zhou and Danbolt 2013), thus it is possible that EAAT2 is being expressed on glial cells near the surface of blood vessels in the dura. Inhibited clearance of glutamate by EAAT2 in the dura could affect sensory transmission by afferent fibres in migraine patients (Laurson et al. 2014). Laurson et al., analyzed the effect of inhibiting EAAT1/2 in the TG on the excitability of temporalis or masseter muscle afferents in rats (Laurson et al. 2014). Co-administration of EAAT1/2 inhibitor TFB-TBOA ((3S)-3-[[3-[[4-(trifluoromethyl)benzoyl]amino]phenyl]methoxy]-l-aspartic acid) and glutamate into the TG potentiated the effects of glutamate alone at inducing neuronal discharge (Laurson et al. 2014). These data indicate that elevated blood concentrations of glutamate could act on both ionotropic and metabotropic receptors expressed by dural vascular tissues.

2.4.3. Sex-related Differences in Response to MSG

Men and women appear to respond to both oral and intravenous MSG differently. Early experiments aimed at understanding mechanisms of MSG symptoms complex found that women were more responsive than men to oral MSG and that men needed a higher dose to provoke a negative effect of MSG (Ambos et al. 1968; Tarasoff and Kelly 1993). When injected subcutaneously into the forehead or intramuscularly into the masseter muscle, glutamate also evoked a higher pain rating score in women than men (Cairns et

al. 2001; Svensson et al. 2003; Gazerani et al. 2006). In electrophysiological experiments in rats, the neuronal response to glutamate also differs by sex. Intramuscular injections of glutamate into the masseter muscle provoked a larger increase in discharge of trigeminal afferent fibers in female than in male rats (Cairns et al. 2001). Unfortunately, in many animal models used to assess mechanisms of pain, female rats are not used. This is especially disappointing in studies aimed at understanding migraine with its sexually dimorphic prevalence. In this study both male and female rats were used, but I did not find a sex related difference in their responses to MSG or APV. An estrogen mediated upregulation of peripheral NMDA receptor expression has been used to explain sex-related differences in glutamate-evoked afferent discharge in previous experiments (Dong et al. 2007). In the present study, there was no association between serum estrogen levels and baseline mechanical threshold for dural activation of SpVc neurons. However, my study may have been underpowered to resolve sex-related differences, previous studies that found a sex-related difference in responses to glutamate used larger numbers of animals (Cairns et al. 2001; Cairns et al. 2003).

2.4.4. Animal Models of Migraine

The present study investigated the effect of MSG and a peripherally restricted NMDA receptor antagonist on mechanisms which are proposed to be involved in the pathophysiology of migraine. Examining dural blood flow and neuronal responses of the trigeminovascular system are commonly used to assess potential migraine treatments (Jansen-Olesen et al. 2013; Romero-Reyes and Akerman 2014). Another commonly used animal model is to induce CSD using either KCl or electrical stimulation of the dura

(Jansen-Olesen et al. 2013; Romero-Reyes and Akerman 2014). All of these models have pearls and pitfalls and will likely evolve as the pathophysiology of migraine becomes elucidated. While the importance of a vascular component of migraine is still unclear, assessing a drug's effect on dural vasculature predicts its efficacy in treating migraine headache pain (Williamson et al. 1997; Petersen et al. 2004; Levy and Burstein 2011; Shepherd et al.). Some commonly prescribed migraine drugs, such as triptans, and other drugs, which have failed for reasons other than efficacy, such as CGRP antagonists, have a vasoconstricting effect on dural blood vessels (Williamson et al. 1997; Petersen et al. 2004; Shepherd et al.). A potential explanation of MSG's ability to trigger a headache or migraine headache in those who are susceptible is via a vasodilatory mechanism. The use of animal models studying dural vasodilation for migraine have been discredited in recent years but will likely continue to be used until definitive evidence exists as to whether dural vasodilation is key to migraine pain (Jansen-Olesen et al. 2013; Romero-Reyes and Akerman 2014).

