GENE EXPRESSION CHANGES IN THE BRAINSTEM IN A MOUSE MODEL OF OROFACIAL PAIN

DR LUTFUN NAHAR

NATIONAL UNIVERSITY OF SINGAPORE

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DEDICATION

This thesis is dedicated to my sister and my parents and my parents-in-laws and my family who were always by my side giving me endless support throughout my candidature.

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DECLARATION

I hereby declare that this thesis is original and does not contain any material which has been submitted previously for any other degree or qualification.

DR LUTFUN NAHAR

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SUMMARY

The present study was carried out to examine possible gene expression changes that occur in the brainstem in a mouse facial carrageenan injection model of orofacial pain. Mice that received facial carrageenan injection showed increased mechanical allodynia, demonstrated by increased responses to von Frey hair stimulation of the face. The brainstem was harvested at 3 days post-injection, corresponding to the time of peak responses, and analyzed by Affymetrix Mouse Genome 430 2.0 microarrays. Large number of genes were up or down regulated in the brainstem after carrageenan injection, but the number of genes that showed common change after right or left sided facial carrageenan injection were relatively small. The common genes were then classified and analysed by using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis et al., 2003). Most of them were upregulated and the largest group of genes was in the category of "host defence genes against pathogens". These include chemokine, inflammation related, and endothelial related genes. Of these, increased expression of P-selectin, ICAM-1 and CCL12 after carrageenan injection could be verified by real-time RT-PCR on both the right and left sides, and the increases in P-selectin and ICAM-1 further verified by Western blot analysis and immunohistochemistry. CCL12 is closely related to human MCP-1/CCL2 in structure and may contribute to a signalling system that might cause neuronal hyperexcitability. ICAM-1 is an immunoglobulin like cell adhesion molecule that binds to leukocytes. It recruits immunocytes containing opioids to facilitate the local control of inflammatory pain. P-selectin is a marker for platelet activation and endothelial dysfunction. P-selectin mediates the capturing of leukocytes from the blood stream and rolling of leukocytes along the endothelial surface. It is hypothesize that increased nociceptive input to the

brainstem could attract circulating macrophages into the brain, resulting in neuroinflammation and pain. The present findings suggest that CCL12, ICAM-1, and P-selectin may play a role in orofacial pain.

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ABBREVIATIONS

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ANOVA	Analysis of varience
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
CCL12	Chemokine (C-C motif) ligand-12
CCL2	Chemokine (C-C motif) ligand -2
CCL-5	Chemokine (C-C motif) ligand-5
CCR	Chemotactic cytokine receptor
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX- 2	Cyclooxygenase-2
DAB	Diamino benzidine tetra hydrochloride
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetraacetic acid
IASP	International Association for the Study of Pain
ICAM-1	Intercellular adhesion molecule- 1
IgG	Immunoglobulin G
IL -1b	Interleukin-1b
IL-6	Interleukin-6

αLβ2	Alpha L beta 2
LFA-1	Lymphocytes function- associated antigen-1
MAC-1	Membrane attack complex type-1
MARK	Mitogen-activated protein kinase
MCP	Monocytes chemoattractant protein
mRNA	messenger ribo-nucleic acid
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NS	Nociceptive specific
PBS- TX	Phosphate buffered saline – triton
PCGEM	Parametric test based on cross gene error model
PG	Prostaglandin
PKC	Protein kinase C
PSGL-1	P-selectin glycoprotein ligand-1
PVDF	Polyvinylidene difluoride
qPCR	Quantatitive polymerase chain reaction
RT-PCR	Real-time polymerase chain reaction
Slep	P- selectin
SP	Substance P
TBS	Tris buffered solution
TNF-alpha	Tumour necrosis factor – alpha
VBSNC	Trigeminal brainstem sensory nucler complex

VCAM	Vascular cell adhesion molecule
WDR	Wide dynamic range

LITERATURE REVIEW

PAIN

Pain is defined by the “International Association for the Study of Pain” (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". The World Health Organisation has defined pain as “an unpleasant sensory or emotional experience associated with actual or potential tissue damage, or described in term of such damage (Last updated Oct 19, 2007).

So as a brief, pain can be defined as an unpleasant sensation that can range from mild, localized discomfort to agony. Pain has both physical and emotional components. The physical part of pain results from nerve stimulation. Pain may be contained to a discrete area, as in an injury, or it can be more diffuse, as in disorders like –fibro myalgia (Cimen et al., 2009).

It is a major symptom in many medical conditions, which significantly interferes with a person’s quality of life and general functions. This is a subjective experience, one difficult to measure or quantify but one having great interest regarding which therapy should be applied as well as its effectiveness (Garralda and Saez, 2009).

According to duration, intensity, type (dull, burning, or stabbing), source, or location in the body, pain can be characterized in various ways. Diagnosis of the diseases also depends on the pain characters. The pain which is immediate and short in duration, and mostly results from disease, inflammation, or injury to tissues, is known as acute pain. Chronic pain is continuous pain that persists and beyond the time of normal healing. It ranges from mild to severe and can last for weeks, months,

or years to a life time. Studies have shown that the pathophysiology of chronic pain shows alterations of normal physiological pathways, giving rise to hyperalgesia or allodynia (Riedel and Neeck, 2001)

The study of pain has in recent years attracted many different fields such as pharmacology, neurobiology,dentistry etc. Pain medicine is now a separate subspecialty figuring under some medical specialties like anaesthesiology and neurology.

NOCICEPTION

Nociception refers to the noxious stimulus originating from the sensory receptor. This information is carried into the central nervous system (CNS) by the primary afferent neuron.

Pain sensation is perceived in the cortex, usually as a result of incoming nociceptive input. Nociceptive input does not always relate closely to pain. CNS has the ability to alter or modulate nociceptive input before it reaches the cortex for recognition. Modulation of nociceptive input can either increase or decrease the perception of pain (Okeson, 2005).

A recent Study has shown that the physiology of nociception involves a complex interaction of peripheral and central nervous system structures, extending from the skin, the viscera and the musculoskeletal tissues, then integration in the spinal cord and information is transferred to thalamus before reaches to the somatosensory (cerebral) cortex (Riedel and Neeck, 2001). The same study also shows that modulation of nociception occurs at all levels of the neuraxis. The N-methyl-D-

aspartate (NMDA) and opioid receptor systems are the two most important systems for the modulation of nociception. Moreover, antinociception show a close distribution pattern in nearly all CNS regions, and activation of NMDA receptors has been found to contribute to the hyperalgesia associated with nerve injury or inflammation (Riedel and Neeck, 2001).

The afferents that terminate in the spinal trigeminal nucleus contain neuropeptides and amino acids (such as, SP, glutamate), and the gas nitric oxide are the excitatory neurotransmitters in central nociceptive transmission (Sessle, 2000)

PAIN HYPERSENSITIVITY

Increased sensitivity of pain pathways is known as pain hypersensitivity. Two mechanism are known to be in pain hypersensitivity- peripheral and central sensitization**.** Sensitization here means an increase in the excitability of neurons, thereby becoming more sensitive to stimuli or sensory inputs.

PERIPHERAL SENSITIZATION

Peripheral sensitization is a reduction in threshold and an increase in responsiveness of the peripheral ends of nociceptors, the high-threshold peripheral sensory neurons that transfer input from peripheral targets such as skin, muscles, joints and the visceras, though peripheral nerves to the CNS (Woolf and Scholz, 2000)

Around the site of tissue damage or inflammation, sensitization arises due to the action of inflammatory chemicals or mediators, such as ATP, can directly activate the ends of the peripheral nociceptors, signalling the presence of inflamed tissue and

producing pain (Woolf et al., 2001).A recent study shows that peripheral inflammation increased the synaptic expression of NMDA receptors in the dorsal horn of the spinal cord (Yang et al., 2009).

CENTRAL SENSITIZATION

Central sensitization is an increase in the excitability of neurons within the central nervous system, so that normal inputs begin to produce abnormal responses.

Central sensitization also has two phases:

- An immediate but relatively transient phase, which depends on changes to existing proteins, and
- A slower onset but longer-lasting phase, which relies on new gene expression.

The early phase reflects changes in synaptic connections within the spinal cord, after a signal has been received from nociceptors. The central terminals of the nociceptors release a host of signal molecules, including the excitatory amino acid synaptic transmitter glutamate, neuropeptides (SP and calcitonin gene-related peptide, CGRP) and synaptic modulators including brain-derived neurotrophic factor

(BDNF) (Woolf, 2000).

It is likely that NMDA receptors play a role in central sensitization. Influx of calcium ions through the NMDA receptor could result in increased activation of calcium dependent kinase, resulting in increased phosphorylation of AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptors, and increased efficacy of synaptic transmission between primary and secondary neurons in the pain pathway, resulting in hyperalgesia. Central sensitization might also be due to changes in AMPA

receptors density on the post-synaptic membrane or increased synaptic contacts between primary and secondary neurons in the nociceptive pathway (Woolf and Thompson, 1991).

HYPERALGESIA

Hyperalgesia is an increased sensitivity (increased responsiveness) to pain, whereby noxious stimuli produce an exaggerated and prolonged pain which may be caused by damage to nociceptors or peripheral nerves.

Primary hyperalgesia describes pain sensitivity that occurs directly in the damaged tissues. Secondary hyperalgesia describes pain sensitivity that occurs in surrounding undamaged tissues.

Primary hyperalgesia is characterized by the presence of enhanced pain to heat and mechanical stimuli, whereas secondary hyperalgesia is characterized by enhanced pain to only mechanical stimuli. The changes responsible for secondary hyperalgesia have two different components:

(I) A change in the modality of the sensation evoked by low – threshold mechanoreceptors, from touch to pain – this is known as allodynia. And

(II) An increase in the magnitude of the pain sensation evoked by mechanical sensitive nociceptors (LaMotte et al., 1991; Cervero et al., 1994).

Nociceptors sensitization and central sensitization are considered to underlie the development of primary hyperalgesia and secondary hyperalgesia, respectively (Urban and Gebhart, 1999). Increased release of SP from primary afferents (Otsuka and Yanagisawa, 1987, McCarson and Krause 1996) and increased expression of the substance P receptor, neurokinin-1 in the dorsal spinal cord have been reported after peripheral inflammation in rats and mice (Allen et al., 2003). SP enhances glutamate-

and NMDA- induced activities in spinal cord dorsal horn neurons (Liu et al., 1997). In addition, glutamate, acting at a spinal NMDA receptor has itself been shown to be involved in the development of secondary hyperalgesia (Jang et al., 2004). NMDA receptor activation also induces the expression of the immediate early genes c-fos which, in turn, could lead to changes in the expression of other genes (Ro et al., 2007), such as those involved in the production of NOS or PKC which are implicated in the maintenance of hyperalgesia(Urban and Gebhart, 1999).

The peripheral mechanism of hyperalgesia is considered to be the result of nociceptors sensitization. In injured tissue bradykinin, histamine, prostaglandin (PG), protons and nerve growth factor are released, which are possible agents causing nociceptor sensitization, since blocking of these agents suppresses sensitization.

Secondary hyperalgesia differs from primary hyperalgesia in important ways. The zone of secondary hyperalgesia describes the region immediately surrounding the injured tissue but does not include the injured tissue. Any change in pain sensation in this region must be due to sensitization spreading from the zone of injury or to changes in processing in the CNS.

Central sensitization plays a major role in secondary hyperalgesia. Many of the insight acquired about secondary hyperalgesia have been gained from studies with capsaisin. Capsaisin is a naturally occurring vanilloid that selectively deactivates, and ultimately damages several types of fine sensory C and A-delta fibres. It causes intense pain and a large zone of secondary hyperalgesia when applied topically or intradermally to the skin (Simone et al., 1989). Studies by Koppert et al., (2001),

Klede et al., (2003), and Sang et al., (1996) also suggest that central sensitization plays a major role in secondary hyperalgesia.

TRIGEMINAL NERVE

The chief mediator of somatic sensation from the mouth and face is the fifth cranial nerve – the trigeminal nerve. Sensory information from the face and body is processed by parallel pathway in the CNS. Trigeminal nerve is the largest cranial nerve, which innervates the face superficially in the region forward of a line drawn vertically from the ears across the top of the head and superior to the level of the lower border of the mandible. The fifth cranial nerve is primarily a sensory nerve, but it also has motor functions.

DISTRIBUTION OF THE TRIGEMINAL NERVE

It has three major branches (Figure1):

- Ophthalmic nerve, V1.
- Maxillary nerve, V2.
- Mandibular nerve, V3.

