Decreased Face Primary Motor Cortex (Face-M1) Excitability Induced by Noxious Stimulation of the Molar Tooth Pulp is Dependent on the Functional Integrity of Face-M1 Astrocytes

By

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Abstract

Acute inflammatory dental pain is a prevalent orofacial pain condition that is often associated with altered orofacial sensorimotor functions. The underlying motor cortex mechanisms are unclear. The aims were to examine whether application of the inflammatory irritant mustard oil (MO) to the rat maxillary molar tooth pulp affects face-M1 excitability manifested as a change in intracortical microstimulation threshold intensity for evoking jaw-opening muscle electromyographic activity, and if subsequent application of the astrocytic inhibitor methionine sulfoximine (MSO) to the face-M1 can modulate the MO-induced effects. Compared with control, pulpal MO application induced significantly decreased face-M1 excitability. Subsequent MSO application to the face-M1 attenuated the MO-induced effects. These novel findings suggest that acute noxious stimulation of the dental pulp is associated with decreased face-M1 excitability that is, at least in part, dependent on the functional integrity of face-M1 astrocytes and may be related to mechanisms underlying limited jaw movements in acute pain conditions.
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<td>Adenosine 1</td>
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<td>Acotinase</td>
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<td>AG</td>
<td>Agranular</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>AP</td>
<td>Anteroposterior</td>
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<td>Adenosine triphosphate</td>
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<td>B2</td>
<td>Bradykinin receptor 2</td>
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<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<td>Calcitonin gene-related peptide</td>
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<td>Diffuse noxious inhibitory control</td>
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<td>FA</td>
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<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
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<td>G</td>
<td>Granular</td>
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<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric acid</td>
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<td>Glial fibrillary acidic protein</td>
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<td>Gu</td>
<td>Gustatory</td>
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<td>IASP</td>
<td>International Association for the Study of Pain</td>
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<tr>
<td>IBA1</td>
<td>Ionized calcium-binding adapter molecule 1</td>
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<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
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<tr>
<td>ICMS</td>
<td>Intracortical microstimulation</td>
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<tr>
<td>IM</td>
<td>Intramuscular</td>
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<td>IP</td>
<td>Intraperitoneal</td>
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<td>Intravenous</td>
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<td>JNK</td>
<td>c-jun-N-terminal kinase</td>
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<td>KA</td>
<td>Kainate</td>
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<td>LAD</td>
<td>Left anterior digastric</td>
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<td>LTP</td>
<td>Long term potentiation</td>
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<td>M1</td>
<td>Primary motor cortex</td>
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<td>MDH</td>
<td>Medullary dorsal horn</td>
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<td>MeAIB</td>
<td>Methylamino-isobutyric acid</td>
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<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
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<td>ML</td>
<td>Mediolateral</td>
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<td>MO</td>
<td>Mustard oil</td>
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<td>NK1</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>OEG</td>
<td>Olfactory ensheathing glia</td>
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<td>P2X</td>
<td>Purinoceptor subtype 2X</td>
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<td>p38</td>
<td>MAPK P38 mitogen-activated protein kinases</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<td>PIP2</td>
<td>Phosphatidylinositol bisphosphate</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>RAD</td>
<td>Right anterior digastric</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S1</td>
<td>Primary somatosensory cortex</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>TBOA</td>
<td>Threo-beta-benzylxoyaspartate</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
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<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMS</td>
<td>Transcranial magnetic stimulation</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TrkA</td>
<td>Tyrosine receptor kinase type 1</td>
</tr>
<tr>
<td>TRPA1</td>
<td>Transient receptor potential ankyrin 1</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid 1</td>
</tr>
<tr>
<td>VBSNC</td>
<td>Trigeminal brainstem somatosensory complex</td>
</tr>
<tr>
<td>VPM</td>
<td>Ventroposteriormedial nucleus</td>
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Chapter 1

Introduction

Dental pain is one of the most prevalent orofacial pain conditions, and it is a major reason why patients seek dental care. As noted in subsequent sections, it is well known that motor functions may be affected by dental pain. Clinical or experimentally-induced orofacial pain (including dental pain) results in smaller and slower movements as well as an inhibition in agonist muscle activity. The primary motor cortex representing the orofacial region (face-M1) plays an important role in the generation and control of orofacial motor functions (e.g., jaw opening, tongue protrusion, mastication). The face-M1 also receives somatosensory inputs from the orofacial region (including the teeth) either directly through the thalamus or indirectly through the face primary somatosensory cortex (face-S1) that provide somatosensory feedback and feedforward information that is crucial for modulating orofacial motor functions. It has also been shown that changes in sensory inputs (including nociceptive inputs) or motor outputs can induce neuroplastic changes within the face-M1 that are crucial to how humans and animals adapt (or not) to an altered oral state. Furthermore, emerging evidence reviewed below, suggests that non-neural cells (e.g., astrocytes) may be involved in the mechanisms underlying neuroplasticity in the trigeminal nociceptive afferent pathways. However, it is unclear whether acute dental pain is also associated with neuroplastic changes in face-M1 function and, if such changes do occur, whether astrocytes are involved. Accordingly, the objectives of the present thesis study were to use an established rat model of acute dental pain and utilise electrophysiological and pharmacological approaches to test whether experimental acute noxious stimulation of the rat maxillary molar tooth pulp is associated with neuroplastic changes within the face-M1 manifested as changes in face-M1 excitability, and whether these changes depend on the functional integrity of face-M1 astrocytes. This literature review will first discuss pain including acute dental pain and an acceptable model of acute inflammatory pulpal pain. Then it will outline the inputs relayed by peripheral and central somatosensory pathways to the face-S1 and face-M1, as well as the neural and glial processes that contribute to peripheral and central sensitization. This will be followed by how inputs to the face-S1 can modulate motor outputs of the face-M1 as well as the neuroplasticity of the face-M1 and possible underlying mechanisms. Since the rat is the animal used in this thesis project, emphasis will be placed on the rat face-M1 and face-S1.
Finally, since face-M1 excitability can be studied by examining movements evoked by intracortical microstimulation (ICMS) of the face-M1, the ICMS technique used in this study will be described.

1. Acute Dental Pain

The International Association for the Study of Pain (IASP) defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Pain can be classified based on its cause or duration. Pain lasting for hours or days is termed acute pain, while pain lasting over three months and beyond the time of healing is generally considered to be chronic pain. Pain caused by a lesion or disease of the somatosensory nervous system is known as neuropathic pain, and pain from inflammation due to injury of peripheral tissues is known as inflammatory pain; pain can also be associated with diseases such as cancer (Katz, 1998; Merskey and Bogduck, 1994; Renn and Dorsey, 2005; Wall and Melzack, 2013).

1.1 Epidemiology of Acute Dental Pain

Dental pain is one of the most prevalent orofacial pain conditions and it is a major reason why patients seek dental care (Jain et al. 2013; Lipton et al. 1993; Sessle 2005; Wall and Melzack, 2013). In the United States, the prevalence of dental pain in the general population has been reported to be around 12% (Lipton et al., 1993). Dental pain can be severe, leading to disruption of daily activities including missed work, sleep disruption, difficulty eating, weight loss, and mood alterations and thereby affecting quality of life of the patients (Acs et al., 1999; Heavilin et al., 2011; Locker and Grushka, 1987; Reisine, 1988). In addition, acute dental pain in some patients can develop into chronic pain. For example, development of acute dental pain into chronic pain occurs in 3-6% of the patients after dental root canal treatment, in 0-3% after tooth extraction, and in 13% after dental implant surgery (Abarca et al., 2006; Dao and Mellor, 1998; Gregg, 2000; Marbach and Raphael, 2000; Nixdorf et al., 2010).

1.2 Etiology and Clinical Manifestations of Acute Dental Pain

Acute dental pain can be of non-odontogenic origin (e.g., angina pectoris, sinusitis) but is mainly of odontogenic origin. The most common odontogenic cause of acute dental pain is pulpitis (pulpal inflammation) which can be produced by caries, trauma, or tooth fracture, as well as
mechanical, thermal, or chemical stimuli. Lesions arising from caries first penetrate the dentine to induce dentinal pain, causing increased sensitivity to thermal stimuli and sweet substances (Cummins 2009; Napenas 2013; Wall and Melzack, 2013). Once the carious lesion penetrates deeper into the tooth and reaches the tooth pulp, pulpal inflammation occurs, which may result in increased sensitivity to mechanical and thermal stimuli such as chewing, touch and temperature (Cummins 2009; Napenas 2013; Wall and Melzack, 2013). This increased sensitivity can be manifested as alldynia (pain produced by a stimulus that is usually non-noxious), hyperalgesia (increased pain in response to a noxious stimulus), spontaneous and referred pain as well as pain spread (Chiang et al., 2012; Sessle, 2006, 2011). Furthermore, motor dysfunction is one of the most common clinical signs of acute (and chronic) pain conditions, and in the case of orofacial pain (including dental pain) is often manifested as limited jaw movement (Huge et al., 2011; Sessle, 2000).

2. Dental Pulp Innervation, Major Sensorimotor Pathways and Processes of Acute Dental Pain

2.1 Peripheral Nociceptive Pathways and Processes

2.1.1 Dental pulp innervation

The dental pulp is highly innervated, predominantly by fast-conducting, myelinated Aδ and Aβ primary afferent nerve fibres and slow-conducting, unmyelinated primary afferent C-fibres (Bueltman et al., 1972; Fried and Hildebrand, 1981a, 1981b). In addition, there are efferent fibres which are predominantly sympathetic efferent fibres that regulate blood flow and can also influence the excitability of the afferent fibres (Byers, 1984; Byers and Narhi, 1999). The A-fibres terminate mainly at the pulp-dentin border in the coronal portion of the pulp and are more concentrated in the pulp horns. The C-fibres terminate mainly in the core of the pulp and can extend into the cell-free zone under the odontoblastic layer (Bender, 2000; Byers and Dong, 1983). In the coronal dentin, both A- and C-fibres branch extensively and form the subodontoblastic plexus of Raschkow. Some of the nerve fibres ramify and end around the odontoblastic layer, while other nerve fibres enter the dentin tubules along the odontoblastic processes (Bergenholtz et al., 2003; Nancy and Ten Cate, 2003). The axons of the A- and C-fibres follow the distribution of the blood supply within the pulp and exit the pulp through the
apical foramen. Primary afferent fibres from the maxillary teeth project through alveolar branches to form the maxillary branch of the fifth (V) cranial nerve (i.e. the trigeminal nerve) while afferent fibres from the mandibular teeth project through alveolar branches to the mandibular branch of the trigeminal nerve. These somatosensory primary afferent fibres have their cell bodies primarily in the trigeminal ganglion and project to the trigeminal brainstem sensory nuclear complex (VBSNC).

2.1.2 Nociceptors

The small-diameter slow-conducting, unmyelinated C-fibres and the fast-conducting myelinated Aδ-fibres have free nerve endings which function as nociceptors. Each free nerve ending has membrane receptors that can be activated by one or more types of stimuli including mechanical, thermal (hot, cold) and chemical. Of particular interest to the present study is the transient receptor potential ankyrin 1 (TRPA1) channel which has been shown to be present on nociceptive primary afferent endings and involved in the nociceptive afferent responses to acute noxious stimuli and endogenous inflammatory mediators as well as various exogenous inflammatory irritants, including allyl isothiocyanate which is present in mustard oil (MO) (Bandell et al., 2004; Bautista et al., 2006; Chiang et al. 2012; Jordt et al, 2004; Sessle, 2011). Studies confirm the dense expression of TRPA1 channels in the sensory nerve endings of the dental pulp including the maxillary molar tooth pulp (Kim and Cavanagh, 2007; Nilius et al., 2007; Park et al., 2006). TRPA1 channels can be activated by a large variety of stimuli such as bradykinin (Phospholipase-C -dependent), noxious mechanical or cold stimuli, as well as by pungent painful stimuli including MO. MO is a potent agonist of TRPA1 channels and can directly activate the channels and excite the nociceptors (Bautista et al., 2006; Nilius et al, 2007) (Fig.1-1). Consequently excitation of nociceptors may result in generation of action potentials in the nociceptive primary afferent nerve fibres that conduct the signals into the central nervous system (CNS) to result in acute pain sensation (see sections below). Somatosensory primary afferents from the orofacial tissues including the dental pulp have their cell bodies primarily in the trigeminal ganglion and project to the VBSNC to synapse on second-order neurons (Dubner et al., 1978; Sessle, 2006).
Fig. 1-1 Possible mechanisms of TRPA1 channel activity associated with nociceptive processing in primary afferent endings. TRPA1 channel can be activated by different peripheral stimuli including MO and its function is coupled to the TRPV1 channel and both channels are regulated by phospholipase-C - dependent sensitization pathways. (ACh: Acetylcholine, ATP: Adenosine triphosphate, 5HT: Serotonin, NGF: Nerve growth factor, GPCR: G protein-coupled receptor, B2: Bradykinin receptor 2, TrkA: Tyrosine receptor kinase type 1, TRPA1: Transient receptor potential ankyrin 1, TRPV1: Transient receptor potential vanilloid 1, BK: Bradykinin, PIP2: Phosphatidylinositol bisphosphate, PKC: Protein kinase C, PLC: Phospholipase C) (Adapted from Bautista et al., 2006; Nilius et al., 2007).

2.1.2.1 Peripheral Sensitization

Peripheral sensitization refers to an increased excitability of nociceptive endings induced by the action of inflammatory mediators or chemicals released into the affected tissue following peripheral injury or inflammation (Coutaux et al, 2005; Dray, 1995; Lam et al, 2005; Lund and Sessle, 1994; Rang et al, 1991; Svensson and Sessle, 2004; Woolf, 2011). Damage to the dental pulp tissue (such as by dental drilling, tooth pulp exposure or application of inflammatory irritants substances such as MO) can induce the so-called neurogenic inflammation (Bautista et al., 2006) through the release of chemical mediators such as bradykinins, histamine, serotonin, prostaglandins, tumour necrosis factor α (TNFα) from mast cells, macrophages and immune cells (Hahn and Liewehr, 2007; Jontell et al., 1998; Manuja et al., 2010; Svensson and Sessle, 2004); nerve endings can also release neurotransmitters such as glutamate, and neuropeptides such as
substance-P, calcitonin gene-related peptide (CGRP) as well as neurotrophins such as nerve growth factor; and the sympathetic efferents innervating peripheral blood vessels can release noradrenaline. All of these mediators can contribute either directly or through the action of second messengers to the increased excitability of the nociceptor-endings. The increased excitability of the nociceptive endings can be associated with their spontaneous activity, lowered activation thresholds and increased responsiveness to noxious and innocuous stimuli. (Adachi et al., 2010; Coutaux et al., 2005; Dray, 1995; Lam et al, 2005; Lund and Sessle, 1994; Matsuura et al., 2013; Rang et al, 1991; Seltzer et al., 1963; Svensson and Sessle, 2004; Woolf, 2011). These features can contribute to the clinical manifestation of allodynia, hyperalgesia and spontaneous pain. As a result of the pulp tissue damage, chemical mediators may also spread and induce changes in nerve endings of neighbouring nociceptive primary afferent nerve fibres that can contribute to the clinical manifestation of pain spread (Chiang et al., 2012; Sessle, 2006, 2011).

A related and significant process is the contribution of bradykinin to peripheral sensitization. Bradykinin is a peptide produced by the kinin system in response to tissue injury or inflammation. It has been shown that MO application to the dental pulp can also induce pulpal inflammation (Chiang et al., 1998; Narhi et al., 1997). Bradykinin has dual effects on nociceptors; it can result in acute excitation as well as long-lasting hypersensitivity to mechanical and thermal stimuli (Bautista et al., 2006). It has been shown that the inflammatory hypersensitivity actions of bradykinin are mediated by TRPA1 channels. Bradykinin binds to G-protein coupled receptors on the surface of primary afferent fibres activating phospholipase-C (PLC). This leads to the hydrolysis of membrane phosphatidylinositol bisphosphate (PIP2), activation of protein kinase-C (PKC), and release of calcium from intracellular stores. Subsequently, transient receptor potential vanilloid 1 (TRPV1) is sensitized, leading to channel opening, calcium influx and activation of TRPA1 (Bautista et al., 2006; Nilius et al., 2007). Consequently, activation of TRPA1 results in generation of action potentials that propagate along nociceptive primary afferent nerve fibres into the CNS to result in acute pain (see sections below).

2.1.2.2 Role of Glial Cells

Glial cells are non-neuronal cells present in the central and peripheral nervous systems. Satellite glia are present in the sensory ganglia of the peripheral nervous system. Glial cells have been
known to provide a support function to neurons, maintaining the chemical environment around them as well as protecting them and assisting in their repair and regeneration upon injury, inflammation or infection (Hanisch and Kettenmann, 2007; Haydon and Carmignoto, 2006; Parpura and Zorec, 2010). While these cells do not fire electrical impulses, they can release several neurotransmitters and growth factors that may influence the activity of neurons or act on cells of the immune system to aid in the repair and regeneration of process of neurons (Chiang et al., 2011; Verkhratsky and Butt, 2013). Recent studies indicate that these cells also play an important role in the pathogenesis of pain, whereby glia may be involved in processes producing pain through modulation of nociceptive processing and transmission (Chiang et al., 2011; Scholz and Wolf, 2007).