Laser Doppler flowmetry and intravital microscopy are commonly used to measure indirect changes in dural blood flow. Both methods have limitations, largely due to the experimental conditions needed for their use (Akerman et al. 2013). Several measures were taken to overcome the limitations of laser Doppler flowmetry in this thesis. In the laser Doppler flowmetry experiments, the bone over the middle meningeal artery was thinned (closed window) rather than removed entirely to allow for a more physiologically accurate measurement of dural blood flow (Akerman et al. 2013).

Following this protocol, the animal was allowed to stabilize after the skull was thinned

before being the experimental protocol. The reason behind this is that the drilling of the skull could activate dural afferent fibres and induce vasodilation in itself (Akerman et al. 2013). The laser Doppler was also calibrated with each use to ensure accuracy of flux measurements. In some experiments the dural flux was measured during and after administration of pentobarbital to measure the biological zero flux (the flux once the animal has died), this is also used as an indicator of accuracy (Rajan et al. 2009). Appropriate responses to these tests, and a consistent experimental setup and procedure validate the use of laser Doppler flowmetry to measure changes in blood flow.

The activation of the trigeminal afferent system is perhaps the most agreed upon mechanism in migraine pathophysiology as is thus studied extensively.

Electrophysiological studies often are used to examine neuronal excitability in the SpVc or the TG in response to CSD, or electrical or chemical stimulation of the dura (Romero-Reyes and Akerman 2014). CSD has a pathophysiological connection to migraine with aura. The phenomenon has been measured during migraine attacks which are preceded by aura (Hadjikhani et al. 2001). Electrical or chemical stimulation, often with inflammatory soup, are not physiologically translatable but induce reproducible activation of the trigeminal afferent system and increases in dural blood flow (Romero-Reyes and Akerman 2014). These models are based upon seminal studies that discovered that electrical, mechanical, and chemical stimulation of the dura in awake patients induced unilateral pain similar to that of migraine headaches (Ray and Wolff

1940; Wolff et al. 1953; Romero-Reyes and Akerman 2014). A drug's ability to modulate the activity of trigeminovascular neurons either peripherally or centrally is often a good indicator of its usefulness in treating headache pain (Goadsby and Hoskin 1996; Hoskin et al. 1996).

In vivo electrophysiology, while quite useful in understanding neuronal responses in real-time, is not without its limitations. In the in-vivo electrophysiology experiments in this thesis, both the dura and brainstem were exposed, this invasive surgery will modulate the activity of sensory neurons. Recent studies have also suggested that dural afferent fibres may have collateral branches that travel through cranial sutures to innervate the periosteum (Zhao and Levy 2014). If this proves to be correct, the removal of the periosteum, which is necessary to access the dura, may inadvertently damage the dural afferent fibres as well.

2.4.5. Potentially Confounding Factors and Limitations

Isoflurane was used to anaesthetise the animals for Doppler flowmetry and electrophysiology. Isoflurane induces unconsciousness, amnesia, immobility, and analgesia; the exact mechanisms of its actions are unknown. Molecular targets of isoflurane include the NMDA and AMPA receptor, as well as sodium and calcium channels, and the GABA_A (gamma-aminobutyric acid subtype A) receptor (Rudolph and Antkowiak 2004). The effect of isoflurane on these targets could confound the effects of MSG due to altered sensory transmission both peripherally and centrally (Puil and

Gimbarzevsky 1987; Savola et al. 1991). Isoflurane also modulates cerebral blood flow differently depending on its mean arterial concentration (MAC)(Maekawa et al. 1986; Lemkiul et al. 2013). Its effects on cerebral metabolism and smooth muscle to induce vasodilation result in cerebral vasoconstriction at low doses (<0.5 MAC) and cerebral vasodilation at high doses (1+ MAC)(Maekawa et al. 1986; Lemkiul et al. 2013). The MAC of isoflurane was not measured in these experiments but was maintained within a range based on the arterial blood pressure and heart rate of the animal. Because of the confounding effects of anaesthesia on blood flow, it is not recommended to compare laser Doppler measurements taken under different anaesthetic agents (Akerman et al. 2013).