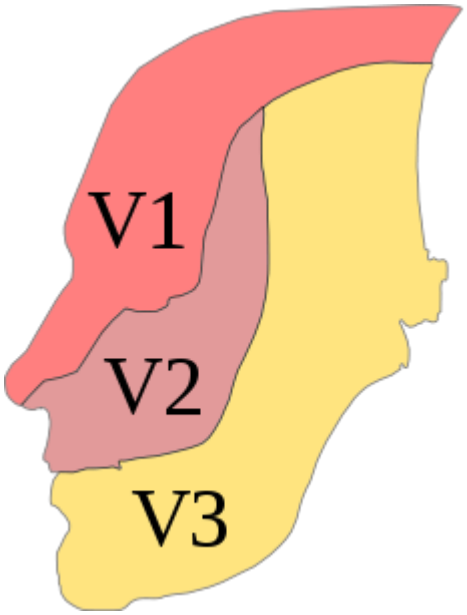


Figure 1: Shows dermatome distribution of the branches of the Trigeminal nerve, V1; Ophthalmic nerve, V2; Maxillary nerve and V3; Mandibular nerve (Wikipedia).

BRANCHES OF THE TRIGEMINAL NERVE

Ophthalmic and maxillary nerves are purely sensory while the mandibular nerve has both sensory and motor functions.

The **ophthalmic** nerve carries sensory information from the scalp and forehead, the upper eyelid, the conjunctiva and cornea of the eye, the nose (including the tip of the nose), the nasal mucosa, the frontal sinuses, and parts of the meninges (the dura mater and blood vessels).

The **maxillary** nerve carries sensory information from the lower eyelid and cheek, the nares and upper lip, the upper teeth and gums, the nasal mucosa, the palate and roof of the pharynx, the maxillary, ethmoid and sphenoid sinuses, and parts of the meninges.

The **mandibular** nerve carries sensory information from the lower lip, the lower teeth and gums, the chin and jaw, parts of the external ear, and part of the meninges. The deeper structures of the orofacial region are innervated by branches of the same cranial nerve.

In classical anatomy, the trigeminal nerve is said to have general somatic afferent (sensory) components, as well as special visceral efferent (motor) components. The motor branches of the trigeminal nerve control the movement of eight muscles, including the four muscles of mastication (Okeson, 2005).

TRIGEMINAL GANGLION

The three branches converge on the trigeminal ganglion (also called the semilunar or Gasserian ganglion), that is located within Meckel's cave, and contains the cell bodies of incoming sensory nerve fibres. The trigeminal ganglion is analogous to the dorsal root ganglia of the spinal cord, which contain the cell goodies of incoming sensory fibres from the rest of the body. From the trigeminal ganglion, a single large sensory root enters the brainstem at the level of the pons. Motor fibers pass through the trigeminal ganglion on their way to peripheral muscles, but their cell bodies are located in the motor nucleus of the fifth cranial nerve.

A variety of peptides are known to be present in the ganglion. For humans, these include CGRP, SP, somatostatin, galanin and enkephalins (Del Fiacco and Quartu, 1994). Besides the peptides, another transmitter for the trigeminal ganglion and dorsal root ganglion, is likely to be glutamate (Wanaka et al., 1987).

TRIGEMINAL NUCLEUS

The impulses carried by the trigeminal nerve enter directly into the brainstem in the region of the pons to synapse in the trigeminal spinal tract nucleus. This region of the brainstem is structurally very similar to the dorsal horn of the spinal cord. It is also considered as an extension of the dorsal horn and is sometimes referred to as the medullary dorsal horn. Trigeminal nucleus complex consists of the main sensory trigeminal nucleus and the spinal tract of the trigeminal nucleus. The main sensory trigeminal nucleus receives periodontal and some pulpal afferents.

The spinal tract is divided into three parts:

- Subnucleus oralis,
- Subnucleus interpolaris, and
- Subnucleus caudalis, which corresponds to the medullary dorsal horn.

The subnucleus caudalis has especially been implicated in trigeminal nociceptive mechanism on the basis of electrophysiologic observations of nociceptive neurons.

The subnucleus oralis appears to be a significant area of the trigeminal brainstem complex with regard to oral pain mechanisms (Okeson, 2005).

ASCENDING TRIGEMINOTHALAMIC TRACTS

Trigeminal divisions V1, V2 and V3 are responsible for cutaneous innervation of the face. The spinal trigeminal tract extends from C3 to the level of the trigeminal nerve in the midpons (which is homologous to the dorsolateral tract of Lissauer) and receives pain, temperature and light touch input. Pain fibres from the spinal trigeminal tract terminate in the caudal third of the spinal trigeminal nucleus (pars caudalis), convey general somatic afferent information from the face, oral cavity and dura mater to the thalamus(Okeson, 2005). It divides into two parts:

- Ventral trigeminothalamic tract, and
- Dorsal trigeminothalamic tract.

Each consists of a chain of three neurons, which have their 1st order neuron in the sensory ganglion of cranial nerves VII, IX and X.

OROFACIAL PAIN

The diagnosis and treatment of facial pain remains a great challenge for oral and maxillofacial surgeons. The pain syndromes are classified according to the IASP (International Association for the Study of Pain). The pain syndromes that the maxillofacial surgeons most frequently confronted with are idiopathic trigeminal neuralgia, atypical facial pain, and temporomandibular joint pain (Claeys et al., 1992).

Facial pain has many causes, including idiopathic factors, trigeminal neuralgia, dental problems, temporomandibular joint disorders, cranial abnormalities, and infections. The clinical diagnosis of facial pain is sometimes difficult to establish

because clinical manifestations commonly overlap. Therefore, a careful evaluation of the patient history and a thorough physical examination are essential (Yoon et al., 2009).

Facial pain with focal autonomic sign is mostly primary and belongs to the group of idiopathic trigeminal autonomic cephalalgias, but can occasionally be secondary. Neuralgias are often primary. Pure facial pain is most often due to sinusitis and the chewing apparatus, but may also be due to a multitude of other causes (Siccoli et al., 2006).

The most frequent conditions that produce secondary facial pain are myofacial pain syndrome, sinusitis, cervical vertebral lesions, post herpetic neuralgias, malignant head and neck tumours and encephalic vascular lesions of the pain pathway (Ramirez et al., 1989).

MECHANISM OF OROFACIAL PAIN

The pain pathway includes the trigeminal nerve, trigeminal nucleus, thalamus and cerebral cortex. The sensory input from the face and orofacial region is carried by the fifth cranial nerve, the trigeminal nerve. The cell bodies of the trigeminal afferent neurons are located in the Gasserian ganglion. The impulses carried by the trigeminal nerve enter directly into the brainstem in the region of the pons to synapse in the trigeminal spinal tract nucleus (Okeson, 2005). This region of the brainstem is structurally very similar to the dorsal horn of the spinal cord. Trigeminal nucleus complex consists of the main sensory trigeminal nucleus and the spinal tract of the trigeminal nucleus. Impulses then convey to the cerebral cortex via thalamus.

Study shows that small-diameter nociceptive afferents, such as, A-delta or C nerve fibres (free nerve endings) respond to craniofacial noxious stimuli, project to the trigeminal (V) brainstem complex where they can excite nociceptive neurons, [categorized as either nociceptive-specific (NS) or wide dynamic range (WDR)]. These neurons project to other brainstem regions or to the contralateral thalamus. The lateral and medial thalamus contains NS and WDR neurons which have properties and connections with the overlying cerebral cortex or other thalamic regions (Sessle, 1999**).**

A review study shows that the trigeminal brainstem sensory nuclear complex (VBSNC) plays a crucial role in craniofacial nociceptive transmission (Sessle, 2000). Impairment of the trigeminal nociceptive system due to demyelination and/or axonal dysfunction on the symptomatic side (locate this defect close to the root entry zone in the brainstem) in patient with trigeminal neuralgia (Obermann et al., 2007).

A recent study shows that glutamate and capsaicin have effects on trigeminal nociception, activation and peripheral sensitization of deep craniofacial nociceptive afferents (Lam et al., 2009).

OROFACIAL PAIN AND GENE EXPRESSION

Inflammation of the peripheral tissues show increased spontaneous and evoked activity (Menetrey and Besson, 1982; Calvino et al., 1987; Schaible et al., 1987), decreased thresholds to noxious stimulation (Menetrey and Besson, 1982; Hylden et al., 1989; Neugebauer and Schaible, 1990), and enlarged receptive fields (Calvino et al., 1987; Neugebauer and Schaible,1990) caused by sensitization of spinal cord sensory cells (Menetrey and Besson, 1982; Calvino et al., 1987; Schaible et al., 1987).

Tissue injury is followed by initiation of various inflammatory mediators and hyperalgesic substances such as PGs (Chichorro et al., 2004), cytokines and chemokines (Cunha et al., 2008). These tissue injuries integrate the release of mediators and hyperalgesic substances, which initiate inflammatory response which is also associated with sensitization of nociceptors and subsequent changes in the excitability of the central neurons and provoke central sensitization. Nociceptors sensitization and central sensitization are considered to underlie the development of primary hyperalgesia and secondary hyperalgesia respectively (Urban and Gebhart, 1999). Recent findings have identified a CNS neuroimmune response that may play a major role in neuronal hypersensitivity. Neuroimmune activation involves the activation of non-neuronal cells such as endothelial and glial cells, which when stimulated leads to enhanced production of a host of inflammatory mediators (Rutkowski and DeLeo, 2002a; Moalem and Tracey, 2006).

In tissue injury, microglia has an important role in the genesis of enhanced nociceptive behaviour (Yeo et al., 1995). An increase in the expression of the microglial marker OX42 (monoclonal antibody) has been shown in the spinal cord after formalin injection in the hind paw (Fu et al., 1999). Increased OX42 immunostaining has also been found in the spinal trigeminal nucleus after facial formalin injection in rats (Yeo et al., 2001).

In terms of inflammatory pain, it was known that glial cells can release a variety of algesic substances that may enhance pain transmission by neurons (Sommer, 2003; Watkins and Maier, 2003). These include proinflammatory cytokines such as inter leukin- 1b (IL- 1b), IL-6 and tumour necrosis factor alpha (TNF-α) (Raghavendra et al., 2004), chemokines such as CC-chemokine ligand-5 (CCL-5) and CCL-2 (Chan et al., 2006), cyclooxygenase (COX) products (Marriott et al., 1991; Stella et al., 1994) and NO (Simmon et al., 1992; Agullo et al., 1995). Chemokines are not stored within the cells but are synthesized in response to a variety of agents, including proinflammatory cytokines (Furie et al., 1995). IL-6 plays an important role in controlling leukocyte recruitment pattern during acute inflammation (Hurst et al., 2001). IL-6 secretion is in turn induced by many other inflammatory mediators including IL-1β, TNF-α and PGE2. IL-6 itself induces the release of chemokines CCL-2 and IL-8 (Rittner et al., 2006). Inhibition of microglia by p38 mitogen-activated protein kinase (MAPK) inhibitors (Svensson et al., 2003) or minocycline (Cho et al., 2006) resulted in attenuation of hyperalgesia, after intradermal or intraplantar injection of formalin in rats.

It was found that chemokines such as CCL-5 and CCL-2 (Chan et al., 2006) are present in the CNS neuroimmune cascade that ensues after injury to peripheral

nerves, and CCL-2 is a key mediator of microglial activation in neuropathic pain states (Thacker et al., 2008). Chemokines are synthesized at the site of injury and establish a concentration gradient through which immune cells migrate. Central sensitization through activation of immune mediators, and macrophage traffic across the blood-brain barrier are thought to play a key role in the development and maintenance of radicular pain (Rutkowski et al., 2002b) and morphine tolerance or withdrawal-induced hyperalgesia (Raghavendra et al., 2002). Moreover, it was demonstrated that microglial Toll-like receptor 4 and MAPK pathway are critical for glial control of neuropathic pain (Tanga et al., 2005, Suter et al., 2007). Besides attracting or activating glial cells, chemokines may also contribute directly to nociception (Boddeke, 2001).

Vascular endothelium also plays an important role by promoting inflammation through upragulation of adhesion molecules such as intercellular adhesion molecule (ICAM), E-selectin, and P-selectin that bind to the circulating leukocytes and facilitate migration of leukocytes into the CNS. Leukocytes can produce cytotoxic molecules that promote cell death (Wen et al., 2006). Peripheral inflammatory pain increases blood-brain barrier permeability and altered expression of tight junction protein such as ICAM-1 in endothelial cells of the thalamus and cortex (Huber et al., 2006). Increased expression of ICAM and VCAM, both indicators of endothelial activation, and increased migration of S100A8 and S100A9 expressing neutrophils into the spinal cord have also been detected after carrageenan-induced inflammation of rat hind paw (Mitchell et al., 2008). Peripheral carrageenan injection shows rapid induction of COX-2 expression in vascular endothelial cells in the CNS (Ibuki et al., 2003).

MICROARRAY ANALYSIS

Massive data acquisition technologies, such as genome sequencing, high-throughput drug screening, and DNA arrays are in the process of revolutionizing Biology and Medicine. A microarray provides an unprecedented capacity for whole genome profiling.

DNA microarrays have been used to examine changes in coding mRNA in a wide variety of pathological conditions. Besides coding mRNA, there is also much recent interest in the role of small, non-coding, micro RNA (miRNA) in regulating gene expression.