It has been shown that acute noxious stimulation induced by pulp exposure, MO application to the molar tooth pulp, or molar extraction can result in activation of satellite cells within the trigeminal ganglion (Gunjigake et al., 2009; Matsuura et al., 2013; Stephenson and Byers, 1995). Similarly, acute noxious stimulation of other orofacial tissues (nitric oxide proton stimulation of the trigeminal nerve as well as capsaicin injection into the temporomandibular joint) innervated by one of the trigeminal divisions can produce intracellular changes (e.g., increased expression of protein kinase and phosphatase) in trigeminal ganglion neurons and satellite glial cells within all three divisions of the trigeminal nerve, hence possibly contributing to peripheral mechanisms that play a role in the spread of pain in the orofacial region (Freeman et al., 2008; Thalakoti et al., 2007). In addition, release of neuropeptides including substance P and CGRP from trigeminal ganglion neurons during peripheral sensitization induced by peripheral tissue injury and inflammation can activate satellite glial cells which in turn can release chemical mediators (e.g., chemokines and cytokines) that can act on trigeminal ganglion neurons influencing their excitability (Takeda et al., 2009; Vause and Durham, 2009). Moreover, gap junctions which exist between satellite glial cells and trigeminal ganglion neurons appear to play a role in spreading excitation between the satellite glial cells as well as trigeminal ganglion neurons (Garrett and Durham, 2008; Ohara et al., 2008; Thalakoti et al., 2007). These gap junctions are permeable to Ca$^{2+}$, initiating Ca$^{2+}$ waves in adjacent satellite glial cells which are associated with an increase in expression of connexin channels CX26, 36, 40, or 43 thereby causing an increase in trigeminal neuron excitability (Vit et al., 2006). These findings suggest that satellite glial cells may play an important role in modulating the activity of primary afferents in orofacial pain conditions.
2.2 Central Nociceptive Pathways and Processes

2.2.1 Central Nociceptive Pathways

Somatosensory primary afferents from the orofacial tissues with cell bodies primarily in the trigeminal ganglion, project in a somatotopic manner to the VBSNC to synapse on second-order neurons. Somatosensory primary afferents enter the brainstem and ascend or descend the trigeminal spinal tract and give off collateral branches that terminate in different subdivisions of the VBSNC: the main sensory nucleus and the spinal tract nucleus which is further divided into three subnuclei (oralis, interpolaris, and caudalis). Subnucleus caudalis extends into the cervical spinal cord where it merges with the spinal dorsal horn. Since clear functional and anatomical similarities exist between subnucleus caudalis and the spinal dorsal horn, the former has often been termed the ‘medullary dorsal horn’ (MDH). While most orofacial nociceptive primary afferent nerve fibres terminate in laminae I, II, V, and VI of the MDH, some orofacial nociceptive primary afferent nerve fibres terminate in the main sensory nucleus and subnucleus oralis as well as subnucleus interpolaris (Dallel et al., 1990; Hayashi et al., 1984; Sessle 2000, 2006). The main sensory nucleus and subnucleus oralis are involved in tactile sensation from the orofacial region (Dubner et al., 1978; Sessle, 2000; Svensson and Sessle, 2004). The VBSNC is somatotopically organized such that second-order neurons that receive mandibular afferent inputs are located in the dorsal region of each nucleus or subnucleus of the VBSNC; the ophthalmic afferents are located in the ventral region, the maxillary afferents are located in between, and oral and perioral structures are located more medially (Dubner et al., 1978; Svensson and Sessle, 2004).

Second-order neurons in the VBSNC project to other brainstem regions such as the reticular formation as well as cranial nerve motor nuclei contributing to circuitry responsible for autonomic and muscle reflex responses to orofacial stimuli. Other second-order neurons in the VBSNC project intrinsically, terminating within the VBSNC; these include neurons which project from the MDH and terminate onto nociceptive neurons in the subnucleus oralis to influence their activity (Sessle, 2006). Other second-order neurons project to higher brain centres including the thalamus, either directly or indirectly through polysynaptic pathways that involve the reticular formation (Dubner et al., 1978; Sessle, 2000; Svensson and Sessle, 2004).
Furthermore, second-order neurons project to the superior colliculus which plays a role in directing eye movements, to the periaqueductal grey which plays a role in the descending modulation of pain, and to the cerebellum which plays an important part in motor control (Dostrovsky, 2006; Sessle, 2006, 2009). In addition, there is a segmental circuitry within the VBSNC as well as descending circuitry that arises from cerebral cortical and subcortical regions (e.g., periaqueductal gray, nucleus raphe magnus, amygdala and other parts of the limbic system, lateral hypothalamus, lateral habenular nucleus, basal ganglia, anterior pre-TECTAL nucleus, red nucleus, cerebellum and the face-S1 and face-M1), and projects to the VBSNC and these circuits can also modulate VBSNC second-order neurons and nociceptive transmission (Dubner et al., 1978; Fields and Basbaum, 1984; Maixner et al., 2001; Sessle, 2000; Svensson and Sessle, 2004; Woolf, 2011). Second-order neurons which project from the VBSNC to the thalamus terminate onto neurons in the lateral thalamus including the ventrobasal complex (i.e., ventroposterior medial, VPM, nucleus), as well as the posterior nuclear group and the medial thalamus (Dubner et al., 1978; Sessle, 2000; Svensson and Sessle, 2004). The neurons in the VPM are somatotopically arranged, and many are relay neurons that project to contralateral cortical regions including the face-S1, S2, cingulate gyrus and insula. The face-S1, the main cortical receptive region involved in somatosensory processing, is also somatotopically organized, whereby each region in the face-S1 receives peripheral inputs from specific areas in the orofacial region (Dubner et al., 1978; Henry et al., 2005; Remple et al., 2003). It is important to note that the face-M1 also receives somatosensory information either directly from the thalamus or indirectly through face-S1 (Chakrabarti and Alloway, 2006; Dubner et al., 1978; Hoffer et al., 2005; Iyengar et al., 2007; Miyashita et al., 1994; Rocco-Donovan et al., 2011).

The S1 is the main cortical receptive region involved in innocuous somatosensory processing. Human pain imaging studies have reported that noxious stimulation of orofacial tissues can activate the face-S1 (and other regions within the so called ‘pain network’) as defined by functional magnetic resonance imaging (fMRI) and positron emission tomography (PET). For example, electrical stimulation of the tooth pulp results in increased fMRI-defined activation of the S1 bilaterally (Jantsch et al., 2005; Weigelt et al., 2010). Painful thermal stimulation of the facial skin overlying the masseter muscle has been associated with increased fMRI-defined activation of the contralateral S1 (de Leeuw et al., 2006), and injection of hypertonic saline into the masseter muscle has been associated with significant increases in regional cerebral blood
flow in the contralateral face-S1 (Kupers et al., 2004). These findings are consistent with other pain studies involving other body parts and showing increased activation of S1 and other regions within the pain network following different types of experimentally induced acute noxious stimuli (Apkarian et al., 2005; Davis et al., 1995; Peyron et al., 2000; Woolf, 2011).

2.2.1.1 Neuroplasticity and Central Sensitization in the Trigeminal Afferent System

Central sensitization is thought to reflect neuroplastic changes that can arise from an increased excitability of nociceptive neurons within the CNS whereby nociceptive neurons become more sensitive to sensory inputs. This can result in abnormal and increased pain responses. Increased excitability can be induced by a barrage of nociceptive inputs from the nociceptive afferents to VBSNC nociceptive neurons such as that occurs following nerve injury or inflammation, peripheral noxious stimulation or deafferentation (Sessle, 2000, 2006, 2011; Svensson and Sessle, 2004; Woolf, 2011). These neuroplastic changes can enhance the synaptic efficacy between converging nociceptive afferent inputs and VBSNC nociceptive neurons. The increased excitability of nociceptive neurons can be manifested as an increase in nociceptive neuronal mechanoreceptive field (RF) size, a decrease in mechanical activation threshold and an increase in spontaneous activity and in responses to noxious RF stimuli. Of importance for this thesis, it has been shown that application of the inflammatory irritant MO to the rat molar tooth pulp can significantly increase neuronal responses of nociceptive neurons within the MDH as well as within the thalamus and S1 (Calford and Tweedale 1991; Chiang et al 2007; Katz et al., 1999; Kawamura et al., 2010; Okada-Ogawa et al., 2009; Park et al., 2006; Zhang et al., 2006). These changes have been implicated as important processes underlying the clinical manifestation of hyperalgesia, allodynia, spontaneous pain and pain spread and referral in acute (and chronic) pain conditions following injury or inflammation of orofacial tissues (Sessle, 2000, 2006, 2011; Svensson and Sessle, 2004; Woolf, 2011).

One important neurotransmitter that is crucial for central sensitization is glutamate. Glutamate is the major excitatory neurotransmitter in the CNS. Glutamate can activate multiple receptors in the CNS including ionotropic amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors, and kainate (KA) receptors as well as metabotropic (G-protein coupled) receptors (mGluR) (Latremoliere and Woolf, 2009). Owing to
the importance of NMDA receptors to central sensitization, the action of NMDA receptor antagonists including MK-801 have been shown to block and suppress central sensitization in the MDH as well as spinal cord (Chiang et al. 1998; Latremoliere and Woolf, 2009; Ma and Woolf, 1995; Woolf and Thompson, 1991). Another molecule critical for central sensitization (induced by capsaicin injection to the hindpaw of rats) is substance P, which acts on the neurokinin (NK1) G-protein coupled receptors expressed by spinothalamic, spinoparabrachial, and spinoperiaqueductal gray neurons as well as neurons in the MDH. It is responsible for long-lasting membrane depolarization, and is potentiated by CGRP receptor activation, leading to protein kinase A (PKA) and protein kinase C (PKC) activation (Latremoliere and Woolf, 2009; Sun et al., 2003, 2004).

2.2.1.2 Acute Orofacial Pain and Reflex Motor Responses

Studies have shown that experimentally-induced noxious stimulation of orofacial tissues in animals can affect orofacial motor functions. High-intensity stimuli (e.g., MO application to the tooth pulp) can activate the jaw-opening reflex while some stimuli can evoke transient inhibitory effects through the recruitment of central opioid inhibitory mechanisms (Dubner et al., 1978; Lund et al., 2001; Sessle, 2006; Svensson and Sessle, 2004). These reflex effects reflect brainstem neural circuits activated by the noxious stimuli. It has been shown that application of MO to the molar tooth pulp of anaesthetised rats can activate neurons in the MDH, which in turn project to brainstem cranial motor nuclei, the motor neurons of which supply the jaw muscles (as well as other craniofacial muscles), thereby eliciting increased reflexive electromyographic (EMG) activity in the jaw-opening (anterior digastric) and jaw-closing (masseter) muscles. However, this reflexive activity lasts only 30-60 seconds (Narita et al., 2012; Sunakawa et al., 1999). Similar studies in humans have shown that noxious stimulation of orofacial tissues (e.g., electrical stimulation of the lip and oral mucosa; glutamate-evoked jaw or neck muscle pain) elicits a transient increased EMG activity in the jaw-closing (masseter) muscles (Bratzlavsky, 1978; Cadden, 2007; Sessle and Schmitt, 1972; Svensson et al., 2004).

2.2.1.3 Role of Glial Cells

In the CNS, glial cells far outnumber neurons and are divided into several subtypes including astroglia (astrocytes), resident microglia, perivascular microglia, and oligodendrocytes, each of which has distinct functions. Microglia and astroglia have been shown to play a role in
nociceptive processing in the CNS (Chiang et al., 2011, 2012; Gao and Ji, 2010; Inoue and Tsuda, 2009; Milligan and Watkins, 2009; Ren and Dubner, 2008). However, it is not known how microglial and astroglial processes impact orofacial pain-related changes in face-M1 function and no study has addressed the role of glial cells in dental pain-related changes in face-M1.

2.2.1.3.1 Microglia

Microglia constitute ~10% of the total cells in the CNS. In addition to reacting to CNS injury, infection, or pathology, emerging lines of evidence indicate that microglia sculpt the structure of the CNS, refine neuronal circuitry and network connectivity, and contribute to plasticity. These physiological functions of microglia in the normal CNS begin during development and persist into maturity (Salter and Beggs, 2014).

Studies indicate that upregulation of microglial markers including complement receptor type 3, known as OX-42, occurs in inflammatory pain models. In one model, experimental tooth movement induced masseter muscle allodynia which was associated with increased expression of OX-42 in microglia present in the MDH (Liu et al., 2009). Pre-treatment with minocycline (by i.p. injection), a p38 MAPK inhibitor (p38 MAPK is a prerequisite for cytokine synthesis and release from microglia) attenuated the tooth movement-induced masseter muscle allodynia (Liu et al., 2009). Increased expression of OX-42 in microglia in the MDH also occurred after subcutaneous formalin injection into the left lateral face. The period of microglial activation corresponded closely to that showing enhanced nociceptive behavior after perioral formalin injection (Cadet et al., 1995; Yeo et al., 2001). Moreover, central sensitization in the MDH induced by MO application to the molar tooth pulp can be blocked by prior application of SB230580 (an inhibitor of p38 MAPK) or minocycline to the MDH (Chiang et al., 2010c; Xie et al., 2007). It is important to note that microglial inhibitors do not affect baseline neuronal nociceptive response properties, but instead exert their effect by blocking the hyperexcitable state associated with central sensitization of the neurons (Chiang et al., 2011; Milligan and Watkins, 2009; Watkins et al., 2001).

Purinergic P2X receptors, especially P2X₄ and P2X₇, are primarily located in microglia (Cotrina and Nedergaard, 2009), and recent studies have revealed the role of microglial P2X receptor
mechanisms in central sensitization induced by MO application to the molar tooth pulp (Chiang et al., 2005; Xie et al., 2007). Application of P2X₇ receptor blockers to the MDH (periodate oxidized ATP or brilliant blue G) could significantly attenuate central sensitization induced by MO application to the tooth pulp (Li et al., 2008). Furthermore, medullary application of ATP could induce central sensitization in cases where all other P2X and A1 (except P2X₄ and P2X₇) receptor subtypes have been blocked by suramin and DPCPX (Itoh et al., 2009). Brief activation of P2X₇ may also play a protective (antisensitization) role; this includes the P2X₇-mediated release of proinflammatory cytokines which may inhibit astrocytic gap junction activity (Meme et al., 2004, 2006) as well as inhibit glutamate uptake and glutamine synthesis ultimately suppressing downstream inflammatory changes (Lo et al., 2008). However, under extreme conditions (e.g., tissue damage), P2X₇ receptor activation may prompt microglia to produce and release an exaggerated amount of proinflammatory cytokines as well as ATP, and set off astrocytes to release chemokines, cytokines, and glutamate which may cause pathological changes and eventually lead to apoptosis and gliosis (Miller et al., 2009; Milligan and Watkins, 2009; Saab et al., 2010). Blockade or deletion of P2X₄ has also been shown to potently attenuate pain states (Chessell et al., 2005; Tsuda et al., 2003). The role of microglial P2X₄ receptors appears to be closely related to brain-derived neurotrophic factor (BDNF), which is released from microglia following P2X₄ receptor activation (Trang et al., 2009; Tsuda et al., 2009; Ulmann et al., 2008). Finally, activated microglia release TNFα which can activate the intracellular signalling protein c-jun-N-terminal kinase (JNK), which in part through the involvement of astrocytes, may accentuate MDH neuronal excitability (Abbadie et al., 2009; Gao et al., 2009; McMahon and Malcongio, 2009).

In the cerebral cortex, microglia have been shown to undergo activation following λ-carrageenan-induced inflammatory pain (CIP, injected into the plantar surface of the right hind paw). Elevated expression of ICAM-1, a molecule that plays a role in immune-mediated cell-cell adhesive interactions as well as intracellular transduction pathways, was detected in specific cortical regions (frontal and parietal cortex) which correlated directly with increased expression of cortical microglia (Huber et al, 2006). This suggests the region-specific increases in microglial activation may potentially play a role in a central-mediated responses following CIP. Studies have also demonstrated that microglia in the forebrain undergo robust activation following peripheral inflammation induced by Complete Freund’s Adjuvant (CFA) injected into the plantar
surface of the left hind paw (Raghavendra et al., 2004). This also suggests that cortical microglia may play a role in hyperexcitable pain states. On the other hand, a recent study demonstrated that microglia became selectively activated in the spinal cord but not in the S1 following common peroneal nerve ligation in mice (Zhang et al., 2008). However, it is important to note that the only measure of microglial involvement in the latter study was morphological.

2.2.1.3.2 Astrocytes

Astrocytes are the most abundant of glial cells in the CNS. They modulate synaptic transmission as well as play an important role in linking blood flow to neuronal activity (Chiang et al., 2011; Giuame et al., 2010; Haydon and Carmignoto, 2006; Iadecola and Nedergaard, 2007; McMahon and Malcongio, 2009; Parpura and Zorec, 2010). They express numerous neurotransmitter receptors including NMDA and non-NMDA, metabotropic glutamatergic, substance P, and cytokine receptors. They can also interact with the vasculature, neurons, and surrounding astroglia by modulating the uptake of K⁺, the uptake and release of glucose-derived neurotransmitters [glutamate and gamma amino butyric acid (GABA)], and the release of gliotransmitters including ATP, glutamate, BDNF and D-serine that result in the modulation of neuronal excitability and neuronal functions (Hayden and Carmignoto, 2006; Kang et al., 2008; Parpura and Zorec, 2010).

Astrocytes are fundamental for controlling homeostasis of several key neurotransmitters in the – most notably glutamate, GABA and adenosine. Astrocytes remove neurotransmitters from the synaptic cleft, thereby terminating synaptic transmission and preventing possible toxicity. In addition, astrocytes catabolise these neurotransmitters into intermediates, which are then shuttled back to neurons to be transformed into active molecules, thus maintaining synaptic transmission (Chiang et al., 2011; Danbolt, 2001; Eulenberg and Gomeza, 2010; Hertz et al., 1999; Verkhratsky and Butt, 2013).

Glutamate can be synthesised only in astrocytes and not in neurons since neurons lack the enzyme pyruvate carboxylase that produces oxaloacetate, a link in the tricarboxylic acid cycle in astrocytes (See Fig.1-2). Oxaloacetate interacts with acetyl co-enzyme A derived from pyruvate, which is in turn metabolized in the tricarboxylic acid cycle to form α-ketoglutarate, a direct precursor of glutamate (Hertz and Zielke, 2004). Fluoroacetate and its metabolite fluorocitrate are potent inhibitors of astrocytes at low doses (Fonnum et al., 1997; Wei et al., 2008).
Fluoroacetate combines with acetyl coenzyme A to form fluoroacetyl coenzyme A which can substitute for acetyl coenzyme A in the tricarboxylic acid cycle and react with citrate synthetase to produce fluorocitrate, a metabolite which binds tightly to aconitase bringing the astrocytic tricarboxylic cycle to a halt (See Fig.1-2) (Proudfoot et al., 2006). Studies have shown that intrathecal administration of fluoroacetate can attenuate MDH central sensitization induced by the application of MO to the rat tooth pulp but has no effect on baseline properties of MDH nociceptive neurons (Xie et al., 2007). Studies have also shown that intrathecal administration of fluoroacetate can attenuate MDH central sensitization induced by transection of the inferior alveolar nerve but has no effect on baseline properties of MDH nociceptive neurons (Okada-Ogawa et al., 2009). It has also been shown that fluoroacetate-attenuation of MDH central sensitization can be prevented by co-administration with glutamine (Okada-Ogawa et al., 2009).