Potentially confounding variables were addressed in the histological assay. The lack of expression of GluRs and CGRP on PGP 9.5 identified axons, apart from those innervating dural blood vessels, was a surprising result. Previous studies have identified 5HT receptors along axons of dural afferent fibres (Harriott and Gold 2008), but to my knowledge this is the first time the expression of GluRs has been assessed at the level of the dura. A potential explanation of the results is that GluRs are localized around the axonal endings because of the important role they play in synaptic transmission. CGRP expression was identified on dural blood vessels but also not on axons distal to blood vessels. Eftekhari et al., suggest that CGRP is expressed in smaller unmyelinated C-fibres in the dura and not other types of nerve fibres (Eftekhari et al. 2013). An alternative explanation of my results is that the axons that I identified were not of the C-fibre population because they are inherently harder to resolve in confocal images. The use of

an axonal tracer such as Fast Blue applied to the dura may have made nerve fibres easier to identify and resulted in a different outcome. To ensure the specificity of secondary antibodies used in immunohistochemistry, the primary antibodies were omitted to ensure non-specific labelling. Slices of TG, brainstem, and brain were also stained with antibodies against each GluR, CGRP as well as EAAT1-3 to ensure the epitope of interest was identified in areas where it has previously identified. Controls, both negative and positive, resulted in the appropriate outcome (i.e. lack of staining when primary antibodies were omitted, and expression of each antibody when assessed in either the TG, brainstem or brain) which validated the findings.

The result from experiments in saline treated animals suggests that a volume effect may have contributed to the observed mechanical sensitization of SpVc neurons with dural input. Mechanically sensitive trigeminal afferent fibres densely innervate dural blood vessels, so it is possible that distention may have contributed to mechanical sensitization. However, when the dural flux was measured following the administration of saline, it induced a decrease in the flux of the MMA, even though the volume of saline administered in the laser Doppler experiments was larger than that in the electrophysiology experiments. Further, saline, unlike MSG, did not affect the ongoing discharge of the SpVc neurons. It is, therefore, possible that repeated mechanical activation of the dura with the electronic von Frey hair was responsible for the sensitization. Interestingly, co-administration of APV with MSG not only reversed MSG-induced mechanical sensitization but actually appeared to increase the mechanical activation threshold of SpVc neurons with dural receptive fields. This could be

interpreted to indicate that mechanical sensitization is dependent on the local release of glutamate, perhaps from the terminal endings of dural afferent fibres, as has been found for the temporalis muscle (Gazerani et al. 2010).

Chapter 3: Conclusion

3.1. Conclusion

It has been proposed that migraine sufferers have a hyper-excitabile brain (Burstein et al. 2015), which could explain how ingestion of MSG could trigger a migraine-like headache. This theory postulates that migraine is a disorder of thresholds, meaning that in migraineurs the level of activation along 'headache pain pathways' necessary to trigger an attack is lower than in healthy subjects (Romero-Reyes and Akerman 2014). Theoretically, these people have sensory systems that are already primed so that MSG could initiate a migraine-like headache rather than the low-grade headache seen in their healthy counterparts. The results of this study provide evidence that MSG induces excitation of trigeminovascular neurons and dural vasodilation. It also suggests that this effect (vasodilation) is mediated by the peripheral NMDA receptor which is expressed in the dural blood vessels. The MSG induced neuronal excitability can be attenuated with the use of a peripherally restricted glutamate receptor antagonist. A peripherally acting glutamate receptor antagonist would likely be useful as a treatment for migraine headache based on the results from this study as well as from promising pre-clinical studies (Chan and MaassenVanDenBrink 2014). The use of a peripherally restricted NMDA antagonist, such as APV, could potentially help to elucidate the pathophysiology of migraine. If it was effective as an abortive treatment, it would suggest that migraine headache is generated in the peripheral nervous system rather than the central. As with other abortive migraine drugs its use would likely be more effective if taken prior to the

onset of central sensitization as identified by the patient by allodynia. Central sensitization which is thought to occur approximately 30-60 minutes after the initial onset of the headache is thought to play a role in the efficacy of most abortive pharmacotherapy (Burstein et al. 2015). If unsuccessful as an abortive therapy, a peripheral NMDA receptor antagonist may also be useful as a prophylactic agent. Migraine sufferers have higher levels of glutamate in their plasma, having an NMDA receptor antagonist on board regularly may inhibit the 'priming' of the peripheral trigeminovascular system which could occur due to the abnormal levels of glutamate. This prophylactic may be especially effective in migraine patients who have altered glutamate homeostasis due to genetic abnormalities.