Using the mRNA of a given cell, at a given time, under a given set of conditions, DNA microarrays can provide a snapshot of the level of expression of all the genes in the cell. Such snapshots can be used to study fundamental biological phenomena such as development or evolution, to determine the function of new genes, to infer the role that individual genes or group of genes may play in diseases, and to monitor the effect of drugs and other compounds on gene expression. The quality of gene expression data obtained from microarrays can vary greatly with platforms and procedures used such as Real – Time qPCR- the Gold Standard for Validation (Morey et al., 2006). Validating these results using real – time qPCR provides more definitive quantitative analysis.

CRITICAL STEPS IN MICROARRAY ANALYSIS OF GENE EXPRESSION AND VALIDATIONS

STEP 1: MICROARRAY BENCHWORK:

1. Sample collection, RNA isolation
2. RNA quality control (Bioanalyzer)
3. RNA to Biotin –labelled cRNA
4. GeneChip hybridization (e.g. Affymetrix Platform)
5. Gene Chip quality control

STEP 2: PREPROCESSING (GCOS)

1. Detection call
2. Signal intensity
3. Normalization
4. Array concordance (GENESIFTER Intensity plots)

STEP 3: COMPUTATIONAL BIOLOGY

(SPOTFIRE, GENESHIFTER, GENESPRING, PARTEK)

1. Analysis of variance (ANOVA- 1 WAY)
2. False Discovery Rate of 5%, Benjamini and Hochberg (1995).
3. Post - Hoc Test

STEP 4: DATA MINING AND FILTERING

(SPOTFIRE, GENESIFTER, GENESPRING, PARTEK etc)

1. Heat Maps (Visualization tool)

2. Scatter Plots
3. Hierachical Clustering (Sample wise, Gene wise)
4. Principal Component Analysis
5. Venn Diagrams

STEP 5: BIOLOGICAL INTEPRETATION AND VALIDATION

Real-Time qPCR which is the Gold Standard for Validation (Morey et al., 2006).

REAL-TIME POLYMERASE CHAIN REACTION

In molecular biology, real time polymerase chain reaction (PCR), also called quantitative real time polymerase chain reaction (q-PCR) or kinetic PCR, is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification of a specific sequence in a DNA sample.

Real Time PCR is one of the most sensitive and reliably quantitative methods for gene expression analysis.

The procedure follows the general principle of PCR; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in real time after each amplification cycle. Two common methods of quantification are:

(1) The use of fluorescent dyes that intercalate with double-stranded DNA, and

(2) Modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA.

Cells in all organisms regulate gene expression and turnover of gene transcripts (messenger RNA, abbreviated to mRNA), and the number of copies of an mRNA transcript of a gene in a cell or tissue is determined by the rates of its expression and degradation.

There are numerous applications for real-time PCR in the laboratory. It is commonly used for both diagnostic and basic research. Diagnostic real-time PCR is applied to rapidly detect nucleic acids that are diagnostic of infectious diseases, cancer, and genetic abnormalities. The introduction of real-time PCR assays to the clinical Microbiology laboratory has significantly improved the diagnosis of infectious diseases (Sails, 2009).

In research settings, real-time PCR is mainly used to provide quantitative measurements of gene transcription. The technology may be used in determining how the genetic expression of a particular gene changes over time, such as the response of tissue and cell cultures to an administration of a pharmacological agent, progression of cell differentiation, or in response to changes in environmental conditions.

P-SELECTIN

P-Selectin are single chain transmembrane glycoproteins (Figure 2) which share similar properties to c-type lectins due to a related amino terminus and calcium-dependent binding (Cleator, 2006).

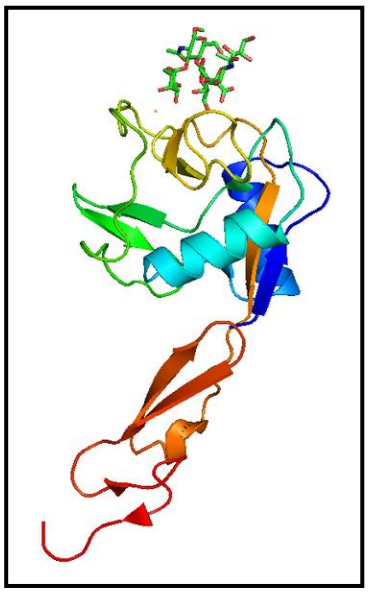


Figure 2: P-selectin lectin chain (Wikipedia)

During an inflammatory response, stimuli such as histamine and thrombin cause endothelial cells to mobilize P-selectin from stores inside the cell to the cell surface.

As the leukocyte rolls along the blood vessel wall, the distal lectin-like domain of the selectin binds to certain carbohydrate groups presented on proteins (such as PSGL-1) on the leukocyte, which slows the cell and allows it to leave the blood vessel and enter the site of infection (Aplin and Howe, 1998). The low-affinity nature of selectins is what allows the characteristic "rolling" action attributed to leukocytes during the

leukocyte adhesion cascade (Dept of Biomedical Engineering, University of Virginia. "Inflammation: The Leukocyte Adhesion Cascade).

P-selectin is a marker for platelet activation (Makin et al., 2003), as well as a marker for endothelial dysfunction (Krska et al., 2003). P-selectin found stored in the Weibel-palade bodies of endothelial cells and in the membranes of the α-granules of platelets (Stenberg et al., 1985; McEver et al., 1989). Endothelial cells express lectins that interact with leukocyte counter receptors and mediate the initial adhesion of leukocytes and their rolling along endothelial surfaces (Robinson et al., 1999).

Upregulation of P-selectin by vascular endothelium promotes inflammation by binding to circulating leukocytes, thus facilitating their migration into the CNS (Danton et al., 2003). A recent study has shown that expression of P-selectin was upregulated on vascular endothelium of inflammed lymph nodes and subcutaneous paw tissues (Mousa et al., 2000). P-selectin receptors express on T-lymphocytes bind to endothelial cells by a specific interaction with P-selectin *in vitro* (Machelska et al., 1998). P-Selectin also plays a role in the recruitment of β-endorphin containing immunocytes into inflammed subcutaneous paw tissues (Mousa et al., 2000).

CHEMOKINE (C-CMOTIF) 12

Chemokines are large family of cytokines, or proteins secreted by cells that control the recruitment of leukocytes in immune and inflammatory responses (Sarafi et al., 1997). Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells, they are therefore called chemotactic cytokines.

Some chemokines are considered pro-inflammatory and can be induced during an immune response to promote cells of the immune system to the site of infection, while others are considered homeostatic and are involved in controlling the migration of cells during normal processes of tissue maintenance or development. These proteins exert their biological effects by interacting with G protein linked transmembrane receptors called chemokine receptors that are selectively found on the surfaces of their target cells. The main sources of chemokine release are from astrocytes and microglia/macrophages (Flugel et al., 2001).

The major role of chemokines is to guide the migration of cells like lymphocytes. Cells that are attracted by chemokines follow a signal of increasing chemokine concentration towards the source of the chemokine. Some chemokines are inflammatory and functions mainly as chemoattractants for leukocytes, monocytes, neutrophils and other effectors cells from the blood.

Chemokines are not only found in the immune system or expressed in inflammatory condition, but also present in the brain in both glial cells and neurons. Chemokine have several character, that define neurotransmitter, they modify the induce release of neurotransmitters or neuropeptides and they might act as neurotransmitter or neuromodulators and can cross the blood brain barrier (Rostene et al., 2007).

Chemokine (c-c motif) ligand 12 (CCL-12) is a small cytokine belonging to the CC chemokine family that has been described in mice. CCL-12 specifically attracts eosinophils, monocytes and lymphocytes (Jia et al., 1996). CCL-12 also known as monocyte chemoattractant protein–5 (MCP – 5) is most closely related to human

chemokine MCP – 1/CCL-2 in structure (66% amino acid identity) (Sarafi et al., 1997). Thus function of CCL-12 and CCL-2 are assumed to be similar. CCL-2 is a ligand for chemotactic cytokine receptor 2 (CCR-2) (Moore et al., 2006).

INTERCELLULAR ADHESION MOLECULE 1 (ICAM- 1)

Intercellular adhesion molecules (ICAMs) are proteins located on the cell surface involved with the binding with other cells or with the extracellular matrix in the process called 'cell adhesion'. These proteins are typically transmembrane receptors and are composed of three domains:

- An intracellular domain that interacts with the cytoskeleton,
- A transmembrane domain, and
- An extracellular domain

ICAM-1 is an immunoglobulin-like cell adhesion molecule that binds to leukocyte beta-2 integrin (Miklossy et al., 2006). Endothelial ICAM-1 interacts with LFA-1 and Mac-1, and mediates leukocyte adherence, transendothelial migration and movement of activated lymphocytes into sites of inflammation (Miklossy et al., 2006). ICAM-1 also plays an important role in immune-mediated cell to cell adhesive interactions, intracellular signal transduction pathways through outside-in signalling events and may play a primary role in regulating blood brain barrier (BBB) function and structure (Huber et al., 2006). ICAM-1 is also a marker for endothelial dysfunction (Krska et al., 2003) and systemic inflammation (Mateos-Cáreres et al., 2002). A recent study has shown that expression of ICAM-1 on vascular endothelium

recruits immunocytes containing opioids to facilitate the local control of inflammatory pain (Machelska et al., 2002).

AIMS OF THE PRESENT STUDY

The aims of the project were:

(a) To evaluate the gene expression changes that occur in the brainstem after facial carrageenan injection in a mouse model of orofacial pain, by DNA microarray analysis (Affymetrix Mouse Genome 430 2.0 microarrays),

(b) To identify differential changes in coding mRNA in the spinal trigeminal nucleus / brainstem after facial carrageenan induced hyperalgesia and validate the findings by RT-PCR, immunohistochemistry and Western blot analysis. Also to evaluate differential changes in micro RNA, and

(c) To examine a possible relation between changes in micro RNA and mRNA in the spinal trigeminal nucleus / brainstem after facial carrageenan induced hyperalgesia. Genes that might be involved in pain can be targeted to reduce pain in the mice and probably applicable to human.

MATERIALS AND METHODS

ETHICAL CONCERNS

Animals in the present study were cared for and treated according to the ethical standards and guidelines for investigations of experimental pain in animals prescribed by the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP 1983).

All procedures involving the mice were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore (NUS), and adhered to the guidelines of the Committee for Research and Ethical Issues of IASP.

ANIMALS

Twenty-four adult male C57BL/6J (B6) mice, about 6-8 weeks of age and weighing approximately 20-30 g at arrival were purchased from the Laboratory Animal Centre, Singapore. The mice were housed in stainless steel cages (4 mice per cage) in the animal house with an ambient temperature water and food were available *ad libitum*. Each mice will be subjected to facial carrageenan injection and behavior testing done for the next 3 days after injection before harvesting the brainstem.

The mice were randomly divided into three groups,

- Right treated
- Left treated, and
- Control

Considering the discomforting disorder, the number of animals restricted to the bare minimum necessary, average of eight mice per group was used. The treated groups were injected with carrageenan on the facial area-i.e. the trigeminal nerve distribution area.

Refinement of procedures had been fine-tuned to the best of abilities because the procedure had been carried out on several occasions. Every effort was made to treat the animal humanely. However, using of analgesics and sedatives to reduce stress and pain of animals after facial injection was avoided because it may affect the test results. All mice were labelled with a coded number tag on their tails, to allow the behavioural responses of an individual mouse to be followed at different time intervals.

FACIAL CARRAGEENAN INJECTION

All the treated mice received a facial injection of 50 µl carrageenan (2 mg / 50 µl saline of lambda carrageenan, Sigma) in the subcutaneous tissue over the right/left trigeminal nerve distribution area – ophthalmic, maxillary and mandibular regions, while the mice were still under anaesthesia. The injection of carrageenan produced a constant swelling and caused allodynia in the injected area in the days following the injection (Ng and Ong, 2001; Vahidy et al., 2006)

ASSESSMENT OF RESPONSES TO MECHANICAL STIMULATIONS

The testing procedure consisted of assessment of the animal's responses to mechanical stimulation of the face. The mice were assessed for responses to von Frey hair stimulation of the face before injections, and from day 1 to day 3 after injections (Yeo et al,). All assessment procedures were carried out in a blinded manner and this method has been quoted to be a good model for pain study in contrast to itch (Shimada and Lamotte, 2008).

STIMULUS

For mechanical stimulation a von Frey hair (Touch- Test Sensory Evaluator, North Coast Medical, Morgan Hill, USA) was used. The von Frey hair consisted of plastic monofilament of length 4 cm delivering a force required to bend was approximately 1.4 gm (or converted to log units 4.17 log units). The test stimulus was applied over the subcutaneous tissue of the right and left maxillary region. Pilot observations showed that each stimulus evoked a behavioural response when applied to the face of normal animals.