One of the main functions of astrocytes however is the uptake of extracellular glutamate and GABA in the synaptic region through the action of glutamate transporters (e.g., glutamate transporter-3, and glutamate aspartate transporter) and GABA transporters (e.g., GAT-1 and GAT-3) (Conti et al., 2004; Danbolt, 2001; Eulenburg and Gomez, 2010). Once in the astrocytes, GABA is converted by the tricarboxylic acid cycle to α-ketoglutarate, a direct precursor of glutamate (Dennis et al., 1977; Coulter and Eid, 2012). Glutamate is then converted by glutamine synthetase to glutamine, an important precursor to glutamate and GABA. Glutamine is then trafficked through transporters into glutamatergic neuronal terminals to replenish the glutamate transmitter pool (Kanamori and Ross, 2006). This function, which is specific to astrocytes, is known as the glutamate-glutamine shuttle (Hertz and Zielke, 2004) (See Fig.1-2). In inhibitory neurons, glutamate is decarboxylated by glutamic acid decarboxylase to form GABA replenishing the GABA transmitter pool. This is known as the GABA-glutamate-glutamine shuttle and is also specific to astrocytes (Coulter and Eid, 2012; Verkhratsky and Butt, 2013).

It has been shown that the glutamate-glutamine shuttle is a crucial mechanism in nociceptive neuronal central sensitization and that is specific to astrocytes (Chiang et al., 2007, 2008, 2011, 2012; Tsuboi et al., 2011; Verkhratsky and Butt 2013). Hence, any chemical substance that is capable of disrupting this mechanism may affect the availability of glutamate and as a result, the production of central sensitization in acute and inflammatory pain states. For example, methylamino-isobutyric acid (MeAIB), a selective inhibitor of glutamine uptake into presynaptic terminals (See Fig.1-2), has been shown to suppress MDH central sensitization induced by MO.
application to the tooth pulp (Chiang et al., 2008). Of particular interest is the astrocytic inhibitor methionine sulfoximine (MSO). MSO is an inhibitor of the astroglial glutamine synthetase and can disrupt the conversion of glutamate to glutamine (See Fig. 1-2). It has been shown that MSO can prevent or reverse MDH central sensitization induced by MO application to the tooth pulp (Chiang et al., 2007). It has also been shown that MSO-attenuation of MDH central sensitization can be prevented by co-administration with glutamine (Chiang et al., 2008). Studies have also shown that MSO can attenuate MDH central sensitization induced by chronic pulpitis (Tsuboi et al., 2011). Noteworthy is that MSO application to the MDH can also attenuate the decreased face-M1 excitability induced by MO application to the tooth pulp (Pun et al., 2013), pointing to the role of the MDH in transmitting sensory information to the face-S1 and face-M1 and the influence of noxious afferent inputs on the excitability of face-M1. The involvement of astrocytes in central sensitization is further supported by studies whereby central sensitization induced by chronic pulpitis or trigeminal nerve injury has been associated with immunoractivity of MDH astrocytes that could last 4 weeks (Okada-Ogawa et al., 2009; Tsuboi et al., 2011).

It is important to note that administration of MeAIB or MSO has no effect on baseline properties of MDH nociceptive neurons but once injury occurs and astrocytes become activated (reactive), MeAIB or MSO can attenuate MDH central sensitization induced by the application of MO to the rat tooth pulp (Chiang et al., 2005, 2007, 2008, 2011).

Another aspect of astrocytes involves the ability of glutamate and ATP to evoke astrocytic transients and oscillatory waves of cytoplasmic Ca\(^{2+}\), which may spread to adjacent astrocytes through gap junctions and hemichannels. The resulting activated astrocytic network may in turn release gliotransmitters (glutamate, ATP, D-serine) that can modulate synaptic transmission (Kang et al., 2008; Nagy et al., 2004; Scemes and Giaume, 2006; Scemes et al., 2009). It should also be noted that gap junctions and hemichannels are present between neurons and astrocytes as well. Studies show that intrathecal administration of carbenoxolone, a potent blocker of the hemichannel protein Cx-43 and plasmalemmal channel protein pannexin-1, can completely block central sensitization in the MDH induced by MO application to the rat tooth pulp (Chiang et al., 2010b). This suggests that spatio-temporal features of central sensitization may be brought about by intercellular Ca\(^{2+}\) wave-mediated excitation of neurons and non-neuronal cells, which involve gap junctions and hemichannels (Fellin, 2009; Giaume et al., 2010).
Fig.1-2 Astrocytic glutamate-glutamine shuttle. MeAIB is a specific blocker of the neuronal glutamine transporter and can inhibit glutamine (Gln) uptake into presynaptic terminals. Threo-beta-Benzyloxyaspartate (TBOA) is a selective blocker of glutamate transporters thereby blocking glutamate uptake into the astrocyte. Fluoroacetate (FA) is a specific inhibitor of Acotinase (Ac) which is a vital component of the tricarboxylic acid (TCA) cycle. MSO inhibits glutamine synthetase (GS) which converts glutamate (Glu) to glutamine (Gln) thereby disrupting the glutamate-glutamine shuttle.

In a recent pilot study in 2 rats, we have shown that within 30 min of MO application to the molar tooth pulp there was an increased immunoreactivity to glial fibrillary acidic protein (GFAP) (a specific marker of astrocytes) within layer V of the contralateral face-M1 (Varathan et al., 2014). It has also been shown that intraplantar application of CFA results in peripheral inflammation that is associated with astrocyte activation within the cortical forebrain (Raghavendra et al., 2004). In addition, astrocytes have also been shown to be involved in long-term potentiation (LTP) of neuronal excitation in the anterior cingulate cortex following induction of peripheral inflammation produced by CFA injected into the hind paw of rats (Ikeda et al., 2013). The LTP could be inhibited by the application of an NMDA receptor antagonist (anastrocytic toxin) to the anterior cingulate cortex. This suggests that the activation of astrocytes
in the anterior cingulate cortex plays a crucial role in the LTP of neuronal excitation during pain hypersensitivity induced by peripheral inflammation.

2.3 Cortical Sensorimotor Integration

The face-M1 is main cortical region involved in the generation and control of orofacial motor functions. The face-M1 receives somatosensory information from the orofacial region either directly through the thalamus or indirectly through the face-S1 (Avivi-Arber et al., 2011; Chakrabarti and Alloway, 2006; Dubner et al., 1978; Hoffer et al., 2005; Iyengar et al., 2007; Miyashita et al., 1994; Rocco-Donovan et al, 2011). These somatosensory inputs play a crucial role in the modulation of orofacial motor functions. Since the face-M1 receives somatosensory inputs and the face-S1 assists in modulating motor outputs, the face-S1 and the face-M1 are collectively termed the face sensorimotor cortex (Avivi-Arber et al., 2011).

2.4 Cortical Motor Pathways

Axons descending from the face-M1 (and face-S1) can project directly but mainly indirectly (through multisynaptic relays in subcortical regions which include the basal ganglia, cerebellum, red nucleus, and reticular formation) to cranial nerve motor nuclei and synapse onto motor neurons which innervate orofacial muscles (Haque et al., 2010; Hatanaka et al., 2005). It is important to note that face-M1 neurons that project to cranial nerve motor nuclei are somatotopically organized whereby each region in the face-M1 represents a muscle or a group of muscles (Avivi-Arber et al., 2010a, 2010b; Kakei et al, 1999). The cranial nerve motor nuclei include the trigeminal motor nucleus, the motor neurons of which innervate most jaw muscles, and the hypoglossal nucleus, the motor neurons of which innervate the extrinsic and intrinsic muscles of the tongue (Dubner et al., 1978; Lund and Kolta, 2006; Lund et al., 2009; Sessle, 2006, 2009). In addition, intracortical interneurons form an extensive intracortical network (see below) that modulates motor output from the sensorimotor cortex (Asanuma, 1989; Schieber, 2001).
2.4.1 Features of the Primary Motor Cortex

2.4.1.1 Morphological Features

The M1 and the adjacent S1 consist of six horizontal layers (I-VI), each with a different neuronal and non-neuronal (glial) composition and connectivity (Donoghue and Wise, 1982; Kaas, 1983; Welker, 1976; Welker and Woolsey, 1974; Woolsey and Van der Loos, 1970; Woolsey et al., 1975). There are several identifiable cell types in the cerebral cortex but this section focuses on the main types and projections in rats, the animals used in this thesis study. The granular cortex is characterised by a granular layer IV of densely packed cells that is characteristic of the S1. Layer IV is the main input layer receiving somatosensory inputs from thalamic somatosensory nuclei as well as some intra-hemispheric cortical inputs. Layer IV is further characterised by discrete areas of small densely packed and darkly stained aggregates of neurons (i.e., 'barrels'; e.g., barrels representing rodents’ whiskers). These cell-dense areas contain neurons with a specific peripheral RF and are distinctly separated from each other by less dense 'septa' referred to as dysgranular areas. Neurons within the dysgranular area receive exteroceptive as well as proprioceptive inputs from the orofacial tissues and in comparison with the granular area, they have larger and more complex RFs (Chapin and Lin, 1984; Welker, 1976; Welker et al., 1984). The M1, located more medial and rostral to the S1, is characterised by large pyramidal cells within layer V and a lack of a prominent granular layer IV, and therefore is referred to as the agranular cortex. At the border between S1 and M1 there is a transition zone where layer IV gradually thins. Layer V gives rise to all of the principal cortical efferent projection sites such as the basal ganglia and brainstem motor neuronal and interneuronal regions involved in orofacial muscle control. Layers I-III are the main interhemispheric cortical input layers. Layer I has prominent reciprocal horizontal projections (including between M1 and S1) of axons originating from multiple cortical and subcortical regions and synapsing on apical dendrites of pyramidal cells. Layer I also has a small number of GABAergic neurons. Layer III functions as the main corticocortical output layer (For review, see: Asanuma, 1989; Burish, 2008; Cauller, 1994; Douglas and Martin, 2004; Iyengar et al., 2007; Kaas et al., 2006; Mountcastle, 1997). While the pyramidal neurons project to brainstem motor neurons, their dendrites and axon collaterals extend vertically and horizontally (for ~1-3 mm) within all cortical layers, but mainly within layers II-III and V, and synapse on neighbouring pyramidal cells through excitatory glutamatergic connections. In addition, there are stellate cells which are intracortical interneurons...
with GABA as their main neurotransmitter, and they provide inhibitory inputs to adjacent pyramidal neurons. Altogether, these intrinsic connections form an extensive intracortical network of excitatory and inhibitory connections that can contribute to the excitation and inhibition of motor efferents and thereby affect the excitability of pyramidal neurons (Monkeys: DeFelipe et al., 1986; Huntley and Jones, 1991b; Kwan et al., 1987; Rats: Aroniadou and Keller, 1993; Hall and Lindholm, 1974; Huntley, 1997a; Sanderson et al., 1984; for reviews, see Keller, 1993; Mountcastle, 1997; Schieber, 2001).

2.4.1.2 Functional Features

Studies utilizing ICMS have revealed that each part of the body is represented somatotopically within the M1 whereby the trunk and limbs are represented medially and the orofacial region is represented more laterally (Adachi et al., 2008; Avivi-Arber et al., 2010a, 2010b; Neafsey et al., 1986; Tandon et al., 2008). Orofacial muscles are characterised by bilateral representations but with a contralateral predominance (Avivi-Arber et al., 2010a, 2010b). Electrophysiological studies utilising ICMS or single neuron recordings in conjunction with M1 lesioning or reversible cold block techniques in animals underscore the crucial role of face-M1 in the generation and control of orofacial motor functions. Furthermore, these studies have revealed that the somatosensory system, including the face-S1, also contributes to the control of orofacial movements by providing peripheral feedback from the orofacial tissues including the teeth (Asanuma, 1989; Ebner, 2005; Hiraba, 1999, 2004; Hiraba et al., 1997, 2000, 2007; Huang et al., 1989; Plowman et al., 2010; Sapienza et al., 1981).

2.4.1.3 Neuroplasticity of the Face-M1

One striking feature of the face-M1, and the nervous system in general, is its capacity to undergo neuroplastic changes and be remodelled throughout life. Neuroplasticity may involve structural (e.g., synaptogenesis, dendritic branching) and/or functional (e.g., increased or decreased neuronal excitability, reorganization of motor representations) changes (Greenough et al., 1985; Kleim et al., 1996; Monfils et al., 2004; Rioult-Pedotti et al., 1998; Teskey et al., 2007; Withers and Greenough, 1989). Neuroplastic changes may occur at a peripheral level, subcortical level, or cerebral cortical level and involve molecular, cellular, and synaptic mechanisms. Changes may have fast-onset or slow-onset and they may be either short-lived or long-lasting. M1 neuroplasticity may occur as a result of changes in sensory inputs or in association with altered
motor functions including learning of a novel motor skill. Notably, M1 neuroplasticity has been found to occur with motor function recovery following central or peripheral injury and pain. Neuroplastic changes within M1 may be reflected as a functional reorganization of motor representations and/or changes in cortical excitability (Avivi-Arber et al., 2011; Buonomano and Merzenich, 1998; Donoghue, 1995, 1997; Ebner, 2005; Ridding and Rothwell, 1997; Sanes and Donoghue, 2000; Sessle, 2006; Sessle et al., 2007). However, very limited published data are available on the neuroplastic capabilities of face-M1 following intraoral manipulations and experimentally-induced noxious stimulation and no study has addressed whether acute dental pain can induce neuroplastic changes within face-M1.

2.4.1.3.1 Neuroplasticity Associated with Modified Motor Function

Peripheral injury to motor nerves has been shown to produce neuroplastic changes in the face-M1 areas representing the vibrissae in rats (Buonomano and Merzenich, 1998; Ebner, 2005; Sanes and Donoghue, 2000). Injury to the facial nerve which supplies motor innervation to the vibrissae results in decreased vibrissal representation as well as expansion of the neighbouring forelimb and eyelid representations into the deprived vibrissal representation within the contralateral M1 (Donoghue et al., 1990; Huntley, 1997b; Sanes et al., 1988, 1990; ; Toldi et al., 1996). Furthermore, the ICMS threshold intensities required to evoke a response from the expanded representations decreased, suggesting an increased excitability in vibrissal-M1 (Sanes et al., 1990). These changes can occur within 1 hour after the nerve transection, last for at least 4 months (Donoghue et al., 1990; Sanes et al., 1988) and can be preceded by a rapid-onset (4 minutes) and transient (hours to 1 day) changes in the ipsilateral vibrissal representation (Toldi et al., 1996). Tongue-force training in rats is associated with increased excitability of the face-M1 but no changes in motor map representations (Guggenmos et al. 2009). Training monkeys in a novel tongue-protrusion task is associated with significant neuroplastic changes within face-M1 representing the tongue, specifically a significant increase in the motor representation of tongue-protrusion and a decrease in the motor representation of tongue-retrusion (Sessle et al., 2005, 2007). Analogous studies in humans have shown that training in a tongue-protrusion task is associated with an increased tongue representation within face-M1 which reverses after one or two weeks of no training (Svensson et al., 2003, 2006). Furthermore, increased excitability of the face-M1 occurs after a mere 15 minutes of training humans in the same tongue task, while tongue-training tasks which include different and more complex directional parameters are
associated with increased excitability in a number of cortical motor representation sites (Boudreau et al., 2007, 2013). Similarly, fMRI studies in humans have shown increased activity in the face-M1 during tongue-task training (Arima et al., 2011).

2.4.1.3.2 Neuroplasticity Associated with Modified Somatosensory Inputs

Sensory deprivation or perturbation of orofacial sensory inputs to the face-M1 has been shown to induce neuroplastic changes within the face-M1 (Avivi-Arber et al., 2011; Buonomano and Merzenich, 1998; Ebner, 2005).

2.4.1.3.2.1 Peripheral Deafferentiation

Unilateral transection of the rat lingual nerve which supplies sensory innervation to the tongue results in significant time-dependent changes in the tongue motor representation within the face-M1 (Adachi et al., 2007). Injury to the infraorbital nerve which supplies sensory innervation to the vibrissae produces a significant increase in ICMS threshold intensities required to evoke vibrissal movements; this suggests a decreased vibrissal-M1 excitability occurred but there were no changes in motor representations (Franchi, 2001). In humans, transcranial magnetic stimulation (TMS) studies have shown that lingual nerve block is associated with decreased excitability of the tongue-M1, reflected in an increased threshold intensity required for TMS to evoke tongue movements (Halkjaer et al., 2006) while local anaesthesia of the lower facial skin produces an increased excitability (reflected in increased motor-evoked potentials) of jaw-M1 (Yildiz et al., 2004).

2.4.1.3.2.2 Dental Trimming and Extraction

Tooth extraction results in peripheral soft tissue injury and irreversible deafferentiation of the pulp and periodontal ligament, thereby altering somatosensory inputs into the face-S1 and face-M1. One study has shown that tooth extraction produces a significant increase in the number of sites within face-M1 and face-S1 from which ICMS can evoke right anterior digastric (RAD) EMG activity, a lateral shift of the RAD and left anterior digastric (LAD) centres of gravity within face-M1, shorter onset latencies of ICMS-evoked genioglossus (GG) activities within face-M1 and face-S1, and an increased number of sites within face-M1 from which ICMS simultaneously evoked RAD and GG activities (Avivi-Arber et al., 2010b). In contrast, altering
the rat dental occlusion by trimming the mandibular incisors out of occlusal contacts with opposing maxillary teeth results in a significantly decreased representation in the face-M1 (Lee et al, 2005). In another study, altering the rat dental occlusion by moving the maxillary molar teeth using an orthodontic appliance can also result in a significantly decreased representation in the face-M1 (Sood et al., 2009). These neuroplastic changes in the face-M1 associated with dental manipulations provide evidence that face-M1 has the ability to change and adapt following changes in somatosensory inputs.