3.2. Future Studies

Many questions remain unanswered when it comes to migraine, these include curiosities into the pathogenesis of migraine and also what drugs would be most effective in a preventing or alleviating migraine pain. Future work based upon the results of this thesis could include testing the effect of chronic administration of APV on the expression of GluRs in the peripheral and central trigeminovascular system. This may infer whether an APV-like drug could be useful as a prophylactic. APV should also be used in electrophysiological experiments using animal models of migraine such as CSD or electrical stimulation of the dura. Finally, it would be interesting to use APV in animals which have a genetic mutation which would downregulate EAAT2 expression. Genetic models are quite expensive so alternatively an antibody against EAAT2 or a drug

(ex. dihydrokainic acid, TFB-TBOA) could be used to transiently decrease EAAT2 expression or activity, respectively. Positive results from these studies would support the use of an APV-like drug in a clinical study.

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Appendices

Appendix A

Immunohistochemistry Controls

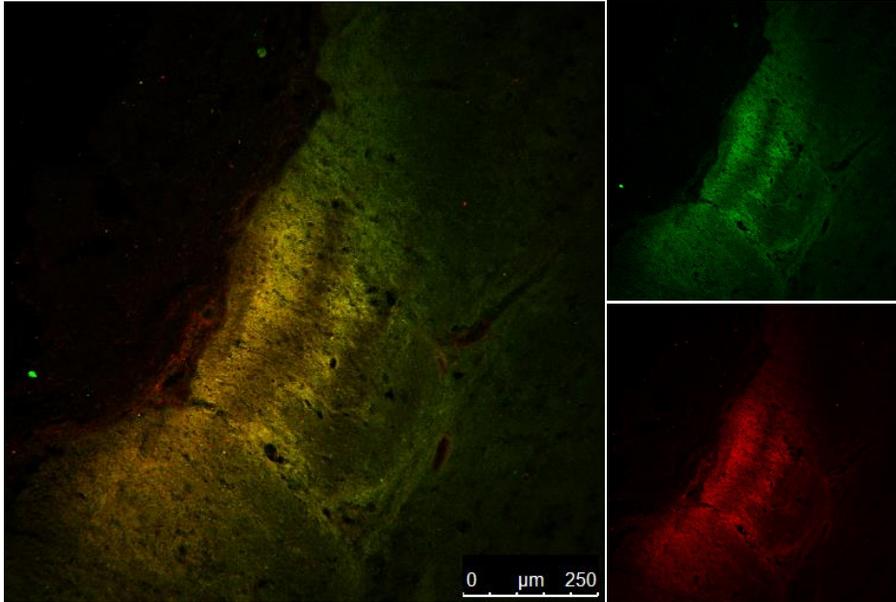


Figure A1: Photomicrographs show the expression of excitatory amino acid transporter 1 (EAAT1) in the brainstem. EAAT1 expression is identified by the green label and astrocytes labelled with glutamine synthetase (GS) is identified by the red label.