TESTING PROCEDURES

To observe the response to mechanical stimulations, all the mice were tested individually in a deep rectangular stainless steel tank (60cm×40cm×25cm= 60000cm^3). Before the actual stimulation session began, the mice were habituated to the tank for at least 5-10 min. The experimenter reached into the tank with a von Frey hair to habituate the mice to the reaching movements for 5–10 min before testing. The mice were observed during this time, to ensure that they were able to move freely, and

had no obvious motor deficits. After the mice were adapted, a series of mechanical stimuli were started. The test stimulations were administered when the mice were in a no locomotion state, with four paws placed on the ground, neither moving nor freezing, but exhibiting sniffing behaviour. A new stimulus was applied only when the mouse resumed this position and at least 30 secs after the preceding stimulation. The carrageenan injected area of the face was probed 20 times with the von Frey hair filament. The response scoring procedure used in this study has been modified from Vos et al, 1994 (Table 1).

Response category	Detection	Withdrawal	Escape/Attack	Grooming
No response	0	0	0	0
Non-aversive response	1	0	0	0
Mild-aversive response	1	1	0	0
Strong-aversive response	1	1	1	0
Prolong-aversive behaviour	1	1	1	1

Table 1: Responses scoring system (by Vos et al,1994).

CATEGORIES OF RESPONSE

The number of any immediate response exhibited by the mouse after each stimulation, was recorded in each of the following four categories as previously described (Vos et al., 1994)

(1) **Detection:** Mouse turns head toward stimulating object, and the stimulating object is then explored (sniffing, licking),

(2) **Withdrawal reaction:** Mouse turns head slowly away or pulls it briskly backward when stimulation is applied,

(3) **Escape/Attack:** Mouse avoids further contact with the stimulating object, either passively or actively by attacking (biting or grabbing movements) the stimulating object,

(4) **Asymmetric face grooming/scratching:** Mouse displays an uninterrupted series of face-wash strokes directed to the stimulated facial area. Each stroke was counted as one response.

The number of face grooming / scratching was totalled, to give the 'total responses' after 20 stimulations with the von Frey hair. The mean and standard deviation of the total responses were then calculated for each treatment group, and the significant differences between the means elucidated using independent t-test. $P < 0.05$ was considered significant.

METHOD OF ANAESTHESIA

The agent, dose, volume and route of administration in the mice species given below (Table 2):

	Agent	Dose	Volume	Route of administration
Mice	Ketamine +medepomidine	Ketamine 75 mg/kg +medepomidine 1 mg/kg	0.1 ml/10 g body weight	Intraperitonial

Table 2: Method of anaesthesia.

MICROARRAY DATA COLLECTION GENE EXPRESSION

The portion of the brainstem containing the spinal trigeminal nucleus (Figure 3) i.e. relay neurons for nociception from the orofacial region, was dissected out from carrageenan-injected- and control mice with the help of a scalpel, with reference to an atlas, (Paxinos and Franklin, 2001).

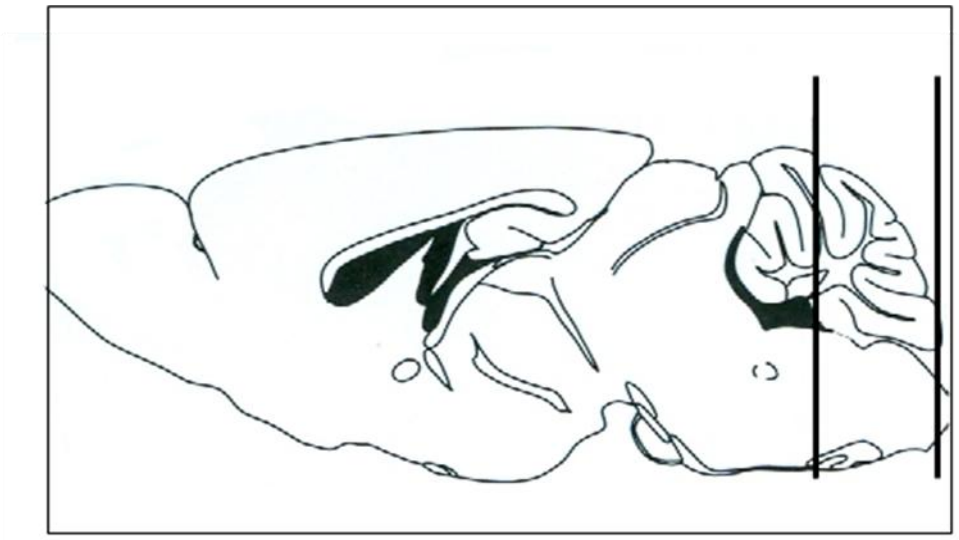


Figure 3: Lateral view of a mouse brain. The extent of the spinal trigeminal nucleus is delimited by the vertical lines (Paxinos and Franklin, 2001).

Gene expression profiles of brainstem tissue isolated from carrageenan injected mice and control mice were compared using Affymetrix Mouse Genome 430 2.0 microarrays (Affymetrix, CA, USA). Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol, and RNeasy® Mini Kit (Qiagen, Inc., CA, USA) was used to clean up the RNA. The RNA was stored at -80 °C. Total RNA was then submitted to the BFIG Core Facility Lab (National University of Singapore, Department of Paediatrics), where RNA quality was analyzed using an Agilent 2100 Bioanalyzer, and cRNA was generated and labelled using the one-cycle target labelling method, cRNA from each mouse was hybridized to a single array according to standard Affymetrix protocols.

Altogether, total of sixteen microarrays were used – four for the right brainstem of mice receiving right sided facial carrageenan injection, four for the right side of untreated controls, and similarly for the left brainstem of mice receiving left sided

facial carrageenan injection and corresponding controls. Initial image analysis of the microarray chips was performed with Affymetrix GCOS 1.2 software. The data were exported into GeneSpring v7.3 (Agilent Technologies, CA, USA) software for analysis using parametric test based on cross gene error model (PCGEM). One-way ANOVA approach was used to identify differentially expressed genes. Differentially expressed genes were then classified based on their known biological functions using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis et al., 2003).

REAL- TIME POLYMERASE CHAIN REACTION

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol, and RNeasy® Mini Kit (Qiagen, Inc., CA, USA) was used to clean up the RNA. The RNA was later treated with Dnase I (Applied Biosystems, CA, USA) according to manufacturer's protocol. The samples were then reverse transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Bio systems, CA, USA). Reaction conditions were 25^{o}C for 10 min, 37^{o}C for 120 min and 85^{o}C for 5 secs. RT-PCR amplification was then carried out in the 7500 RT- PCR system (Applied Bio systems, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Bio systems, CA, USA) and gene-specific primers and probes according to manufacturer's protocols. ß-actins were used as internal control, and all primers and probes were synthesized by Applied Bio systems. The PCR conditions were: an initial incubation at 50^{o}C for 2 min and 95^{o}C for 10 min followed by 40 cycles at 95^{o}C for 15 s and 60^{o}C for 1 min. All reactions were carried out in triplicate. The threshold cycle, CT, which correlates inversely with the levels of target mRNA,

was measured as the number of cycles at which the reporter fluorescence emission exceeds the preset threshold level. The amplified transcripts were quantified using the comparative CT method as described previously (Livak and Schmittgen, 2001), with the formula for relative fold change = $2^{-\Delta\Delta CT}$. The mean was calculated and significant differences analysed using Student's t-test. $P < 0.05$ was considered significant.

IMMUNOHISTOCHEMISTRY OF P-SELECTIN AND ICAM-1

Four adult male C57BL/6J (B6) mice were used for this portion of study. The mice were injected with carrageenan in the facial area and sacrificed at 3 days after injection. The mice were deeply anesthetized and perfused through the left ventricle with a solution of 4% Paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected out, and blocks containing the brainstem sectioned at 40 µm using a freezing microtome. The sections were washed for 3 h in phosphate-buffered saline containing 0.1% Triton (PBS-TX) to remove traces of fixative, and immersed for 1 h in a solution of 5% normal rabbit serum and 1% bovine serum albumin in PBS-TX to block non-specific binding of antibodies. They were then incubated overnight with goat polyclonal antibodies to P-selectin (Selp), and intercellular adhesion molecule 1 (ICAM-1) (Santa Cruz, diluted 1:100). Negative controls were carried out by incubation with P-selectin or ICAM-1 antigen-absorbed antibodies. The sections were then washed three times in PBS, and incubated for 1 h at room temperature in 1:200 dilution of biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, USA). This was followed by three changes of PBS to remove unreacted secondary antibody. The sections were then reacted for 1 h at room temperature with an avidin-biotinylated horseradish peroxidase complex. The reaction

was visualized by treatment for 5 min in 0.05% 3.3-diaminobenzidine tetrahydrochloride (DAB) solution in Tris buffered saline containing 0.05% hydrogen peroxide. The colour reaction was stopped with several washes of Tris buffer. Sections were counterstained with methyl green before cover slipping. Light micrographs were captured using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). The location of the spinal trigeminal nucleus is shown in (Figure 4).

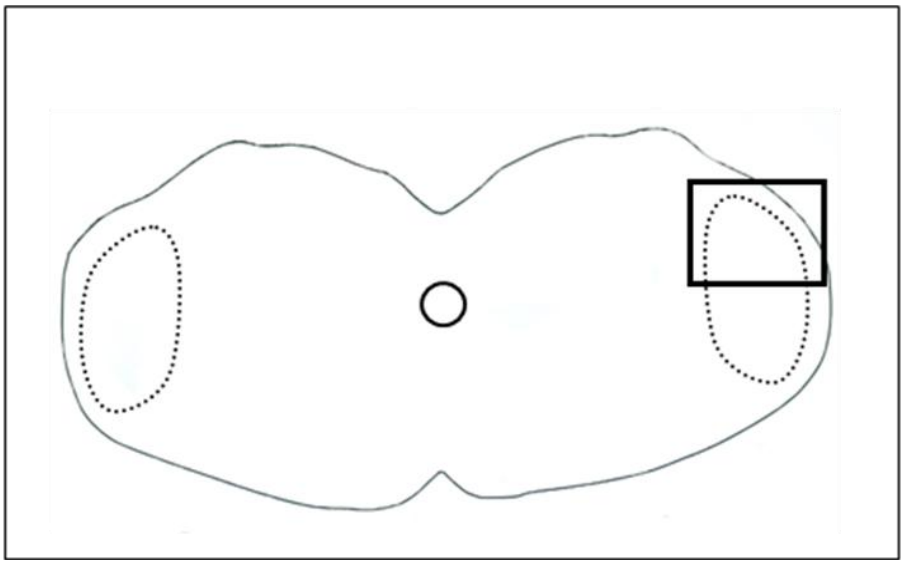


Figure 4: A mouse brainstem. The spinal trigeminal nucleus is demarcated by the dotted line. The square box indicates the approximate region where images in figure 11 were obtained.

The density of staining was analysed using MetaMorph software (Fatemi et al., 2001). The mean density was calculated and significant differences analysed using Student's t-test. $P < 0.05$ was considered significant.

WESTERN BLOT ANALYSIS

A further six adult male C57BL/6J (B6) mice were used for this portion of study. The first 3 mice were injected with carrageenan in the right side of the face and sacrificed 3 days after injection, while the other 3 mice were used as controls. They were deeply anesthetized and decapitated, a portion of the right brainstem containing the spinal trigeminal nucleus was removed and homogenized in 10 volumes of ice-cold lysis buffer (150 mM sodium chloride, 50 mM Tris hydrochloride, 0.25 mM EDTA, 1 % Triton X-100, 0.1% sodium orthovanadate, and 0.1% protease inhibitor cocktail, pH 7.4). After centrifugation at 10,000 g for 10 min at 4^{o}C, the supernatant was collected. The protein concentrations in the preparation were then measured using the Bio-Rad protein assay kit. The homogenates (40 μg) were resolved in 10% SDS-polyacrylamide gels under reducing conditions and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites on the PVDF membrane were blocked by incubating with 5% non-fat milk in 0.1% Tween-20 TBS (TTBS) for 1 h. The PVDF membrane was then incubated overnight in polyclonal antibody to P-selectin (1:200) and ICAM-1 (1:200) in 1% bovine serum albumin in TTBS. Negative controls were carried out by incubation with P-selectin or ICAM-1 antigen-absorbed antibodies. After washing with TTBS, the membrane was incubated with horseradish peroxidase-conjugated anti-goat IgG (1:2,000 in TTBS, Pierce, Rockford, IL) for 1 h at room temperature. Immunoreactivity was visualized using a chemiluminescent substrate (Supersignal West Pico, Pierce, Rockford, IL). Loading controls were carried out by incubating the blots at 50°C for 30 min with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-hydrochloride, pH 6.7), followed by reprobing with a mouse monoclonal antibody to β-actin (Sigma;

diluted 1:5,000 in TTBS) and horseradish peroxidase-conjugated anti-mouse IgG (1:5,000 in TTBS, Pierce). Exposed films containing blots were scanned and the densities of the bands measured, using Gel-Pro Analyzer 3.1 program (Media Cybernetics, Silver Spring, MD). The densities of the P-selectin and ICAM-1 bands were normalized against those of β-actin, and the mean ratios calculated. Significant differences between the values from the carrageenan injected and control animal were then analyzed, using the Student's t-test. $P < 0.05$ was considered significant.