2.4.1.3.2.3 Orofacial Pain

Very little is known of the effect of acute orofacial pain on face-M1 neuroplasticity, and the information that is available comes from studies focusing on orofacial muscle pain. Experimental noxious stimulation induced by application of the algesic chemical glutamate to the tongue of anaesthetised rats has been shown to result in decreased face-M1 excitability (Adachi et al, 2008). In humans, topical application of capsaicin to the tongue results in a decreased ability of the subjects to perform a novel tongue-protrusion task that is also associated with decreased tongue-M1 excitability (Boudreau et al., 2007, 2010). On the other hand, injection of hypertonic saline into the human masseter muscle has been shown to result in an initial transient increase in fMRI signal intensity in the contralateral M1 which has been followed by a continuous decrease in signal intensity. These findings have been considered to suggest that the initial increased M1 activity may underlie nocifensive escape-like motor patterns, but once the initial threat subsides, longer-term reductions in M1 activation may occur to minimize jaw movement and further tissue damage (Nash et al., 2010; Sae-Lee et al., 2008; Stohler, 1999; Svensson and Graven-Neilsen, 2001). To date, no study has addressed the effect of acute dental pain on face-M1 excitability.

2.4.1.3.2.4 Face-M1 Neuroplasticity as a Reflection of Neuroplasticity within the Trigeminal Afferent System

Since M1 receives somatosensory input through the ascending trigeminal system and since this input can modulate M1 motor outputs (see sections above), it is possible that M1 neuroplasticity reflects, at least in part, changes in other cortical areas (e.g., S1) as well as in subcortical (i.e., thalamus, brainstem) relays of ascending somatosensory information to the face-M1. Changes in subcortical areas can be induced by a barrage of nociceptive inputs from the nociceptive afferents to VBSNC nociceptive neurons such as that occurs following nerve injury or
inflammation, peripheral noxious stimulation or deafferentation (Sessle, 2000, 2006, 2011; Svensson and Sessle, 2004; Woolf, 2011).

2.4.1.4 Mechanisms Underlying Face-M1 Neuroplasticity

There is a clear lack of studies related to the mechanisms underlying face-M1 neuroplasticity and manifested as changes in face-M1 excitability. Most of the published studies on neuroplasticity have focused on limb-M1 neuroplasticity, and some data are available from studies of the vibrissal-M1.

Several features of the sensorimotor cortex may contribute to its neuroplasticity; these include an extensive network of excitatory and inhibitory connections. Excitatory connections are usually monosynaptic connections of pyramidal axon collaterals extending horizontally for several millimeters (Keller and Asanuma, 1993; Mountcastle, 1997; Schieber, 2001). Inhibitory interneurons constitute about 30% of M1 neurons (Jones, 1993). Within M1, the functional organization of skeletal muscles is characterised by spatial contiguity of sensory inputs and motor outputs (for reviews, see above and Sanes and Donoghue, 2000; Sanes and Schieber, 2001; Sessle et al., 2007; Tehovnik et al., 2006). The face-M1 receives a large amount of peripheral somatosensory inputs either directly from the thalamus (Dubner et al., 1978; Iyengar et al., 2007) or indirectly from the face-S1 through extensive intra-and inter-cortical excitatory and inhibitory neural connections (Chakrabarti and Alloway, 2006; Hoffer et al., 2005; Miyashita et al., 1994; Rocco-Donovan et al., 2011). These somatosensory inputs can play a crucial role in modulating motor outputs in response to changes in somatosensory inputs. Different mechanisms may be involved in different neuroplastic processes at different time points, or they may operate simultaneously. Mechanisms underlying sensorimotor cortex neuroplasticity may be short-term or long-term mechanisms. Short-term mechanisms include potentiation of pre-existing connections by unmasking (e.g., disinhibition) of existing intracortical excitatory synaptic connections which are normally masked by inter- and intra-hemispheric lateral (e.g., GABAergic) inhibition (Farkas et al., 2000; Jacobs and Donoghue, 1991) as well as by increased neuronal excitability (Aou et al., 1992). Long-term mechanisms include enhanced gene expression (Kleim et al., 1996), dendritic branching (Greenough et al., 1985; Jones et al., 1996; Monfils et al., 2004), and synaptogenesis (Kleim et al., 2002a, 2004, 1996).
2.4.1.4.1 Role of Glial Cells

Very limited data is available on the role of glial cells in M1 neuroplasticity and no study to date has investigated whether glial cells play a role in the mechanisms underlying pain-induced face-M1 neuroplasticity and no study has addressed the role of the astrocytic glutamate-glutamine shuttle in M1 neuroplasticity. Glial cells in the cerebral cortex have been shown to become activated following peripheral manipulation affecting sensory inputs or motor functions (see sections above). Morphological and functional changes in M1 astrocytes may occur on a time scale comparable to the neuronal activity changes following peripheral nerve injury. For example, environmental enrichment can lead to a significantly increased number of astrocytes within layer I of limb-M1 (Ehninger and Kempermann, 2003). Transection of the facial motor nerve in adult rats is associated with increased immunoreactivity of various astroglial markers in layers I/II and III/V of the M1 as early as one hour after the nerve transection and lasts for 2-5 hours. This suggests that M1 astrocytes may play a role in modulating M1 activity at early and late stages following peripheral motor nerve injury (Laskawi et al., 1997).

2.4.1.4.2 Glia and Motor Function

Studies have shown that glial cells also play a role in modulating motor function. In the spinal cord, transplantation of olfactory ensheathing glia (OEG) into the spinal cord of spinally transected adult rats successfully led to structural and functional recovery (Ramon-Cueto et al., 2000; Ziegler et al., 2011); OEG-transplanted animals recovered locomotor functions and sensorimotor reflexes and they also manifested voluntary hindlimb movements, were able to support their own body weight, and their hindlimbs responded to light skin contact and proprioceptive stimuli. A similar pilot study was conducted in humans in which olfactory mucosa was transplanted into spinal cord lesions caused by traumatic injuries. Following transplantation, subjects experienced significant improvement in motor function as well as recovery in sensation below the level of injury (Lima et al., 2006).

Studies have also shown that glial cell-derived neurotrophic factor (GDNF) plays an important role in restoring motor function. Chronic controlled infusion of GDNF into the lateral ventricle or striatum of rhesus monkeys that had neural deficits modelling the terminal stages of Parkinson’s disease significantly improved motor function in the monkeys (Gash et al, 1996; Grondin et al., 2002, 2003). GDNF infusion was associated with pronounced regulation and
regeneration of nigral dopamine neurons and their processes innervating the striatum, thereby promoting the restoration of the nigrostriatal dopaminergic system (Grondin et al., 2002, 2003). In a similar study in humans, intraputaminal infusion of GDNF into patients inflicted with Parkinson’s disease significantly improved motor function including balance and gait and speed of hand movements (Gill et al., 2003; Slevin et al., 2010). GDNF infusion was associated with a significant increase in dopamine storage in the putamen, suggesting a direct effect of GDNF on dopamine function (Gill et al., 2003).

Astrocytes have been shown to modulate motor function in trigeminal brainstem motor circuits. Trigeminal sensorimotor circuits involved in mastication rely on rhythmic neuronal bursting which is enhanced and promoted when extracellular calcium concentrations decrease (Kadala et al., 2013). In vitro studies have shown that astrocytes modulate rhythmogenesis in the trigeminal sensorimotor circuit involved in mastication by influencing extracellular calcium concentrations in the trigeminal main sensory nucleus (Morquette et al., 2013). This suggests that rhythmogenesis in trigeminal sensorimotor circuits responsible for masticatory movements is dependent on neuron-astrocyte interactions.

3. The Intracortical Microstimulation Technique

Electrical stimulation of the sensorimotor cortex has long been used to investigate the functional and organisational features of the sensorimotor cortex. The ICMS technique, developed by Asanuma and his colleagues, provides good spatial (at the neuron level, µm) and temporal (msec) resolution; however, the invasive nature of this procedure limits its use to animals (Asanuma, 1989; Buolton et al., 1989, Cheney, 2002; Patterson and Kesner, 1981; Yeomans, 1990).

ICMS is an extracellular stimulation technique in which a microelectrode conveys small cathode electrical currents to a localized area within the cortex. These currents can for example excite pyramidal tract neurons within layers V-VI of the cortex and generate action potentials that propagate along fibres of the corticobulbar (or corticospinal) tracts which in turn synapse on and activate corticocortical interneurons and motor neurons within the brainstem (or spinal cord) motor nuclei. The motor neurons project to the neuromuscular junction of muscles to evoke a muscle response that can be either observed visually or recorded by an electromyograph (Asanuma, 1989; Greenshaw, 1998; Miles et al., 2004).
Parameters of ICMS-evoked EMG activities include its threshold (the lowest ICMS intensity reliably evoking EMG activity), onset latency (the time between the onset of the ICMS stimulus and the ensuing evoked EMG activity), and duration, amplitude, and area under the curve of the activity. The amplitude and the area under the curve of a rectified EMG waveform are indicators of the contractile force of the muscle). Under analogous stimulation parameters (e.g., intensity, frequency, duration), alterations in the parameters of ICMS-evoked EMG responses may suggest changes in the excitability of the corticobulbar (or corticospinal) tracts (Asanuma, 1989; Greenshaw, 1998; Ranck, 1975). Changes in experimental parameters including cortical depth at which ICMS takes place, the level of consciousness, previous stimulations, and posture of the body part/muscle can also have an effect on the characteristics of ICMS-evoked EMG responses and may therefore influence the excitability of the corticobulbar and corticospinal tracts (Asanuma, 1989; Graziano et al., 2002b; Greenshaw, 1998; Neafsey et al., 1986; Sessle and Wiesendanger, 1982; Tandon et al., 2008; Tehovnik et al., 2006).

3.1 Extent of ICMS Current Spread

ICMS activates the group of neurons in the proximity of the stimulating microelectrode. The scope of neuronal activation is dictated by two factors: the effective transmission of the stimulating current which directly activates neighbouring neurons, as well the indirect excitation transmitted through transynaptic connections (Asanuma et al., 1976; Avivi-Arber, 2009; Gustafsson and Jankowska, 1976; Ranck, 1975; Stoney et al., 1968a; for reviews, see Asanuma, 1989; Tehovnik, 1996; Tehovnik et al., 2006). The distance that an ICMS current can spread effectively to activate neighbouring neurons is dependent upon the ICMS intensity and the excitability of the neuronal tissue, the neuronal density, as well as axonal diameter and myelination of the neurons (Asanuma et al., 1976; Stoney et al., 1968a; for reviews, see Asanuma, 1989; Tehovnik, 1996; Tehovnik et al., 2006). For example, in awake rats, a 20μA ICMS train (0.2 msec duration pulses at 300Hz) can activate pyramidal cells within a 0.25 mm radius (Sapienza et al., 1981) while a 50μA ICMS train (0.2 msec duration, 300HZ) can activate pyramidal tract neurons within a 0.5 mm radius (Neafsey et al., 1986; Ranck, 1975). The axon collaterals of pyramidal tract neurons project 1-3 mm horizontally (Aroniadou and Keller, 1993) and can project even further through interneurons that project to adjacent pyramidal tract neurons. However, studies have shown that interneurons are mostly inhibitory, and would therefore restrict the spread of synaptically mediated excitation past monosynaptic connections.
of pyramidal neurons (Asanuma et al., 1976). Thus, stimulating currents can diffuse horizontally through monosynaptic connections to activate remote neurons and as a result, indirectly expand their effective spread (Asanuma, 1989; Gustafsson and Jankowska, 1976; Ranck, 1975; Tehovnik, 1996; Tehovnik et al., 2006). Moreover, descending projections from the sensorimotor cortex to the cranial nerve motor nuclei and spinal cord may further broaden the range of activity of excitatory and inhibitory motor connections, and as a consequence, affect the extent of ICMS-evoked EMG responses in muscles.

The use of ICMS currents at threshold intensity aims at activating the most excitable projection neurons in the vicinity of the tip of the stimulating microelectrode. A combination of neuronal elements, including cell bodies and axons, can be activated (Asanuma et al., 1976; Gustafsson and Jankowska, 1976; McIntyre and Grill, 2000; Nowak and Bullier, 1998a; Rattay, 1999; Stoney et al., 1968a; Stoney et al., 1968b; Swadlow, 1992; Tehovnik et al., 2006). Cortical layer III primarily contains horizontal connections, while layer V is the main corticobulbar and corticospinal output layer; hence, the lowest ICMS threshold intensity required to evoke an EMG muscle response can be achieved by activating layer V neurons (Asanuma et al., 1976; Hall and Lindholm, 1974; Sapienza et al., 1981). On the other hand, higher ICMS threshold intensities are required to evoke an EMG muscle response when stimulating layer III neurons, predominantly because of indirect activation of pyramidal tract neurons (Asanuma et al., 1976). Furthermore, whereas each corticobulbar tract neuron projects extensively to several motor neuron pools within a specific brainstem motor nucleus or within different motor nuclei, adjacent corticobulbar tract neurons congregate and project to a certain motor neuron pool that innervates a particular muscle such that low-threshold ICMS can evoke short-latency muscle activity in the most heavily innervated muscle. On the other hand, a higher ICMS intensity can excite a number of motor neuron pools thus activating more than one muscle (Asanuma, 1989; Mountcastle, 1997; Schieber, 2001).

Excessive ICMS currents (e.g., in cats, 6 pulses at >80 μA; 0.2 msec duration at 500Hz) can yield noxious effects which may result in tissue damage. Increased activation of lateral inhibitory connections can also occur, such that their summation exceeds the excitatory effect on pyramidal neurons, and in doing so, the effective spread of stimulating currents through trans-synaptic connections may be blocked (Asanuma and Arnold, 1975).
The number and duration of repetitive stimulations (i.e. stimulation train) are other important stimulation parameters. Temporal summation can occur as a result of repetitive stimulation, subsequently leading to a gradual increase in the amplitude and duration of successive ICMS-evoked motor neuron excitatory post-synaptic potentials. As a consequence, repetitive ICMS stimulation using long-duration (30-40 msec), high frequency (300-400 Hz) and long trains (> 10 pulses) can evoke EMG responses in an individual muscle at a relatively low ICMS threshold intensity (Asanuma et al., 1976; Ranck, 1975).

3.2 Effect of General Anaesthesia

The ICMS threshold intensity for evoking muscle activity can be influenced by several factors including the level of consciousness or alertness of the animal (Asanuma et al., 1968; Graziano et al., 2002b; Sessle and Wiesendanger, 1982; Wong et al., 1978). ICMS is frequently used in anaesthetised animals. Ketamine HCl, is a commonly used dissociative general anaesthetic during ICMS procedures as it is one of the few general anaesthetics that does not abolish ICMS-evoked muscle activities (Nudo et al., 2003). Ketamine functions as a non-competitive blocker of NMDA receptors (Ebert et al., 1997; Yamakura et al., 2001). NMDA receptors are present in the membranes of dendrites and cell bodies of cortical neurons, but since they are not present in axonal branches, Ketamine HCl primarily affects cell bodies and dendrites. Because ICMS primarily excites axons (Nowak and Bullier, 1998a; Nowak and Bullier, 1998b), the effect of Ketamine HCl on ICMS-evoked muscle activities is relatively minimal. However, cell bodies and dendrites of brainstem motor neurons activated by corticobulbar tracts can be affected by Ketamine HCl, and studies have shown that deeper states of general anaesthesia induced by Ketamine HCl and other anaesthetics may influence ICMS-evoked muscle activities manifested as increased threshold intensities and longer onset latencies (Gioanni and Lamarche, 1985; Sapienza et al., 1981; Sirisko and Sessle, 1983; Tandon et al., 2008). Nonetheless, low ICMS intensities have been shown to evoke EMG activities in orofacial muscles in anaesthetised as well as awake animals (Huang et al., 1989b; Sapienza et al., 1981; Tandon et al., 2008). It has also been shown that relatively similar patterns of EMG activity can be obtained by spike-triggered averaging in awake animals and repetitive ICMS in anaesthetised animals (Cheney, 2002). Thus, data collected from ICMS experiments conducted on anaesthetised animals may be comparable to those obtained from studies with awake animals. Therefore, under Ketamine general anaesthesia, ICMS can be an appropriate technique to assess cortical excitability.
Nevertheless, it is imperative to regulate the administration of the anaesthetic and maintain it at a stable level that allows for EMG activity to be evoked by relatively low ICMS intensities (Nudo et al., 2003). It is also important to note that anaesthesia levels must be carefully regulated and maintained across all experimental and control groups to ensure comparability of the results across the study groups.

4. Statement of the Problem and Study Objectives

Dental pain is one of the most prevalent orofacial pain conditions, and it is a major reason patients seek dental care (Jain et al. 2013; Lipton et al. 1993; Sessle 2005; Wall and Melzack, 2013). It is well known that motor functions may be affected by dental pain. Clinical or experimentally induced orofacial pain (including dental pain), in humans or animals, has been shown to result in limited jaw movements (e.g., smaller and slower movements as well as an inhibition in agonist muscle activity) (Lund et al., 1991; Murray and Peck, 2007; Sae-Lee et al., 2008; Stohler, 1999; Svensson and Graven-Neilsen, 2001). The face-M1 plays an important role in the generation and control of orofacial motor functions. Somatosensory inputs (including pain information) from the orofacial region (including the teeth) to the face-M1, play a crucial role in modulating orofacial motor functions.

Studies reviewed above have shown that acute experimental dental pain induced by MO application to the rat molar tooth pulp results in central sensitization (increased excitability) of MDH neurons which can be prevented or reversed by administration of an astrocytic inhibitor (e.g., MSO) to the MDH. These studies suggest that MDH central sensitization and associated neuroplasticity are dependent on the functional integrity of medullary astrocytes. Emerging evidence from human and animal studies reviewed above suggests that acute orofacial pain may also be associated with decreased face-M1 excitability but the effect of acute dental pain on face-M1 excitability is still unclear. Furthermore, no study has addressed the role— if any— of cortical astrocytes in any such effects. Nevertheless, this information is of clinical significance since a better understanding of the face-M1 mechanisms underlying acute dental pain and its effects on orofacial sensorimotor functions may assist in the development of improved treatment strategies to effectively alleviate acute dental pain and prevent the development and maintenance of chronic pain and associated motor dysfunctions.
4.1 Objectives

The objectives of the present thesis were to use an established rat model of acute dental pain and utilise electrophysiological and pharmacological approaches to test whether experimental acute noxious stimulation of the rat maxillary molar tooth pulp is associated with excitability changes within the face-M1 and whether these changes depend on the functional integrity of face-M1 astrocytes. Changes in cortical excitability may be manifested as changes in ICMS threshold intensities required for evoking EMG activity in a muscle. In the present study, we chose a face-M1 site with a low ICMS threshold intensity required for evoking EMG activity in the RAD (jaw-opening muscle), and we chose RAD since preliminary data revealed that at the selected ICMS site (at 3.0 mm anterior and 3.0 mm lateral to Bregma), RAD had the most consistent low (≤30µA) ICMS threshold intensity. Therefore, experiments focused only on changes in ICMS-evoked EMG parameters related to the RAD.