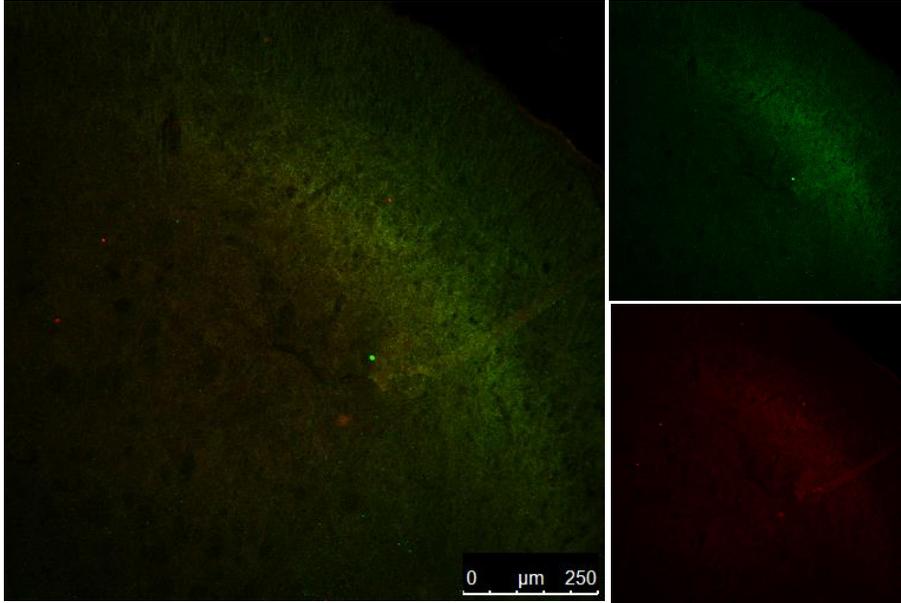


Figure A2: Photomicrographs show the expression of excitatory amino acid transporter 2 (EAAT2) in the brainstem. EAAT2 expression is identified by the green label and astrocytes labelled with glutamine synthetase (GS) is identified by the red label.

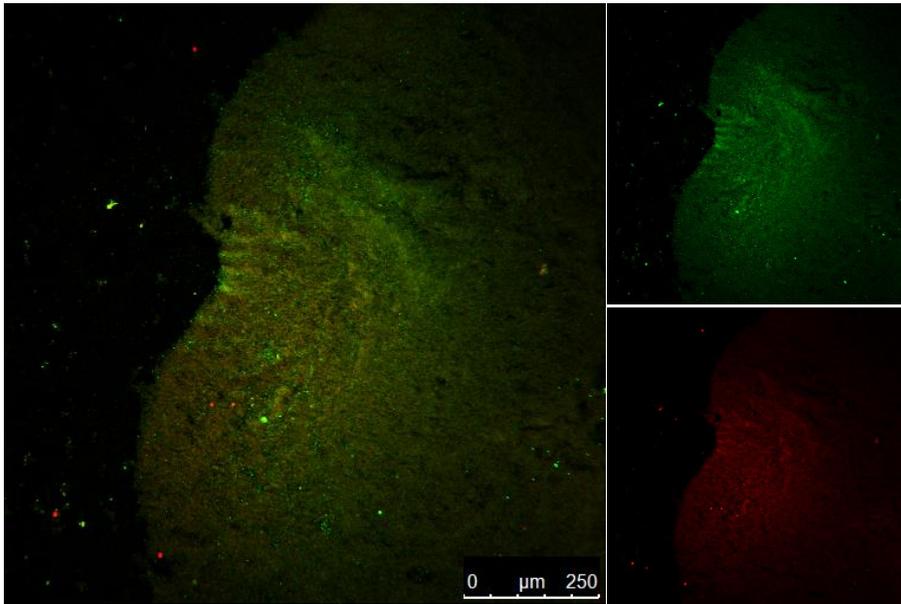


Figure A3: Photomicrographs show the expression of excitatory amino acid transporter 3 (EAAT3) in the brainstem. EAAT3 expression is identified by the green label and astrocytes labelled with glutamine synthetase (GS) is identified by the red label.

Appendix B

The following images depict the response of individual caudalis neurons with dural input following the administration of 50mg/kg MSG either alone or co-administered with APV (5 or 50mg/kg). The neuronal discharge was measured for a period of five minutes immediately before and after administration of drugs, cumulated and plotted.