RESULTS

PAIN RESPONSES AFTER FACIAL CARRAGEENAN INJECTION

The number of face strokes after facial carrageenan injection with peak responses was recorded at 3 days after injection. The carrageenan injected mice in this experiment likewise showed increasing responses up to the third day after carrageenan injection (Data shown in tables 3 and 4) with significantly increased responses compared to control mice at post injection days 1, 2 and 3 (Figure 5, 6, 7, and 8). They were sacrificed on the 3rd post injection day and the brainstem harvested for microarray analysis. Data was analysed by Student's t-test, P value< 0.05 was considered significant.

AVERAGE RESPONSES AND STANDARD DEVIATION OF RIGHT TREATED VS RIGHT CONTROL

Responses	Right Treated		Right Control	
	Average	**SD**	**Average**	**SD**
Before injection	6	0.82	8.25	1.50
Day 1	20.25	4.35	7.75	3.40
Day 2	23.50	3.87	6.50	1.73
Day 3	30.75	3.30	8.75	1.71

Table 3: Average responses (no of face strokes) and standard deviation of right treated and right control mice at different time points.

AVERAGE RESPONSES AND STANDARD DEVIATIONS OF LEFT TREATMENT VS LEFT CONTROL

Responses	Left Treated		Left Control	
	Average	**SD**	**Average**	**SD**
Before injection	4.50	1.91	3.25	1.26
Day 1	20	1.83	4.50	2.08
Day 2	23.75	0.96	7	2.16
Day 3	28	0.82	7.25	2.22

Table 4: Average responses (no of face strokes) and standard deviations left treated and left control mice at different time points.

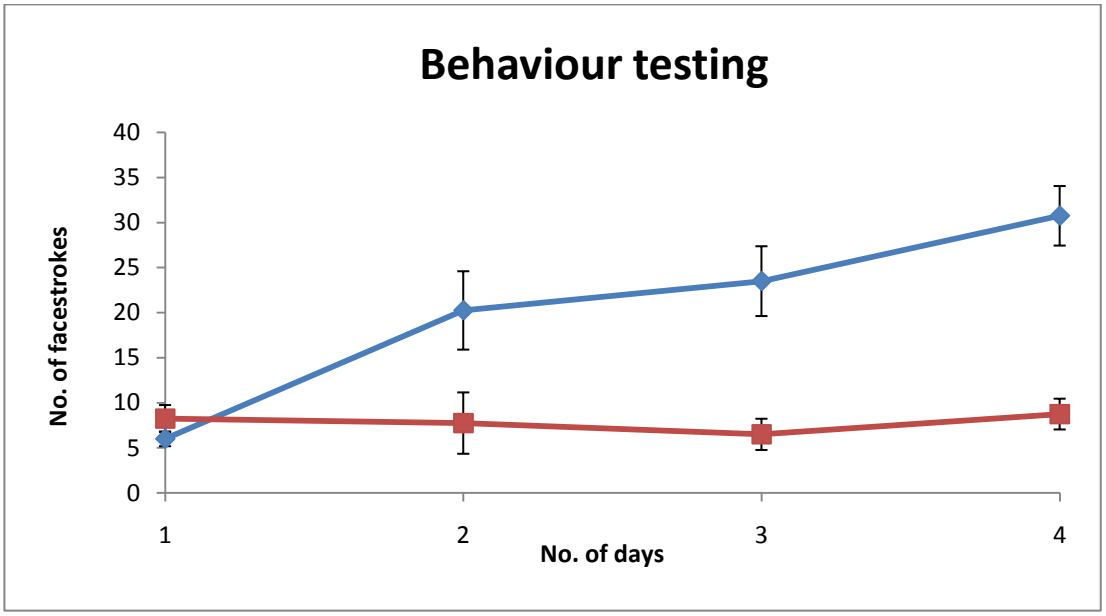


Figure 5: Responses to von Frey hair stimulation of the face after tissue inflammation induced by right sided carrageenan injection vs. right control. The Y axis represents number of face strokes to von Frey hair stimulation of the carrageenan injected areas of the face. The X axis represents the no of days; BI: before injection, 1D, 2D, 3D refer to 1 day, 2 days, and 3 days after injection. Red line: Untreated control. Blue line: After facial carrageenan injection. Analysed by student t-test P value <0.05, considered significant.

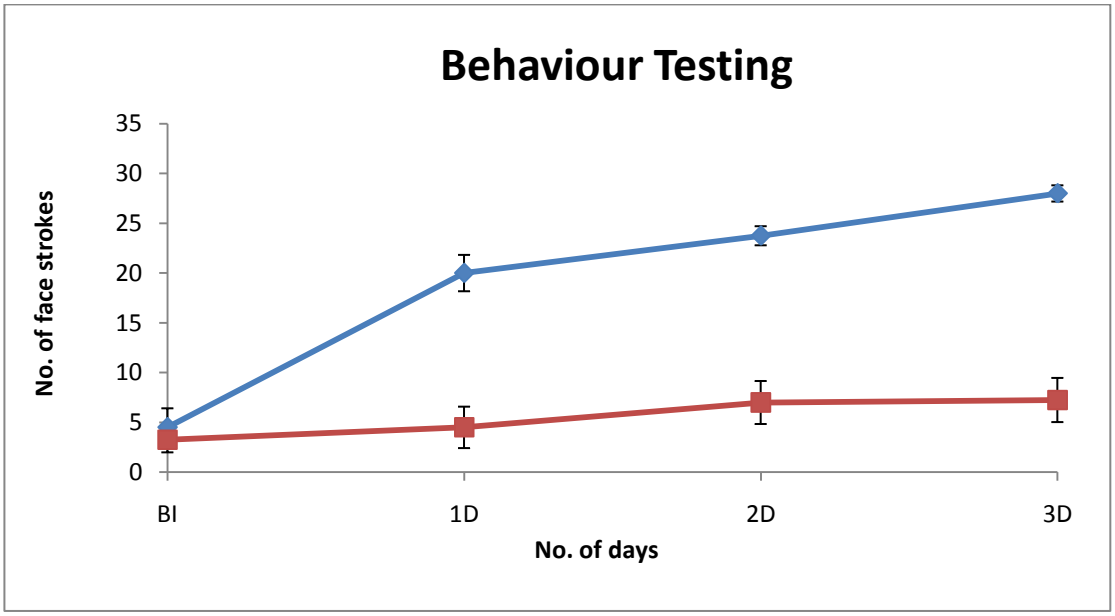


Figure 6: Responses to von Frey hair stimulation of the face after tissue inflammation induced by left sided carrageenan injection vs. left control. The Y axis represents number of face strokes to von Frey hair stimulation of the carrageenan injected areas of the face. The X axis represents the no of days ;BI: before injection, 1D, 2D, 3D refer to 1 day, 2 days, and 3 days after injection. Red line: Untreated control. Blue line: After facial carrageenan injection. Analysed by Student's t-test, $P < 0.05$ considered significant.

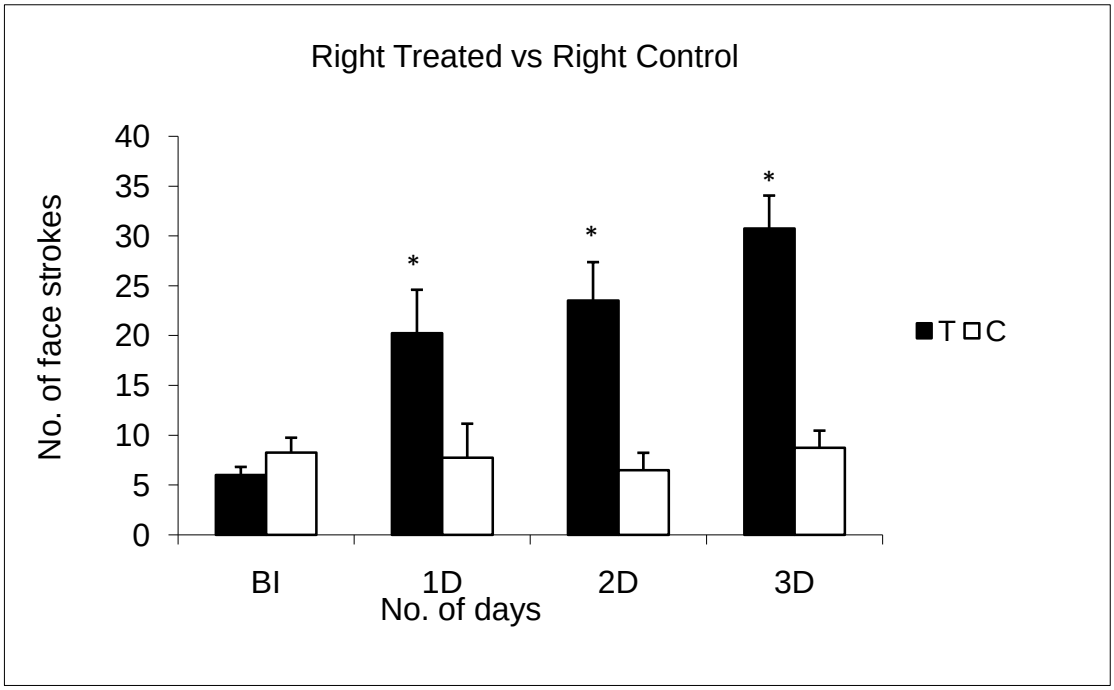


Figure 7: Responses to von Frey hair stimulation of the face after tissue inflammation induced by right sided carrageenan injection vs. right control. The Y axis represents number of responses to von Frey hair stimulation of the carrageenan injected areas of the face. BI: before injection, 1D, 2D, 3D refer to 1 day, 2 days, and 3 days after injection. C: Untreated control. T: After facial carrageenan injection. Analysed by Student's t-test. Asterisks indicate significant difference ($P < 0.05$).

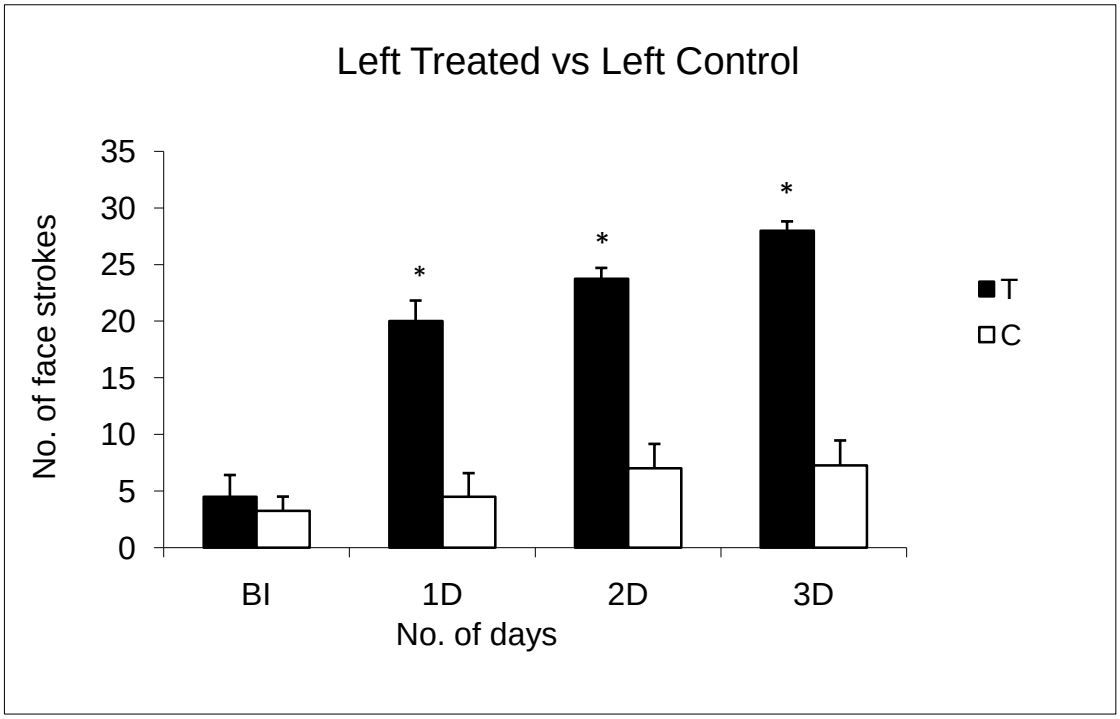


Figure 8: Responses to von Frey hair stimulation of the face after tissue inflammation induced by left sided carrageenan injection vs. left normal. The Y axis represents number of responses to von Frey hair stimulation of the carrageenan injected areas of the face. BI: before injection, 1D, 2D, 3D refer to 1 day, 2 days, and 3 days after injection. C: Untreated control. T: After facial carrageenan injection. Analysed by Student's t-test. Asterisks indicate significant difference ($P < 0.05$).