The specific aims were to use ICMS and recordings of evoked EMG activity in the RAD to determine whether:

1. Application of the inflammatory irritant MO to the rat maxillary molar tooth pulp can affect face-M1 excitability manifested as an altered ICMS threshold intensity required to evoke EMG activity in the RAD.

2. Subsequent application of the astrocytic inhibitor MSO to the face-M1 can influence MO-induced effects observed in experiments related to objective 1.

4.2 Hypotheses

Our hypotheses were:

1. MO application to the tooth pulp will result in decreased face-M1 excitability reflected as an increase in the ICMS threshold intensity required to evoke EMG activity in the RAD.

2. Subsequent application of MSO to the face-M1 will attenuate the MO-induced changes in face-M1 excitability.
Chapter 2

Materials and Methods

All experimental procedures were approved by The University of Toronto Animal Care Committee, in accordance with the Canadian Council on Animal Care Guidelines and the regulations of The Ontario Animals for Research Act (R.S.O 1990).

Each experiment in experimental and control animals was performed in a random order to reduce potential experimenter bias. Each step of the experimental procedures and data analyses was completed by one investigator (Laith Awamleh or Henry Pun), and followed strict standard protocols to ensure consistency in procedures and blinded data analysis.

1. Animals

Studies have shown that responses to pain may be influenced by sex (through estrogen cycling) as well as age (Boudreau et al., 2007; Cairns et al. 2001; Dao and LeResche, 2000; Franchi et al., 2006; Hattemer et al., 2007; Huntley, 1997b; Jonasson, 2005; Shiga et al., 2012; Yao et al., 2002). Thus, all experiments were performed on young adult male Sprague-Dawley rats (Charles River, Montreal, QC, Canada). All rats were 200-250g upon arrival to our laboratory, and 300-400g on the day of the experiment. Rats were housed under constant temperature (21±1 °C), humidity (50±5 %) and a 12-hour light/dark cycle in transparent boxes (27 x 45 x 20 cm) containing a Polyvinyl Chloride tube (used as an environmental enrichment, a shelter, and a gnawing device). In accordance with standard experimental protocols in the laboratory (Avivi-Arber et al., 2010a, 2010b), rats were housed individually and fed a soft diet (Rodent diet #2018M, Harlan Teklad) to prevent social masking of pain behaviour that may also affect brain structure and function and to ensure adequate food and water intake.

2. Study Groups and General Study Design

To address the study objectives and test whether application of the inflammatory irritant mustard oil (MO) to the maxillary molar tooth pulp can induce changes in face-MI excitability, and whether these changes can be affected by subsequent application of the astrocytic inhibitor
methionine sulfoximine (MSO) to the face-M1. The study consisted of 40 rats divided into a ‘naïve’ group (n=8) and ‘experimental’ group (n=32). In all rats, baseline intracortical microstimulation (ICMS) threshold intensities for evoking right anterior digastric (RAD) electromyographic (EMG) activity were monitored every 15 minutes for 30 minutes. The experimental group was further divided into two groups: the ‘MO’ group (n=16) received MO to the exposed molar tooth pulp; the control ‘sham MO’ group (n=16) received saline or mineral oil (i.e., vehicle control) to the exposed right maxillary molar tooth pulp. In both groups ICMS threshold intensities were monitored 1 minute, 5 minutes, and 15 minutes after MO or vehicle application to the tooth pulp. Rats in the ‘MO’ group were further divided into two subgroups: the ‘MO/MSO’ subgroup (n=8) received MSO to the face-M1 and the ‘MO/PBS’ subgroup (n=8) received phosphate-buffered saline (PBS) to the face-M1. The control ‘sham MO’ group was also divided into 2 more control subgroups: the ‘sham MO/MSO’ subgroup (n=8) received MSO to the face-M1; and the ‘sham MO/PBS’ subgroup (n=8) received PBS to the face-M1. After the MSO or PBS application to the face-M1, ICMS threshold intensities were monitored every 15-30 minutes for 2 hours. The ‘naïve’ group (n=8) received no dental pulp exposure, no MO/saline/mineral oil application to the tooth pulp, and no MSO/PBS application to the face-M1. ICMS threshold intensities were monitored at time points corresponding to the ICMS time points of the experimental groups (See Fig.2-1).

![Fig.2-1 Study groups and subgroups, general study design and timeline](image-url)
3. Rat Preparation

3.1 Anaesthesia

All experimental procedures were performed under general anaesthesia supplemented by local anaesthesia. General anaesthesia was induced through the intramuscular administration (IM) of Ketamine HCl (175 mg/kg, Ketaset®, Ayerst Veterinary Laboratories, Ontario, Canada) and Xylazine (25 mg/kg, Rompun®, Bayer, Ontario, Canada). This was followed by femoral intravenous (IV) infusion of Ketamine HCl regulated by an infusion pump (PHD 2000, model 11 Plus, Harvard Apparatus, Inc., Holliston, MA, USA) at 75 mg/kg/hr during molar tooth drilling, EMG electrode implantation, and craniotomy. The infusion rate was decreased to 25 mg/kg/hr during ICMS. The ICMS experiment typically lasted 3 hours, and the rat was continuously monitored and the infusion rate adjusted to ensure that the rat remained within a narrow window of as light as possible anaesthetic depth to maintain face-M1 excitability. At this state of general anaesthesia, spontaneous jaw and tongue muscle twitches occurred at a rate of at least 4-5 twitches per minute, but a noxious pinch to the hind paw did not induce a withdrawal response. In addition, local anaesthesia (0.1ml, 2% Lidocaine in 1:100,000 epinephrine, Lignocaine, Lignospan Standard®, Septodont, Ontario, Canada) was administered subcutaneously at each surgical site (but not the tooth pulp) before any incision was made to block afferent inputs that may affect M1 excitability either by direct effect on M1 or through diffuse noxious inhibitory control (DNIC) mechanisms whereby a heterotopic stimulus can alter the sensitivity to a noxious stimulus (i.e., ‘pain inhibits pain’) (Hu, 1990; Le bars, 1979; Luft et al., 2002).

A heating pad (Model 73A, YSI, Ohio, USA) regulated by feedback from a rectal thermometer maintained the rat’s physiological body temperature at 37-38 °C. Heart rate (330-350 beats/min) was monitored through an electrocardiogram (EKG), and a pulse oximeter (8600, Nonin, MN, USA) monitored O₂ saturation levels. The fur covering the surgical sites (right hind limb, head, and submandibular region) was shaved with a fur trimmer.
3.2 Dental Pulp Exposure

While the animal was lying in a supine position, the coronal pulp of the right maxillary first molar tooth was exposed using a round carbide bur attached to a high speed dental hand piece (Model D7950, Kavo, Germany) under intermittent saline irrigation. To ensure pulp exposure and consistent cavity depth, the tooth was drilled until slight bleeding from the tooth pulp occurred. Thereafter, the cavity was covered with a saline-soaked cotton pellet.

3.3 EMG Electrode Implantation

An incision was made in the submandibular region to expose the anterior digastric (jaw-opening) and masseter (jaw-closing) muscles bilaterally. As per standard ICMS experimental protocols in the laboratory (Adachi et al., 2007, 2008; Avivi-Arber et al., 2010a, 2010b), a pair of EMG electrodes (40 gauge, single stranded, Teflon-insulated stainless-steel wires with 0.5-1 mm exposed tip) (Cooner wire, Chatsworth, CA, USA) was then inserted into each of the left and right anterior digastric (LAD, RAD), the left and right masseter, the genioglossus (GG, tongue-protrusion muscle), the trapezius (shoulder elevation muscle), and the right extensor digitorum (wrist extension muscle). It is important to note that as mentioned earlier (Introduction section 4.1), the focus of the present study was on the RAD muscle, however, electrodes were also implanted into these other muscles to assist in mapping the face-M1 area and finding a low-threshold ICMS site for evoking RAD EMG activity (see below). To ensure that the EMG electrode placement was successful, and that the condition of the muscle had not deteriorated during the experiment, immediately following the placement of the EMG electrodes and once again before the termination of the experiment, a constant-current stimulation train (33.2 msec, 12x0.2msec pulses, 333Hz) delivered to each muscle had to evoke an observed muscle twitch at a threshold stimulation intensity of ≤200µA. Upon termination of the experiment, any muscle stimulation threshold >200µA indicated possible EMG electrode displacement or deterioration in the state of the muscle in which case the data collected from that experiment was excluded from the study.

3.4 Craniotomy

The rat was placed in a stereotaxic apparatus (Model 1340, David Kopf, Tujunga, CA, USA) which included a bite bar and two ear rods to immobilize the rat’s head in a prone position. Topical lidocaine ointment (5% Xylocaine, AstraZeneca, Mississauga, ON, Canada) was applied
to the ear rods before their insertion into the external auditory meatus. A midline incision was then made and the scalp tissue was elevated to expose the skull. Bone wax was applied to the skull to stop any bleeding. The Bregma was marked as the zero reference point (See Fig.2-2). Under intermittent water irrigation, a round carbide bur attached to a low-speed dental hand piece (Model D7950, Kavo, Germany) was then used to cut through the bone to expose the left hemisphere of the brain 0.0-5.0 mm anterior to Bregma and 1.0-5.0 mm lateral to the sagittal suture. Attention was paid during drilling to avoid damaging the sagittal sinus medially and the orbital and temporal bones and associated soft tissues laterally. The dura covering the proposed ICMS microelectrode penetration site (2.5-3.5 mm anterior and 2.5-3.5 mm lateral to Bregma) was then removed to allow for smooth microelectrode penetration and subsequent application of MSO or PBS to the face-M1 ICMS site. Vaseline was then used to create a well around the exposed area to contain any substances subsequently applied to the face-M1 (See Fig.2-2). Mineral oil maintained at 37 °C was applied to the exposed cortical surface to keep it moist at all times.

Fig.2-2 Superior view of the left hemisphere exposed after craniotomy. Arrows indicate the location of the Bregma as a reference point as well as the location of the Vaseline well around the ICMS microelectrode penetration site. The well ensured localized administration of MSO and PBS around the ICMS microelectrode penetration site.
4. Intracortical Microstimulation (ICMS)

ICMS is a well-established technique whereby a fine microelectrode inserted into the deep cortical layers delivers electrical currents that can evoke muscle activity which can be observed and/or recorded electromyographically (See introduction for details). Systematic microelectrode penetrations and recordings of ICMS-evoked EMG activity have been used to map the motor representations of skeletal muscles within M1 (e.g. Adachi et al., 2007; Asanuma 1989; Avivi-Arber et al., 2010a, 2010b; Neafsey et al., 1986). Consistent with previous studies (e.g., Adachi et al., 2008; Avivi-Arber 2010a, 2010b), ICMS was used in the present study to examine changes in face-M1 excitability manifested as changes in ICMS-evoked EMG parameters (e.g., threshold intensities required to evoke EMG activity and onset latencies of evoked EMG activity).

4.1 Intracortical Microstimulation Procedure

Glass-insulated tungsten microelectrodes (1-3MΩ, 250–450pF, 10-20µm exposed tip, 125µm shank diameter and 300µm outer diameter) (Alpha-Omega Engineering, Nazareth, Israel) were used for ICMS. A stainless-steel rod placed under the skin of the neck was used as a reference electrode. A micropositioner (Kopf model 2260, David Kopf Instruments, Tujunga, California) was used to manipulate the horizontal and vertical position of the microelectrode. The microelectrode was positioned in a face-M1 site at which ICMS could evoke low-threshold (≤30µA, see section 4.2) RAD EMG activity. Based on previous studies, the location of the ICMS penetration site was approximately 3.0 mm anterior and 3.0 mm lateral to Bregma and at a depth of 1.8-2.4 mm, coinciding with layers V-VI of the agranular cortex of the face-M1 (See section 6) (Adachi et al., 2007, 2008; Avivi-Arber et al., 2010a, 2010b; Lee et al., 2006). Areas of the cortex with major blood vessels were not penetrated to avoid haemorrhage.

4.2 Intracortical Microstimulation Parameters

ICMS parameters were similar to those used previously in many ICMS studies in anaesthetised rats (Adachi et al., 2007, 2008; Avivi-Arber et al., 2010a, 2010b; Franchi, 2001, 2002; Huntley, 1997b; Kleim et al., 1998; Neafsey et al., 1986). Sequencer and script codes in Spike2 and CED-1401 Plus System (Cambridge Scientific Instruments, UK) were established to generate monophasic, cathodal, constant-current stimulation trains of 333Hz [i.e., 33.2msec trains comprised of 12pulses of short (0.2msec) duration, with 2.8msec inter-pulses intervals] delivered
through a stimulus isolator (Model A365, World Precision Instruments, Stevenage, UK) to the monopolar microelectrode. The use of biphasic pulses consisting of a negative and a positive phase respectively or discharging the microelectrode electronically after each monophasic pulse could better balance the electrical current delivered to the tissue and limit possible tissue damage resulting from accumulation of electrical charges and microelectrode polarization (Asanuma and Arnold, 1975; Asanuma et al., 1976). In the present study, microelectrode polarization and tissue damage were minimized by using short monophasic ICMS trains with pulse durations of 0.2msec, which is shorter than the chronaxie reported for pyramidal cells (Ranck, 1975; Stoney et al., 1968a), and ICMS intensities of ≤60μA. In addition, the use of cathodal currents, as opposed to anodal currents, further minimized damage to the microelectrode tip (as a result of positive ions removal), and allowed for more effective excitation of deep cortical cells (i.e., pyramidal cells) (Asanuma and Sakata, 1967; Ranck, 1975; Rattay, 1999; Stoney et al., 1968a).

Two trains of ICMS were delivered at 1sec intervals, starting at 60μA ICMS intensity. If an ICMS intensity of 60μA could evoke RAD EMG activity (See Fig.2-4), the intensity was gradually decreased in increments of 10μA until EMG activity could not be evoked in the RAD muscle. Then the ICMS intensity was gradually increased in increments of 2μA to determine the lowest ICMS intensity required to evoke EMG activity. This stimulation intensity was defined as the “ICMS threshold intensity”. A cortical site was considered a low-threshold ICMS site for evoking RAD EMG activity if the ICMS threshold intensity was ≤30μA. The same procedure was carried out to determine the ICMS threshold intensity at each measurement time interval over the duration of the experiment.

Once a site was defined as a low-threshold ICMS site for evoking RAD EMG activity, the ICMS threshold intensity at that site was monitored every 15 minutes for 30 minutes to confirm a stable ICMS threshold intensity. Once a stable ICMS threshold intensity was confirmed it was defined as the ‘baseline ICMS threshold intensity’.

4.3 MO/Mineral Oil/Saline Application to the Tooth Pulp

Once a stable ICMS threshold intensity was confirmed, a small piece of a dental paper point soaked with either MO (allyl isothiocyanate, >93%, Sigma Aldrich, USA) or vehicle control (mineral oil or saline) was inserted into the exposed molar tooth pulp and thereafter the cavity
was sealed off with a dental filling material (3M™ ESPE™ Cavit™) to prevent MO leakage into the oral cavity. Preliminary data revealed that the effect of MO application to the molar tooth pulp on the ICMS threshold intensities required to evoke RAD EMG activity peaks 15 minutes later. Therefore, the ICMS threshold intensity for evoking RAD EMG activity was measured 1 minute, 5 minutes, and 15 minutes after MO, mineral oil or saline application to the pulp.

4.4 MSO/PBS Application to the Face-M1

MSO is an inhibitor of glutamine synthetase and can disrupt astrocytic and neuronal functions (Chiang et al., 2007; Shaw and Bains, 1998; Shaw et al., 1999). Previous studies from our laboratory whereby MSO was applied to the medulla following MO application to the molar tooth pulp have shown that MSO superfusion at high doses of 0.25 to 4 mM produced strong depression of neuronal function, and that only a dose of 0.1mM MSO superfused over the medulla attenuated MO-induced neuronal central sensitization without disturbing normal neuronal function (receptive field size, threshold, and response (Chiang et al., 2007). In addition, preliminary data from the present study indicated that MSO concentrations below 0.1mM had no effect on the MO-induced changes in ICMS threshold intensity for evoking RAD EMG activity. Therefore, and since the effect of MO application to the molar tooth pulp on ICMS threshold intensities peaks 15 minutes after MO application to the molar tooth pulp, in the present study, 15 minutes following MO application to the molar tooth pulp, a 0.1mM dose of MSO (Sigma Aldrich, USA) or PBS (vehicle control; Wisent Inc., Canada) was topically applied to the cortical surface around the microelectrode penetration site. The ICMS threshold intensities for evoking RAD EMG activity were then measured every 15-30 minutes for 2 hours. During the experiment, PBS and MSO were kept in a water bath (Isotemp 102, Fisher Scientific, Ottawa, ON, Canada) at 37°C.

4.5 Electrolytic Lesion

At the termination of the ICMS experiment, an electrolytic lesion was placed at a depth of 3.6 mm (i.e., well below the ICMS site) by passing cathodal DC current – 10µA for 7sec. – (Adachi et al., 2007; Avivi-Arber 2010a, 2010b; Iriki et al., 1991; Toda and Taoka, 2004). The lesion served as a landmark for subsequent histological verification of the ICMS sites within the face-M1.
5. Perfusion, Extraction, and Fixation of the Brain

At the termination of the experiment, the rat was deeply anaesthetized by an overdose of ketamine HCl. A transverse incision opened the pericardium of the rat to expose the heart. A perfusion metal needle with a ball tip was then inserted into the aorta through the apex of the left ventricle and the tip of the catheter was secured in place with a suture. The right atrium was then cut open, and 250ml saline solution was perfused through the needle to the aorta to clear the capillaries and wash the blood out of the tissues (including the brain). Once the blood was flushed out, 10% buffered formalin (Fisher Scientific, New Jersey, USA) was perfused for tissue fixation. After the perfusion was complete, the brain of the rat was exposed, extracted, and fixed in 10% buffered formalin for a minimum period of 24 hours.