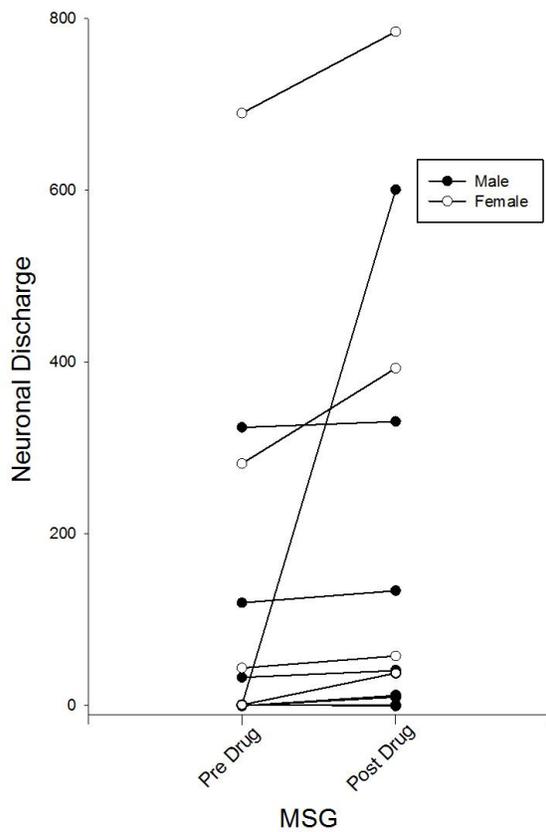


Figure B1: MSG induced changes in neuronal discharge of caudalis neurons. The total number of action potentials recorded before and after drug administration are plotted for animals that received MSG alone (N=6 per sex).

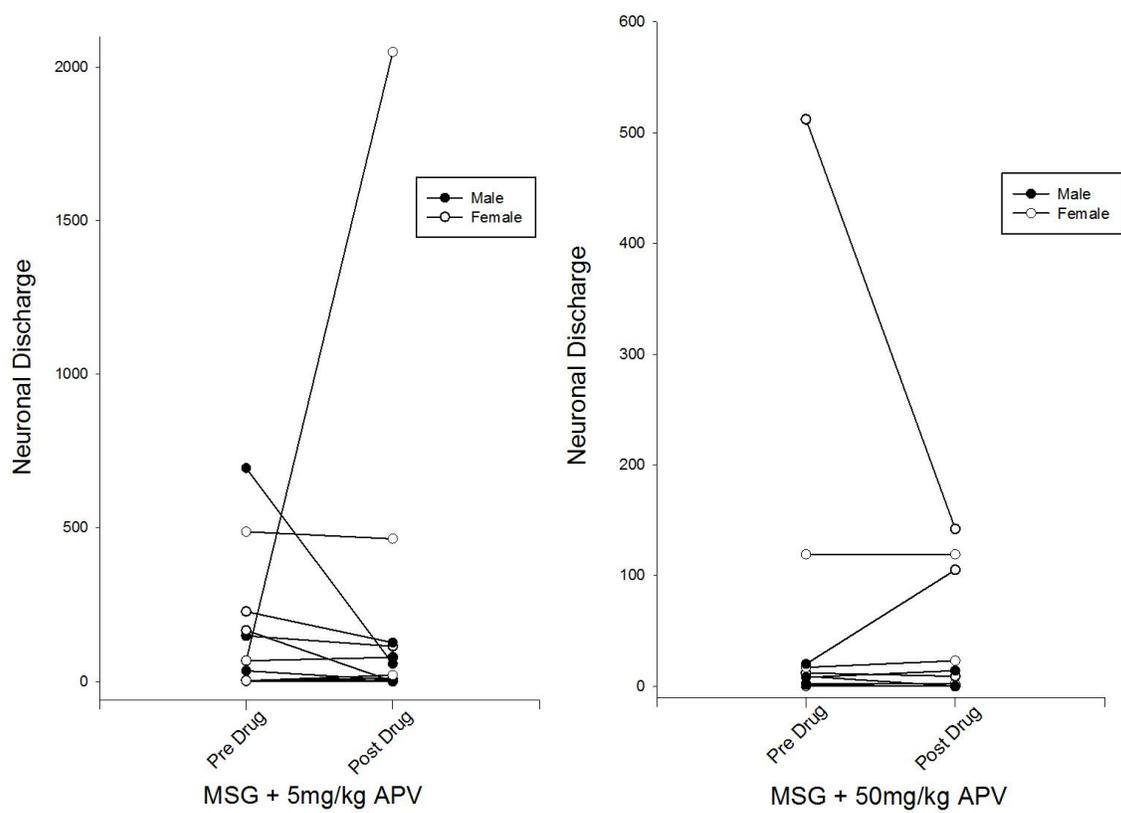


Figure B2: Co-administration of MSG and APV induced decreases in neuronal discharges of caudalis neurons. The total number of action potentials recorded before and after drug administration are plotted for animals that received MSG + 5mg/kg APV (left) or MSG + 50mg/kg APV (right). N=6 per sex for each treatment.