MICROARRAY ANALYSIS

The carrageenan injected mice in this experiment showed increasing responses up to the third day after carrageenan injection, with significantly increased responses compared to control mice at post-injection days 1, 2 and 3. They were sacrificed on the 3rd post-injection day and the brainstem harvested for microarray analysis.

A total of 539 and 646 genes were found to have greater than 2-fold changes in the right or left halves of the brainstem, after right or left sided carrageenan injection compared to untreated animals. Of these, genes of uncertain ontology were ignored, leaving 173 and 161 known genes with more than two fold change after right or left sided carrageenan injection. Thereafter, identical genes resulted from both right and left facial carrageenan injection were identified, which helped to minimise the impact of false positives that could have arise by chance. This results in a relatively small list of genes (22 genes). Most of them showed upregulation after carrageenan injection. They were classified using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis et al., 2003) (Table 5 and 6). Expected many of them fell into the categories of "response to stress", "defence response", "response to biotic stimulus", "cell adhesion" and "leukocyte adhesion". Five genes in common in these categories were selected for further validation in the present study, these were P-selectin, ICAM-1, Chemokine (C-C) motif ligand 12 (CCL12, MCP-5), Angiogenin 4 (Ang4) and cathelicidin antimicrobial peptide (Camp).

Gene Ontology	Gene	Gene symbol	Fold Change (Mean ± SD)	
			Right Injected	Left Injected
Developmental Process / Anatomical Structure Development	Prostaglandin-endoperoxidase synthase 2	Ptgs2	13.7 ± 6.9	6.7 ± 2.7
	Angiogenin 4	Ang4	5.1 ± 0.9	3.8 ± 0.9
	Von Willebrand factor homolog	Vwf	4.8 ± 1.6	2.3 ± 0.6
	Suppressor of cytokine signalling 3	Socs3	3.8 ± 2.6	2.1 ± 0.5
	Homeo box, msh-like 2	Msx2	2.9 ± 0.6	2.2 ± 0.7
Anatomical / Structure Morphogenesis	Angiogenin 4	Ang4	As above	As above
	Von Willebrand factor homolog	Vwf	As above	As above
	Suppressor of cytokine signalling 3	Socs3	As above	As above
	Homeo box, msh-like 2	Msx2	As above	As above
Response to Stress	Prostaglandin-endoperoxidase synthase 2	Ptgs2	As above	As above
	Selectin, platelet	Selp	12.3 ± 7.2	2.6 ± 2.4
	Chemokine (C-C motif) ligand 12	Ccl12	8.9 ± 2.5	14.2 ± 7.1
	Von Willebrand factor homolog	Vwf	As above	As above
System / Organ Development	Prostaglandin-endoperoxidase synthase 2	Ptgs2	As above	As above
	Angiogenin 4	Ang4	As above	As above
	Von Willebrand factor homolog	Vwf	As above	As above
	Homeo box, msh-like 2	Msx2	As above	As above
Cell Adhesion / Biological Adhesion	Selectin, platelet	Selp	As above	As above
	Von Willebrand factor homolog	Vwf	As above	As above
	Intercellular adhesion molecule	Icam1	4.5 ± 1.4	2.8 ± 1.3
Defence Response	Selectin, platelet	Selp	As above	As above
	Chemokine (C-C motif) ligand 12	Ccl12	As above	As above
	Angiogenin 4	Ang4	As above	As above
Negative Regulation of Cellular / Biological Process	Angiogenin 4	Ang4	As above	As above
	Suppressor of cytokine signalling 3	Socs3	As above	As above
	Homeo box, msh-like 2	Msx2	As above	As above
Organ Morphogenesis	Angiogenin 4	Ang4	As above	As above
	Von Willebrand factor homolog	Vwf	As above	As above
	Homeo box, msh-like 2	Msx2	As above	As above
Response to Wounding	Selectin, platelet	Selp	As above	As above
	Chemokine (C-C motif) ligand 12	Ccl12	As above	As above
	Von Willebrand factor homolog	Vwf	As above	As above
Cell-Cell Adhesion	Selectin, platelet	Selp	As above	As above
	Intercellular adhesion molecule	Icam1	As above	As above
Enzyme Linked Receptor Protein Signalling Pathway	Suppressor of cytokine signalling 3	Socs3	As above	As above
	Homeo box, msh-like 2	Msx2	As above	As above
Leukocyte Adhesion	Selectin, platelet	Selp	As above	As above
Response to Biotic Stimulus	Intercellular adhesion molecule	Icam1	As above	As above
	Angiogenin 4	Ang4	As above	As above

Table 5: Upregulated genes in the brainstem after facial carrageenan injection. These are genes that show up regulation by more than two folds after both right – or left sided carrageenan injection, compared to brainstem specimens of the same side of untreated control mice. They are grouped into functional classes using DAVID software.

Gene Ontology	Gene	Gene Symbol	Fold Change (Mean ± SD)	
			Right Injected	Left Injected
Developmental Process	Sclerostin domain containing 1	Sostdc1	-2.5 ± 0.5	-2.8 ± 0.7
	Myosin, heavy polypeptide 1 skeletal muscle	Myh1	-2.3 ± 0.6	-8.0 ± 0.2
	Heat shock protein 1A	Hspa1a	-2.1 ± 0.3	-2.3 ± 0.2
Anatomical Structure / System / Organ Development	Sclerostin domain containing 1	Sostdc1	As above	As above
	Myosin, heavy polypeptide 1, skeletal muscle	Myh1	As above	As above
Negative Regulation of Cellular / Biological Process	Sclerostin domain containing 1	Sostdc1	As above	As above
	Heat shock protein 1A	Hspa1a	As above	As above
Response to Biotic Stimulus	Cathelicidin antimicrobial peptide	Camp	-4.3 ± 0.4	-2.8 ± 0.6
	Heat shock protein 1A	Hspa1a	As above	As above
Anatomical Structure Morphogenesis	Sclerostin domain containing 1	Sostdc1	As above	As above
Cell Adhesion / Biological Adhesion / Cell-Cell Adhesion	Cadherin 3	Cdh3	-2.7 ± 1.4	-3.7 ± 0.2
Defence Response	Cathelicidin antimicrobial peptide	Camp	As above	As above
Organ Morphogenesis / Enzyme Linked Receptor Protein Signalling Pathway	Sclerostin domain containing 1	Sostdc1	As above	As above
Response to Stress	Heat shock protein 1A	Hspa1a	As above	As above

Table 6: Down regulated genes in the brainstem after facial carrageenan injection. These are genes that show down regulation by more than two folds after both right – or left sided carrageenan injection, compared to brainstem specimens of the same side of untreated control mice. They are grouped into functional classes using DAVID software.

VALIDATION OF DIFFERENTIALLY EXPRESSED GENES IDENTIFIED BY MICROARRAY ANALYSIS

Real time RT-PCR was used to validate the results of the microarray analysis for the selected genes, P-selectin, ICAM-1, CCL-12, Ang4 and Camp. Only 3 genes, P-selectin, ICAM-1 and CCL-12 were validated. All of them showed upregulation after either right or left sided carrageenan injection, thus validating the results of the microarray analysis.

The relative fold change (Figure 9 and 10) of P-selectin, ICAM-1 and CCL12 for the right treatment were 25.3, 3 and 4.30 respectively, while the relative fold change of P-selectin, ICAM-1 and CCL12 for the left treatment were 10.80, 5.90 and 3.80 respectively (Data shown in table 7 and 8).

REAL-TIME PCR CALCULATION (RIGHT SIDE)

Genes	Fold Change	Stdev
CCL12 Control	1	0.27
CCL12 Treated	4.32	0.43
ICAM Control	1	0.20
ICAM Treated	3.02	0.67
P-selectin Control	1	1.04
P-selectin Treated	25.27	1.69

Table 7: Real time RT- PCR analysis: Fold changes in common genes CCL12, ICAM-1 and P- selectin of right treated vs. right untreated control mice (Average and standard deviation).

REAL-TIME PCR CALCULATION (LEFT SIDE)

Genes	Fold Change	St dev
CCL12 Control	1	0.50
CCL12 Treated	3.75	0.41
ICAM-1 Control	1	1.37
ICAM-1 Treated	5.90	1.51
P-selectin Control	1	1.11
P-selectin Treated	10.82	1.17

Table 8: Real time RT- PCR analysis: Fold changes in common genes CCL12, ICAM-1 and P- selectin of left treated vs. left untreated control (Average and standard deviation).

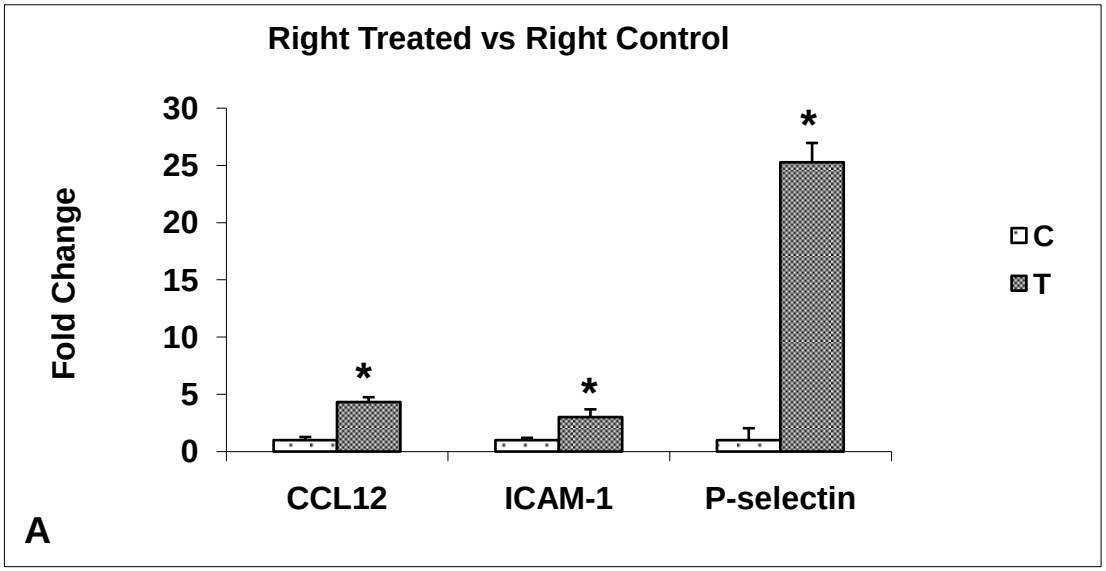


Figure 9: Real time RT-PCR analysis of changes in common genes, P-selectin, ICAM-1, and CCL12 in the mouse brainstem after facial carrageenan injection. Right sided carrageenan injection. C: Untreated control. T: After facial carrageenan injection. Analysed by Student's t- test. Asterisks indicate significant difference ($P < 0.05$).

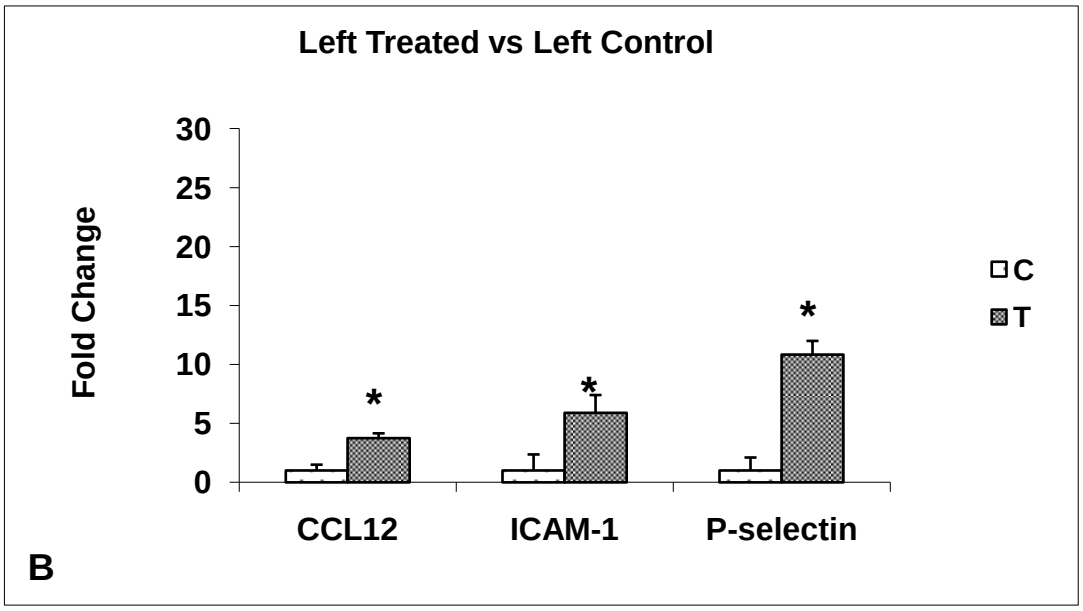


Figure 10: Real time RT-PCR analysis of changes in common genes, P-selectin, ICAM-1, and CCL12 in the mouse brainstem after facial carrageenan injection. Left sided carrageenan injection. C: Untreated control. T: After facial carrageenan injection. Analysed by Student's t-test. Asterisks indicate significant difference ($P < 0.05$).