6. Histological Verification of ICMS Site

Coronal brain sections (100µm thick) were cut with a vibratome (Model 3000, TPI, Missouri, USA). Sections were stained with Nissl stain which is an aniline stain that binds to ribonucleic acid (RNA) of the nucleolus and ribosomes in the cytoplasm of neuronal cell bodies. This results in a darkly stained nucleolus and stained cell body of each neuron that reveals the general cytoarchitecture of the brain and helps in delineating the borders between cortical and subcortical regions and between the granular and agranular cortical regions (Kadar et al. 2009) (see Results Fig.3-1).

Histological sections (along with a ruler for calibration) were scanned into a computer with a flatbed scanner having a resolution of 1200 dots per inch (dpi). A public domain Image-J software program (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006) was used to measure the vertical and mediolateral distance of the electrolytic lesion from the cortical surface and the midline respectively. This position was used to adjust the measurement recorded during the experiment by the micropositioner-micromanipulator. The adjusted penetration depths determined from the histological sections were then used to verify that the ICMS sites were within the grey matter of the cytoarchitectonically defined face-M1. The primary motor cortex (M1) is defined as the agranular cortex and is located medial and rostral to the primary somatosensory cortex (S1) which is defined as the granular cortex since it is characterized by a granular layer IV, which does not exist in M1 (Donoghue and Wise, 1982). The Paxinos atlas (Paxinos and Watson,
1998) and Swanson atlas (Swanson, 2004) were used to assist in identifying brain anatomical landmarks and thereby ensure that the ICMS penetration sites in all the rats were at around the same brain region of 2.5-3.5 mm anterior and 2.5-3.5 mm lateral to Bregma and within the cortical grey area. If an ICMS site was found to be outside this area, the associated data were excluded from the data analysis. In addition, histological slides were checked for any cortical tissue damage caused by repeated ICMS, in which case the associated data were excluded from the data analysis.

7. Data Acquisition and Analysis

EMG activity was amplified using a gain of x1000 and filtered (bandpass 100~1 kHz) by an alternating current (AC) amplifier (model 1700, A-M system, Washington, USA). The signals were digitized at 5kHz by an analogue-to-digital converter (CED 1401 plus, Cambridge Electronic Design, Cambridge, UK) which was operated on a personal computer. Customized software written in Spike2 script (CED, Cambridge Electronic Design, Cambridge, UK), and LabView (National Instruments, Austin, TX, USA) was used to analyze data files off-line (Adachi et al., 2007; Avivi-Arber et al., 2010a, 2010b). The 2 ICMS-evoked RAD EMG responses, corresponding to the series of 2 stimulation trains, were rectified, averaged and smoothed by a 4-msec moving-average window (Fig. 2-4) (Baker and Lemon, 1995; Myers et al., 2003; for review, see Cheney, 2002).

Two EMG recording parameters were analyzed: ICMS threshold intensity and onset latency. The computer algorithm used 2 criteria to define ICMS-evoked EMG activity as a positive response:

1. The rectified, averaged and smoothed EMG responses had a peak activity exceeding the mean value of the initial 10msec signal by 2 standard deviations (SD, 95% confidence interval) and with an onset latency ≤40msec (Adachi et al., 2007, 2008; Avivi-Arber et al., 2010a, 2010b) (See Fig.2-5).

2. At least one of the two ICMS-evoked responses met the first criterion (Hodges and Bui, 1996).
Since movement artifacts or EKG may obscure computer-identified onset of EMG activity, each of the computer-identified EMG responses was checked visually to ensure a correct identification of the positive ICMS response (Hodges and Bui, 1996).

**Fig. 2-3** Example of a set of ICMS-evoked EMG responses recorded from RAD of a single rat. ICMS evoked RAD EMG activity at 60µA, 50µA, 40µA, 30µA, 20µA, and 17µA stimulus intensities. RAD EMG activity recorded at 22.2 and 34.5 seconds represents jaw activity during spontaneous swallowing. ICMS: intracortical microstimulation, RAD: right anterior digastric, EMG: electromyographic.

**Fig.2-4** Example of an ICMS-evoked EMG response in the RAD after being rectified, averaged and smoothed by a 4msec-moving average window. 2SD: 2 standard deviations from the mean.
8. Statistical Analyses

8.1 Sample Size Calculation

To determine the number of rats needed per group to provide statistically based data, a sample size calculation was performed, as outlined below.

Sample size calculation:

\[ \frac{(u+v)^2}{\sigma_1^2 + \sigma_2^2} \]  \quad (Kirkwood and Sterne, 2003)

\[ (\mu_1 - \mu_2) \]

\(u\) – one-sided percentage point of the normal distribution corresponding to 100 - % the power

\(v\) – percentage point of the normal distribution corresponding to the (two-sided) significance level

\(\sigma_1, \sigma_2\) – SD

\(\mu_1 - \mu_2\) – difference between means

The number of rats required in each group to obtain 95% power \((u=1.64)\), significant at a level of 5% \((v=1.96)\) with a 45% difference between means \((\mu_1 - \mu_2=0.45)\), and an SD of 25% in each group \((\sigma_1 = \sigma_2 = 0.25)\), is:

\[ = \frac{(1.64+1.96)^2(0.25^2+0.25^2)}{(0.45)^2} \]

\[ = 8 \]

For 5 groups and subgroups (MO/MSO, MO/PBS, sham MO/MSO, sham MO/PBS, naïve), with 8 rats per group/subgroup, a total of 40 rats was needed to find significant differences between the study groups and subgroups.

8.2 Analysis of Significance

SigmaPlot 11 (Systat Software, San Jose, CA, USA) was used to perform the statistical analysis. The first series of data analysis was concerned with the effects of chemicals used (MO/mineral oil/saline to the molar tooth pulp, MSO/PBS to the face-M1; i.e., the independent variables) on
the ICMS threshold intensity for evoking RAD EMG activity and onset latency of RAD EMG activity evoked by ICMS at 60µA (i.e., the dependent variables) within each experimental group and control group or subgroup at different time points. The means of the % change from baseline of the ICMS threshold intensities (±SEM) and onset latencies (±SEM) at baseline and at each time point after each chemical application (MO/mineral oil/saline, MSO/PBS) were compared through repeated-measures ANOVA followed by post-hoc Bonferroni-adjusted pairwise comparisons as appropriate. The second series of data analysis compared the means of the % change from baseline of the ICMS threshold intensities (±SEM) and onset latencies (±SEM) across all experimental and control groups and subgroups at each time point by the use of repeated-measures ANOVA, and results were followed by post-hoc Bonferroni-adjusted pairwise comparisons as appropriate. In addition, mineral oil is the standard vehicle control for MO application to the molar tooth pulp (Chiang et al., 1998). However, in the present study, saline as well as mineral oil were used as vehicle controls for MO. Therefore, we tested whether there was any significant difference between the effect of saline versus mineral oil on mean ICMS threshold intensities (±SEM) as well as on mean onset latencies (±SEM) through repeated measures ANOVA followed by post-hoc Bonferroni-adjusted pairwise comparisons as appropriate. In all analyses, a probability level of p < 0.05 was considered to reflect statistical significance.
Chapter 3

Results

1. Histological verification of ICMS sites

Histological verification of ICMS sites confirmed that all ICMS sites fell close to coronal plane AP $3.0 \pm 0.2$ mm as defined by Swanson atlas (Swanson, 2004), at ML coordinate $3.0 \pm 0.2$ mm and a depth ranging between 1.8-2.4 mm. These ICMS sites coincided with the cytoarchitectonically defined layers V-VI of the face-M1 agranular cortex (Fig. 3-1). In addition, the histological analysis revealed no apparent evidence of cortical tissue damage as a result of the repeated electrical stimulations required for determining the ICMS threshold intensities over the 3-hour duration of each experiment.

**Fig. 3-1a.** A Nissl-stained coronal section (100µm thickness) from a representative rat sensorimotor cortex at AP plane 3.0mm anterior to bregma showing electrolytic lesion placed at 3.6 mm depth which is deeper than the ICMS site. The lesion was used to verify the location of the ICMS site within layer V-V1 of the agranular cortex that coincides with face-M1. (AP: anteroposterior, ICMS: intracortical microstimulation, AG: agranular cortex, G: granular cortex, Gu: gustatory region).

**b.** A schematic diagram indicating the layers of the sensorimotor cortex and the borders between the M1 (agranular), S1 (granular) and gustatory (Gu) cortex. (M1: primary motor cortex, S1: primary somatosensory cortex, (Adapted from Swanson atlas (Swanson, 2004),
2. ICMS and RAD EMG activity

2.1 Baseline Data

2.1.1 RAD EMG activity

Prior to ICMS, baseline RAD EMG activity that was monitored over a period of 30 minutes revealed a stable EMG activity with at least 4-5 spontaneous jaw and tongue muscle twitches per minute. This indicated an adequate state of general anaesthesia.

2.1.2 ICMS threshold intensities

In all rats, the baseline mean ICMS threshold intensities for evoking RAD EMG activities were monitored for 30 minutes and revealed stable intensities that ranged between 10-30 µA (25.3 ± 1.5 µA, Mean ± SEM) with no statistically significant differences in intensities across the study groups and subgroups (Repeated-measures ANOVA; P= 0.17) (Table 3-1).

2.1.3 EMG onset Latencies

The baseline mean onset latencies of RAD EMG activities evoked by ICMS (60µA) were in the range of 8-25 milliseconds (17.5 ± 2.1 milliseconds). The baseline mean onset latencies were stable with no statistically significant differences across the study groups and subgroups (Repeated-measures ANOVA; p= 0.80) (Table 3-1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Threshold intensity (Mean ± SEM) (µA)</th>
<th>Onset Latency (Mean ± SEM) (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO (n=16)</td>
<td>24.3 ± 1.4</td>
<td>17.9 ± 2.3</td>
</tr>
<tr>
<td>Sham MO (n=16)</td>
<td>24.7 ± 1.4</td>
<td>17.9 ± 2.0</td>
</tr>
<tr>
<td>Naïve (n=8)</td>
<td>27.7 ± 1.4</td>
<td>16.8 ± 2.1</td>
</tr>
</tbody>
</table>

**Table 3-1.** Baseline mean ICMS threshold intensities for evoking RAD EMG activities and mean onset latencies of RAD EMG activities evoked by ICMS (60µA). There were no statistically significant differences across the three groups. (ICMS: intracortical microstimulation, RAD: right anterior digastric, EMG: electromyographic, MO: mustard oil).
2.2 Effects of mineral oil versus saline application to the molar tooth pulp

2.2.1 Effect on RAD EMG activity

Neither saline nor mineral oil application to the molar tooth pulp had any effect on the observed RAD EMG activity.

2.2.2 Effect on ICMS threshold intensities

Over the 15 minutes of monitoring time, there were no statistically significant differences in mean ICMS threshold intensities for evoking RAD EMG activities between saline and mineral oil application to the molar tooth pulp (Repeated-measures ANOVA; p =0.19) (Table 3-2).

2.2.3 Effect on EMG onset latencies

There were no statistically significant differences in the mean onset latencies of RAD EMG activities evoked by ICMS (60µA) between saline and mineral oil application to the molar tooth pulp (Repeated-measures ANOVA; p=0.93) (Table 3-2).

Since there were no statistically significant differences between saline and mineral oil in the mean ICMS threshold intensities and the mean onset latencies, data from both groups were pooled into one group termed “Sham MO”.

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<table>
<thead>
<tr>
<th>Group</th>
<th>Time after substance application to the pulp</th>
<th>Threshold intensity (Mean ± SEM) (µA)</th>
<th>Onset latency (Mean ± SEM) (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline to pulp</td>
<td>Baseline</td>
<td>23.7 ± 1.2</td>
<td>17.4 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>1 min.</td>
<td>23.7 ± 1.3</td>
<td>16.3 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>5 min.</td>
<td>23.9 ± 1.3</td>
<td>18.6 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>15 min.</td>
<td>23.4 ± 1.2</td>
<td>19.5 ± 3.3</td>
</tr>
<tr>
<td>Mineral oil to pulp</td>
<td>Baseline</td>
<td>25.7 ± 1.6</td>
<td>18.3 ± 2.4</td>
</tr>
<tr>
<td>(n=8)</td>
<td>1 min.</td>
<td>26.2 ± 1.2</td>
<td>19.1 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>5 min.</td>
<td>26.3 ± 1.2</td>
<td>16.9 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>15 min.</td>
<td>26.3 ± 1.4</td>
<td>17.2 ± 2.8</td>
</tr>
</tbody>
</table>

Table 3-2. Mean ICMS threshold intensities and mean onset latencies of RAD EMG activities evoked by ICMS (60µA), at baseline, 1 minute, 5 minutes, and 15 minutes following saline or mineral oil application to the molar tooth pulp. There were no statistically significant differences in the mean ICMS threshold intensities or the mean onset latencies between groups receiving saline or mineral oil application to the molar tooth pulp. (ICMS: intracortical microstimulation, RAD: right anterior digastric, EMG: electromyographic).

### 2.3 Effects of MO or vehicle application to the molar tooth pulp

#### 2.3.1 Effect on RAD EMG activity

MO application to the molar tooth pulp resulted in a transient increase in RAD EMG activity starting at about 3.3 seconds following MO application to the tooth pulp, and lasting about 12 seconds (Fig.3-2).
Fig. 3-2. RAD EMG activity before and following MO application to the right maxillary molar tooth pulp. A transient increase in RAD EMG activity was observed following MO application to the molar tooth pulp, starting at about 2-4 seconds after MO application to the tooth pulp and lasting to about 12 seconds. (RAD: right anterior digastric, EMG: electromyographic, MO: mustard oil).

2.3.2 Effect on ICMS threshold intensities

In comparison with baseline, MO application to the molar tooth pulp resulted in significantly increased mean ICMS threshold intensities for evoking RAD EMG activities; there was a 25.5% increase at 1 minute (Repeated-measures ANOVA, post-hoc Bonferroni, p=0.005), a 46.3% increase at 5 minutes (p<0.001), and a 53.9% increase at 15 minutes (p<0.001) of MO application to the molar tooth pulp. In contrast to MO, in comparison to baseline, vehicle (sham MO) application to the right molar tooth pulp had no statistically significant effect on mean ICMS threshold intensities (Repeated-measures ANOVA, p=1.00). There were also no statistically significant differences in mean ICMS threshold intensities between the sham MO group and the naïve group at any of the studied time points (p = 1.00). However, the MO-induced increases in mean ICMS threshold intensities were also significantly larger than the mean ICMS threshold intensities measured in the sham MO and naïve rats at corresponding 1 minute (Repeated-measures ANOVA, post-hoc Bonferroni, sham MO p=0.006, naïve p=0.04), 5 minutes (sham MO p < 0.001, naïve p=0.035) and 15 minutes (sham MO p < 0.001, naïve p=0.01) time points (Fig.3-3).
2.3.3 Effect on EMG onset latencies

In comparison with baseline, neither MO nor vehicle control application to the molar tooth pulp had any statistically significant effects on the mean onset latencies of RAD EMG activities evoked by ICMS (60µA) (Repeated-measures ANOVA; \( p = 0.86 \)). There were also no statistically significant differences in the mean onset latencies across MO, sham MO, and naïve groups (Repeated-measures ANOVA; \( p = 0.86 \)) (Fig.3-4).

2.4 Effects of MSO or vehicle application to the face-M1 following MO application to the molar tooth pulp

2.4.1 Effect on ICMS threshold intensities

As mentioned above in section 2.3.2, in comparison to baseline, MO application to the molar tooth pulp resulted, 15 minutes later, in a 53.9% increase in the mean ICMS threshold intensities for evoking RAD EMG activities. Subsequent application of MSO to the face-M1 significantly attenuated these MO-induced increases in the mean ICMS threshold intensities (Fig.3-3). In comparison to the MO-induced increase in ICMS threshold intensity, MSO application to the face-M1 (MO/MSO group) significantly decreased the mean ICMS threshold intensities by 26.9% within the first 15 minutes, 27.1% within the first 30 minutes, and 41.5% within the first 60 minutes (Repeated-measures ANOVA, post-hoc Bonferroni, \( p = 0.026, p = 0.024, p = 0.005 \), respectively). In contrast to MSO, PBS application to the face-M1 (MO/PBS group) had no statistically significant effects on the MO-induced changes in mean ICMS threshold intensities (Repeated-measures ANOVA; \( p = 1.00 \)). In comparison to PBS application to the face-M1 (MO/PBS group), MSO application to the face-M1 (MO/MSO group) resulted, 15, 30, and 60 minutes later, in a significantly decreased mean ICMS threshold intensities (Repeated-measures ANOVA, \( p = 0.029, p = 0.029, p = 0.004 \), respectively). At the 60 minutes time point following MSO application to the face-M1 (MO/MSO), there were no significant differences between the MO/MSO group and any of the control groups (Repeated-measures ANOVA, \( p = 0.13 \)). However, thereafter, in some rats, mean ICMS threshold intensities started to gradually increase and at the 120 minutes time point, the mean change in ICMS threshold intensities reached 40.5% of baseline level; at this time point there were no statistically significant differences in the mean ICMS threshold intensities across all study groups (Repeated-measures ANOVA; \( p = 1.00 \)).
2.4.2 Effect on EMG onset latencies

Neither MSO nor PBS application to the face-M1 had any statistically significant effects on the mean onset latencies of RAD EMG activities evoked by ICMS (60µA) (Repeated-measures ANOVA; p = 0.93) (Fig. 3-4).

2.5 Effects of MSO or vehicle application to the face-M1 following saline/mineral oil application to the molar tooth pulp

2.5.1 Effect on ICMS threshold intensities

As mentioned above, application of saline or mineral oil (vehicle control) to the molar tooth pulp had no effect on the mean ICMS threshold intensities for evoking RAD EMG activities. Subsequent application of MSO or PBS (vehicle control) to the face-M1 also had no statistically significant effect on the mean ICMS threshold intensities, which remained low for at least 120 minutes and were comparable to the ICMS threshold intensities measured in the naïve group at the same time points (Repeated-measures ANOVA; p = 0.13) (Fig. 3-3).