IMMUNOHISTOCHEMISTRY OF P-SELECTIN AND ICAM-1

Light staining for P-selectin was observed in the brainstem of untreated mice (Figure 11A). In contrast, mice that received facial carrageenan injection showed significantly increased density of staining compared to untreated mice (Figure 11C). Mice that received right facial carrageenan injection also showed increased density of staining compared to its contralateral side (Figure 11E). Sections which were incubated with antigen absorbed antibody showed absence of labelling, indicating specificity of the antibody (Figure 11G).

Likewise, light staining to ICAM-1 was observed in the brainstem of untreated mice (Figure 11B). In contrast, mice that received facial carrageenan injection showed significantly increased density of staining compared to untreated mice (Figure 11D). Mice that received right facial carrageenan injection also showed increased density of staining compared to its contralateral side (Figure 11F). Sections which were incubated with antigen absorbed antibody showed absence of labelling, indicating specificity of the antibody (Figure 11H).

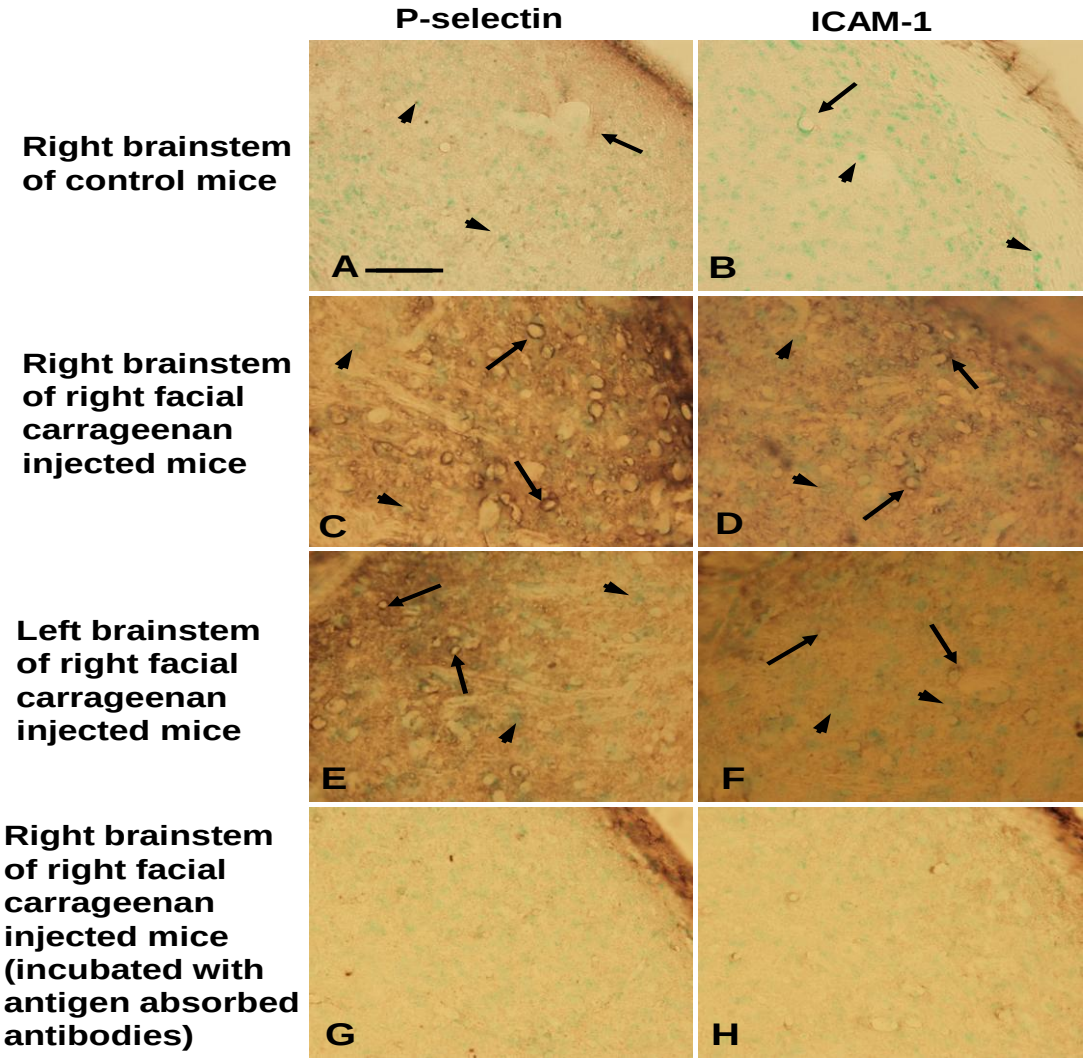


Figure 11: Light micrographs of sections of the spinal trigeminal nucleus after right sided facial carrageenan injection. A, C and E: sections incubated with antibody to P-selectin, showing increased staining on the blood vessels of a treated mouse (C, arrows), compared to an untreated control (A, arrow), and its contralateral side (E, arrows). G: section incubated with antigen-absorbed P-selectin antibody, showing absence of labelling. B, D and F: sections incubated with antibody to ICAM-1, showing increased staining on the blood vessels of a treated mouse (D, arrows), compared to an untreated control (B, arrow), and its contralateral side (F, arrows). H: section incubated with antigen-absorbed ICAM-1 antibody, showing absence of labelling. Counter-staining of methyl green (stain for nuclear) are shown in the images as green circular structure. Scale: A-H = 60 µm.

The density of staining analysed using MetaMorph software (Fatemi et al., 2001). The mean density was calculated and possible significant differences analysed using Student's t-test. $P < 0.05$ was considered significant (Figure 12 and 13)

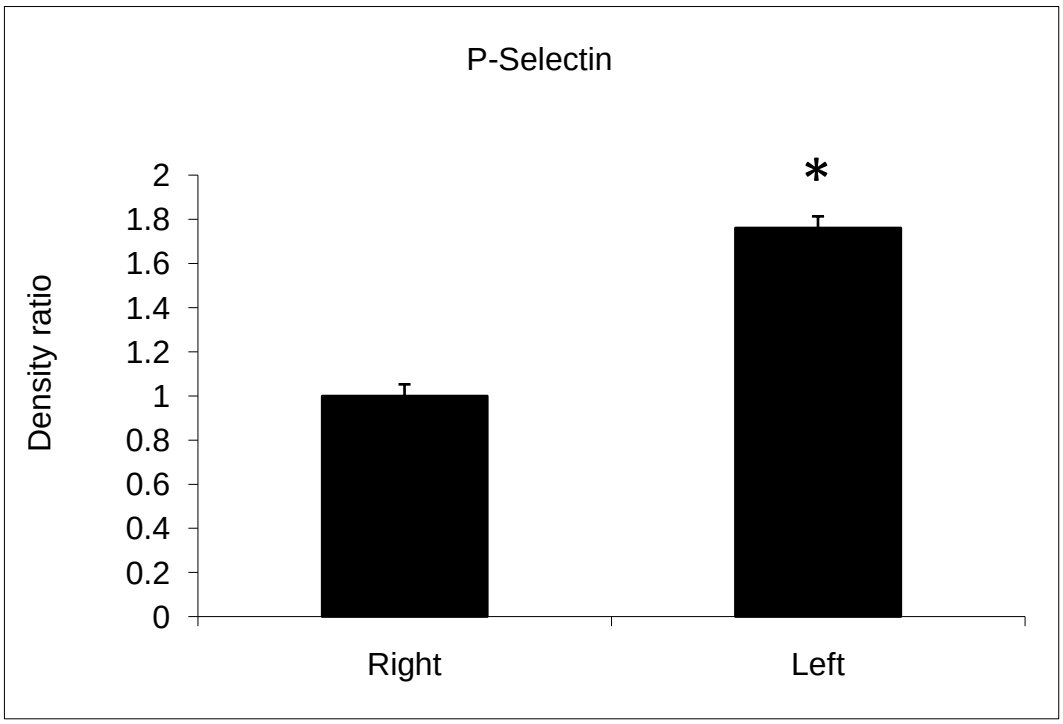


Figure 12: Ratio of densities of P- selectin on the right side of the brainstem, compared to the left side. The mean density was calculated and possible significant differences analysed using Student's t-test. Asterisks indicate significant difference ($P < 0.05$).

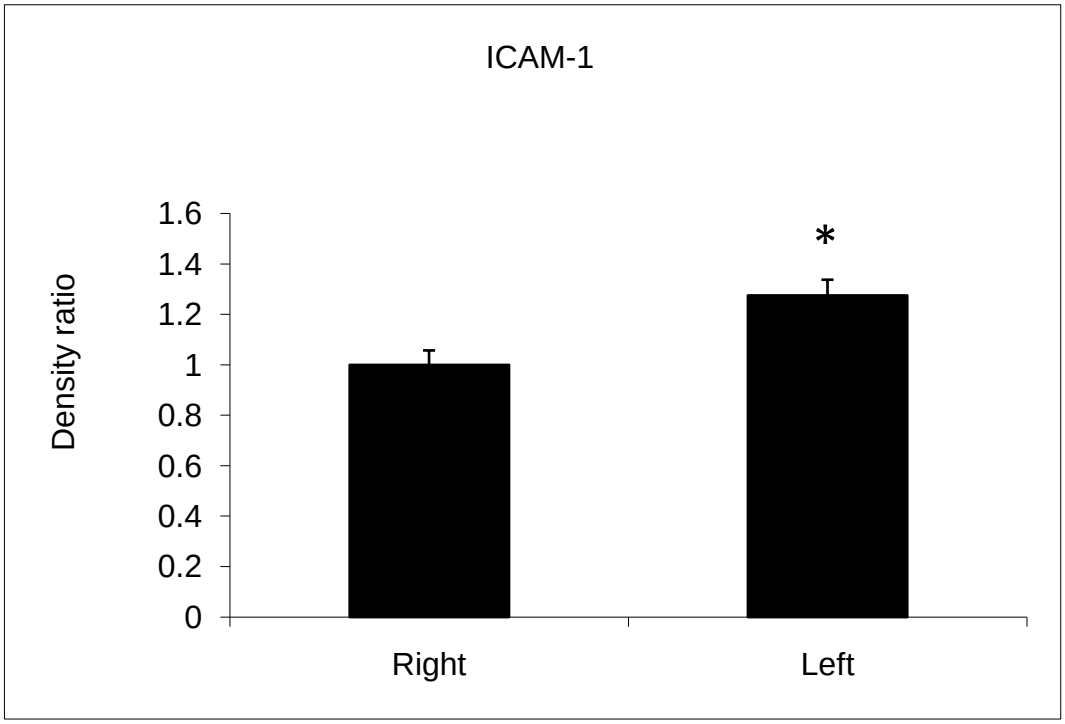


Figure 13: Ratio of densities of ICAM-1 on the right side of the brainstem, compared to the left side. The mean density was calculated and possible significant differences analysed using Student's t-test. Asterisks indicate significant difference ($P < 0.05$).

WESTERN BLOT ANALYSIS OF P-SELECTIN AND ICAM-1

The antibodies to P-selectin and ICAM-1 detected bands at around 140 kDa and 90 kDa respectively, consistent with the expected molecular weights of these proteins (Walter et al., 1997; Yang et al., 1999). No band was detected in the negative control. The antibody to β-actin reacted against a band at 42 kDa. Significant increase in the density ratios of P-selectin and ICAM-1 to β-actin bands were observed in Western blots of the 3 days post-carrageenan-injected mice, compared to the control mice indicating upregulation of P-selectin and ICAM-1 at this time point.

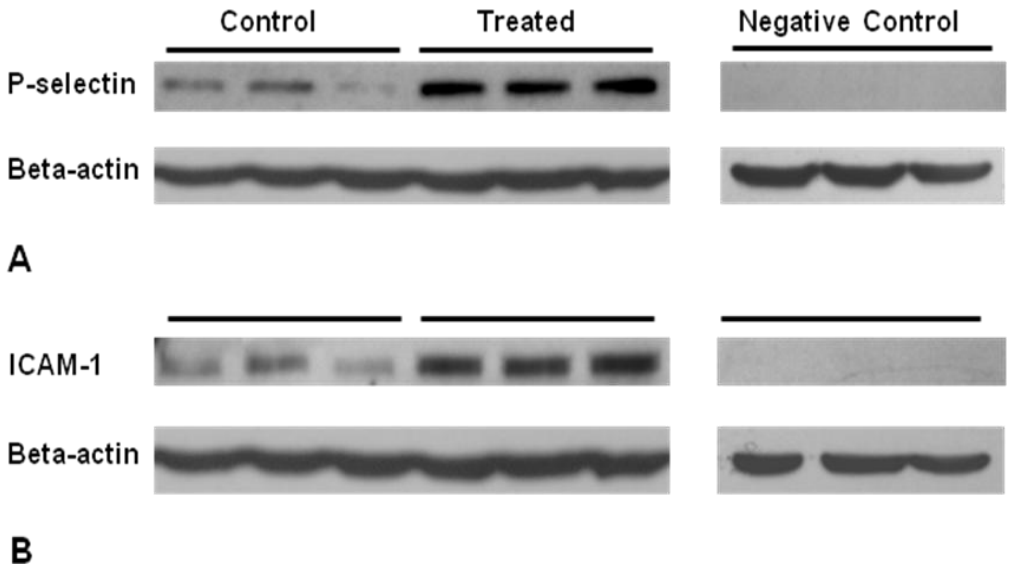
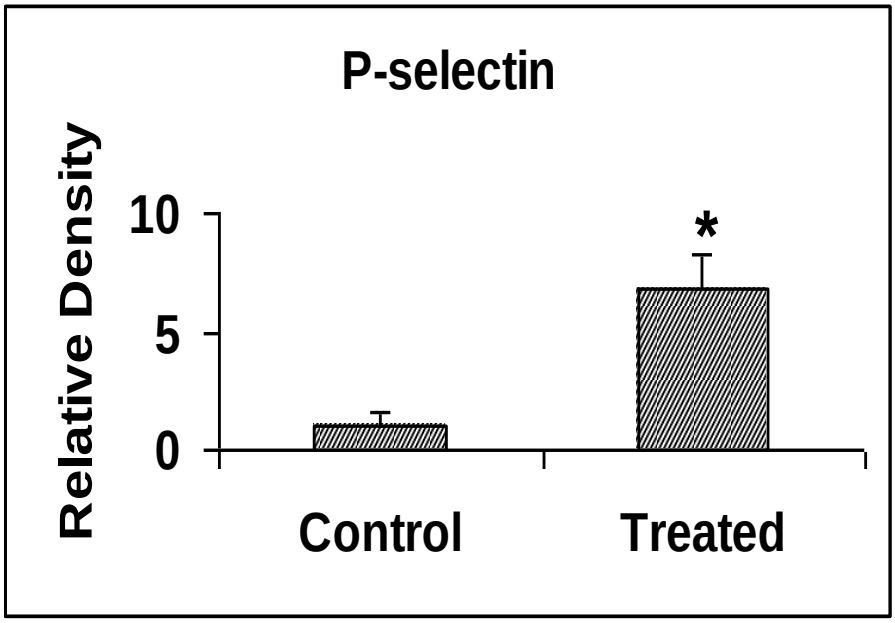


Figure 14: (A and B) Western blot analysis of homogenates of the brainstem for untreated and 3-day post-facial carrageenan injected mice. A, B: Left panels: The antibody to P-selectin (A) and ICAM-1 (B) detected single bands at ~140 kDa and ~90 kDa respectively. Right panels: No bands were detected in negative controls that were incubated with P-selectin or ICAM-1 antigen-absorbed antibodies.



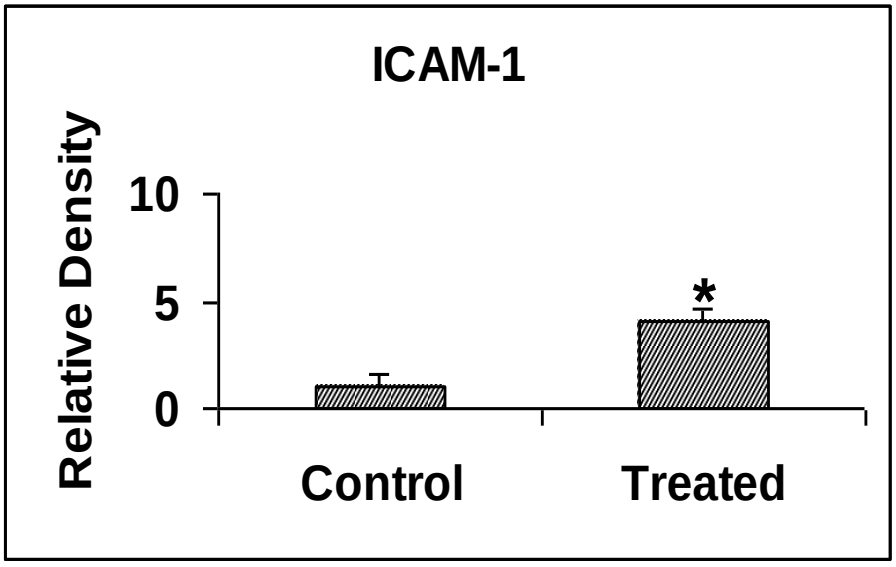


Figure 15: Quantification of western blots. P-selectin and ICAM-1 bands were normalized to β-actin. Significant increase in the density of detected bands was observed in carrageenan-injected mice, compared to untreated controls. Analyzed by Student's t-test. Asterisks indicate significant difference ($P < 0.05$).

DISCUSSION

&

CONCLUSION

The present study was carried out to examine changes in gene expression in the mouse brainstem after carrageenan-induced hyperalgesia. Facial injection of carrageenan resulted in increased nociception, as demonstrated by increased responses to von Frey hair stimulation. Previous studies showed that such increased responses peaked at about 3-4 days after injection. The brainstem was harvested 3 days after injection, corresponding to the peak time of hyperalgesia. Large numbers of genes showed two or more fold changes in the brainstem after carageenan injected mice compared with the controls. Mice that had been injected with carrageenan on the right side were compared to controls that were injected with saline on the same side, and likewise for the left side. The number of these genes was relatively small, with both sides having 22 genes in common. Most of them showed up regulation after carrageenan injection. The functions of the genes were classified by DAVID software (Dennis et al., 2003). Surprisingly, the largest groups of genes were found to belong to the category of host defence genes against pathogens. These include chemokine, inflammation related and endothelial related genes. Of the above genes, the increase in expression of P-selectin, ICAM-1, and CCL2 could be verified by real time RT-PCR on both the right and left sides. P-selectin and ICAM-1 could also be verified by immunohistochemistry, with significantly greater staining on endothelial cells on the side of the injection, compared to the opposite side, consistent with the microarray and RT-PCR results. In addition, difference in the number of face strokes between right and left facial carrageenan- injected mice seems to correlate with the difference in the general regulation of the genes in both right and left spinal trigeminal nucleus. Fold change of genes in the right facial carrageenan- injected mice seems to be up regulated in a higher level compared to left facial carrageenan injected mice, i.e. 12.3 fold

change difference in p-selectin of right facial carrageenan-injected mice compared to 2.6 fold change difference in left facial carrageenan-injected mice, and 4.5 fold change difference in ICAM-1 of right facial carrageenan-injected mice compared to 2.8 fold change difference in left facial carrageenan-injected mice. These higher degrees of up regulation of these genes could correlate with the increase in the number of face strokes. However, the reason for this difference is not known.

P-selectin is important in macrophage adhesion to blood vessels (Danton and Dietrich, 2003). Endothelial cells express selectins that interact with leukocyte counter receptors and mediate the initial adhesion of leukocytes and their rolling along endothelial surfaces (Robinson et al., 1999). It was found that selectins mediate the initial phase of immunocytes extravasations into inflamed sites, and anti-selectin treatment inhibits the infiltration of immunocytes (Machelska et al., 1998). Up regulation of P-selectin by vasculature endothelium promotes inflammation by binding to circulating leukocytes, thus facilitating their migration into the CNS (Danton and Dietrich, 2003). ICAM-1 is a marker for endothelial dysfunction (Krska et al., 2003) and systemic inflammation (Foster et al., 2003). It plays an important role in immune-mediated cell-cell adhesive interactions, intracellular signal transduction pathways through outside-in signalling events and may play a primary role in regulating BBB function and structure (Huber et al., 2006). Upregulation of adhesion molecules including P-selectin, E-selectin and ICAM may enhance the migration of leukocytes into the CNS (Danton and Dietrich, 2003). Antibody against ICAM-1 blocked neutrophils accumulation and thermal hyperalgesia (Foster et al., 2003).

Besides endothelial markers, changes were also observed in chemokines and cytokines. CCL-12 also known as MCP-5, is most closely related to human MCP-1/CCL-2 in structure (66% amino acid identity) (Sarafi et al., 1997). CCL2 is a ligand for chemotatic cytokine receptor 2 (CCR-2) (Moore et al., 2006). Both of these are involved in macrophage migration (McTigue et al., 1998). Intrathecal administration of MCP-1 induced mechanical allodynia, suggesting that MCP-1 has a role in the development of mechanical allodynia induced by nerve injury (Tanaka et al., 2004). MCP-1/CCR2 may also contribute to neuronal hyperexcitability and neuropathic pain after chronic nerve compression injury (White et al., 2005).

One possibility is that increased neuronal activity can cause the upregulation of endothelial markers in blood vessels, such as P-selectin and ICAM-1. Increased release of SP from primary afferents (McCarson and Krause, 1996; Otsuka and Yanagisawa, 1987) has been reported after peripheral inflammation in rats and mice (Allen et al., 2003), and it is possible that this could induce the expression of P-selectin and ICAM-1 in brain endothelial cells. SP has been shown to induce human dermal microvascular endothelial cells to express P-selectin, ICAM-1 and VCAM-1, suggesting that it plays a role in recruiting cells to the inflamed skin (Quinlan et al., 1999a, 1999b; Miyazaki et al., 2006). It is conceivable that changes in amino acid neurotransmitters could also result in a form of neurovascular coupling - a recent study showed that seizure-like activity induces the expression of P-selectin and ICAM-1 in endothelium of small-medium size brain blood vessels (Librizzi et al., 2007).

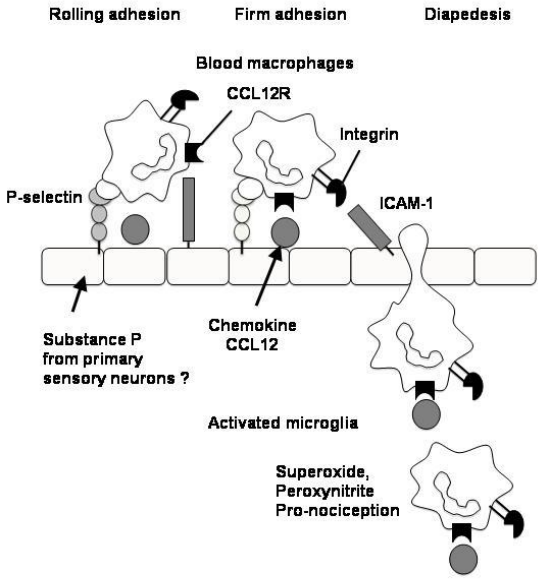


Figure 16: Hypothetical interaction of neuronal activity, blood vessels and macrophage responses in pain.

Increased P-selectin and ICAM-1 expression might result in recruitment of blood monocytes into the brain (Ling, 1979), where they could produce pro-nociceptive substances in the CNS, such as superoxide (Cathcart, 2004), or peroxynitrite (Yeo et al., 2008) (Figure 16). A possible role of P-selectin in the development of hyperalgesia after facial carrageenan injection was therefore elucidated. Carrageenan-injected mice treated with P-selectin inhibitor showed decreased nociception, demonstrated by decreased responses to von Frey hair. These findings support the notion that increased P-selectin expression contributes to pro- rather than anti- nociception, probably through the enhancement of macrophage/monocyte entry into the brain. This is consistent with a previous study

from this laboratory which showed prolonged macrophage/monocytes reaction in the caudal spinal trigeminal nucleus after facial formalin injection (Yeo et al., 2001).

In the periphery, ICAM-1 is up regulated in the vascular endothelium, simultaneously with an enhanced immigration of opioid-containing immune cells into inflammed paw tissue. The increase in ICAM-1 has been reported to have an anti nociceptive effect (Machelska et al., 2002). However, another study showed that hyperalgesia due to nerve growth factor (NGF) injection into the paw was dependent on local endothelial cell activation and increased ICAM-1 expression, and that antibody to ICAM-1 had an anti nociceptive effect (Foster et al., 2003).

In conclusion, the results indicate an intimate relationship between neurons, glial cells, and blood vessel cells of the brain, and that the CNS vascular reaction to neuronal stimulus could play an important role in central sensitization of nociception, after peripheral inflammation, and that increased nociceptive input to the brainstem could attract circulating macrophages into the brain, resulting in neuroinflammation and pain. The present result suggests that CCL-12, ICAM-1, and P-selectin may play a role in orofacial pain.

FUTURE STUDIES

AND

POSSIBILITIES

Knowledge of CNS gene expression changes after tissue injury or inflammation and further studies which attempt to reverse facial carrageenan induced hyperalgesia using inhibitors to differentially expressed genes could pave the way for the identification of novel drug targets for the clinical treatment of human orofacial pain.

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