2.5.2 Effect on EMG onset latencies

Neither MSO nor PBS application to the face-M1 following vehicle application to the molar tooth pulp had any statistically significant effects on the mean onset latencies of RAD EMG activities evoked by ICMS (60µA) (Repeated-measures ANOVA; p = 0.87) (Fig. 3-4).
Fig. 3-3. The % changes from baseline in the mean ICMS threshold intensities for evoking RAD EMG activities are plotted for all study groups as a function of time and show the effects of MO or vehicle control (sham-MO) application to the maxillary molar tooth pulp, and subsequent application of MSO or PBS (control) to the face-M1.

* In comparison with baseline, vehicle control (sham MO) or naïve, MO application to the molar tooth pulp resulted within 1 minute, 5 minutes, and 15 minutes, in a significantly increased ICMS threshold intensities for evoking RAD EMG activities (Repeated-measures ANOVA; p < 0.05). ** In comparison with PBS (MO/PBS), MSO application to the face-M1 resulted, within 15 minutes, 30 minutes, and 60 minutes, in a significantly decreased ICMS threshold intensities for evoking RAD EMG activities (Repeated-measures ANOVA; p < 0.05). (ICMS: intracortical microstimulation, RAD: right anterior digastric, EMG: electromyographic, MO: mustard oil, MSO: methionine sulfoximine, PBS: phosphate-buffered saline.)
Fig. 3-4. Mean onset latencies of RAD EMG activities evoked by ICMS (60µA). Mean onset latencies of RAD EMG activities evoked by ICMS (60µA) were compared across all study groups at baseline, upon application of MO or vehicle (sham MO) to the molar tooth pulp, and
upon applying MSO or PBS to the face-M1. MO or vehicle (sham MO) application to the molar tooth pulp did not result in any significant changes in mean onset latencies (Repeated-measures ANOVA; p = 0.86). MSO or PBS application to the face-M1 also did not result in any significant changes in mean onset latencies (Repeated-measures ANOVA; MO/MSO and MO/PBS p = 0.93, sham MO/MSO and sham MO/PBS p = 0.87) (ICMS: intracortical microstimulation, RAD: right anterior digastric, EMG: electromyographic, MO: mustard oil, MSO: methionine sulfoximine, PBS: phosphate buffered saline).
Chapter 4
Discussion

The present study has provided the first documentation that application of the inflammatory irritant MO (but not vehicle control) to the rat’s right maxillary molar tooth pulp results in a transient (12-60 seconds) increased RAD EMG activity that is associated with decreased excitability of the contralateral face-M1, reflected in a significant increase (53.9% within 15 minutes) in the mean ICMS threshold intensities required to evoke RAD EMG activity. The present study has also demonstrated that subsequent topical application of the astrocytic inhibitor MSO (but not vehicle control) around the ICMS site can attenuate the MO-induced effects, reflected in a significant decrease in ICMS threshold intensities that decreased towards baseline levels within 1 hour. In contrast, neither MO application to the molar tooth pulp, nor subsequent MSO application to the face-M1 had any significant effect on the mean onset latencies of RAD EMG activities evoked by ICMS. Together, these novel findings suggest that acute inflammatory dental pain may be associated with decreased face-M1 excitability that is at least partially attributed to intracortical mechanisms dependent on the functional integrity of face-M1 astrocytes. These cortical changes may be related to the mechanisms by which acute dental pain is associated in humans with limited jaw movements. Furthermore, such changes may underlie the mechanisms by which acute pain develops into chronic pain. Therefore, understanding these mechanisms is important for the development of better therapeutic approaches for the treatment of acute pain and the prevention of its development into a chronic pain condition.

1. General features of RAD ICMS-evoked EMG activity within face-M1

At baseline, all rats in all study groups showed similar parameters of ICMS-evoked RAD EMG activity (i.e., threshold intensity and onset latency). The location of face-M1 sites studied coincided with cytoarchitectonic features of the agranular cortex that, consistent with previous studies in rats, were characterized by a lack of a granular layer IV and were associated with ICMS-defined motor representations of jaw and tongue muscles (Avivi-Arber et al, 2010a, 2010b; Donoghue and Wise, 1982; Neafsey et al., 1986; Paxinos and Watson, 1998; Swanson, 2004). These ICMS sites were defined by a short-train ICMS, and consistent with our previous
studies (Avivi-Arber et al., 2010a, 2010b; Adachi et al., 2008) had a low baseline ICMS threshold intensity (< 30µA) for evoking RAD EMG activity.

The baseline onset latencies of ICMS-evoked RAD EMG activities were also consistent with our previous studies (Adachi et al., 2008; Avivi-Arber et al, 2010a, 2010b) and ranged between 8-25 milliseconds (17.5 ± 2.1 milliseconds) at 60µA ICMS. Onset latency is a reflection of several factors including conduction velocity, distance and number of synapses along the projection pathways from the face-M1 to the RAD. The conduction velocity is directly proportional, but not limited, to the diameter of the nerve fibre and its myelination; the largest diameter myelinated fibers are the fastest conducting (Chapman and Yeomans, 1994; Waxman, 1980). Pyramidal tract neurons vary in their diameter and myelination and therefore differ in their conduction velocity. At 60µA, the onset latency reflects conduction through the fastest nerve fibre with the shortest traveling distance and smallest number of synapses. Our findings of a range of onset latencies may suggest that RAD is activated through multisynaptic as well as more direct corticobulbar projections (Grinevich et al., 2005; Jones, 1976; Rathelot and Strick, 2006; Wise and Jones, 1977a; Zhang and Sasamoto, 1990).

We have also found that changing the ICMS intensity can modulate the onset latency of the ICMS-evoked RAD EMG activity. Increased ICMS intensity was associated with shorter onset latencies. Consistent with previous studies, these findings may indicate that RAD was represented within a number of spatially related face-M1 loci (Avivi-Arber et al., 2010a, 2010b; Grinevich et al., 2005; Hatanaka et al., 2005; Miyashita et al., 1994; Zhang and Sasamoto, 1990). Considering the ‘all-or-none’ principle of neuronal firing and muscle fibre contraction, by which the response of a single neuron or muscle fiber is independent of the strength of the stimulus, once the ICMS intensity exceeded threshold intensity, the face-M1 motor-output neuron generated an action potential that propagated to and activated brainstem motor neurons to evoke an RAD EMG response; with more direct corticobulbar projections, the onset latency would have remained more stable and would have been less dependent on the ICMS intensity. However, with multisynaptic corticobulbar projections, increased ICMS intensity could have a larger extent of current spread and therefore could excite more RAD motor-output neurons and could have increased the likelihood to identify the motor output neuron/s with the shortest onset latency. Theoretically, there could have been instances whereby the ICMS site was in a close proximity to a pyramidal tract neuron with a more direct corticobulbar projection/s; in such
cases, the onset latency at threshold and suprathreshold ICMS intensities would have remained constant.

2. Acute Dental Pain Model

MO is a small-fibre excitant and inflammatory irritant which has been found to induce local inflammation and pain and so it has been widely used to induce dental nociceptive afferent inputs into the CNS to study the underlying peripheral and central nociceptive mechanisms in animals (Chiang et al., 1998, 2002, 2005, 2007, 2008, 2010; Park et al., 2001, 2006; Wang et al., 2013 Xie et al., 2007; Zhang et al., 2006). Allyl isothiocyanate, the substance present in MO, produces nociceptive effects (allodynia, hyperalgesia, and pain spread) by activating the TRPA1 channels expressed in sensory neurons (including dental pulp neurons) either directly or through the release of the inflammatory mediator bradykinin (Bandell et al., 2004; Bautista et al., 2006; Jordt et al, 2004; Kim et al, 2007).

3. Acute Dental Pain Associated with Neuroplasticity and Central Sensitization within the Trigeminal Afferent System

MO applied to the molar tooth pulp at volumes and concentrations comparable to those used in the present study can activate primary nociceptive afferents and induce long-lasting (hours) trigeminal neuroplastic changes reflected in ‘central sensitization’ (increased excitability) of nociceptive neurons within the MDH. This central sensitization can be manifested as increased neuronal RF field, decreased activation threshold, and increased responses to noxious and innocuous mechanical stimuli which can contribute to the clinical manifestation of allodynia, hyperalgesia, and pain spread or referral that characterize acute (and chronic) pain conditions (Chiang et al., 1998, 2002, 2005, 2007, 2008, 2010b, 2010c; Sessle, 2011). Noxious stimulation-induced central sensitization has not been restricted to the MDH but also occurs at higher relay centres such as the thalamus and face-S1 by way of projections from the MDH (Park et al., 2006; Sessle, 2000; Zhang et al., 2006).

In humans, brain imaging has revealed that experimental pain activates brain regions analogous to those reported in rats in brain regions implicated in many types of pain conditions including toothache and orofacial pain. For example, noxious electrical stimulation of the tooth pulp results in a strong activation of the S1 bilaterally (Jantsch et al., 2005; Weigelt et al., 2010). Painful
thermal stimulation of the facial skin overlying the masseter muscle is associated with activation of the contralateral S1 (de Leeuw et al., 2006), and injection of hypertonic saline into the masseter muscle is associated with significant increases in regional cerebral blood flow in the contralateral face-S1 (Kupers et al., 2004).

4. Acute Dental Pain and Associated Motor Functions

Human imaging studies have also revealed the involvement of M1 in the pain network (Becerra et al., 1999; Brügger et al., 2011, 2012; de Leeuw et al., 2006). The face-M1 is the main cortical motor output region that plays a crucial role in the initiation and control of orofacial motor functions (Avivi-Arber et al., 2011; Sessle, 2001). Somatosensory inputs (including noxious stimuli) from the orofacial tissues (including the teeth), can also be relayed to the face-M1 either directly through the thalamus or indirectly through the face-S1 (Chakrabarti and Alloway, 2006; Dubner et al., 1978; Hoffer et al., 2005; Iyengar et al., 2007; Miyashita et al., 1994; Rocco-Donovan et al., 2011). These sensory inputs to the face-M1 provide important feedback and feedforward information that can assist (or interfere) in modulating orofacial motor outputs. Therefore, it is possible that in the present study, some of the changes observed within face-M1 and described in the following sections, were the result of altered sensory transmission within face-S1 or subcortical relay stations (see sections below).

4.1 Increased RAD EMG Activity

Consistent with previous studies (Narita et al., 2012; Sunakawa et al., 1999), we have found that acute noxious stimulation induced by MO application to the rat maxillary molar tooth pulp can result in a transient increased EMG activity in the AD muscles that can last 12-60 seconds. These findings are consistent with previous studies showing that MO application to the molar tooth pulp of anaesthetised rats can activate neurons in the MDH, which have projections to brainstem cranial motor nuclei, including the trigeminal motor nucleus, the motor neurons of which supply the jaw muscles (Dostrovsky 2006; Sessle, 2006, 2009). Thus, MO application to the tooth pulp can activate a brainstem circuit that can result in a reflexive transient increase in EMG activity in jaw muscles, including the AD. Such increased muscle activity may have clinical implications as it may facilitate nocifensive escape behaviour to protect the injured tissue.
4.2 Decreased Face-M1 Excitability

In the present study, we have found that acute noxious stimulation induced by MO application to the rat maxillary molar tooth pulp results, within 15 minutes, in decreased face-M1 excitability that can last for at least 1-2 hours. This finding is consistent with previous studies in rats and humans whereby acute noxious stimulation of other orofacial tissues (e.g., tongue or jaw muscles) can induce decreased face-M1 excitability. For example, glutamate infusion into the tongue of anaesthetized rats has been shown to result in decreased tongue-M1 excitability (Adachi et al., 2008). Topical application of capsaicin to the tongue in humans results in decreased ability to perform a novel tongue protrusion-task that is associated with decreased tongue-M1 excitability (Boudreau et al., 2007, 2010). A fMRI study in humans has shown that experimental pain induced by injection of hypertonic saline into the masseter muscle results in an initial transient increase in fMRI signal intensity, followed by a continuous decrease in signal intensity within the face-M1 (Nash et al., 2010). These findings have clinical significance and point to the involvement of face-M1 in modulating early as well as late pain-related alterations in orofacial motor functions. It can be speculated from the present and earlier studies that the initial increases in fMRI-defined M1 activity and muscle activity as a result of noxious orofacial stimulation may underlie the nocifensive escape-like motor patterns, but once the initial threat subsides, longer-term reductions in M1 activity may occur to minimize jaw movement and further tissue damage (Nash et al., 2010; Sae-Lee et al., 2008; Stohler, 1999; Svensson and Graven-Neilsen, 2001).

It is noteworthy that another study has demonstrated that tongue-M1 excitability in rats is not affected by hypertonic saline infusion into the tongue (Murray et al., 2006), and a TMS study in humans has shown that neither hypertonic saline-evoked masseter muscle pain nor capsaicin-evoked cheek skin pain is associated with any changes in human jaw-M1 excitability (Romaniello et al., 2000). Moreover, in contrast to the findings of Boudreau et al., 2010 (see above), capsaicin application to the tongue has been reported to have no effect on human tongue-M1 excitability (Halkjaer et al., 2006). These differences in findings of the effects of experimentally induced acute orofacial pain on face-M1 excitability may be explained by differences in methodological features or approaches; e.g., the pre-contraction of the masseter muscle required in the Romaniello study may have masked any inhibitory effect associated with pain (Halkjaer et al., 2006; Le Pera et al., 2001). It is also possible that experimental pain may
differentially modulate M1 excitability depending on the type of noxious stimulus and the orofacial tissue involved.

Studies focussing on limb pain and limb-M1 indicate that limb pain may also be associated with differing and complex effects on M1 excitability. In humans, decreased limb-M1 excitability has been shown to be associated with capsaicin-induced skin pain (Cheong et al., 2003; Farina et al., 2001), hypertonic saline-induced muscle pain (Le Pera et al., 2001; Svensson et al., 2003) as well as with noxious thermal stimulation of the hand (Dube and Mercier, 2011). In contrast, noxious electrical stimulation of the finger has been reported to induce increased limb-M1 excitability for distal hand muscles, and simultaneous decrease in limb-M1 excitability for proximal upper arm muscles (Kofler et al., 1998). More studies are needed to clarify the effects of different types of noxious stimuli to different orofacial tissues on the excitability of the face-M1.

4.2.1 Mechanisms Underlying Changes in Face-M1 Excitability

Although studies have examined neuroplasticity of limb-M1 as well as vibrissal-M1, few studies have examined the mechanisms underlying face-M1 neuroplasticity. Therefore, we can only speculate on the mechanisms underlying our findings.

Consistent with other acute orofacial pain studies in animals and in humans (Adachi et al., 2008; Boudreau et al., 2007, 2010), the present study has shown that acute noxious stimulation induced by MO application to the rat maxillary molar tooth pulp may be associated with short-onset (seconds-minutes) and long-lasting (hours) increases in ICMS threshold intensities for evoking RAD EMG activity. We have also shown for the first time that the MO-induced face-M1 changes can be attenuated by subsequent application of the astrocytic inhibitor MSO directly to the face-M1. These novel findings suggest that the observed changes may be, at least in part, the result of excitability changes within the face-M1 and that mechanisms involving non-neural (glial) cells may have contributed. Noteworthy is that MSO application to the MDH can also modulate the MO-induced decreased face-M1 excitability (Pun et al., 2013) pointing to the role of the MDH astrocytes in modulating face-M1 excitability. Therefore, we cannot rule out the possibility that these changes also reflect excitability changes in subcortical relays of descending motor output (e.g., trigeminal brainstem motor nuclei) or changes in other cortical (e.g., S1) and subcortical (i.e., thalamus, brainstem) relays of ascending somatosensory information to the face-M1 (Dubner et al., 1978; Kaas et al., 2008; Katz et al., 1999; Landgren and Olsson, 1976; Lund
et al., 1984; Sessle et al., 1976; Sunakawa et al., 1999; Yu et al., 1995). The following paragraphs discuss our novel findings and possible mechanisms underlying M1 excitability.

4.2.1.1 Face-M1 Architecture

Several features of the M1 neural (and non-neural) network may contribute to the substrate for altered cortical excitability. The face-M1 has an extensive network of excitatory and inhibitory neural connections. Excitatory connections are characterized by monosynaptic connections of pyramidal axon collaterals projecting horizontally for several millimetres (for reviews, see Keller and Asanuma, 1993; Mountcastle, 1997; Schieber, 2001). Neighbouring excitatory regions of M1 are connected via inhibitory interneurons. Inhibitory interneurons constitute about 30% of M1 neurons and they form a horizontal network of inhibitory connections that play an important role in modulating M1 motor outputs (Jones, 1993). Within M1, the functional organization of skeletal muscles is characterized by spatial contiguity of sensory inputs and motor outputs (for reviews, see above and Sanes and Donoghue, 2000; Sanes and Schieber, 2001; Sessle et al., 2007; Tehovnik et al., 2006). The face-M1 receives a large amount of peripheral somatosensory inputs either directly from the thalamus including the thalamic motor nuclei (Aldes, 1988; Cicirata et al., 1968a, 1968b; Dubner et al., 1978; Hatanaka et al., 2005; Iyengar et al., 2007; Miyashita et al., 1994), or indirectly from the face-S1 through extensive intra-and inter-cortical excitatory and inhibitory neural connections (Chakrabarti and Alloway, 2006; Hoffer et al., 2005; Miyashita et al., 1994; Rocco-Donovan et al., 2011). As discussed below, these somatosensory inputs can play a crucial role in modulating motor outputs in response to changes in somatosensory inputs.

4.2.1.2 Changes in Ascending Somatosensory Relay Stations

As described earlier (section 2 of this chapter, and chapter 1), experimental acute noxious stimulation of the dental pulp has been associated with neuroplastic changes at different trigeminal nociceptive relay regions including the trigeminal brainstem sensory nuclei (Chiang et al., 1998, 2002, 2005, 2007, 2008, 2010b, 2010c; Okada-Ogawa, 2009; Sessle, 2011; Tsuboi, 2011), thalamus (Katz et al., 1999; Park et al., 2006; Zhang et al., 2006) and face-S1 (de Leeuw et al., 2006; Jantsch et al., 2005; Kupers et al., 2004; Weigelt et al., 2010). Since face-M1 receives somatosensory inputs from the face-S1 and thalamus, in the present study, drilling and MO application to the tooth pulp caused peripheral injury and inflammatory irritation that could
have resulted in a sustained increase in excitatory inputs to the thalamus and S1 and consequently sustained inhibitory inputs to the face-M1. Such changes in somatosensory inputs could have increased the release of inhibitory neurotransmitters and through long-term depression induced decreased synaptic efficacy and decreased face-M1 excitability (Jones, 1993; Monfils and Teskey, 2004a; Teskey et al., 2007). Therefore, decreased face-M1 excitability could have impeded the ability to evoke motor responses by ICMS of face-M1 sites that previously could have been excited by low ICMS threshold intensities and thus, ICMS required higher intensity currents to evoke a motor response.

4.2.1.3 Changes in Descending Motor Relays

ICMS of M1 evokes EMG responses through activation of brainstem motor neurons. However, many of the corticobulbar projections are multisynaptic pathways involving several subcortical relay stations (e.g., basal ganglia and red nucleus) (Haque et al., 2010; Hatanaka et al., 2005) and therefore, any changes in subcortical synaptic efficacy could have contributed to the changes observed within face-M1 excitability in the present study. In addition, somatosensory inputs can also reflexly modulate the activity of brainstem motor neurons controlling the RAD muscle (Narita et al., 2012; Sunakawa et al., 1999). Therefore, we cannot rule out the possibility that MO induced changes at other subcortical or cortical areas and that the observed changes in face-M1 excitability were, at least in part, a reflection of altered subcortical synaptic efficacy. Although we did not test excitability changes in brainstem motor nuclei, subcortical changes in synaptic efficacy are less likely since altered subcortical synaptic efficacy can be expected to be associated with an altered onset latency of ICMS-evoked RAD EMG activity (Asanuma et al., 1976; Butovas and Schwarz, 2003; Ranck, 1975; Ridding and Rothwell, 1997; Stoney et al., 1968b; Tehovnik et al., 2006), and in the present study, no changes were observed in the mean onset latencies of RAD following MO application to the molar tooth pulp. Furthermore, it is also possible that statistical analysis failed to reveal any significant changes in onset latency due to the large inter-subject variability; future studies using a larger sample size might reveal significant differences in onset latency.
Role of Astrocytes in Modulating Face-M1 Excitability

The present study has shown that acute noxious stimulation of the molar tooth pulp produces a significant decrease in face-M1 excitability that can be reversed toward baseline within 1 hour following application of the astrocytic inhibitor MSO to the face-M1. This finding suggests that astrocytes may be involved in the mechanisms determining face-M1 excitability.

An important feature of the face-M1 architecture and the nervous system in general is the close morphological relations between neural synapses and astrocytes. Although the astrocyte network is spread throughout the cortical layers I-VI, different types of astrocytes are present at different cortical layers. Astrocytes are not just immunological and metabolic supporters for neuronal function. By virtue of their close spatial relationship with neural networks, their receptors for several mediators (e.g., glutamate, GABA, Ach, ATP, cytokines), their ability to release and remove excitatory (e.g., glutamate) and inhibitory (e.g., GABA) neurotransmitters to or from the extracellular space, and actions involved in regulating these neurotransmitters, astrocytes can sense and modulate neuronal activity and thereby may play a role in modulating face-M1 excitability (Giaume et al., 2010; Haydon and Carmignoto, 2006; Iadecola and Nedergaard, 2007; Kang et al., 2008; McMahon and Malcogno, 2009; Parpura and Zorec, 2010; Verkhratsky and Butt 2013).

Processes specific to astrocytes are the glutamate/glutamine shuttle (or cycle) and the GABA/glutamate/glutamine shuttle (Chiang et al., 2011; Danbolt, 2001; Hertz and Zielke, 2004, Kanamori and Ross, 2006; Verkhratsky and Butt 2013) (See introduction, Fig.1-1). Glutamate is the main excitatory neurotransmitter in the CNS and is also a precursor of GABA, the main inhibitory neurotransmitter in the CNS. The majority of glutamate and GABA in the synapses is taken up by astrocytes. Within the astrocytes, GABA is metabolized to glutamate. Glutamate is then converted by the enzyme glutamine synthetase to glutamine. Glutamine is then transported back to the excitatory neurons where it is converted by mitochondrial glutaminase to glutamate. In inhibitory neurons, glutamate is decarboxylated by glutamic acid decarboxylase to form GABA. Therefore, inhibition of the shuttle at inhibitory synapses will rapidly decrease neuronal GABA production, deplete pre-synaptic GABA, and thus reduce inhibitory transmission. On the other hand, inhibition of the shuttle at excitatory synapses will result in decreased excitatory transmission.
It has recently been shown that in comparison to naïve or sham rats, rats receiving orthodontic tooth movement or molar teeth extraction show significant face-M1 neuroplasticity manifested as decreases in jaw or tongue motor representations (25-50%) that could last up to 28 days (Varathan et al., 2014). In a series of subsequent pilot studies, it has been shown that compared with control, orthodontic tooth movement or molar teeth extraction are associated with significant (15-50%) increases in both astroglial and microglial labelling within the face-M1 (Varathan et al., 2014). In the case of MO application to the molar tooth pulp, a pilot study in 2 rats has revealed that within 30 min of MO application to the tooth pulp there is an increased GFAP (astrocytic) but mainly IBA1 (microglia) immunoreactivity within layer V of the contralateral face-M1. In another study, transection of the facial motor nerve in adult rats has been associated with increased activation of astrocytes in layers I/II and III/V of the M1 as early as one hour after the nerve transection and could last for 2-5 hours (Laskawi et al., 1997). These studies reveal that several different types of orofacial manipulations can induce neuroplastic changes in face-M1 which may be related to how humans adapt their orofacial sensorimotor behaviours in clinically related situations involving the teeth. These studies also suggest that glial cells may be integrally involved in the mechanisms underlying the induction and consolidation of these face-M1 neuroplastic changes. These studies are also consistent with a study whereby intraplantar application of CFA results in peripheral inflammation and hypersensitivity that is associated with astrocytic activation within the cortical forebrain. However, this activation occurs only at the subacute phase (i.e. at 4 days) and is preceded by an earlier (at 4hrs) microglial activation supporting a view that microglia play a role in the initiation phase and astrocytes in the maintenance of the limb hypersensitivity (Raghavendra et al., 2004).

In the present study MSO application to the face-M1 attenuated the MO-induced decrease in face-M1 excitability. MSO is a potent inhibitor of glutamine synthetase which is present exclusively in astrocytes (Miyake and Kitamura, 1992; Norenberg and Martinez-Hernandez, 1979). Inhibition of glutamine synthetase can alter the levels of several neurochemicals including glutamine, glutamate, and GABA (Hertz and Zielke, 2004) and consequently affect neuronal activity. In the present study, MSO was applied to the superficial face-M1 area surrounding the microelectrode so the extent of its absorption into deep layers is unknown. It has been shown that transection of the facial motor nerve in adult rats can result in increased activation of astrocytes in layers I/II and III/V of the M1 as early as one hour after the nerve transection and can last for
2-5 hours (Laskawi et al., 1997). Environmental enrichment can lead to a significantly increased number of astrocytes within layer I of limb-M1 (Ehninger and Kempermann, 2003). Cortical layer I contains prominent horizontal projections of axons originating from multiple cortical (including S1) and subcortical regions and synapsing on apical dendrites of layer III and V pyramidal cells. Layer III is the primary origin and termination of intracortical connections including between S1 and M1 and both layers I and in particular layer III contain GABA
ergic interneurons (Douglas and Martin, 2004). Thus, MSO does not necessarily have to penetrate the deeper cortical layers to exert its effect.

Therefore, one possible explanation for the MO-induced decreased face-M1 excitability and the subsequent reversal of the MO-induced effects by an astrocytic inhibitor is that acute noxious stimulation of the rat maxillary molar tooth pulp results in increased and sustained sensory inputs to the face-S1 that are relayed to the face-M1 through intracortical neurons including those within the superficial layer I. Astrocytes and neurons form the so-called tripartite synapse constructed from a pre-synaptic terminal, a post-synaptic neuronal membrane and surrounding astrocyte processes. Astrocytes can distinguish the intensity of neuronal activity through changes in astrocytic Ca2+ oscillations that are frequency encoded; the frequency can increase following an increase in synaptic activity (Verkhratsky and Butt, 2013). GABA released from the GABA
ergic (inhibitory) pre-synaptic terminal can bind to specific receptors located in both the post-synaptic neuronal membrane and the astrocytic membrane. This triggers changes in the frequency of astroglial Ca2+ oscillations resulting in the release of more glutamine from astrocytes that is transported to the inhibitory neuron to produce more GABA. Increased frequency of Ca2+ oscillations can cause activation of neighbouring astrocytes and shuttling of more glutamine from astrocytes to synapses of GABA
ergic (inhibitory) interneurons. Subsequently, as a result of increased GABA synthesis, the increased inhibition of synaptic transmission results in decreased face-M1 excitability. Within such a process, MSO application to the face-M1 can block the GABA-glutamate-glutamine shuttle in the reactive astrocytes and result in depletion of synaptic glutamine, decreased GABA synthesis and disinhibition (i.e., increase) of neuronal activity, thereby attenuating the MO-induced decrease in face-M1 excitability (Jenstad et al., 2009; Ortinski, 2010; Solbu et al., 2010; Verkhratsky and Butt 2013).

The present study has found that MSO application to the face-M1 significantly attenuated the MO-induced decrease in face-M1 excitability. In contrast, neither vehicle application to the
molar tooth pulp nor subsequent application of MSO to the face-M1 has any significant effects on the ICMS thresholds intensities for evoking RAD EMG activity. These findings are in line with previous studies of acute (MO-induced) or chronic orofacial pain models that have shown that comparable concentrations of MSO interfered with central sensitization occurring in MDH nociceptive neurons but did not affect the basal nociceptive neuronal properties within the MDH (Chiang et al., 2007; Tsuboi et al., 2011; Xie et al., 2007). These findings suggest that MSO action on astrocytic glutamine synthetase has no effect on baseline properties of neurons but once injury occurs and astrocytes become activated (reactive) they provided inhibitory effects on neighbouring neurons, and it is at this stage that inhibition or reversal of astrocyte activation by MSO can attenuate or reverse the effects of astrocytes on neighbouring neurons.

It should be noted that several published studies focusing on other brain regions (e.g., hippocampus) have shown that at physiological conditions doses (1.5 mM) that are much higher than those used in the present study (0.1 mM) can disrupt GABAergic but not glutamatergic synaptic function and induce gliosis (Fricke et al., 2007; Liang et al., 2006; Yang and Cox, 2011).

5. Clinical Implications

Acute dental pain is one of the most prevalent orofacial pain conditions and is a major reason why patients seek dental care (Jain et al. 2013; Lipton et al. 1993; Sessle 2005; Wall and Melzack, 2013). One of the most common clinical signs and a main complaint in acute and chronic pain conditions is orofacial motor dysfunction (Huge, 2011; Sessle, 2000). Furthermore, in some patients, acute orofacial pain can be transformed into a chronic orofacial pain condition (Gregg, 2000; Ji et al., 2013; Marbach, 2000; Nixdorf, 2010) and novel treatment approaches put a large emphasis on motor function including M1 stimulation as a treatment modality in several chronic pain conditions (Leo et al., 2007). Therefore, a better understanding of the face-M1 mechanisms underlying acute dental pain and its effects on orofacial sensorimotor functions is important for the development of improved treatment strategies that not only effectively alleviate acute dental pain, but can also prevent the development and maintenance of chronic pain and associated motor dysfunctions.

Consistent with previous animal and human studies and in support of the ‘Pain Adaptation Model’, in the present study, the decreased face-M1 excitability induced by noxious stimulation
of the rat dental tooth pulp may explain clinical manifestation of limited jaw movement as a protective mechanism to prevent further tissue injury in patients suffering from acute (and chronic) orofacial pain conditions (Lund et al., 1991; Murray and Peck, 2007; Sae-Lee et al., 2008; Stohler, 1999; Svensson and Graven-Neilsen, 2001).

Although differences exist between rodent and human glial cells (Smith and Dragunow, 2014; Verkhratsky and Butt, 2013), our novel findings that an astrocytic inhibitor applied directly to the face-M1 can reverse the decreased face-M1 excitability induced by noxious stimulation of the rat dental tooth pulp may provide important insights into the potential role of astrocytes in modulating face-M1 excitability in acute orofacial pain conditions. Future research is suggested to focus on astrocytes as a potential therapeutic target for the management of acute orofacial pain and the prevention of its development into a chronic pain state.

6. Study Limitations and Future Directions

ICMS is considered to be an appropriate technique for investigating face-M1 excitability (Adachi et al., 2008). Nevertheless, ICMS parameters such as cortical depth at which ICMS is applied, level of consciousness of the animal, as well as previous stimulations can all have an effect on the features of the ICMS-evoked EMG activity and therefore may influence face-M1 excitability (Asanuma, 1989; Graziano et al., 2002b; Greenshaw, 1998; Neafsey et al., 1986; Sessle and Wiesendanger, 1982; Tandon et al., 2008; Tehovnik et al., 2006). Accordingly, attempts were made to minimize potential variability related to these parameters. Similar stimulation parameters were utilized in all experiments and in all rats the administration of general anaesthetic was carefully regulated and maintained at a stable level that allowed for EMG activity to be evoked by relatively low ICMS intensities. A histological check was also completed to verify that the repetitive stimulations at the set ICMS intensities did not cause any cortical damage. Comparable experimental conditions as well as general anaesthesia levels were also applied to all study groups to ensure comparability of data collected across the study groups.

The gradual increase in ICMS threshold intensities for evoking RAD EMG activity after 1 hour following MSO application to the face-M1 may reflect a gradual buildup of general anesthetic, and/or a deterioration in the ICMS microelectrode and/or damage to the cerebral cortical tissue as a result of the repeated ICMS.
Whether MSO spread was limited to face-M1 region around the microelectrode penetration site cannot be determined with certainty. However, a vaseline dam was applied around the ICMS microelectrode to limit the spread of MSO and to ensure that it was contained to the face-M1 region adjacent to the microelectrode penetration site.

The use of only male rats precluded any assessment of possible sex differences that have been demonstrated in the sensory and motor effects of orofacial noxious stimuli in rats and humans (Cairns et al., 2001, 2002, 2003; Komiyama et al. 2005). Future studies could make use of both male and female rats to investigate possible sex differences in the effects of noxious stimulation of the tooth pulp on face-M1 excitability and the effects of MSO.

MSO (0.1-0.3 mM) application to cortical slices in vitro has been shown to induce glutamate and glutamine release that produced a sustained cortical neuronal depolarization which could be partially blocked by an NMDA antagonist (Albrecht and Norenberg, 1990; Shaw et al., 1999; Zielinska et al., 2004). Thus, it is possible that the MSO effects on face-M1 excitability that were documented in the present in vivo study were caused by direct action of MSO on face-M1 neuronal excitability. However, only a low dose of MSO (0.1mM) was used in the present study, and the lack of any observed significant effects of MSO on the basal physiologic face-M1 excitability, in contrast to the findings that it significantly attenuates MO-induced decrease in face-M1 excitability, may suggest that MSO acted indirectly by inhibiting astrocytes and not by directly acting on face-M1 neurons.

The present study investigated the effect of MSO on face-M1 excitability following MO application to the molar tooth pulp. Future studies could investigate whether MSO application to the face-M1 prior to MO application to the molar tooth pulp would modulate face-M1 excitability by blocking the MO-induced changes in face-M1 excitability.

In the MDH, central sensitization may be associated with microglial as well as astrocytic function, and administration of microglial or astrocytic inhibitors has been shown to attenuate central sensitization (Chessell et al., 2005; Chiang et al., 2005, 2007, 2008, 2010b, 2010c, 2011; Li et al., 2008; Lo et al., 2008; Meme et al., 2004, 2006; Milligan and Watkins, 2009; Tsuda et al., 2003; Watkins et al., 2001 Xie et al., 2007). In the cortex, microglia have been shown to undergo activation following CIP (Huber et al., 2006). Studies also demonstrate that microglia in the forebrain undergo robust activation following CFA-induced peripheral inflammation.
(Raghavendra et al., 2004). In the present study, we only explored the potential role of face-M1 astrocytes in modulating face-M1 excitability following noxious stimulation of the tooth pulp with MO. Future studies could investigate whether microglia may also play a role in modulating face-M1 excitability in this as well as other orofacial pain models.

In a recent pilot study in 2 rats, we have shown that within 30 min of MO application to the molar tooth pulp there was an increased GFAP (astrocytic) immunoreactivity within layer V of the contralateral face-M1 (Varathan et al., 2014). It has been documented that GFAP does not label all astrocytes and often reveal only their major processes and within the cortex only 15-20% of the astrocytes show GFAP immunoreactivity (Verkhratsky and Butt 2013). Future studies with a larger number of animals and different postoperative time points could investigate immunohistochemical labelling of cortical microglia as well as astrocytes at different cortical layers and thereby add further insights into differential time-dependent role of face-M1 glial cells in face-M1 changes occurring in orofacial pain models.

Future studies could investigate whether noxious stimulation of orofacial tissues has effects on other muscle (LAD, GG, limb) representations in M1 to determine how specific the MO effects are to the RAD, or if they occur in other muscle representations. Future studies could also investigate whether noxious stimulation of orofacial tissues has effects on face-S1 sensory input or motor output sites (Avivi-Arber et al., 2010a, 2010b) that are comparable to or different from its effects on face-M1 documented in the present study, and whether subsequent application of MSO has an effect on face-S1 excitability and the potential role of face-S1 astrocytes in any face-S1 changes.

Future studies are planned to investigate the potential role of microglia and astrocytes in chronic orofacial pain models and whether they play a role also in the mechanisms underlying acute pain transformation into chronic pain.

7. Conclusions

The present study represents the first documentation that: 1) Acute noxious stimulation of the dental pulp is associated with decreased face-M1 excitability; 2) This face-M1 excitability change is dependent on the functional integrity of face-M1 astrocytes; 3) The excitability
changes may be mediated by intracortical mechanisms but may also involve subcortical mechanisms.

These novel findings provide a new perspective on the mechanisms by which the function of face-M1 may be influenced in pain conditions and suggest that face-M1 astrocytes may present important influences in the control of sensorimotor behaviours mediated by face-M1. These findings thereby point to future research directions to address the role of face-M1 astrocytes and microglia in the control of sensorimotor functions in normal and pathological conditions and offer glial cells as a new and promising target for improved therapeutic intervention and prevention of acute and chronic pain conditions.
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